



Contents lists available at ScienceDirect

## Regulatory Toxicology and Pharmacology

journal homepage: [www.elsevier.com/locate/yrtph](http://www.elsevier.com/locate/yrtph)

## Hazard assessment of nitrosamine and nitramine by-products of amine-based CCS: Alternative approaches

H.E. Buist\*, S. Devito, R.A. Goldbohm, R.H. Stierum, J. Venhorst, E.D. Kroese

TNO, PO Box 360, 3700 AJ Zeist, Netherlands

### ARTICLE INFO

#### Article history:

Received 16 August 2013

Available online xxx

#### Keywords:

Carbon capture and storage

Carcinogenicity

Cell transformation assay

Mutagenicity

Nitramines

Nitrosamines

Predictive *in vitro* models

Predictive *in vivo* models

QSARs

Transgenic animal test

### ABSTRACT

Carbon capture and storage (CCS) technologies are considered vital and economic elements for achieving global CO<sub>2</sub> reduction targets, and is currently introduced worldwide (for more information on CCS, consult for example the websites of the International Energy Agency (<http://www.iea.org/topics/ccs/>) and the Global CCS Institute (<http://www.globalccsinstitute.com/>)). One prominent CCS technology, the amine-based post-combustion process, may generate nitrosamines and their related nitramines as by-products, the former well known for their potential mutagenic and carcinogenic properties. In order to efficiently assess the carcinogenic potency of any of these by-products this paper reviews and discusses novel prediction approaches consuming less time, money and animals than the traditionally applied 2-year rodent assay. For this, available animal carcinogenicity studies with N-nitroso compounds and nitramines have been used to derive carcinogenic potency values, that were subsequently used to assess the predictive performance of alternative prediction approaches for these chemicals. Promising cancer prediction models are the QSARs developed by the Helguera group, *in vitro* transformation assays, and the *in vivo* initiation-promotion, and transgenic animal assays. All these models, however, have not been adequately explored for this purpose, as the number of N-nitroso compounds investigated is yet too limited, and therefore further testing with relevant N-nitroso compounds is needed.

© 2015 Published by Elsevier Inc.

### 1. Introduction

Carbon capture and storage (CCS; alternatively referred to as carbon capture and sequestration), is a means of mitigating the contribution of fossil fuel emissions to climate change. The increase of the CO<sub>2</sub> concentration in our atmosphere leads to climate change and ocean acidification. CCS is based on capturing CO<sub>2</sub> from large emissions sources such as fossil fuel power plants, and storing it subsurface, from where it will not enter the atmosphere.

A leading CCS technology is the amine-based post-combustion process, specifically for retrofit options for existing large scale stationary emitters (IPCC, 2005). Post-combustion capture involves separating the CO<sub>2</sub> from other exhaust gases after combustion of the fossil fuel. A CO<sub>2</sub> rich gas stream, such as a power plant's flue gas, is brought into contact with a "lean" amine solution in an absorber. The amine solution binds CO<sub>2</sub> as it passes through, while other gases remain in the flue gas. The CO<sub>2</sub> in the resulting CO<sub>2</sub>-saturated amine solution is then removed from the amines, dried and conditioned, after which it is ready for carbon storage.

The amines themselves are continuously recycled and re-used. While post-combustion CO<sub>2</sub> capture is technically available for fossil fuel power plants, it has not yet been commercially used for large-scale CO<sub>2</sub> removal (IPCC, 2005).

One of the drawbacks associated with post-combustion amine based CCS technology is, however, the formation of potentially harmful by-products: i.e. degradation products from reactions during the capturing process that are subsequently emitted into the atmosphere. Of particular concern among these by-products are nitrosamines and their related nitramines (de Koeijer et al., 2013), both by-products of CCS, of which the former are well known for their potential mutagenic as well as carcinogenic properties (IARC, 2012). So far the nitrosamines dimethylnitrosamine (NDMA), diethylnitrosamine (NDEA), diethanolnitrosamine (NDELA), nitrosomorpholine (NMOR), nitrosopiperidine (NPIP) and nitrosopiperazine (NPIPz), and the nitramines methylnitramine (MA-NO<sub>2</sub>), dimethylnitramine (DMA-NO<sub>2</sub>) and methylethyl-nitramine (MEA-NO<sub>2</sub>) have been identified as by-products formed in post-combustion amine based CCS technology (Bråten et al., 2008). Structurally, nitrosamines and nitramines are related, the difference being that the former possesses a N-nitroso-group and the latter a N-nitro-group (see Fig. 1). Nitrosamines are N-nitroso compounds of which the radical groups are alkyl or aryl groups

\* Corresponding author. Fax: +31 88 866 87 66.

E-mail address: [Harrie.Buist@tno.nl](mailto:Harrie.Buist@tno.nl) (H.E. Buist).

**Table 1**  
Basic information on the N-nitroso compounds and nitramines discussed in this paper (sorted in alphabetic order on abbreviation).

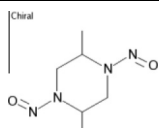
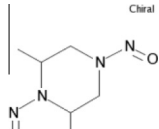
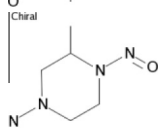
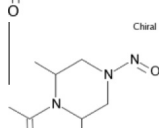
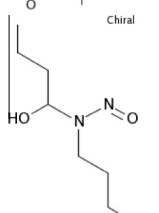
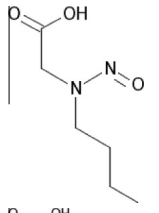
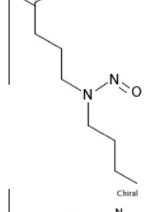
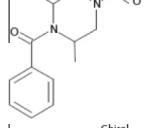
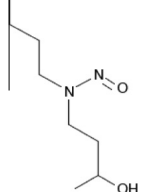
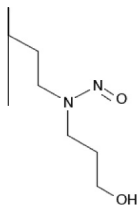
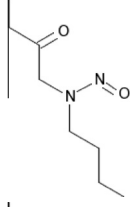
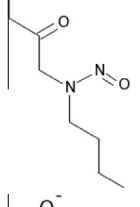
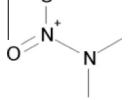
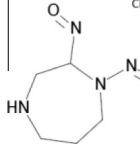
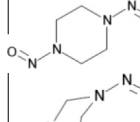
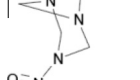
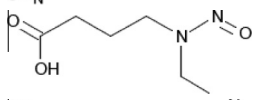
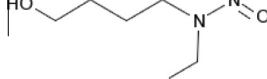
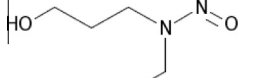
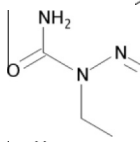
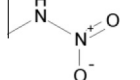
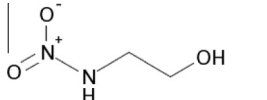
Name	Abbreviation	CAS-RN	MW	Structure
2,5-Dimethyl-1,4-dinitrosopiperazine	2,5-DMDNPIPz	55556-88-2	172.19	
2,6-Dimethyl-1,4-dinitrosopiperazine	2,6-DMDNPIPz	55380-34-2	172.19	
2-Methyl-1,4-dinitrosopiperazine	2-MDNPIPz	55556-94-0	158.16	
1-Nitroso-4-acetyl-3,5-dimethylpiperazine	AcNM2PIPz	75881-17-3	185.22	
Butylhydroxybutylnitrosamine	BBN	78619-31-5	174.24	
N-butyl-N-(carboxymethyl)nitrosamine	BCMN	61864-02-6	160.17	
Butyl(3-carboxypropyl)nitrosamine	BCPN	38252-74-3	188.12	
1-Nitroso-4-benzoyl-3,5-dimethylpiperazine	BeNM2PIPz	61034-40-0	247.29	
N-Butyl-N-(3-hydroxybutyl)nitrosamine	BHBN-3	40911-07-7	174.24	

Table 1 (continued)

Name	Abbreviation	CAS-RN	MW	Structure
N-Butyl-N-(3-hydroxypropyl)nitrosamine	BHPN	51938-13-7	160.12	
N-Butyl-N-(2-oxobutyl)nitrosamine	BOBN-2	61734-90-5	172.22	
N-Butyl-N-(3-oxobutyl)nitrosamine	BOBN-3	61734-89-2	172.22	
N-methyl-N-nitro-methanamine	DMA-NO <sub>2</sub>	4164-28-7	90.08	
1,4-Dinitroso-1,4-diazepane	DNhPIPz	55557-00-1	158.16	
1,4-Dinitrosopiperazine	DNPIPz	140-79-4	144.13	
N,N-dinitrosopentamethylenetetramine	DNPT	101-25-7	185.18	
N-Ethyl-N-(3-carboxypropyl)nitrosamine	ECPN	54897-63-1	160.17	
N-Ethyl-N-(4-hydroxybutyl)nitrosamine	EHBN	54897-62-0	146.19	
N-Ethyl-N-(3-hydroxypropyl)nitrosamine	EHPN	61734-88-1	132.16	
N-nitroso-N-ethylurea	ENU	759-73-9	117.10	
N-nitro-methanamine	MA-NO <sub>2</sub>	598-57-2	76.05	
Ethanolnitramine	MEA-NO <sub>2</sub>	74386-82-6	106.04	

(continued on next page)

Table 1 (continued)

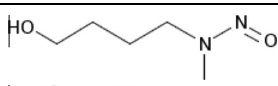
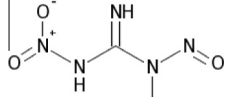
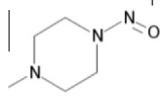
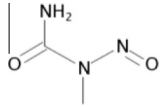
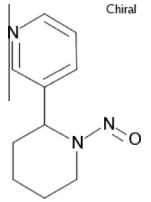
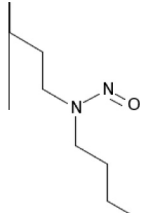
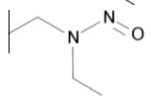
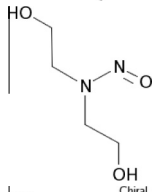
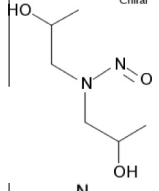
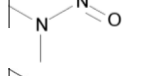
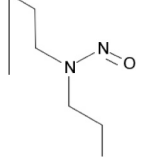
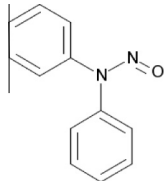
Name	Abbreviation	CAS-RN	MW	Structure
N-Methyl-N-(4-hydroxybutyl)nitrosamine	MHBN	51938-16-0	132.16	
N-methyl-N-nitro-N-nitrosoguanidine	MNNG	70-25-7	147.10	
N-Nitroso-N'-methylpiperazine	NMPIPz	16339-07-4	129.16	
N-nitroso-N-methylurea	MNU	684-93-5	103.08	 Chiral
N'-Nitrosoanabasine	NAB	1133-64-8	191.23	
N-nitrosodi-n-butylamine	NDBA	924-16-3	158.24	
N-nitrosodiethylamine	NDEA	55-18-5	102.14	
N-nitrosodiethanolamine	NDELA	1116-54-7	131.14	 Chiral
N-nitrosodiisopropanolamine	NDiPLA	53609-64-6	162.19	 Chiral
N-nitrosodimethylamine	NDMA	62-75-9	74.08	
N-nitrosodi-n-propylamine	NDPA	621-64-7	130.19	
N-nitrosodiphenylamine	NDPhA	86-30-6	198.08	

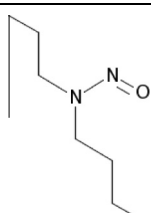
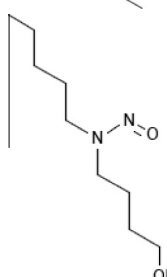
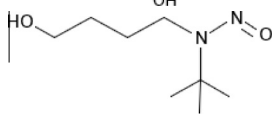


Table 1 (continued)

Name	Abbreviation	CAS-RN	MW	Structure
N-nitrosoethylbutylamine	NEBA	4549-44-4	130.19	
N-nitrosoethylphenylamine	NEPhA	612-64-6	150.18	
1-Nitroso-3,5-dimethylpiperazine	NM2PIPz	67774-31-6	143.19	
3,4,5-Trimethyl-nitrosopiperazine	NM3PIPz	75881-18-4	157.21	
N-nitrosomethylbutylamine	NMBA	7068-83-9	116.16	
N-nitrosomethylethylamine	NMEA	10595-95-6	88.11	
N-nitrosomorpholine	NMOR	59-89-2	16.12	
N-nitrosomethylpropylamine	NMPA	924-46-9	102.14	
N-nitrosomethylphenylamine	NMPhA	614-00-6	136.15	
N-nitrosoglyphosphate	NNG	56516-72-4	198.07	
4-(methylnitrosamino)-1-(3-pyridyl)-1-Butanone	NNK	64091-91-4	207.23	
N'-nitrosornicotine	NNN	64162-58-9	177.09	
N-nitrosopiperidine	NPIP	100-75-4	114.15	
N-nitrosopiperazine	NPIPz	5632-47-3	115.13	
N-nitrosopyrrolidine	NPYR	930-55-2	100.12	

(continued on next page)

Table 1 (continued)

Name	Abbreviation	CAS-RN	MW	Structure
N-Propyl-N-butyl nitrosamine	PBN	25413-64-3	144.21	
N-Pentyl-N-(4-hydroxybutyl) nitrosamine	PeHBN	61734-86-9	188.27	
N-tert-butyl-N-(4-hydroxybutyl) nitrosamine	t-BBN	61734-87-0	174.24	

(WHO, 1978). In principle, every nitramine has a nitrosamine counterpart and vice versa: e.g. dimethylnitramine (DMA-NO<sub>2</sub>) and NDMA.

Considering their potential mutagenic as well as carcinogenic properties, it is important that the issue of potentially harmful nitrosamine and nitramine by-products of this new CO<sub>2</sub>-emission reducing technology is well addressed. The potential human exposure as well as the mutagenic properties and carcinogenic potencies of the specific nitrosamines and nitramines generated need to be adequately assessed to enable conclusions about the risk to human health. As the amine technology will continue to develop within the coming decades, new nitrosamine and nitramine structures with unknown mutagenic and carcinogenic properties may be generated as by-products. In this paper we will exclusively focus on alternative approaches to predict the carcinogenic properties of these compounds, i.e. to search for alternatives to the time-consuming, costly and impractical 2-year rodent assay traditionally applied in carcinogenicity assessment. First, we will shortly describe the human health profile of nitrosamines and other N-nitroso compounds, and nitramines, followed by reviewing available data on mutagenic and carcinogenic properties as their most critical effects. Table 1 presents all N-nitroso compounds and nitramines discussed in this paper and their structures. Subsequently, we will review and discuss alternative methodologies that were used to predict their carcinogenic properties, and explore the potential of these methodologies to predict the carcinogenic potency of nitrosamine or nitramine structures, and their potential use in place of the classical *in vivo* animal bioassay. Supplemental material is provided in two Appendices (A and B), accessible via the online version, at <http://dx.doi.org/10.1016/j.yrtph.2014.01.017>.

## 2. Human health toxicity profiles of N-nitroso compounds and CCS nitramines

### 2.1. Short term effects

#### 2.1.1. Genotoxicity

Regarding short term toxicity, most data available concern the genotoxic properties of N-nitroso compounds and nitramines.

Appendix A of the supplemental material presents a summary table of all the study results and references. In Table 2 the genotoxicity profiles of those N-nitroso compounds and nitramines are presented. From this inventory it is clear that all the listed N-nitroso compounds are positive in one or the other genotoxicity test. However, although NPIPz is positive when tested in the presence of metabolic activation in an Ames test with *Salmonella typhimurium* strain 1535 (Zeiger and Sheldon, 1978), this positive result is canceled by the negative result obtained with the *in vitro* HPRT gene mutation test with Chinese Hamster Ovary cells in the presence of metabolic activation (Jones et al., 1981). In a comparable test with Chinese Hamster Lung cells, the CCS nitrosamines NMOR (Robichová et al., 2004) and NDELA (Liu and Russell, 2008) did test positive. Still NPIPz is carcinogenic (see Table 3), and it might be a (very) weak mutagen. The fact that it is the least potent carcinogen of the CCS nitrosamines supports this last notion, while also the main target organ of NPIPz (the nasal cavity, same as for the structurally related DNPIPz) supports, or at least does not contradict, a comparable mechanism of carcinogenicity as its genotoxic congener. Except for ENU and MNU, all N-nitroso compounds completely tested in cytogenic tests or indicator tests for chromosome damage needed metabolic activation to be positive.

The nitramines DMA-NO<sub>2</sub>, MA-NO<sub>2</sub> and MEA-NO<sub>2</sub> appear to be negative in indicator tests for chromosome damage. For the gene mutation tests the picture is more varied, some N-nitroso compounds only score positive in the presence of metabolic activation, others also in its absence. The nitramines DMA-NO<sub>2</sub>, MA-NO<sub>2</sub> and MEA-NO<sub>2</sub> are mutagenic, even without metabolic activation. Unfortunately, for those nitrosamines scoring negative in carcinogenicity assays (EHPN, PeHBN, t-BBN, and BHBN-3) no genotoxicity data could be found in public literature.

#### 2.1.2. Effects other than genotoxicity

Acute oral toxicity data retrieved from public literature are listed in Table 4. Oral LD<sub>50</sub>'s range from 0.3 to 60 mmol/kg bw, with an average of 12 mmol/kg bw. The three nitramines included in the list all have a LD<sub>50</sub> of around 10 mmol/kg bw and are in the mid-range of N-nitroso compound acute toxicity.

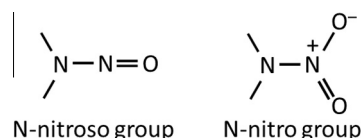


Fig. 1. Structure of N-nitroso (nitrosamine) and N-nitro (nitramine) groups.

Only for a few nitramines data on skin irritation and corrosion, eye irritation, and skin sensitization are available, all generated by Fjellsbø et al. (2011, 2013). The first three endpoints were tested using OECD guidelines under GLP conditions, while the last was assessed using the VITASENS assay as described by Hooyberghs et al. (2008), which has no regulatory status. The nitramines, MA-NO and MEA-NO<sub>2</sub> tested negative for skin irritation and corrosion, positive for ocular corrosion and were classified as non-sensitizers in the VITASENS assay. DMA-NO<sub>2</sub> also tested negative for skin irritation and positive for eye corrosion, but could not or was not tested in the other two assays.

## 2.2. Long term effects

### 2.2.1. Carcinogenicity

Human and animal studies on the carcinogenicity of N-nitroso compounds and CCS nitramines were collected in order to derive carcinogenic potency values for nitroso substances emitted to the air in the CCS process. However, human data on CCS nitramines could not be retrieved from public literature and none of the epidemiological dose–response studies we retrieved from public literature could link observed carcinogenic effects clearly to a single nitrosamine; only one study seemed to evaluate exposure to one specific nitrosamine, NDMA, but unfortunately, it is not suitable to derive a carcinogenic potency value since the investigated German cohort was not only exposed to NDMA but also to NMOR as well as to a number of nitrosamines which have not been measured (Straif et al., 1998, 2000; Weiland et al., 1996). Therefore the carcinogenic potency values derived for all N-nitroso compounds and CCS nitramines are based on animal data only (see Appendix B of the supplemental material).

In order to assess the relative carcinogenic potency of nitr(os)amines emitted to the air in the CCS process, preferably inhalation studies, performed with the same strain of animals, with guideline-conform protocols under identical experimental conditions should be used. However, in most studies available the oral route of administration has been investigated. In that case one needs to extrapolate the carcinogenic potency value obtained via the oral route to the respiratory route. Differences in toxicokinetic behavior between the oral and respiratory route (first pass effect, differences in biotransformation enzyme make up between the portals of entry, differences in uptake) may complicate this extrapolation. In particular when local tumors occur (i.e. tumors occurring at sites that are exposed even when there is no systemic uptake), oral and respiratory doses are difficult to compare. Among toxicological risk assessors there is consensus that only systemic effects may be extrapolated from one route to the other (see a.o. ECHA, 2012b). Consequently, no respiratory potency values were derived for the few N-nitroso compounds for which only oral studies were available that just showed local effects (e.g. esophagus tumors).

In principle, in absence of suitable human data, data from the most sensitive animal species should be used to derive human carcinogenic potency values, unless the tumors observed are irrelevant for humans. The vast majority of the studies encountered investigated carcinogenicity in rats. In case other species had been studied as well, the rat often was the most sensitive species (e.g. for NDELA, NMEA, NMPA, NDPA, NDBA, NPYR and NPIP: see

Appendix B of the supplemental material). To avoid species bias when evaluating the predictive value of tests, the rat was chosen as the species to derive carcinogenic potency values from. As a matter of fact, in the few cases that other species were more sensitive than the rat, the values derived from them were not very different from those derived from rats (see Table 3).

The incidence per unit dose/concentration ( $I_{\text{dose/conc}}$ ) was chosen as potency value, because it shows a direct proportional relationship with carcinogenic potency, making it easier to use than alternative measures like the  $T_{25}^1$  or the  $TD_{50}^2$  which show an inversely proportional relation with carcinogenic potency. For the calculation of  $I_{\text{dose}}$  (for oral studies) and  $I_{\text{conc}}$  (for respiratory studies), it was assumed that the general population would be exposed daily for lifetime. The incidence of tumor-bearing animals per mmol/kg bw/day for oral studies was calculated as follows<sup>3</sup>:

$$I_{\text{dose}} = [I_e - I_c] / [(D/AS \times (X_{\text{po}}/L) \times (X_{\text{pe}}/L) \times (\text{hours per day}/24) \times (\text{days per week}/7)]$$

For inhalation studies a similar formula was used<sup>4</sup>:

$$I_{\text{conc}} = [I_e - I_c] / [(C \times (X_{\text{po}}/L) \times (X_{\text{pe}}/L) \times (\text{hours per day}/24) \times (\text{days per week}/7)]$$

To estimate the extra lifetime risk of cancer in humans under lifespan conditions on the basis of results in animal experiments, it was assumed that no difference exists between experimental animals and man with respect to toxicokinetics, mechanism of tumor induction, target susceptibility, etc., unless specific information is available which justifies a different approach. Furthermore, it was assumed that the average human being lives 75 years, inhales 20 m<sup>3</sup> per day, is exposed 24 h per day, 7 days per week, 52 weeks per year, for lifetime. These proceedings are very similar to those used by e.g. the EU Scientific Committee on Occupational Exposure Limits (SCOEL) (Bolt and Huici-Montagud, 2008), the European Chemicals Agency (ECHA, 2012b) and the Dutch Health Council (Gezondheidsraad, 2012).

Since inhalation will be the main route of exposure for nitrosamines associated with CCS, and often only oral studies are available,  $I_{\text{dose}}$  for systemic tumors is extrapolated to  $I_{\text{conc}}$  using the following formula<sup>5</sup>:

$$I_{\text{conc}} = I_{\text{dose}} \times RV/BW \times RAbs/OAbs$$

<sup>1</sup> The chronic dose rate which will give 25% of the animals tumors at a specific tissue site, after correction for spontaneous incidence, within the standard life-time of that species.

<sup>2</sup> Daily dose-rate in mg/kg/body weight/day for life to induce tumors in half of test animals that would have remained tumor-free at zero dose.

<sup>3</sup>  $I$  is estimated tumor incidence;  $I_e$  and  $I_c$  are tumor incidences in exposed and control animals, respectively;  $AS$  is the allometric scaling factor,  $D$  is the daily dose (mmol/kg bw/day) in the lowest dose group showing a statistically significant increase in number of animals with a specific tumor, consistent with the dose–response;  $X_{\text{po}}$  and  $X_{\text{pe}}$  are observation and exposure period, respectively;  $L$  is the standard lifespan for the animal species in question.

<sup>4</sup>  $C$  is the exposure concentration (mmol/m<sup>3</sup>) in the lowest dose group showing a statistically significant increase in number of animals with a specific tumor, consistent with the dose–response, and allometric scaling is not applied because it is proportional to the respiratory rate and thus implicitly taken into account.

<sup>5</sup>  $RV$  is respiratory volume, 20 m<sup>3</sup>/day by default for the general population;  $BW$  is body weight, 70 kg by default for the general population;  $RAbs$  is percentage absorption of the nitrosamine via the respiratory route and  $OAbs$  is percentage absorption of the nitrosamine via the oral route, if no measured values are available, by default a ratio of 2 is used for  $RAbs/OAbs$ . It should be noted the  $I_{\text{conc}}$  for the general population can be simply converted into an occupational one by correcting for differences in exposure duration (assumed to be continuous throughout life time (75 years) for the general population and intermittent (e.g. 8 h/day, 5 days/week, 48 weeks/year for 40 years) and respiratory volume (10 m<sup>3</sup>/8 h for workers) (see e.g. ECHA, 2012b).

**Table 2**  
Overall genotoxicity test results for the N-nitroso compounds and nitramines discussed in this paper.

Nitr(os)amine	Cytogenic tests			Gene mutation tests			Overall genotoxicity		
	No MA	MA	Overall	No MA	MA	Overall	No MA	MA	Overall
<i>Alkyl-nitr(os)amines</i>									
<b>MA-NO<sub>2</sub></b>	eq.	neg	eq.	pos	pos	pos	pos	pos	pos
<b>NDMA</b>	neg	pos	pos	neg	pos	pos	neg	pos	pos
<b>DMA-NO<sub>2</sub></b>	neg	neg	neg	pos	pos	pos	pos	pos	pos
<b>NDEA</b>	neg	pos	pos	neg	pos	pos	pos	pos	pos
NMEA					pos	pos		pos	pos
NMPA					pos	pos		pos	pos
NMBA					pos	pos		pos	pos
NEBA					pos	pos		pos	pos
NDPA	neg	neg	neg		pos	pos	neg	pos	pos
NDBA					pos	pos		pos	pos
<i>Alcohol-nitr(os)amines</i>									
<b>MEA-NO<sub>2</sub></b>	neg	neg	neg	pos	pos	pos	pos	pos	pos
<b>NDELA</b>	neg	pos	pos		pos	pos	neg	pos	pos
<i>Heterocyclic nitrosamines</i>									
<b>NPIP</b>	pos	pos	pos		pos	pos	pos	pos	pos
NPYR		pos	pos	pos	pos	pos	pos	pos	pos
<b>NPIPz</b>	neg	neg	neg		neg			neg	neg
<b>DNPIPz</b>	neg	pos	pos		pos	pos	neg	pos	pos
NMPIPz		pos	pos	neg	pos	pos	neg	pos	pos
NM <sub>2</sub> PIPz				neg	pos	pos	neg	pos	pos
NBeM <sub>2</sub> PIPz				neg	neg	neg	neg	neg	neg
<b>NMOR</b>	neg	pos	pos	neg	pos	pos	neg	pos	pos
<i>Phenyl-nitrosamines</i>									
NMPhA					pos	pos		pos	pos
NDPhA		pos	pos	eq.	eq.	eq.	eq.	pos	pos
<i>Nitroso-ureas</i>									
MNU	pos		pos	pos		pos	pos		pos
ENU	pos		pos	pos		pos	pos		pos

MA = metabolic activation.

Also *in vitro* tests executed with cells that clearly have xenobiotic metabolizing capacity (e.g. hepatocytes) were considered to have been executed in presence of metabolizing activity. Positive tests are shaded. Nitr(os)amines demonstrated to be produced in the CCS process are bold-faced.

The following test were considered to be "cytogenic tests": CA *in vitro*, Comet *in vitro*, MN *in vitro*, MN *in vivo*, Comet *in vivo*, MN *in vivo*. "Gene mutation tests" were considered to be GM Bacteria, Germ line *in vivo*, GM *in vitro*, GM *in vivo* and UDS *in vivo*. In one category, a result was considered positive if at least one test in the category tested positive (pos); if no test was positive and one or more test were equivocal, the category was labeled "equivocal" (eq.); if no test was positive or equivocal and one or more tests were negative, the category was labeled "negative" (neg), else the category was left blank (no data).

The data and the corresponding literature references on which this table is based are summarized in [Appendix A of the supplemental material](#).

In view of the uncertainties inherent to the procedure followed to derive limit values, all calculations were rounded off to two significant figures only. Whenever specific values needed for the calculations were not available from the original articles, default values were used. These values and their sources are listed in [Table 1 of Appendix B of the supplemental material](#).

The studies available for the derivation of cancer potency values as well as the selection of the most suitable study to derive them and the exact calculation are described in [Appendix B of the supplemental material](#). The potency values are listed in [Table 3](#).

### 2.2.2. Effects other than carcinogenicity

No general studies on repeated dose toxicity of N-nitroso compounds or nitramines were encountered in public literature.

Likewise data on reproductive and developmental toxicity are very scarce, however, since the vast majority of these N-nitroso compounds are genotoxic carcinogens, carcinogenicity is most likely the leading health effect for risk assessment purposes: For that reason e.g. the EU requires no data on reproductive and developmental toxicity for genotoxic carcinogens (ECHA, 2012a).

Only the nitroso-urea ENU has been amply investigated for reproductive and developmental toxicity (see a.o. Akiyama et al., 2008; Katayama et al., 2001, 2000a, 2000b, 2002; Kennedy and O'Bryan, 2006; Lee et al., 2009; Sussman et al., 2001). It was proven to be a developmental toxicant and toxic for reproduction. For other N-nitroso compounds and the CCS nitramines hardly any data are available for these endpoints. An *ex vivo* study with

**Table 3**

Respiratory carcinogenic potencies of N-nitroso compounds and nitramines based on rat data. If data on other more sensitive species were available, the potency derived from the most sensitive species is listed as well.

Compound	MW	$I_{\text{conc}}$ based on				Rank (oral studies) <sup>a</sup>
		Oral route (rat)		Respiratory route (rat)		
		(mmol/m <sup>3</sup> ) <sup>-1</sup>	(mg/m <sup>3</sup> ) <sup>-1</sup>	(mmol/m <sup>3</sup> ) <sup>-1</sup>	(mg/m <sup>3</sup> ) <sup>-1</sup>	
<i>Alkyl-nitr(os)amines</i>						
DMA-NO <sub>2</sub>	90.08	$7.4 \times 10^1$	$8.2 \times 10^{-1}$	–	–	30
MA-NO <sub>2</sub>	76.05	$1.2 \times 10^1$	$1.6 \times 10^{-1}$	–	–	36
NDBA	158.24	$1.2 \times 10^3$	$7.3 \times 10^0$	–	–	4
NDEA	102.14	$1.1 \times 10^3$	$1.1 \times 10^1$	–	–	5
NDMA	74.08	$3.0 \times 10^2$	$4.0 \times 10^0$	$4.2 \times 10^3$	$5.7 \times 10^1$	18
NDPA	130.19	$4.1 \times 10^2$	$3.1 \times 10^0$	–	–	13
NEBA	130.19	$3.0 \times 10^1$	$2.3 \times 10^{-1}$	–	–	33
NMBA	116.16	–	–	–	–	–
		Hamster: $5.5 \times 10^2$	$4.8 \times 10^0$	–	–	–
NMEA	88.11	$4.5 \times 10^2$	$5.1 \times 10^0$	–	–	12
NMPA	102.14	–	–	–	–	–
		Hamster: $6.0 \times 10^2$	$5.9 \times 10^0$	–	–	–
PBN	144.21	$1.3 \times 10^2$	$9.1 \times 10^{-1}$	–	–	23
<i>Alcohol-, aldehyd- and carboxylnitr(os)amines</i>						
BBN	174.24	$9.4 \times 10^2$	$5.4 \times 10^0$	–	–	8
BHBN-3	174.24	neg	neg	–	–	–
BHPN	160.12	neg	neg	–	–	–
EHBN	146.19	$1.1 \times 10^2$	$7.7 \times 10^{-1}$	–	–	26
		Mouse: $1.3 \times 10^2$	$9.2 \times 10^{-1}$	–	–	–
EHPN	132.16	neg	neg	–	–	–
MHBN	132.16	$8.7 \times 10^1$	$6.6 \times 10^{-1}$	–	–	29
NDELA	131.14	$4.0 \times 10^1$	$3.0 \times 10^{-1}$	–	–	32
PeHBN	188.27	neg	neg	–	–	–
t-BBN	174.24	neg	neg	–	–	–
NDiPLA	162.19	$1.6 \times 10^2$	$1.0 \times 10^0$	–	–	22
BOBN-2	172.22	$1.1 \times 10^2$	$6.5 \times 10^{-1}$	–	–	25
BOBN-3	172.22	$2.2 \times 10^1$	$1.3 \times 10^{-1}$	–	–	34
BCMN	160.17	neg	neg	–	–	–
BCPN	188.12	$2.4 \times 10^2$	$1.3 \times 10^0$	–	–	21
ECPN	160.17	$1.1 \times 10^2$	$7.1 \times 10^{-1}$	–	–	25
<i>Phenyl-nitrosamines</i>						
NDPhA	198.08	$1.2 \times 10^0$	$5.8 \times 10^{-3}$	–	–	38
NMPhA	136.15	$1.10 \times 10^3$	$7.7 \times 10^0$	–	–	5
NEPhA	150.18	–	–	–	–	–
<i>Heterocyclic nitrosamines</i>						
2,5-DMDNPIPz	172.19	$3.8 \times 10^2$	$2.2 \times 10^0$	–	–	14
2,6-DMDNPIPz	172.19	$9.8 \times 10^2$	$5.7 \times 10^0$	–	–	7
2-MDNPIPz	158.16	$7.2 \times 10^2$	$4.5 \times 10^0$	–	–	10
AcNM2PIPz	185.22	–	–	–	–	–
BeNM2PIPz	247.29	$1.5 \times 10^1$	$6.3 \times 10^{-2}$	–	–	35
DNhPIPz	158.16	$9.2 \times 10^2$	$5.8 \times 10^0$	–	–	9
DNPIPz	144.13	$3.1 \times 10^2$	$2.2 \times 10^0$	–	–	17
		mouse: $4.6 \times 10^2$	$3.2 \times 10^0$	–	–	–
DNPT	185.18	neg	neg	–	–	–
NMPIPz	129.16	$4.3 \times 10^1$	$3.3 \times 10^{-1}$	$4.6 \times 10^2$	$3.5 \times 10^0$	31
NAB	191.23	–	–	–	–	–
NM2PIPz	143.19	$2.5 \times 10^2$	$1.8 \times 10^0$	–	–	20
NM3PIPz	157.21	$3.0 \times 10^2$	$1.9 \times 10^0$	–	–	18
NMOR	116.12	$2.1 \times 10^3$	$1.8 \times 10^1$	$4.1 \times 10^2$	$3.5 \times 10^0$	3
NNK	207.23	$3.0 \times 10^3$	$1.5 \times 10^1$	–	–	2
NNN	177.09	$1.2 \times 10^2$	$7.0 \times 10^{-1}$	–	–	24
NPIP	114.15	$6.9 \times 10^2$	$6.0 \times 10^0$	–	–	11
NPIPz	115.13	$6.6 \times 10^0$	$5.7 \times 10^{-2}$	–	–	37
NPYR	100.12	$1.1 \times 10^2$	$1.1 \times 10^0$	–	–	25
<i>Nitroso-ureas and -guanidines</i>						
ENU	117.10	$3.9 \times 10^3$	$3.3 \times 10^1$	–	–	1
MNU	103.08	$3.3 \times 10^2$	$3.2 \times 10^0$	–	–	16
MNNG	147.10	$3.5 \times 10^2$	$2.4 \times 10^0$	–	–	15
NNG	198.07	–	–	–	–	–

$I_{\text{conc}}$  = incidence of tumor bearing animals per unit of concentration (lifetime continuous exposure).

The derivation of these carcinogenic potency values and the studies on which they are based are described and referenced in [Appendix B of the supplemental material](#).

<sup>a</sup> Based on the values displayed in the third column.

NDMA conducted by [Annola et al. \(2009\)](#) demonstrated that the human fetus can be exposed to NDMA from the maternal circulation and that NDMA is not metabolized in full-term human

placenta from healthy non-smoking, non-drinking mothers. This suggests that NDMA may cause developmental toxicity by direct interaction with the fetus.



**Table 4**  
Oral LD<sub>50</sub> for selected N-nitroso compounds and nitramines in rats.

Nitr(os)amine	LD <sub>50</sub>		References
	mmol/kg bw	mg/kg/bw	
<i>Alkyl-nitr(os)amines</i>			
DMA-NO <sub>2</sub>	8.50	770	Fjellsbø et al. (2011)
MA-NO <sub>2</sub>	11.00	840	Fjellsbø et al. (2011)
NDBA	7.60	1200	Druckrey et al. (1967)
NDEA	2.80	290	Druckrey et al. (1967)
NDMA	0.54	40	Druckrey et al. (1967)
NDPA	3.70	480	Druckrey et al. (1967)
NMBA	2.90	340	Druckrey et al. (1967)
NMEA	1.00	88	Druckrey et al. (1967)
NMPA	9.50	970	Druckrey et al. (1967)
<i>Alcohol-nitr(os)amines</i>			
MEA-NO <sub>2</sub>	9.10	970	Fjellsbø et al. (2011)
BBN	10.00	1740	Druckrey et al. (1967)
NDELA	60.00	7870	Druckrey et al. (1967)
<i>Phenyl-nitrosamines</i>			
NDPhA	15.00	2970	Druckrey et al. (1967)
NEPhA	0.30	45	Druckrey et al. (1967)
NMPhA	2.10	290	Druckrey et al. (1967)
<i>Heterocyclic nitrosamines</i>			
DNPIPz	1.10	160	Druckrey et al. (1967)
NMPIPz	7.80	1010	Druckrey et al. (1967)
NMOR	2.80	330	Druckrey et al. (1967)
NPIP	1.70	190	Druckrey et al. (1967)
NPYR	9.00	900	Druckrey et al. (1967)
<i>Nitroso-ureas</i>			
ENU	2.00	230	Druckrey et al. (1967)
MNU	1.10	110	Druckrey et al. (1967)

### 2.3. Target-organs of tumor formation

Based on the studies described in [Appendix B of the supplemental material](#), an inventory was made of the principle target organs of tumor formation in the carcinogenicity studies with CCS nitrosamines, other N-nitroso compounds and nitramines. [Table 5](#) displays the results of this effort for rat carcinogenicity studies, which constitute the majority of the available studies.

When comparing target-organs for carcinogenicity for these compounds, the data clearly show that many organs are potentially at risk. It is not evident from the data what determines the specific targeting.

Many CCS nitrosamines and also the nitramine DMA-NO<sub>2</sub> have the liver as main target organ in oral studies. Also for other carcinogens the liver is often the main target organ after oral administration, which is probably related to the relatively high dose this organ will face and its high concentration of a broad spectrum of xenobiotic metabolizing enzymes, including many CYPs.

The second most frequent target organs are the esophagus and the nasal cavity. There are indications that CYP2A3 may play a role in this organ preference: CYP2A3 is reported to be present in rat nasal and lung tissue but not in the liver ([Honkakoski and Negishi, 1997](#); [Robottom-Ferreira et al., 2003](#)), and it is also present in the esophagus ([Ribeiro Pinto et al., 2001](#); [Robottom-Ferreira et al., 2003](#)). Unfortunately no data on the interaction between the CCS nitrosamines mainly targeting the nasal mucosa, DNPIPz and NPIPz, and CYPs are available.

From the overview one remarkable observation is the absence of tumors of the upper part of the digestive tract for the group of alcohol-, keto-, and carboxynitrosamines, as compared to the other groups. Also, a relatively high number of these nitrosamines have the bladder as their main target for cancer induction. Another remarkable observation is that piperazine derivatives as a group clearly do not target the liver.

Both DMA-NO<sub>2</sub> and MA-NO<sub>2</sub> target the Central Nervous System (CNS) ([Hassel and Frei, 1987](#); [Mirvish et al., 1980](#); [Scherf et al.,](#)

[1989](#)), which is the only and main target organ of the latter compound ([Hassel and Frei, 1987](#); [Scherf et al., 1989](#)), while for the former the liver is the most sensitive for tumor development ([Druckrey et al., 1967, 1961](#); [Goodall and Kennedy, 1976](#); [Mirvish et al., 1980](#)).

There seem to be some species differences in N-nitroso compound carcinogenicity as can be seen from the data on hamster and mouse compared to those on rats, although the number of hamster and mouse studies is too small to draw firm conclusions. For example, via the oral route the main target organ of NMPA and NMBA is the esophagus in rats ([Lijinsky et al., 1983](#)) while it is the liver in the hamster ([Lijinsky and Kovatch, 1988](#)). Furthermore, the main target organ of NDELA via the oral route in the rat is the liver, while in the mouse it is the lung ([Hecht et al., 1989](#)).

Based on the few available respiratory rat carcinogenicity studies, also some route differences in organ specificity are apparent: e.g. for NDMA the main target via the respiratory route is the nasal cavity and via the oral route the liver. This issue, and its importance for the extrapolation to humans, is touched upon in more detail in the discussion (Section 4).

Besides route and species, also animal strain and even dose-regime appear to be of influence: e.g. a single high oral dose of NDMA led to kidney tumors, while prolonged exposure mainly induced liver tumors ([Magee and Barnes, 1967](#)).

Fairly small differences in chemical structure may change organ specificity. For example, NDMA and NDEA induce tumors at many sites, e.g. in liver, kidney and upper digestive tract, but not in the bladder ([Peto et al., 1991a,b](#)), which is the major target organ of NDBA ([Druckrey et al., 1967](#)). Also the piperazine derivatives show a clear target-organ shift with relatively small structural changes: replacing a 4-methylgroup in NM<sub>3</sub>PIPz with a 4-acetylgroup (AcNM<sub>2</sub>PIPz) results in the complete disappearance of lymphomas and leukemia, and the appearance of esophagus and tongue tumors ([Singer et al., 1981](#)).

Overall, a great variety of organs may be targeted by carcinogenic CCS nitrosamines and nitramines, of which the liver is the most frequent one, followed by the esophagus and the nasal cavity. Although some generalizations appear possible, the nature of the target organs is determined by many different factors and the present database only shows some tendencies that do not allow firm conclusions.

## 3. Alternative methodologies for predicting carcinogenicity of N-nitroso compounds and nitramines

### 3.1. Approaches using chemical structure

#### 3.1.1. Structural classes and carcinogenic potency

From [Table 3](#) one can observe that estimated potencies of N-nitroso compounds and nitramines span over 3 orders of magnitude. Potency values of the alkyl nitrosamines (based on oral studies) have a smaller potency range of only 50-fold (comparing NDBA and NEBA), of which NDBA appears the most potent carcinogen on a mmol/m<sup>3</sup> basis. Actually, all these nitrosamines cluster within one order of magnitude potency difference, only NEBA being about one order less potent than the second least potent one of this group. There is no other evident trend, e.g. with alkyl chain length.

None of the alkanol nitrosamines has a higher potency than NDEA, and the potency range of nitrosamines listed here is somewhat similar: about 10-fold. It should be recognized though that all 'negative' nitrosamines in this group may in fact be relatively weak carcinogens, as the experimental design used for these nitrosamines is not sensitive enough to conclude that they are not carcinogenic. Thus, should these negative nitrosamines have been tested

**Table 5**  
Overview of target organs of carcinogenesis of N-nitroso compounds.

Compound	Oral																			Respiratory							
	Oc	Oe	GSt	Fst	Di	Na	PEx	Tr	Lu	Pe	Li	He	Bl	Ki	Ov	Ty	Ma	Te	Ut	CNS	HPS	Na	Li	Th	CNS		
<i>Alkyl-nitr(os)amines</i>																											
<b>MA-NO<sub>2</sub></b>																					X		--	--	--	--	
<b>DMA-NO<sub>2</sub></b>		X				X					X			X							X		--	--	--	--	
<b>NDMA</b>								X			X			X								X	--	--	--	--	
<b>NDEA</b>		X									X												--	--	--	--	
NMEA		X				X			X		X			X									--	--	--	--	
NMPA	X	X		X																			--	--	--	--	
NMBA	X	X		X		X																	--	--	--	--	
NEBA	X	X									X												--	--	--	--	
NDPA	X	X		X		X					X												--	--	--	--	
NPBA (PBN)		X									X												--	--	--	--	
NDBA	X	X		X							X		X										--	--	--	--	
<i>Alcohol- keto- and carboxy- nitrosamines</i>																											
<b>NDELA</b>						X					X			X				X					--	--	--	--	
NDiPLA								X			X												--	--	--	--	
MHBN									X														--	--	--	--	
EHBN													X										--	--	--	--	
EHPN													X										--	--	--	--	
BBN													X										--	--	--	--	
PeHBN																							--	--	--	--	
t-BBN																							--	--	--	--	
BHBN-3																							--	--	--	--	
BCPN													X										--	--	--	--	
ECPN													X										--	--	--	--	
BCMN													X										--	--	--	--	
<i>Heterocyclic nitrosamines</i>																											
<b>BHPN</b>																							--	--	--	--	
<b>BOBN-3</b>											X												--	--	--	--	
<b>BOBN-2</b>											X												--	--	--	--	
<b>NPIP</b>		X		X				X			X												--	--	--	--	
<b>NMOR</b>		X									X												X	X	X	X	
NPYR											X							X					--	--	--	--	
<b>NPIPz</b>											X												--	--	--	--	
<b>DNPIPz</b>								X			X										X		--	--	--	--	
MNPIPz											X												X	--	--	--	--
M <sub>3</sub> NPIPz											X												--	--	--	--	
M <sub>2</sub> NPIPz											X												--	--	--	--	
AcM <sub>2</sub> NPIPz	X	X																					--	--	--	--	
BeM <sub>2</sub> NPIPz				X																			--	--	--	--	
2,5-DMDNPIPz		X									X										X		--	--	--	--	
DNhPIPz		X									X										X		--	--	--	--	
2-MDNPIPz		X									X												--	--	--	--	
2,6-DMDNPIPz		X									X												--	--	--	--	
NNK							X				X		X										--	--	--	--	
NNN		X	X			X					X												--	--	--	--	
<i>Phenylnitrosamines</i>																											
NMPhA		X		X						X													--	--	--	--	
NDPhA													X										--	--	--	--	
<i>Nitrosoureas and -guanidines</i>																											
ENU					X			X	X						X		X		X	X	X		--	--	--	--	
<i>Nitrosamines and nitramines</i>																											
<b>MNU</b>	X			X	X						X			X							X		--	--	--	--	
<b>MNNG</b>			X	X	X					X													--	--	--	--	

-- = no data; Bl = bladder; CNS = Central Nervous System, Di = digestive tract (after stomach), Fst = forestomach, He = heart, HPS = haematopoietic system, Ki = kidneys, Li = liver, Lu = lungs, Ma = mammary gland, Na = nasal cavity, Oc = oral cavity, Oe = oesophagus, Ov = ovary, PEx: pancreas, exocrine, Te = testis, Th = thyroid, Tr = trachea, Ty = thymus, Ut = uterus. Empty cells indicate no effects were observed on the organ mentioned in the column heading. The most sensitive organ is indicated by a shaded cell. The CCS nitrosamines and nitramines are printed in bold face. The studies on which these data are based are described and referenced in [Appendix B of the supplemental material](#).

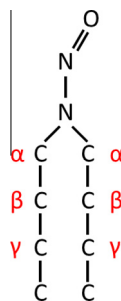


Fig. 2. The  $\alpha$ -,  $\beta$ - and  $\gamma$ -positions in the N-nitroso compound structure.

in a study design with a higher resolution, i.e. closer to OECD guidelines, the potency range of this group may well expand into the lower potency region. From direct comparison of potencies of the respective pairs NMBA/MHBN, NEBA/EHBN, and NDBA/BBN, there is no clear effect of alkyl group hydroxylation on carcinogenic potency discernible; this also holds for the NDEA/NDELA and NDPA/NDiPLA pairs.

Also, all cyclic nitrosamines have lower potency estimates than NDEA, except NMOR that shows a comparable carcinogenic potency. Potencies of NPIP, and NPYR are within about one order of magnitude of NMOR. The structural changes in the NPIPz analogues show substantial impact on the carcinogenic potency set off against NPIPz itself: compare e.g. NPIPz with NM<sub>2</sub>PIPz or NM<sub>3</sub>PIPz.

The most potent N-nitroso compounds, e.g. NMOR, NDEA, NPIP and BBN do not show a clear structural similarity, the same is valid for the least potent N-nitroso compounds NPIPz, NEBA, and NDELA. Thus, from this data a clear relationship between structure and carcinogenic potency is not evident.

### 3.1.2. SARs and QSARs

In recent years a limited number of studies have been performed on predicting the carcinogenic potency of N-nitroso compounds using chemical structures as a basis. In a mechanistic study by Zhao et al. (2005) the carcinogenic activity of nitroso compounds was correlated with the reactivity of these compounds at two sites in their molecular structure. Next to bioactivation by  $\alpha$ -hydroxylation or hydrolysis to form alkyldiazonium ions – the commonly accepted route for carcinogenesis – the formation of  $\beta$ - and  $\gamma$ -positioned esters as a key determinant for toxification was postulated (see Fig. 2). Based on the calculated lower activation energy of  $\beta$ -metabolites of methylethyl nitrosamine as a model compound, it was concluded that they display a higher reactivity than  $\gamma$ -metabolites. The ability of N-nitroso compounds to form reactive intermediates from  $\beta$ - or  $\gamma$ -metabolites may potentially provide a means to rank the carcinogenic potency of this compound class by means of their activation energies. However, the accuracy of such a correlation has not yet been described. Therefore the practical use of this study for the prediction of N-nitroso compound carcinogenic potency is unclear.

Other studies on the carcinogenic potency of N-nitroso compounds focus less on mechanistic aspects, and instead involve various methods to derive Quantitative Structure Activity Relationships (QSAR) and classification models. In QSAR studies, the *in vivo* carcinogenic potencies of N-nitroso compounds are correlated with their molecular properties (descriptors). This allows predictions regarding the carcinogenic potency of new structures. Classification models, on the other hand, merely provide a categorization of compounds into carcinogenic and non-carcinogenic

classes. The Carcinogenic Potency Database (CPDB)<sup>6</sup> is used as major data source for most studies. This database contains TD<sub>50</sub> values as a potency measure.

Support vector machines (SVM) and linear discriminant analysis (LDA) were used by Luan et al. (2005) to classify 148 N-nitroso compounds as carcinogenic or non-carcinogenic.<sup>7</sup> The dataset used in this study, with about 80% of compounds classified as carcinogenic, was randomly divided in a training (118) and test set (30). Equal percentages of non-carcinogenic entities were maintained in both sets. A wide range of molecular descriptors was calculated and used to generate both LDA and SVM prediction models. The most optimal LDA model with a total accuracy of 89.8% (test and training set) was obtained using seven descriptors. Using identical descriptors, a nonlinear SVM model was built providing an overall accuracy of 95.2%. Thus both methods gave satisfactory results in overall predictive power, with a slightly better performance by SVM. However, when zooming in on the non-carcinogenic compounds of the test set the predictive power was less: 71.4% and 57.1% for LDA and SVM respectively. For the test and training set combined, the predictivity of the LDA model for non-carcinogenic compounds (68.6%) was lower than that of SVM (84.4%).

In the most recent studies on building a classification model, Yuan and colleagues linked molecular descriptors to the carcinogenicity of N-nitroso compounds. The studies, focusing on overall (Yuan et al., 2011) and liver-specific (Yuan et al., 2012) tumorigenic potential in rats, follow a similar approach to Helguera et al. (2007, 2008a,b, 2010; see below) by incorporating molecular TOPS-MODE<sup>8</sup> and Abraham solute descriptors. Neither of the studies was restricted to a specific route of administration or gender. Thus, a larger dataset could be used. In the 2011 study, 95 carcinogenic and 16 non-carcinogenic compounds were used, whereas the liver-specific study entailed 32 hepatocarcinogenic, 60 carcinogenic non-hepatocarcinogenic and 16 non-carcinogenic nitroso compounds. Non-carcinogenic and non-hepatocarcinogenic compounds were combined into one class of “non-hepatocarcinogens”, the other class being the hepatocarcinogens. Both models were assessed by means of a test set (37 and 27 compounds, respectively) and by leave-one-out cross validation. Overall prediction accuracies of

<sup>6</sup> <http://potency.berkeley.edu/cpdb>.

<sup>7</sup> SVM is a method that constructs hyperplanes in a multidimensional space such that samples belonging to two different classes are separated in space. Any new sample is mapped in that space, and depending on which side of the plane it falls, belongs to one of the classes. LDA is a method to find a linear combination of features (descriptors) which characterizes or separates two or more classes of samples. The resulting combination may be used as a linear classifier to categorize new samples, in this case nitroso compounds.

<sup>8</sup> TOPS-MODE (TOPological Substructural MOlecular Design) measures the concentration of structural or physicochemical properties in regions of different sizes in the molecule.



respectively 90% (overall carcinogenicity) and 94% (hepatocarcinogenicity) were reported, with acceptable statistical quality. However, the predictive value for the non-(hepto)carcinogenic group was significantly lower. Reported values for the test and training set were 87.5% and 65% in the 2011 study, and 71.4% and 76% in the 2012 study.

Helguera et al. (2007) reported a QSAR study using the TOPS-MODE approach. Several multilinear regression models were built based on  $TD_{50}$  values of 35 nitroso compounds.<sup>9</sup> Only compounds orally administered to male rats via the gavage route were taken into account, as species differences and administration routes may impact the measured  $TD_{50}$ . The best model was obtained using 5 parameters, yielding a model able to explain 84% of the experimental variance (1 outlier: 1-(2-hydroxyethyl)-1-nitrosourea) and showed good statistical quality. An analogous model was subsequently generated for female rats, applying a dataset of 26 compounds, which accounted for more than 86% of the variance in the experimental activity (Helguera et al., 2008b). In two follow-up studies, the TOPS-MODE approach was further explored. Abraham solute descriptors were incorporated in the model to reflect the effect of solvent on the carcinogenicity of nitroso-compounds orally administered to male and female rats via drinking water (Helguera et al., 2008a, 2010). The resulting QSAR models (dataset of resp. 39 and 56 nitroso compounds) displayed a performance and quality similar to that of the first study reported. A nice add-on of the TOPS-MODE approach is the ability to identify quantitative contributions of substructures to the studied endpoint. Thus, properties such as the length of the alkyl chain and substitution patterns can be used as alerts for the carcinogenic potential of nitroso compounds.

The  $TD_{50}$  data from the 2007 Helguera study were extracted and extended with 12 other compounds with identical endpoint by Harju et al. (2010). Using 33 compounds as training set and the remaining 7 as test set, a QSAR model was built with ADMEWORKS™ using interactive multi-linear regression and the most relevant descriptors as assessed by a genetic algorithm.<sup>10</sup> The final model had a satisfactory overall performance of 82%. Further analysis of the dataset, however, showed an underrepresentation of low carcinogenic nitroso compounds, i.e. the  $TD_{50}$  values did not fulfill the requirements for a normal distribution of data. Recommendations were therefore made to rebuild the model once additional data become available to confirm the validity of the prediction model and used descriptors.

The subsequent report by this group (Harju et al., 2011) describes this effort. The structures (ChemIDplus) and  $TD_{50}$  data (CPDB) of 92 N-nitroso compounds were collected and curated. The harmonic mean of the most potent  $TD_{50}$  value of each available study was used as quantitative measure as opposed to the earlier used lowest  $TD_{50}$  value obtained from rats. The remaining set of 58 N-nitroso compounds all contained alkyl or aryl N-nitroso groups<sup>11</sup> as the only structural alert for mutagenicity and carcinogenicity, and are – as far as known – bioactivated by CYPs. For this data set a large number of physicochemical and structural descriptors was calculated with ADMEWORKS™. By using self-organizing maps, a neural network based on unsupervised learning, a training (41 nitrosamines) and test set (17 nitrosamines) were defined. The best descriptors for model building were derived by a variable selection procedure and subsequent Multiple Linear Regression. A maximum of 6 descriptors was allowed to prevent overfitting. The best

model had an  $R^2$  of 0.72 for the training set which falls within the acceptable limit. However, the model completely failed to predict the test set ( $Q^2 = -0.69$ ). This may be due to the heterogeneous nature of the historical  $TD_{50}$  data of the CPDB: non-standardized test protocols, different laboratories, and different application methods. Furthermore, it should be emphasized that the harmonic mean is based on all available positive studies, while in human risk assessment it is customary to use the valid study with the highest carcinogenic potency (which in this case would correspond to the lowest  $TD_{50}$ ).

In summary, the availability of a large, structurally diverse dataset with consistently measured biological data is essential for generating a prediction model with a broad ('global') applicability domain. Often such data are lacking. This is also true for N-nitroso compounds. Apart from a small, diverse dataset this compound class additionally suffers from a misbalance between carcinogenic and non-carcinogenic entities, and the fact that  $TD_{50}$  values derived from differing experimental setups are used. The latter includes differences in the organ-specificity of tumors but also the potential dependency of a measured endpoint on differences in species, sex and administration route. Hence, the approach taken by Helguera to generate separate prediction models. One can thus conclude that despite the apparent good quality of the generated models, it is difficult to assess how these models will perform for new compounds – especially non-carcinogenic ones. Regardless, it is clear that new predictions rely heavily on the application domain of the model. An approach other than striving for a global prediction model would be the development of a local model. This would entail inclusion of a single mechanism of action in model building as described by the Zhao study and the 2011 NILU report. None of the other QSAR studies described have explicitly taken the mechanism of action into account. The studied compounds do, however, have different molecular pathways to become carcinogenic. N-nitrosoureas undergo non-enzymatic decomposition: breakage of the amide bond generates unstable carbamic acid and carbonium ions, resulting in alkylation and carbomoylation reactions of various biological macromolecules including DNA (Faustino et al., 2005; Golding et al., 1997). N-nitrosamines on the other hand require biotransformation by cytochromes P450 (CYP) to exert their full carcinogenic effect. Thus, predictive model building may benefit from taking activation routes into account. However, as N-nitrosamine bioactivation involves a large battery of CYP isozymes, including CYPs 1A1, 1A2, 1B1, 2A6, 2C8, 2C19, 2E1, 3A4 and 3A5 (see e.g. Cooper and Porter, 2000; Duarte et al., 2005; Emmert et al., 2006; Fujita and Kamataki, 2001a,b; Kushida et al., 2000), a CYP-specific 3D-modeling approach, pursued for individual compounds by DeVore and Scott (2012), will probably not be feasible, also taking into account that some CYPs may be deactivating and that other xenobiotic metabolizing enzymes may be involved as well.

### 3.2. Approaches using mutagenicity or alternative carcinogenicity tests

#### 3.2.1. Introduction

Public literature was screened for papers on genotoxicity of N-nitroso compounds for which carcinogenicity data were acquired and for alternative carcinogenicity test results involving N-nitroso compounds. The papers retrieved were screened for their predictive potential with respect to N-nitroso compound carcinogenic potency.

#### 3.2.2. Bacterial mutation tests

One of the main aims of making this inventory, was to investigate whether it would be possible to predict carcinogenic potency of N-nitroso compounds based on mutagenic potency. Since our objective was to find a quantitative prediction of potency,

<sup>9</sup> Taken from the published peer-reviewed papers from public literature included in the CPDB, excluding the NTP studies also contained in the CPDB. The authors stated they aimed to test the developed models on these additional data. So far no such efforts have been reported in public literature.

<sup>10</sup> Genetic algorithms encompass a variety of search paradigms inspired by nature: selection and recombination operators are applied to evolve a solution (best combination of descriptors) to a problem (variance in  $TD_{50}$  value).

<sup>11</sup> SA\_21 from the Benigni-Bossa rulebase for mutagenicity and carcinogenicity.

the results of the available tests were expressed as much as possible in a quantitative measure of mutagenicity: number of revertants per  $\mu\text{mol}$  of N-nitroso compound exposure. When the papers publishing the results of these tests did not mention potency figures, it was attempted to calculate them from the published data. Most of the bacterial mutagenicity studies were executed with *S. typhimurium* strains, some with *Escherichia coli* strains. To avoid species based bias, only the *Salmonella* results were considered.

In Table 6 the N-nitroso compounds are ranked for their mutagenic potency. Unfortunately, only for a few N-nitroso compounds the required data are available, covering only a small range of mutagenic potencies. Most of the tests have been executed with some sort of metabolic activation (221 of the 239 tests). Conspicuous is the wide range of mutagenic potencies found for most N-nitroso compounds. This is a reflection of the variety of tests with respect to strain, and metabolic activation source (S9 mixes of rats induced with different drugs, bacteria transfected with various human CYPs and CYP reductase). Upon analysis of the combined data, no apparent relationship exists between mutagenic and carcinogenic potency, not even when contemplating broad potency classes (see Fig. 3). This may be in part due to the heterogeneous nature of the dataset.

Guttenplan (1987) has studied the relation between mutagenic potency of a series of nitrosamines tested with the *S. typhimurium* strain TA100 in the presence of S9 and carcinogenic potency classes attributed by Lijinsky (1984), based on a subjective judgment of relative potency. He did not find any relationship when the mutagenic potency was expressed as number of revertants per  $\mu\text{mol}$  nitrosamine. However, he did find a positive correlation between carcinogenic potency classes and mutagenic efficiency, defined by him as the number of revertants per  $\mu\text{mol}$  nitrogen produced in an assay designed to measure  $\alpha$ -hydroxylation of nitrosamines. However, neither measures showed any relationship with the carcinogenic potencies listed in Table 3.

Fujita and Kamataki (2001a, 2001b) have studied the relation between metabolic activation by various CYP isozymes and nitrosamine mutagenicity by transfecting *S. typhimurium* strain YG7108 with different human CYPs and CYP reductase. They have expressed mutagenic potency as the number of revertants per nmol nitrosamine per pmol CYP around the Minimum Effect Concentration of the nitrosamine. The nitrosamines investigated were NNK, NDEA, NPYR, NPIP, NMOR, NNN, NABS, NATB, NDBA, NDMA, NDPA, NEBA, NMBA, NMEA and NMPA. Nitrosamine mutagenic potency in the presence of human CYPs did not clearly correlate positively with their carcinogenic potency except for CYP3A4, with Pearson ( $r$ ) and Spearman ( $\rho$ ) correlation coefficients  $>0.6$ . However, even for this CYP, no clear separation between carcinogenic potency classes could be made based on the mutagenic potency of the nitrosamines in the YG7108 strain expressing it (see Fig. 4). Overall mutagenic potency parameters in this study did not correlate or even correlated negatively with carcinogenic potency (see Table 7).

Also using strain YG7108, the mutagenic potencies determined by Hakura et al. (2005) for the nitrosamines NDBA, NDEA, NDMA, NDPA and NMOR in the presence of human or rat S9 mix did not show a positive correlation with their carcinogenic potency ( $r = -0.59$  to  $-0.12$ ,  $\rho = -0.9$  to  $-0.3$ ). Also the mutagenic potencies derived by Cooper and Porter, 2000 for NDBA, NDEA, NDMA and NDPA using strains YG7104 and YG7108 and human CYP2E1 showed a negative correlation with carcinogenic potency ( $r = -0.49$  and  $-0.39$ , respectively,  $\rho = -0.8$  and  $-0.4$ , respectively). To conclude, Wagner et al. (2012) determined mutagenic potencies for NDMA, NMOR, NPIP and NPYR using strain YG7108 and rat S9 and did not find a correlation with carcinogenic potency ( $r = -0.16$ ,  $\rho = 0.2$ ).

Summarizing, mutagenic potencies derived from Ames tests do not seem to correlate well with N-nitroso compound carcinogenic potency. However, the present test set of N-nitroso compounds shows a narrow range of carcinogenic potencies (all within one order of magnitude), while mutagenic potencies vary up to three orders of magnitude. Therefore, testing with a good number of N-nitroso compounds representing a broader range of carcinogenic potencies could yield better results when using a well-defined and consistent AMES testing protocol.

### 3.2.3. In vitro mammalian mutation tests

Only one systematic exploration of *in vitro* mammalian cell mutagenicity systems for the prediction of carcinogenic potency of N-nitroso compounds has been traced. Jones et al. (1981) described the relationship between carcinogenicity and mutagenicity measured in Chinese hamster V79 cells co-cultivated with primary rat hepatocytes. They found a good correlation between the carcinogenic potency index<sup>12</sup> and the mutagenic potency index<sup>13</sup> for the 6-thioguanine marker ( $r = 0.85$ ). They used a dataset of 26 N-nitroso compounds, most of which have a potency deviating at most a factor 2.7 from the linear regression line determined, with the notable exception of NDMA, which had circa 7 times higher carcinogenic potency index. The mutagenic potency derived by Jones et al. (1981) was plotted against the more commonly used carcinogenic potency we have calculated from oral rat studies and expressed as  $I_{\text{conc}}$  in  $(\text{mmol}/\text{m}^3)^{-1}$ . No correlation between the two parameters was observed (see Fig. 5,  $r = -0.06$  and  $\rho = 0.17$ ).

Another dataset was published by Bradley et al. (1981) and also contained a number of N-nitroso compounds. Various study designs were used, with different selective agents (8-azaguanine, 6-thioguanine, ouabain) or none and different activation systems (microsomes/S9, hepatocytes) or none. The mutagenic potency ( $1/D_{10}$  in  $\text{nM}^{-1}$ ) of the most frequently used marker, 8-azaguanine was plotted against the carcinogenic potency we have calculated from oral rat studies. As with the Jones study, no apparent relationship was observed (see Fig. 6).

The results of neither study could be used in an obvious way to discriminate between carcinogenic potency classes. This lack of correlation may be due to interlaboratory variation, since potencies for the same mutagen may vary between 30 and 60-fold (based on  $n = 2$ ) (Bradley et al., 1981). However, N-nitroso compounds with approximately the same carcinogenic potency showed a variation in mutagenic potency over 3 orders of magnitude. Therefore, interlaboratory variation cannot be the only explanation. Concluding, in the form as reported by Bradley et al. (1981) and Jones et al. (1981), this test appears not to be able to predict carcinogenic potency of N-nitroso compounds.

### 3.2.4. In vitro transformation assays

Dunkel et al. (1981) compared the neoplastic transformation responses to chemical carcinogens of BALB/c 3T3 cells, Syrian Hamster Embryo Cells (SHE) and Rauscher Murine Leukemia Virus infected Fischer 344 Rat Embryo cells (RMLV-FRE). The authors included a number of N-nitroso compounds in their evaluation: MNNG, NDEA, NDMA, NDPhA and NEPhA. The compounds were tested without bioactivation. The test with RMLV-FRE only gives qualitative results, and can therefore not be used for quantitative prediction. The results with the other two tests are shown in Table 8. Without bioactivation even the qualitative performance of the two transformation tests for the selected N-nitroso compounds

<sup>12</sup> Defined as  $\ln(1 + \frac{100}{D \times T})$ , in which  $D$  is the total dose in mol/rat and  $T$  is the time (weeks) for death of 50% of the animals from tumours.

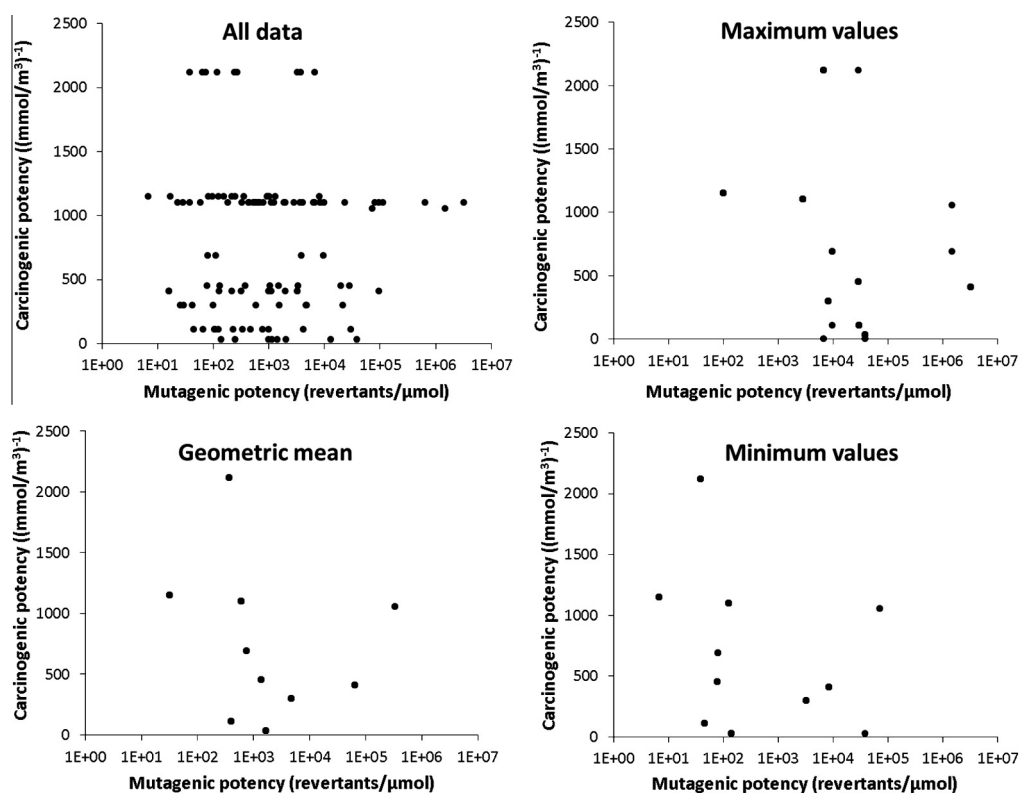
<sup>13</sup> Defined as  $1/D_{10}$ , in which  $D_{10}$  was defined as the concentration in nM of the compound that yielded mutant frequencies 10 times higher than the spontaneous mutant frequency.

**Table 6**Ames tests with nitrosamines of known carcinogenic potency for which mutagenic potency could be expressed in revertants/ $\mu\text{mol}$ .

Nitrosamine	Number of tests	Mutagenic potency (rev/ $\mu\text{mol}$ )			Carcinogenic potency ([ $\text{mmol}/\text{m}^3$ ] $^{-1}$ )	
		Range	Geometric mean	Ranking		Ranking
<b>NDEA</b>	53	330–110,000	2200	1	1100	3
NEBA	11	140–38,000	1700	2	30	9
NMEA	11	79–29,000	1400	3	452	5
<b>NPIP</b>	17	3900–9600	760	4	687	4
NDPA	21	220–96,000	620	5	409	6
<b>NDMA</b>	26	43–22,000	490	6	297	7
NPYR	17	110–30,000	400	7	108	8
<b>NMOR</b>	16	240–6700	370	8	2118	1
NDBA	17	97–8200	250	9	1150	2

The CCS nitrosamines and nitramines are printed in bold face.

The studies on which the mutagenic potencies are based, are summarized and referenced in [Appendix A of the supplemental material](#). The derivation of the carcinogenic potency values and the studies on which they are based are described and referenced in [Appendix B of the supplemental material](#).

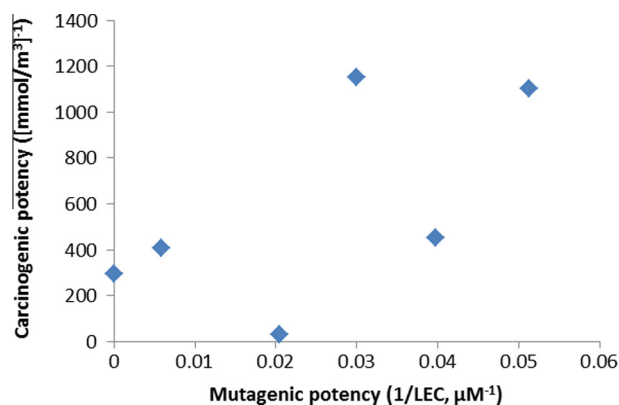


**Fig. 3.** Plot of mutagenic potency of N-nitroso compounds in bacterial reverse mutation tests against their carcinogenic potency. All data represent the mutagenic potency data of every Ames test of each N-nitroso compound we retrieved from public literature. The references to these studies are given in [Table 9 of Appendix A of the supplemental material](#). Maximum and minimum values and geometric mean refer to the mutagenic potencies of the individual N-nitroso compounds. Carcinogenic potency data are the oral values listed in [Table 3](#) of this paper.

is poor, so they certainly are not useful in a quantitative prediction. NDEA was also tested in the presence of hepatocytes and found to be positive ([Dunkel et al., 1981](#)). However, in this form the test will probably not be useful in quantitative predications because of its bad resolution: the maximum response was 4 transformed colonies in 664 surviving colonies for a concentration of 2.5 mg/mL of this potent carcinogen (at the next concentration, 5 mg/mL, survival dropped from approximately 100% to approximately 10%).

The Detailed Review Paper (DRP) number 31 of the Organization for Economic Cooperation and Development ([OECD, 2007](#)) analysed the performance of three models used in Cell Transformation Assays (CTAs) to screen the carcinogenic potential of chemicals: the Syrian hamster embryo (SHE) cells, and the mouse cell lines BALB/c 3T3 and C3H/10T $^{1/2}$  ([OECD, 2007](#);

[Vasseur and Lasne, 2012](#)). The report compared CTA results collected from public literature to results from recent genotoxicity tests using mammalian and non-mammalian cell systems. The performance of the CTAs in predicting carcinogenic potential has been established for several hundreds of chemicals, comprising organic and inorganic substances. In this report ([OECD, 2007](#)) several N-nitroso compounds are listed. Unfortunately the report only published the categorical conclusion for each test result taken from public literature. Therefore, the source data of the N-nitroso compound results published in the OECD report were analyzed. Most of the original publications retrieved reported either the number of foci induced per culture dish or flask or the percentage of transformed cells or both. For each concentration reported the number of foci/dish (flask) or % cells transformed per mM concentration



**Fig. 4.** Relationship between mutagenic potency in the *S. typhimurium* strain YG7108 hCYP3A4 and carcinogenic potency of N-nitroso compounds. The mutagenic potency is expressed as 1 divided by the Lowest Effect Concentration (LEC), and the data are derived from Fujita and Kamataki (2001a, 2001b). The carcinogenic potency data are the oral values listed in Table 3 of this paper.

were calculated. The maximum of these values calculated for each tested N-nitroso compound per cell line in one publication was taken as a measure of its transformation potency as measured in that publication. When more than one set of testing conditions were used, only the “standard conditions” were considered. The values thus calculated are listed in Table 11 of Appendix B.

Most of the transformation assays were executed without metabolic activation, a few assays did include metabolic activation in some form. For one publication hepatocytes had been added to the exposed cells, for another S9 fraction, while two publications used a cell line clone expressing human CYP2A6. On average, there is a very good correlation between the transformation potency of the various N-nitroso compounds in the three assays ( $r = 0.98$ – $1.00$ ). Furthermore, it is apparent that MNNG shows the highest transformation potency in the three transformation assays, much higher than would be expected based on its carcinogenic potency: it is number four in carcinogenic potency of the 8 N-nitroso compounds tested, with a potency ten times lower than that of number 1 (ENU), while in transformation potency it is number 1 with a potency 10 times higher than number 2 (NDMA). MNNG is the most tested N-nitroso compound in the three CTA assays, since it is often used as a positive control. Therefore, MNNG was used as

**Table 7**  
Correlation between mutagenic potency and carcinogenic potency of selected nitrosamines.

Parameter	Corr. coeff.	
	$r$	$\rho$
CYP1A1	−0.29	+0.09
CYP1A2	−0.46	+0.14
CYP1B1	−0.39	−0.20
CYP2A6	−0.10	+0.26
CYP2C8	+0.11	+0.26
CYP2C9	−0.10	+0.59
CYP2C19	+0.10	+0.45
CYP2D6	−0.13	+0.13
CYP2E1	+0.04	−0.09
CYP3A4	+0.62	+0.66
CYP3A5	−0.13	+0.13
Maximum mutagenic potency <sup>a</sup>	−0.01	+0.09
Average mutagenic potency <sup>a</sup>	−0.12	−0.14
Geometric mean mutagenic potency <sup>a</sup>	−0.44	−0.37

The mutagenic potencies are derived from Fujita and Kamataki (2001a, 2001b), the derivation of the carcinogenic potency values and the studies on which they are based, are described and referenced in Appendix B of the supplemental material.

<sup>a</sup> Calculated as 1/Lowest Effect Concentration (negatives set to 0).

a yard stick of transformation potency in order to analyze the results of the tested N-nitroso compounds across the three assays, expressing their transformation potency in each assay relative to the average potency of MNNG in that assay (see Table 9).

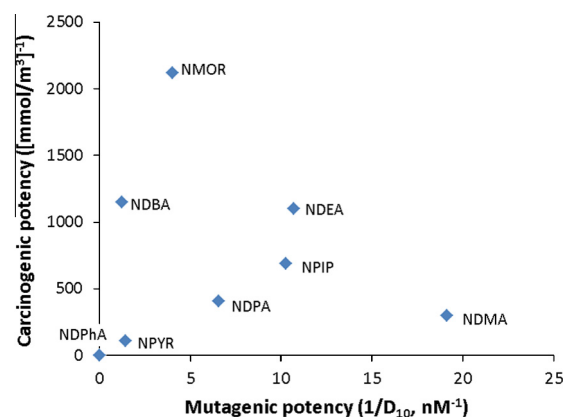
The mean relative potency of the tested N-nitroso compounds over all three assays executed without metabolic activation does not correlate at all with their carcinogenic potency ( $r = -0.23$  and  $\rho = -0.10$ ). However, in spite of the diverse nature of the metabolic activation employed, a good association was observed for the relative potencies determined in the presence of metabolic activation ( $r = 0.95$  and  $\rho = 0.8$ ; see Fig. 7). Since the number of N-nitroso compounds is rather low ( $n = 4$ ) and the degree of association is mainly determined by one data point (NNK), more N-nitroso compounds need to be tested in a cell transformation assay (CTA) in the presence of metabolic activation in order to pass a definitive judgment.

Kowalski et al. (2000) evaluated an *in vitro* transformation test for predicting the rodent carcinogenicity  $TD_{50}$  (taken from the CPDB) against a testing database of 64 chemicals including both genotoxic and non-genotoxic carcinogens and carcinogens that normally require addition of a S-9 microsomal fraction for detection in the bacterial mutagenicity assay. The assay uses focus formation in a stable, bovine papillomavirus type 1 (BPV-1) DNA carrying C3H/10T½-mouse embryo fibroblast cell line (T1). Ninety-two percent of the compounds were correctly predicted as carcinogens, promoters, or non-carcinogens (NC). The authors listed the lowest transforming dose in the assay for the positive substances. Two N-nitroso compounds were mentioned: NDEA and MNNG, with lowest transforming doses of 0.05 mg/mL and 0.01 μg/mL, respectively. However, this clearly is not in line with the observed carcinogenic potencies of the CPDB: i.e. the  $TD_{50}$  in the rat for MNNG is 0.803 mg/kg bw/day, while for NDEA it is 0.0265 mg/kg bw/day. It confirms the remarkable potency of MNNG in cell transformation assays already noted in the assays with cell lines not transfected with viral genes (see above).

### 3.2.5. *In vivo* initiation assay

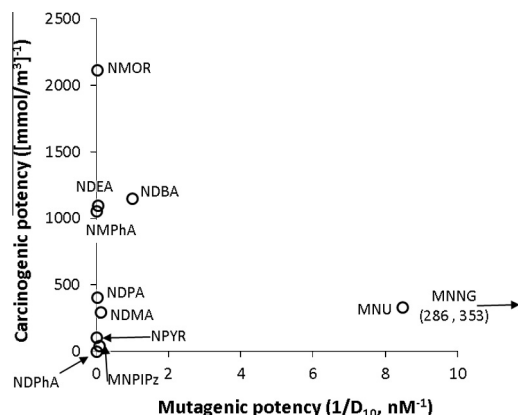
Only few studies were identified investigating the relation between *in vivo* mutagenic potency and carcinogenic potency.

Sakai et al. (2002) investigated the initiating activities of 26 chemicals by single intragastric administration in an *in vivo* 5 week initiation model in male Fischer 344 rats by evaluation of the induction of glutathione S-transferase placental form (GST-P)



**Fig. 5.** Relationship between mutagenic potency of N-nitroso compounds in Chinese hamster V79 cells using thioguanine as marker gene and their carcinogenic potency. The mutagenic potency is expressed as 1 divided by the concentration in nM that yields mutant frequencies 10 times higher than the spontaneous mutant frequency ( $D_{10}$ ). These mutagenic potency data are derived from Jones et al. (1981). The carcinogenic potency data are the oral values listed in Table 3 of this paper.





**Fig. 6.** Relationship between mutagenic potency of N-nitroso compounds in Chinese hamster V79 cells using 8-azaguanine as marker gene and their carcinogenic potency. The mutagenic potency is expressed as 1 divided by the concentration in nM that yields mutant frequencies 10 times higher than the spontaneous mutant frequency ( $D_{10}$ ). These mutagenic potency data are derived from Bradley et al. (1981). The carcinogenic potency data are the oral values listed in Table 3 of this paper.

positive foci in the liver as end-point marker for hepatocarcinogenesis. Among the substances tested were NDEA, NDMA, NDiPLA, BBN, MNNG and MNU. The test was able to correctly identify genotoxic and non-genotoxic carcinogens as well as mutagenic non-carcinogens. Some association was found between the number of induced foci and the carcinogenic potency of these N-nitroso compounds ( $r = 0.68$ ,  $\rho = 0.20$ ), but which appears to strongly depend on one data point (see Fig. 8 and Table 10). However, when only considering those N-nitroso compounds which principally target the liver (all nitrosamines), a very good correlation is observed. The number of data points ( $n = 3$ ) is too small to draw definitive conclusions, but this test does seem to hold promise for a quantitative prediction, especially for nitrosamines targeting the liver.

Also Fukushima et al. (2005) studied induction of liver lesions in male F344 rats by NDMA provided in a wide range of dose levels in drinking water, i.e. from 0.001 to 10 ppm (c. 0.142–1420  $\mu\text{g}/\text{kg}$  bw/day), for a period of 16 weeks. A dose related and statistically significant increase of GST-P-positive foci, in the 1 and 10 ppm dose groups (c. 142 and 1420  $\mu\text{g}/\text{kg}$  bw/day, respectively) was observed, corresponding to a relative increase of 3460 at the highest dose. This relative increase is circa 10 times higher than observed for NDMA by Sakai et al. (2002), which is probably related to the difference in dosing route and regime (single gavage of a relatively high dose (5 mg/kg bw) versus 16 week repeated administration via the drinking water). No increment in foci could be detected with the lower doses (0.001, 0.01, and 0.1 ppm (c. 0.142, 1.42 and 14.2  $\mu\text{g}/\text{kg}$  bw/day, respectively)).

### 3.2.6. *In vivo* mutation assays

Sanner and Dybing (2005) have compared the lowest effective dose (LED) giving a response in an *in vivo* genotoxic test after oral or inhalation exposure with the carcinogenic dose descriptor  $T_{25}$ . The authors used a variety of *in vivo* genotoxicity assays: micronucleus, sister chromatid exchange, DNA adducts, chromosomal aberrations and Comet. The 34 genotoxic carcinogens in this analysis represented different classes of carcinogens and different genotoxic endpoints, exhibiting carcinogenic and mutagenic potencies covering four orders of magnitude, including one nitrosamine: NDELA. A linear correlation was found between the log of the lowest effective dose (LED) for *in vivo* genotoxicity after oral administration or inhalation exposure and the log of the lowest dose descriptor  $T_{25}$

**Table 8**

Carcinogenicity prediction by two transformation assays (data from (Dunkel et al., 1981)).

N-nitroso compound	Balb/3T3	SHE	Carcinogenic potency ( $[\text{mmol}/\text{m}^3]^{-1}$ ) <sup>a</sup>
NDEA	±	–	1100
MNNG	+	+	350
NDMA	+	–	300
NDPhA	–	±	1

<sup>a</sup> Based on oral rat carcinogenicity studies (see Table 5).

for tumor formation ( $r = 0.84$ ). Including 8 non-genotoxic substances in the analyses did not substantially change the degree of correlation ( $r = 0.82$ ). The median of the ratio LED/ $T_{25}$  was 1.05% and for 90% of the substances this ratio fell in the range of 0.21–9.2, which shows that the numerical value of the LED is similar to the numerical value of the  $T_{25}$  within 1.5 orders of magnitude.

Hernandez et al. (2011) performed a literature search for dose–response data in various *in vivo* genotoxicity and carcinogenicity studies. They applied the benchmark dose (BMD) approach using the dose–response modeling program PROAST on dose–response data from 18 compounds in the micronucleus assay (MN) and the *in vivo* transgenic rodent mutation assay (TGM). They compared their BMD<sub>10</sub> values to the BMD<sub>10</sub> from carcinogenicity studies in mice. The dataset contained 18 mostly genotoxic<sup>14</sup> carcinogenic compounds with potencies in the mouse spanning 4 orders of magnitude, including two N-nitroso compounds: MNU and NDMA. The authors observed a positive correlation between the logarithm of the lowest BMD<sub>10</sub> from the genotoxicity tests (MN and TGM) and the logarithm of the tissue-matched<sup>15</sup> carcinogenicity BMD<sub>10</sub> ( $r = 0.54$  and  $0.59$ , respectively). However, the correlation between the lowest overall genotoxicity BMD<sub>10</sub> and the lowest tumor BMD<sub>10</sub> was poor ( $r = 0.43$ ) and the correlation between the lowest TGM BMD<sub>10</sub> and the lowest tumor BMD<sub>10</sub> was even poorer ( $r = 0.22$ ). Since in risk assessment carcinogenic risk values are usually based on the tumors that appear in the highest frequency per unit dose, this means the evaluated tests cannot be used to predict carcinogenic potency thus derived, based on the available data. The authors blame this on the lack of proper dose–response studies due to the focus on qualitative hazard identification. Another cause could be the variety in species/strains treated, route of administration, exposure duration, tissues examined and genes used.

Of all the *in vivo* mutagenicity data for N-nitroso compounds that could be collected, only the *in vivo* transgenic rodent mutation assay (TGM) yielded relatively sufficient data for a quantitative analysis.

When plotting the maximum mutagenic potency value against the oral carcinogenic potency of the N-nitroso compounds a reasonable correlation is observed ( $r = 0.74$  and  $\rho = 0.43$ , see Fig. 9). However, it all depends on one data point for ENU and no obvious carcinogenic potency classes could be discriminated using mutagenic potency determined in lacZ transgenic mice. On the other hand, the tobacco-specific nitrosamine NNK seems to be an outlier. When this data point is removed, the correlation improves dramatically, but still is dominated by one data point. However, without a good mechanistic reason for the exclusion of NNK, its removal remains arbitrary.

Concluding, potentially *in vivo* mutagenic potency may predict the oral carcinogenic potency of N-nitroso compounds. However, the database needs to be increased considerably using a uniform study protocol in order to verify this.

<sup>14</sup> Except perhaps benzene and dichloroacetic acid.

<sup>15</sup> Meaning genotoxic potency and carcinogenic potency were compared for identical tissues only.

**Table 9**  
Transformation potencies of N-nitroso compounds.

Met Act	Compound	Cell lines						Primary cells		
		Balb/c 3T3			C3H/10T½			SHE		
		n	Mean transformation potency <sup>a</sup>	Relative transformation potency	n	Mean transformation potency <sup>a</sup>	Relative transformation potency	n	Mean transformation potency <sup>b</sup>	Relative transformation potency <sup>c</sup>
No	ENU	2	$1.4 \times 10^0$	$3.4 \times 10^{-4}$	1	$3.5 \times 10^0$	$4.7 \times 10^{-2}$	1	$2.9 \times 10^{-1}$	$4.0 \times 10^{-4}$
	MNNG	6	$4.2 \times 10^3$	$1.0 \times 10^0$	3	$7.5 \times 10^1$	$1.0 \times 10^0$	4	$7.2 \times 10^2$	$1.0E \times 10^0$
	MNU	2	$8.1 \times 10^0$	$1.9 \times 10^{-3}$	1	$1.9 \times 10^{-2}$	$2.5 \times 10^{-4}$	1	$2.4 \times 10^1$	$3.3 \times 10^{-2}$
	NDEA	2	$6.5 \times 10^0$	$1.5 \times 10^{-3}$	2	$7.7 \times 10^{-2}$	$1.0 \times 10^{-3}$	2	$1.1 \times 10^{-1}$	$1.5 \times 10^{-4}$
	NDELA	1	$4.8 \times 10^{-2}$	$1.1 \times 10^{-5}$						
	NDMA	3	$5.3 \times 10^1$	$1.3 \times 10^{-2}$				1	$1.4 \times 10^2$	$2.0 \times 10^{-1}$
	NDPhA	1	$2.0 \times 10^2$	$4.7 \times 10^{-2}$	1	$1.1 \times 10^0$	$1.7 \times 10^{-2}$	1	$0.0 \times 10^0$	$0.0 \times 10^0$
	NEPhA	1	$9.4 \times 10^1$	$2.2 \times 10^{-2}$				1	$1.1 \times 10^2$	$1.5 \times 10^{-1}$
	NNK				2	$1.2 \times 10^{-1}$	$1.7 \times 10^{-3}$			
	Total		18			11			11	
Yes	MNU	1	$1.6 \times 10^0$	$3.8 \times 10^{-4}$						
	NDEA				1	$1.8 \times 10^{-1}$	$2.4 \times 10^{-3}$			
	NDMA	1	$8.4 \times 10^{-1}$	$2.0 \times 10^{-4}$	1	$3.3 \times 10^{-1}$	$4.5 \times 10^{-3}$			
	NNK				2	$5.6 \times 10^{-1}$	$7.5 \times 10^{-3}$			
Total		2			4					

Source data listed in Table 11 of Appendix A of the supplemental material. The transformation potency in a study is the maximum of the values calculated for each concentration tested. The mean is the average of the transformation potencies of all studies available for a particular cell line and nitrosamine.

<sup>a</sup> Number of foci/dish (flask)/mM.

<sup>b</sup> % Transformation/mM.

<sup>c</sup> The mean transformation potency in a cell line divided by the mean transformation potency of MNNG in that cell line; n = number of studies.

### 3.2.7. Summary of predictive performance of mutagenicity or alternative carcinogenicity tests

The results of some of the mutagenicity and alternative carcinogenicity tests discussed in the previous sections show a reasonable correlation with carcinogenic potency based on oral rat carcinogenicity studies. These tests are the combination of three *in vitro* cell transformation assays with the BALB/c 3T3, the SHE or the C3H/10T½ cell line executed in the presence of metabolic activation system, the *in vivo* GST-P assay and the *in vivo* lacZ mutation assay in transgenic mice. However, as shown in Table 11, the available N-nitroso compound datasets for these tests are too small to draw definitive conclusions.

We have also tested the predictive performance of the tests discussed in the previous sections against N-nitroso compounds potency data from the CPDB (inverse harmonic minimum TD<sub>50</sub>'s for oral rat carcinogenicity studies), but this did not lead to significantly better or worse predictions (data not shown).

## 4. Discussion

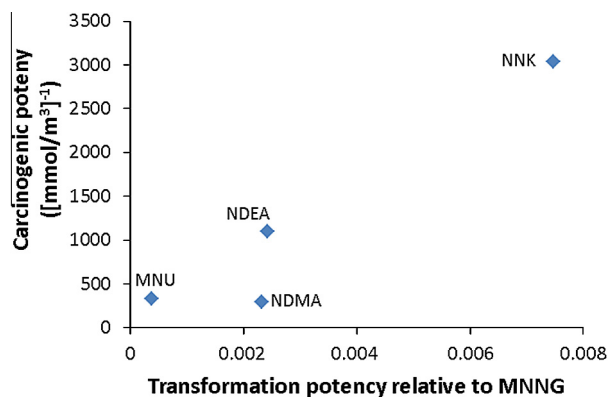
Based on the available evidence, genotoxic carcinogenicity is the leading health effect for both the CCS nitrosamines as well as the CCS nitramines. All CCS nitrosamines and nitramines, except NPIPz, are genotoxic. Every CCS nitrosamines and nitramines that was tested for carcinogenicity is carcinogenic, even NPIPz. Most N-nitroso compounds and nitramines have a carcinogenic potency within two orders of magnitude of each other in oral studies. Notable exceptions are NDPhA, which is more than 4 orders of magnitude less potent than the most potent N-nitroso compound NNK, and NPIPz which is more than 3 orders of magnitude less potent.

Many nitrosamines and also the nitramine DMA-NO<sub>2</sub> have the liver as main target organ for carcinogenicity in oral studies. Among the CCS substances the exceptions are the piperazines DNPIPz and NPIPz and the nitramine MA-NO<sub>2</sub>, which is most potent in the CNS, although it does target the liver as well. The tested piperazines do not target the liver, and most of them induce nasal cavity tumors. For some other nitrosamines the esophagus is the main target: NMPA, NMBA, and NEBA. In general, tissues at

the portals of entry, such as the oral cavity, esophagus, forestomach (after oral exposure), and the nasal cavity, trachea, and lung (after inhalation) appear at risk as well. However, the only more or less clear trends discernible from these carcinogenicity data are the tendency to induce nasal cavity tumors for nitrosopiperazines and bladder tumors for those (hydroxy)butyl nitrosamines that proved carcinogenic under the test conditions.

Based on the process chemistry involved in the CCS systems, the general population is potentially exposed via the environment through inhalation (de Koeijer et al., 2013). Although no data on workplace exposure are available, also occupational exposure to nitramines and nitrosamines as by-products of the CCS process may occur via this route (Gentry et al., 2013). For most CCS nitrosamines, as well as for most other N-nitroso compounds, only oral studies are available. That raises the question of how reliable extrapolation from oral to respiratory cancer values is. At present only for two CCS nitrosamines and one other nitrosamine oral as well as respiratory studies are available: NDMA, NMOR and NMPIPz.

The respiratory cancer risk value of NDMA derived from oral studies is circa 14× lower than the one directly derived from respiratory studies, and also the critical target organ is different: the liver after oral exposure and the olfactory mucosa after respiratory exposure. The epidemiological evidence by Straif et al. (2000) suggests that the cancer risk value derived from oral rat studies is closer to the real human respiratory cancer risk of NDMA than the one derived from the respiratory rat studies: Carrier et al. (2011) reanalyzed the data presented by Straif et al. (2000) and found that the threshold inducing an excess risk of mortality from cancers associated with exposure to nitrosamines for an average exposure period of approximately 10 years in a German cohort, corresponds to an average concentration between 2.5 µg/m<sup>3</sup> and 15 µg/m<sup>3</sup> of total nitrosamines (NDMA + NMOR). Overall, exposure to NDMA and NMOR was about equally high. Based on the NDMA carcinogenic potency value of 57 (mg/m<sup>3</sup>)<sup>-1</sup> derived from the respiratory rat carcinogenicity study, one would expect an excess cancer mortality of 57/1000 × 2.5/2 × 10/40 × 100 = 1.8% cancer deaths already at the lower threshold concentration of 2.5 µg/m due to NDMA exposure alone, while based on the NDMA potency of 4 (mg/m<sup>3</sup>)<sup>-1</sup> derived from the oral rat study one would expect an excess



**Fig. 7.** Correlation between relative transformation potency in the presence of metabolic activation and carcinogenic potency of some N-nitroso compounds. The relative transformation potency data are taken from Table 9 and the carcinogenic potency data are the oral values listed in Table 3.

mortality of 0.15%. Furthermore, in this epidemiological study the most common nitrosamine induced cancers are upper gastrointestinal tract (oesophageal and oral) cancers. Therefore, NDMA-induced local tumors in the nose observed in respiratory carcinogenicity studies in rats may not be relevant for humans.

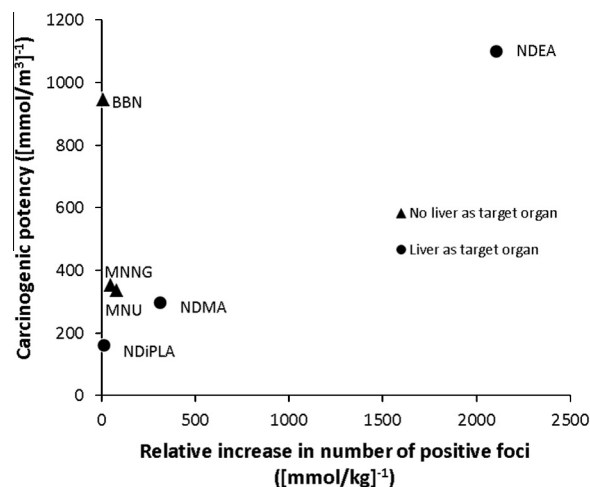
For NMOR both routes of exposure are more in line with each other: the critical target organ is the same, the liver, and the predicted human respiratory cancer risk values are in the same order of magnitude, the one derived from the oral studies being 5× higher than the one derived from the rat respiratory study.

NMPIPz targets the nasal cavity in the rat, both after oral and respiratory administration: via the latter route, where this target site constitutes the portal of entry, it is ten times as potent as the extrapolation from the oral route suggests (see Table 3).

Concluding, for one nitrosamine the oral rat study seems to be more predictive of the human respiratory cancer potency than the respiratory study (NDMA), for another there is reasonable agreement between the oral and respiratory rat study with respect to carcinogenic potency (NMOR) while for the third the oral rat study underestimates carcinogenic potency via the respiratory route (NMPIPz). Overall, based on this limited and somewhat contradictory evidence for these three nitrosamines, there is not sufficient evidence to depart from the simple absorption based oral to respiratory route extrapolation for systemic toxic effects.

N-nitroso compounds may differ quite substantially in carcinogenic potency (see Fig. 10): the potency range for the group described here stretches more than 3 orders of magnitude. Some of the presently negatively tested nitrosamines may turn out to be very low-potent carcinogens after all (even weaker than the weakest one identified here), when tested in regulatorily acceptable testing protocols. Also apparent from the data is that relatively small changes in molecular structure may result in quite different target organs for carcinogenicity, or in substantial potency changes.

Most of the available carcinogenicity data come from studies in which one or just a few of the substances presently investigated were tested. The majority of these studies were not designed for specifically estimating cancer potency of the investigated compound, but merely to identify its carcinogenic properties. Therefore, only few of these studies meet guideline-standards for estimating potency, and many suffer from shortcomings in this respect, such as an insufficient number of animals used, the absence of concurrent controls, a too short period of exposure and/or observation, testing (far) beyond the maximum tolerable dose, and incomplete toxicological examinations (including histopathological detail) and/or reporting (for references and more



**Fig. 8.** Relation between carcinogenic potency and increase in GST-P positive foci. The GST-P data are derived from Sakai et al. (2002) and are listed in Table 10 and the carcinogenic potency data are the oral values listed in Table 3.

details see Appendix B of the supplemental material). The following substances have rat oral cancer risk values based on appropriate studies: NDMA (Peto et al., 1991a,b), NDEA (Peto et al., 1991a,b), NMEA (Lijinsky and Reuber, 1980), DNPIPz (taking into account the study by Lijinsky and Taylor (1975) and the study by Takano et al. (1982), when combining its different exposure/observation time groups), NDPhA (Cardy et al., 1979) and ENU (Maekawa et al., 1984). NDELA has an appropriate oral study on male rats only (Berger et al., 1987), NMOR has an appropriate oral study only including female rats (Lijinsky et al., 1988). All the other studies were less appropriate. Also the few available inhalation studies by Klein et al. (for NDMA (1991), NMOR (1990) and NMPIPz (1999)) were less fit for potency estimation, especially with respect to the number of doses and the number of animals tested. The few available dermal studies (NDEA: Iversen (1980) and Hoffmann and Graffi (1964); NDELA: Hoffman et al. (1983); NDPhA: Iversen (1980)) are not suitable for deriving cancer risk values. Clearly, one should recognize that most of the available studies are less appropriate for potency estimation, and that this may hamper the establishment of firm conclusions.

There are only two nitramines for which we could allocate carcinogenicity data, MA-NO<sub>2</sub>, and DMA-NO<sub>2</sub>, whose potency was in the same order of magnitude. On this basis it is not possible to validate any prediction model for this class of compounds. For N-nitroso compounds, on the other hand, carcinogenicity data are available for a substantial number of substances, covering a potency range of well over 3 orders of magnitude, including some very weak, if not non-carcinogenic compounds. Therefore, predictive models for this chemical group could be evaluated, as summarized below.

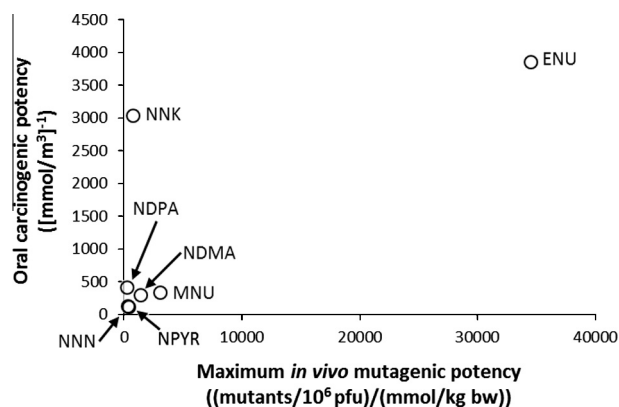
With regard to *in silico* prediction models, both classification models as well as QSARs have been developed, all using the CPDB as the major source of *in vivo* carcinogenicity data. The reported sensitivity of the classification models is rather good, but their specificity is quite poor. This latter result was to be expected given the limited number of non-carcinogenic N-nitroso compounds. Additionally these 'non-carcinogenic' N-nitroso compounds may in fact be very low-potent carcinogens as the performed tests are not adequate to definitively classify compounds as 'non-carcinogenic'. The QSAR models developed by the groups of Helguera et al. (2007, 2008a, 2008b, 2010) and Harju et al. (2010), respectively, showed a good predictive performance when the lowest TD<sub>50</sub> values in the CPDB were selected as measure of carcinogenic potency. They have attempted to reduce bias by

**Table 10**  
Results initiation assay with N-nitroso compounds.

Nitrosamine	Dose (mg/kg)	Carcinogenicity target organ(s)	Test group		Control group		Potency (relative increase in no. of foci/(mmol/kg))
			n	No. of foci (Mean ± SD)	n	No. of foci Mean ± SD	
BBN	450	Bladder	6	29.5 ± 5.7	18	1.8 ± 1.5	6.3
NDEA	10	Liver	12	123.6 ± 30.8	9	0.6 ± 0.7	2104.1
NDMA	5	Liver	10	46.1 ± 21.1	19	2.2 ± 2.3	310.5
NDiPLA	500	Liver, kidney	16	75.5 ± 26.8	18	1.8 ± 1.5	13.6
MNU	35	Stomach	12	43.0 ± 22.6	20	1.6 ± 2.0	79.2
MNNG	80	Stomach	12	41.1 ± 16.4	20	1.6 ± 2.0	47.2

n = number of animals in group.

The potency values are calculated by the authors of this paper by dividing the number of foci observed by Sakai et al. by the administered doses (converted to mmol/kg).



**Fig. 9.** Correlation between *in vivo* gene mutation in the transgenic rodent mutation assay and carcinogenic potency of N-nitroso compounds. pfu = plaque forming unit. The transgenic rodent mutation assay (TGM) data are listed in Table 8 of Appendix A of the supplemental material. For each N-nitroso compound the maximum of the potency values over all available studies is used. The carcinogenic potency data are the oral values listed in Table 3.

limiting the carcinogenicity data to specific study designs and protocols, such as oral gavage carcinogenicity studies with male rats. As their models were not validated with an external validation set, predictions for non-tested N-nitroso compounds are still to be interpreted with caution. In a subsequently developed QSAR model by NILU (Harju et al., 2011), the harmonic mean of the most potent TD<sub>50</sub> values in rats was taken as measure of carcinogenic potency, and just N-nitroso compounds with only their trade-mark structural alert of the N-nitroso group were selected. This model satisfactorily predicted the potency of the training set compounds, but was non-predictive for a predefined test set. Thus, the confined QSAR model of Helguera et al. (2008a) appears most suitable for generating a first estimate of the TD<sub>50</sub> of an untested nitrosamine. The training sets used to develop these models, though, consist of rather diverse chemical structures with just a few nitrosamine structures similar to those identified in the CCS process, making predictions for new untested compounds that structurally relate to these compounds more uncertain. Therefore, a way forward with respect to making better predictive QSAR models essentially entails adequately addressing the above complicating factors. Thus, N-nitroso compounds should preferably be modeled as separate classes characterized by a common bioactivation reaction underlying their toxicity induction. At the same time, all potency values should be derived from a single species, exposed via the same route. However, should this result in a limited training set, the criteria may need to be less strict in order to obtain a sufficient number of chemicals in the set.

Potentially, biological models may produce better predictions than purely chemical models. There are quite a number of *in vitro* and *in vivo* biological models that could provide adequate

**Table 11**  
Datasets of tests with a reasonable correlation with carcinogenic potency.

Compound	Tests			Total
	CTA + MA	lacZ mouse	GST-P assay	
NDMA	X	X	X	3
NDEA	X		X	2
MNU	X	X	X	3
NNK	X	X		2
NDPA		X		1
NNN		X		1
NPYR		X		1
ENU		X		1
MNNG			X	1
NDiPLA			X	1
BBN			X	1
Total	4	7	6	

Based on the data described in Section 3.2.

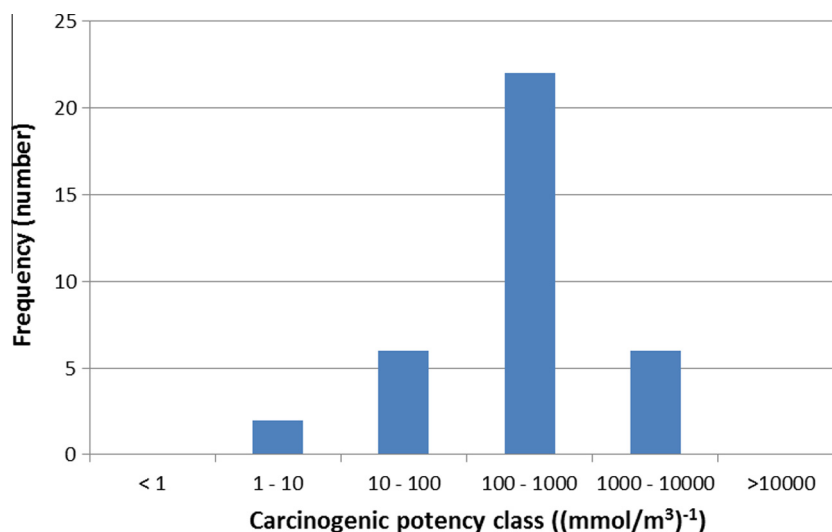
predictions of the carcinogenic potency of N-nitroso compounds. Firstly, of course, the many tests that assess mutagenic or genotoxic potential and potency. They could well reflect the chemical's carcinogenic potency, especially when focusing on a specific group of structurally related, genotoxic carcinogens. This especially holds for short-term *in vivo* models in which in principle all potential target organs may be investigated for mutations or pre-neoplastic lesions, indicative of the carcinogenic potency of the respective N-nitroso compound in that specific organ. But before stepping into *in vivo* studies, which actually should be seen as a last resort in this respect, first *in vitro* options should be explored.

Clearly, bacterial mutagenicity data are most abundantly available for this exploration. However, attempts to correlate mutagenic potency in any bacterial model to carcinogenic potency of N-nitroso compounds have not been recovered from public literature, and our own attempts to correlate mutagenic potency of N-nitroso compounds in all available Ames tests with carcinogenic potency were not successful. Though this may in part be due to the heterogeneity of the available data, also standardized mutagenicity data from Fujita and Kamataki, 2001a did not give any satisfying correlation with carcinogenic potency values. From this it must be concluded that this bacterial assay cannot serve as predictive model.

With regard to *in vitro* mammalian mutagenicity tests specifically focusing on N-nitroso compounds, only one attempt by Jones et al. (1981) with Chinese hamster V79 cells could be identified. They generally found a good correlation between carcinogenicity potency values and mutagenicity measured in V79 cells co-cultivated with primary rat hepatocytes for 26 N-nitroso compounds. However, they formulated a rather unconventional potency value, defined as  $\ln(1 + \frac{100}{D \times T^3})$ ,<sup>16</sup> which is difficult to interpret biologically.

<sup>16</sup> In which D is the total dose in mol/rat and T is the time (weeks) to death of 50% of the animals from tumors.





**Fig. 10.** Frequency distribution of respiratory carcinogenic potencies of the N-nitroso compounds described in this paper, derived from oral rat studies. Based on the data listed in Table 3.

When using the underlying original mutagenicity data together with our potency values, we were not able to find any correlation though. The absence of any other attempts in this field suggests that many options are still waiting to be explored. Quite a number of *in vitro* assays with mammalian cells potentially are suitable for this purpose, given their high true prediction rate of *in vivo* carcinogenicity potential (Kirkland, 2011).

We have explored the dose–response data for *in vitro* cell transformation assays (CTAs) and found a promising association between carcinogenic potency and transformation potency relative to the positive control MNNG, but only when metabolic activation was applied. As the dataset only comprises four N-nitroso compounds, this work needs to be further extended before a proper judgment about the predictive value of these assays can be made.

Toxicogenomic studies on N-nitroso compounds and on other well-known carcinogens that included a few N-nitroso compounds show that toxicogenomic read outs in *in vitro* or *in vivo* models apparently have the potential to distinguish genotoxic carcinogens, non-genotoxic carcinogens, and non-carcinogens (see e.g. Magkoufopoulou et al., 2012; Ellinger-Ziegelbauer et al., 2008), but attempts to predict carcinogenic potency on the basis of toxicogenomic data were not encountered, leaving this an option for future research.

Also for *in vivo* models detecting mutagenic potency we could not find any attempt to specifically predict N-nitroso compound carcinogenic potency. In all the studies retrieved, only a few N-nitroso compounds were included in a group of structurally diverse carcinogens. In the only initiation–promotion model study that involved more than two N-nitroso compounds, performed by Sakai et al. (2002), who used six N-nitroso compounds, indeed a promising association was found between the number of induced GST-P positive foci and the carcinogenic potency of the hepatocarcinogenic nitrosamines (NDEA, NDMA, and NDiPLA). It should be noted that only a weak association was found when including the non-hepatocarcinogens (BBN, MNU and MNNG).

Furthermore, an association was found between mutation induction in transgenic mice using the Lac Z gene and the carcinogenic potencies of the tested N-nitroso compounds. Thus, also this *in vivo* model may hold promise as prediction model for carcinogenic potency. However, the number of N-nitroso compounds tested was limited, there was an unexplained outlier and the association depended very strongly on one data point (leverage point).

## 5. Conclusions

Overall, no single model was identified that resulted in adequate and correct predictions of the carcinogenic potency of N-nitroso compounds as a group. Some models seem to hold promise in this respect, such as the QSARs developed by the Helguera group, the *in vitro* transformation assays, and the *in vivo* initiation–promotion, and transgenic animal assays. Most of the discussed models, however, have not been adequately explored for this purpose, as the number of N-nitroso compounds investigated is yet too limited. Therefore, further systematic data generation is needed to definitely assess their predictive power. As far as biological models are concerned, first preference should be given to *in vitro* models, for pragmatic as well as ethical reasons. Therefore, creating an extended dataset for one of the CTAs executed in the presence of a bioactivating system (e.g. rat S9), including a wide variety of N-nitroso compounds with respect to potency and chemical class and all CCS nitramines so far identified, would be a good first step in developing a model for N-nitroso compound and nitramine carcinogenic potency prediction. Based on the available data, none of the three evaluated CTAs is clearly preferred, as they show, at least for N-nitroso compounds, a strong correlation with each other. The SHE assay and the BALB/c 3T3 assay are currently in the process of being included in an OECD testing guideline, while the C3H/10T½-assay is not (Vasseur and Lasne, 2012), so one of the first two (or both) would be the first option to be followed up. Should the *in vitro* CTA model not provide the desired certainty and accuracy, one could embark on extending the datasets for one of the *in vivo* tests. In this case, the transgenic animal assay would be preferred, since it is already useable for the evaluation of various potential target organs. Once the cancer potency prediction of individual models has been established, combining these models should be explored to see whether this will raise the predictive capacity even further.

Though the carcinogenicity data on nitramines are too few to validate any prediction model, it may be anticipated that any prediction model (or combination of models) identified as suitable for nitrosamines might also predict carcinogenic potency for untested nitramines, noting their potential interconversion *in vivo* and the comparable carcinogenic potency and target-organs of DMA-NO<sub>2</sub> and NDMA when tested via the oral administration.

## Acknowledgments

The following people are gratefully acknowledged for critically reviewing parts of the manuscript: E.D. Schoen, W.R. Leeman, M.M.H. van Lipzig. This research has been carried out in the context of the CATO-2-program. CATO-2 is the Dutch national research program on CO<sub>2</sub> Capture and Storage technology (CCS). The program is financially supported by the Dutch government (Ministry of Economic Affairs) and the CATO-2 consortium parties.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.yrtph.2014.01.017>.

## References

- Akiyama, K., Akimaru, S., Asano, Y., Khalaj, M., Kiyosu, C., Masoudi, A.A., Takahashi, S., Katayama, K., Tsuji, T., Noguchi, J., Kunieda, T., 2008. A new ENU-induced mutant mouse with defective spermatogenesis caused by a nonsense mutation of the Syntaxin 2/Epimorphin (Stx2/Epim) gene. *J. Reprod. Dev.* 54, 122–128.
- Annola, K., Heikkinen, A.T., Partanen, H., Woodhouse, H., Segerback, D., Vahakangas, K., 2009. Transplacental transfer of nitrosodimethylamine in perfused human placenta. *Placenta* 30, 277–283.
- Berger, M.R., Schmaehl, D., Zerban, H., 1987. Combination experiments with very low doses of three genotoxic N nitrosamines with similar organotropic carcinogenicity in rats. *Carcinogenesis* (Lond.) 8, 1635–1644.
- Bolt, H.M., Huici-Montagud, A., /lhword> 2008. Strategy of the scientific committee on occupational exposure limits (SCOEL) in the derivation of occupational exposure limits for carcinogens and mutagens. *Arch. Toxicol.* 82, 61–64.
- Bradley, M.O., Bhuyan, B., Francis, M.C., Langenbach, R., Peterson, A., Huberman, E., 1981. Mutagenesis by chemical agents in V79 Chinese hamster cells: a review and analysis of the literature. A report of the gene-tox program. *Mutat. Res.* 87, 81–142.
- Bråten, H.B., Bunkan, A.J., Bache-Andreassen, L., Solimannejad, M., Nielsen, C.J., 2008. Final Report on a Theoretical Study on the Atmospheric Degradation of Selected Amines. NILU Report OR 77/2008 ed. NILU, Norwegian Institute for Air Research, Kjeller, Norway. Downloadable from the internet: <<http://www.nilu.no/DesktopModules/NiluWeb.UserControls/Resources/File.aspx?filename=77-2008-UiO-co2-capture.pdf&filetype=file>>.
- Cardy, R.H., Lijinsky, W., Hildebrandt, P.K., 1979. Neoplastic and nonneoplastic urinary bladder lesions induced in Fischer 344 rats and B6C3F1 hybrid mice by N-nitrosodiphenylamine. *Ecotoxicol. Environ. Saf.* 3, 29–35.
- Carrier, G., Desrosiers, M., Adib, G., 2011. Cancer Risk Assessment for Workers Exposed to Nitrosamines in a Warehouse of Finished Rubber Products in the Eastern Townships (Quebec, Canada). Institut National de Sante Publique du Quebec, Quebec, Canada.
- Cooper, M.T., Porter, T.D., 2000. Mutagenicity of nitrosamines in methyltransferase-deficient strains of *Salmonella typhimurium* coexpressing human cytochrome P450 2E1 and reductase. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* 454, 45–52.
- de Koeijer, G., Talstad, V.R., Nepstad, S., Tønnessen, D., Falk-Pedersen, O., Mære, Y., Nielsen, C., 2013. Health risk analysis for emissions to air from CO<sub>2</sub> Technology Centre Mongstad. *Int. J. Greenhouse Gas Control* 18, 200–207.
- DeVore, N.M., Scott, E.E., 2012. Nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone binding and access channel in human cytochrome P450 2A6 and 2A13 enzymes. *J. Biol. Chem.* 287, 26576–26585.
- Druckrey, H., Preussmann, R., Ivankovic, S., Schmahl, D., 1967. Organotropic carcinogenic effects of 65 various N-nitroso-compounds on BD rats. *Z. Krebsforsch.* 69, 103–201.
- Druckrey, H., Preussmann, R., Schmaehl, D., Muller, M., 1961. Erzeugung von Magenkrebs durch Nitrosamide an Ratten. *Naturwissenschaften* 48, 165.
- Duarte, M.P., Palma, B.B., Laires, A., Oliveira, J.S., Rueff, J., Kranendonk, M., 2005. *Escherichia coli* BTC, a human cytochrome P450 competent tester strain with a high sensitivity towards alkylating agents: involvement of alkyltransferases in the repair of DNA damage induced by aromatic amines. *Mutagenesis* 20, 199–208.
- Dunkel, V.C., Pienta, R.J., Sivak, A., Traul, K.A., 1981. Comparative neoplastic transformation responses of BALB/c 3T3 cells, Syrian hamster embryo cells, and Rauscher murine leukemia virus-infected Fischer 344 rat embryo cells to chemical carcinogens. *J. Natl Cancer Inst.* 67, 1303–1315.
- ECHA, 2012a. Chapter R.7a: endpoint specific guidance, in: Anonymous Guidance on Information Requirements and Chemical Safety Assessment., Version: 2.1 ed. European Chemicals Agency, Helsinki, Finland.
- ECHA, 2012b. Chapter R.8: characterisation of dose [concentration]–response for human health, in: Anonymous Guidance on Information Requirements and Chemical Safety Assessment, Version: 2.1 ed. European Chemicals Agency, Helsinki, Finland.
- Ellinger-Ziegelbauer, H., Gmuender, H., Bandenburg, A., Ahr, H.J., 2008. Prediction of a carcinogenic potential of rat hepatocarcinogens using toxicogenomics analysis of short-term in vivo studies. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* 637, 23–39.
- Emmert, B., Bunger, J., Keuch, K., Muller, M., Emmert, S., Hallier, E., Westphal, G.A., 2006. Mutagenicity of cytochrome P450 2E1 substrates in the Ames test with the metabolic competent *S. typhimurium* strain YG7108pin3ERb5. *Toxicology* 228, 66–76.
- Faustino, C., Garcia-Rio, L., Leis, J.R., Norberto, F., 2005. Decomposition of N'-benzoyl-N-nitrosoureas in aqueous media. *Eur. J. Org. Chem.*, 154–161
- Fjellsbø, L.M., Van Rompay, A.R., Hooyberghs, J., Nelissen, I., Dusinska, M., 2013. Screening for potential hazard effects from four nitramines on human eye and skin. *Toxicol. In Vitro* 27, 1205–1210
- Fjellsbø, L.M.B., Magdolenova, Z., Ravnum, S., Pran, E.R., Dusinska, M., 2011. Chapter E – acute toxicity, cytotoxicity, irritation, sensitization and corrosion, in: Dye, C., Fjellsbø, L.M.B., Dusinska, M. (Eds.), Nitramine Analysis Procedures Development and Screening Toxicity Study. NILU, Norwegian Institute for Air Research, Kjeller, Norway, pp. 45–64. Downloadable from the internet: <<http://www.gassnova.no/gassnova2/frontend/files/CONTENT/Rapporter/Nitramineanalysisprocedureve lopmentandscreeningtoxicitystudy NILU.pdf>>.
- Fujita, K., Kamataki, T., 2001a. Predicting the mutagenicity of tobacco-related N-nitrosamines in humans using 11 strains of *Salmonella typhimurium* YG7108, each coexpressing a form of human cytochrome P450 along with NADPH-cytochrome P450 reductase. *Environ. Mol. Mutagen.* 38, 339–346.
- Fujita, K., Kamataki, T., 2001b. Role of human cytochrome P450 (CYP) in the metabolic activation of N-alkylnitrosamines: application of genetically engineered *Salmonella typhimurium* YG7108 expressing each form of CYP together with human NADPH-cytochrome P450 reductase. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* 483, 35–41.
- Fukushima, S., Wanibuchi, H., Morimura, K., Nakae, D., Tsuda, H., Imaida, K., Shirai, T., Tatematsu, M., Tsukamoto, T., Hirose, M., Furukawa, F., 2005. Lack of potential of low dose N-nitrosodimethylamine to induce preneoplastic lesions, glutathione S-transferase placental form-positive foci, in rat liver. *Cancer Lett.* 222, 11–15.
- Gentry, P.R., House-Knight, T., Harris, A., Greene, T., Campleman, S., 2013. Potential occupational risk of amines in carbon capture for power generation. *Int. Arch. Occup. Environ. Health*, 1–16.
- Gezondheidsraad, 2012. Leidraad Berekening Risicogetallen Voor Carcinogene Stoffen. (Guidance on the Calculation of Risk Values for Carcinogenic Substances.), publicatiernr. 2012/16 ed. Gezondheidsraad, The Hague, Netherlands.
- Golding, B.T., Bleasdale, C., McGinnis, J., Muller, S., Rees, H.T., Rees, N.H., Farmer, P.B., Watson, W.P., 1997. The mechanism of decomposition of N-methyl-N-nitrosourea (MNU) in water and a study of its reactions with 2'-deoxyguanosine, 2'-deoxyguanosine 5'-monophosphate and d(GTGCAC). *Tetrahedron* 53, 4063–4082.
- Goodall, C.M., Kennedy, T.H., 1976. Carcinogenicity of dimethylnitramine in NZR rats and NZO mice. *Cancer Lett.* 1, 295–298.
- Guttenplan, J.B., 1987. Structure-activity relationships in metabolism and mutagenicity of N-nitrosamines. *IARC Sci. Publ.* 84, 129–131.
- Hakura, A., Shimada, H., Nakajima, M., Sui, H., Kitamoto, S., Suzuki, S., Satoh, T., 2005. *Salmonella*/human S9 mutagenicity test: a collaborative study with 58 compounds. *Mutagenesis* 20, 217–228.
- Harju, M., Ravnum, S., Fjellsbø, L.M.B., Dusinska, M., Heimstad, E.S., 2010. Alternative approaches to standard toxicity testing, TQP ID 9 – 257430120 – NILU ed. NILU, Norwegian Institute for Air Research, Kjeller, Norway. Downloadable from the internet: <<http://www.nilu.no/DesktopModules/NiluWeb.UserControls/Resources/File.aspx?filename=14-2011-AMINE9.pdf&filetype=file>>.
- Harju, M., Ravnum, S., Pran, E.R., Grossberndt, S., Fjellsbø, L.M.B., Dusinska, M., Heimstad, E.S., 2011. Alternative approaches to standard toxicity testing, TQP ID 9 – OPTION – 257430181 – NILU ed. NILU, Norwegian Institute for Air Research, Kjeller, Norway. Can be ordered from NILU via the internet: <<http://www.nilu.no/Default.aspx?tabid=62&ctl=PublicationDetails&mid=764&publicationid=26456>>.
- Hassel, M., Frei, E., 1987. Metabolism of N nitrosodimethylamine in target and nontarget organs of the rat. Fourth Symposium of the Deutschen Krebsgesellschaft, Section Experimentelle Krebsforschung (German Cancer Society, Section of Experimental Cancer Research), Heidelberg, West Germany, March 18–21, 1987. *J. Cancer Res. Clin. Oncol.* 113, S23.
- Hecht, S.S., Lijinsky, W., Kovatch, R.M., Chung, F.L., Saavedra, J.E., 1989. Comparative tumorigenicity of N-nitroso-2-hydroxymorpholine, N-nitrosodiethanolamine and N-nitrosomorpholine in A/J mice and F344 rats. *Carcinogenesis* 10, 1475–1477.
- Helguera, A.M., Cordeiro, M.N.D.S., Perez, M.A.C., Combes, R.D., Gonzalez, M.P., 2008a. Quantitative structure carcinogenicity relationship for detecting structural alerts in nitroso-compounds: species: rat; sex: male; route of administration: water. *Toxicol. Appl. Pharmacol.* 231, 197–207.
- Helguera, A.M., Gonzalez, M.P., Cordeiro, M.N.D.S., Perez, M.A.C., 2008b. Quantitative structure-carcinogenicity relationship for detecting structural alerts in nitroso compounds: species, rat; sex, female; route of administration, gavage. *Chem. Res. Toxicol.* 21, 633–642.
- Helguera, A.M., Gonzalez, M.P., D S Cordeiro, M.N., Perez, M.A., 2007. Quantitative structure carcinogenicity relationship for detecting structural alerts in nitroso-compounds. *Toxicol. Appl. Pharmacol.* 221, 189–202.

- Helguera, A.M., Perez-Machado, G., Cordeiro, M.N., Combes, R.D., 2010. Quantitative structure–activity relationship modelling of the carcinogenic risk of nitroso compounds using regression analysis and the TOPS-MODE approach. *SAR QSAR Environ. Res.* 21, 277–304.
- Hernandez, L.G., Slob, W., van Steeg, H., van Benthem, J., 2011. Can carcinogenic potency be predicted from in vivo genotoxicity data?: a meta-analysis of historical data. *Environ. Mol. Mutagen.* 52, 518–528.
- Hoffman, D., Rivenson, A., Adams, J.D., Juchatz, A., Vinchkoski, N., Hecht, S.S., 1983. Effects of route of administration and dose on the carcinogenicity of N-nitrosodiethanolamine in the Syrian golden hamster. *Cancer Res.* 43, 2521–2524.
- Hoffmann, F., Graffi, A., 1964. Carcinoma of the paranasal sinuses in mice after application of diethylnitrosamine drops to the skin of the back. *Acta Biol. Med. Ger.* 12, 623–625.
- Honkakoski, P., Negishi, M., 1997. The structure, function, and regulation of cytochrome P450 2A enzymes. *Drug Metab. Rev.* 29, 977–996.
- Hooyberghs, J., Schoeters, E., Lambrechts, N., Nelissen, I., Witters, H., Schoeters, G., Van Den Heuvel, R., 2008. A cell-based in vitro alternative to identify skin sensitizers by gene expression. *Toxicol. Appl. Pharmacol.* 231, 103–111.
- IARC, 2012. Chemical agents and related occupations. IARC Monogr. Eval. Carcinog. Risks Hum. 100, 9–562.
- IPCC, 2005. IPCC Special Report on Carbon Dioxide Capture and Storage. Prepared by Working Group III of the Intergovernmental Panel on Climate Change. In: Metz, B., Davidson, O., de Coninck, H.C., Loos, M., Meyer, L.A. (Eds.), Cambridge University Press, New York, USA.
- Iversen, O.H., 1980. Tumorigenicity of N-nitroso-diethyl-, -dimethyl and -diphenyl-amines in skin painting experiments. A study utilizing the tetrazolium test and skin applications on hairless mice. *Eur. J. Cancer* 16, 695–698.
- Jones, C.A., Marlino, P.J., Lijinsky, W., Huberman, E., 1981. The relationship between the carcinogenicity and mutagenicity of nitrosamines in a hepatocyte-mediated mutagenicity assay. *Carcinogenesis (Lond.)* 2, 1075–1078.
- Katayama, K., Ishigami, N., Uetsuka, K., Nakayama, H., Doi, K., 2000a. Ethylnitrosourea (ENU)-induced apoptosis in the rat fetal tissues. *Histol. Histopathol.* 15, 707–711.
- Katayama, K., Ishigami, N., Suzuki, M., Ohtsuka, R., Kiatipattanasakul, W., Nakayama, H., Doi, K., 2000b. Teratologic studies on rat perinates and offspring from dams treated with ethylnitrosourea (ENU). *Exp. Anim.* 49, 181–187.
- Katayama, K., Ueno, M., Takai, H., Ejiri, N., Uetsuka, K., Nakayama, H., Doi, K., 2002. Ethylnitrosourea induces apoptosis and growth arrest in the trophoblastic cells of rat placenta. *Biol. Reprod.* 67, 431–435.
- Katayama, K., Uetsuka, K., Ishigami, N., Nakayama, H., Doi, K., 2001. Apoptotic cell death and cell proliferative activity in the rat fetal central nervous system from dams administered with ethylnitrosourea (ENU). *Histol. Histopathol.* 16, 79–85.
- Kennedy, C.L., O'Bryan, M.K., 2006. N-ethyl-N-nitrosourea (ENU) mutagenesis and male fertility research. *Hum. Reprod. Update* 12, 293–301.
- Kirkland, D., 2011. Improvements in the reliability of in vitro genotoxicity testing. *Expert Opin. Drug Metab. Toxicol.* 7, 1513–1520.
- Klein, R.G., Janowsky, I., Pool-Zobel, B.L., Schmezer, P., Hermann, R., Amelung, F., Spiegelhalder, B., Zeller, W.J., 1991. Effects of long-term inhalation of N-nitrosodimethylamine in rats. *IARC Sci. Publ.*, 322–328.
- Klein, R.G., Schmezer, P., Hermann, R., Waas, P., Spiegelhalder, B., Bartsch, H., 1999. Strong nasal carcinogenicity and genotoxicity of 1-nitroso-4-methylpiperazine after low dose inhalation in rats. *Carcinogenesis* 20, 1629–1631.
- Klein, R.G., Spiegelhalder, B., Preussmann, R., 1990. Inhalation carcinogenesis of N-nitrosomorpholine (NMOR) in rats and hamsters. *Exp. Pathol.* 40, 189–195.
- Kowalski, L.A., Laitinen, A.M., Mortazavi-Asl, B., Wee, R.K., Erb, H.E., Assi, K.P., Madden, Z., 2000. In vitro determination of carcinogenicity of sixty-four compounds using a bovine papillomavirus DNA-carrying C3H/10T½ cell line. *Environ. Mol. Mutagen.* 35, 300–311.
- Kushida, H., Fujita, K., Suzuki, A., Yamada, M., Nohmi, T., Kamataki, T., 2000. Development of a Salmonella tester strain sensitive to promutagenic N-nitrosamines: expression of recombinant CYP2A6 and human NADPH-cytochrome P450 reductase in *S. typhimurium* YG7108. *Mutat. Res.* 471, 135–143.
- Lee, M., Chang, C., Lee, Y., Wu, Y., Tseng, H., Tung, Y., Wu, M., Chen, Y., Kuo, L., Stephenson, D., Hung, S., Wu, J., Chang, C., Chen, Y., Chern, Y., 2009. Longitudinal evaluation of an N-ethyl-N-nitrosourea-created murine model with normal pressure hydrocephalus. *PLoS One* 4.
- Lijinsky, W., 1984. Species differences in nitrosamine carcinogenesis. *J. Cancer Res. Clin. Oncol.* 108, 46–55.
- Lijinsky, W., Kovatch, R.M., 1988. Comparative carcinogenesis by nitrosomethylalkylamines in Syrian hamsters. *Cancer Res.* 48, 6648–6652.
- Lijinsky, W., Kovatch, R.M., Riggs, C.W., Walters, P.T., 1988. Dose-response study with N-nitrosomorpholine in drinking water of F-344 rats. *Cancer Res.* 48, 2089–2095.
- Lijinsky, W., Reuber, M.D., 1980. Carcinogenicity in rats of nitrosomethyl-ethylamines labeled with deuterium in several positions. *Cancer Res.* 40, 19–21.
- Lijinsky, W., Reuber, M.D., Saavedra, J.E., Singer, G.M., 1983. Carcinogenesis in F344 rats by N-nitrosomethyl-n-propylamine derivatives. *J. Natl Cancer Inst.* 70, 959–963.
- Lijinsky, W., Taylor, H.W., 1975. Carcinogenicity of methylated dinitrosopiperazines in rats. *Cancer Res.* 35, 1270–1273.
- Liu, C., Russell, R.M., 2008. Nutrition and gastric cancer risk: an update. *Nutr. Rev.* 66, 237–249.
- Luan, F., Zhang, R., Zhao, C., Yao, X., Liu, M., Hu, Z., Fan, B., 2005. Classification of the carcinogenicity of N-nitroso compounds based on support vector machines and linear discriminant analysis. *Chem. Res. Toxicol.* 18, 198–203.
- Maekawa, A., Ogiu, T., Matsuoka, C., Onodera, H., Furuta, K., Kurokawa, Y., Takahashi, M., Kokubo, T., Tanigawa, H., Hayashi, Y., 1984. Carcinogenicity of low doses of N-ethyl-N-nitrosourea in F344 rats; a dose-response study. *Gann* 75, 117–125.
- Magee, P.N., Barnes, J.M., 1967. Carcinogenic nitroso compounds. *Adv. Cancer Res.* 10, 163–246.
- Magkoufopoulou, C., Claessen, S.M.H., Tsamou, M., Jennen, D.G.J., Kleinjans, J.C.S., van Delft, J.H.M., 2012. A transcriptomics-based in vitro assay for predicting chemical genotoxicity in vivo. *Carcinogenesis* 33, 1421–1429.
- Mirvish, S.S., Bulay, O., Runge, R.G., Patil, K., 1980. Study of the carcinogenicity of large doses of dimethylnitramine, N-nitroso-L-proline, and sodium nitrite administered in drinking water to rats. *J. Natl Cancer Inst.* 64 (6), 1435–1442.
- OECD, 2007. Detailed review paper on cell transformation assays for detection of chemical carcinogens. OECD Environment, Health and Safety Publications. Series on Testing and Assessment 31. OECD, Paris, France.
- Peto, R., Gray, R., Brantom, P., Grasso, P., 1991a. Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. *Cancer Res.* 51, 6452–6469.
- Peto, R., Gray, R., Brantom, P., Grasso, P., 1991b. Effects on 4080 rats of chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine: a detailed dose–response study. *Cancer Res.* 51 (6415), 6451.
- Ribeiro Pinto, L.F., Moraes, E., Albano, R.M., Silva, M.C., Godoy, W., Glisovic, T., Lang, M.A., 2001. Rat oesophageal cytochrome P450 (CYP) monooxygenase system: comparison to the liver and relevance in N-nitrosodiethylamine carcinogenesis. *Carcinogenesis* 22, 1877–1883.
- Robichová, S., Robichova, S., Slamenova, D., Gabelova, A., Sedlak, J., Jakubikova, J., 2004. An investigation of the genotoxic effects of N-nitrosomorpholine in mammalian cells. *Chem. Biol. Interact.* 148, 163–171.
- Robottom-Ferreira, A.B., Aquino, S.R., Queiroga, R., Albano, R.M., Ribeiro Pinto, L.F., 2003. Expression of CYP2A3 mRNA and its regulation by 3-methylcholanthrene, pyrazole, and p-ionone in rat tissues. *Braz. J. Med. Biol. Res.* 36, 839–844.
- Sakai, H., Tsukamoto, T., Yamamoto, M., Kobayashi, K., Yuasa, H., Imai, T., Yanai, T., Masegi, T., Tatematsu, M., 2002. Distinction of carcinogens from mutagens by induction of liver cell foci in a model for detection of initiation activity. *Cancer Lett.* 188, 33–38.
- Sanner, T., Dybing, E., 2005. Comparison of carcinogenic and in vivo genotoxic potency estimates. *Basic Clin. Pharmacol. Toxicol.* 96, 131–139.
- Scherf, H.R., Frei, E., Wiessler, M., 1989. Carcinogenic properties of N-nitrosodimethylamine and N-nitromethylamine in the rat. *Carcinogenesis* 10, 1977–1981.
- Singer, S.S., Singer, G.M., Saavedra, J.E., Reuber, M.D., Lijinsky, W., 1981. Carcinogenesis by derivatives of 1-nitroso-3,5-dimethylpiperazine in rats. *Cancer Res.* 41, 1034–1038.
- Straif, K., Weiland, S.K., Bungers, M., Holtherrich, D., Taeger, D., Yi, S., Keil, U., 2000. Exposure to high concentrations of nitrosamines and cancer mortality among a cohort of rubber workers. *Occup. Environ. Med.* 57, 180–187.
- Straif, K., Weiland, S.K., Werner, B., Chambless, L., Mundt, K.A., Keil, U., 1998. Workplace risk factors for cancer in the German rubber industry: Part 2. Mortality from non-respiratory cancers. *Occup. Environ. Med.* 55, 325–332.
- Sussman, H.E., Bauer, M.J., Shi, X., Judice, S.A., Albertini, R.J., Walker, V.E., 2001. Transplacental mutagenicity of N-ethyl-N-nitrosourea at the hprt locus in T-lymphocytes of exposed B6C3F1 mice. *Environ. Mol. Mutagen.* 38, 30–37.
- Takano, T., Shirai, T., Ogiso, T., Tsuda, H., Baba, S., Ito, N., 1982. Sequential changes in tumor development induced by 1,4-dinitrosopiperazine in the nasal cavity of F344 rats. *Cancer Res.* 42 (4236), 4240.
- Vasseur, P., Lasne, C., 2012. OECD Detailed Review Paper (DRP) number 31 on cell transformation assays for detection of chemical carcinogens: main results and conclusions. *Mutat. Res.* 744, 8–11.
- Wagner, E.D., Hsu, K., Lagunas, A., Mitch, W.A., Plewa, M.J., 2012. Comparative genotoxicity of nitrosamine drinking water disinfection byproducts in Salmonella and mammalian cells. *Mutat. Res.* 741, 109–115.
- Weiland, S.K., Mundt, K.A., Keil, U., Kraemer, B., Birk, T., Person, M., Bucher, A.M., Straif, K., Schumann, J., Chambless, L., 1996. Cancer mortality among workers in the German rubber industry: 1981–1991. *Occup. Environ. Med.* 53, 289–298.
- WHO, 1978. Environmental Health Criteria 5. Nitrates, nitrites and n-nitroso compounds. ISBN 92 4 154065 6. World Health Organization, Geneva, Switzerland.
- Yuan, J., Pu, Y., Yin, L., 2011. Predicting carcinogenicity and understanding the carcinogenic mechanism of N-nitroso compounds using a TOPS-MODE approach. *Chem. Res. Toxicol.* 24 (2269), 2279.
- Yuan, J., Pu, Y., Yin, L., 2012. QSAR study of liver specificity of carcinogenicity of N-nitroso compounds. *Ecotoxicol. Environ. Saf.* 84, 282–292.
- Zeiger, E., Sheldon, A.T., 1978. The mutagenicity of heterocyclic N-nitrosamines for *Salmonella-typhimurium*. *Mutat. Res.* 57, 1–10.
- Zhao, L., Zhong, R., She, Y., Dai, Q., 2005. Ab initio study on the reactivity of non- $\alpha$ -metabolites of alkyl nitrosamines. *Acta Phys. Chim. Sin.* 21, 883–887.



# Atmospheric Chemistry of Amines

Modelling nitrosamine and nitramine formation in the atmospheric gas phase photo-oxidation of Cansolv DC103 amine.

Claus Nielsen

Department of Chemistry

University of Oslo

September 2014



This page is intentionally left blank

CONFIDENTIAL

# Atmospheric Chemistry of Amines

Modelling nitrosamine and nitramine formation in the atmospheric gas phase photo-oxidation of Cansolv DC103 amine.

Claus Nielsen

Department of Chemistry, University of Oslo

September 2014.

CONFIDENTIAL

This page is intentionally left blank

CONFIDENTIAL

## Table of Contents

Executive Summary .....	3
Nitrosamine and nitramine formation in atmospheric amine photo-oxidation.....	5
1 Literature .....	10

CONFIDENTIAL



This page is intentionally left blank

CONFIDENTIAL

## Executive Summary

The present report is an anonymized extract of the report "Atmospheric Chemistry of Amines - A theoretical study of the OH initiated atmospheric gas phase photo-oxidation of DC103" intended for dispersion modeling purpose only.

The atmospheric gas phase photo-oxidation of the DC103 amine is dominated by reaction with OH radicals. The rate coefficient for the OH radical reaction with DC103 is not reported in the literature; a best estimate based on available available data for structurally similar compounds suggests room temperature rate coefficient  $k_{OH} = 2.5 \times 10^{-10} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$  at 298 K. The average atmospheric lifetime of DC103 with respect to reaction with OH is consequently estimated to be around 70 min in the Boundary Dam area.

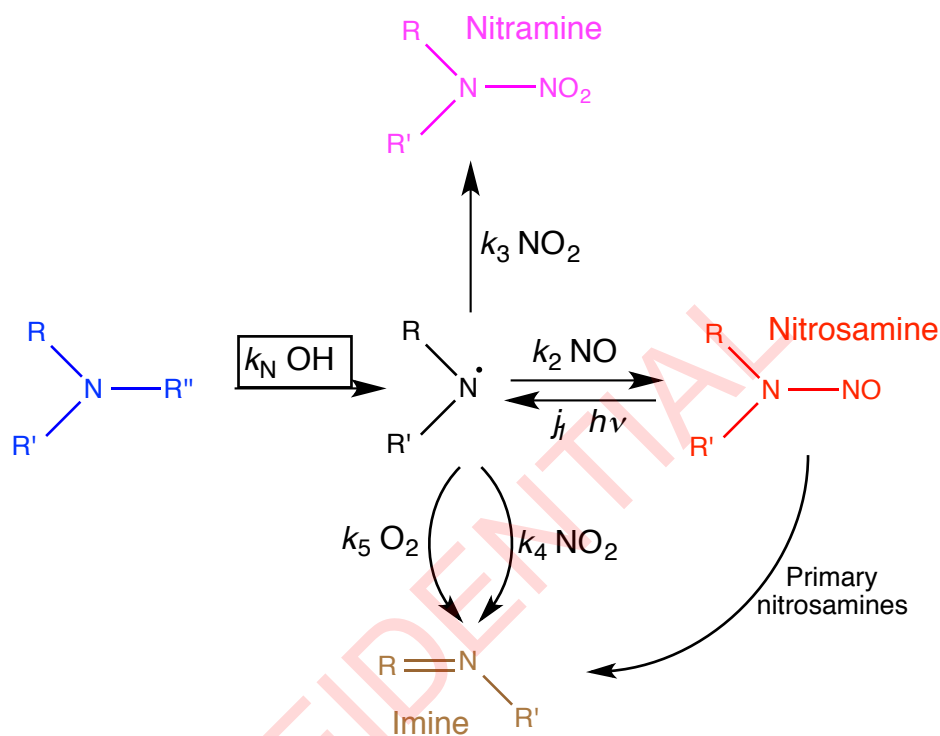
The study shows that the DC103 potential to form first generation nitrosamines and nitramines is smaller than that of dimethylamine,  $(\text{CH}_3)_2\text{NH}$ , but larger than that of MEA,  $\text{NH}_2\text{CH}_2\text{CH}_2\text{OH}$ . The amount of nitramines formed in the atmospheric photo-oxidation of DC103 is conservatively estimated to be respectively 2.8 % after 2 h, and 3.4 % after complete photo-oxidation in the Boundary Dam area. For comparison, the numbers for dimethylamine are 1.4 % after 2h and 3.9 % after complete photo-oxidation under the same conditions.

This page is intentionally left blank

CONFIDENTIAL

## Nitrosamine and nitramine formation in atmospheric amine photo-oxidation

Estimates of nitrosamine and nitramine formation in the OH initiated photo-oxidation of DC103 is based on the general mechanism shown in Scheme 1, in which nitrosamine formation from primary amines has been included. Primary nitrosamines do, however, isomerize and react with O<sub>2</sub> followed by N-N bond scission within seconds to give the corresponding imines.<sup>1</sup>



Scheme 1. Atmospheric photo-oxidation scheme for DC103 showing the routes to nitramines and nitrosamines.

The photolysis rate coefficient,  $j_1$ , is proportional to NO<sub>2</sub> photolysis rate coefficient,  $j_{\text{NO}_2}$ :  $j_{\text{Rel}} = j_1/j_{\text{NO}_2} = 0.37$ .<sup>2</sup>

The initial amine reaction with OH radicals constitutes the rate-limiting step in the formation of nitrosamines, nitramines and imines. The amount of nitrosamine and nitramine formed at a given time in the atmospheric oxidation of DC103 depends on the rate coefficient of the initial hydrogen abstraction reaction, the branching ratio in the reaction (here:  $k_N/k_1$ ), and the branching in the subsequent amino radical reactions:

$$B_{\text{Nitramine}} = k_3 \cdot [\text{NO}_2] / (k_2 \cdot [\text{NO}] + k_3 \cdot [\text{NO}_2] + k_4 \cdot [\text{NO}_2] + k_5 \cdot [\text{O}_2])$$

$$B_{\text{Nitrosamine}} = k_2 \cdot [\text{NO}] / (k_2 \cdot [\text{NO}] + k_3 \cdot [\text{NO}_2] + k_4 \cdot [\text{NO}_2] + k_5 \cdot [\text{O}_2])$$

$$B_{\text{Imine}} = (k_4 \cdot [\text{NO}_2] + k_5 \cdot [\text{O}_2]) / (k_2 \cdot [\text{NO}] + k_3 \cdot [\text{NO}_2] + k_4 \cdot [\text{NO}_2] + k_5 \cdot [\text{O}_2])$$

The total yield of amino radicals formed in the DC103 + OH reaction is estimated to be ~37 % ( $k_N/k_1 = 0.37$ ), which is comparable to that of the  $(\text{CH}_3)_2\text{NH} + \text{OH}$  reaction ( $k_N/k_1 = 0.42$ ).<sup>2</sup>

The Boundary Dam area (48°59'N) is characterized by NO<sub>x</sub>-levels in the region 2-4 ppb. Table 1 summarises the estimated average NO<sub>2</sub>-photolysis rate coefficient, and the NO and NO<sub>2</sub> mixing ratios in the Boundary Dam area.

Table 1. Assumed annual average values of the NO<sub>2</sub> photolysis rate, OH radical concentration, and NO and NO<sub>2</sub> volume mixing ratios in the Boundary Dam area.

$j_{\text{NO}_2}$	/s <sup>-1</sup>	$1.6 \times 10^{-3}$
OH	/molecules cm <sup>-3</sup>	$1.0 \times 10^6$
NO	/ppb	2.2
NO <sub>2</sub>	/ppb	3.7

Estimates of the branching in the DC103 amino radical reactions with O<sub>2</sub>, NO and NO<sub>2</sub> ( $k_2/k_3$ ,  $k_4/k_3$ ,  $k_5/k_3$ ) are given in Table 2 together with other parameters needed in estimating the annual average nitramine yield and the steady state nitrosamine mixing ratio.

The average wind speed in the Boundary Dam area is around 4 m s<sup>-1</sup>. During 2 hours an air parcel will therefore, on average, travel 30 km. It is of interest to know not only the theoretical maximum nitramine yield, but also the nitramine yield during the first hours after emission. Table 2 includes the calculated nitramine yield 2 hours after emission as well as the maximal yield (100 % photo-oxidized amine). The results reported in Table 2, are based on annual averages listed in Table 1.1.

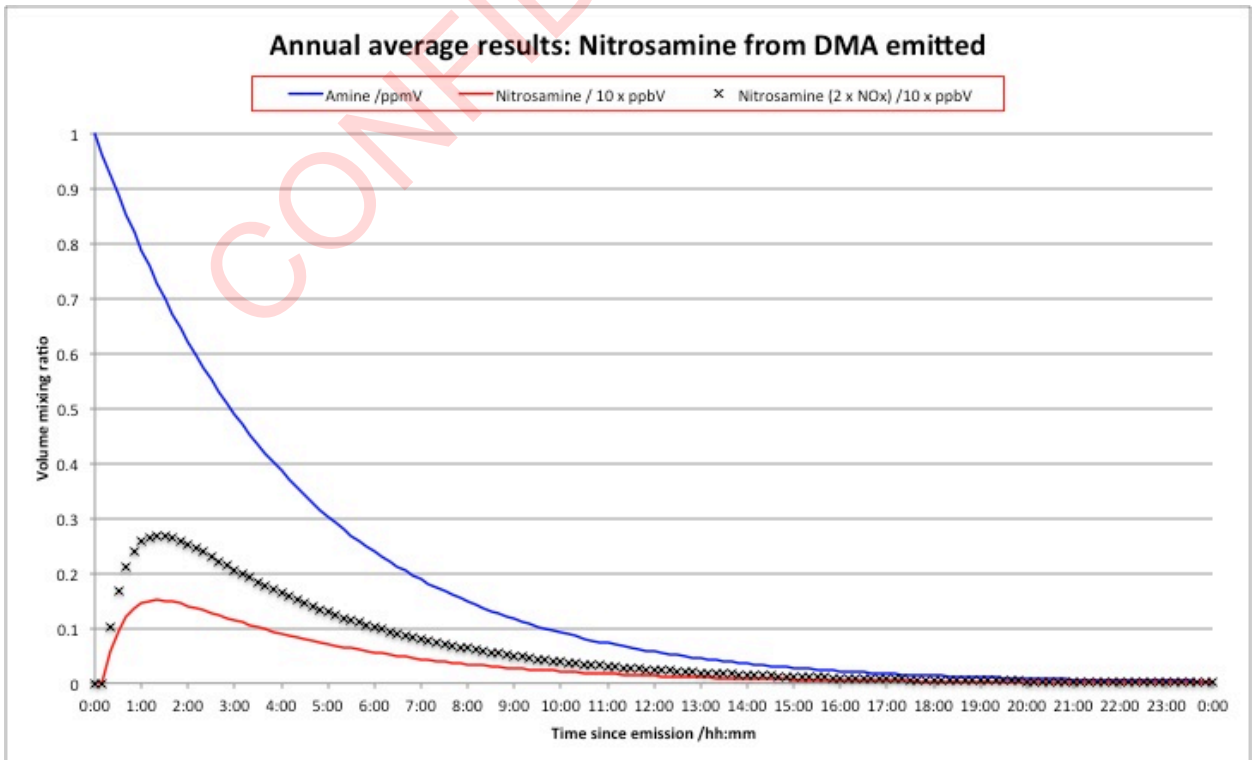
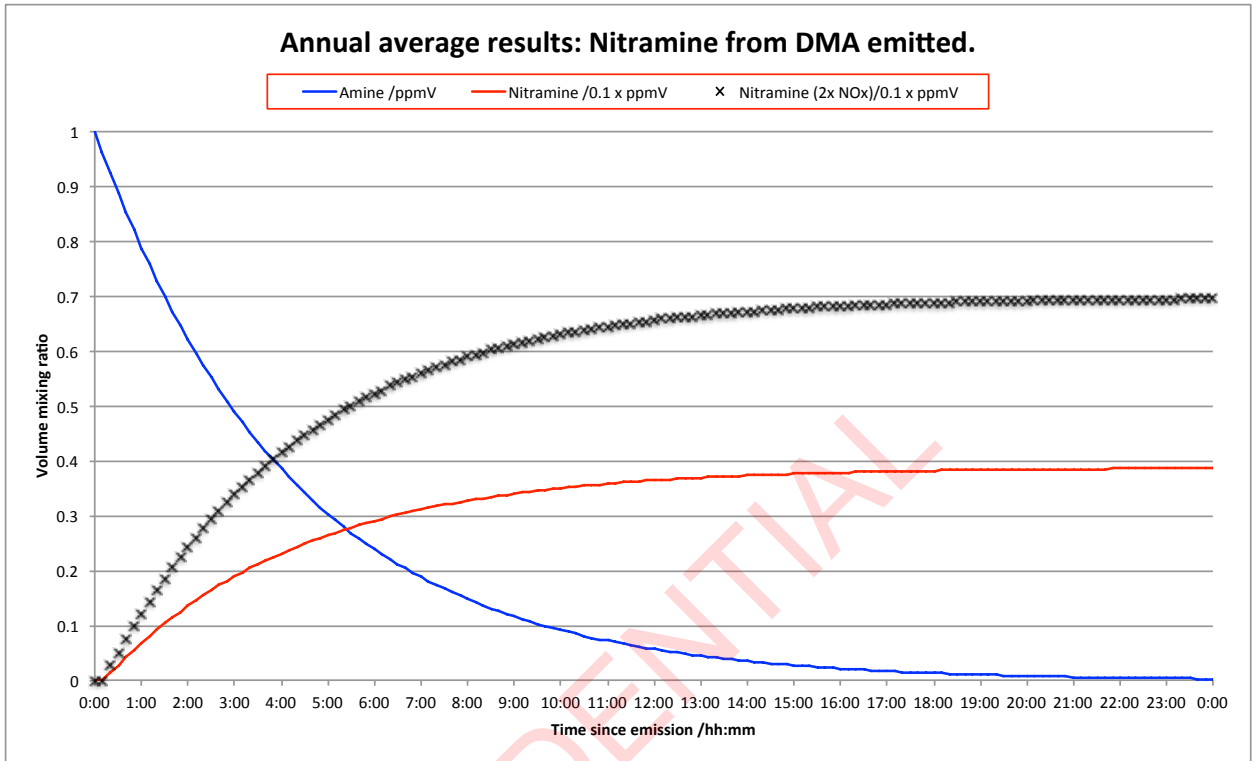
Table 2. Reaction rate coefficients and branching ratios for formation of the corresponding amino radical and its reactions with NO, O<sub>2</sub> and NO<sub>2</sub>. See Scheme 1 for definition of rate coefficients.

Amine	$k_{\text{Amine+OH}}^a$	$J_{\text{rel}}^b$	$k_N/k_1$	$k_2/k_3^b$	$k_4/k_3^b$	$k_5/k_3^b$	Nitramine yield	
							2h	$\infty$
DC103	$2.5 \times 10^{-10}$	0.34	0.37	0.67	0.20	$1.7 \times 10^{-7}$	2.8 %	3.4 %
MEA <sup>c</sup>	$7.6 \times 10^{-11}$	0.34	0.09	0.67	0.20	$1.7 \times 10^{-7}$	0.3 %	0.8 %
$(\text{CH}_3)_2\text{NH}$	$6.5 \times 10^{-11}$	0.34 <sup>d</sup>	0.42 <sup>d</sup>	0.67 <sup>d</sup>	0.20 <sup>d</sup>	$1.7 \times 10^{-7d}$	1.4 %	3.9 %

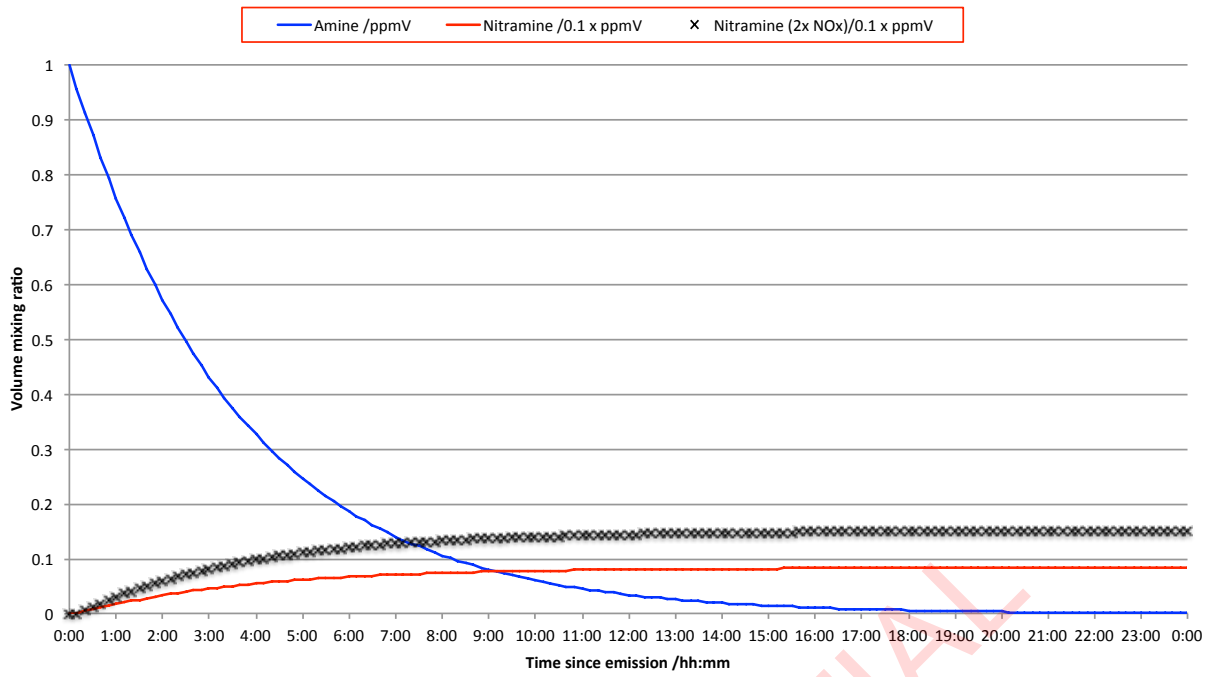
<sup>a</sup> Unit: cm<sup>3</sup> molecule<sup>-1</sup> s<sup>-1</sup>. <sup>b</sup> Assumed to be the same in DC103, MEA and  $(\text{CH}_3)_2\text{NH}$ . <sup>c</sup> From ref. <sup>3</sup> <sup>d</sup> From Ref. 2.

## Box model results

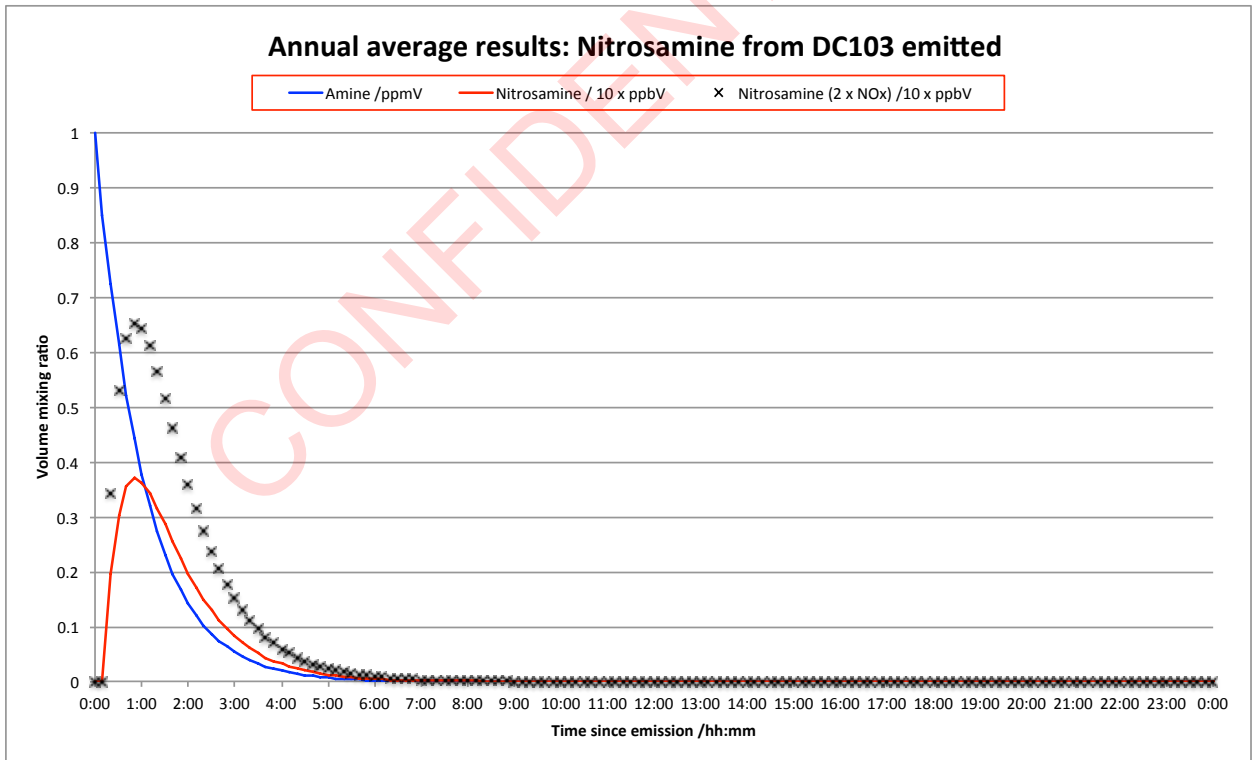
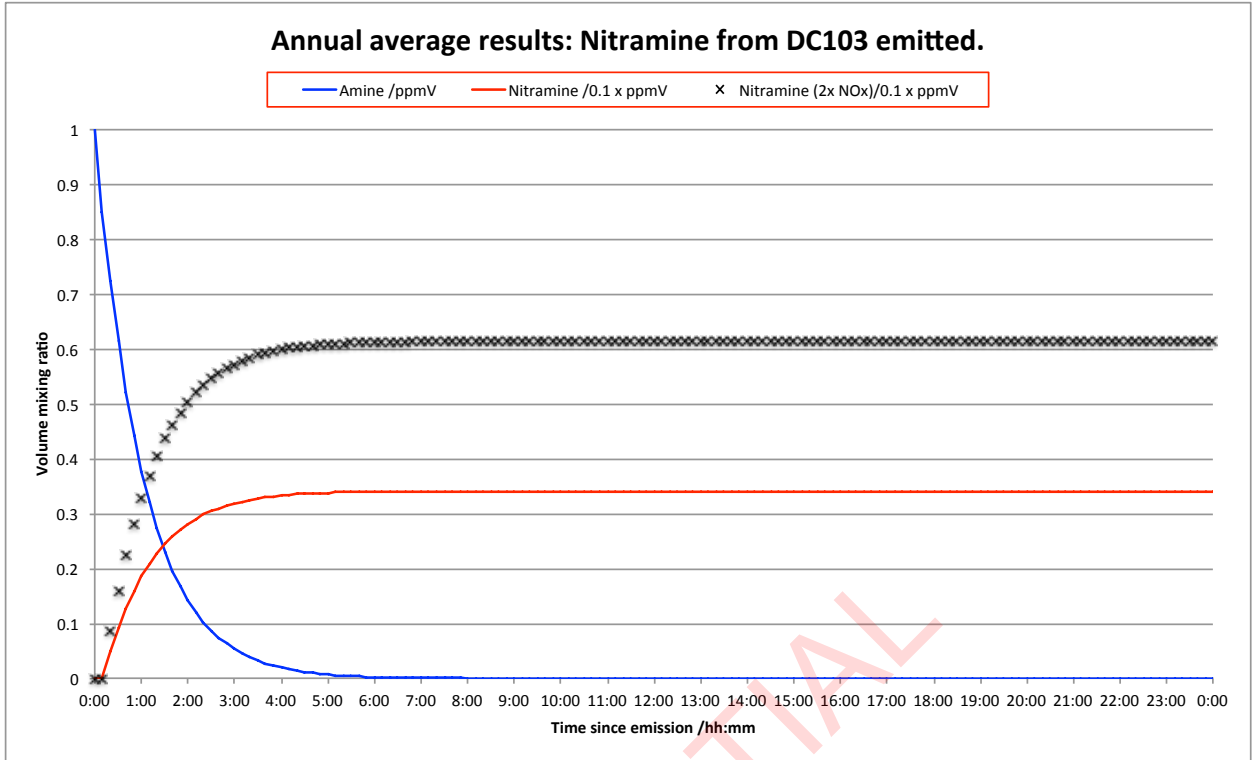
The results from simple box model calculations of nitramine and nitrosamine mixing ratios in an closed air parcel following emission are shown below. The box model is based on the parameters given in Table 1.



### Annual average results: Nitramine from MEA emitted.



CONFIDENTIAL





## Literature

1. Nielsen, C. J.; D'Anna, B.; Karl, M.; Aursnes, M.; Boreave, A.; Bossi, R.; Bunkan, A. J. C.; Glasius, M.; Hansen, A.-M. K.; Hallquist, M.; Kristensen, K.; Mikoviny, T.; Maguta, M. M.; Müller, M.; Nguyen, Q.; Westerlund, J.; Salo, K.; Skov, H.; Stenstrøm, Y.; Wisthaler, A. *Summary Report: Photo-oxidation of Methylamine, Dimethylamine and Trimethylamine. Climit project no. 201604*; NILU OR 2/2011, ISBN 978-82-425-2357-0; NILU: 2011.
2. Nielsen, C. J.; Herrmann, H.; Weller, C., Atmospheric chemistry and environmental impact of the use of amines in carbon capture and storage (CCS). *Chem. Soc. Rev.* **2012**, *41* (19), 6684-6704.
3. Nielsen, C. J.; D'Anna, B.; Dye, C.; George, C.; Graus, M.; Hansel, A.; Karl, M.; King, S.; Musabila, M.; Müller, M.; Schmiedbauer, N.; Stenstrøm, Y.; Wisthaler, A. *Atmospheric Degradation of Amines (ADA). Summary Report: Gas phase photo-oxidation of 2-aminoethanol (MEA)*; NILU OR 8/2010, ISBN 978-82-425-2172-9; NILU: 2010.

CONFIDENTIAL

**FINAL REPORT**

**Test Facility Study No. 20172120**

**Combined 28-Day Repeated Dose Toxicity Study with the  
Reproduction/Developmental Toxicity Screening Test of DiHEP Aqueous  
Solution by Oral Gavage in Rats**

**SPONSOR:**

Shell International B.V.  
Carel van Bylandtlaan 16  
2596 HR The Hague  
The Netherlands

**TEST FACILITY:**

Charles River Laboratories Den Bosch BV  
Hambakenwetering 7  
5231 DD 's-Hertogenbosch  
The Netherlands

**TABLE OF CONTENTS**

LIST OF APPENDICES .....	5
QUALITY ASSURANCE STATEMENT .....	6
COMPLIANCE STATEMENT AND REPORT APPROVAL.....	8
1. RESPONSIBLE PERSONNEL.....	9
1.1. Test Facility .....	9
1.2. Individual Scientists (IS) at Test Facility.....	9
1.3. Sponsor .....	9
2. SUMMARY .....	10
3. INTRODUCTION .....	12
4. MATERIALS AND METHODS .....	13
4.1. Test Item .....	13
4.2. Test Item Characterization.....	13
4.3. Reserve Samples .....	13
4.4. Test Item Inventory and Disposition.....	13
4.5. Dose Formulation and Analysis.....	14
4.5.1. Preparation of Test Item.....	14
4.5.2. Sample Collection and Analysis .....	14
4.6. Test System.....	14
4.6.1. Receipt .....	14
4.6.2. Justification for Test System and Number of Animals.....	14
4.6.3. Animal Identification .....	14
4.6.4. Environmental Acclimation .....	15
4.6.5. Selection, Assignment, Replacement, and Disposition of Animals.....	15
4.6.6. Husbandry .....	15
4.7. Experimental Design.....	16
4.7.1. Administration of Test Materials .....	17
4.7.2. Justification of Route and Dose Levels .....	17
4.8. In-life Procedures, Observations, and Measurements – F <sub>0</sub> -Generation.....	18
4.8.1. Mortality/Moribundity Checks – F <sub>0</sub> -Generation.....	18
4.8.2. Clinical Observations – F <sub>0</sub> -Generation .....	18
4.8.3. Body Weights – F <sub>0</sub> -Generation .....	19
4.8.4. Food Consumption – F <sub>0</sub> -Generation .....	19
4.8.5. Water Consumption – F <sub>0</sub> -Generation.....	19
4.8.6. Functional Tests – F <sub>0</sub> -Generation .....	19
4.8.7. Estrous cycle determination – F <sub>0</sub> -Generation .....	20
4.8.8. Cohabitation/Mating Procedure – F <sub>0</sub> -Generation .....	20
4.8.9. General Reproduction Data – F <sub>0</sub> -Generation.....	20
4.9. In-life Procedures, Observations, and Measurements – F <sub>1</sub> -Generation.....	20
4.9.1. Mortality/Moribundity Checks – F <sub>1</sub> -Generation.....	20
4.9.2. Clinical Observations – F <sub>1</sub> -Generation .....	20
4.9.3. Body Weights – F <sub>1</sub> -Generation .....	21
4.9.4. Sex – F <sub>1</sub> -Generation .....	21
4.9.5. Anogenital Distance – F <sub>1</sub> -Generation .....	21
4.9.6. Areola/Nipple Retention – F <sub>1</sub> -Generation .....	21
4.9.7. Culling – F <sub>1</sub> -Generation .....	21
4.10. Laboratory Evaluations.....	21

4.10.1.	Clinical Pathology.....	21
4.11.	Terminal Procedures – F <sub>0</sub> -Generation .....	24
4.11.1.	Unscheduled Deaths.....	24
4.11.2.	Scheduled Euthanasia .....	24
4.11.3.	Necropsy – F <sub>0</sub> -Generation.....	25
4.11.4.	Organ Weights – F <sub>0</sub> -Generation.....	25
4.11.5.	Tissue Collection and Preservation – F <sub>0</sub> -Generation.....	25
4.11.6.	Histology – F <sub>0</sub> -Generation .....	27
4.11.7.	Histopathology – F <sub>0</sub> -Generation .....	27
4.12.	Terminal Procedures – F <sub>1</sub> -Generation .....	27
4.12.1.	Method of Euthanasia - F <sub>1</sub> -Generation .....	27
4.12.2.	Unscheduled Deaths - F <sub>1</sub> -Generation.....	27
4.12.3.	Scheduled Euthanasia - F <sub>1</sub> -Generation .....	27
5.	CONSTRUCTED VARIABLES.....	28
5.1.	Parental Variables .....	28
5.2.	Reproduction and Developmental Variables .....	28
6.	STATISTICAL ANALYSIS .....	29
6.1.	Parametric .....	29
6.2.	Non-Parametric .....	29
6.3.	Incidence .....	29
7.	COMPUTERIZED SYSTEMS .....	29
8.	RETENTION OF RECORDS, SAMPLES, AND SPECIMENS .....	30
9.	RESULTS.....	31
9.1.	Parental Data.....	31
9.1.1.	Mortality .....	31
9.1.2.	Clinical Observations.....	31
9.1.3.	Body Weights and Body Weight Gains .....	31
9.1.4.	Food Consumption.....	32
9.1.5.	Functional Tests .....	32
9.1.6.	Haematology .....	32
9.1.7.	Coagulation .....	32
9.1.8.	Clinical Chemistry .....	33
9.1.9.	Gross Pathology .....	33
9.1.10.	Organ Weights .....	34
9.1.11.	Histopathology .....	34
9.2.	Reproduction Data .....	35
9.2.1.	Estrous Cycle .....	35
9.2.2.	Mating Index.....	35
9.2.3.	Precoital Time.....	36
9.2.4.	Number of Implantation Sites.....	36
9.2.5.	Fertility Index.....	36
9.3.	Developmental Data.....	36
9.3.1.	Gestation Index and Duration .....	36
9.3.2.	Parturition/Maternal Care .....	36
9.3.3.	Post-Implantation Survival Index .....	36
9.3.4.	Litter Size.....	37
9.3.5.	Live Birth Index.....	37
9.3.6.	Viability Index .....	37

9.3.7.	Lactation Index .....	38
9.3.8.	Clinical Signs .....	38
9.3.9.	Body Weights.....	38
9.3.10.	Sex Ratio.....	38
9.3.11.	Anogenital Distance.....	38
9.3.12.	Areola/Nipple Retention .....	38
9.3.13.	Clinical Biochemistry (T4 levels).....	39
9.3.14.	Macroscopy.....	39
10.	DISCUSSION.....	40
11.	CONCLUSION .....	42

**LIST OF APPENDICES**

Appendix 1 Summary Tables

Appendix 2 Individual Tables

Appendix 3 Test Item Characterization

Appendix 4 Histopathology Report

Appendix 5 Summary of the Dose Range Finder

Appendix 6 Study Numbers used for Data Collection

Appendix 7 Study plan, Last Amended Study Plan, and Deviations



**QUALITY ASSURANCE STATEMENT**

Study title: Combined 28-Day Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test of DiHEP Aqueous Solution by Oral Gavage in Rats.

This report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data. The Test Facility inspection program was conducted in accordance with Standard Operating Procedure. During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

**Test Facility Study No. 20172120**

<b>Type of Inspections</b>	<b>Phase/Process</b>	<b>Start Inspection date</b>	<b>End Inspection date</b>	<b>Reporting date to TFM and SD*</b>	
<b>Study</b>	Final Study Plan	16-Jan-2019	16-Jan-2019	16-Jan-2019	
	Littering/Culling	18-Mar-2019	18-Mar-2019	18-Mar-2019	
	Study Plan Amendment 01	21-May-2019	21-May-2019	21-May-2019	
	Data Review - Formulations	20-Aug-2019	20-Aug-2019	04-Sep-2019	
	Report - Materials and Methods	04-Sep-2019	06-Sep-2019	09-Sep-2019	
	Data Review - Technical Operations	05-Sep-2019	06-Sep-2019	09-Sep-2019	
	Data Review - Necropsy	05-Sep-2019	06-Sep-2019	09-Sep-2019	
	Report - Results	05-Sep-2019	09-Sep-2019	09-Sep-2019	
	Data Review - Clinical Pathology	05-Sep-2019	06-Sep-2019	09-Sep-2019	
	Data Review - Fetal Pathology	06-Sep-2019	06-Sep-2019	09-Sep-2019	
	Data Review - Histology/Pathology				
	Working Group	06-Sep-2019	06-Sep-2019	09-Sep-2019	
	Phase Report - Pathology	06-Sep-2019	06-Sep-2019	09-Sep-2019	
	Statistics	09-Sep-2019	09-Sep-2019	09-Sep-2019	
	Final Report	16-Sep-2019	16-Sep-2019	16-Sep-2019	
	<b>Process</b>	<b>Animal Facilities</b>	21-Jan-2019	31-Jan-2019	04-Feb-2019
		Test Item Handling Exposure Observations/Measurements Specimen Handling			
		<b>Clinical pathology</b>	13-Feb-2019	22-Feb-2019	22-Feb-2019
	Observations/Measurements Specimen Handling				

<b>Test Item Formulation</b> Test Item Handling	19-Feb-2019	28-Feb-2019	07-Mar-2019
<b>Fetal Pathology</b> Observations/Measurements Specimen Handling	26-Feb-2019	08-Mar-2019	08-Mar-2019
<b>Test Item Receipt</b> Test Item Handling	04-Mar-2019	15-Mar-2019	15-Mar-2019
<b>Necropsy</b> Observations/Measurements Specimen Handling	12-Mar-2019	21-Mar-2019	22-Mar-2019
<b>Histology</b> Specimen Handling	26-Mar-2019	29-Mar-2019	29-Mar-2019

\*TFM=Test Facility Management SD = Study Director



R. Dassen  
 Quality Assurance Auditor

**COMPLIANCE STATEMENT AND REPORT APPROVAL**

The study was performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

This study was conducted in accordance with the procedures described herein. All deviations authorized/acknowledged by the Study Director are documented in the Study Records. The report represents an accurate and complete record of the results obtained. There were no deviations from the above regulations that affected the overall integrity of the study or the interpretation of the study results and conclusions.



D. van den Oetelaar, MSc  
Study Director

**1. RESPONSIBLE PERSONNEL**

**1.1. Test Facility**

Study Director	D. van den Oetelaar, MSc
Test Facility Management	H.H. Emmen, MSc

**1.2. Individual Scientists (IS) at Test Facility**

Histopathology	E.J.M. Lambregts, DVM
----------------	-----------------------

**1.3. Sponsor**

Sponsor Representative	M. Rooseboom, PhD, ERT
------------------------	------------------------

## 2. SUMMARY

The objectives of this study were to determine the potential toxic effects of Dihydroxyethylpiperazine (DiHEP) Aqueous Solution when given orally by gavage for a minimum of 28 days to Wistar Han rats, and to evaluate the potential to affect male and female reproductive performance such as gonadal function, mating behaviour, conception, parturition and early postnatal development.

In addition, parental, reproduction (up to and including implantation) and developmental (from implantation onwards) No Observed Adverse Effect Levels (NOAELs) were evaluated.

The dose levels in this study were selected to be 0, 100, 300 and 1000 mg/kg/day, based on information provided by the sponsor (see section 4.7.2). The tolerability of 1000 mg/kg/day was confirmed in a dose range finder (Test Facility Study No. 521627, see Appendix 5).

The study design was as follows:

Text Table 1  
Experimental Design

Group No.	Test Item Id.	Dose Level (mg/kg)	Dose Volume (mL/kg) <sup>a</sup>	Corrected Test Item Concentration (mg/mL) <sup>b</sup>	Number of Animals	
					Males	Females
1	-	0 (Control) <sup>c</sup>	2.07	-	10	10
2	DiHEP Aqueous Solution	100	0.21	482.9	10	10
3		300	0.62		10	10
4		1000	2.07		10	10

Id.= identification.

<sup>a</sup> Dose volume was calculated as dose level (mg/kg) / corrected test item concentration (mg/ml).

<sup>b</sup> The test item consisted of 43.9% DiHEP, test item concentration was corrected for % DiHEP and specific gravity (factor: 1.1). See section 4.5.1 for details.

<sup>c</sup> Test-item treated animals received undiluted test item and consequently, no vehicle was used. Control animals were dosed with water (Elix) at the same dose volume as Group 4.

The following parameters and end points were evaluated in this study: mortality/morbidity, clinical signs, functional observations, body weight and food consumption, estrous cycle determination, clinical pathology, measurement of thyroid hormone T4 (F<sub>0</sub>-males), gross necropsy findings, organ weights and histopathologic examinations.

In addition, the following reproduction/developmental parameters were determined: mating and fertility indices, precoital time, number of implantation sites, gestation index and duration, parturition, maternal care, sex ratio and early postnatal pup development (mortality, clinical signs, body weights, sex, anogenital distance, areola/nipple retention and macroscopy, measurement of thyroid hormone T4 (PND 4 and PND 14-16 pups)).

No parental toxicity was observed up to 1000 mg/kg, test item-related findings were limited to non-adverse changes as described below:

Clinical signs included slight salivation directly after dosing in animals at 1000 mg/kg, which was regarded as a physiological response to the taste of the test item rather than a sign of systemic toxicity. In addition, piloerection and hunched posture were noted on multiple consecutive days for a few animals at 1000 mg/kg/day. At the incidence observed, and since the findings were transient, these were considered not toxicologically significant.

The slight, not statistically significant, changes in mean grip strength (females at 1000 mg/kg/day), total movements (males and females at 100 and 1000 mg/kg/day) and ambulations (males at 100 and 1000 mg/kg/day and females at all dose levels) were not supported by clinical observations or other functional observation tests, were slight in nature (within the normal range for rats of this age and strain), and had no supportive morphological

correlates in examined neuronal tissues. These effects were therefore considered not to represent an adverse effect on neurobehaviour.

Thymus weight (absolute and relative) was decreased in all treated males in a dose-related manner. No statistical significance was achieved, all values remained within the historical control range and this decrease was not accompanied by any anatomic pathology alterations. As such, this finding was considered non-adverse.

Microscopic test item-related findings were present in the stomach of males at 1000 mg/kg/day, consisting of (multi) focal erosions of the glandular mucosa. Based on the low degrees (up to slight) and absence of concomitant test item-related microscopic changes, these findings were regarded as non-adverse.

No reproduction or developmental toxicity was observed up to the highest dose level tested (1000 mg/kg/day).

In conclusion, based on the results of this combined 28-day repeated dose toxicity study with the reproduction/developmental toxicity screening test, the Parental, Reproduction and Developmental No Observed Adverse Effect Levels (NOAEL) for DiHEP Aqueous Solution were established to be at least 1000 mg/kg/day.



### 3. INTRODUCTION

The objectives of this study were to determine the potential toxic effects of DiHEP Aqueous Solution when given orally by gavage for a minimum of 28 days to Wistar Han rats, and to evaluate the potential to affect male and female reproductive performance such as gonadal function, mating behaviour, conception, parturition and early postnatal development.

In addition, parental, reproduction (up to and including implantation) and developmental (from implantation onwards) No Observed Adverse Effect Levels (NOAELs) were evaluated.

The design of this study was based on the following study guidelines:

- OECD 422, Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, 2016.
- EPA OPPTS 870.3650, Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, 2000.

In addition, the procedures described in this study plan essentially conform to the following guidelines:

- OECD 421, Reproduction/Developmental Toxicity Screening Test, 2016.
- EPA OPPTS 870.3550, Reproduction/Developmental Toxicity Screening Test, 2000.
- EC No 440/2008, B.7 Repeated Dose (28 days) Toxicity (oral), 2008.
- OECD 407, Repeated Dose 28-day Oral Toxicity Study in Rodents, 2008.
- EPA OPPTS 870.3050, Repeated Dose 28-day Oral Toxicity Study in Rodents, 2000.

The Study Director signed the study plan on 16 Jan 2019, and dosing of the main study was initiated on 05 Feb 2019. The in-life phase of the main study was completed on 02 Apr 2019. The experimental start date was 18 Jan 2019, and the experimental completion date was 11 Jun 2019. The study plan, last amended study plan and deviations are presented in [Appendix 7](#).

For data collection in the dose range finder, an additional Test Facility Study No. was generated (No. 521627). For a complete overview of all used study numbers, see [Appendix 6](#). The entire study is reported under Test Facility Study No. 20172120.

## 4. MATERIALS AND METHODS

### 4.1. Test Item

Identification:	DiHEP Aqueous Solution <sup>1</sup>
Batch (Lot) Number:	D2921BF000
Expiry date:	31 August 2020 (expiry date)
Physical Description:	Yellow aqueous liquid
Purity/Composition:	See Certificate of Analysis <sup>2</sup>
Storage Conditions:	At room temperature protected from light

#### Additional information

Test Facility Test Item Number:	209813/A
Purity/Composition correction factor:	Correct for percentage solid matter and additionally for percentage DiHEP.
Test item handling:	No specific handling conditions required
Chemical name (IUPAC, synonym or trade name):	1,4-Bis(2-hydroxyethyl)piperazine Dihydroxyethylpiperazine
CAS number:	122-96-3
Molecular formula:	C <sub>8</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>
Molecular weight:	174.24
Irritant or corrosive:	Yes
pH:	13
Specific gravity / density:	1.1 kg/m <sup>3</sup> at 20°C

### 4.2. Test Item Characterization

The Sponsor provided to the Test Facility documentation of the identity, purity, composition, and stability for the test item. The characterization of the test item was conducted in a GLP quality environment. A Certificate of Analysis was provided to the Test Facility and is presented in [Appendix 3](#).

### 4.3. Reserve Samples

For the used batch (lot) of test item, a reserve sample (about 0.5 gram) was collected and maintained under the appropriate storage conditions by the Test Facility. The sample will be destroyed after the expiry date.

### 4.4. Test Item Inventory and Disposition

Records of the receipt, distribution, and storage of test item were maintained. With the exception of reserve samples, all unused Sponsor-supplied test item was discarded after completion of the scheduled program of work. Records of the decisions made will be kept at the Test Facility.

---

<sup>1</sup> DiHEP stands for Dihydroxyethylpiperazine.

<sup>2</sup> For Certificate of Analysis see [Appendix 3](#).

#### **4.5. Dose Formulation and Analysis**

##### **4.5.1. Preparation of Test Item**

The test item, DiHEP Aqueous Solution was administered as received. An adequate amount of the test item was dispensed into daily aliquots, which were stored in a controlled temperature area set to maintain 21°C until use.

Test item dosing formulations were kept at room temperature until dosing.

Adjustment was made for specific gravity of the test item. A factor of 43.9 was used to correct for the purity/composition of the test item (based on the 48.9% solid matter of which 89.7% is DiHEP).

Any residual volumes were discarded.

##### **4.5.2. Sample Collection and Analysis**

The test item was used as received from the Sponsor; therefore, samples for dose formulation analysis were not collected by the Test Facility.

#### **4.6. Test System**

##### **4.6.1. Receipt**

On 16 Jan 2019, female Crl: WI(Han) rats were received and on 30 Jan 2019, male Crl:WI(Han) rats were received from Charles River Deutschland, Sulzfeld, Germany. At initiation of dosing, males were 10-11 weeks old and weighed between 268 and 331 g and females were 13-14 weeks old and weighed between 201 and 246 g.

A health inspection was performed before the initiation of dosing.

##### **4.6.2. Justification for Test System and Number of Animals**

The Wistar Han rat was chosen as the animal model for this study as it is an accepted rodent species for toxicity testing by regulatory agencies. Charles River Den Bosch has general and reproduction/developmental historical data in this species from the same strain and source. This animal model has been proven to be susceptible to the effects of reproductive toxicants.

The total number of animals used in this study was considered to be the minimum required to properly characterize the effects of the test item. This study has been designed such that it does not require an unnecessary number of animals to accomplish its objectives.

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

This study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of project license AVD2360020172866 approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

##### **4.6.3. Animal Identification**

Prior to start of the pretest period (females) or treatment period (males), each animal was identified using earmark and tattoo. Prior to the pretest period, reserve females were numbered R1 through R8 at random by indelible marker. Any reserve female replacing an allocated female prior to treatment received identification by earmark and tattoo.

Pups were identified on postnatal day (PND) 1. They were randomized per litter and individually identified by means of subcutaneous injection of Indian ink. When general hair growth blurred the identification, the pups were identified by tattoo on the feet.

#### **4.6.4. Environmental Acclimation**

The animals were allowed to acclimate to the Test Facility toxicology accommodation for 6 days prior to start of the pretest period (females) or 6 days before the commencement of dosing (males).

#### **4.6.5. Selection, Assignment, Replacement, and Disposition of Animals**

A total of 40 females was selected at randomization before initiation of the pretest phase. Any selected female classified as not having regular estrous cycles during the pretest phase was replaced before initiation of dosing by one of the 8 additional females having regular estrous cycles. A total of 40 females with regular estrous cycles continued in the study. The supernumerary females were removed from the study, and their estrous cycle results were kept in the raw data but not reported.

Animals were assigned to groups by a computer-generated random algorithm according to body weights, with all animals within  $\pm 20\%$  of the sex mean. Males and females were randomized separately.

#### **4.6.6. Husbandry**

##### **4.6.6.1. Housing**

On arrival and following the pretest (females only) and pre-mating period, animals were group housed (up to 5 animals of the same sex and same dosing group together) in polycarbonate cages (Macrolon, MIV type, height 18 cm).

During the mating phase, males and females were cohabitated on a 1:1 basis in Macrolon plastic cages (MIII type, height 18 cm).

During the post-mating phase, males were housed in their home cage (Macrolon plastic cages, MIV type, height 18 cm) with a maximum of 5 males/cage. Females were individually housed in Macrolon plastic cages (MIII type, height 18 cm).

During the lactation phase, females were housed in Macrolon plastic cages (MIII type, height 18 cm). Pups were housed with the dam, except during locomotor activity monitoring of the dams, when the pups were kept warm in their home cage using bottles filled with warm water. In order to avoid hypothermia of pups, pups were not left without their dam or a bottle filled with warm water for longer than 30-40 minutes.

During locomotor activity monitoring, animals were housed individually in a Hi-temp polycarbonate cage (Ancare corp., USA; dimensions: 48.3 x 26.7 x 20.3 cm) without cage-enrichment, bedding material, food and water.

The cages contained appropriate bedding (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) and were equipped with water bottles. The rooms in which the animals were kept were documented in the study records.

Animals were separated during designated procedures/activities.

Each cage was clearly labeled with a color-coded cage card indicating Test Facility Study No., group, animal number(s), and sex.

**4.6.6.2. Environmental Conditions**

Target temperatures of 18 to 24°C with a relative target humidity of 40 to 70% were maintained. The actual daily mean temperature during the study period was 20 to 21°C with an actual daily mean relative humidity of 45 to 51%. A 12-hour light/12-hour dark cycle was maintained. Ten or greater air changes per hour with 100% fresh air (no air recirculation) were maintained in the animal rooms.

**4.6.6.3. Food**

Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) was provided *ad libitum* throughout the study, except during designated procedures. During motor activity measurements, animals had no access to food for a maximum of 2 hours.

The feed was analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis were provided by the supplier and are on file at the Test Facility.

It is considered that there were no known contaminants in the feed that would interfere with the objectives of the study.

**4.6.6.4. Water**

Municipal tap water was freely available to each animal via water bottles. During motor activity measurements, animals had no access to water for a maximum of 2 hours.

Periodic analysis of the water was performed, and results of these analyses are on file at the Test Facility.

It is considered that there were no known contaminants in the water that would interfere with the objectives of the study.

**4.6.6.5. Animal Enrichment**

For psychological/environmental enrichment and nesting material, animals were provided with paper (Enviro-dri, Wm. Lilico & Son (Wonham Mill Ltd), Surrey, United Kingdom).

**4.6.6.6. Veterinary Care**

Veterinary care was available throughout the course of the study; however, no examinations or treatments were required.

**4.7. Experimental Design**

Text Table 2  
Experimental Design

Group No.	Test Item Id.	Dose Level (mg/kg)	Dose Volume (mL/kg) <sup>a</sup>	Corrected Test Item Concentration (mg/mL) <sup>b</sup>	Number of Animals		Animal Numbers	
					Males	Females	Males	Females
1	-	0 (Control) <sup>c</sup>	2.07	-	10	10	01-10	41-50
2	DiHEP	100	0.21	482.9	10	10	11-20	51-60
3	Aqueous	300	0.62		10	10	21-30	61-70
4	Solution	1000	2.07		10	10	31-40	71-80

Id.= identification.

<sup>a</sup> Dose volume was calculated as dose level (mg/kg) / corrected test item concentration (mg/ml).

<sup>b</sup> The test item consisted of 43.9% DiHEP, test item concentration was corrected for % DiHEP and specific gravity (factor: 1.1). See section 4.5.1 for details.

<sup>c</sup> Test-item treated animals received undiluted test item and consequently, no vehicle was used. Control animals were dosed with water (Elix) at the same dose volume as Group 4.

The following 5 animals/sex/group were selected for functional tests, clinical pathology, collection of full list of organs/tissues at macroscopic examination, organ weights (full list) and histopathology (full list), see also respective paragraphs:

Group No.	Animal numbers	
	Males	Females <sup>a</sup>
1	01-05	41, 44, 45, 47, 50
2	11-15	52, 54, 56, 57, 58
3	21-25	62, 64, 66, 68, 70
4	31-35	71, 73, 74, 79, 80

<sup>a</sup> Females with living pups.

#### 4.7.1. Administration of Test Materials

The test item or water (Elix) were administered to the appropriate animals by once daily oral gavage 7 days a week for a minimum of 28 days. Males were treated for 29 days, i.e. 14 days prior to mating, during mating and up to and including the day before scheduled necropsy. Females that delivered were treated for 51-56 days, i.e. 14 days prior to mating (with the objective to cover at least two complete estrous cycles), the variable time to conception, the duration of pregnancy and at least 14 days after delivery, up to and including the day before scheduled necropsy. Females which failed to deliver were treated for 42-52 days.

The first day of dosing was designated as Day 1.

On Day 2 of dosing, the males of Group 2 received a dose volume corresponding with a dose level of 1000 mg/kg instead of 100 mg/kg. Based on the available results, the single dose at the same level as Group 4 animals did not impact the study outcome.

Female Nos. 43 (control), 55, 59 (100 mg/kg/day), 61, 62, 67 (300 mg/kg/day), 77, 78 (1000 mg/kg/day), were not dosed on one occasion as these females were littering at the moment of dosing. The omission of one day of dosing over a period of several weeks was considered not to affect the toxicological evaluation.

The dose volume for each animal was based on the most recent body weight measurement. The doses were given using a plastic feeding tube.

A dose control system (DCS) was used as additional check to verify the dosing procedure according to Standard Operating Procedures.

Pups were not treated directly but were potentially exposed to the test item in utero, via maternal milk, or from exposure to maternal urine/feces.

#### 4.7.2. Justification of Route and Dose Levels

The oral route of administration was selected because this is the recommended route by OECD TG 422, REACH regulation and ECHA guidelines. In addition, the test material is a non-volatile aqueous solution.

The dose levels were selected based on information provided by the Sponsor (data on file at Sponsor site), and in an attempt to produce graded responses to the test item.

A previously performed acute toxicity study with DiHEP Aqueous Solution via oral gavage in Sprague Dawley (SD) rats indicated a low acute toxicity (LD50<sub>males</sub> was 20,093 mg/kg and LD50<sub>females</sub> was 18,738 mg/kg).

In addition, multiple studies were performed with several structural analogs:

- A dietary 7-day toxicity study was performed with a hydroxypiperazine solution (containing 12-20% piperazine, 38-47% hydroxypiperazine, 16-25%



dihydroxypiperazine, 17-26% water). During this study Wistar han rats received 590, 1420, and 3720 mg/kg for males and 680, 1610 and 3970 mg/kg for female rats. A slight body weight decrease was observed in females fed 3970 mg/kg/day but was not observed in females fed lower doses or in males. This body weight decrease was statistically significant after days 1 and 4 but not day 7. Remaining parameters were considered unaffected by treatment.

- A dietary 90-day toxicity study was performed with analog piperazine. During this study, SD rats received 400, 1200 and 2394 mg/kg by dietary administration. A dose related decrease in body-weight gain (a decrease of 10% in high dose animals when compared with concurrent control) was noted. Remaining parameters were considered unaffected by treatment.
- A dietary 90-day toxicity study was performed with analog anhydrous piperazine. Rats received 1000, 3000 and 10000 ppm (corresponding to 50, 150 and 500 mg/kg/day piperazine base). At 10000 ppm histopathological degenerative changes were noted in the liver and kidney, at 3000 ppm similar changes were noted to a lesser extent and at 1000 ppm no adverse effects were noted. In addition, at 10000 ppm a decrease in body weight gain was noted (statistically significant in females only). Remaining parameters were considered unaffected by treatment.
- A developmental toxicity study was performed, in which pregnant SD females received 0, 105, 420 and 2100 mg/kg piperazine base during days 6-15 by oral gavage. In high-dose females, excessive salivation, lethargy and a reduction in body weight gain and food consumption were noted. Remaining parameters, including pre- and post-implantation loss, litter size and sex ratio, were considered unaffected by treatment.
- In a dietary two generation study with piperazine dihydrochloride, SD rats received 0, 5000, 12000 and 25000 ppm (corresponding to 0, 125, 300 and 625 mg/kg piperazine base). The mid-dose was considered as LOAEL, with effects mainly on fertility (i.e. reduced pregnancy index and decreased number of implantation sites). These effects were not observed in the developmental toxicity study, which is be considered to support that the effect on fertility are the main effect of piperazine on reproduction.

Based on the observed low acute toxicity of DiHEP Aqueous Solution and the effects noted in the studies performed with structural analogs, 0, 100, 300 and 1000 mg/kg were selected as dose levels for this study.

The high-dose level should produce some toxic effects, but not death nor obvious suffering. The mid-dose level is expected to produce minimal to moderate toxic effects. The low-dose level should produce no observable indications of toxicity.

#### **4.8. In-life Procedures, Observations, and Measurements – F<sub>0</sub>-Generation**

##### **4.8.1. Mortality/Moribundity Checks – F<sub>0</sub>-Generation**

Throughout the study, animals were observed for general health/mortality and moribundity twice daily, in the morning and at the end of the working day. Animals were not removed from the cage during observation, unless necessary for identification or confirmation of possible findings.

##### **4.8.2. Clinical Observations – F<sub>0</sub>-Generation**

Clinical observations were performed once daily, beginning during the first administration of the test item and lasting throughout the dosing periods up to the day prior to necropsy.

During the dosing period, these observations were performed after dosing at no specific time point, but within a similar time period after dosing for the respective animals (no peak effect of occurrence of clinical signs was observed in the dose range finder (Test Facility Study No. 521627, see [Appendix 5](#))).

The time of onset, grade and duration of any observed sign was recorded. Signs were graded for severity and the maximum grade was predefined at 3 or 4. Grades were coded as slight (grade 1), moderate (grade 2), severe (grade 3) and very severe (grade 4). For certain signs, only its presence (grade 1) or absence (grade 0) was scored. In the data tables, the scored grades were reported, as well as the percentage of animals affected in summary tables.

#### **4.8.2.1. Arena Observations – F<sub>0</sub>-Generation**

Clinical observations were conducted in a standard arena beginning before the first administration of the test item and then once weekly throughout treatment.

#### **4.8.3. Body Weights – F<sub>0</sub>-Generation**

Animals were weighed individually on the first day of treatment (prior to dosing), and weekly thereafter. Mated females were weighed on Days 0, 4, 7, 11, 14, 17, and 20 post-coitum and during lactation on PND 1, 4, 7, and 13.

A terminal weight was recorded on the day of scheduled necropsy (fasted for males and non-fasted for females).

#### **4.8.4. Food Consumption – F<sub>0</sub>-Generation**

Food consumption was quantitatively measured weekly, except for males and females which were housed together for mating and for females without evidence of mating. Food consumption of mated females was measured on Days 0, 4, 7, 11, 14, 17, and 20 post-coitum and during lactation on PND 1, 4, 7, and 13.

#### **4.8.5. Water Consumption – F<sub>0</sub>-Generation**

Subjective appraisal was maintained during the study, but no quantitative investigation was introduced as no effect was expected or noted at visual inspection of the water bottles.

#### **4.8.6. Functional Tests – F<sub>0</sub>-Generation**

Functional tests were performed on the selected 5 males during Week 4 of treatment and the selected 5 females during the last week of lactation (i.e. PND 10-13). These tests were performed after completion of clinical observations (including arena observation, if applicable).

The following tests were performed (abbreviations mentioned in the respective tables are indicated between brackets):

- Hearing ability (HEARING) (Score 0 = normal/present, score 1 = abnormal/absent).
- Pupillary reflex (PUPIL L/R) (Score 0 = normal/present, score 1 = abnormal/absent).
- Static righting reflex (STATIC R) (Score 0 = normal/present, score 1 = abnormal/absent).
- Fore- and hind-limb grip strength, recorded as the mean of three measurements per animal (Series M4-10, Mark-10 Corporation, J.J. Bos, Gouda, The Netherlands).
- Locomotor activity (recording period: 1-hour under normal laboratory light conditions, using a computerized monitoring system, Kinder Scientific LLC, Poway, USA). Total movements and ambulations were reported. Ambulations represent

movements characterized by a relocation of the entire body position like walking, whereas total movements represent all movements made by the animals, including ambulations but also smaller or more fine movements like grooming, weaving or movements of the head.

#### **4.8.7. Estrous cycle determination – F<sub>0</sub>-Generation**

Estrous cycles were evaluated by examining the vaginal cytology of samples obtained by vaginal lavage.

Daily vaginal lavage was performed for all females beginning 14 days prior to treatment (pretest period), the first 14 days of treatment and during mating until evidence of copulation was observed. Vaginal lavage was continued for those females with no evidence of copulation until termination of the mating period.

On the day of necropsy, a vaginal lavage was also taken to determine the stage of estrous.

#### **4.8.8. Cohabitation/Mating Procedure – F<sub>0</sub>-Generation**

After 14 days of treatment, animals were cohabitated on a 1:1 basis within the same treatment group, avoiding sibling mating. Detection of mating was confirmed by evidence of sperm in the vaginal lavage or by the appearance of an intravaginal copulatory plug. This day was designated Day 0 post-coitum. Once mating had occurred, the males and females were separated.

Detection of mating was not confirmed in first instance for female no. 78. Evidence of mating was obtained by palpation and indirectly by delivery of a litter. Apparently, mating was overlooked in the assessment of the vaginal lavage, which explains the continued estrous during the mating in this female. The mating date of this animal was estimated at 21 days prior to the actual delivery date. This day was designated Day 0 post-coitum.

#### **4.8.9. General Reproduction Data – F<sub>0</sub>-Generation**

From the mating period onwards, the following parameters were recorded for each female: male number paired with, mating date, confirmation of pregnancy and delivery day.

Females were allowed to litter normally. Postnatal day (PND) 1 is defined as the day when a litter is found completed (i.e. membranes and placentas cleaned up, nest built and/or feeding of pups started). The day prior to PND 1 is considered to be the day when the female started to deliver and is defined as PND 0 and used for recording of delivery. Females that were littering were left undisturbed.

Cage debris of pregnant females was examined for evidence of premature delivery and pregnant females were examined to detect signs of difficult or prolonged parturition or deficiencies in maternal care.

### **4.9. In-life Procedures, Observations, and Measurements – F<sub>1</sub>-Generation**

#### **4.9.1. Mortality/Moribundity Checks – F<sub>1</sub>-Generation**

Pups were observed daily for general health/mortality. The number of live and dead pups was determined on PND 1 and daily thereafter. Pups were not removed from the cage during observation, unless necessary for identification or confirmation of possible findings.

#### **4.9.2. Clinical Observations – F<sub>1</sub>-Generation**

Clinical observations were performed at least once daily for all pups. Only days on which clinical signs were present between the first and last litter check were given in the respective report tables.

**4.9.3. Body Weights – F<sub>1</sub>-Generation**

Live pups were weighed individually on PND 1, 4, 7 and 13.

**4.9.4. Sex – F<sub>1</sub>-Generation**

Sex was externally determined for all pups on PND 1 and 4.

**4.9.5. Anogenital Distance – F<sub>1</sub>-Generation**

Anogenital distance (AGD) was measured for all live pups on PND 1. The AGD was normalized to the cube root of body weight.

**4.9.6. Areola/Nipple Retention – F<sub>1</sub>-Generation**

All male pups in each litter were examined for the number of areola/nipples on PND 13.

**4.9.7. Culling – F<sub>1</sub>-Generation**

To reduce variability among the litters, on PND 4 eight pups from each litter of equal sex distribution (if possible) were selected. Blood samples were collected from two of the surplus pups (if possible from one male and one female pup). Selective elimination of pups, e.g. based upon body weight or AGD, was not done.

**4.10. Laboratory Evaluations****4.10.1. Clinical Pathology****4.10.1.1. Sample Collection**

Blood of F<sub>0</sub>-animals was collected on the day of scheduled necropsy. Samples were collected, between 7.00 and 10.30 a.m., from the retro-orbital sinus under anesthesia using isoflurane in the animal facility. Due to clotting of non-serum samples of individual animals, additional blood samples were obtained in necropsy room. After collection all samples were transferred to the appropriate laboratory for analysis.

F<sub>0</sub>-males were fasted overnight with a maximum of 24 hours before blood sampling, but water was available. F<sub>0</sub>-females were not fasted overnight.

Blood of F<sub>1</sub>-animals was collected on PND 4 and PND 14-16, if possible. This was performed in the necropsy room.

On PND 4 at culling, blood was collected from two surplus pups per litter (if possible) by decapitation, between 7.00 and 10.30 a.m., and samples were pooled to one sample per litter. If available, blood was collected from one male and one female pup. If only one surplus pup per litter was available at culling, as much blood as possible was collected from this single pup.

On PND 14-16, separate blood samples were collected from two pups per litter (from one male and one female). Blood was drawn, between 7.00 and 10.30 a.m., by aorta puncture under anaesthesia using isoflurane as part of the necropsy procedure.

Samples were collected according to the following table.

Text Table 3  
Samples for Clinical Pathology Evaluation

Animals	Time Point	Hematology	Coagulation	Clinical Chemistry	Thyroid Hormone
Selected F <sub>0</sub> -animals (5/sex/group) <sup>a</sup>	On the day of scheduled necropsy	X	X	X	X
Non-selected F <sub>0</sub> -animals (≤ 5/sex/group) <sup>a, b</sup>	On the day of scheduled necropsy	-	-	-	X
2 pups/litter	PND 4	-	-	-	X
2 pups/litter	PND 14-16	-	-	-	X

X = Collected sample; - = Not applicable.

<sup>a</sup> See section 4.7 for details of the selected F<sub>0</sub>-animals.

#### 4.10.1.2. Hematology

Blood samples at a target volume of 0.5 mL were collected into tubes containing K<sub>3</sub>-EDTA as anticoagulant. Samples were analyzed for the parameters specified in the following table.

Text Table 4  
Hematology Parameters

White blood cells (WBC)	Red Blood Cell Distribution Width (RDW)
Neutrophils (absolute)	Haemoglobin
Lymphocytes (absolute)	Haematocrit
Monocytes (absolute)	Mean corpuscular volume (MCV)
Eosinophils (absolute)	Mean corpuscular haemoglobin (MCH)
Basophils (absolute)	Mean corpuscular haemoglobin concentration (MCHC)
Red blood cells	Platelets
Reticulocytes (absolute)	

A blood smear was prepared from each hematology sample. Blood smears were labeled, stained, and stored. In case additional examination of blood smears was deemed necessary, the smears were subsequently evaluated.

#### 4.10.1.3. Coagulation

Blood samples at a target volume of 0.45 mL were collected into tubes containing Citrate as anticoagulant. Samples were processed for plasma, and plasma was analyzed for the parameters listed in the following table.

Text Table 5  
Coagulation Parameters

Prothrombin Time (PT)	Activated Partial Thromboplastin Time (APTT)
-----------------------	--

#### 4.10.1.4. Clinical Chemistry

Blood samples at a target volume of 0.5 mL (plasma) were collected into tubes containing Li-Heparin as anticoagulant. Serum samples at a target volume of 0.25 mL for males and 1.0 mL for females were collected in tubes without anticoagulant. Blood samples were processed for plasma or serum (bile acids), which was analyzed for the parameters specified in the following table.

Text Table 6  
Clinical Chemistry Parameters

Alanine aminotransferase (ALAT)	Creatinine
Aspartate aminotransferase (ASAT)	Glucose
Alkaline Phosphatase (ALP)	Cholesterol
Total protein	Sodium
Albumin	Potassium
Total Bilirubin	Chloride
Bile Acids	Calcium
Urea	Inorganic Phosphate (Inorg. Phos)

**4.10.1.5. Thyroid hormone**

Blood samples at a target volume of 1.0 mL (F<sub>0</sub>-animals), 0.5 mL (pooled PND 4 pups) and 1.0 mL (PND 14-16 pups) were collected into tubes without anticoagulant. Blood samples were processed for serum, and serum was analyzed for total Thyroxine (T4).

Measurement of total T4 was conducted for F<sub>0</sub>-males and PND 14-16 pups PND 4 pups.

For the F<sub>0</sub>-generation, assessment of T4 (females) and Thyroid Stimulating Hormone (TSH; both sexes) was considered not relevant because no treatment-related changes in T4 were noted in F<sub>0</sub>-males, no adverse effects on thyroid histopathology were noted and no treatment related changes in thyroid weight were recorded (see section 9.1.8 for more details).

Assessment of T4 for PND 4 pups and TSH for PND 14-16 pups was considered not relevant because no treatment-related changes in T4 were noted in pups at PND 14-16 (see section 9.3.13 for more details).

Serum samples retained for possible future analysis were maintained by the Test Facility in the freezer ( $\leq -75^{\circ}\text{C}$ ). Under these storage conditions, samples are stable for 6 months. Any remaining sample will be discarded.



**4.11. Terminal Procedures – F<sub>0</sub>-Generation**

Terminal procedures are summarized in the following table.

Text Table 7  
Terminal Procedures

Group No.	(Non) Selected Animals	No. of Animals		Scheduled Euthanasia	Necropsy Procedures			Histology and histopathology
		M	F		Necropsy	Tissue Collection	Organ Weights	
1	Selected	5	5	Males: after a minimum of 28 days of administration  Females: PND 14-16	X	X <sup>a</sup>	X <sup>b</sup>	Full list
	Non-selected	5	5					Gross lesions Reproductive tissues <sup>c</sup>
2	Selected	5	5					Gross lesions Target tissues <sup>d</sup> Reproductive tissues <sup>c</sup>
	Non-selected	5	5					Gross lesions Reproductive tissues <sup>c</sup>
3	Selected	5	5					Gross lesions Target tissues <sup>d</sup> Reproductive tissues <sup>c</sup>
	Non-selected	5	5					Gross lesions Reproductive tissues <sup>c</sup>
4	Selected	5	5					Full list
	Non-selected	5	5					Gross lesions Reproductive tissues <sup>c</sup>

X = Conducted procedure.

<sup>a</sup> See Tissue Collection and Preservation table for listing of tissues (section 4.11.5).

<sup>b</sup> See Organ Weights table for listing of tissues (section 4.11.4).

<sup>c</sup> Reproductive tissues were applicable for males that failed to sire and females that failed to deliver pups (i.e. non-pregnant females). See Tissue Collection and Preservation table for listing of tissues (section 4.11.5).

<sup>d</sup> Adrenal glands and Stomach of selected males of Group 2 and 3, based on possible treatment-related changes in these tissues..

**4.11.1. Unscheduled Deaths**

No animals died during the course of the study.

**4.11.2. Scheduled Euthanasia**

Animals surviving until scheduled euthanasia were weighed, and deeply anaesthetized using isoflurane and subsequently exsanguinated and subjected to a full *post mortem* examination.

Scheduled necropsies were conducted on the following days:

Males: Following completion of the mating period (a minimum of 28 days of administration).

Females which delivered: PND 14-16.

Females which failed to deliver (Nos. 42, 46, 51, 53, 63 and 72): With evidence of mating: Post-coitum Days 25-27.

All males surviving to scheduled necropsy were fasted overnight with a maximum of 24 hours before necropsy. Water was available. F<sub>0</sub>- females were not fasted.

#### 4.11.3. Necropsy – F<sub>0</sub>-Generation

All animals were subjected to a full *post mortem* examination, with special attention being paid to the reproductive organs.

Necropsy procedures were performed by qualified personnel with appropriate training and experience in animal anatomy and gross pathology. A veterinary pathologist, or other suitably qualified person, was available.

The numbers of former implantation sites were recorded for all paired females.

In case no macroscopically visible implantation sites were present, non-gravid uteri were stained using the Salewski technique in order to detect any former implantation sites and the number of corpora lutea was recorded in addition (for exceptions, see [Appendix 7](#)).

#### 4.11.4. Organ Weights – F<sub>0</sub>-Generation

The organs identified in the table below were weighed at necropsy for all scheduled euthanasia animals. Paired organs were weighed together. In the event of gross abnormalities, in addition to the combined weight, the weight of the aberrant organ was taken and recorded in the raw data. Organ to body weight ratios (using the terminal body weight) were calculated.

Text Table 8  
Organs Weighed at Necropsy for all selected animals

Brain	Heart
Cervix <sup>a</sup>	Kidney <sup>b</sup>
Epididymis <sup>b</sup>	Liver
Gland, adrenal <sup>b</sup>	Ovaries <sup>b</sup>
Gland, coagulation <sup>b, c</sup>	Spleen
Gland, parathyroid <sup>d</sup>	Testes <sup>b</sup>
Gland, prostate	Thymus
Gland, seminal vesicle <sup>b</sup>	Uterus
Gland, thyroid	

<sup>a</sup> Weighed together with the uterus.

<sup>b</sup> Paired organ weight.

<sup>c</sup> Weighed together with the seminal vesicles.

<sup>d</sup> Weighed together with the thyroid.

Text Table 9

Organs Weighed at Necropsy for all remaining animals (incl. males that failed to sire and females that failed to deliver pups).

Epididymis <sup>a</sup>	Gland, seminal vesicle <sup>a</sup>
Gland, coagulation <sup>a, b</sup>	Gland, thyroid
Gland, parathyroid <sup>c</sup>	Testes <sup>a</sup>
Gland, prostate	

<sup>a</sup> Paired organ weight.

<sup>b</sup> Weighed together with the seminal vesicles.

<sup>c</sup> Weighed together with the thyroid.

#### 4.11.5. Tissue Collection and Preservation – F<sub>0</sub>-Generation

Representative samples of the tissues identified in the table below were collected from all animals and preserved in 10% neutral buffered formalin (neutral phosphate buffered 4% formaldehyde solution, Klinipath, Duiven, The Netherlands), unless otherwise indicated.

Text Table 10  
 Tissue Collection and Preservation for all selected animals

Animal identification Artery, aorta Body cavity, nasopharynx Bone marrow Bone, femur Bone, sternum Brain (eight levels) Cervix Epididymides <sup>a</sup> Esophagus Eye <sup>a</sup> Gland, adrenal Gland, coagulation Gland, Harderian <sup>a, b</sup> Gland, lacrimal Gland, mammary Gland, parathyroid <sup>c</sup> Gland, pituitary Gland, prostate Gland, salivary Gland, seminal vesicle Gland, thyroid Gross lesions/masses Gut-associated lymphoid tissue Heart Kidney	Large intestine, cecum Large intestine, colon Large intestine, rectum Larynx Liver Lung Lymph node (mandibular and mesenteric site) Muscle, skeletal Nerve, optic <sup>a, b</sup> Nerve, sciatic Ovaries Pancreas Skin Small intestine, duodenum Small intestine, ileum Small intestine, jejunum Spinal cord Spleen Stomach Testes <sup>a</sup> Thymus Tongue Trachea Urinary bladder Uterus Vagina
--	--

- <sup>a</sup> Preserved in modified Davidson’s fixative and transferred to formalin after fixation for at least 24 hours.
- <sup>b</sup> Only collected if present in the routine section of the eye.
- <sup>c</sup> Only collected if present in the routine section of the thyroid.

Text Table 11  
 Tissue Collection and Preservation for all remaining animals (incl. males that failed to sire and females that failed to deliver pups).

Animal identification Cervix Epididymis <sup>a</sup> Gland, coagulation Gland, mammary Gland, parathyroid <sup>b</sup> Gland, pituitary Gland, prostate	Gland, seminal vesicle Gland, thyroid Gross lesions/masses Ovaries Testes <sup>a</sup> Uterus Vagina
--	--

- <sup>a</sup> Preserved in modified Davidson’s fixative and transferred to formalin after fixation for at least 24 hours.
- <sup>b</sup> Only collected if present in the routine section of the thyroid.

#### 4.11.6. Histology – F<sub>0</sub>-Generation

The following tissues were embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin:

Selected animals:	Tissues identified in <a href="#">Text Table 10</a> (except animal identification, aorta, nasopharynx, esophagus, harderian gland, lacrimal gland, salivary gland, larynx, optic nerve, pancreas, skin and tongue).
Males that failed to sire (except for males which were selected) and females that failed to deliver pups:	Cervix, epididymis, coagulation gland, prostate gland, seminal vesicles, ovaries, testes, uterus and vagina.
Remaining animals:	Gross lesions/masses.

#### 4.11.7. Histopathology – F<sub>0</sub>-Generation

All tissues as defined under Histology – F<sub>0</sub>-Generation (section [4.11.6](#)) were examined by a board-certified toxicological pathologist with training and experience in laboratory animal pathology. Target tissues identified by the study pathologist during microscopic evaluation were communicated to the Study Director; tissues were evaluated and reported.

For the testes of all selected males of Groups 1 and 4 and all males that failed to sire, a detailed qualitative examination was made, taking into account the tubular stages of the spermatogenic cycle.

A peer review on the histopathology data was performed by a second pathologist.

#### 4.12. Terminal Procedures – F<sub>1</sub>-Generation

##### 4.12.1. Method of Euthanasia - F<sub>1</sub>-Generation

Pups, younger than 7 days were euthanized by decapitation.

All remaining pups (PND 14-16), except for the two pups per litter selected for blood collection were euthanized by an intraperitoneal injection of sodium pentobarbital (Euthasol® 20%).

The pups selected for blood collection on PND 14-16 were anesthetized using isoflurane followed by exsanguination.

##### 4.12.2. Unscheduled Deaths - F<sub>1</sub>-Generation

Pup that died before scheduled termination were examined externally and sexed (both externally and internally). The stomach of pups not surviving to the scheduled necropsy date was examined for the presence of milk, if possible. If possible, defects or cause of death were evaluated.

##### 4.12.3. Scheduled Euthanasia - F<sub>1</sub>-Generation

On PND 4, the surplus pups (> 8 pups per litter) were euthanized by decapitation. From two surplus pups per litter, blood was collected, if possible. For details see also section [4.10.1](#).

All remaining pups were euthanized on PND 14-16. Sex was determined both externally and internally. Descriptions of all external abnormalities were recorded. Particular attention was paid to the external reproductive genitals to examine signs of altered development.

In addition, blood was collected from two pups per litter (see also section [4.10.1](#)), and the thyroid from two pups per litter (one male and one female pup) was preserved in 10%

buffered formalin. The pups selected for (complete) blood sampling were the same pups as selected for thyroid preservation.

## 5. CONSTRUCTED VARIABLES

All results presented in the tables of the report were calculated using values as per the raw data rounding procedure and may not be exactly reproduced from the individual data presented.

### 5.1. Parental Variables

Body Weight Gains:	Calculated against the body weight on Day 1 (pre-mating, mating and lactation periods) or Day 0 (post-coitum period).
Relative Food Consumption	Calculated against the body weight for scheduled intervals.
Organ Weight Relative to Body Weight:	Calculated against the terminal body weight.

### 5.2. Reproduction and Developmental Variables

For each group, the following calculations were performed. Group mean values of precoital time and duration of gestation were calculated from individual values of F<sub>0</sub>-females, the remaining group values were calculated from the total number in each group.

Mating index (%):	$\frac{\text{Number of females mated}}{\text{Number of females paired}} \times 100$
Precoital time:	Number of days between initiation of cohabitation and confirmation of mating
Fertility index (%):	$\frac{\text{Number of pregnant females}}{\text{Number of females mated}} \times 100$
Gestation index (%):	$\frac{\text{Number of females with living pups on Day 1}}{\text{Number of pregnant females}} \times 100$
Duration of gestation:	Number of days between confirmation of mating and the beginning of parturition
Post-implantation survival index (%):	$\frac{\text{Total number of offspring born}}{\text{Total number of uterine implantation sites}} \times 100$
Live birth index (%):	$\frac{\text{Number of live offspring on Day 1 after littering}}{\text{Total number of offspring born}} \times 100$
Percentage live males at First Litter Check (%):	$\frac{\text{Number of live male pups at First Litter Check}}{\text{Number of live pups at First Litter Check}} \times 100$
Percentage live females at First Litter Check (%):	$\frac{\text{Number of live female pups at First Litter Check}}{\text{Number of live pups at First Litter Check}} \times 100$

$$\text{Viability index (\%)}: \frac{\text{Number of live offspring on Day 4 before culling}}{\text{Number live offspring on Day 1 after littering}} \times 100$$

$$\text{Lactation index (\%)}: \frac{\text{Number of live offspring on Day 13 after littering}}{\text{Number live offspring on Day 4 (after culling)}} \times 100$$

## 6. STATISTICAL ANALYSIS

All statistical tests were conducted at the 5% significance level. All pairwise comparisons were conducted using two sided tests and were reported at the 1% or 5% levels.

Numerical data collected on scheduled occasions for the listed variables were analyzed as indicated according to sex and occasion. Descriptive statistics number, mean and standard deviation (or %CV or SE when deemed appropriate) were reported whenever possible. Inferential statistics were performed according to the comparison matrix below when possible, but excluded semi-quantitative data, and any group with less than 3 observations.

The following pairwise comparisons were made:

Group 2 vs. Group 1

Group 3 vs. Group 1

Group 4 vs. Group 1

### 6.1. Parametric

Datasets with at least 3 groups (the designated control group and at least 2 other groups) were compared using Dunnett-test (many-to-one-t-test).

For the motor activity data set (at least 3 groups) parametric (ANOVA) tests on group means were applied with Bonferroni correction for multiple testing. Mixed modelling techniques, comparing six different covariance structures, were used in order to select the best fitting statistical model (for deviation, see [Appendix 7](#)).

### 6.2. Non-Parametric

Datasets with at least 3 groups was compared using a Steel-test (many-to-one rank test).

### 6.3. Incidence

An overall Fisher's exact test was used to compare all groups at the 5% significance level. The above pairwise comparisons were conducted using Fisher's exact test whenever the overall test is significant.

## 7. COMPUTERIZED SYSTEMS

Critical computerized systems used in the study are listed below and/or presented in the appropriate Phase Report. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data. The Study Numbers used to collect online data are presented in [Appendix 6](#).

Text Table 12  
Critical Computerized Systems

System name	Version No.	Description of Data Collected and/or Analyzed
ToxData <sup>a</sup>	8.0	In-life phase (Mortality; Clinical signs; Body weights; Food consumption; Functional tests, Organ weights; Reproduction parameters; Observations pups <sup>b</sup> ) data collection
REES Centron	SQL 2.0	Temperature and humidity (animal and laboratory facilities) data collection
Deviation Information Library	2.1.61	Deviations
ADVIA® 2120i	6.3.2-MS	Haematology data collection
STA Compact®	107.07	Clotting Parameters data collection
AU400	9.1	Clinical biochemistry data collection
MotorMonitor II	16224-11GLP	Motor activity measurement data collection
Pathdata	6.2e2	Histopathology data collection
IMMULITE® 1000	5.22	Thyroid hormone data collection

<sup>a</sup> For logistic reasons, data was captured under separate Study numbers, see [Appendix 6](#).

<sup>b</sup> Only at first and last litter check, and in case of clinical pup findings also on the respective days in between.

## 8. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS

All study-specific raw data, electronic data, documentation, study plan, samples, specimens, and final report(s) from this study were archived at the Test Facility by no later than the date of final report issue.

After two years of archiving, all study-specific raw data, electronic data, documentation, study plan and final reports will be transferred to Iron Mountain Germany, Harpener Hellweg 31, D-44805 Bochum, Germany. They shall be indexed by using the Test Facility Project No. allowing unequivocal identification and providing the necessary information (e.g. test system, test item, date of Toxicology and Ecology for the time period set by the GLP regulations). In order to index and before the actual transfer to Iron Mountain Germany the test facility will connect with the sponsor. Records of transfer will be retained by test facility. The Sponsor will be informed about the transfer and will receive a digital copy of the record of transfer. The sponsor will ensure that all study material is expediently returned to Charles River Den Bosch if requested by GLP monitoring authorities for audit.

## 9. RESULTS

### 9.1. Parental Data

#### 9.1.1. Mortality

(Appendix 2)

No mortality occurred during the study period.

#### 9.1.2. Clinical Observations

(Appendix 1 and Appendix 2)

No test item-related clinical signs were noted during the observation period up to 300 mg/kg/day and no findings were noted during the weekly arena observations up to 1000 mg/kg/day.

At 1000 mg/kg/day, one male showed piloerection on 4 consecutive days and one female showed hunched posture for 6 consecutive days, on 4 occasions combined with piloerection. At the incidence observed, and since the findings were transient, these were considered not toxicologically significant.

Salivation seen after dosing among most males and few females of the 1000 mg/kg/day dose group during week 4 and/or 5 of the treatment period was considered not toxicologically relevant, taking into account the nature and minor severity of the effect and its time of occurrence (i.e. after dosing). This sign was considered to be a physiological response related to taste of the test item rather than a sign of systemic toxicity.

Note to clinical signs tables: For males, “Repro period” represents the mating phase. For females, “Repro period” represents the mating, post coitum and lactation phase.

#### 9.1.3. Body Weights and Body Weight Gains

(Appendix 1 and Appendix 2)

Body weights and body weight gain were considered to have been unaffected by treatment with the test item up to 1000 mg/kg/day.

Body weight gain of control females was slightly lower when compared with historical control data<sup>3</sup> on Day 8 and Day 15 of treatment (Day 1 of the mating period), resulting in the statistical significance noted for mean body weight gain of females at 300 and 1000 mg/kg/day on Day 1 of the mating period. The body weight development of females up to 1000 mg/kg/day remained within normal limits and was therefore considered unaffected by the test item.

The statistical significant higher mean body weight of females at 300 mg/kg/day on Day 17 post coitum was considered unrelated to treatment with the test item, as no trend was apparent regarding dose and duration of treatment.

---

<sup>3</sup> Historical control data for body weight gain for female Wistar Han rats (period 2017 - wk 4 2019).  
Mean body weight gain Day 8 (%) = 2, P5 – P95 = -2 – 6 (n=474).  
Mean body weight gain Day 15 (%) = 4, P5 – P95 = -1 – 6 (n=474).



#### 9.1.4. Food Consumption

(Appendix 1 and Appendix 2)

Food consumption before or after correction for body weight considered to be unaffected by treatment with the test item up to 1000 mg/kg/day.

The statistically significant changes in relative food consumption over Days 7-11 and 14-20 for females at 100 mg/kg/day and over Days 17-20 for females at 300 mg/kg/day were considered to be unrelated to treatment with the test item, as no trend was apparent regarding dose and duration of treatment.

#### 9.1.5. Functional Tests

(Appendix 1 and Appendix 2)

Hearing ability, pupillary reflex and static righting reflex were normal in all examined animals. Forelimb and hind limb grip strength of males were unaffected by treatment with the test item up to 1000 mg/kg/day.

In females at 1000 mg/kg/day, mean grip strength of the fore legs appeared higher than concurrent controls (18%). No statistical significance was achieved and values remained well within historical control range<sup>4</sup>.

Total movements were slightly decreased in males and females at 100 and 1000 mg/kg/day as compared with concurrent control (up to 35%). Ambulations were slightly decreased in males at 100 and 1000 mg/kg/day and females at all dose levels compared with concurrent controls (up to 41%). For males, no dose-related trend was apparent, but for females, a dose-related trend was observed for the decreased ambulations. No statistical significance was achieved and values remained within the historical control range<sup>5</sup>. All groups showed a similar habituation profile with very high activity in the first interval that decreased over the duration of the test period.

#### 9.1.6. Haematology

(Appendix 1 and Appendix 2)

Haematological parameters of treated rats were considered not to have been affected by treatment with the test item up to 1000 mg/kg/day.

The statistically significant change in the mean neutrophil level of males at 100 mg/kg/day was considered to be unrelated to treatment with the test item in the absence of a dose-related trend.

#### 9.1.7. Coagulation

(Appendix 1 and Appendix 2)

Coagulation parameters of treated females were considered not to have been affected by treatment with the test item up to 1000 mg/kg/day.

---

<sup>4</sup> Historical control data of female Wistar Han rats (period 2015-2018):

Grip strength fore leg (gram): Mean = 1009; P5 - P95 = 551 – 1458 (n=400).

<sup>5</sup> Historical control data of Wistar Han rats (period 2015 - 2018):

Total movements: Males: Mean = 3592; P5 - P95 = 1798 – 5617 (n=445).

Females: Mean = 3372; P5 - P95 = 1529 – 5618 (n=400).

Ambulations: Males: Mean = 806; P5 - P95 = 302 – 1295 (n=445).

Females: Mean = 817; P5 - P95 = 317 – 1440 (n=400).

Prothrombin time (PT) of males at 100, 300 and 1000 mg/kg/day was decreased with statistical significance when compared with controls (all 0.94x of control). As there was no dose response and mean values remained within normal limits<sup>6</sup>, this was considered to be of no toxicological relevance.

#### 9.1.8. Clinical Chemistry

(Appendix 1 and Appendix 2)

The following (not statistically significant) changes distinguished treated from control animals. Relative changes in mean values as compared to the concurrent control group are indicated between parentheses:

- Mean urea levels were dose-dependently increased in treated animals (up to 1.1x of control for both sexes). Values generally remained within the historical control range<sup>7</sup>.
- Mean glucose levels in males at 1000 mg/kg were decreased (0.8x of control). Values generally remained within the historical control range<sup>8</sup>.

As no statistical significance was achieved for any of the differences, mean values remained within normal limits and differences were minimal, the above mentioned changes were considered not toxicologically relevant.

#### Thyroid hormone analyses:

Serum T4 levels in F<sub>0</sub>-males were considered not to be affected by treatment with the test item.

Mean serum T4 levels were decreased with statistical significance for males at 300 mg/kg/day (0.8x of control). Since values generally remained within the historical control range<sup>9</sup> and no dose-related trend was observed, this was considered unrelated to treatment with the test item.

#### 9.1.9. Gross Pathology

(Appendix 1 and Appendix 2)

Macroscopic observations at necropsy did not reveal any alterations that were considered to have arisen as a result of treatment with the test item.

A macroscopic finding of note was recorded in two females of the 100 mg/kg/day group (Nos. 52 and 57) and one female of the 300 mg/kg/day group (No. 70). This finding consisted of watery clear fluid of the thoracic cavity. Since this finding was absent in the high dose group, and since there were no other macroscopic findings of organs of the thoracic cavity, or related clinical findings, this finding was considered to be unrelated to the test item.

The remainder of the recorded macroscopic findings were within the range of background gross observations encountered in rats of this age and strain.

---

<sup>6</sup> Historical control data of male Wistar Han rats (period 2017 - wk 4 2019):  
PT (s): Mean = 18.2; P5 - P95 = 16.2 – 21.2 (n=252).

<sup>7</sup> Historical control data of urea (mmol/L) in Wistar Han rats (period 2017 - wk 4 2019):  
Males: mean = 6.7, P5 – P95 = 4.8 – 9.2 (n=270).  
Females: mean = 10.1, P5 – P95 = 8.4 – 12.0 (n=210).

<sup>8</sup> Historical control data of glucose (mmol/L) in Wistar Han rats (period 2017 - wk 4 2019):  
Males: mean = 9.05, P5 – P95 = 6.40 – 12.22 (n=270).

<sup>9</sup> Historical control data of male Wistar Han rats (period 2017 - 2019):  
Total T4 (ug/dL): Mean = 4.51; P5 - P95 = 2.85 – 6.37 (n=557).

**9.1.10. Organ Weights**[\(Appendix 1 and Appendix 2\)](#)

Thymus weights of test item-treated males were lower in a dose-related manner. This organ weight change was not statistically significantly different from the concurrent controls and group mean values remained within background ranges. However, individual organ weight values of 1/5 males of the 300 mg/kg/day group and 2/5 males of the 1000 mg/kg/day group were at the lower range<sup>10</sup>. Therefore a possible test item relationship at these dose levels could not be excluded.

The apparent dose-related decrease noted for absolute and relative spleen weight of treated males was attributed to a relatively high concurrent control value<sup>11</sup>. This change was therefore considered a chance finding, unrelated to the test item.

Any other differences, including those that reached statistical significance (i.e. absolute weight of adrenal glands in males at 300 mg/kg/day and relative weight of the heart in males at 100 mg/kg/day, absolute weight of kidneys in females at 1000 mg/kg/day and relative weight of thymus in females at 100 mg/kg/day) were considered not to be test item-related due to the direction of the change, lack of a dose-related pattern, and/or general overlap and variability in individual values.

**9.1.11. Histopathology**[\(Appendix 4\)](#)

Test item-related microscopic findings after treatment with DiHEP Aqueous Solution were noted in the stomach of males at 1000 mg/kg/day and are summarized in [Text Table 13](#).

Text Table 13  
Summary Test Item-Related Microscopic Findings – Scheduled Euthanasia Animals (Day 29)

Dose level (mg/kg/day):	Males			
	0	100	300	1000
STOMACH <sup>a</sup>	5	5	5	5
<i>Erosion glandular mucosa, focal</i>				
Minimal	-	-	-	2
Slight	-	-	-	1

<sup>a</sup> = Number of tissues examined from each group.

(Multi) focal erosions of the glandular mucosa of the stomach were noted in 1000 mg/kg/day males (up to slight degree). These erosions were all located at the pyloric area.

The remainder of the recorded microscopic findings, including the low degrees of vacuolation of the zona fasciculata of the adrenal glands of males, were within the range of background pathology encountered in rats of this age and strain. There was no test item-related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations.

<sup>10</sup> Historical control data for thymus weight in male Wistar Han rats (period 2017 – wk 4 2019):

Absolute mean = 0.364, P5 – P95 = 0.243 – 0.503 (n=255).

Relative mean = 0.107, P5 – P95 = 0.071 – 0.144 (n=255).

<sup>11</sup> Historical control data for spleen weight in male Wistar Han rats (period 2017 – wk 2019):

Absolute mean = 0.578, P5 – P95 = 0.464 – 0.721 (n=245).

Relative mean = 0.170, P5 – P95 = 0.141 – 0.204 (n=245).

Reproductive performance

Text Table 14

Correlation of Histopathology Findings with In-Life Reason for Males that Failed to Sire and Females that Failed to Deliver Healthy Pups.

Group	Dose level mg/kg bw/day	Female/Male Nos.	In-Life Reason	Histopathology
1	0	42 / 2	Not pregnant	Implantation site and necrosis of placenta of the uterus (observed as nodule)
		46 / 6	Not pregnant	
2	100	51 / 11	Not pregnant	-
		53 / 13	Not pregnant	-
3	300	63 / 23	Not pregnant	-
4	1000	72 / 32	Not pregnant	-

There were 2/10 couples of the control group, 2/10 couples of 100 mg/kg/day group, 1/10 couples of the 300 mg/kg/day group and 1/10 couples of the 1000 mg/kg/day group with no offspring. Female No. 42 showed evidence of a former pregnancy, in the form of the presence of a necrotic placenta. For the other couples, no abnormalities which could account for the lack of offspring were seen in the reproductive organs.

There were no morphological findings in the reproductive organs of either sex which could be attributed to the test item. Stage aware evaluation of the testes did not show any indication for abnormal spermatogenesis. The testis revealed normal progression of the spermatogenic cycle and the expected cell associations and proportions in the various stages of spermatogenesis were present.

**9.2. Reproduction Data**

Note: For one female of the control group (No. 42), no implantation sites were noted at necropsy. As no Salewski staining was performed (see [Appendix 7](#)), the total number of implantation sites could not be determined and was not included in the tables. During the microscopic evaluation one implantation site was noted, providing evidence that this female was in fact pregnant.

**9.2.1. Estrous Cycle**

([Appendix 2](#))

Length and regularity of the estrous cycle were considered not to have been affected by treatment with the test item.

All females had regular cycles of 4 days, with exception of one female at 1000 mg/kg/day (No. 79; with normal litter) for which the estrous stage could not be determined during the pre-mating phase. Given the incidental nature and absence of an apparent correlation to pregnancy status, this finding did not indicate a relation with the test item.

**9.2.2. Mating Index**

([Appendix 1](#) and [Appendix 2](#))

Mating index was not affected by treatment with the test item. All females showed evidence of mating, the mating index was therefore 100% for all groups.

**9.2.3. Precoital Time**

(Appendix 1 and Appendix 2)

Precoital time was not affected by treatment with the test item. Most females showed evidence of mating within 4 days, except for one control female, for which mating took 13 days.

**9.2.4. Number of Implantation Sites**

(Appendix 1 and Appendix 2)

Number of implantation sites was considered not to be affected by treatment with the test item.

**9.2.5. Fertility Index**

(Appendix 1 and Appendix 2)

The fertility index was considered not to be affected by treatment. The fertility indices were 90% for the control, 300 and 1000 mg/kg/day groups and 80% for the 100 mg/kg/day group.

A total of 1/10 control females, 2/10 females at 100 mg/kg/day and 1/10 females at 300 and 1000 mg/kg/day each were not pregnant. In the absence of a dose-related incidence and based on the occurrence within the control group, these non-pregnancies were considered unrelated to the test item.

**9.3. Developmental Data**

Note: For one female of the control group (No. 42), no implantation sites were noted at necropsy. As no Salewski staining was performed (see Appendix 7), the total number of implantation sites could not be determined and was not included in the tables. During the microscopic evaluation one implantation site was noted, providing evidence that this female was in fact pregnant. This female was excluded from the calculation of the post-implantation survival index.

**9.3.1. Gestation Index and Duration**

(Appendix 1 and Appendix 2)

Gestation index and duration of gestation were considered not to be affected by treatment with the test item up to 1000 mg/kg/day.

Except for one control female, all pregnant females had live offspring. The gestation indices were 89% for the control group and 100% for all treated groups.

During microscopic evaluation, an implantation site and necrosis of placenta of the uterus were noted for control Female No. 42. As this occurred in a single female of the control group, the failed pregnancy was considered a chance finding.

**9.3.2. Parturition/Maternal Care**

No signs of difficult or prolonged parturition were noted among the pregnant females. Examination of cage debris of pregnant females revealed no signs of abortion or premature birth. No deficiencies in maternal care were observed.

**9.3.3. Post-Implantation Survival Index**

(Appendix 1 and Appendix 2)

The total number of offspring born compared to the total number of uterine implantations was considered not to be affected by treatment with the test item.

Post-implantation survival index (total number of offspring born as percentage of total number of uterine implantation sites) was 95, 97, 94 and 89% for the control, 100, 300 and 1000 mg/kg groups, respectively. The survival index at 1000 mg/kg/day was slightly lower than concurrent controls, however as this occasionally occurs in controls and the survival index remained within historical control range<sup>12</sup> this was considered a change finding.

For Female No. 61 (300 mg/kg/day), the number of pups was slightly higher than the number of implantations. This phenomenon is observed from time to time and is caused by normal resorption of these areas during (the 16 days of) lactation. No toxicological relevance was attached to this finding in the current study.

#### **9.3.4. Litter Size**

(Appendix 1 and Appendix 2)

Litter size was considered not affected by treatment with the test item up to 1000 mg/kg/day.

Live litter sizes were 11.1, 14.1, 12.0 and 12.9 living pups/litter for the control, 100, 300 and 1000 mg/kg/day groups, respectively.

#### **9.3.5. Live Birth Index**

(Appendix 1 and Appendix 2)

The number of live offspring on Day 1 after littering compared to the total number of offspring born was not affected by treatment.

Live birth indices (number of live offspring on PND 1 as percentage of total number of offspring born) were 100% for the control, 100 and 1000 mg/kg/day groups and 99% for the 300 mg/kg/day group.

One pup of the 300 mg/kg/day group (Litter No. 65) was found dead at first litter check. No toxicological relevance was attributed to this dead pup since the mortality incidence did not show a dose-related trend and remained within the range considered normal for pups of this age.

#### **9.3.6. Viability Index**

(Appendix 1 and Appendix 2)

The number of live offspring on Day 4 before culling compared to the number of offspring on Day 1 was not affected by treatment.

Viability indices (number of live offspring on PND 4 before culling as percentage of number of live offspring on PND 1) were 99% for the control group and 100% for the 100, 300 and 1000 mg/kg/day groups.

One pup of the control group (Litter No. 48) went missing on PND 4, which was most likely cannibalised. The incidence remained within the range considered normal for pups of this age and given the occurrence in control group only this mortality was not attributed to the test item.

---

<sup>12</sup> Historical control data for post-implantation survival index for Wistar Han rats (period 2015 – wk 4 2019): Mean = 92, P5 – P95 = 83 – 98 (n=118).

**9.3.7. Lactation Index**

(Appendix 1 and Appendix 2)

The number of live offspring on Day 13 after littering compared to the number of live offspring on Day 4 (after culling) was not affected by treatment with the test item up to 1000 mg/kg/day. The lactation indices were 98% for the control for the control group and 100% for the 100, 300 and 1000 mg/kg/day groups.

One pup of the control group (Female No. 48) went missing on PND 6, which was most likely cannibalised. The incidence remained within the range considered normal for pups of this age and given the occurrence in control group only this mortality was not attributed to the test item.

One pup at 300 mg/kg/day (Litter No. 61) died spontaneously on PND 16. No toxicological relevance was attributed to this since the mortality incidence did not show a dose-related trend and remained within the range considered normal for pups of this age. Note: as this pup died on PND 16 it was not included in the tables as breeding loss.

**9.3.8. Clinical Signs**

(Appendix 2)

No clinical signs occurred among pups that were considered to be related to treatment with the test item.

The nature and incidence of observed clinical signs remained within the range considered normal for pups of this age, and were therefore considered not to be toxicologically relevant.

Note: Only days on which clinical signs were present between first and last litter check are presented in the table.

**9.3.9. Body Weights**

(Appendix 1 and Appendix 2)

Mean body weights of male and female pups at 1000 mg/kg/day were slightly lower when compared with concurrent controls (up to 0.91x of control). As all values were within normal limits and body weights of these pups were similar to concurrent control on Day 13 of lactation, this slight decrease at the beginning of the lactation period was considered not toxicologically relevant.

**9.3.10. Sex Ratio**

(Appendix 1 and Appendix 2)

Sex ratio was unaffected by treatment with the test item.

**9.3.11. Anogenital Distance**

(Appendix 1 and Appendix 2)

Anogenital distance (absolute and normalized for body weight) in male and female pups was considered not to be affected by treatment with the test item.

**9.3.12. Areola/Nipple Retention**

(Appendix 1 and Appendix 2)

Treatment up to 1000 mg/kg/day had no effect on areola/nipple retention. For none of the examined male pups nipples were observed at PND 13.

**9.3.13. Clinical Biochemistry (T4 levels)**

(Appendix 1 and Appendix 2)

Serum T4 levels in male and female pups at PND 14-16 were considered not to be affected by treatment with the test item. All values remained within the historical control range<sup>13</sup>.

**9.3.14. Macroscopy**

(Appendix 2)

No macroscopic findings were noted among pups that were considered to be related to treatment with the test item.

The nature and incidence of macroscopic findings remained within the range considered normal for pups of this age, and were therefore considered not to be related to treatment.

---

<sup>13</sup> Historical control data for total T4 in PND 14-16 pups of Wistar Han rats (period 2017 – wk4 2019):  
Males mean = 5.93, P5 – P95 = 4.28 – 8.03 (n=499)  
Females mean = 5.57, P5 – P95 = 4.04 – 7.50 (n=499)



## 10. DISCUSSION

Wistar Han rats were treated with undiluted DiHEP Aqueous Solution by daily oral gavage at dose levels of 100, 300 and 1000 mg/kg. Concurrent controls (10 rats/sex) received Elix water.

Males were treated for 2 weeks prior to mating, during mating, and up to termination (for a total of 29 days). Females that delivered offspring were treated for 2 weeks prior to mating, during mating, during post-coitum, and at least 13-15 days of lactation (for a total of 51-56 days). Females that failed to deliver pups were treated for 42-52 days.

### *Parental results*

No parental toxicity was observed up to the highest dose level tested (1000 mg/kg/day) and no mortality occurred. Test item-related findings were limited to non-adverse changes as described below:

Test item-related clinical signs included slight salivation directly after dosing among most males and few females of the 1000 mg/kg/day dose group during week 4 and/or 5 of treatment. This was regarded as a physiological response to the taste of the test item rather than a sign of systemic toxicity. In addition, one male showed piloerection on 4 consecutive days and one female showed hunched posture for 6 consecutive days, on 4 occasions combined with piloerection. At the incidence observed, and since the findings were transient, these were considered not toxicologically significant.

The slight, not statistically significant, changes in mean grip strength (females at 1000 mg/kg/day), total movements (males and females at 100 and 1000 mg/kg/day) and ambulations (males at 100 and 1000 mg/kg/day and females at all dose levels) were not supported by clinical observations or other functional observation tests, were slight in nature (within the normal range for rats of this age and strain), and had no supportive morphological correlates in examined neuronal tissues. These effects were therefore considered not to represent an adverse effect on neurobehaviour.

Thymus weight (absolute and relative) was decreased in all treated males in a dose-related manner. No statistical significance was achieved, all values remained within the historical control range and this decrease was not accompanied by any anatomic pathology alterations. As such, this finding was considered non-adverse.

Microscopic test item-related findings were present in the stomach of males at 1000 mg/kg/day, consisting of (multi) focal erosions of the glandular mucosa. Based on the low degrees (up to slight) and absence of concomitant test item-related microscopic changes, these findings were regarded as non-adverse.

No toxicologically significant changes were noted in any of the remaining parameters investigated in this study (i.e. body weight, food consumption, clinical laboratory investigations (including male T4 thyroid hormone levels) and macroscopic examination).

### *Reproductive results*

No reproduction toxicity was observed up to the highest dose level tested (1000 mg/kg/day).

No test item-related changes were noted in any of the reproductive parameters investigated in this study (i.e. mating and fertility indices, precoital time, number of implantations, estrous cycle, spermatogenic profiling, and histopathological examination of reproductive organs).

*Developmental results*

No developmental toxicity was observed up to the highest dose level tested (1000 mg/kg/day).

No toxicologically relevant changes were noted in any of the developmental parameters investigated in this study (i.e. gestation, viability and lactation indices, duration of gestation, parturition, sex ratio, maternal care and early postnatal pup development consisting of mortality, clinical signs, body weight, anogenital distance, areola/nipple retention, T4 thyroid hormone levels and macroscopic examination).

## **11. CONCLUSION**

Based on the results of this combined 28-day repeated dose toxicity study with the reproduction/developmental toxicity screening test, the Parental, Reproduction and Developmental No Observed Adverse Effect Levels (NOAEL) for DiHEP Aqueous Solution were established to be at least 1000 mg/kg/day.

**Appendix 1**  
**Summary Tables**

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.1 CLINICAL SIGNS SUMMARY MALES

SIGN (MAX. GRADE) (LOCATION)	WEEK:	PRE MATING	REPRO PERIOD
		DAY:	DAY:
		1.....	1.....
		12345671234567	123456712345671
<b>GROUP 1 (CONTROL)</b>			
No clinical signs noted			
<b>GROUP 2 (100 MG/KG)</b>			
Various			
Broken (1)	G:	.....	.....1111
(Tail apex)	%:	.....	.....1111
<b>GROUP 3 (300 MG/KG)</b>			
No clinical signs noted			
<b>GROUP 4 (1000 MG/KG)</b>			
Skin / fur			
Piloerection (1)	G:	.....	.....1111..
	%:	.....	.....1111..
Secretion / excretion			
Salivation (3)	G:	.....	.....11111
	%:	.....	.....45689

G: Median value of the highest individual daily grades

%: Percent of affected animals (0=less than 5%, 1=between 5% and 15%,..., A=more than 95%)

.: Observation performed, sign not present

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.1 CLINICAL SIGNS SUMMARY FEMALES

SIGN (MAX. GRADE) (LOCATION)	WEEK:	PRE MATING	REPRO PERIOD
		1.....	1..... 4.....
	DAY:	12345671234567	12345671234567123456712345671234567
<b>GROUP 1 (CONTROL)</b>			
No clinical signs noted			
<b>GROUP 2 (100 MG/KG)</b>			
No clinical signs noted			
<b>GROUP 3 (300 MG/KG)</b>			
Skin / fur			
Scabs (3)	G:	..... 1	11.....
(Cheek left)	%:	..... 1	11.....
Scabs (3)	G:	..... 1	11.....
(Cheek right)	%:	..... 1	11.....
<b>GROUP 4 (1000 MG/KG)</b>			
Posture			
Hunched posture (1)	G:	.....	1111111.....
	%:	.....	1111111.....
Skin / fur			
Piloerection (1)	G:	.....	11111.....
	%:	.....	11111.....
Secretion / excretion			
Salivation (3)	G:	.....	1111111.. 1.....
	%:	.....	1111121.. 1.....

G: Median value of the highest individual daily grades  
 %: Percent of affected animals (0=less than 5%, 1=between 5% and 15%,..., A=more than 95%)  
 .: Observation performed, sign not present

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.2 BODY WEIGHTS (GRAM) SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAY 1	MEAN	294	295	294	295
WEEK 1	ST.DEV	13.4	14.9	18.5	12.7
	N	10	10	10	10
DAY 8	MEAN	316	318	312	312
WEEK 2	ST.DEV	15.9	17.5	19.9	19.5
	N	10	10	10	10
<b>MATING PERIOD</b>					
DAY 1	MEAN	333	334	330	330
WEEK 1	ST.DEV	18.9	21.1	20.5	24.8
	N	10	10	10	10
DAY 8	MEAN	342	348	344	342
WEEK 2	ST.DEV	20.4	23.2	22.4	25.3
	N	10	10	10	10
DAY 15	MEAN	358	365	361	358
WEEK 3	ST.DEV	21.2	27.6	25.5	29.0
	N	10	10	10	10

## FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAY 1	MEAN	225	222	226	223
WEEK 1	ST.DEV	10.7	11.7	15.1	9.8
	N	10	10	10	10
DAY 8	MEAN	225	225	228	228
WEEK 2	ST.DEV	13.3	11.0	15.7	7.9
	N	10	10	10	10
<b>MATING PERIOD</b>					
DAY 1	MEAN	230	233	239	237
WEEK 1	ST.DEV	11.9	12.2	14.6	6.4
	N	10	10	10	10
DAY 8	MEAN	271			271
WEEK 2	ST.DEV	---			---
	N	1			1
DAY 15	MEAN				294
WEEK 3	ST.DEV				---
	N				1
DAY 22	MEAN				330
WEEK 4	ST.DEV				---
	N				1

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.2 BODY WEIGHTS (GRAM) SUMMARY FEMALES

### F0-GENERATION

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>POST COITUM</b>					
DAY 0	MEAN	229	231	240	237
	ST.DEV.	11.4	10.8	15.5	5.0
	N	9	8	9	8
DAY 4	MEAN	243	246	254	248
	ST.DEV.	12.0	11.9	15.4	8.1
	N	9	8	9	8
DAY 7	MEAN	251	252	261	255
	ST.DEV.	12.5	13.4	17.8	8.2
	N	9	8	9	8
DAY 11	MEAN	264	265	276	272
	ST.DEV.	10.9	13.1	17.6	11.9
	N	9	8	9	8
DAY 14	MEAN	273	276	288	281
	ST.DEV.	11.2	13.3	18.5	13.0
	N	9	8	9	8
DAY 17	MEAN	292	300	318 *	306
	ST.DEV.	16.5	14.4	26.6	13.0
	N	9	8	9	8
DAY 20	MEAN	325	342	350	345
	ST.DEV.	24.2	15.5	26.5	19.5
	N	9	8	9	8
<b>LACTATION</b>					
DAY 1	MEAN	255	252	267	266
	ST.DEV.	11.8	18.5	19.0	12.2
	N	8	8	9	9
DAY 4	MEAN	268	269	285	283
	ST.DEV.	7.9	18.8	20.0	12.8
	N	8	8	9	9
DAY 7	MEAN	277	276	293	291
	ST.DEV.	12.3	15.6	22.4	13.7
	N	8	8	9	9
DAY 13	MEAN	290	294	306	300
	ST.DEV.	13.2	15.9	18.3	11.4
	N	8	8	9	9

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level  
Explanations for excluded data are listed in the tables of the individual values

28Jun19 08h33



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.3 BODY WEIGHT GAIN (%) SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAY 1	MEAN	0	0	0	0
WEEK 1	ST.DEV	0.0	0.0	0.0	0.0
	N	10	10	10	10
DAY 8	MEAN	7	8	6	6
WEEK 2	ST.DEV	1.7	2.4	1.3	3.1
	N	10	10	10	10
<b>MATING PERIOD</b>					
DAY 1	MEAN	13	13	12	12
WEEK 1	ST.DEV	2.8	4.3	2.7	4.3
	N	10	10	10	10
DAY 8	MEAN	16	18	17	16
WEEK 2	ST.DEV	3.3	5.4	3.1	4.6
	N	10	10	10	10
DAY 15	MEAN	22	23	23	21
WEEK 3	ST.DEV	4.3	7.3	4.1	5.7
	N	10	10	10	10

## FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAY 1	MEAN	0	0	0	0
WEEK 1	ST.DEV	0.0	0.0	0.0	0.0
	N	10	10	10	10
DAY 8	MEAN	0	1	1	2
WEEK 2	ST.DEV	1.9	2.7	2.1	1.9
	N	10	10	10	10
<b>MATING PERIOD</b>					
DAY 1	MEAN	2	5	6 *	6 *
WEEK 1	ST.DEV	2.7	4.2	2.5	2.3
	N	10	10	10	10
DAY 8	MEAN	12			13
WEEK 2	ST.DEV	---			---
	N	1			1
DAY 15	MEAN				23
WEEK 3	ST.DEV				---
	N				1
DAY 22	MEAN				38
WEEK 4	ST.DEV				---
	N				1

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.3 BODY WEIGHT GAIN (%) SUMMARY FEMALES

### F0-GENERATION

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>POST COITUM</b>					
DAY 0	MEAN	0	0	0	0
	ST.DEV.	0.0	0.0	0.0	0.0
	N	9	8	9	8
DAY 4	MEAN	6	6	6	5
	ST.DEV.	1.5	1.9	1.9	2.3
	N	9	8	9	8
DAY 7	MEAN	10	9	9	7
	ST.DEV.	1.8	2.5	2.6	2.4
	N	9	8	9	8
DAY 11	MEAN	16	15	15	15
	ST.DEV.	2.7	3.0	2.9	3.4
	N	9	8	9	8
DAY 14	MEAN	20	20	20	18
	ST.DEV.	3.8	3.1	3.4	3.9
	N	9	8	9	8
DAY 17	MEAN	28	30	33	29
	ST.DEV.	7.0	2.5	7.0	3.8
	N	9	8	9	8
DAY 20	MEAN	42	48	46	45
	ST.DEV.	11.3	3.9	5.5	6.3
	N	9	8	9	8
<b>LACTATION</b>					
DAY 1	MEAN	0	0	0	0
	ST.DEV.	0.0	0.0	0.0	0.0
	N	8	8	9	9
DAY 4	MEAN	5	7	7	6
	ST.DEV.	3.4	2.0	2.0	3.3
	N	8	8	9	9
DAY 7	MEAN	9	10	10	9
	ST.DEV.	1.3	2.6	2.3	3.4
	N	8	8	9	9
DAY 13	MEAN	14	17	15	13
	ST.DEV.	3.6	3.2	3.7	5.1
	N	8	8	9	9

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level  
Explanations for excluded data are listed in the tables of the individual values

28Jun19 08h39

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.4 FOOD CONSUMPTION (G/ANIMAL/DAY) SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAYS 1-8	MEAN	24	25	24	24
WEEKS 1-2	ST.DEV	0.0	0.8	0.5	1.6
	N (CAGE)	2	2	2	2
DAYS 8-15	MEAN	24	25	24	24
WEEKS 2-3	ST.DEV	0.1	1.1	0.0	1.3
	N (CAGE)	2	2	2	2
MEAN OF MEANS OVER PRE MATI...	MEAN	24	25	24	24
<b>MATING PERIOD</b>					
DAYS 1-8	MEAN	25	26	26	25
WEEKS 1-2	ST.DEV	0.5	0.7	0.7	0.2
	N (CAGE)	2	2	2	2
DAYS 8-15	MEAN	25	25	25	24
WEEKS 2-3	ST.DEV	0.4	0.7	0.5	0.0
	N (CAGE)	2	2	2	2
MEAN OF MEANS OVER MATING P...	MEAN	25	25	25	25

## FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAYS 1-8	MEAN	18	18	18	18
WEEKS 1-2	ST.DEV	0.5	0.2	0.7	0.8
	N (CAGE)	2	2	2	2
DAYS 8-15	MEAN	18	17	18	18
WEEKS 2-3	ST.DEV	0.3	0.4	0.8	0.4
	N (CAGE)	2	2	2	2
MEAN OF MEANS OVER PRE MATI...	MEAN	18	18	18	18

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.4 FOOD CONSUMPTION (G/ANIMAL/DAY) SUMMARY FEMALES

### F0-GENERATION

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>POST COITUM</b>					
DAYS 0-4	MEAN	21	21	21	21
	ST.DEV.	1.0	1.6	1.3	2.5
	N	9	8	9	8
DAYS 4-7	MEAN	22	21	22	21
	ST.DEV.	1.2	1.8	2.4	2.1
	N	9	8	9	8
DAYS 7-11	MEAN	22	20	23	21
	ST.DEV.	1.2	1.2	2.1	1.7
	N	9	8	9	7
DAYS 11-14	MEAN	23	22	24	22
	ST.DEV.	1.6	1.2	1.8	3.2
	N	9	8	9	8
DAYS 14-17	MEAN	23	22	24	23
	ST.DEV.	2.3	1.0	1.9	2.0
	N	9	8	9	8
DAYS 17-20	MEAN	28	25	28	27
	ST.DEV.	2.6	2.2	3.8	2.9
	N	9	8	9	8
MEAN OF MEANS		23	22	24	23
<b>LACTATION</b>					
DAYS 1-4	MEAN	35	37	38	36
	ST.DEV.	5.4	9.2	6.5	4.3
	N	8	8	9	9
DAYS 4-7	MEAN	42	44	45	46
	ST.DEV.	5.8	3.1	3.7	4.8
	N	8	8	9	9
DAYS 7-13	MEAN	53	57	56	57
	ST.DEV.	6.1	1.7	3.1	1.9
	N	8	8	9	9
MEAN OF MEANS		43	46	46	46

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level  
Explanations for excluded data are listed in the tables of the individual values

28Jun19 08h41

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.5 RELATIVE FOOD CONSUMPTION (G/KG BODY WEIGHT/DAY) SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAYS 1-8	MEAN	77	79	77	77
WEEKS 1-2	ST.DEV	0.7	3.0	0.3	0.9
	N (CAGE)	2	2	2	2
DAYS 8-15	MEAN	76	79	77	78
WEEKS 2-3	ST.DEV	0.2	3.8	1.8	0.1
	N (CAGE)	2	2	2	2
MEAN OF MEANS OVER PRE MATI...	MEAN	76	79	77	77
<b>MATING PERIOD</b>					
DAYS 1-8	MEAN	73	74	75	74
WEEKS 1-2	ST.DEV	0.3	2.0	0.5	5.7
	N (CAGE)	2	2	2	2
DAYS 8-15	MEAN	69	69	69	68
WEEKS 2-3	ST.DEV	0.1	1.8	0.2	5.3
	N (CAGE)	2	2	2	2
MEAN OF MEANS OVER MATING P...	MEAN	71	72	72	71

## FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>PRE MATING</b>					
DAYS 1-8	MEAN	80	80	80	80
WEEKS 1-2	ST.DEV	1.3	0.4	0.6	3.0
	N (CAGE)	2	2	2	2
DAYS 8-15	MEAN	79	77	80	79
WEEKS 2-3	ST.DEV	0.5	3.0	0.0	1.6
	N (CAGE)	2	2	2	2
MEAN OF MEANS OVER PRE MATI...	MEAN	80	79	80	80

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.5 RELATIVE FOOD CONSUMPTION (G/KG BODY WEIGHT/DAY) SUMMARY FEMALES

### F0-GENERATION

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>POST COITUM</b>					
DAYS 0-4	MEAN	87	84	85	84
	ST.DEV.	3.7	6.0	4.2	9.2
	N	9	8	9	8
DAYS 4-7	MEAN	87	83	86	82
	ST.DEV.	3.1	6.8	6.0	6.9
	N	9	8	9	8
DAYS 7-11	MEAN	83	77 *	84	79
	ST.DEV.	4.7	3.6	5.4	5.6
	N	9	8	9	7
DAYS 11-14	MEAN	84	79	84	79
	ST.DEV.	4.8	3.1	4.4	12.5
	N	9	8	9	8
DAYS 14-17	MEAN	79	73 *	76	76
	ST.DEV.	5.1	2.5	4.4	4.7
	N	9	8	9	8
DAYS 17-20	MEAN	86	73 **	78 *	79
	ST.DEV.	4.9	4.0	7.1	6.0
	N	9	8	9	8
MEAN OF MEANS		84	78	82	80
<b>LACTATION</b>					
DAYS 1-4	MEAN	132	135	135	128
	ST.DEV.	19.9	31.1	24.3	14.4
	N	8	8	9	9
DAYS 4-7	MEAN	152	160	152	158
	ST.DEV.	19.2	12.6	10.7	16.6
	N	8	8	9	9
DAYS 7-13	MEAN	182	195	183	189
	ST.DEV.	20.0	7.3	11.4	4.5
	N	8	8	9	9
MEAN OF MEANS		155	164	157	159

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level  
Explanations for excluded data are listed in the tables of the individual values

28Jun19 08h42

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.6 FUNCTIONAL OBSERVATIONS SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
HEARING SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
PUPIL L SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
PUPIL R SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
STATIC R SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
GRIP FORE GRAM	MEAN	1313	1474	1268	1395
	ST.DEV	155	47	130	216
	N	5	5	5	5
GRIP HIND GRAM	MEAN	720	789	790	769
	ST.DEV	87	83	138	130
	N	5	5	5	5

## FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
HEARING SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
PUPIL L SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
PUPIL R SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
STATIC R SCORE 0/1	MEDIAN	0	0	0	0
	N	5	5	5	5
GRIP FORE GRAM	MEAN	940	1029	1070	1108
	ST.DEV	282	257	157	220
	N	5	5	5	5
GRIP HIND GRAM	MEAN	582	647	620	570
	ST.DEV	88	41	106	85
	N	5	5	5	5

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level  
+/\*\* Steel-test significant at 5% (+) or 1% (++) level

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.7 MOTOR ACTIVITY TEST SUMMARY MALES

<b>Total Movements</b>	<b>CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
MEAN	4006	3366	3812	3068
N	5	5	5	5
STDEV	379	936	499	544

\* indicates a p-value <0.05, \*\* indicates a p-value <0.01

MEAN and STDEV values are calculated per group, from each animal's total Total Movements over all intervals

29May19 12h06



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.7 MOTOR ACTIVITY TEST SUMMARY MALES

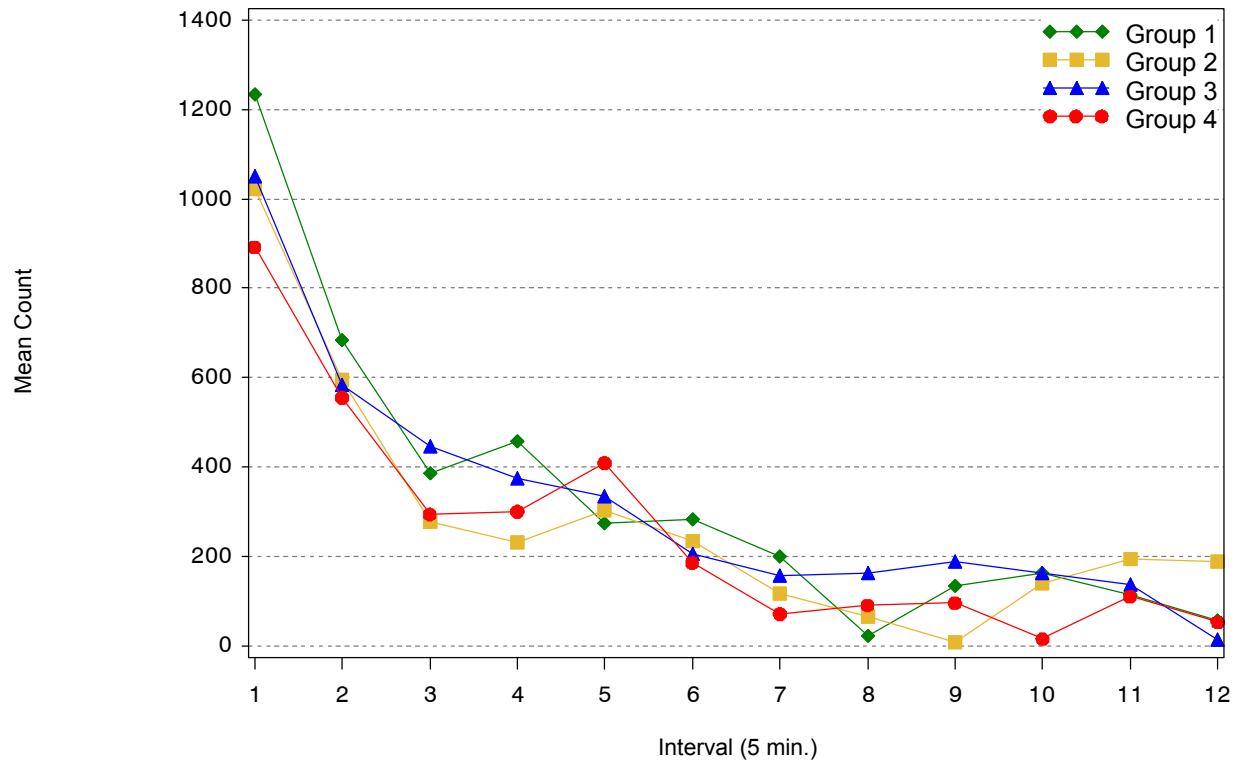
<b>Ambulations</b>	<b>CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
MEAN	910	775	909	867
N	5	5	5	5
STDEV	170	247	237	139

\* indicates a p-value <0.05, \*\* indicates a p-value <0.01

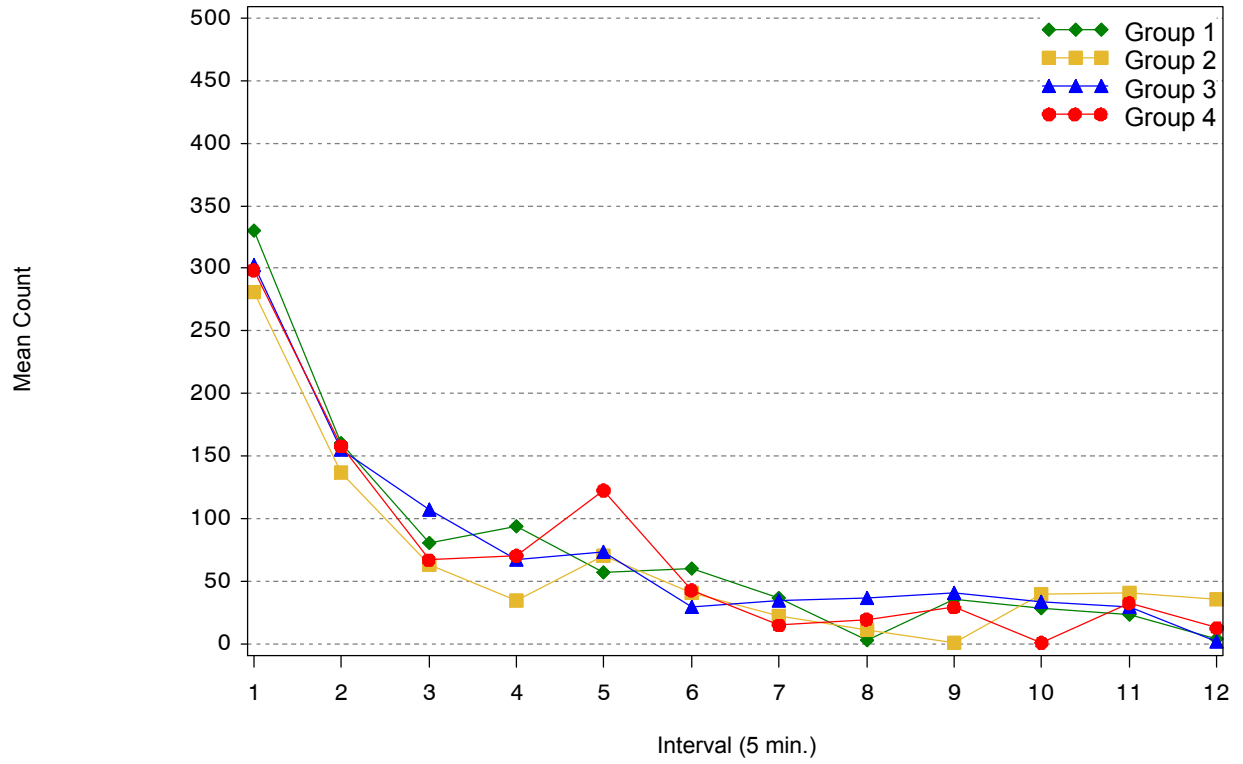
MEAN and STDEV values are calculated per group, from each animal's total Ambulations over all intervals

29May19 12h06

**1.7 MOTOR ACTIVITY: TOTAL MOVEMENTS  
MALES**



**1.7 MOTOR ACTIVITY: AMBULATIONS  
MALES**



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.7 MOTOR ACTIVITY TEST SUMMARY FEMALES

<b>Total Movements</b>	<b>CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
MEAN	4626	3802	4663	3016
N	5	5	5	5
STDEV	1179	707	1255	1715

\* indicates a p-value <0.05, \*\* indicates a p-value <0.01

MEAN and STDEV values are calculated per group, from each animal's total Total Movements over all intervals

29May19 12h07

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.7 MOTOR ACTIVITY TEST SUMMARY FEMALES

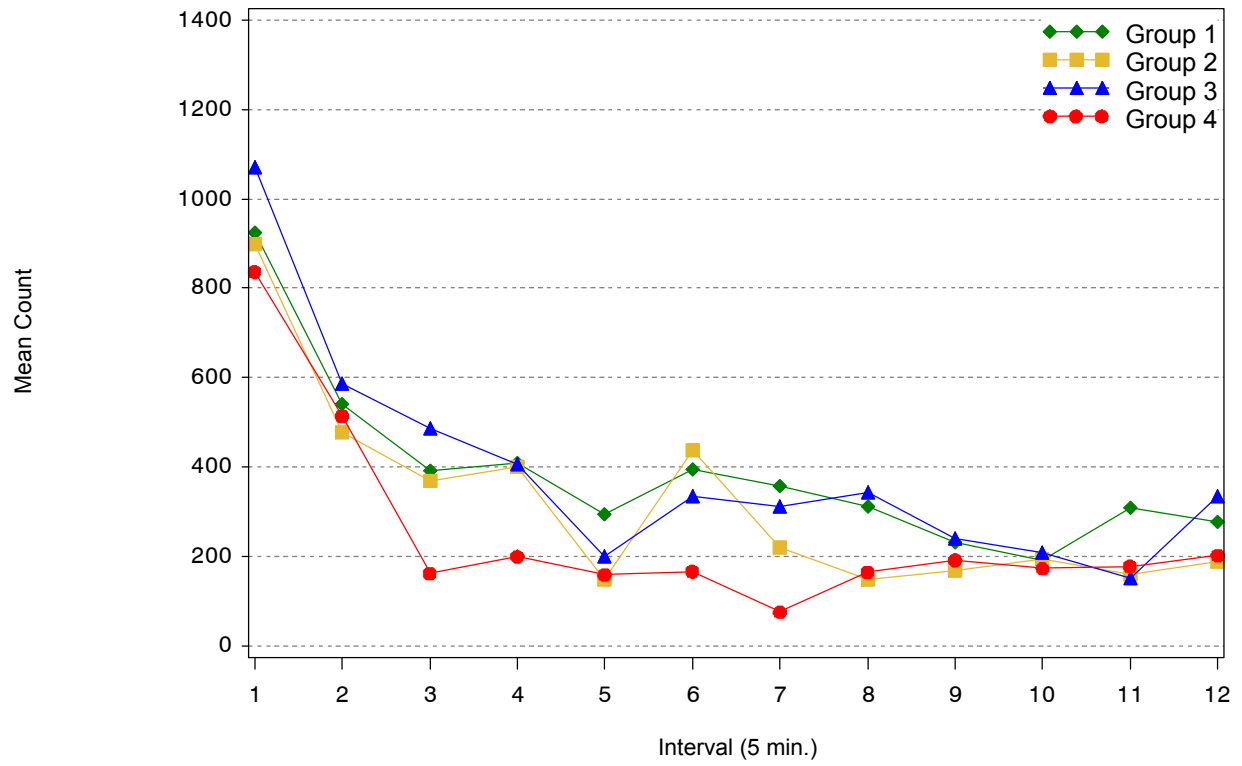
<b>Ambulations</b>	<b>CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
MEAN	1229	1040	1000	728
N	5	5	5	5
STDEV	311	275	218	518

\* indicates a p-value <0.05, \*\* indicates a p-value <0.01

MEAN and STDEV values are calculated per group, from each animal's total Ambulations over all intervals

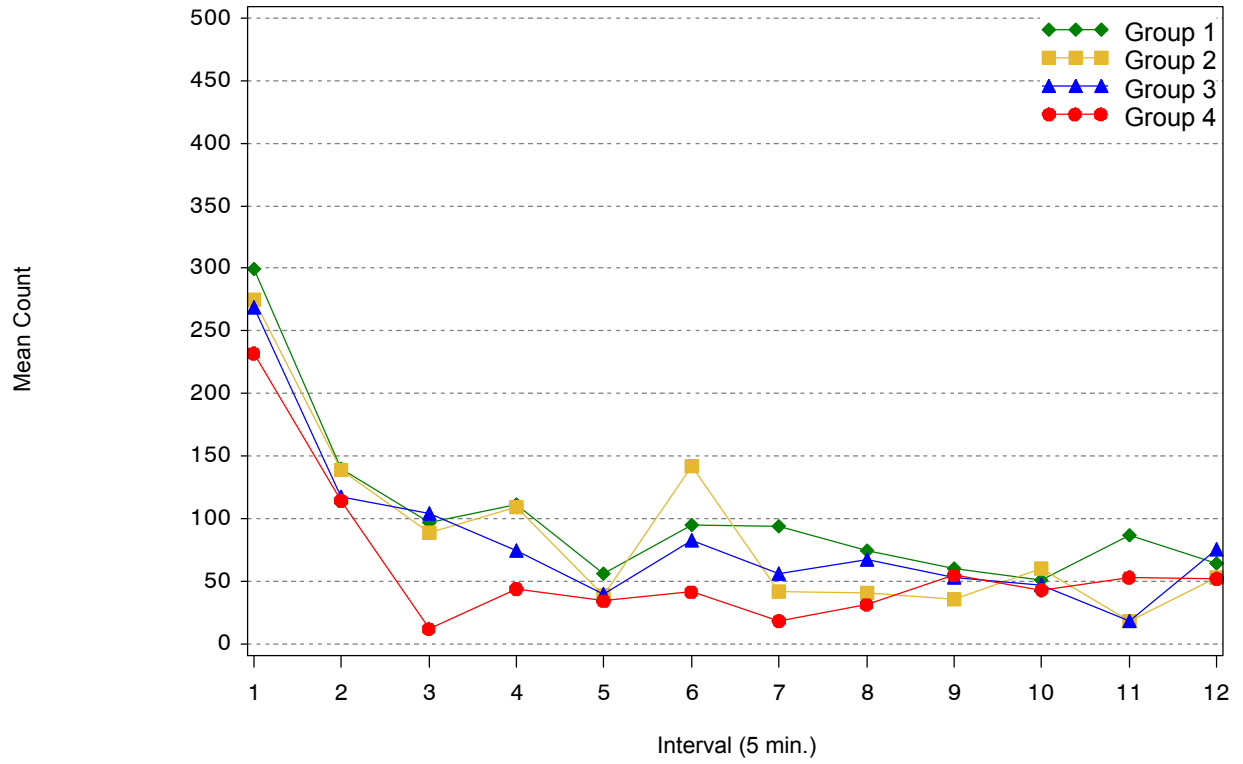
29May19 12h07

**1.7 MOTOR ACTIVITY: TOTAL MOVEMENTS  
FEMALES**



29May19 12h07

**1.7 MOTOR ACTIVITY: AMBULATIONS  
FEMALES**



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.8 HAEMATOLOGY SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
WBC	MEAN	9.0	6.6	6.0	7.8
10E9/L	ST.DEV	1.4	1.7	2.1	2.1
	N	5	5	5	5
Neutrophils	MEAN	1.4	0.9 +	1.1	1.2
10E9/L	ST.DEV	0.3	0.1	0.3	0.2
	N	5	5	5	5
Lymphocytes	MEAN	7.4	5.4	4.7	6.5
10E9/L	ST.DEV	1.4	1.6	1.9	2.2
	N	4	5	5	4
Monocytes	MEAN	0.2	0.1	0.1	0.1
10E9/L	ST.DEV	0.1	0.1	0.0	0.0
	N	5	5	5	5
Eosinophils	MEAN	0.1	0.1	0.1	0.1
10E9/L	ST.DEV	0.1	0.0	0.0	0.1
	N	5	5	5	5
Basophils	MEAN	0.0	0.0	0.0	0.0
10E9/L	ST.DEV	0.0	0.0	0.0	0.0
	N	5	5	5	5
Red blood cells	MEAN	8.93	8.45	8.44	8.94
10E12/L	ST.DEV	0.29	0.49	0.68	0.23
	N	5	5	5	5
Reticulocytes	MEAN	210.0	202.5	247.8	219.2
10E9/L	ST.DEV	24.1	13.7	23.9	24.0
	N	5	5	5	5
RDW	MEAN	11.8	12.2	12.1	12.2
%	ST.DEV	0.3	0.9	0.3	0.5
	N	5	5	5	5
Haemoglobin	MEAN	10.1	9.7	9.3	10.0
mmol/L	ST.DEV	0.5	0.7	0.6	0.3
	N	5	5	5	5
Haematocrit	MEAN	0.476	0.450	0.446	0.478
L/L	ST.DEV	0.026	0.035	0.025	0.015
	N	5	5	5	5
MCV	MEAN	53.3	53.2	52.9	53.4
fL	ST.DEV	1.8	1.5	2.1	1.4
	N	5	5	5	5
MCH	MEAN	1.13	1.14	1.10	1.11
fmol	ST.DEV	0.03	0.04	0.04	0.04
	N	5	5	5	5
MCHC	MEAN	21.18	21.43	20.78	20.85
mmol/L	ST.DEV	0.37	0.67	0.34	0.36
	N	5	5	5	5
Platelets	MEAN	675	690	763	681
10E9/L	ST.DEV	31	133	64	75
	N	5	5	5	5

+/\*\* Steel-test significant at 5% (+) or 1% (\*\*) level

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.8 HAEMATOLOGY SUMMARY MALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<hr/>					
<b>END OF TREATMENT</b>					
PT	MEAN	18.9	17.7 *	17.8 *	17.7 *
s	ST.DEV	0.7	0.9	0.8	0.4
	N	5	4	5	5
APTT	MEAN	16.1	15.0	13.9	16.7
s	ST.DEV	3.2	2.6	2.7	2.0
	N	5	5	5	5

+/\*\* Steel-test significant at 5% (+) or 1% (\*\*) level

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.8 HAEMATOLOGY SUMMARY FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
WBC	MEAN	5.9	5.0	6.2	4.9
10E9/L	ST.DEV	2.1	1.1	1.6	1.4
	N	5	5	5	5
Neutrophils	MEAN	1.6	1.3	1.8	1.3
10E9/L	ST.DEV	0.4	0.4	0.9	0.3
	N	5	5	5	5
Lymphocytes	MEAN	4.0	3.5	4.1	3.4
10E9/L	ST.DEV	1.7	1.0	1.0	1.1
	N	5	5	5	5
Monocytes	MEAN	0.1	0.1	0.1	0.1
10E9/L	ST.DEV	0.1	0.0	0.0	0.0
	N	5	5	5	5
Eosinophils	MEAN	0.2	0.1	0.1	0.1
10E9/L	ST.DEV	0.1	0.0	0.0	0.0
	N	5	5	5	5
Basophils	MEAN	0.0	0.0	0.0	0.0
10E9/L	ST.DEV	0.0	0.0	0.0	0.0
	N	5	5	5	5
Red blood cells	MEAN	7.33	7.22	7.02	7.17
10E12/L	ST.DEV	0.72	0.38	0.29	0.61
	N	5	5	5	5
Reticulocytes	MEAN	223.3	233.4	204.9	215.7
10E9/L	ST.DEV	47.7	31.1	22.0	26.5
	N	5	5	5	5
RDW	MEAN	12.6	12.9	12.4	12.3
%	ST.DEV	1.3	0.8	1.1	0.8
	N	5	5	5	5
Haemoglobin	MEAN	8.8	8.8	8.8	8.6
mmol/L	ST.DEV	0.5	0.5	0.2	0.6
	N	5	5	5	5
Haematocrit	MEAN	0.431	0.434	0.433	0.423
L/L	ST.DEV	0.032	0.019	0.014	0.029
	N	5	5	5	5
MCV	MEAN	59.0	60.0	61.7	59.1
fL	ST.DEV	2.2	1.2	2.2	1.7
	N	5	5	5	5
MCH	MEAN	1.20	1.22	1.25	1.20
fmol	ST.DEV	0.07	0.02	0.05	0.03
	N	5	5	5	5
MCHC	MEAN	20.35	20.26	20.23	20.37
mmol/L	ST.DEV	0.53	0.42	0.26	0.37
	N	5	5	5	5
Platelets	MEAN	717	775	652	705
10E9/L	ST.DEV	20	114	80	117
	N	5	5	5	5

+/\*\* Steel-test significant at 5% (+) or 1% (\*\*) level

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.8 HAEMATOLOGY SUMMARY FEMALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<hr/>					
<b>END OF TREATMENT</b>					
PT	MEAN	17.2	17.3	17.6	16.9
s	ST.DEV	0.8	1.2	0.3	1.0
	N	4	5	4	5
APTT	MEAN	19.2	20.7	21.3	19.1
s	ST.DEV	1.1	1.9	3.0	0.9
	N	4	5	5	5

+/\*\* Steel-test significant at 5% (+) or 1% (\*\*) level

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.9 CLINICAL BIOCHEMISTRY SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
ALAT U/L	MEAN	49.2	46.4	45.1	50.5
	ST.DEV	8.1	11.4	10.3	5.4
	N	5	5	5	5
ASAT U/L	MEAN	86.4	82.3	81.0	90.5
	ST.DEV	7.8	4.6	12.9	9.3
	N	5	5	5	5
ALP U/L	MEAN	151	135	123	160
	ST.DEV	40	23	23	26
	N	5	5	5	5
Total protein g/L	MEAN	62.4	62.8	62.2	61.4
	ST.DEV	1.7	2.0	1.0	3.5
	N	5	5	5	5
Albumin g/L	MEAN	31.7	31.9	31.7	31.2
	ST.DEV	0.5	0.3	0.2	1.3
	N	5	5	5	5
Total bilirubin umol/L	MEAN	2.3	2.2	2.1	2.4
	ST.DEV	0.6	0.2	0.2	0.2
	N	5	5	5	5
Urea mmol/L	MEAN	6.4	6.8	7.0	7.2
	ST.DEV	1.0	1.5	1.3	1.3
	N	5	5	5	5
Creatinine umol/L	MEAN	36.8	39.4	37.6	35.6
	ST.DEV	2.6	2.3	2.5	1.6
	N	5	5	5	5
Glucose mmol/L	MEAN	9.67	8.23	8.69	7.92
	ST.DEV	1.94	1.21	1.67	1.70
	N	5	5	5	5
Cholesterol mmol/L	MEAN	1.77	1.90	1.59	1.66
	ST.DEV	0.18	0.26	0.20	0.43
	N	5	5	5	5
Bile Acids umol/L	MEAN	24.1	16.9	22.3	32.1
	ST.DEV	10.4	5.8	6.9	13.6
	N	5	5	5	5
Sodium mmol/L	MEAN	140.4	140.5	139.9	141.2
	ST.DEV	0.9	0.8	1.1	1.0
	N	5	5	5	5
Potassium mmol/L	MEAN	4.06	3.96	3.89	3.92
	ST.DEV	0.18	0.09	0.14	0.34
	N	5	5	5	5
Chloride mmol/L	MEAN	104	105	104	104
	ST.DEV	1	1	2	1
	N	5	5	5	5
Calcium mmol/L	MEAN	2.54	2.55	2.53	2.54
	ST.DEV	0.03	0.04	0.08	0.04
	N	5	5	5	5

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.9 CLINICAL BIOCHEMISTRY SUMMARY MALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<hr/>					
<b>END OF TREATMENT</b>					
Inorg.Phos	MEAN	1.85	1.78	1.78	1.94
mmol/L	ST.DEV	0.13	0.28	0.10	0.10
	N	5	5	5	5
Total T4	MEAN	5.11	4.73	4.03 *	4.49
ug/dL	ST.DEV	1.05	0.95	0.46	1.07
	N	10	10	10	10

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.9 CLINICAL BIOCHEMISTRY SUMMARY FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
ALAT U/L	MEAN	99.3	108.4	111.0	100.1
	ST.DEV	8.0	8.8	14.6	13.0
	N	5	5	5	5
ASAT U/L	MEAN	104.1	134.5	113.5	113.6
	ST.DEV	16.1	57.9	22.1	22.2
	N	5	5	5	5
ALP U/L	MEAN	364	461	360	333
	ST.DEV	187	57	152	144
	N	5	5	5	5
Total protein g/L	MEAN	56.6	56.7	56.9	56.0
	ST.DEV	3.5	2.9	2.2	1.8
	N	5	5	5	5
Albumin g/L	MEAN	30.5	29.6	30.0	29.5
	ST.DEV	1.4	1.2	1.0	1.0
	N	5	5	5	5
Total bilirubin umol/L	MEAN	2.4	2.0	2.4	1.9
	ST.DEV	0.5	0.4	0.5	0.3
	N	5	5	5	5
Urea mmol/L	MEAN	10.1	10.2	10.9	11.0
	ST.DEV	1.0	0.6	0.9	2.3
	N	5	5	5	5
Creatinine umol/L	MEAN	36.2	37.5	34.9	35.9
	ST.DEV	2.2	1.5	1.9	2.7
	N	5	5	5	5
Glucose mmol/L	MEAN	7.33	6.80	6.54	7.44
	ST.DEV	0.80	0.69	0.71	0.66
	N	5	5	5	5
Cholesterol mmol/L	MEAN	2.25	2.04	2.17	2.13
	ST.DEV	0.22	0.17	0.28	0.16
	N	5	5	5	5
Bile Acids umol/L	MEAN	25.5	27.2	26.3	20.2
	ST.DEV	11.4	13.4	11.6	6.1
	N	5	5	5	5
Sodium mmol/L	MEAN	139.8	140.3	139.7	139.5
	ST.DEV	2.2	1.8	0.6	2.2
	N	5	5	5	5
Potassium mmol/L	MEAN	4.43	4.43	4.85	4.79
	ST.DEV	0.27	0.26	0.21	0.37
	N	5	5	5	5
Chloride mmol/L	MEAN	102	103	103	102
	ST.DEV	2	1	1	2
	N	5	5	5	5
Calcium mmol/L	MEAN	2.50	2.45	2.51	2.51
	ST.DEV	0.05	0.08	0.08	0.07
	N	5	5	5	5

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.9 CLINICAL BIOCHEMISTRY SUMMARY FEMALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<b>END OF TREATMENT</b>					
Inorg.Phos mmol/L	MEAN	0.70	0.81	0.99	0.89
	ST.DEV	0.24	0.50	0.41	0.62
	N	5	5	5	5

---

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.10 MACROSCOPIC FINDINGS SUMMARY MALES

	GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>				
Animals examined	10	10	10	10
Animals without findings	10	9	10	7
Animals affected	0	1	0	3
Liver				
Discolouration	0	0	0	1
Kidneys				
Irregular surface	0	0	0	1
Epididymides				
Nodule(s)	0	0	0	1
Bone				
Tail bent	0	1	0	0

## FEMALES

	GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>				
Animals examined	10	10	10	10
Animals without findings	9	8	4	8
Animals affected	1	2	6	2
Jejunum				
Focus/foci	0	0	1	0
Uterus				
Nodule(s)	1	0	0	0
Contains fluid	0	0	1	1
Contents:	1	0	0	0
Clitoral glands				
Focus/foci	0	1	0	0
Thyroid gland				
Discolouration	0	0	1	1
Thymus				
Focus/foci	1	0	1	0
Mandibular lymph n				
Focus/foci	1	0	0	1
Eyes				
Exophthalmus	1	0	1	1
Body cavities				
Nodule(s)	0	0	1	0
Contains fluid	0	2	1	0

# / ## Fisher's Exact test significant at 5% (#) or 1% (##) level

03Jun19 12h49



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.11 ORGAN WEIGHTS (GRAM) SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
BODY W. (GRAM)	MEAN	335	340	335	332
	ST.DEV	20	25	22	28
	N	10	10	10	10
BRAIN (GRAM)	MEAN	2.00	2.02	2.02	1.98
	ST.DEV	0.07	0.11	0.10	0.07
	N	5	5	5	5
HEART (GRAM)	MEAN	0.962	0.894	0.938	0.869
	ST.DEV	0.073	0.063	0.102	0.048
	N	5	5	5	5
LIVER (GRAM)	MEAN	8.06	7.88	8.15	7.29
	ST.DEV	0.56	0.67	0.70	0.63
	N	5	5	5	5
THYROIDS (GRAM)	MEAN	0.016	0.016	0.016	0.018
	ST.DEV	0.002	0.003	0.003	0.003
	N	10	10	10	10
THYMUS (GRAM)	MEAN	0.385	0.367	0.346	0.300
	ST.DEV	0.064	0.068	0.089	0.089
	N	5	5	5	5
KIDNEYS (GRAM)	MEAN	2.27	2.19	2.32	2.29
	ST.DEV	0.09	0.11	0.30	0.19
	N	5	5	5	5
ADRENALS (GRAM)	MEAN	0.060	0.053	0.070 *	0.058
	ST.DEV	0.008	0.003	0.005	0.004
	N	5	5	5	5
SPLEEN (GRAM)	MEAN	0.652	0.617	0.587	0.525
	ST.DEV	0.122	0.056	0.085	0.044
	N	5	5	5	5
TESTES (GRAM)	MEAN	3.39	3.29	3.39	3.32
	ST.DEV	0.27	0.16	0.24	0.22
	N	10	10	10	10
PROSTATE GLAND (GRAM)	MEAN	0.863	0.836	0.841	0.785
	ST.DEV	0.110	0.126	0.141	0.147
	N	10	10	10	10
EPIDIDYMIDES (GRAM)	MEAN	1.060	1.059	1.075	1.066
	ST.DEV	0.087	0.058	0.074	0.078
	N	10	10	10	10
SEMINAL VESICLES (GRAM)	MEAN	1.339	1.276	1.305	1.172
	ST.DEV	0.140	0.190	0.243	0.214
	N	10	10	10	10

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.11 ORGAN/BODY WEIGHT RATIOS (%) SUMMARY MALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
BODY W. (GRAM)	MEAN	335	340	335	332
	ST.DEV	20	25	22	28
	N	10	10	10	10
BRAIN (%)	MEAN	0.60	0.60	0.59	0.63
	ST.DEV	0.04	0.04	0.04	0.04
	N	5	5	5	5
HEART (%)	MEAN	0.289	0.263 **	0.274	0.277
	ST.DEV	0.010	0.006	0.010	0.013
	N	5	5	5	5
LIVER (%)	MEAN	2.42	2.32	2.39	2.33
	ST.DEV	0.09	0.06	0.06	0.17
	N	5	5	5	5
THYROIDS (%)	MEAN	0.005	0.005	0.005	0.005
	ST.DEV	0.001	0.001	0.001	0.001
	N	10	10	10	10
THYMUS (%)	MEAN	0.117	0.108	0.101	0.095
	ST.DEV	0.025	0.017	0.020	0.025
	N	5	5	5	5
KIDNEYS (%)	MEAN	0.68	0.65	0.68	0.73
	ST.DEV	0.03	0.05	0.05	0.08
	N	5	5	5	5
ADRENALS (%)	MEAN	0.018	0.016	0.021	0.019
	ST.DEV	0.003	0.001	0.001	0.001
	N	5	5	5	5
SPLEEN (%)	MEAN	0.195	0.182	0.172	0.168
	ST.DEV	0.031	0.018	0.022	0.010
	N	5	5	5	5
TESTES (%)	MEAN	1.01	0.97	1.01	1.00
	ST.DEV	0.05	0.05	0.09	0.07
	N	10	10	10	10
PROSTATE GLAND (%)	MEAN	0.258	0.246	0.251	0.237
	ST.DEV	0.028	0.038	0.036	0.043
	N	10	10	10	10
EPIDIDYMIDES (%)	MEAN	0.317	0.312	0.322	0.324
	ST.DEV	0.023	0.019	0.026	0.042
	N	10	10	10	10
SEMINAL VESICLES (%)	MEAN	0.400	0.377	0.391	0.356
	ST.DEV	0.039	0.061	0.076	0.075
	N	10	10	10	10

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.11 ORGAN WEIGHTS (GRAM) SUMMARY FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
BODY W. (GRAM)	MEAN	276	279	290	290
	ST.DEV	15	21	30	25
	N	10	10	10	10
BRAIN (GRAM)	MEAN	1.89	1.90	1.91	1.94
	ST.DEV	0.09	0.05	0.05	0.09
	N	5	5	4	5
HEART (GRAM)	MEAN	0.828	0.830	0.861	0.861
	ST.DEV	0.063	0.059	0.087	0.041
	N	5	5	5	5
LIVER (GRAM)	MEAN	11.84	12.12	11.90	13.20
	ST.DEV	1.28	0.65	0.74	0.58
	N	5	5	5	5
THYROIDS (GRAM)	MEAN	0.017	0.019	0.018	0.018
	ST.DEV	0.004	0.004	0.004	0.004
	N	10	10	10	10
THYMUS (GRAM)	MEAN	0.237	0.187	0.225	0.200
	ST.DEV	0.054	0.022	0.017	0.044
	N	5	5	5	5
KIDNEYS (GRAM)	MEAN	1.87	2.05	2.01	2.09 *
	ST.DEV	0.14	0.17	0.08	0.06
	N	5	5	5	5
ADRENALS (GRAM)	MEAN	0.076	0.081	0.078	0.078
	ST.DEV	0.008	0.007	0.008	0.008
	N	5	5	5	5
SPLEEN (GRAM)	MEAN	0.500	0.507	0.510	0.551
	ST.DEV	0.052	0.037	0.090	0.134
	N	5	5	5	5
OVARIES (GRAM)	MEAN	0.113	0.115	0.117	0.115
	ST.DEV	0.010	0.008	0.013	0.018
	N	5	5	5	5
UTERUS (GRAM)	MEAN	0.360	0.360	0.356	0.345
	ST.DEV	0.038	0.068	0.055	0.031
	N	5	5	5	5

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.11 ORGAN/BODY WEIGHT RATIOS (%) SUMMARY FEMALES

		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>END OF TREATMENT</b>					
BODY W. (GRAM)	MEAN	276	279	290	290
	ST.DEV	15	21	30	25
	N	10	10	10	10
BRAIN (%)	MEAN	0.69	0.65	0.66	0.67
	ST.DEV	0.04	0.02	0.04	0.04
	N	5	5	4	5
HEART (%)	MEAN	0.301	0.286	0.296	0.298
	ST.DEV	0.024	0.018	0.020	0.011
	N	5	5	5	5
LIVER (%)	MEAN	4.30	4.18	4.09	4.57
	ST.DEV	0.37	0.18	0.07	0.12
	N	5	5	5	5
THYROIDS (%)	MEAN	0.006	0.007	0.006	0.006
	ST.DEV	0.001	0.002	0.002	0.001
	N	10	10	10	10
THYMUS (%)	MEAN	0.086	0.065 *	0.077	0.069
	ST.DEV	0.020	0.007	0.005	0.015
	N	5	5	5	5
KIDNEYS (%)	MEAN	0.68	0.71	0.69	0.73
	ST.DEV	0.05	0.05	0.04	0.03
	N	5	5	5	5
ADRENALS (%)	MEAN	0.027	0.028	0.027	0.027
	ST.DEV	0.002	0.003	0.003	0.003
	N	5	5	5	5
SPLEEN (%)	MEAN	0.182	0.175	0.175	0.190
	ST.DEV	0.019	0.012	0.025	0.043
	N	5	5	5	5
OVARIES (%)	MEAN	0.041	0.040	0.040	0.040
	ST.DEV	0.003	0.003	0.003	0.007
	N	5	5	5	5
UTERUS (%)	MEAN	0.131	0.124	0.122	0.120
	ST.DEV	0.014	0.022	0.017	0.012
	N	5	5	5	5

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 12h49

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.12 REPRODUCTION DATA SUMMARY

---

	<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
Females paired	10	10	10	10
Females mated	10	10	10	10
Pregnant females	9	8	9	9
Females with living pups on Day 1	8	8	9	9

---

<b>Mating index (%)</b> (Females mated / Females paired) * 100	100	100	100	100
<b>Fertility index (%)</b> (Pregnant females / Females mated) * 100	90	80	90	90
<b>Gestation index (%)</b> (Females with living pups on Day 1 / Pregnant females) * 100	89	100	100	100

---

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.13 PRECOITAL TIME

### F0-GENERATION - POST COITUM

DAY OF THE PAIRING PERIOD	GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
<b>NUMBER OF FEMALES MATED</b>				
1	2	2	3	3
2	-	4	1	1
3	6	3	4	4
4	1	1	2	2
13	1	-	-	-
MEDIAN PRECOITAL TIME	3	2	3	3
MEAN PRECOITAL TIME	3.7	2.3	2.5	2.5
N	10	10	10	10

+ / ++ Steel-test significant at 5% (+) or 1% (++) level

03Jun19 14h16

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.14 IMPLANTATION SITES SUMMARY FEMALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<b>AT NECROPSY</b>					
Implantations	MEAN	11.8	14.6	12.9	14.4
	ST.DEV	3.2	2.2	1.6	2.0
	N	8	8	9	9

---

+ / ++ Steel-test significant at 5% (+) or 1% (++) level

03Jun19 12h59

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.15 DEVELOPMENTAL DATA

### F0-GENERATION - LACTATION

	GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
LITTERS				
TOTAL	8	8	9	9
DURATION OF GESTATION				
MEAN (+)	21.4	21.3	21.7	21.0
ST.DEV.	0.5	0.5	0.5	0.0
N	8	8	9	9
DEAD PUPS AT FIRST LITTER CHECK				
LITTERS AFFECTED (#)	0	0	1	0
TOTAL	0	0	1	0
MEAN (+)	0.0	0.0	0.1	0.0
ST.DEV.	0.0	0.0	0.3	0.0
N	8	8	9	9
LIVING PUPS AT FIRST LITTER CHECK				
% OF MALES / FEMALES (#)	49 / 51	50 / 50	48 / 52	48 / 52
TOTAL	89	113	108	116
MEAN (+)	11.1	14.1	12.0	12.9
ST.DEV.	3.3	1.9	1.7	1.9
N	8	8	9	9
POSTNATAL LOSS				
% OF LIVING PUPS	1.1	0.0	0.0	0.0
LITTERS AFFECTED (#)	1	0	0	0
TOTAL (#)	1	0	0	0
MEAN (+)	0.1	0.0	0.0	0.0
ST.DEV.	0.4	0.0	0.0	0.0
N	8	8	9	9
CULLED PUPS				
TOTAL	26	49	36	44
LIVING PUPS DAY 4 P.P.				
TOTAL	62	64	72	72
MEAN (+)	7.8	8.0	8.0	8.0
ST.DEV.	0.7	0.0	0.0	0.0
N	8	8	9	9
BREEDING LOSS DAYS 5 - 13 P.P.				
% OF LIVING PUPS AT DAY 4 P.P.	1.6	0.0	0.0	0.0
LITTERS AFFECTED (#)	1	0	0	0
TOTAL (#)	1	0	0	0
MEAN (+)	0.1	0.0	0.0	0.0
ST.DEV.	0.4	0.0	0.0	0.0
N	8	8	9	9
LIVING PUPS DAY 13 P.P.				
% OF MALES / FEMALES (#)	51 / 49	50 / 50	48 / 52	50 / 50
TOTAL	61	64	71	72
MEAN (+)	7.6	8.0	7.9	8.0
ST.DEV.	0.7	0.0	0.3	0.0
N	8	8	9	9

+++ Steel-test significant at 5% (+) or 1% (++) level

# / ### Fisher's Exact test significant at 5% (#) or 1% (###) level

03Jun19 14h19



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.15 DEVELOPMENTAL DATA

	GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
Total number of offspring born	89	113	109	116
Total number of uterine implantation sites	94	117	116	130
Number of live offspring on Day 1 after littering	89	113	108	116
Number of live offspring on Day 4 (before culling)	88	113	108	116
Number of live offspring on Day 4 (after culling)	62	64	72	72
Number of live offspring on Day 13 after littering	61	64	72	72
<b>Post-implantation survival index (%)</b> (Total number of offspring born/Total number of uterine implantation sites) * 100	95	97	94	89
<b>Live birth index (%)</b> (Number of live offspring on Day 1 after littering/Total number of offspring born) * 100	100	100	99	100
<b>Viability index (%)</b> (Number of live offspring on Day 4 (before culling)/Number of live offspring on Day 1 after littering)*100	99	100	100	100
<b>Lactation index (%)</b> (Number of live offspring on Day 13 after littering/Number of live offspring on Day 4 (after culling)) * 100	98	100	100	100

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.16 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

DAY	SEX		GROUP 1 CONTROL	GROUP 2 100 MG/KG	GROUP 3 300 MG/KG	GROUP 4 1000 MG/KG
1	M	MEAN	6.6	6.1	6.7	6.1
		ST.DEV.	0.8	0.4	0.7	0.3
		N	8	8	9	9
	F	MEAN	6.4	5.8	6.4	5.8
		ST.DEV.	0.8	0.4	0.7	0.4
		N	8	8	9	9
	M+F	MEAN	6.5	5.9	6.6	6.0
		ST.DEV.	0.8	0.4	0.6	0.3
		N	8	8	9	9
4	M	MEAN	9.8	8.7	10.2	9.1
		ST.DEV.	1.6	0.5	1.2	0.4
		N	8	8	9	9
	F	MEAN	9.5	8.4	9.8	8.7
		ST.DEV.	1.5	0.5	1.1	0.5
		N	8	8	9	9
	M+F	MEAN	9.6	8.6	10.0	8.9
		ST.DEV.	1.5	0.5	1.2	0.5
		N	8	8	9	9
7	M	MEAN	16.1	15.2	17.3	15.9
		ST.DEV.	2.1	0.8	1.8	0.6
		N	8	8	9	9
	F	MEAN	15.8	14.6	16.4	15.1
		ST.DEV.	2.0	0.6	1.7	0.9
		N	8	8	9	9
	M+F	MEAN	16.0	14.9	16.9	15.5
		ST.DEV.	2.0	0.6	1.8	0.7
		N	8	8	9	9
13	M	MEAN	31.3	30.7	32.7	31.6
		ST.DEV.	2.6	1.3	2.8	1.1
		N	8	8	9	9
	F	MEAN	30.7	29.6	31.7	30.4
		ST.DEV.	2.5	0.8	2.7	1.0
		N	8	8	9	9
	M+F	MEAN	31.0	30.1	32.2	31.0
		ST.DEV.	2.5	1.0	2.8	0.8
		N	8	8	9	9

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 14h19

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.17 ANOGENITAL DISTANCE AND NIPPLE RETENTION PER GROUP

### F0-GENERATION - LACTATION

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
anogenital dist M mm	MEAN	2.61	2.59	2.66	2.65
	ST.DEV.	0.11	0.09	0.14	0.08
	N	8	8	9	9
anogenital dist F mm	MEAN	0.95	1.04	1.00	1.01
	ST.DEV.	0.08	0.20	0.14	0.18
	N	8	8	9	9
Number of nipples	MEAN	0.00	0.00	0.00	0.00
	MEDIAN (+)	0.00	0.00	0.00	0.00
	N	8	8	9	8

# / ## Fisher's Exact test significant at 5% (#) or 1% (##) level  
+ / ++ Steel-test significant at 5% (+) or 1% (++) level

03Jun19 14h19

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.18 CORRECTED ANOGENITAL DISTANCE SUMMARY FEMALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<hr/>					
<b>PND 1</b>					
norm anog dist M	MEAN	1.40	1.42	1.41	1.45
mm	ST.DEV	0.05	0.03	0.07	0.05
	N	8	8	9	9
norm anog dist F	MEAN	0.51	0.58	0.54	0.56
mm	ST.DEV	0.05	0.10	0.08	0.10
	N	8	8	9	9

+ / ++ Steel-test significant at 5% (+) or 1% (++) level

03Jun19 13h42

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.19 CLINICAL BIOCHEMISTRY SUMMARY MALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<b>PUPS (PND 14-16)</b>					
Total T4	MEAN	6.58	6.19	6.11	6.03
ug/dL	ST.DEV	1.35	0.92	0.79	0.69
	N	8	8	9	9

---

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 14h12

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 1

Project 20172120

## 1.19 CLINICAL BIOCHEMISTRY SUMMARY FEMALES

---

		<b>GROUP 1 CONTROL</b>	<b>GROUP 2 100 MG/KG</b>	<b>GROUP 3 300 MG/KG</b>	<b>GROUP 4 1000 MG/KG</b>
<b>PUPS (PND 14-16)</b>					
Total T4	MEAN	5.98	5.93	5.74	5.28
ug/dL	ST.DEV	0.98	0.75	0.61	0.89
	N	8	8	9	9

---

\*/\*\* Dunnett-test based on pooled variance significant at 5% (\*) or 1% (\*\*) level

03Jun19 14h12

**Appendix 2**  
**Individual Tables**

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.1 MORTALITY DATA MALES

---

ANIMAL	SCHEDULED SACRIFICE	TREATMENT FROM	TO
<b>GROUP 1 (CONTROL)</b>			
1	06MAR19	05FEB19	05MAR19
2	06MAR19	05FEB19	05MAR19
3	06MAR19	05FEB19	05MAR19
4	06MAR19	05FEB19	05MAR19
5	06MAR19	05FEB19	05MAR19
6	06MAR19	05FEB19	05MAR19
7	06MAR19	05FEB19	05MAR19
8	06MAR19	05FEB19	05MAR19
9	06MAR19	05FEB19	05MAR19
10	06MAR19	05FEB19	05MAR19
<b>GROUP 2 (100 MG/KG)</b>			
11	06MAR19	05FEB19	05MAR19
12	06MAR19	05FEB19	05MAR19
13	06MAR19	05FEB19	05MAR19
14	06MAR19	05FEB19	05MAR19
15	06MAR19	05FEB19	05MAR19
16	06MAR19	05FEB19	05MAR19
17	06MAR19	05FEB19	05MAR19
18	06MAR19	05FEB19	05MAR19
19	06MAR19	05FEB19	05MAR19
20	06MAR19	05FEB19	05MAR19
<b>GROUP 3 (300 MG/KG)</b>			
21	06MAR19	05FEB19	05MAR19
22	06MAR19	05FEB19	05MAR19
23	06MAR19	05FEB19	05MAR19
24	06MAR19	05FEB19	05MAR19
25	06MAR19	05FEB19	05MAR19
26	06MAR19	05FEB19	05MAR19
27	06MAR19	05FEB19	05MAR19
28	06MAR19	05FEB19	05MAR19
29	06MAR19	05FEB19	05MAR19
30	06MAR19	05FEB19	05MAR19
<b>GROUP 4 (1000 MG/KG)</b>			
31	06MAR19	05FEB19	05MAR19
32	06MAR19	05FEB19	05MAR19
33	06MAR19	05FEB19	05MAR19
34	06MAR19	05FEB19	05MAR19
35	06MAR19	05FEB19	05MAR19
36	06MAR19	05FEB19	05MAR19
37	06MAR19	05FEB19	05MAR19
38	06MAR19	05FEB19	05MAR19
39	06MAR19	05FEB19	05MAR19
40	06MAR19	05FEB19	05MAR19

## FEMALES

---

ANIMAL	SCHEDULED SACRIFICE	TREATMENT FROM	TO
<b>GROUP 1 (CONTROL)</b>			
41	29MAR19	05FEB19	28MAR19
42	19MAR19	05FEB19	18MAR19
43	01APR19	05FEB19	31MAR19
44	29MAR19	05FEB19	28MAR19
45	29MAR19	05FEB19	28MAR19
46	29MAR19	05FEB19	28MAR19
47	28MAR19	05FEB19	27MAR19

03Jun19 13h46



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.1 MORTALITY DATA FEMALES

---

ANIMAL	SCHEDULED SACRIFICE	TREATMENT FROM	TO
<b>GROUP 1 (CONTROL)</b>			
48	01APR19	05FEB19	31MAR19
49	01APR19	05FEB19	31MAR19
50	29MAR19	05FEB19	28MAR19
<b>GROUP 2 (100 MG/KG)</b>			
51	19MAR19	05FEB19	18MAR19
52	29MAR19	05FEB19	28MAR19
53	19MAR19	05FEB19	18MAR19
54	28MAR19	05FEB19	27MAR19
55	02APR19	05FEB19	01APR19
56	29MAR19	05FEB19	28MAR19
57	29MAR19	05FEB19	28MAR19
58	29MAR19	05FEB19	28MAR19
59	01APR19	05FEB19	31MAR19
60	29MAR19	05FEB19	28MAR19
<b>GROUP 3 (300 MG/KG)</b>			
61	02APR19	05FEB19	01APR19
62	01APR19	05FEB19	31MAR19
63	19MAR19	05FEB19	18MAR19
64	29MAR19	05FEB19	28MAR19
65	01APR19	05FEB19	31MAR19
66	29MAR19	05FEB19	28MAR19
67	01APR19	05FEB19	31MAR19
68	29MAR19	05FEB19	28MAR19
69	01APR19	05FEB19	31MAR19
70	29MAR19	05FEB19	28MAR19
<b>GROUP 4 (1000 MG/KG)</b>			
71	28MAR19	05FEB19	27MAR19
72	19MAR19	05FEB19	18MAR19
73	28MAR19	05FEB19	27MAR19
74	29MAR19	05FEB19	28MAR19
75	29MAR19	05FEB19	28MAR19
76	29MAR19	05FEB19	28MAR19
77	01APR19	05FEB19	31MAR19
78	01APR19	05FEB19	31MAR19
79	28MAR19	05FEB19	27MAR19
80	29MAR19	05FEB19	28MAR19

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.2 CLINICAL SIGNS MALES

SIGN (MAX. GRADE) (LOCATION)	WEEK: DAY:	PRE MATING 1..... 12345671234567	REPRO PERIOD 1..... 123456712345671
---------------------------------	---------------	--	---

### GROUP 1 (CONTROL)

**ANIMAL 1**  
No clinical signs noted  
**ANIMAL 2**  
No clinical signs noted  
**ANIMAL 3**  
No clinical signs noted  
**ANIMAL 4**  
No clinical signs noted  
**ANIMAL 5**  
No clinical signs noted  
**ANIMAL 6**  
No clinical signs noted  
**ANIMAL 7**  
No clinical signs noted  
**ANIMAL 8**  
No clinical signs noted  
**ANIMAL 9**  
No clinical signs noted  
**ANIMAL 10**  
No clinical signs noted

### GROUP 2 (100 MG/KG)

**ANIMAL 11**  
No clinical signs noted  
**ANIMAL 12**  
No clinical signs noted  
**ANIMAL 13**  
No clinical signs noted  
**ANIMAL 14**  
Various  
Broken (1)  
(Tail apex)  
**ANIMAL 15**  
No clinical signs noted  
**ANIMAL 16**  
No clinical signs noted  
**ANIMAL 17**  
No clinical signs noted  
**ANIMAL 18**  
No clinical signs noted  
**ANIMAL 19**  
No clinical signs noted  
**ANIMAL 20**  
No clinical signs noted

### GROUP 3 (300 MG/KG)

**ANIMAL 21**  
No clinical signs noted  
**ANIMAL 22**  
No clinical signs noted  
**ANIMAL 23**  
No clinical signs noted  
**ANIMAL 24**  
No clinical signs noted  
**ANIMAL 25**  
No clinical signs noted  
**ANIMAL 26**  
No clinical signs noted  
**ANIMAL 27**  
No clinical signs noted

G: ..... 1111

G: Highest daily grades  
.: Observation performed, sign not present

03Jun19 13h46

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.2 CLINICAL SIGNS MALES

	PRE MATING	REPRO PERIOD
SIGN (MAX. GRADE)	WEEK: 1.....	1.....
(LOCATION)	DAY: 12345671234567	123456712345671

### GROUP 3 (300 MG/KG)

**ANIMAL 28**  
No clinical signs noted  
**ANIMAL 29**  
No clinical signs noted  
**ANIMAL 30**  
No clinical signs noted

### GROUP 4 (1000 MG/KG)

<b>ANIMAL 31</b> Secretion / excretion Salivation (3)	G: .....	111
<b>ANIMAL 32</b> Secretion / excretion Salivation (3)	G: .....	11
<b>ANIMAL 33</b> Secretion / excretion Salivation (3)	G: .....	1..11
<b>ANIMAL 34</b> Secretion / excretion Salivation (3)	G: .....	11111
<b>ANIMAL 35</b> Secretion / excretion Salivation (3)	G: .....	11111
<b>ANIMAL 36</b> Skin / fur Piloerection (1)	G: .....	1111..
Secretion / excretion Salivation (3)	G: .....	1
<b>ANIMAL 37</b> Secretion / excretion Salivation (3)	G: .....	11111
<b>ANIMAL 38</b> Secretion / excretion Salivation (3)	G: .....	1111
<b>ANIMAL 39</b> Secretion / excretion Salivation (3)	G: .....	1111
<b>ANIMAL 40</b> No clinical signs noted		

G: Highest daily grades  
.: Observation performed, sign not present

03Jun19 13h46

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.2 CLINICAL SIGNS FEMALES

SIGN (MAX. GRADE) (LOCATION)	PRE MATING		REPRO PERIOD			
	WEEK:	1.....	1.....	2.....	3.....	4.....
	DAY:	12345671234567	12345671234567	12345671234567	12345671234567	12345671234567
<b>GROUP 1 (CONTROL)</b>						
<b>ANIMAL 41</b>						
No clinical signs noted						
<b>ANIMAL 42</b>						
No clinical signs noted						
<b>ANIMAL 43</b>						
No clinical signs noted						
<b>ANIMAL 44</b>						
No clinical signs noted						
<b>ANIMAL 45</b>						
No clinical signs noted						
<b>ANIMAL 46</b>						
No clinical signs noted						
<b>ANIMAL 47</b>						
No clinical signs noted						
<b>ANIMAL 48</b>						
No clinical signs noted						
<b>ANIMAL 49</b>						
No clinical signs noted						
<b>ANIMAL 50</b>						
No clinical signs noted						
<b>GROUP 2 (100 MG/KG)</b>						
<b>ANIMAL 51</b>						
No clinical signs noted						
<b>ANIMAL 52</b>						
No clinical signs noted						
<b>ANIMAL 53</b>						
No clinical signs noted						
<b>ANIMAL 54</b>						
No clinical signs noted						
<b>ANIMAL 55</b>						
No clinical signs noted						
<b>ANIMAL 56</b>						
No clinical signs noted						
<b>ANIMAL 57</b>						
No clinical signs noted						
<b>ANIMAL 58</b>						
No clinical signs noted						
<b>ANIMAL 59</b>						
No clinical signs noted						
<b>ANIMAL 60</b>						
No clinical signs noted						
<b>GROUP 3 (300 MG/KG)</b>						
<b>ANIMAL 61</b>						
No clinical signs noted						
<b>ANIMAL 62</b>						
No clinical signs noted						
<b>ANIMAL 63</b>						
No clinical signs noted						
<b>ANIMAL 64</b>						
No clinical signs noted						
<b>ANIMAL 65</b>						
Skin / fur						
Scabs (3)	G:	.....	1	11.....		
(Cheek left)						
Scabs (3)	G:	.....	1	11.....		
(Cheek right)						
<b>ANIMAL 66</b>						
No clinical signs noted						

G: Highest daily grades  
.: Observation performed, sign not present

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.2 CLINICAL SIGNS FEMALES

SIGN (MAX. GRADE) (LOCATION)	PRE MATING		REPRO PERIOD			
	WEEK: 1.....	1.....	1.....	2.....	3.....	4.....
	DAY: 1234567	1234567	1234567	1234567	1234567	1234567
<b>GROUP 3 (300 MG/KG)</b>						
<b>ANIMAL 67</b>						
No clinical signs noted						
<b>ANIMAL 68</b>						
No clinical signs noted						
<b>ANIMAL 69</b>						
No clinical signs noted						
<b>ANIMAL 70</b>						
No clinical signs noted						
<b>GROUP 4 (1000 MG/KG)</b>						
<b>ANIMAL 71</b>						
No clinical signs noted						
<b>ANIMAL 72</b>						
Posture						
Hunched posture (1)	G:	.....	.....	.....	1111111..	.....
Skin / fur						
Piloerection (1)	G:	.....	.....	.....	11111....	.....
<b>ANIMAL 73</b>						
Secretion / excretion						
Salivation (3)	G:	.....	.....	.....	11..1.....	.....
<b>ANIMAL 74</b>						
No clinical signs noted						
<b>ANIMAL 75</b>						
No clinical signs noted						
<b>ANIMAL 76</b>						
Secretion / excretion						
Salivation (3)	G:	.....	.....	.....	11111.....	.....
<b>ANIMAL 77</b>						
No clinical signs noted						
<b>ANIMAL 78</b>						
No clinical signs noted						
<b>ANIMAL 79</b>						
Secretion / excretion						
Salivation (3)	G:	.....	.....	.....	1.....	.....
<b>ANIMAL 80</b>						
No clinical signs noted						

G: Highest daily grades  
.: Observation performed, sign not present

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.3 BODY WEIGHTS (GRAM) MALES

---

	PRE MATING		MATING PERIOD		
DAYS	1	8	1	8	15
WEEKS	1	2	1	2	3
ANIMAL					

---

### GROUP 1 (CONTROL)

1	285	304	311	321	339
2	268	284	298	304	327
3	317	337	353	362	374
4	295	324	340	350	364
5	296	321	343	354	368
6	287	306	325	330	331
7	291	311	327	340	361
8	302	334	359	372	395
9	308	326	345	354	371
10	294	312	328	335	351

### GROUP 2 (100 MG/KG)

11	285	315	337	354	377
12	315	343	360	371	391
13	280	307	328	345	366
14	301	322	334	351	363
15	295	308	311	317	323
16	305	337	363	378	403
17	270	282	294	302	314
18	285	310	327	340	360
19	305	323	339	356	371
20	313	330	349	361	378

### GROUP 3 (300 MG/KG)

21	331	357	378	391	415
22	283	296	313	330	342
23	311	328	341	360	372
24	280	300	314	325	342
25	282	307	331	341	361
26	284	301	329	346	362
27	311	326	340	361	381
28	280	299	318	337	357
29	298	315	330	337	353
30	275	293	306	311	321

### GROUP 4 (1000 MG/KG)

31	280	295	309	320	326
32	280	282	294	307	317
33	300	300	323	340	364
34	297	312	331	339	356
35	291	308	319	319	328
36	290	310	326	346	361
37	296	315	324	341	360
38	321	349	384	395	414
39	308	338	356	368	384
40	286	308	330	345	367

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.3 BODY WEIGHTS (GRAM) FEMALES

---

	PRE MATING		MATING PERIOD			
DAYS	1	8	1	8	15	22
WEEKS	1	2	1	2	3	4
ANIMAL						

---

### GROUP 1 (CONTROL)

41	213	214	215	---
42	222	223	229	---
43	239	247	254	---
44	218	220	225	---
45	220	215	234	---
46	243	246	240	271
47	216	212	224	---
48	228	222	225	---
49	234	238	239	---
50	214	217	216	---

### GROUP 2 (100 MG/KG)

51	224	227	232	
52	201	214	226	
53	237	238	240	
54	224	217	230	
55	213	215	215	
56	220	220	227	
57	215	222	232	
58	223	232	249	
59	218	215	222	
60	242	246	255	

### GROUP 3 (300 MG/KG)

61	246	244	255	
62	243	247	255	
63	220	219	228	
64	207	217	227	
65	243	250	263	
66	217	215	236	
67	233	236	243	
68	226	225	236	
69	221	229	228	
70	203	202	219	

### GROUP 4 (1000 MG/KG)

71	224	224	236	---	---	---
72	214	224	230	---	---	---
73	220	223	235	---	---	---
74	236	240	245	---	---	---
75	222	230	236	---	---	---
76	217	224	238	---	---	---
77	219	226	233	---	---	---
78	240	242	245	271	294	330
79	229	226	245	---	---	---
80	208	216	227	---	---	---

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.3 BODY WEIGHTS (GRAM) FEMALES

### F0-GENERATION

DAYS ANIMAL	POST COITUM						LACTATION				
	0	4	7	11	14	17	20	1	4	7	13
<b>GROUP 1 (CONTROL)</b>											
41	212	231	239	258	266	287	316	250	262	274	283
42 <IO>	230	247	256	265	260	258	269	---	---	---	---
43	251	269	274	284	292	310	339	273	276	298	318
44	227	237	248	261	270	301	339	246	272	271	290
45	229	243	248	263	272	288	312	262	266	281	280
46 <NP>	261	264	270	271	268	262	259	---	---	---	---
47	225	240	246	264	280	298	330	267	274	285	296
48	233	243	261	271	280	306	351	250	272	275	290
49	235	250	255	268	281	305	339	257	271	277	292
50	215	228	231	243	257	279	327	237	252	255	274
<b>GROUP 2 (100 MG/KG)</b>											
51 <NP>	237	239	241	246	244	243	246	---	---	---	---
52	224	240	239	252	261	286	325	234	261	264	279
53 <NP>	246	255	266	275	262	262	260	---	---	---	---
54	226	241	244	258	271	289	330	245	259	272	285
55	223	238	248	266	276	292	328	234	250	268	283
56	225	243	256	265	279	304	349	244	261	266	296
57	230	247	250	267	276	299	337	262	274	281	297
58	242	260	265	278	289	317	366	273	291	294	312
59	225	230	238	247	259	288	336	238	254	260	276
60	253	266	277	287	299	325	361	282	303	304	320
<b>GROUP 3 (300 MG/KG)</b>											
61	258	264	269	282	297	317	354	276	294	309	314
62	253	273	279	293	308	336	375	278	303	310	317
63 <NP>	225	244	246	245	244	236	235	---	---	---	---
64	227	242	243	261	278	298	336	263	275	275	288
65	258	277	289	305	320	355	403	304	323	332	338
66	230	246	250	263	278	295	337	249	269	278	303
67	241	252	264	278	285	356	356	259	283	287	308
68	240	256	267	285	295	319	348	279	289	305	319
69	238	250	252	269	274	305	332	250	262	272	286
70	211	228	233	248	260	280	312	245	264	265	282
<b>GROUP 4 (1000 MG/KG)</b>											
71	236	244	250	271	274	299	326	269	276	286	291
72 <NP>	230	244	251	257	243	241	245	---	---	---	---
73	236	251	252	279	286	314	353	274	290	296	302
74	242	247	254	267	276	301	345	246	262	273	282
75	236	257	263	274	289	312	357	265	277	292	305
76	232	242	254	259	266	290	321	249	277	280	301
77	244	257	265	291	302	325	380	285	303	312	303
78	---	---	---	---	---	---	---	268	300	310	322
79	240	254	259	282	289	316	348	273	280	290	302
80	229	234	239	255	264	289	328	263	280	277	290

<IO> Implantation site only  
<NP> Non-pregnant

28Jun19 08h18



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.4 BODY WEIGHT GAIN (%) MALES

	PRE MATING		MATING PERIOD		
DAYS	1	8	1	8	15
WEEKS	1	2	1	2	3
ANIMAL					
<b>GROUP 1 (CONTROL)</b>					
1	0	7	9	13	19
2	0	6	11	13	22
3	0	6	11	14	18
4	0	10	15	19	23
5	0	8	16	20	24
6	0	7	13	15	15
7	0	7	12	17	24
8	0	11	19	23	31
9	0	6	12	15	20
10	0	6	12	14	19
<b>GROUP 2 (100 MG/KG)</b>					
11	0	11	18	24	32
12	0	9	14	18	24
13	0	10	17	23	31
14	0	7	11	17	21
15	0	4	5	7	9
16	0	10	19	24	32
17	0	4	9	12	16
18	0	9	15	19	26
19	0	6	11	17	22
20	0	5	12	15	21
<b>GROUP 3 (300 MG/KG)</b>					
21	0	8	14	18	25
22	0	5	11	17	21
23	0	5	10	16	20
24	0	7	12	16	22
25	0	9	17	21	28
26	0	6	16	22	27
27	0	5	9	16	23
28	0	7	14	20	28
29	0	6	11	13	18
30	0	7	11	13	17
<b>GROUP 4 (1000 MG/KG)</b>					
31	0	5	10	14	16
32	0	1	5	10	13
33	0	0	8	13	21
34	0	5	11	14	20
35	0	6	10	10	13
36	0	7	12	19	24
37	0	6	9	15	22
38	0	9	20	23	29
39	0	10	16	19	25
40	0	8	15	21	28

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.4 BODY WEIGHT GAIN (%) FEMALES

DAYS WEEKS ANIMAL	PRE MATING		MATING PERIOD			
	1	8	1	8	15	22
	1	2	1	2	3	4
<b>GROUP 1 (CONTROL)</b>						
41	0	0	1	---		
42	0	0	3	---		
43	0	3	6	---		
44	0	1	3	---		
45	0	-2	6	---		
46	0	1	-1	12		
47	0	-2	4	---		
48	0	-3	-1	---		
49	0	2	2	---		
50	0	1	1	---		
<b>GROUP 2 (100 MG/KG)</b>						
51	0	1	4			
52	0	6	12			
53	0	0	1			
54	0	-3	3			
55	0	1	1			
56	0	0	3			
57	0	3	8			
58	0	4	12			
59	0	-1	2			
60	0	2	5			
<b>GROUP 3 (300 MG/KG)</b>						
61	0	-1	4			
62	0	2	5			
63	0	0	4			
64	0	5	10			
65	0	3	8			
66	0	-1	9			
67	0	1	4			
68	0	0	4			
69	0	4	3			
70	0	0	8			
<b>GROUP 4 (1000 MG/KG)</b>						
71	0	0	5	---	---	---
72	0	5	7	---	---	---
73	0	1	7	---	---	---
74	0	2	4	---	---	---
75	0	4	6	---	---	---
76	0	3	10	---	---	---
77	0	3	6	---	---	---
78	0	1	2	13	23	38
79	0	-1	7	---	---	---
80	0	4	9	---	---	---

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.4 BODY WEIGHT GAIN (%) FEMALES

### F0-GENERATION

DAYS ANIMAL	POST COITUM							LACTATION			
	0	4	7	11	14	17	20	1	4	7	13
<b>GROUP 1 (CONTROL)</b>											
41	0	9	13	22	25	35	49	0	5	10	13
42 <IO>	0	7	11	15	13	12	17	---	---	---	---
43	0	7	9	13	16	24	35	0	1	9	16
44	0	4	9	15	19	33	49	0	11	10	18
45	0	6	8	15	19	26	36	0	2	7	7
46 <NP>	0	1	3	4	3	0	-1	---	---	---	---
47	0	7	9	17	24	32	47	0	3	7	11
48	0	4	12	16	20	31	51	0	9	10	16
49	0	6	9	14	20	30	44	0	5	8	14
50	0	6	7	13	20	30	52	0	6	8	16
<b>GROUP 2 (100 MG/KG)</b>											
51 <NP>	0	1	2	4	3	3	4	---	---	---	---
52	0	7	7	13	17	28	45	0	12	13	19
53 <NP>	0	4	8	12	7	7	6	---	---	---	---
54	0	7	8	14	20	28	46	0	6	11	16
55	0	7	11	19	24	31	47	0	7	15	21
56	0	8	14	18	24	35	55	0	7	9	21
57	0	7	9	16	20	30	47	0	5	7	13
58	0	7	10	15	19	31	51	0	7	8	14
59	0	2	6	10	15	28	49	0	7	9	16
60	0	5	9	13	18	28	43	0	7	8	13
<b>GROUP 3 (300 MG/KG)</b>											
61	0	2	4	9	15	23	37	0	7	12	14
62	0	8	10	16	22	33	48	0	9	12	14
63 <NP>	0	8	9	9	8	5	4	---	---	---	---
64	0	7	7	15	22	31	48	0	5	5	10
65	0	7	12	18	24	38	56	0	6	9	11
66	0	7	9	14	21	28	47	0	8	12	22
67	0	5	10	15	18	48	48	0	9	11	19
68	0	7	11	19	23	33	45	0	4	9	14
69	0	5	6	13	15	28	39	0	5	9	14
70	0	8	10	18	23	33	48	0	8	8	15
<b>GROUP 4 (1000 MG/KG)</b>											
71	0	3	6	15	16	27	38	0	3	6	8
72 <NP>	0	6	9	12	6	5	7	---	---	---	---
73	0	6	7	18	21	33	50	0	6	8	10
74	0	2	5	10	14	24	43	0	7	11	15
75	0	9	11	16	22	32	51	0	5	10	15
76	0	4	9	12	15	25	38	0	11	12	21
77	0	5	9	19	24	33	56	0	6	9	6
78	---	---	---	---	---	---	---	0	12	16	20
79	0	6	8	18	20	32	45	0	3	6	11
80	0	2	4	11	15	26	43	0	6	5	10

<IO> Implantation site only  
<NP> Non-pregnant

28Jun19 09h03

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.5 FOOD CONSUMPTION (G/ANIMAL/DAY) MALES

---

	PRE MATING		MATING PERIOD	
DAYS	1-8	8-15	1-8	8-15
WEEKS	1-2	2-3	1-2	2-3
CAGE				

---

GROUP 1 (CONTROL)				
1	24	24	25	24
2	24	24	25	25

GROUP 2 (100 MG/KG)				
3	24	24	25	25
4	26	26	26	26

GROUP 3 (300 MG/KG)				
5	24	24	26	25
6	24	24	25	25

GROUP 4 (1000 MG/KG)				
7	23	23	26	24
8	25	25	25	24

## FEMALES

---

	PRE MATING	
DAYS	1-8	8-15
WEEKS	1-2	2-3
CAGE		

---

GROUP 1 (CONTROL)		
9	18	18
10	18	18

GROUP 2 (100 MG/KG)		
11	18	18
12	18	17

GROUP 3 (300 MG/KG)		
13	19	19
14	18	18

GROUP 4 (1000 MG/KG)		
15	19	18
16	18	18

17Jun19 08h57

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.5 FOOD CONSUMPTION (G/ANIMAL/DAY) FEMALES

### F0-GENERATION

DAYS ANIMAL	POST COITUM						LACTATION		
	0-4	4-7	7-11	11-14	14-17	17-20	1-4	4-7	7-13
<b>GROUP 1 (CONTROL)</b>									
41	20	22	22	21	22	27	42	39	50
42 <IO>	21	23	22	21	17	23	---	---	---
43	22	23	21	23	23	30	33	47	58
44	22	22	23	24	24	30	43	43	59
45	21	20	21	23	23	27	32	36	45
46 <NP>	18	21	25	29	18	17	---	---	---
47	21	21	23	26	24	26	31	53	54
48	22	23	23	23	25	29	40	35	44
49	22	22	23	23	24	28	33	43	57
50	20	20	20	23	24	31	29	41	57
<b>GROUP 2 (100 MG/KG)</b>									
51 <NP>	19	18	20	18	18	18	---	---	---
52	21	20	20	21	21	22	50	43	58
53 <NP>	24	24	24	22	20	17	---	---	---
54	20	19	19	22	21	23	34	51	55
55	20	21	22	21	22	23	27	44	57
56	23	25	22	23	21	26	30	40	57
57	21	19	20	21	22	25	33	43	58
58	23	21	21	24	24	27	51	43	59
59	18	20	19	20	21	27	30	42	56
60	20	22	21	22	22	28	36	46	60
<b>GROUP 3 (300 MG/KG)</b>									
61	21	21	22	22	24	24	36	50	56
62	24	25	26	26	26	31	46	42	55
63 <NP>	25	21	19	16	15	17	---	---	---
64	21	23	24	25	23	25	48	40	55
65	22	26	26	26	27	33	38	50	58
66	22	20	20	23	21	26	36	46	59
67	23	24	24	24	24	30	37	47	60
68	22	24	24	26	25	30	28	43	55
69	22	20	22	22	23	23	42	41	50
70	19	19	21	22	23	25	32	43	55
<b>GROUP 4 (1000 MG/KG)</b>									
71	24	18	21	24	22	23	36	56	55
72 <NP>	23	24	23	22	20	21	---	---	---
73	23	20	24	25	25	28	38	46	59
74	18	19	19	21	20	27	30	42	53
75	23	23	23	24	24	30	44	40	57
76	19	21	20	22	23	28	30	45	58
77	20	24	41 (8)	15	26	32	37	50	57
78	---	---	---	---	---	---	37	45	59
79	23	22	22	24	25	27	35	44	57
80	18	21	22	22	21	24	37	46	57

(8) Accidental food loss  
<IO> Implantation site only  
<NP> Non-pregnant

28Jun19 08h28

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.6 RELATIVE FOOD CONSUMPTION (G/KG BODY WEIGHT/DAY) MALES

---

	PRE MATING		MATING PERIOD	
DAYS	1-8	8-15	1-8	8-15
WEEKS	1-2	2-3	1-2	2-3
CAGE				

---

GROUP 1 (CONTROL)				
1	77	76	73	69
2	76	76	73	69

GROUP 2 (100 MG/KG)				
3	76	77	73	67
4	81	82	76	70

GROUP 3 (300 MG/KG)				
5	77	76	75	69
6	77	78	75	69

GROUP 4 (1000 MG/KG)				
7	77	78	79	72
8	78	78	70	65

## FEMALES

---

	PRE MATING	
DAYS	1-8	8-15
WEEKS	1-2	2-3
CAGE		

---

GROUP 1 (CONTROL)		
9	79	79
10	81	80

GROUP 2 (100 MG/KG)		
11	80	80
12	80	75

GROUP 3 (300 MG/KG)		
13	80	80
14	81	80

GROUP 4 (1000 MG/KG)		
15	83	80
16	78	78

17Jun19 09h00

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.6 RELATIVE FOOD CONSUMPTION (G/KG BODY WEIGHT/DAY) FEMALES

### F0-GENERATION

DAYS ANIMAL	POST COITUM						LACTATION		
	0-4	4-7	7-11	11-14	14-17	17-20	1-4	4-7	7-13
<b>GROUP 1 (CONTROL)</b>									
41	84	92	83	80	78	85	162	142	175
42 <IO>	85	89	84	79	67	86	---	---	---
43	81	85	73	80	75	89	121	157	183
44	94	90	87	88	81	88	158	159	204
45	87	82	79	83	80	85	120	128	161
46 <NP>	66	79	93	109	69	64	---	---	---
47	88	84	88	94	79	78	114	185	184
48	91	87	84	83	83	83	146	126	150
49	87	88	87	83	79	82	122	156	196
50	87	85	83	88	85	95	114	161	206
<b>GROUP 2 (100 MG/KG)</b>									
51 <NP>	78	75	79	72	74	75	---	---	---
52	85	85	80	79	75	68	193	164	207
53 <NP>	95	91	85	83	75	64	---	---	---
54	83	78	75	81	72	70	133	186	191
55	82	86	83	76	76	71	109	163	201
56	94	96	81	81	70	74	114	152	191
57	86	76	75	77	72	74	120	152	194
58	87	78	75	83	75	75	175	146	188
59	78	85	75	79	73	80	119	163	202
60	73	78	74	74	69	77	120	152	188
<b>GROUP 3 (300 MG/KG)</b>									
61	79	77	78	75	76	67	122	161	179
62	86	91	90	84	78	84	153	135	174
63 <NP>	100	87	79	66	62	74	---	---	---
64	85	93	93	91	77	74	175	145	192
65	78	89	84	82	77	83	119	151	171
66	88	80	75	83	71	76	134	164	196
67	90	91	86	85	67	85	132	164	196
68	86	89	82	88	79	87	96	140	171
69	86	79	83	82	75	69	162	151	174
70	83	83	85	83	82	80	122	161	196
<b>GROUP 4 (1000 MG/KG)</b>									
71	97	71	78	88	72	70	130	196	190
72 <NP>	94	97	89	89	84	87	---	---	---
73	93	81	84	89	81	79	132	154	194
74	73	75	69	77	68	78	116	153	187
75	90	89	82	83	76	83	160	136	186
76	77	81	77	81	80	88	108	162	193
77	76	91	141 (8)	50	79	84	123	161	186
78	---	---	---	---	---	---	123	146	182
79	90	84	79	83	80	77	126	152	190
80	78	88	86	85	74	74	133	165	196

(8) Accidental food loss  
<IO> implantation site only  
<NP> Non-pregnant

28Jun19 08h30

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.7 FUNCTIONAL OBSERVATIONS MALES

### END OF TREATMENT

ANIMAL	HEARING SCORE 0/1	PUPIL L SCORE 0/1	PUPIL R SCORE 0/1	STATIC R SCORE 0/1	GRIP FORE GRAM	GRIP HIND GRAM
<b>GROUP 1 (CONTROL)</b>						
1	0	0	0	0	1379	627
2	0	0	0	0	1325	723
3	0	0	0	0	1322	853
4	0	0	0	0	1480	737
5	0	0	0	0	1060	658
<b>GROUP 2 (100 MG/KG)</b>						
11	0	0	0	0	1475	769
12	0	0	0	0	1535	927
13	0	0	0	0	1496	753
14	0	0	0	0	1452	787
15	0	0	0	0	1410	707
<b>GROUP 3 (300 MG/KG)</b>						
21	0	0	0	0	1253	773
22	0	0	0	0	1070	683
23	0	0	0	0	1280	909
24	0	0	0	0	1305	633
25	0	0	0	0	1431	951
<b>GROUP 4 (1000 MG/KG)</b>						
31	0	0	0	0	1391	771
32	0	0	0	0	1063	601
33	0	0	0	0	1663	940
34	0	0	0	0	1401	838
35	0	0	0	0	1458	695

## FEMALES

### END OF TREATMENT

ANIMAL	HEARING SCORE 0/1	PUPIL L SCORE 0/1	PUPIL R SCORE 0/1	STATIC R SCORE 0/1	GRIP FORE GRAM	GRIP HIND GRAM
<b>GROUP 1 (CONTROL)</b>						
41	0	0	0	0	761	651
44	0	0	0	0	1407	638
45	0	0	0	0	679	540
47	0	0	0	0	920	635
50	0	0	0	0	931	447
<b>GROUP 2 (100 MG/KG)</b>						
52	0	0	0	0	819	674
54	0	0	0	0	1111	663
56	0	0	0	0	719	599
57	0	0	0	0	1146	690
58	0	0	0	0	1351	607
<b>GROUP 3 (300 MG/KG)</b>						
62	0	0	0	0	883	531
64	0	0	0	0	1116	687
66	0	0	0	0	1103	764
68	0	0	0	0	1289	605
70	0	0	0	0	960	513
<b>GROUP 4 (1000 MG/KG)</b>						
71	0	0	0	0	1016	617
73	0	0	0	0	993	595
74	0	0	0	0	1220	651
79	0	0	0	0	1434	555

03Jun19 13h46



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.7 FUNCTIONAL OBSERVATIONS FEMALES

### END OF TREATMENT

---

ANIMAL	HEARING SCORE 0/1	PUPIL L SCORE 0/1	PUPIL R SCORE 0/1	STATIC R SCORE 0/1	GRIP FORE GRAM	GRIP HIND GRAM
<b>GROUP 4 (1000 MG/KG)</b>						
80	0	0	0	0	877	431

---

03Jun19 13h46

**2.8 MOTOR ACTIVITY TEST - TOTAL MOVEMENTS  
MALES**

	INTERVAL (5 min.)												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
<b>ANIMAL</b>													
<b>Group 1 (CONTROL)</b>													
1	1096	484	274	341	270	288	303	38	356	187	344	153	4134
2	1221	910	419	325	96	33	13	2	41	348	89	31	3528
3	1336	661	405	442	532	439	203	64	0	263	102	95	4542
4	1313	578	295	664	131	374	359	6	274	18	5	6	4023
5	1204	792	538	508	344	275	121	0	0	0	22	0	3804
<b>Group 2 (100 MG/KG)</b>													
11	977	583	346	308	545	439	67	1	0	196	331	137	3930
12	1042	554	141	14	0	48	14	112	3	274	125	2	2329
13	751	608	391	156	372	297	288	149	13	209	499	428	4161
14	1327	680	217	622	589	369	170	58	0	10	2	7	4051
15	1005	548	295	49	3	12	41	0	18	13	7	369	2360
<b>Group 3 (300 MG/KG)</b>													
21	885	566	492	498	411	148	265	8	78	3	2	31	3387
22	1203	521	451	413	187	2	4	12	12	48	459	9	3321
23	769	478	400	424	333	214	304	369	238	44	165	1	3739
24	976	630	452	82	502	129	61	416	613	238	0	5	4104
25	1425	718	434	460	231	538	141	7	4	484	51	14	4507
<b>Group 4 (1000 MG/KG)</b>													
31	1153	676	253	175	398	311	0	0	4	25	19	119	3133
32	729	385	324	244	179	1	91	291	173	28	0	3	2448
33	729	468	283	45	590	65	18	3	22	20	507	116	2866
34	703	446	381	358	91	321	242	153	263	0	1	4	2963
35	1145	801	228	678	785	228	2	4	14	0	24	22	3931

**2.8 MOTOR ACTIVITY TEST - AMBULATIONS  
MALES**

	INTERVAL (5 min.)												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
<b>ANIMAL</b>													
<b>Group 1 (CONTROL)</b>													
1	327	117	58	111	60	87	109	2	126	34	112	15	1158
2	276	216	67	72	5	0	0	0	6	70	1	1	714
3	340	148	88	25	139	92	1	11	0	37	0	4	885
4	339	84	43	147	7	72	74	0	44	0	0	0	810
5	369	234	144	114	71	50	0	0	0	0	0	0	982
<b>Group 2 (100 MG/KG)</b>													
11	309	110	58	17	137	86	14	0	0	36	67	17	851
12	319	124	30	0	0	5	1	32	0	88	10	0	609
13	212	185	128	7	109	37	90	15	2	74	125	95	1079
14	345	182	25	146	104	73	3	6	0	0	0	0	884
15	220	84	76	2	0	0	1	0	1	0	1	65	450
<b>Group 3 (300 MG/KG)</b>													
21	321	159	102	95	100	2	23	1	0	0	0	1	804
22	270	118	79	59	3	0	0	1	0	8	76	1	615
23	281	193	159	89	138	27	138	102	43	21	69	0	1260
24	264	136	118	1	113	0	0	76	158	28	0	0	894
25	375	167	78	92	14	115	12	0	0	112	1	5	971
<b>Group 4 (1000 MG/KG)</b>													
31	396	166	64	19	108	80	0	0	0	2	0	29	864
32	240	88	129	47	54	0	0	66	65	1	0	1	691
33	258	139	5	2	205	5	3	0	0	0	161	32	810
34	255	148	101	98	34	81	71	31	79	0	0	0	898
35	344	249	36	185	211	47	0	0	0	0	1	0	1073

**2.8 MOTOR ACTIVITY TEST - TOTAL MOVEMENTS  
FEMALES**

	INTERVAL (5 min.)												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
<b>ANIMAL</b>													
<b>Group 1 (CONTROL)</b>													
41	876	592	316	125	62	251	136	283	28	6	45	34	2754
44	1054	608	448	370	470	587	386	135	406	291	422	419	5596
45	912	386	413	451	239	465	374	366	213	285	504	454	5062
47	908	471	410	462	415	574	483	473	334	312	356	299	5497
50	871	642	364	634	278	97	406	304	167	65	212	182	4222
<b>Group 2 (100 MG/KG)</b>													
52	958	627	361	391	32	554	201	16	41	5	10	369	3565
54	718	344	311	253	19	576	331	26	7	480	338	238	3641
56	921	649	620	472	466	303	238	294	171	297	232	81	4744
57	1058	406	259	375	30	308	53	192	133	9	44	0	2867
58	838	364	284	505	196	450	280	204	484	172	168	248	4193
<b>Group 3 (300 MG/KG)</b>													
62	749	564	406	246	47	447	324	306	235	451	54	266	4095
64	1370	689	544	532	192	478	452	553	343	228	1	649	6031
66	1097	654	708	500	363	667	410	254	303	344	292	226	5818
68	997	615	401	557	242	70	348	163	38	4	381	523	4339
70	1141	411	371	191	146	5	18	430	280	13	24	1	3031
<b>Group 4 (1000 MG/KG)</b>													
71	950	704	173	48	96	8	8	27	24	2	0	22	2062
73	863	437	95	36	51	49	12	274	7	0	0	145	1969
74	467	178	76	3	4	138	82	115	25	37	33	198	1356
79	764	660	79	595	180	615	0	0	378	423	499	273	4466
80	1138	592	384	314	459	19	272	406	520	399	355	371	5229

**2.8 MOTOR ACTIVITY TEST - AMBULATIONS  
FEMALES**

	INTERVAL (5 min.)												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
<b>ANIMAL</b>													
<b>Group 1 (CONTROL)</b>													
41	253	169	73	29	22	75	18	65	1	0	0	0	705
44	331	119	116	67	90	178	91	1	127	63	129	93	1405
45	285	88	113	111	42	60	94	87	9	62	137	117	1205
47	306	134	92	160	40	135	105	165	104	103	83	58	1485
50	324	190	92	189	85	28	158	54	61	28	86	52	1347
<b>Group 2 (100 MG/KG)</b>													
52	298	191	63	147	2	204	44	0	0	0	0	123	1072
54	174	88	65	58	0	174	59	0	0	135	51	60	864
56	337	245	210	125	134	108	30	91	43	109	14	13	1459
57	333	104	70	93	2	64	1	61	2	3	1	0	734
58	230	64	37	124	55	161	76	52	131	51	22	68	1071
<b>Group 3 (300 MG/KG)</b>													
62	263	112	97	36	0	140	54	83	60	146	13	74	1078
64	300	135	132	110	45	116	76	136	58	49	0	150	1307
66	224	138	111	91	46	155	75	22	62	37	0	6	967
68	228	114	91	136	70	0	74	0	1	0	79	145	938
70	326	86	87	0	35	0	0	92	82	0	0	0	708
<b>Group 4 (1000 MG/KG)</b>													
71	291	165	0	0	5	0	0	0	0	0	0	0	461
73	227	85	0	3	1	0	0	29	0	0	0	34	379
74	111	51	0	0	0	36	19	0	0	0	0	45	262
79	197	121	3	139	52	170	0	0	108	63	141	84	1078
80	334	150	56	77	112	1	71	127	164	151	123	96	1462

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.9 HAEMATOLOGY MALES END OF TREATMENT

ANIMAL	WBC 10E9/L	Neutrophils 10E9/L	Lymphocytes 10E9/L	Monocytes 10E9/L	Eosinophils 10E9/L
<b>GROUP 1 (CONTROL)</b>					
1	8.2	1.5	---	0.2	0.0
2	7.8	1.2	6.4	0.1	0.1
3	10.1	1.1	8.6	0.2	0.2
4	8.0	1.6	6.1	0.2	0.1
5	10.8	1.8	8.6	0.2	0.2
<b>GROUP 2 (100 MG/KG)</b>					
11	8.7	1.0	7.4	0.2	0.1
12	6.2	0.9	5.2	0.1	0.1
13	7.1	0.8	6.1	0.1	0.1
14	3.9	0.9	2.9	0.1	0.1
15	6.8	1.1	5.4	0.2	0.1
<b>GROUP 3 (300 MG/KG)</b>					
21	7.8	1.4	6.2	0.1	0.1
22	2.7	0.8	1.9	0.0	0.0
23	5.7	1.0	4.5	0.1	0.1
24	6.2	1.5	4.5	0.1	0.1
25	7.8	1.0	6.5	0.1	0.1
<b>GROUP 4 (1000 MG/KG)</b>					
31	11.5	1.4	9.8	0.2	0.1
32	6.6	1.2	5.2	0.1	0.1
33	6.8	1.2	5.4	0.1	0.1
34	7.2	1.4	---	0.1	0.0
35	7.1	1.0	5.8	0.1	0.2

## MALES END OF TREATMENT

ANIMAL	Basophils 10E9/L	Red blood cells 10E12/L	Reticulocytes 10E9/L	RDW %	Haemoglobin mmol/L
<b>GROUP 1 (CONTROL)</b>					
1	0.0	9.29	218.7	11.6	10.5
2	0.0	8.56	232.3	12.3	9.3
3	0.0	9.14	224.1	11.6	10.6
4	0.0	8.75	203.8	12.0	10.0
5	0.0	8.89	171.2	11.7	10.1
<b>GROUP 2 (100 MG/KG)</b>					
11	0.0	8.49	212.9	12.3	9.5
12	0.0	8.93	193.2	11.4	9.9
13	0.0	8.50	202.8	11.2	9.8
14	0.0	7.64	185.2	13.2	8.6
15	0.0	8.71	218.6	13.1	10.5
<b>GROUP 3 (300 MG/KG)</b>					
21	0.0	8.03	256.2	12.2	9.1
22	0.0	8.69	205.5	12.0	9.2
23	0.0	8.99	262.7	11.7	10.1
24	0.0	7.47	254.0	12.6	8.4
25	0.0	9.04	260.6	12.1	9.6
<b>GROUP 4 (1000 MG/KG)</b>					
31	0.0	9.08	237.4	11.6	10.5
32	0.0	9.21	192.1	12.7	9.8
33	0.0	8.64	243.8	12.7	9.8
34	0.0	8.96	227.1	12.3	10.0

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.9 HAEMATOLOGY MALES END OF TREATMENT

ANIMAL	Basophils 10E9/L	Red blood cells 10E12/L	Reticulocytes 10E9/L	RDW %	Haemoglobin mmol/L
<b>GROUP 4 (1000 MG/KG)</b>					
35	0.0	8.79	195.4	11.7	9.7

## MALES END OF TREATMENT

ANIMAL	Haematocrit L/L	MCV fL	MCH fmol	MCHC mmol/L	Platelets 10E9/L
<b>GROUP 1 (CONTROL)</b>					
1	0.484	52.1	1.13	21.67	651
2	0.440	51.3	1.09	21.14	695
3	0.512	56.0	1.16	20.63	656
4	0.469	53.6	1.14	21.30	655
5	0.475	53.4	1.13	21.18	720
<b>GROUP 2 (100 MG/KG)</b>					
11	0.455	53.6	1.12	20.83	707
12	0.470	52.6	1.11	21.01	621
13	0.467	54.9	1.15	20.98	507
14	0.389	51.0	1.13	22.12	760
15	0.471	54.0	1.20	22.20	855
<b>GROUP 3 (300 MG/KG)</b>					
21	0.436	54.3	1.14	20.96	814
22	0.438	50.5	1.06	20.92	833
23	0.480	53.4	1.13	21.09	697
24	0.414	55.4	1.12	20.21	697
25	0.461	51.0	1.06	20.74	776
<b>GROUP 4 (1000 MG/KG)</b>					
31	0.499	54.9	1.16	21.06	810
32	0.482	52.3	1.06	20.29	673
33	0.473	54.8	1.13	20.71	662
34	0.477	53.2	1.12	21.06	629
35	0.457	52.0	1.10	21.14	630

## MALES END OF TREATMENT

ANIMAL	PT s	APTT s
<b>GROUP 1 (CONTROL)</b>		
1	18.9	12.9
2	18.0	14.0
3	19.7	20.6
4	19.4	18.4
5	18.6	14.8
<b>GROUP 2 (100 MG/KG)</b>		
11	18.8	18.9
12	17.9	16.2
13	17.2	12.5
14	16.8	13.6
15	---	13.7

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.9 HAEMATOLOGY MALES END OF TREATMENT

ANIMAL	PT s	APTT s
<b>GROUP 3 (300 MG/KG)</b>		
21	16.8	10.6
22	18.4	17.2
23	17.2	12.7
24	18.7	16.1
25	17.7	13.1
<b>GROUP 4 (1000 MG/KG)</b>		
31	17.3	17.8
32	17.8	19.4
33	17.8	16.8
34	17.3	15.3
35	18.1	14.4

## FEMALES END OF TREATMENT

ANIMAL	WBC 10E9/L	Neutrophils 10E9/L	Lymphocytes 10E9/L	Monocytes 10E9/L	Eosinophils 10E9/L
<b>GROUP 1 (CONTROL)</b>					
41	8.5	1.7	6.5	0.1	0.1
44	7.4	2.0	5.0	0.2	0.3
45	5.4	1.8	3.2	0.2	0.2
47	4.3	1.1	2.9	0.1	0.2
50	3.7	1.1	2.4	0.1	0.1
<b>GROUP 2 (100 MG/KG)</b>					
52	6.9	1.3	5.3	0.1	0.1
54	4.4	1.2	3.1	0.1	0.1
56	5.3	2.0	3.2	0.1	0.1
57	4.5	1.3	3.1	0.1	0.0
58	4.1	0.9	3.0	0.1	0.1
<b>GROUP 3 (300 MG/KG)</b>					
62	5.9	0.9	4.9	0.1	0.0
64	6.9	2.4	4.3	0.1	0.1
66	3.8	1.1	2.5	0.1	0.1
68	8.1	3.0	4.8	0.2	0.1
70	6.1	1.8	4.1	0.1	0.1
<b>GROUP 4 (1000 MG/KG)</b>					
71	5.1	1.3	3.6	0.1	0.1
73	6.9	1.6	5.1	0.1	0.1
74	3.3	1.1	2.1	0.1	0.0
79	4.1	0.9	3.0	0.1	0.1
80	5.1	1.6	3.3	0.1	0.1

## FEMALES END OF TREATMENT

ANIMAL	Basophils 10E9/L	Red blood cells 10E12/L	Reticulocytes 10E9/L	RDW %	Haemoglobin mmol/L
<b>GROUP 1 (CONTROL)</b>					
41	0.0	8.26	285.5	13.2	9.5
44	0.0	7.31	246.5	13.7	9.1

19Jun19 15h26



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.9 HAEMATOLOGY FEMALES END OF TREATMENT

ANIMAL	Basophils 10E9/L	Red blood cells 10E12/L	Reticulocytes 10E9/L	RDW %	Haemoglobin mmol/L
<b>GROUP 1 (CONTROL)</b>					
45	0.0	7.41	156.4	10.3	8.5
47	0.0	7.43	208.5	12.9	8.6
50	0.0	6.25	219.5	12.9	8.2
<b>GROUP 2 (100 MG/KG)</b>					
52	0.0	7.80	215.2	12.4	9.4
54	0.0	7.22	208.3	11.8	8.8
56	0.0	7.15	245.5	13.2	8.5
57	0.0	6.73	282.8	12.9	8.2
58	0.0	7.22	215.3	14.0	8.9
<b>GROUP 3 (300 MG/KG)</b>					
62	0.0	7.18	189.3	11.7	8.7
64	0.0	7.02	184.0	12.4	8.7
66	0.0	6.69	195.6	14.2	9.0
68	0.0	6.81	220.3	11.5	8.5
70	0.0	7.41	235.3	12.3	8.9
<b>GROUP 4 (1000 MG/KG)</b>					
71	0.0	7.47	238.8	11.5	9.0
73	0.0	7.45	243.2	11.8	8.8
74	0.0	6.09	199.2	13.6	7.6
79	0.0	7.29	180.3	12.3	8.8
80	0.0	7.57	217.0	12.3	8.9

## FEMALES END OF TREATMENT

ANIMAL	Haematocrit L/L	MCV fL	MCH fmol	MCHC mmol/L	Platelets 10E9/L
<b>GROUP 1 (CONTROL)</b>					
41	0.471	57.0	1.15	20.11	708
44	0.448	61.3	1.25	20.32	696
45	0.423	57.1	1.14	20.02	722
47	0.431	58.0	1.16	20.02	748
50	0.384	61.4	1.31	21.28	712
<b>GROUP 2 (100 MG/KG)</b>					
52	0.458	58.7	1.21	20.62	929
54	0.425	58.8	1.22	20.72	639
56	0.433	60.6	1.19	19.69	845
57	0.408	60.6	1.22	20.15	715
58	0.444	61.5	1.24	20.14	745
<b>GROUP 3 (300 MG/KG)</b>					
62	0.437	60.8	1.21	19.95	741
64	0.427	60.8	1.24	20.38	565
66	0.439	65.6	1.34	20.41	622
68	0.413	60.7	1.24	20.47	732
70	0.449	60.5	1.21	19.93	602
<b>GROUP 4 (1000 MG/KG)</b>					
71	0.432	57.9	1.21	20.87	669
73	0.427	57.3	1.18	20.60	828
74	0.373	61.2	1.25	20.34	703
79	0.442	60.6	1.21	19.96	531
80	0.441	58.3	1.17	20.08	796

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.9 HAEMATOLOGY FEMALES END OF TREATMENT

---

ANIMAL	PT s	APTT s
<b>GROUP 1 (CONTROL)</b>		
41	17.9	20.0
44	16.3	17.5
45	17.8	19.7
47	16.7	19.5
50	---	---
<b>GROUP 2 (100 MG/KG)</b>		
52	16.9	22.8
54	15.4	17.7
56	18.1	21.2
57	18.6	21.0
58	17.4	20.9
<b>GROUP 3 (300 MG/KG)</b>		
62	17.5	20.5
64	17.9	21.6
66	---	26.0
68	17.5	20.6
70	17.3	17.9
<b>GROUP 4 (1000 MG/KG)</b>		
71	15.8	18.4
73	18.1	19.5
74	17.7	18.7
79	16.1	18.6
80	16.8	20.5

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.10 CLINICAL BIOCHEMISTRY MALES END OF TREATMENT

ANIMAL	ALAT U/L	ASAT U/L	ALP U/L	Total protein g/L	Albumin g/L
<b>GROUP 1 (CONTROL)</b>					
1	49.2	97.2	157	62.0	31.2
2	55.3	89.9	132	64.8	31.9
3	48.6	87.2	150	62.9	32.5
4	36.2	79.8	103	60.1	31.2
5	56.7	78.0	211	62.0	31.6
6	---	---	---	---	---
7	---	---	---	---	---
8	---	---	---	---	---
9	---	---	---	---	---
10	---	---	---	---	---
<b>GROUP 2 (100 MG/KG)</b>					
11	57.5	81.5	171	65.7	32.4
12	41.4	90.1	117	63.9	31.9
13	58.6	77.7	141	62.3	32.0
14	32.2	81.6	114	61.0	31.7
15	42.1	80.6	133	61.2	31.6
16	---	---	---	---	---
17	---	---	---	---	---
18	---	---	---	---	---
19	---	---	---	---	---
20	---	---	---	---	---
<b>GROUP 3 (300 MG/KG)</b>					
21	31.4	65.0	116	63.1	31.8
22	50.6	81.8	92	62.7	31.9
23	54.6	100.7	132	62.6	31.7
24	36.9	80.9	120	60.5	31.3
25	51.8	76.5	155	62.2	31.8
26	---	---	---	---	---
27	---	---	---	---	---
28	---	---	---	---	---
29	---	---	---	---	---
30	---	---	---	---	---
<b>GROUP 4 (1000 MG/KG)</b>					
31	59.5	95.2	126	65.2	33.0
32	51.2	87.5	158	59.8	31.3
33	45.7	104.2	200	58.8	29.8
34	48.4	85.3	161	65.0	31.9
35	47.6	80.4	154	58.0	30.2
36	---	---	---	---	---
37	---	---	---	---	---
38	---	---	---	---	---
39	---	---	---	---	---
40	---	---	---	---	---

## MALES END OF TREATMENT

ANIMAL	Total bilirubin umol/L	Urea mmol/L	Creatinine umol/L	Glucose mmol/L	Cholesterol mmol/L
<b>GROUP 1 (CONTROL)</b>					
1	3.1	4.8	36.3	6.52	1.48
2	2.1	7.5	32.7	11.02	1.85
3	2.7	6.4	37.5	11.03	1.73
4	1.9	6.6	39.9	9.07	1.97
5	1.8	6.7	37.5	10.73	1.82

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.10 CLINICAL BIOCHEMISTRY MALES END OF TREATMENT

ANIMAL	Total bilirubin umol/L	Urea mmol/L	Creatinine umol/L	Glucose mmol/L	Cholesterol mmol/L
<b>GROUP 1 (CONTROL)</b>					
6	---	---	---	---	---
7	---	---	---	---	---
8	---	---	---	---	---
9	---	---	---	---	---
10	---	---	---	---	---
<b>GROUP 2 (100 MG/KG)</b>					
11	2.4	6.9	38.1	7.07	1.78
12	2.0	5.7	39.3	8.65	2.31
13	2.0	9.3	43.5	8.75	2.01
14	2.1	5.8	38.1	6.93	1.69
15	2.4	6.1	38.1	9.77	1.72
16	---	---	---	---	---
17	---	---	---	---	---
18	---	---	---	---	---
19	---	---	---	---	---
20	---	---	---	---	---
<b>GROUP 3 (300 MG/KG)</b>					
21	2.0	6.4	36.3	9.61	1.53
22	1.9	8.1	39.9	8.68	1.71
23	2.1	6.0	34.5	7.57	1.51
24	2.0	5.8	36.9	6.67	1.33
25	2.3	8.8	40.5	10.92	1.86
26	---	---	---	---	---
27	---	---	---	---	---
28	---	---	---	---	---
29	---	---	---	---	---
30	---	---	---	---	---
<b>GROUP 4 (1000 MG/KG)</b>					
31	2.4	7.6	34.5	6.91	2.28
32	2.6	7.4	33.9	9.89	1.37
33	2.6	8.8	37.5	5.57	1.64
34	2.3	5.1	35.1	8.29	1.85
35	2.1	7.3	36.9	8.92	1.16
36	---	---	---	---	---
37	---	---	---	---	---
38	---	---	---	---	---
39	---	---	---	---	---
40	---	---	---	---	---

## MALES END OF TREATMENT

ANIMAL	Bile Acids umol/L	Sodium mmol/L	Potassium mmol/L	Chloride mmol/L	Calcium mmol/L
<b>GROUP 1 (CONTROL)</b>					
1	14.8	141.0	3.77	105	2.49
2	30.5	140.9	4.09	104	2.56
3	33.3	139.2	4.28	105	2.56
4	10.9	141.2	4.05	105	2.52
5	30.8	139.8	4.10	102	2.57
6	---	---	---	---	---
7	---	---	---	---	---
8	---	---	---	---	---
9	---	---	---	---	---
10	---	---	---	---	---

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.10 CLINICAL BIOCHEMISTRY MALES END OF TREATMENT

ANIMAL	Bile Acids umol/L	Sodium mmol/L	Potassium mmol/L	Chloride mmol/L	Calcium mmol/L
<b>GROUP 2 (100 MG/KG)</b>					
11	22.7	141.0	3.95	104	2.60
12	12.0	141.5	3.86	106	2.55
13	22.4	140.7	4.07	104	2.57
14	10.0	139.8	3.89	104	2.53
15	17.6	139.5	4.02	105	2.49
16	---	---	---	---	---
17	---	---	---	---	---
18	---	---	---	---	---
19	---	---	---	---	---
20	---	---	---	---	---
<b>GROUP 3 (300 MG/KG)</b>					
21	13.1	140.6	3.97	105	2.64
22	22.9	138.1	4.09	102	2.45
23	18.0	140.5	3.83	106	2.54
24	27.4	140.7	3.81	105	2.47
25	30.1	139.5	3.75	104	2.57
26	---	---	---	---	---
27	---	---	---	---	---
28	---	---	---	---	---
29	---	---	---	---	---
30	---	---	---	---	---
<b>GROUP 4 (1000 MG/KG)</b>					
31	34.6	140.3	3.88	104	2.59
32	43.9	141.6	3.87	103	2.53
33	42.3	142.7	3.73	105	2.50
34	10.2	140.5	4.51	105	2.57
35	29.3	141.0	3.63	105	2.50
36	---	---	---	---	---
37	---	---	---	---	---
38	---	---	---	---	---
39	---	---	---	---	---
40	---	---	---	---	---

## MALES END OF TREATMENT

ANIMAL	Inorg.Phos mmol/L	Total T4 ug/dL
<b>GROUP 1 (CONTROL)</b>		
1	1.96	6.06
2	1.92	4.67
3	1.63	4.04
4	1.89	5.26
5	1.86	6.73
6	---	4.10
7	---	3.64
8	---	6.35
9	---	4.71
10	---	5.51
<b>GROUP 2 (100 MG/KG)</b>		
11	2.09	6.39
12	1.76	3.65
13	2.01	5.07
14	1.67	3.71
15	1.39	3.45

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.10 CLINICAL BIOCHEMISTRY MALES END OF TREATMENT

---

ANIMAL	Inorg.Phos mmol/L	Total T4 ug/dL
--------	----------------------	-------------------

---

### GROUP 2 (100 MG/KG)

16	---	5.69
17	---	4.63
18	---	5.39
19	---	4.86
20	---	4.45

### GROUP 3 (300 MG/KG)

21	1.93	4.31
22	1.78	3.35
23	1.69	3.74
24	1.83	3.74
25	1.69	4.17
26	---	4.73
27	---	3.83
28	---	4.44
29	---	4.48
30	---	3.52

### GROUP 4 (1000 MG/KG)

31	1.96	4.50
32	1.89	2.73
33	2.08	5.50
34	1.96	4.81
35	1.80	3.54
36	---	4.69
37	---	6.23
38	---	5.41
39	---	3.50
40	---	3.99

## FEMALES END OF TREATMENT

---

ANIMAL	ALAT U/L	ASAT U/L	ALP U/L	Total protein g/L	Albumin g/L
--------	-------------	-------------	------------	----------------------	----------------

---

### GROUP 1 (CONTROL)

41	108.9	116.9	385	58.7	31.3
44	90.5	89.0	185	56.4	30.3
45	98.0	93.6	158	60.6	32.0
47	92.9	95.4	543	55.7	30.5
50	106.0	125.6	547	51.4	28.2

### GROUP 2 (100 MG/KG)

52	118.2	99.6	434	61.1	31.2
54	94.6	97.7	556	53.5	28.0
56	106.7	111.2	466	57.2	30.0
57	110.2	128.5	440	54.6	29.0
58	112.1	235.7	410	57.1	29.8

### GROUP 3 (300 MG/KG)

62	100.4	90.9	399	59.0	30.8
64	118.5	105.7	429	54.9	29.4
66	118.6	111.2	278	56.5	29.4
68	91.2	109.2	149	59.5	31.3
70	126.2	150.4	547	54.7	29.0

### GROUP 4 (1000 MG/KG)

71	117.6	95.9	512	53.8	28.1
----	-------	------	-----	------	------

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.10 CLINICAL BIOCHEMISTRY FEMALES END OF TREATMENT

ANIMAL	ALAT U/L	ASAT U/L	ALP U/L	Total protein g/L	Albumin g/L
<b>GROUP 4 (1000 MG/KG)</b>					
73	81.2	110.9	218	55.3	29.0
74	100.9	151.2	236	56.8	30.0
79	98.4	98.3	230	55.5	29.7
80	102.6	111.9	468	58.6	30.6

## FEMALES END OF TREATMENT

ANIMAL	Total bilirubin umol/L	Urea mmol/L	Creatinine umol/L	Glucose mmol/L	Cholesterol mmol/L
<b>GROUP 1 (CONTROL)</b>					
41	2.3	9.1	35.9	7.50	2.26
44	2.3	10.2	35.9	7.64	2.37
45	3.3	9.1	38.8	7.74	2.11
47	2.0	10.6	37.5	7.86	2.53
50	2.3	11.4	32.9	5.91	1.97
<b>GROUP 2 (100 MG/KG)</b>					
52	2.4	9.8	35.9	6.70	2.19
54	2.0	10.9	38.2	7.28	1.99
56	2.0	9.6	38.8	7.53	1.82
57	2.2	10.8	35.9	5.73	1.95
58	1.4	10.1	38.8	6.78	2.24
<b>GROUP 3 (300 MG/KG)</b>					
62	1.9	10.9	36.9	7.65	2.41
64	2.4	11.1	32.9	5.98	1.76
66	2.1	12.2	35.9	6.01	2.00
68	3.2	10.2	32.9	6.82	2.38
70	2.2	9.9	35.9	6.23	2.29
<b>GROUP 4 (1000 MG/KG)</b>					
71	2.0	11.5	32.7	6.43	2.16
73	2.3	9.5	35.5	8.22	2.09
74	1.7	8.6	37.3	7.73	2.31
79	2.0	14.5	39.6	7.54	2.23
80	1.7	10.9	34.4	7.26	1.88

## FEMALES END OF TREATMENT

ANIMAL	Bile Acids umol/L	Sodium mmol/L	Potassium mmol/L	Chloride mmol/L	Calcium mmol/L
<b>GROUP 1 (CONTROL)</b>					
41	45.7	141.6	4.89	103	2.55
44	19.9	138.5	4.44	100	2.51
45	19.0	140.9	4.37	104	2.50
47	21.8	136.6	4.17	99	2.53
50	20.9	141.5	4.30	104	2.41
<b>GROUP 2 (100 MG/KG)</b>					
52	27.0	142.2	4.09	101	2.58
54	14.4	137.5	4.55	103	2.43
56	18.6	139.8	4.78	104	2.45

19Jun19 15h26

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.10 CLINICAL BIOCHEMISTRY FEMALES END OF TREATMENT

---

ANIMAL	Bile Acids umol/L	Sodium mmol/L	Potassium mmol/L	Chloride mmol/L	Calcium mmol/L
<b>GROUP 2 (100 MG/KG)</b>					
57	49.0	140.6	4.34	104	2.38
58	27.1	141.3	4.37	104	2.40
<b>GROUP 3 (300 MG/KG)</b>					
62	45.7	139.7	5.07	101	2.56
64	17.0	139.8	4.70	103	2.59
66	20.0	140.3	4.63	104	2.38
68	28.1	140.1	4.76	104	2.52
70	20.9	138.7	5.07	104	2.52
<b>GROUP 4 (1000 MG/KG)</b>					
71	21.7	138.2	4.57	100	2.55
73	20.1	136.3	5.39	100	2.56
74	13.3	139.9	4.83	103	2.40
79	29.5	141.6	4.43	104	2.55
80	16.4	141.3	4.74	104	2.50

## FEMALES END OF TREATMENT

---

ANIMAL	Inorg.Phos mmol/L
<b>GROUP 1 (CONTROL)</b>	
41	0.79
44	1.07
45	0.43
47	0.64
50	0.57
<b>GROUP 2 (100 MG/KG)</b>	
52	1.64
54	0.36
56	0.76
57	0.79
58	0.50
<b>GROUP 3 (300 MG/KG)</b>	
62	1.24
64	1.20
66	0.47
68	1.42
70	0.64
<b>GROUP 4 (1000 MG/KG)</b>	
71	1.59
73	1.54
74	0.32
79	0.54
80	0.46

19Jun19 15h26



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.11 MACROSCOPIC FINDINGS MALES

### ALL NECROPSIES

ANIMAL	ORGAN	FINDING	DAY OF DEATH
<b>GROUP 1 (CONTROL)</b>			
1		No findings noted	Scheduled sacrifice, 06Mar2019
2		No findings noted	Scheduled sacrifice, 06Mar2019
3		No findings noted	Scheduled sacrifice, 06Mar2019
4		No findings noted	Scheduled sacrifice, 06Mar2019
5		No findings noted	Scheduled sacrifice, 06Mar2019
6		No findings noted	Scheduled sacrifice, 06Mar2019
7		No findings noted	Scheduled sacrifice, 06Mar2019
8		No findings noted	Scheduled sacrifice, 06Mar2019
9		No findings noted	Scheduled sacrifice, 06Mar2019
10		No findings noted	Scheduled sacrifice, 06Mar2019
<b>GROUP 2 (100 MG/KG)</b>			
11		No findings noted	Scheduled sacrifice, 06Mar2019
12		No findings noted	Scheduled sacrifice, 06Mar2019
13		No findings noted	Scheduled sacrifice, 06Mar2019
14	Bone	Tail, tail apex: tail bent.	Scheduled sacrifice, 06Mar2019
15		No findings noted	Scheduled sacrifice, 06Mar2019
16		No findings noted	Scheduled sacrifice, 06Mar2019
17		No findings noted	Scheduled sacrifice, 06Mar2019
18		No findings noted	Scheduled sacrifice, 06Mar2019
19		No findings noted	Scheduled sacrifice, 06Mar2019
20		No findings noted	Scheduled sacrifice, 06Mar2019
<b>GROUP 3 (300 MG/KG)</b>			
21		No findings noted	Scheduled sacrifice, 06Mar2019
22		No findings noted	Scheduled sacrifice, 06Mar2019
23		No findings noted	Scheduled sacrifice, 06Mar2019
24		No findings noted	Scheduled sacrifice, 06Mar2019
25		No findings noted	Scheduled sacrifice, 06Mar2019
26		No findings noted	Scheduled sacrifice, 06Mar2019
27		No findings noted	Scheduled sacrifice, 06Mar2019
28		No findings noted	Scheduled sacrifice, 06Mar2019
29		No findings noted	Scheduled sacrifice, 06Mar2019
30		No findings noted	Scheduled sacrifice, 06Mar2019
<b>GROUP 4 (1000 MG/KG)</b>			
31	Liver	Discolouration, red-brown.	Scheduled sacrifice, 06Mar2019
32	Kidneys	Cranial pole, right side middle part irregular surface.	Scheduled sacrifice, 06Mar2019
33		No findings noted	Scheduled sacrifice, 06Mar2019
34		No findings noted	Scheduled sacrifice, 06Mar2019
35	Epididymides	Left side, tail: nodule(s), d=10x6 mm, yellowish, soft.	Scheduled sacrifice, 06Mar2019
36		No findings noted	Scheduled sacrifice, 06Mar2019
37		No findings noted	Scheduled sacrifice, 06Mar2019
38		No findings noted	Scheduled sacrifice, 06Mar2019
39		No findings noted	Scheduled sacrifice, 06Mar2019
40		No findings noted	Scheduled sacrifice, 06Mar2019

### FEMALES

#### ALL NECROPSIES

ANIMAL	ORGAN	FINDING	DAY OF DEATH
<b>GROUP 1 (CONTROL)</b>			
41		No findings noted	Scheduled sacrifice, 29Mar2019
42	Uterus	Right horn: nodule(s), d=8x7 mm, tan, reddish, hard. Right horn: contents: greenish, gelatinous.	Scheduled sacrifice, 19Mar2019

03Jun19 13h46

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.11 MACROSCOPIC FINDINGS FEMALES

### ALL NECROPSIES

ANIMAL	ORGAN	FINDING	DAY OF DEATH
<b>GROUP 1 (CONTROL)</b>			
	Thymus	Right side: focus/foci, isolated, reddish.	
	Mandibular lymph n	Both sides: focus/foci, isolated, dark red.	
	Eyes	Left side: exophthalmus.	
43		No findings noted	Scheduled sacrifice, 01Apr2019
44		No findings noted	Scheduled sacrifice, 29Mar2019
45		No findings noted	Scheduled sacrifice, 29Mar2019
46		No findings noted	Scheduled sacrifice, 29Mar2019
47		No findings noted	Scheduled sacrifice, 28Mar2019
48		No findings noted	Scheduled sacrifice, 01Apr2019
49		No findings noted	Scheduled sacrifice, 01Apr2019
50		No findings noted	Scheduled sacrifice, 29Mar2019
<b>GROUP 2 (100 MG/KG)</b>			
51		No findings noted	Scheduled sacrifice, 19Mar2019
52	Body cavities	Thoracic cavity: contains fluid, watery-clear.	Scheduled sacrifice, 29Mar2019
53		No findings noted	Scheduled sacrifice, 19Mar2019
54		No findings noted	Scheduled sacrifice, 28Mar2019
55		No findings noted	Scheduled sacrifice, 02Apr2019
56		No findings noted	Scheduled sacrifice, 29Mar2019
57	Cliitoral glands Body cavities	Left side: focus/foci, d=6x2 mm, tan. Thoracic cavity: contains fluid, watery-clear.	Scheduled sacrifice, 29Mar2019
58		No findings noted	Scheduled sacrifice, 29Mar2019
59		No findings noted	Scheduled sacrifice, 01Apr2019
60		No findings noted	Scheduled sacrifice, 29Mar2019
<b>GROUP 3 (300 MG/KG)</b>			
61		No findings noted	Scheduled sacrifice, 02Apr2019
62		No findings noted	Scheduled sacrifice, 01Apr2019
63	Uterus	Contains fluid.	Scheduled sacrifice, 19Mar2019
64	Jejunum	Focus/foci, d=2x1 mm, reddish.	Scheduled sacrifice, 29Mar2019
65	Body cavities	Abdominal cavity, uterine adipose tissue, right side: nodule(s), d=5x2 mm, tan, hard.	Scheduled sacrifice, 01Apr2019
66	Thyroid gland Eyes	Both sides: discolouration, pale. Right side: exophthalmus.	Scheduled sacrifice, 29Mar2019
67	Thymus	Right side: focus/foci, many, reddish.	Scheduled sacrifice, 01Apr2019
68		No findings noted	Scheduled sacrifice, 29Mar2019
69		No findings noted	Scheduled sacrifice, 01Apr2019
70	Body cavities	Thoracic cavity: contains fluid, watery-clear.	Scheduled sacrifice, 29Mar2019
<b>GROUP 4 (1000 MG/KG)</b>			
71		No findings noted	Scheduled sacrifice, 28Mar2019
72	Uterus Mandibular lymph n	Contains fluid. Both sides: focus/foci, isolated, dark red.	Scheduled sacrifice, 19Mar2019
	Eyes	Left side: exophthalmus.	
73		No findings noted	Scheduled sacrifice, 28Mar2019
74	Thyroid gland	Both sides: discolouration, pale.	Scheduled sacrifice, 29Mar2019
75		No findings noted	Scheduled sacrifice, 29Mar2019
76		No findings noted	Scheduled sacrifice, 29Mar2019
77		No findings noted	Scheduled sacrifice, 01Apr2019
78		No findings noted	Scheduled sacrifice, 01Apr2019
79		No findings noted	Scheduled sacrifice, 28Mar2019
80		No findings noted	Scheduled sacrifice, 29Mar2019

03Jun19 13h46

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN WEIGHTS (GRAM)

### MALES

#### END OF TREATMENT

ANIMAL	BODY W. (GRAM)	BRAIN (GRAM)	HEART (GRAM)	LIVER (GRAM)
<b>GROUP 1 (CONTROL)</b>				
1	317	1.94	0.963	7.64
2	304	2.00	0.837	7.44
3	351	2.10	1.024	8.42
4	347	2.03	0.993	7.99
5	345	1.92	0.993	8.81
6	310	---	---	---
7	335	---	---	---
8	368	---	---	---
9	348	---	---	---
10	326	---	---	---
<b>GROUP 2 (100 MG/KG)</b>				
11	353	2.15	0.898	8.51
12	364	2.07	0.982	8.42
13	341	1.86	0.887	7.88
14	337	2.05	0.898	7.78
15	306	1.99	0.803	6.83
16	374	---	---	---
17	292	---	---	---
18	335	---	---	---
19	345	---	---	---
20	357	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
21	383	2.08	1.105	9.05
22	320	1.95	0.863	7.59
23	344	2.13	0.914	8.38
24	318	2.05	0.852	7.31
25	341	1.88	0.956	8.40
26	337	---	---	---
27	351	---	---	---
28	330	---	---	---
29	331	---	---	---
30	299	---	---	---
<b>GROUP 4 (1000 MG/KG)</b>				
31	303	2.04	0.838	7.48
32	291	1.87	0.865	6.97
33	338	1.94	0.945	7.08
34	334	2.03	0.874	8.29
35	302	2.02	0.821	6.63
36	330	---	---	---
37	335	---	---	---
38	383	---	---	---
39	359	---	---	---
40	346	---	---	---

### MALES

#### END OF TREATMENT

ANIMAL	THYROIDS (GRAM)	THYMUS (GRAM)	KIDNEYS (GRAM)	ADRENALS (GRAM)
<b>GROUP 1 (CONTROL)</b>				
1	0.021	0.484	2.24	0.071
2	0.018	0.397	2.18	0.048
3	0.016	0.383	2.39	0.059
4	0.016	0.317	2.31	0.062
5	0.016	0.343	2.21	0.058

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN WEIGHTS (GRAM)

### MALES

#### END OF TREATMENT

ANIMAL	THYROIDES (GRAM)	THYMUS (GRAM)	KIDNEYS (GRAM)	ADRENALS (GRAM)
<b>GROUP 1 (CONTROL)</b>				
6	0.016	---	---	---
7	0.017	---	---	---
8	0.015	---	---	---
9	0.016	---	---	---
10	0.014	---	---	---
<b>GROUP 2 (100 MG/KG)</b>				
11	0.017	0.463	2.18	0.056
12	0.015	0.348	2.35	0.054
13	0.013	0.407	2.16	0.054
14	0.015	0.298	2.06	0.048
15	0.013	0.317	2.23	0.054
16	0.014	---	---	---
17	0.015	---	---	---
18	0.014	---	---	---
19	0.023	---	---	---
20	0.018	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
21	0.019	0.469	2.73	0.076
22	0.015	0.230	2.10	0.067
23	0.016	0.307	2.54	0.076
24	0.013	0.339	2.01	0.068
25	0.017	0.385	2.22	0.064
26	0.013	---	---	---
27	0.019	---	---	---
28	0.021	---	---	---
29	0.015	---	---	---
30	0.011	---	---	---
<b>GROUP 4 (1000 MG/KG)</b>				
31	0.014	0.359	2.54	0.061
32	0.016	0.204	2.30	0.053
33	0.015	0.310	2.18	0.060
34	0.019	0.411	2.40	0.061
35	0.015	0.218	2.05	0.055
36	0.023	---	---	---
37	0.018	---	---	---
38	0.019	---	---	---
39	0.016	---	---	---
40	0.022	---	---	---

### MALES

#### END OF TREATMENT

ANIMAL	SPLEEN (GRAM)	TESTES (GRAM)	PROSTATE GLAND (GRAM)	EPIDIDYMIDES (GRAM)
<b>GROUP 1 (CONTROL)</b>				
1	0.689	3.40	0.945	1.141
2	0.502	2.84	0.623	0.968
3	0.833	3.90	0.767	1.212
4	0.606	3.40	0.858	1.001
5	0.630	3.46	0.908	1.041
6	---	3.22	0.828	0.988
7	---	3.26	0.863	1.083
8	---	3.54	0.965	1.132
9	---	3.57	1.007	1.098
10	---	3.28	0.870	0.940

03Jun19 13h46

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN WEIGHTS (GRAM)

### MALES

#### END OF TREATMENT

ANIMAL	SPLEEN (GRAM)	TESTES (GRAM)	PROSTATE GLAND (GRAM)	EPIDIDYMIDES (GRAM)
<b>GROUP 2 (100 MG/KG)</b>				
11	0.624	3.44	0.792	1.071
12	0.617	3.42	0.837	1.160
13	0.561	3.25	1.018	1.007
14	0.704	3.09	0.724	1.107
15	0.577	3.06	0.675	1.022
16	---	3.47	0.939	1.068
17	---	3.17	0.865	0.999
18	---	3.17	0.816	0.972
19	---	3.44	1.016	1.096
20	---	3.35	0.677	1.089
<b>GROUP 3 (300 MG/KG)</b>				
21	0.665	3.51	1.090	1.145
22	0.461	3.03	0.867	1.032
23	0.632	3.28	0.922	1.176
24	0.638	3.79	0.899	1.095
25	0.538	3.29	0.567	1.028
26	---	3.44	0.931	0.960
27	---	3.21	0.861	1.066
28	---	3.59	0.809	1.185
29	---	3.59	0.737	1.012
30	---	3.13	0.731	1.054
<b>GROUP 4 (1000 MG/KG)</b>				
31	0.557	3.37	0.625	1.048
32	0.476	3.12	0.614	1.025
33	0.564	3.19	0.919	1.014
34	0.551	3.17	0.852	1.092
35	0.478	3.23	0.766	1.214
36	---	3.34	0.876	1.173
37	---	3.24	0.884	1.006
38	---	3.72	0.718	0.992
39	---	3.72	0.582	0.994
40	---	3.12	1.017	1.100

### MALES

#### END OF TREATMENT

ANIMAL	SEMINAL VESICLES (GRAM)
<b>GROUP 1 (CONTROL)</b>	
1	1.479
2	1.200
3	1.279
4	1.218
5	1.510
6	1.269
7	1.450
8	1.496
9	1.365
10	1.119
<b>GROUP 2 (100 MG/KG)</b>	
11	1.263
12	1.086
13	0.974
14	1.379
15	1.330

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN WEIGHTS (GRAM) MALES END OF TREATMENT

---

ANIMAL	SEMINAL VESICLES (GRAM)
--------	----------------------------

---

### GROUP 2 (100 MG/KG)

16	1.332
17	1.236
18	1.098
19	1.621
20	1.440

### GROUP 3 (300 MG/KG)

21	1.029
22	1.337
23	1.743
24	1.434
25	1.233
26	1.312
27	1.209
28	1.621
29	1.152
30	0.976

### GROUP 4 (1000 MG/KG)

31	1.248
32	0.905
33	1.115
34	1.281
35	1.374
36	1.584
37	1.160
38	1.176
39	0.942
40	0.933

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN/BODY WEIGHT RATIOS (%)

### MALES

#### END OF TREATMENT

ANIMAL	BODY W. (GRAM)	BRAIN (%)	HEART (%)	LIVER (%)
<b>GROUP 1 (CONTROL)</b>				
1	317	0.61	0.304	2.41
2	304	0.66	0.275	2.45
3	351	0.60	0.292	2.40
4	347	0.59	0.286	2.30
5	345	0.56	0.288	2.55
6	310	---	---	---
7	335	---	---	---
8	368	---	---	---
9	348	---	---	---
10	326	---	---	---
<b>GROUP 2 (100 MG/KG)</b>				
11	353	0.61	0.254	2.41
12	364	0.57	0.270	2.31
13	341	0.55	0.260	2.31
14	337	0.61	0.266	2.31
15	306	0.65	0.262	2.23
16	374	---	---	---
17	292	---	---	---
18	335	---	---	---
19	345	---	---	---
20	357	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
21	383	0.54	0.289	2.36
22	320	0.61	0.270	2.37
23	344	0.62	0.266	2.44
24	318	0.64	0.268	2.30
25	341	0.55	0.280	2.46
26	337	---	---	---
27	351	---	---	---
28	330	---	---	---
29	331	---	---	---
30	299	---	---	---
<b>GROUP 4 (1000 MG/KG)</b>				
31	303	0.67	0.277	2.47
32	291	0.64	0.297	2.39
33	338	0.57	0.280	2.10
34	334	0.61	0.262	2.48
35	302	0.67	0.272	2.20
36	330	---	---	---
37	335	---	---	---
38	383	---	---	---
39	359	---	---	---
40	346	---	---	---

### MALES

#### END OF TREATMENT

ANIMAL	THYROIDS (%)	THYMUS (%)	KIDNEYS (%)	ADRENALS (%)
<b>GROUP 1 (CONTROL)</b>				
1	0.007	0.153	0.71	0.022
2	0.006	0.131	0.72	0.016
3	0.005	0.109	0.68	0.017
4	0.005	0.091	0.67	0.018
5	0.005	0.099	0.64	0.017

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN/BODY WEIGHT RATIOS (%)

### MALES

#### END OF TREATMENT

ANIMAL	THYROIDS (%)	THYMUS (%)	KIDNEYS (%)	ADRENALS (%)
<b>GROUP 1 (CONTROL)</b>				
6	0.005	---	---	---
7	0.005	---	---	---
8	0.004	---	---	---
9	0.005	---	---	---
10	0.004	---	---	---
<b>GROUP 2 (100 MG/KG)</b>				
11	0.005	0.131	0.62	0.016
12	0.004	0.096	0.65	0.015
13	0.004	0.119	0.63	0.016
14	0.004	0.088	0.61	0.014
15	0.004	0.104	0.73	0.018
16	0.004	---	---	---
17	0.005	---	---	---
18	0.004	---	---	---
19	0.007	---	---	---
20	0.005	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
21	0.005	0.122	0.71	0.020
22	0.005	0.072	0.66	0.021
23	0.005	0.089	0.74	0.022
24	0.004	0.107	0.63	0.021
25	0.005	0.113	0.65	0.019
26	0.004	---	---	---
27	0.005	---	---	---
28	0.006	---	---	---
29	0.005	---	---	---
30	0.004	---	---	---
<b>GROUP 4 (1000 MG/KG)</b>				
31	0.005	0.118	0.84	0.020
32	0.005	0.070	0.79	0.018
33	0.004	0.092	0.65	0.018
34	0.006	0.123	0.72	0.018
35	0.005	0.072	0.68	0.018
36	0.007	---	---	---
37	0.005	---	---	---
38	0.005	---	---	---
39	0.004	---	---	---
40	0.006	---	---	---

### MALES

#### END OF TREATMENT

ANIMAL	SPLEEN (%)	TESTES (%)	PROSTATE GLAND (%)	EPIDIDYMIDES (%)
<b>GROUP 1 (CONTROL)</b>				
1	0.217	1.07	0.298	0.360
2	0.165	0.93	0.205	0.318
3	0.237	1.11	0.219	0.345
4	0.175	0.98	0.247	0.288
5	0.183	1.00	0.263	0.302
6	---	1.04	0.267	0.319
7	---	0.97	0.258	0.323
8	---	0.96	0.262	0.308
9	---	1.03	0.289	0.316
10	---	1.01	0.267	0.288

03Jun19 13h46



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN/BODY WEIGHT RATIOS (%)

### MALES

#### END OF TREATMENT

ANIMAL	SPLEEN (%)	TESTES (%)	PROSTATE GLAND (%)	EPIDIDYMIDES (%)
<b>GROUP 2 (100 MG/KG)</b>				
11	0.177	0.97	0.224	0.303
12	0.170	0.94	0.230	0.319
13	0.165	0.95	0.299	0.295
14	0.209	0.92	0.215	0.328
15	0.189	1.00	0.221	0.334
16	---	0.93	0.251	0.286
17	---	1.09	0.296	0.342
18	---	0.94	0.244	0.290
19	---	1.00	0.294	0.318
20	---	0.94	0.190	0.305
<b>GROUP 3 (300 MG/KG)</b>				
21	0.174	0.92	0.285	0.299
22	0.144	0.95	0.271	0.323
23	0.184	0.95	0.268	0.342
24	0.201	1.19	0.283	0.344
25	0.158	0.97	0.166	0.301
26	---	1.02	0.276	0.285
27	---	0.91	0.245	0.304
28	---	1.09	0.245	0.359
29	---	1.08	0.223	0.306
30	---	1.05	0.244	0.353
<b>GROUP 4 (1000 MG/KG)</b>				
31	0.184	1.11	0.206	0.346
32	0.164	1.07	0.211	0.352
33	0.167	0.94	0.272	0.300
34	0.165	0.95	0.255	0.327
35	0.158	1.07	0.254	0.402
36	---	1.01	0.265	0.355
37	---	0.97	0.264	0.300
38	---	0.97	0.187	0.259
39	---	1.04	0.162	0.277
40	---	0.90	0.294	0.318

### MALES

#### END OF TREATMENT

ANIMAL	SEMINAL VESICLES (%)
<b>GROUP 1 (CONTROL)</b>	
1	0.467
2	0.395
3	0.364
4	0.351
5	0.438
6	0.409
7	0.433
8	0.407
9	0.392
10	0.343
<b>GROUP 2 (100 MG/KG)</b>	
11	0.358
12	0.298
13	0.286
14	0.409
15	0.435

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN/BODY WEIGHT RATIOS (%)

### MALES

#### END OF TREATMENT

---

ANIMAL	SEMINAL VESICLES (%)
--------	----------------------

---

#### GROUP 2 (100 MG/KG)

16	0.356
17	0.423
18	0.328
19	0.470
20	0.403

#### GROUP 3 (300 MG/KG)

21	0.269
22	0.418
23	0.507
24	0.451
25	0.362
26	0.389
27	0.344
28	0.491
29	0.348
30	0.326

#### GROUP 4 (1000 MG/KG)

31	0.412
32	0.311
33	0.330
34	0.384
35	0.455
36	0.480
37	0.346
38	0.307
39	0.262
40	0.270

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN WEIGHTS (GRAM) FEMALES END OF TREATMENT

ANIMAL	BODY W. (GRAM)	BRAIN (GRAM)	HEART (GRAM)	LIVER (GRAM)
<b>GROUP 1 (CONTROL)</b>				
41	258	1.87	0.854	10.75
42	260	---	---	---
43	296	---	---	---
44	292	1.86	0.873	13.53
45	276	2.02	0.730	10.37
46	258	---	---	---
47	288	1.94	0.883	12.36
48	284	---	---	---
49	282	---	---	---
50	262	1.77	0.802	12.19
<b>GROUP 2 (100 MG/KG)</b>				
51	233	---	---	---
52	283	1.94	0.743	11.01
53	253	---	---	---
54	287	1.87	0.874	12.34
55	286	---	---	---
56	291	1.84	0.794	12.58
57	293	1.86	0.865	12.09
58	296	1.96	0.875	12.57
59	271	---	---	---
60	301	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
61	309	---	---	---
62	309	1.94	0.910	12.83
63	229	---	---	---
64	289	1.84	0.809	11.97
65	342	---	---	---
66	274	1.91	0.793	10.94
67	291	---	---	---
68	301	---	0.992	12.33
69	272	---	---	---
70	281	1.94	0.803	11.43
<b>GROUP 4 (1000 MG/KG)</b>				
71	285	1.83	0.814	12.76
72	231	---	---	---
73	303	1.87	0.891	13.52
74	276	1.97	0.864	12.84
75	302	---	---	---
76	297	---	---	---
77	305	---	---	---
78	323	---	---	---
79	297	2.05	0.911	14.08
80	284	1.98	0.827	12.79

## FEMALES END OF TREATMENT

ANIMAL	THYROIDS (GRAM)	THYMUS (GRAM)	KIDNEYS (GRAM)	ADRENALS (GRAM)
<b>GROUP 1 (CONTROL)</b>				
41	0.019	0.196	1.78	0.072
42	0.014	---	---	---
43	0.020	---	---	---
44	0.013	0.253	2.11	0.085
45	0.016	0.310	1.77	0.079

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN WEIGHTS (GRAM) FEMALES END OF TREATMENT

ANIMAL	THYROIDS (GRAM)	THYMUS (GRAM)	KIDNEYS (GRAM)	ADRENALS (GRAM)
<b>GROUP 1 (CONTROL)</b>				
46	0.013	---	---	---
47	0.018	0.172	1.79	0.079
48	0.018	---	---	---
49	0.024	---	---	---
50	0.010	0.252	1.91	0.064
<b>GROUP 2 (100 MG/KG)</b>				
51	0.024	---	---	---
52	0.015	0.158	1.96	0.081
53	0.023	---	---	---
54	0.020	0.201	1.83	0.091
55	0.016	---	---	---
56	0.016	0.203	2.02	0.072
57	0.016	0.169	2.21	0.079
58	0.022	0.206	2.24	0.080
59	0.015	---	---	---
60	0.020	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
61	0.011	---	---	---
62	0.012	0.249	1.93	0.073
63	0.024	---	---	---
64	0.024	0.229	2.12	0.077
65	0.015	---	---	---
66	0.018	0.227	1.96	0.083
67	0.017	---	---	---
68	0.022	0.208	2.05	0.089
69	0.017	---	---	---
70	0.021	0.210	2.00	0.069
<b>GROUP 4 (1000 MG/KG)</b>				
71	0.021	0.175	2.03	0.068
72	0.014	---	---	---
73	0.021	0.208	2.06	0.085
74	0.024	0.139	2.07	0.087
75	0.018	---	---	---
76	0.018	---	---	---
77	0.021	---	---	---
78	0.015	---	---	---
79	0.020	0.224	2.18	0.076
80	0.012	0.252	2.14	0.075

## FEMALES END OF TREATMENT

ANIMAL	SPLEEN (GRAM)	OVARIES (GRAM)	UTERUS (GRAM)
<b>GROUP 1 (CONTROL)</b>			
41	0.517	0.098	0.380
42	---	---	---
43	---	---	---
44	0.581	0.114	0.384
45	0.452	0.123	0.389
46	---	---	---
47	0.461	0.120	0.345
48	---	---	---
49	---	---	---
50	0.487	0.112	0.300

03Jun19 13h46

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN WEIGHTS (GRAM) FEMALES END OF TREATMENT

---

ANIMAL	SPLEEN (GRAM)	OVARIES (GRAM)	UTERUS (GRAM)
<b>GROUP 2 (100 MG/KG)</b>			
51	---	---	---
52	0.474	0.112	0.341
53	---	---	---
54	0.523	0.115	0.316
55	---	---	---
56	0.529	0.128	0.319
57	0.461	0.108	0.346
58	0.547	0.114	0.479
59	---	---	---
60	---	---	---
<b>GROUP 3 (300 MG/KG)</b>			
61	---	---	---
62	0.577	0.129	0.339
63	---	---	---
64	0.438	0.124	0.319
65	---	---	---
66	0.479	0.100	0.320
67	---	---	---
68	0.632	0.125	0.451
69	---	---	---
70	0.426	0.105	0.352
<b>GROUP 4 (1000 MG/KG)</b>			
71	0.484	0.123	0.313
72	---	---	---
73	0.510	0.106	0.346
74	0.530	0.089	0.332
75	---	---	---
76	---	---	---
77	---	---	---
78	---	---	---
79	0.784	0.119	0.339
80	0.445	0.138	0.396

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN/BODY WEIGHT RATIOS (%) FEMALES END OF TREATMENT

ANIMAL	BODY W. (GRAM)	BRAIN (%)	HEART (%)	LIVER (%)
<b>GROUP 1 (CONTROL)</b>				
41	258	0.72	0.331	4.17
42	260	---	---	---
43	296	---	---	---
44	292	0.64	0.299	4.63
45	276	0.73	0.264	3.76
46	258	---	---	---
47	288	0.67	0.307	4.29
48	284	---	---	---
49	282	---	---	---
50	262	0.67	0.307	4.66
<b>GROUP 2 (100 MG/KG)</b>				
51	233	---	---	---
52	283	0.69	0.263	3.90
53	253	---	---	---
54	287	0.65	0.305	4.30
55	286	---	---	---
56	291	0.63	0.273	4.32
57	293	0.63	0.295	4.12
58	296	0.66	0.296	4.25
59	271	---	---	---
60	301	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
61	309	---	---	---
62	309	0.63	0.294	4.15
63	229	---	---	---
64	289	0.64	0.280	4.14
65	342	---	---	---
66	274	0.70	0.289	3.99
67	291	---	---	---
68	301	---	0.330	4.10
69	272	---	---	---
70	281	0.69	0.286	4.07
<b>GROUP 4 (1000 MG/KG)</b>				
71	285	0.64	0.286	4.48
72	231	---	---	---
73	303	0.62	0.294	4.46
74	276	0.71	0.313	4.65
75	302	---	---	---
76	297	---	---	---
77	305	---	---	---
78	323	---	---	---
79	297	0.69	0.307	4.74
80	284	0.70	0.292	4.51

## FEMALES END OF TREATMENT

ANIMAL	THYROIDS (%)	THYMUS (%)	KIDNEYS (%)	ADRENALS (%)
<b>GROUP 1 (CONTROL)</b>				
41	0.008	0.076	0.69	0.028
42	0.005	---	---	---
43	0.007	---	---	---
44	0.004	0.087	0.72	0.029
45	0.006	0.112	0.64	0.029

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN/BODY WEIGHT RATIOS (%)

### FEMALES

#### END OF TREATMENT

ANIMAL	THYROIDS (%)	THYMUS (%)	KIDNEYS (%)	ADRENALS (%)
<b>GROUP 1 (CONTROL)</b>				
46	0.005	---	---	---
47	0.006	0.060	0.62	0.027
48	0.006	---	---	---
49	0.008	---	---	---
50	0.004	0.096	0.73	0.024
<b>GROUP 2 (100 MG/KG)</b>				
51	0.010	---	---	---
52	0.005	0.056	0.69	0.029
53	0.009	---	---	---
54	0.007	0.070	0.64	0.032
55	0.006	---	---	---
56	0.005	0.070	0.69	0.025
57	0.005	0.058	0.75	0.027
58	0.007	0.070	0.76	0.027
59	0.005	---	---	---
60	0.007	---	---	---
<b>GROUP 3 (300 MG/KG)</b>				
61	0.004	---	---	---
62	0.004	0.081	0.62	0.024
63	0.011	---	---	---
64	0.008	0.079	0.73	0.027
65	0.004	---	---	---
66	0.006	0.083	0.72	0.030
67	0.006	---	---	---
68	0.007	0.069	0.68	0.030
69	0.006	---	---	---
70	0.007	0.075	0.71	0.025
<b>GROUP 4 (1000 MG/KG)</b>				
71	0.007	0.061	0.71	0.024
72	0.006	---	---	---
73	0.007	0.069	0.68	0.028
74	0.009	0.050	0.75	0.031
75	0.006	---	---	---
76	0.006	---	---	---
77	0.007	---	---	---
78	0.005	---	---	---
79	0.007	0.075	0.73	0.026
80	0.004	0.089	0.75	0.026

### FEMALES

#### END OF TREATMENT

ANIMAL	SPLEEN (%)	OVARIES (%)	UTERUS (%)
<b>GROUP 1 (CONTROL)</b>			
41	0.200	0.038	0.147
42	---	---	---
43	---	---	---
44	0.199	0.039	0.131
45	0.164	0.045	0.141
46	---	---	---
47	0.160	0.042	0.120
48	---	---	---
49	---	---	---
50	0.186	0.043	0.115

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.12 ORGAN/BODY WEIGHT RATIOS (%) FEMALES END OF TREATMENT

---

ANIMAL	SPLEEN (%)	OVARIES (%)	UTERUS (%)
<b>GROUP 2 (100 MG/KG)</b>			
51	---	---	---
52	0.168	0.040	0.121
53	---	---	---
54	0.182	0.040	0.110
55	---	---	---
56	0.182	0.044	0.109
57	0.157	0.037	0.118
58	0.185	0.039	0.162
59	---	---	---
60	---	---	---
<b>GROUP 3 (300 MG/KG)</b>			
61	---	---	---
62	0.187	0.042	0.110
63	---	---	---
64	0.152	0.043	0.110
65	---	---	---
66	0.175	0.036	0.117
67	---	---	---
68	0.210	0.042	0.150
69	---	---	---
70	0.152	0.037	0.125
<b>GROUP 4 (1000 MG/KG)</b>			
71	0.170	0.043	0.110
72	---	---	---
73	0.168	0.035	0.114
74	0.192	0.032	0.120
75	---	---	---
76	---	---	---
77	---	---	---
78	---	---	---
79	0.264	0.040	0.114
80	0.157	0.049	0.140







# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.14 GENERAL REPRODUCTION DATA

### F0-GENERATION - POST COITUM

FEMALE NUMBER	MALE NUMBER	MATING DATE	PREGNANT	SCHEDULE	DELIVERY RECORDED	NECROPSY DATE
<b>GROUP 1 (CONTROL)</b>						
41	1	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
42 <IO>	2	22FEB2019	YES	---	---	19MAR2019
43	3	22FEB2019	YES	BREEDING	16MAR2019	01APR2019
44	4	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
45	5	20FEB2019	YES	BREEDING	14MAR2019	29MAR2019
46 <NP>	6	04MAR2019	NO	---	---	29MAR2019
47	7	20FEB2019	YES	BREEDING	13MAR2019	28MAR2019
48	8	23FEB2019	YES	BREEDING	16MAR2019	01APR2019
49	9	22FEB2019	YES	BREEDING	16MAR2019	01APR2019
50	10	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
<b>GROUP 2 (100 MG/KG)</b>						
51 <NP>	11	21FEB2019	NO	---	---	19MAR2019
52	12	21FEB2019	YES	BREEDING	14MAR2019	29MAR2019
53 <NP>	13	20FEB2019	NO	---	---	19MAR2019
54	14	20FEB2019	YES	BREEDING	13MAR2019	28MAR2019
55	15	23FEB2019	YES	BREEDING	17MAR2019	02APR2019
56	16	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
57	17	21FEB2019	YES	BREEDING	14MAR2019	29MAR2019
58	18	21FEB2019	YES	BREEDING	14MAR2019	29MAR2019
59	19	22FEB2019	YES	BREEDING	16MAR2019	01APR2019
60	20	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
<b>GROUP 3 (300 MG/KG)</b>						
61	21	23FEB2019	YES	BREEDING	17MAR2019	02APR2019
62	22	22FEB2019	YES	BREEDING	16MAR2019	01APR2019
63 <NP>	23	20FEB2019	NO	---	---	19MAR2019
64	24	21FEB2019	YES	BREEDING	14MAR2019	29MAR2019
65	25	22FEB2019	YES	BREEDING	16MAR2019	01APR2019
66	26	20FEB2019	YES	BREEDING	14MAR2019	29MAR2019
67	27	22FEB2019	YES	BREEDING	16MAR2019	01APR2019
68	28	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
69	29	23FEB2019	YES	BREEDING	16MAR2019	01APR2019
70	30	20FEB2019	YES	BREEDING	14MAR2019	29MAR2019
<b>GROUP 4 (1000 MG/KG)</b>						
71	31	20FEB2019	YES	BREEDING	13MAR2019	28MAR2019
72 <NP>	32	22FEB2019	NO	---	---	19MAR2019
73	33	20FEB2019	YES	BREEDING	13MAR2019	28MAR2019
74	34	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
75	35	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
76	36	22FEB2019	YES	BREEDING	15MAR2019	29MAR2019
77	37	23FEB2019	YES	BREEDING	16MAR2019	01APR2019
78	38	23FEB2019	YES	BREEDING	16MAR2019	01APR2019
79	39	20FEB2019	YES	BREEDING	13MAR2019	28MAR2019
80	40	21FEB2019	YES	BREEDING	14MAR2019	29MAR2019

<IO> Implantations only

<NP> Non-pregnant

28Jun19 08h13

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.15 IMPLANTATION SITES FEMALES

### AT NECROPSY

---

ANIMAL      Implantations

---

#### GROUP 1 (CONTROL)

41	11.0
42	---
43	9.0
44	15.0
45	7.0
46	---
47	9.0
48	14.0
49	13.0
50	16.0

#### GROUP 2 (100 MG/KG)

51	---
52	14.0
53	---
54	13.0
55	14.0
56	17.0
57	11.0
58	14.0
59	17.0
60	17.0

#### GROUP 3 (300 MG/KG)

61	12.0
62	13.0
63	---
64	14.0
65	14.0
66	13.0
67	15.0
68	10.0
69	14.0
70	11.0

#### GROUP 4 (1000 MG/KG)

71	11.0
72	---
73	14.0
74	16.0
75	14.0
76	14.0
77	16.0
78	17.0
79	12.0
80	16.0

Female No. 42: implantations only

03Jun19 13h46

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

LITTER	DURATION OF GESTATION	FIRST LITTER CHECK					P.NATAL LOSS			LIVING PUPS		
		DEAD M	PUPS F	LIVING M	PUPS F	TOT.	DAYS M	0 - 4 F	CULLED PUPS TOT.	DAY 4 M	P.P. F	TOT.
<b>GROUP 1 (CONTROL)</b>												
41	21	0	0	3	7	10	0	0	2	3	5	8
43	22	0	0	5	3	8	0	0	0	5	3	8
44	21	0	0	9	6	15	0	0	7	4	4	8
45	22	0	0	3	3	6	0	0	0	3	3	6
47	21	0	0	5	4	9	0	0	1	4	4	8
48	21	0	0	5	9	14	0	1	5	4	4	8
49	22	0	0	5	8	13	0	0	5	4	4	8
50	21	0	0	9	5	14	0	0	6	4	4	8
TOTAL		0	0	44	45	89	0	1	26	31	31	62
N	8	8	8	8	8	8	8	8	8	8	8	8
MEAN	21.4	0.0	0.0	5.5	5.6	11.1	0.0	0.1	3.3	3.9	3.9	7.8
ST.DEV.	0.5	0.0	0.0	2.3	2.3	3.3	0.0	0.4	2.8	0.6	0.6	0.7

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

LITTER	DURATION OF GESTATION	FIRST LITTER CHECK					P.NATAL LOSS			LIVING PUPS		
		DEAD M	PUPS F	LIVING M	PUPS F	TOT.	DAYS M	0 - 4 F	CULLED PUPS TOT.	DAY 4 M	P.P. F	TOT.
<b>GROUP 2 (100 MG/KG)</b>												
52	21	0	0	5	9	14	0	0	6	4	4	8
54	21	0	0	6	7	13	0	0	5	4	4	8
55	22	0	0	9	4	13	0	0	5	4	4	8
56	21	0	0	9	8	17	0	0	9	4	4	8
57	21	0	0	6	5	11	0	0	3	4	4	8
58	21	0	0	6	8	14	0	0	6	4	4	8
59	22	0	0	7	9	16	0	0	8	4	4	8
60	21	0	0	8	7	15	0	0	7	4	4	8
TOTAL		0	0	56	57	113	0	0	49	32	32	64
N	8	8	8	8	8	8	8	8	8	8	8	8
MEAN	21.3	0.0	0.0	7.0	7.1	14.1	0.0	0.0	6.1	4.0	4.0	8.0
ST.DEV.	0.5	0.0	0.0	1.5	1.8	1.9	0.0	0.0	1.9	0.0	0.0	0.0

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

LITTER	DURATION OF GESTATION	FIRST LITTER CHECK					P.NATAL LOSS			LIVING PUPS		
		DEAD M	PUPS F	LIVING M	PUPS F	TOT.	DAYS M	0 - 4 F	CULLED PUPS TOT.	DAY 4 M	P.P. F	TOT.
<b>GROUP 3 (300 MG/KG)</b>												
61	22	0	0	4	9	13	0	0	5	4	4	8
62	22	0	0	6	7	13	0	0	5	4	4	8
64	21	0	0	7	6	13	0	0	5	4	4	8
65	22	1	0	6	6	12	0	0	4	4	4	8
66	22	0	0	7	4	11	0	0	3	4	4	8
67	22	0	0	8	6	14	0	0	6	4	4	8
68	21	0	0	6	4	10	0	0	2	4	4	8
69	21	0	0	3	10	13	0	0	5	3	5	8
70	22	0	0	5	4	9	0	0	1	4	4	8
TOTAL		1	0	52	56	108	0	0	36	35	37	72
N	9	9	9	9	9	9	9	9	9	9	9	9
MEAN	21.7	0.1	0.0	5.8	6.2	12.0	0.0	0.0	4.0	3.9	4.1	8.0
ST.DEV.	0.5	0.3	0.0	1.6	2.2	1.7	0.0	0.0	1.7	0.3	0.3	0.0

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

LITTER	DURATION OF GESTATION	FIRST LITTER CHECK					P.NATAL LOSS			LIVING PUPS		
		DEAD M	PUPS F	LIVING M	PUPS F	TOT.	DAYS M	0 - 4 F	CULLED PUPS TOT.	DAY 4 M	P.P. F	TOT.
<b>GROUP 4 (1000 MG/KG)</b>												
71	21	0	0	7	4	11	0	0	3	4	4	8
73	21	0	0	5	9	14	0	0	6	4	4	8
74	21	0	0	7	6	13	0	0	5	4	4	8
75	21	0	0	6	7	13	0	0	5	4	4	8
76	21	0	0	4	6	10	0	0	2	4	4	8
77	21	0	0	8	7	15	0	0	7	4	4	8
78	21	0	0	7	9	16	0	0	8	4	4	8
79	21	0	0	4	8	12	0	0	4	4	4	8
80	21	0	0	8	4	12	0	0	4	4	4	8
TOTAL		0	0	56	60	116	0	0	44	36	36	72
N	9	9	9	9	9	9	9	9	9	9	9	9
MEAN	21.0	0.0	0.0	6.2	6.7	12.9	0.0	0.0	4.9	4.0	4.0	8.0
ST.DEV.	0.0	0.0	0.0	1.6	1.9	1.9	0.0	0.0	1.9	0.0	0.0	0.0



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

---

LITTER	BREEDING LOSS		LIVING PUPS		TOT.
	DAYS 5 M	- 13 F	DAY 13 M	P.P. F	
<b>GROUP 1 (CONTROL)</b>					
41	0	0	3	5	8
43	0	0	5	3	8
44	0	0	4	4	8
45	0	0	3	3	6
47	0	0	4	4	8
48	0	1	4	3	7
49	0	0	4	4	8
50	0	0	4	4	8
<hr/>					
TOTAL	0	1	31	30	61
N	8	8	8	8	8
MEAN	0.0	0.1	3.9	3.8	7.6
ST.DEV.	0.0	0.4	0.6	0.7	0.7

---

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

---

LITTER	BREEDING LOSS		LIVING PUPS		TOT.
	DAYS 5 M	- 13 F	DAY 13 M	P.P. F	
<b>GROUP 2 (100 MG/KG)</b>					
52	0	0	4	4	8
54	0	0	4	4	8
55	0	0	4	4	8
56	0	0	4	4	8
57	0	0	4	4	8
58	0	0	4	4	8
59	0	0	4	4	8
60	0	0	4	4	8
TOTAL	0	0	32	32	64
N	8	8	8	8	8
MEAN	0.0	0.0	4.0	4.0	8.0
ST.DEV.	0.0	0.0	0.0	0.0	0.0

---

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

LITTER	BREEDING LOSS		LIVING PUPS		TOT.
	DAYS 5 M	- 13 F	DAY 13 M	P.P. F	
<b>GROUP 3 (300 MG/KG)</b>					
61	0	0	3	4	7
62	0	0	4	4	8
64	0	0	4	4	8
65	0	0	4	4	8
66	0	0	4	4	8
67	0	0	4	4	8
68	0	0	4	4	8
69	0	0	3	5	8
70	0	0	4	4	8
TOTAL	0	0	34	37	71
N	9	9	9	9	9
MEAN	0.0	0.0	3.8	4.1	7.9
ST.DEV.	0.0	0.0	0.4	0.3	0.3

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.16 DEVELOPMENTAL DATA PER LITTER FEMALES

### F0-GENERATION - LACTATION

---

LITTER	BREEDING LOSS		LIVING PUPS		TOT.
	DAYS 5 M	- 13 F	DAY 13 M	P.P. F	
<b>GROUP 4 (1000 MG/KG)</b>					
71	0	0	4	4	8
73	0	0	4	4	8
74	0	0	4	4	8
75	0	0	4	4	8
76	0	0	4	4	8
77	0	0	4	4	8
78	0	0	4	4	8
79	0	0	4	4	8
80	0	0	4	4	8
<hr/>					
TOTAL	0	0	36	36	72
N	9	9	9	9	9
MEAN	0.0	0.0	4.0	4.0	8.0
ST.DEV.	0.0	0.0	0.0	0.0	0.0

---

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.17 BODY WEIGHTS OF PUPS PER LITTER (GRAM)

### F0-GENERATION - LACTATION

LITTER	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 1 (CONTROL)</b>					
41	M	6.4	9.1	14.3	27.1
	F	6.1	8.7	14.0	26.7
	M+F	6.2	8.8	14.1	26.9
43	M	7.6	11.3	17.5	32.3
	F	7.7	11.4	18.2	33.0
	M+F	7.6	11.3	17.8	32.6
44	M	6.1	8.4	14.1	29.8
	F	5.6	8.0	13.0	27.7
	M+F	5.9	8.3	13.6	28.7
45	M	7.8	12.6	19.7	35.6
	F	7.2	11.5	18.4	34.1
	M+F	7.5	12.1	19.1	34.9
47	M	6.6	10.5	18.1	33.4
	F	6.6	10.2	17.4	32.2
	M+F	6.6	10.4	17.8	32.8
48	M	5.7	8.3	14.5	30.1
	F	5.4	8.0	14.5	29.7
	M+F	5.5	8.1	14.5	29.9
49	M	6.5	10.0	16.5	31.8
	F	6.4	9.5	15.9	31.2
	M+F	6.4	9.7	16.2	31.5
50	M	5.8	8.1	14.5	30.1
	F	6.1	8.3	15.2	30.9
	M+F	5.9	8.2	14.8	30.5
<b>GROUP 2 (100 MG/KG)</b>					
52	M	5.8	8.3	14.5	30.5
	F	5.6	8.0	14.0	29.3
	M+F	5.7	8.1	14.2	29.9
54	M	6.6	9.1	15.6	30.9
	F	5.9	8.6	14.8	29.3
	M+F	6.2	8.8	15.2	30.1
55	M	6.8	9.5	16.4	32.0
	F	6.5	8.9	15.3	30.0
	M+F	6.7	9.3	15.8	31.0
56	M	5.4	7.9	14.6	30.5
	F	5.2	7.6	13.7	28.6
	M+F	5.3	7.7	14.1	29.5
57	M	6.0	8.9	15.4	30.4
	F	5.9	8.9	15.6	30.7
	M+F	6.0	8.9	15.5	30.5
58	M	6.0	8.7	15.1	30.5
	F	5.5	8.3	14.6	29.6
	M+F	5.7	8.5	14.8	30.1
59	M	6.1	8.6	14.1	28.2
	F	6.3	8.9	14.7	28.5
	M+F	6.2	8.8	14.4	28.3

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.17 BODY WEIGHTS OF PUPS PER LITTER (GRAM)

### F0-GENERATION - LACTATION

LITTER	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 2 (100 MG/KG)</b>					
60	M	6.0	8.9	16.1	32.9
	F	5.4	8.3	14.5	30.5
	M+F	5.7	8.6	15.3	31.7
<b>GROUP 3 (300 MG/KG)</b>					
61	M	7.2	10.9	19.2	35.4
	F	6.8	10.3	17.8	34.3
	M+F	6.9	10.5	18.5	34.8
62	M	7.2	10.6	18.1	33.1
	F	6.6	10.0	16.3	31.5
	M+F	6.9	10.3	17.2	32.3
64	M	6.0	8.8	15.9	30.7
	F	5.5	8.5	14.9	29.4
	M+F	5.8	8.7	15.4	30.1
65	M	6.8	11.5	19.4	36.8
	F	6.4	10.8	18.0	35.0
	M+F	6.6	11.1	18.7	35.9
66	M	7.3	11.1	18.1	33.6
	F	7.2	10.9	18.3	33.8
	M+F	7.3	11.0	18.2	33.7
67	M	6.9	10.4	17.4	33.7
	F	6.8	10.2	16.9	33.0
	M+F	6.8	10.3	17.2	33.3
68	M	5.9	8.4	14.1	27.0
	F	5.5	8.0	13.6	26.5
	M+F	5.7	8.2	13.8	26.8
69	M	5.8	8.9	15.3	31.3
	F	5.7	8.8	14.5	30.2
	M+F	5.7	8.8	14.8	30.6
70	M	7.5	11.4	18.2	33.1
	F	7.1	11.0	17.7	32.1
	M+F	7.3	11.2	17.9	32.6
<b>GROUP 4 (1000 MG/KG)</b>					
71	M	5.9	9.1	15.8	31.0
	F	5.6	8.8	15.8	30.7
	M+F	5.8	9.0	15.8	30.8
73	M	6.2	8.9	15.8	31.1
	F	5.6	8.2	14.7	29.7
	M+F	5.8	8.4	15.3	30.4
74	M	6.2	9.1	15.1	29.9
	F	5.9	8.7	14.8	29.5
	M+F	6.0	8.9	15.0	29.7
75	M	6.2	8.7	15.2	31.5
	F	6.1	8.7	14.8	30.6
	M+F	6.1	8.7	15.0	31.0

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.17 BODY WEIGHTS OF PUPS PER LITTER (GRAM)

### F0-GENERATION - LACTATION

---

LITTER	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 4 (1000 MG/KG)</b>					
76	M	6.5	9.8	16.7	33.0
	F	6.0	9.1	15.2	31.1
	M+F	6.2	9.4	16.0	32.1
77	M	6.2	9.2	16.8	33.4
	F	5.7	8.4	15.2	30.8
	M+F	5.9	8.8	16.0	32.1
78	M	6.1	8.9	15.6	31.8
	F	5.3	8.0	13.3	28.6
	M+F	5.6	8.4	14.4	30.2
79	M	6.5	9.7	16.3	31.1
	F	6.6	9.9	16.8	31.8
	M+F	6.6	9.8	16.5	31.4
80	M	5.6	8.8	16.2	31.5
	F	5.7	8.6	15.3	31.0
	M+F	5.6	8.7	15.7	31.2

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 1 (CONTROL)</b>						
41	1	M	6.4	9.1	14.0	26.9
	2	M	6.5	9.2	14.7	27.1
	3	M	6.4	8.9	14.1	27.3
	4	F	6.1	8.8	14.1	26.7
	5	F	6.4	8.8	13.7	26.9
	6	F	6.1	8.8	14.1	26.7
	7	F	5.9	8.7	14.5	27.3
	8	F	5.8	8.2	13.6	26.0
	9	F	6.2	8.9	--	--
	10	F	6.0	8.8	--	--
43	1	M	5.9	8.1	11.7	22.6
	2	M	8.2	12.7	19.9	35.6
	3	M	7.6	11.5	18.3	34.4
	4	M	8.1	12.3	19.0	34.2
	5	M	8.0	11.9	18.7	34.7
	6	F	7.9	11.1	17.5	32.1
	7	F	7.8	11.8	19.0	34.0
	8	F	7.4	11.3	18.0	32.9
44	1	M	6.2	8.4	13.9	29.7
	2	M	6.4	9.0	14.5	29.9
	3	M	6.5	8.9	15.0	31.6
	4	M	5.6	7.8	13.1	28.0
	5	M	5.9	7.9	--	--
	6	M	6.2	8.6	--	--
	7	M	5.5	7.4	--	--
	8	M	6.4	9.3	--	--
	9	M	6.0	8.5	--	--
	10	F	6.2	8.8	14.6	30.2
	11	F	4.8	7.3	12.6	27.2
	12	F	5.3	8.0	13.4	28.1
	13	F	5.1	7.1	11.5	25.1
	14	F	6.3	9.0	--	--
	15	F	5.6	7.9	--	--
45	1	M	7.7	12.3	19.4	35.8
	2	M	8.0	12.9	20.1	35.9
	3	M	7.6	12.7	19.7	35.2
	4	F	7.4	11.8	18.6	34.8
	5	F	7.3	11.7	18.8	34.4
	6	F	6.9	11.1	17.9	33.0
47	1	M	7.0	11.1	19.3	35.0
	2	M	6.4	9.8	17.4	31.9
	3	M	6.6	11.1	18.1	33.5
	4	M	6.9	10.6	17.7	33.0
	5	M	6.2	10.1	--	--
	6	F	6.6	10.8	18.3	32.8
	7	F	6.9	10.8	17.8	32.7
	8	F	6.3	8.5	15.6	30.2
	9	F	6.5	10.7	18.0	32.9
48	1	M	6.1	9.1	15.5	31.6
	2	M	5.8	8.4	14.5	30.4
	3	M	5.3	7.5	13.5	28.3
	4	M	5.5	8.4	14.3	30.1
	5	M	5.8	8.3	--	--
	6	F	5.9	8.6	14.8	30.3
	7	F	5.8	8.8	14.6	29.5
	8	F	5.6	8.4	--	--
	9	F	5.6	7.9	14.1	29.3



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 1 (CONTROL)</b>						
	10	F	5.5	7.9	--	--
	11	F	5.4	7.9	--	--
	12	F	5.4	--	--	--
	13	F	5.4	7.9	--	--
	14	F	4.4	6.3	--	--
49	1	M	6.8	9.9	16.8	31.6
	2	M	6.6	10.3	17.0	32.8
	3	M	6.3	10.0	16.1	32.0
	4	M	6.3	9.6	16.2	30.9
	5	M	6.7	10.3	--	--
	6	F	6.4	10.0	16.8	32.2
	7	F	6.3	9.2	15.8	31.3
	8	F	6.2	9.4	16.0	31.4
	9	F	6.3	8.9	14.9	29.9
	10	F	6.6	9.6	--	--
	11	F	6.1	9.2	--	--
	12	F	6.4	9.5	--	--
	13	F	6.7	9.9	--	--
50	1	M	4.9	6.6	11.5	26.2
	2	M	6.3	9.0	15.7	31.8
	3	M	6.4	9.0	16.2	32.6
	4	M	5.9	8.2	14.4	29.8
	5	M	5.5	7.2	--	--
	6	M	6.3	8.5	--	--
	7	M	6.4	8.9	--	--
	8	M	5.1	7.1	--	--
	9	M	5.8	8.1	--	--
	10	F	6.3	8.7	15.9	31.8
	11	F	5.9	7.9	14.7	30.2
	12	F	6.0	8.5	15.4	31.5
	13	F	5.9	8.1	14.8	30.2
	14	F	6.3	8.5	--	--
<b>GROUP 2 (100 MG/KG)</b>						
52	1	M	6.2	8.8	15.6	31.8
	2	M	5.4	7.9	13.8	29.8
	3	M	5.9	8.2	14.2	29.9
	4	M	5.9	8.3	14.4	30.6
	5	M	5.8	8.3	--	--
	6	F	5.8	8.2	14.0	30.0
	7	F	5.1	7.0	12.2	26.3
	8	F	5.6	8.2	14.5	30.2
	9	F	6.0	8.4	15.1	30.6
	10	F	5.7	8.0	--	--
	11	F	5.2	7.3	--	--
	12	F	5.3	8.3	--	--
	13	F	5.6	8.4	--	--
	14	F	5.8	8.2	--	--
54	1	M	6.6	9.3	15.8	31.4
	2	M	6.4	8.6	15.3	31.0
	3	M	6.7	9.1	15.3	29.9
	4	M	6.8	9.5	15.8	31.3
	5	M	6.4	9.0	--	--
	6	M	6.5	9.0	--	--
	7	F	5.7	8.0	14.0	28.3
	8	F	6.3	8.8	15.0	29.7
	9	F	6.2	8.8	15.3	29.5
	10	F	5.9	9.0	14.8	29.7

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 2 (100 MG/KG)</b>						
	11	F	5.5	8.1	--	--
	12	F	5.9	8.7	--	--
	13	F	6.1	8.7	--	--
55	1	M	7.0	9.7	17.0	33.2
	2	M	7.0	9.6	16.7	32.0
	3	M	6.6	9.2	16.2	31.9
	4	M	6.7	9.1	15.7	30.7
	5	M	7.1	9.5	--	--
	6	M	6.3	9.3	--	--
	7	M	6.9	9.7	--	--
	8	M	7.1	9.7	--	--
	9	M	6.9	9.7	--	--
	10	F	6.7	9.1	15.3	30.1
	11	F	6.7	9.3	15.9	31.0
	12	F	6.9	9.5	16.2	31.0
	13	F	5.5	7.5	13.6	27.9
56	1	M	5.5	7.7	14.1	30.2
	2	M	5.8	8.5	15.0	31.8
	3	M	5.5	8.4	14.9	31.0
	4	M	5.4	7.7	14.2	28.8
	5	M	5.3	7.9	--	--
	6	M	4.9	7.0	--	--
	7	M	5.3	7.5	--	--
	8	M	5.8	8.5	--	--
	9	F	5.5	8.2	13.8	29.3
	10	F	4.9	7.3	12.9	28.2
	11	F	4.8	6.5	12.2	26.5
	12	F	5.9	8.6	15.7	30.3
	13	F	4.9	7.1	--	--
	14	F	5.4	7.8	--	--
	15	F	5.6	7.8	--	--
	16	M	5.4	8.0	--	--
	17	F	4.7	7.1	--	--
57	1	M	6.1	9.0	15.0	30.0
	2	M	6.2	9.0	15.7	31.1
	3	M	5.9	8.7	15.2	30.7
	4	M	6.0	9.0	15.7	29.8
	5	M	6.1	9.1	--	--
	6	M	5.8	8.4	--	--
	7	F	5.9	8.8	15.5	30.4
	8	F	5.8	8.9	15.1	30.1
	9	F	5.9	9.2	16.0	31.3
	10	F	5.9	8.8	15.6	30.8
	11	F	5.9	8.8	--	--
58	1	M	5.8	7.9	14.0	28.8
	2	M	5.8	8.9	15.4	31.1
	3	M	6.1	9.1	15.3	30.4
	4	M	6.3	9.5	15.7	31.8
	5	M	6.4	8.8	--	--
	6	M	5.6	8.1	--	--
	7	F	6.0	8.6	15.7	30.4
	8	F	5.5	8.1	14.5	29.6
	9	F	5.5	8.3	14.5	29.8
	10	F	4.9	7.5	13.6	28.7
	11	F	5.6	8.1	--	--
	12	F	5.7	8.0	--	--
	13	F	5.3	8.3	--	--
	14	F	5.5	9.1	--	--

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
--------	-----	-----	-------	-------	-------	--------

#### GROUP 2 (100 MG/KG)

59	1	M	5.9	7.8	13.4	26.5
	2	M	5.9	8.6	14.8	29.2
	3	M	5.6	7.8	13.1	28.1
	4	M	6.2	8.9	14.9	29.0
	5	M	6.1	8.7	--	--
	6	M	6.5	9.0	--	--
	7	M	6.5	9.7	--	--
	8	F	6.7	9.4	15.0	29.5
	9	F	6.7	9.3	16.3	29.7
	10	F	5.9	8.6	13.8	27.3
	11	F	6.4	8.5	13.7	27.3
	12	F	6.1	8.3	--	--
	13	F	6.4	9.5	--	--
	14	F	6.7	9.4	--	--
	15	F	7.0	9.9	--	--
	16	F	5.1	7.1	--	--

60	1	M	5.9	9.5	16.4	33.3
	2	M	6.0	9.0	16.1	33.0
	3	M	6.0	9.0	15.9	32.8
	4	M	6.0	9.0	15.8	32.3
	5	M	5.3	7.1	--	--
	6	M	6.2	9.1	--	--
	7	M	6.3	9.5	--	--
	8	M	6.3	9.3	--	--
	9	F	6.3	9.2	16.1	33.8
	10	F	5.5	8.4	15.1	30.6
	11	F	5.0	7.4	13.3	28.8
	12	F	4.6	7.7	13.5	28.9
	13	F	5.6	8.4	--	--
	14	F	5.6	8.4	--	--
	15	F	5.3	8.3	--	--

#### GROUP 3 (300 MG/KG)

61	1	M	7.6	11.4	19.9	35.6
	2	M	7.0	10.5	18.4	34.7
	3	M	6.5	10.4	18.7	35.0
	4	M	7.5	11.4	19.7	36.2
	5	F	7.1	10.6	18.3	34.8
	6	F	6.4	9.9	17.4	33.3
	7	F	7.0	10.6	18.1	34.5
	8	F	6.5	9.8	17.5	34.4
	9	F	6.6	10.4	--	--
	10	F	7.4	10.8	--	--
	11	F	6.7	10.3	--	--
	12	F	6.3	9.6	--	--
	13	F	7.0	10.7	--	--

62	1	M	7.4	11.0	18.4	33.3
	2	M	7.3	11.1	18.5	34.2
	3	M	7.5	10.6	18.0	33.6
	4	M	7.3	10.4	17.3	31.3
	5	M	6.7	10.3	--	--
	6	M	7.1	10.3	--	--
	7	F	5.4	8.6	14.5	29.2
	8	F	6.9	10.2	17.1	32.4
	9	F	6.8	10.5	17.5	33.4
	10	F	6.6	9.4	16.1	30.9
	11	F	6.8	10.2	--	--
	12	F	6.8	10.2	--	--

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 3 (300 MG/KG)</b>						
	13	F	7.2	10.7	--	--
64	1	M	6.1	8.7	16.1	30.7
	2	M	5.4	8.0	14.9	29.9
	3	M	5.9	8.9	16.0	30.9
	4	M	6.1	9.2	16.4	31.4
	5	M	5.9	8.7	--	--
	6	M	5.9	8.6	--	--
	7	M	6.7	9.7	--	--
	8	F	5.4	8.1	14.5	29.0
	9	F	5.7	8.9	15.6	30.4
	10	F	5.7	8.8	15.4	29.8
	11	F	5.2	7.9	14.1	28.3
	12	F	5.8	8.7	--	--
	13	F	5.4	8.4	--	--
65	1	M	6.6	11.6	19.4	36.9
	2	M	6.8	11.2	18.6	35.2
	3	M	6.7	11.8	19.0	36.4
	4	M	7.7	12.1	20.5	38.7
	5	M	--	--	--	--
	6	M	7.2	12.8	--	--
	7	M	5.7	9.6	--	--
	8	F	6.4	11.2	18.1	35.0
	9	F	6.1	10.7	17.9	34.9
	10	F	7.1	11.6	19.3	36.5
	11	F	5.9	9.9	16.6	33.6
	12	F	6.8	11.0	--	--
	13	F	6.3	10.2	--	--
66	1	M	7.0	10.8	17.5	32.9
	2	M	7.4	11.2	18.4	34.1
	3	M	7.6	11.2	18.8	33.2
	4	M	6.6	10.3	17.7	34.0
	5	M	7.8	11.4	--	--
	6	M	7.5	11.3	--	--
	7	M	7.4	11.3	--	--
	8	F	7.0	10.6	17.9	33.2
	9	F	7.5	11.4	18.8	33.8
	10	F	6.9	10.8	18.2	34.0
	11	F	7.2	10.8	18.3	34.2
67	1	M	7.0	10.3	17.4	33.8
	2	M	7.1	10.8	18.1	34.6
	3	M	6.6	9.8	16.5	32.6
	4	M	6.8	10.3	17.7	33.7
	5	M	6.6	10.3	--	--
	6	F	6.9	10.5	16.9	32.9
	7	M	6.5	10.1	--	--
	8	M	7.2	10.8	--	--
	9	M	7.1	10.4	--	--
	10	F	6.7	10.5	16.7	32.5
	11	F	6.8	10.3	17.2	33.3
	12	F	6.3	10.0	16.8	33.1
	13	F	6.9	9.9	--	--
	14	F	6.9	10.0	--	--
68	1	M	5.9	9.0	15.6	27.8
	2	M	5.9	8.2	13.4	26.6
	3	M	5.5	7.8	12.9	25.7
	4	M	5.9	8.6	14.5	27.8
	5	M	5.7	8.0	--	--

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 3 (300 MG/KG)</b>						
	6	M	6.2	8.7	--	--
	7	F	5.8	7.9	13.7	26.9
	8	F	6.0	9.3	15.1	28.4
	9	F	5.4	8.2	14.0	26.9
	10	F	4.9	6.7	11.4	23.9
69	1	M	5.9	8.8	14.9	30.8
	2	M	5.6	8.7	15.2	30.9
	3	M	6.0	9.2	15.9	32.1
	4	F	5.4	8.7	14.4	30.2
	5	F	5.5	7.9	13.9	29.2
	6	F	5.8	9.0	13.9	31.1
	7	F	5.6	8.6	15.5	30.0
	8	F	5.4	8.7	14.8	30.3
	9	F	6.0	9.3	--	--
	10	F	6.0	9.1	--	--
	11	F	6.1	9.1	--	--
	12	F	5.3	8.2	--	--
	13	F	6.0	9.1	--	--
70	1	M	7.3	11.0	17.7	33.2
	2	M	7.4	11.5	18.7	33.4
	3	M	7.3	11.0	17.6	32.4
	4	M	7.5	11.5	18.7	33.4
	5	M	8.0	12.2	--	--
	6	F	7.2	11.4	18.2	32.5
	7	F	7.4	11.2	18.0	33.0
	8	F	6.8	10.8	17.2	31.6
	9	F	7.1	10.6	17.3	31.4
<b>GROUP 4 (1000 MG/KG)</b>						
71	1	M	6.1	9.3	16.4	31.7
	2	M	6.5	9.6	16.6	32.5
	3	M	5.6	8.8	16.0	30.7
	4	M	5.6	8.2	14.1	28.9
	5	M	5.9	9.6	--	--
	6	M	5.8	9.0	--	--
	7	M	5.8	8.9	--	--
	8	F	5.8	9.1	16.3	31.5
	9	F	5.2	8.4	15.4	30.5
	10	F	5.5	8.6	15.7	30.4
	11	F	6.0	9.1	15.8	30.5
73	1	M	6.0	8.4	14.7	29.2
	2	M	6.9	9.4	17.2	32.9
	3	M	5.7	8.1	14.7	30.7
	4	M	6.1	9.4	16.7	31.6
	5	M	6.1	9.1	--	--
	6	F	5.4	8.4	15.2	30.4
	7	F	6.0	8.7	15.8	31.7
	8	F	5.7	7.9	14.4	29.3
	9	F	5.3	7.6	13.5	27.5
	10	F	5.5	8.4	--	--
	11	F	5.5	8.2	--	--
	12	F	5.4	7.8	--	--
	13	F	5.6	8.3	--	--
	14	F	5.7	8.1	--	--
74	1	M	6.1	9.0	15.0	28.9
	2	M	5.6	8.5	14.5	29.2
	3	M	6.1	9.2	15.5	30.7

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 4 (1000 MG/KG)</b>						
	4	M	6.5	9.3	15.4	30.6
	5	M	6.7	9.9	--	--
	6	F	5.6	8.8	14.3	29.3
	7	M	6.3	9.4	--	--
	8	M	5.8	8.6	--	--
	9	F	6.1	8.6	14.9	29.1
	10	F	6.2	9.3	15.4	30.3
	11	F	5.9	8.6	14.7	29.2
	12	F	5.8	8.5	--	--
	13	F	5.5	8.6	--	--
75	1	M	6.5	9.1	15.1	31.0
	2	M	6.1	8.6	14.6	30.6
	3	M	6.4	8.9	14.8	31.3
	4	M	6.7	9.5	16.1	33.1
	5	M	5.8	8.4	--	--
	6	M	5.5	7.6	--	--
	7	F	5.4	7.8	13.5	28.5
	8	F	6.4	9.2	15.6	31.8
	9	F	6.3	9.2	15.5	31.9
	10	F	6.0	8.4	14.5	30.1
	11	F	5.8	7.9	--	--
	12	F	6.1	9.3	--	--
	13	F	6.6	9.1	--	--
76	1	M	6.7	10.0	17.4	34.3
	2	M	6.6	10.1	16.6	33.1
	3	M	7.0	10.3	17.6	34.0
	4	M	5.8	8.8	15.2	30.7
	5	F	6.3	9.7	16.1	32.1
	6	F	6.0	8.5	14.7	31.0
	7	F	6.5	9.9	17.1	33.4
	8	F	4.9	7.4	13.0	27.9
	9	F	6.7	10.0	--	--
	10	F	5.6	8.9	--	--
77	1	M	6.5	10.2	18.1	35.3
	2	M	6.1	9.7	17.4	34.6
	3	M	5.8	8.8	15.7	31.5
	4	M	6.1	8.4	15.8	32.3
	5	M	6.3	9.3	--	--
	6	M	5.6	7.7	--	--
	7	M	6.3	9.5	--	--
	8	M	6.5	9.7	--	--
	9	F	5.6	8.4	15.2	30.4
	10	F	5.7	8.3	14.8	30.3
	11	F	5.9	8.7	16.2	32.0
	12	F	5.1	7.9	14.7	30.4
	13	F	6.2	8.5	--	--
	14	F	6.2	9.2	--	--
	15	F	5.2	7.5	--	--
78	1	M	6.2	9.2	15.4	32.8
	2	M	6.1	8.3	14.5	30.6
	3	M	6.4	9.3	15.3	31.2
	4	M	6.4	9.6	17.0	32.4
	5	M	6.2	9.1	--	--
	6	M	6.3	9.2	--	--
	7	M	4.9	7.6	--	--
	8	F	4.0	5.4	9.4	23.1
	9	F	5.5	8.2	14.7	30.6
	10	F	5.5	8.4	14.3	30.1

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.18 BODY WEIGHTS OF PUPS (GRAM)

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	DAY 1	DAY 4	DAY 7	DAY 13
<b>GROUP 4 (1000 MG/KG)</b>						
	11	F	5.5	8.5	14.8	30.6
	12	F	5.8	9.1	--	--
	13	F	4.8	7.5	--	--
	14	F	5.7	8.7	--	--
	15	F	5.1	7.9	--	--
	16	F	5.6	8.4	--	--
79	1	M	6.4	9.7	16.8	31.8
	2	M	7.3	10.6	17.6	32.5
	3	M	5.4	8.5	14.2	28.6
	4	M	6.9	10.0	16.4	31.4
	5	F	7.1	10.3	17.2	32.4
	6	F	6.3	9.2	15.8	30.0
	7	F	6.5	9.9	16.9	32.1
	8	F	6.8	10.2	17.4	32.7
	9	F	6.7	10.3	--	--
	10	F	6.3	9.6	--	--
	11	F	6.5	9.0	--	--
	12	F	6.9	10.4	--	--
80	1	M	5.8	9.2	16.4	31.7
	2	M	5.7	8.9	15.7	30.9
	3	M	5.6	8.9	16.2	31.0
	4	M	6.0	9.3	16.3	32.3
	5	M	5.3	8.2	--	--
	6	M	5.8	9.3	--	--
	7	M	4.7	7.5	--	--
	8	M	5.7	8.8	--	--
	9	F	6.1	9.1	15.9	32.1
	10	F	5.3	8.2	14.7	30.7
	11	F	5.3	8.1	14.5	29.3
	12	F	6.0	9.0	16.1	31.8

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.19 ANOGENITAL DISTANCE AND NIPPLE RETENTION PER LITTER FEMALES

### F0-GENERATION - LACTATION

LITTER	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 1 (CONTROL)</b>			
41	2.51	1.00	0.00
42	--	--	--
43	2.64	0.94	0.00
44	2.48	0.91	0.00
45	2.74	0.92	0.00
46 <NP>	--	--	--
47	2.68	0.78	0.00
48	2.62	0.96	0.00
49	2.72	1.01	0.00
50	2.47	1.06	0.00
MEAN	2.61	0.95	0.00
N	8	8	8
<b>GROUP 2 (100 MG/KG)</b>			
51 <NP>	--	--	--
52	2.52	1.01	0.00
53 <NP>	--	--	--
54	2.70	0.80	0.00
55	2.66	1.49	0.00
56	2.48	0.99	0.00
57	2.59	1.01	0.00
58	2.63	0.89	0.00
59	2.63	1.07	0.00
60	2.47	1.02	0.00
MEAN	2.59	1.04	0.00
N	8	8	8
<b>GROUP 3 (300 MG/KG)</b>			
61	2.86	1.36	0.00
62	2.88	1.02	0.00
63 <NP>	--	--	--
64	2.65	0.93	0.00
65	2.45	0.91	0.00
66	2.63	0.91	0.00
67	2.67	0.94	0.00
68	2.63	1.03	0.00
69	2.50	0.93	0.00
70	2.67	0.94	0.00
MEAN	2.66	1.00	0.00
N	9	9	9
<b>GROUP 4 (1000 MG/KG)</b>			
71	2.71	0.78	0.00
72 <NP>	--	--	--
73	2.56	1.32	0.00
74	2.55	1.03	0.00
75	2.65	1.05	0.00
76	2.67	0.99	--
77	2.81	0.85	0.00
78	2.66	0.91	0.00
79	2.68	1.27	0.00
80	2.60	0.90	0.00
MEAN	2.65	1.01	0.00
N	9	9	8

<NP> Non-pregnant

28Jun19 08h17



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 1 (CONTROL)</b>					
41	1	M	2.52	--	0.00
	2	M	2.45	--	0.00
	3	M	2.57	--	0.00
	4	F	--	1.02	--
	5	F	--	0.90	--
	6	F	--	0.96	--
	7	F	--	0.95	--
	8	F	--	1.12	--
	9	F	--	0.99	--
	10	F	--	1.08	--
43	1	M	2.53	--	0.00
	2	M	2.83	--	0.00
	3	M	2.73	--	0.00
	4	M	2.52	--	0.00
	5	M	2.59	--	0.00
	6	F	--	0.81	--
	7	F	--	1.05	--
	8	F	--	0.96	--
44	1	M	2.43	--	0.00
	2	M	2.92	--	0.00
	3	M	2.47	--	0.00
	4	M	2.25	--	0.00
	5	M	2.60	--	--
	6	M	2.54	--	--
	7	M	2.30	--	--
	8	M	2.57	--	--
	9	M	2.23	--	--
	10	F	--	0.90	--
	11	F	--	0.80	--
	12	F	--	0.87	--
	13	F	--	1.00	--
	14	F	--	1.00	--
	15	F	--	0.89	--
45	1	M	2.84	--	0.00
	2	M	2.53	--	0.00
	3	M	2.85	--	0.00
	4	F	--	0.98	--
	5	F	--	1.00	--
	6	F	--	0.77	--
47	1	M	2.80	--	0.00
	2	M	2.60	--	0.00
	3	M	2.65	--	0.00
	4	M	2.61	--	0.00
	5	M	2.73	--	--
	6	F	--	0.84	--
	7	F	--	0.72	--
	8	F	--	0.77	--
	9	F	--	0.78	--
48	1	M	2.53	--	0.00
	2	M	2.57	--	0.00
	3	M	2.61	--	0.00
	4	M	2.76	--	0.00
	5	M	2.62	--	--
	6	F	--	0.96	--
	7	F	--	0.94	--
	8	F	--	1.11	--
	9	F	--	0.92	--
	10	F	--	0.98	--

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 1 (CONTROL)</b>					
	11	F	--	1.03	--
	12	F	--	1.00	--
	13	F	--	0.76	--
	14	F	--	0.93	--
49	1	M	2.77	--	0.00
	2	M	2.75	--	0.00
	3	M	2.79	--	0.00
	4	M	2.67	--	0.00
	5	M	2.63	--	--
	6	F	--	1.07	--
	7	F	--	0.95	--
	8	F	--	1.05	--
	9	F	--	0.98	--
	10	F	--	1.13	--
	11	F	--	1.10	--
	12	F	--	0.88	--
	13	F	--	0.93	--
50	1	M	2.60	--	0.00
	2	M	2.66	--	0.00
	3	M	2.47	--	0.00
	4	M	2.69	--	0.00
	5	M	2.29	--	--
	6	M	2.34	--	--
	7	M	2.35	--	--
	8	M	2.40	--	--
	9	M	2.42	--	--
	10	F	--	0.82	--
	11	F	--	1.34	--
	12	F	--	1.01	--
	13	F	--	1.06	--
	14	F	--	1.09	--
<b>GROUP 2 (100 MG/KG)</b>					
52	1	M	2.63	--	0.00
	2	M	2.35	--	0.00
	3	M	2.36	--	0.00
	4	M	2.70	--	0.00
	5	M	2.56	--	--
	6	F	--	1.19	--
	7	F	--	0.88	--
	8	F	--	1.21	--
	9	F	--	1.07	--
	10	F	--	1.05	--
	11	F	--	1.06	--
	12	F	--	0.98	--
	13	F	--	0.87	--
	14	F	--	0.75	--
54	1	M	2.67	--	0.00
	2	M	2.65	--	0.00
	3	M	2.73	--	0.00
	4	M	2.76	--	0.00
	5	M	2.74	--	--
	6	M	2.67	--	--
	7	F	--	0.82	--
	8	F	--	0.90	--
	9	F	--	0.86	--
	10	F	--	0.71	--
	11	F	--	0.77	--
	12	F	--	0.69	--

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 2 (100 MG/KG)</b>					
	13	F	--	0.84	--
55	1	M	2.79	--	0.00
	2	M	3.29	--	0.00
	3	M	2.48	--	0.00
	4	M	2.66	--	0.00
	5	M	2.81	--	--
	6	M	2.46	--	--
	7	M	2.85	--	--
	8	M	2.45	--	--
	9	M	2.19	--	--
	10	F	--	1.51	--
	11	F	--	1.78	--
	12	F	--	1.50	--
	13	F	--	1.17	--
56	1	M	2.60	--	0.00
	2	M	2.59	--	0.00
	3	M	2.33	--	0.00
	4	M	2.58	--	0.00
	5	M	2.50	--	--
	6	M	2.47	--	--
	7	M	2.44	--	--
	8	M	2.45	--	--
	9	F	--	1.01	--
	10	F	--	0.98	--
	11	F	--	0.85	--
	12	F	--	0.87	--
	13	F	--	0.92	--
	14	F	--	0.92	--
	15	F	--	1.12	--
	16	M	2.40	--	--
	17	F	--	1.26	--
57	1	M	2.50	--	0.00
	2	M	2.67	--	0.00
	3	M	2.53	--	0.00
	4	M	2.55	--	0.00
	5	M	2.65	--	--
	6	M	2.64	--	--
	7	F	--	1.18	--
	8	F	--	1.04	--
	9	F	--	0.79	--
	10	F	--	1.08	--
	11	F	--	0.96	--
58	1	M	2.65	--	0.00
	2	M	2.55	--	0.00
	3	M	2.69	--	0.00
	4	M	2.62	--	0.00
	5	M	2.63	--	--
	6	M	2.65	--	--
	7	F	--	0.95	--
	8	F	--	0.89	--
	9	F	--	0.90	--
	10	F	--	0.86	--
	11	F	--	0.83	--
	12	F	--	0.91	--
	13	F	--	0.83	--
	14	F	--	0.93	--
59	1	M	2.46	--	0.00
	2	M	2.58	--	0.00

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 2 (100 MG/KG)</b>					
	3	M	2.67	--	0.00
	4	M	2.67	--	0.00
	5	M	2.69	--	--
	6	M	2.57	--	--
	7	M	2.79	--	--
	8	F	--	1.23	--
	9	F	--	1.14	--
	10	F	--	1.14	--
	11	F	--	1.07	--
	12	F	--	1.06	--
	13	F	--	0.98	--
	14	F	--	1.07	--
	15	F	--	0.99	--
	16	F	--	0.98	--
60	1	M	2.42	--	0.00
	2	M	2.63	--	0.00
	3	M	2.68	--	0.00
	4	M	2.44	--	0.00
	5	M	2.23	--	--
	6	M	2.35	--	--
	7	M	2.50	--	--
	8	M	2.48	--	--
	9	F	--	0.97	--
	10	F	--	1.05	--
	11	F	--	0.97	--
	12	F	--	0.98	--
	13	F	--	1.08	--
	14	F	--	1.11	--
	15	F	--	1.00	--
<b>GROUP 3 (300 MG/KG)</b>					
61	1	M	3.21	--	0.00
	2	M	2.91	--	0.00
	3	M	2.62	--	0.00
	4	M	2.71	--	0.00
	5	F	--	1.26	--
	6	F	--	1.73	--
	7	F	--	1.39	--
	8	F	--	1.11	--
	9	F	--	1.49	--
	10	F	--	1.06	--
	11	F	--	1.18	--
	12	F	--	1.72	--
	13	F	--	1.27	--
62	1	M	2.91	--	0.00
	2	M	2.80	--	0.00
	3	M	2.95	--	0.00
	4	M	2.83	--	0.00
	5	M	2.99	--	--
	6	M	2.78	--	--
	7	F	--	1.12	--
	8	F	--	1.05	--
	9	F	--	1.05	--
	10	F	--	1.09	--
	11	F	--	0.91	--
	12	F	--	0.93	--
	13	F	--	0.99	--
64	1	M	2.64	--	0.00
	2	M	2.53	--	0.00

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 3 (300 MG/KG)</b>					
	3	M	2.70	--	0.00
	4	M	2.64	--	0.00
	5	M	2.71	--	--
	6	M	2.68	--	--
	7	M	2.66	--	--
	8	F	--	1.00	--
	9	F	--	0.82	--
	10	F	--	0.96	--
	11	F	--	0.94	--
	12	F	--	0.89	--
	13	F	--	0.98	--
65	1	M	2.54	--	0.00
	2	M	2.36	--	0.00
	3	M	2.47	--	0.00
	4	M	2.43	--	0.00
	5	M	--	--	--
	6	M	2.30	--	--
	7	M	2.60	--	--
	8	F	--	0.98	--
	9	F	--	0.81	--
	10	F	--	1.00	--
	11	F	--	0.77	--
	12	F	--	1.01	--
	13	F	--	0.87	--
66	1	M	2.70	--	0.00
	2	M	2.68	--	0.00
	3	M	2.69	--	0.00
	4	M	2.54	--	0.00
	5	M	2.57	--	--
	6	M	2.57	--	--
	7	M	2.69	--	--
	8	F	--	0.84	--
	9	F	--	1.02	--
	10	F	--	0.88	--
	11	F	--	0.89	--
67	1	M	2.62	--	0.00
	2	M	2.50	--	0.00
	3	M	2.49	--	0.00
	4	M	2.84	--	0.00
	5	M	2.90	--	--
	6	F	--	1.01	--
	7	M	2.51	--	--
	8	M	2.85	--	--
	9	M	2.63	--	--
	10	F	--	0.86	--
	11	F	--	1.01	--
	12	F	--	0.99	--
	13	F	--	0.96	--
	14	F	--	0.82	--
68	1	M	2.45	--	0.00
	2	M	2.56	--	0.00
	3	M	2.76	--	0.00
	4	M	2.68	--	0.00
	5	M	2.51	--	--
	6	M	2.79	--	--
	7	F	--	1.06	--
	8	F	--	1.10	--
	9	F	--	1.00	--
	10	F	--	0.96	--

03Jun19 14h43

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 3 (300 MG/KG)</b>					
69	1	M	2.53	--	0.00
	2	M	2.51	--	0.00
	3	M	2.45	--	0.00
	4	F	--	0.74	--
	5	F	--	0.98	--
	6	F	--	1.01	--
	7	F	--	1.11	--
	8	F	--	0.99	--
	9	F	--	0.89	--
	10	F	--	1.00	--
	11	F	--	0.98	--
	12	F	--	0.80	--
	13	F	--	0.78	--
70	1	M	2.63	--	0.00
	2	M	2.78	--	0.00
	3	M	2.54	--	0.00
	4	M	2.73	--	0.00
	5	M	2.68	--	--
	6	F	--	0.82	--
	7	F	--	1.01	--
	8	F	--	0.98	--
	9	F	--	0.93	--
<b>GROUP 4 (1000 MG/KG)</b>					
71	1	M	2.67	--	0.00
	2	M	2.80	--	0.00
	3	M	2.72	--	0.00
	4	M	2.66	--	0.00
	5	M	2.73	--	--
	6	M	2.74	--	--
	7	M	2.63	--	--
	8	F	--	0.84	--
	9	F	--	0.63	--
	10	F	--	0.85	--
	11	F	--	0.81	--
73	1	M	2.87	--	0.00
	2	M	1.92	--	0.00
	3	M	2.67	--	0.00
	4	M	2.61	--	0.00
	5	M	2.73	--	--
	6	F	--	1.41	--
	7	F	--	1.39	--
	8	F	--	1.16	--
	9	F	--	1.06	--
	10	F	--	1.22	--
	11	F	--	1.34	--
	12	F	--	1.33	--
	13	F	--	1.66	--
	14	F	--	1.30	--
74	1	M	2.67	--	0.00
	2	M	2.37	--	0.00
	3	M	2.48	--	0.00
	4	M	2.70	--	0.00
	5	M	2.60	--	--
	6	F	--	1.15	--
	7	M	2.51	--	--
	8	M	2.52	--	--
	9	F	--	1.15	--

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 4 (1000 MG/KG)</b>					
	10	F	--	1.06	--
	11	F	--	0.98	--
	12	F	--	0.99	--
	13	F	--	0.84	--
75	1	M	2.77	--	0.00
	2	M	2.68	--	0.00
	3	M	2.50	--	0.00
	4	M	2.56	--	0.00
	5	M	2.63	--	--
	6	M	2.74	--	--
	7	F	--	1.09	--
	8	F	--	0.90	--
	9	F	--	1.11	--
	10	F	--	1.04	--
	11	F	--	0.98	--
	12	F	--	1.14	--
	13	F	--	1.12	--
76	1	M	2.68	--	
	2	M	2.58	--	
	3	M	2.95	--	
	4	M	2.47	--	
	5	F	--	1.12	
	6	F	--	0.97	
	7	F	--	1.02	
	8	F	--	0.93	
	9	F	--	1.01	
	10	F	--	0.89	
77	1	M	2.97	--	0.00
	2	M	2.84	--	0.00
	3	M	2.86	--	0.00
	4	M	2.86	--	0.00
	5	M	2.67	--	--
	6	M	2.77	--	--
	7	M	2.80	--	--
	8	M	2.68	--	--
	9	F	--	0.89	--
	10	F	--	0.80	--
	11	F	--	0.79	--
	12	F	--	0.80	--
	13	F	--	0.98	--
	14	F	--	0.74	--
	15	F	--	0.94	--
78	1	M	2.84	--	0.00
	2	M	2.66	--	0.00
	3	M	2.64	--	0.00
	4	M	2.62	--	0.00
	5	M	2.59	--	--
	6	M	2.74	--	--
	7	M	2.54	--	--
	8	F	--	0.69	--
	9	F	--	0.85	--
	10	F	--	1.00	--
	11	F	--	1.01	--
	12	F	--	0.92	--
	13	F	--	0.84	--
	14	F	--	1.07	--
	15	F	--	0.94	--
	16	F	--	0.90	--

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.20 ANOGENITAL DISTANCE AND NIPPLE RETENTION

### F0-GENERATION - LACTATION

---

LITTER	PUP	SEX	anogenital dist M mm	anogenital dist F mm	Number of nipples
<b>GROUP 4 (1000 MG/KG)</b>					
79	1	M	2.82	--	0.00
	2	M	2.64	--	0.00
	3	M	2.16	--	0.00
	4	M	3.08	--	0.00
	5	F	--	1.51	--
	6	F	--	1.23	--
	7	F	--	1.39	--
	8	F	--	1.12	--
	9	F	--	1.14	--
	10	F	--	0.89	--
	11	F	--	1.18	--
	12	F	--	1.72	--
80	1	M	2.71	--	0.00
	2	M	2.53	--	0.00
	3	M	2.54	--	0.00
	4	M	2.61	--	0.00
	5	M	2.61	--	--
	6	M	2.57	--	--
	7	M	2.64	--	--
	8	M	2.62	--	--
	9	F	--	1.00	--
	10	F	--	0.91	--
	11	F	--	0.86	--
	12	F	--	0.84	--



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.21 CORRECTED ANOGENITAL DISTANCE

### PND 1

---

LITTER	norm anog dist M mm	norm anog dist F mm
--------	---------------------------	---------------------------

---

#### GROUP 1 (CONTROL)

41	1.35	0.55
43	1.34	0.48
44	1.36	0.51
45	1.38	0.48
47	1.43	0.42
48	1.47	0.55
49	1.46	0.54
50	1.37	0.58

#### GROUP 2 (100 MG/KG)

52	1.40	0.57
54	1.44	0.44
55	1.40	0.80
56	1.41	0.57
57	1.43	0.56
58	1.45	0.50
59	1.44	0.58
60	1.36	0.58

#### GROUP 3 (300 MG/KG)

61	1.48	0.72
62	1.49	0.54
64	1.46	0.53
65	1.29	0.49
66	1.36	0.47
67	1.40	0.50
68	1.46	0.58
69	1.39	0.52
70	1.36	0.49

#### GROUP 4 (1000 MG/KG)

71	1.50	0.44
73	1.39	0.74
74	1.39	0.57
75	1.44	0.57
76	1.43	0.54
77	1.53	0.48
78	1.46	0.52
79	1.44	0.68
80	1.46	0.50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

## 2.22 CLINICAL BIOCHEMISTRY MALES PUPS (PND 14-16)

---

LITTER	Total T4 ug/dL
--------	-------------------

---

### GROUP 1 (CONTROL)

41	8.59
43	7.88
44	5.62
45	7.08
47	7.36
48	6.02
49	5.19
50	4.87

### GROUP 2 (100 MG/KG)

52	6.23
54	6.61
55	7.16
56	5.87
57	6.10
58	7.45
59	5.60
60	4.52

### GROUP 3 (300 MG/KG)

61	5.83
62	5.10
64	6.29
65	5.82
66	5.94
67	5.89
68	6.61
69	5.58
70	7.89

### GROUP 4 (1000 MG/KG)

71	6.12
73	6.58
74	5.84
75	5.27
76	5.40
77	7.04
78	6.10
79	6.79
80	5.11

## FEMALES PUPS (PND 14-16)

---

LITTER	Total T4 ug/dL
--------	-------------------

---

### GROUP 1 (CONTROL)

41	5.32
43	7.11
44	5.39
45	5.42
47	7.41
48	5.94
49	6.65
50	4.62

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

## 2.22 CLINICAL BIOCHEMISTRY FEMALES PUPS (PND 14-16)

---

LITTER	Total T4 ug/dL
--------	-------------------

---

### GROUP 2 (100 MG/KG)

52	6.35
54	6.57
55	5.25
56	5.15
57	5.86
58	6.54
59	6.85
60	4.88

### GROUP 3 (300 MG/KG)

61	5.94
62	6.32
64	4.77
65	4.93
66	6.08
67	5.18
68	6.06
69	6.21
70	6.21

### GROUP 4 (1000 MG/KG)

71	5.99
73	5.04
74	4.56
75	3.64
76	4.58
77	5.93
78	5.46
79	6.12
80	6.22

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 1 (CONTROL)</b>			
LITTER 41 15MAR2019	1	M DAY 14 Planned Necropsy	FLC Wound nose DAY 2 Scabs snout DAY 3 Scab snout DAY 4 Scab snout LLC No findings MACRO No findings
	2	M DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3	M DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	6	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	7	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	10	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
LITTER 43 16MAR2019	1	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	6	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	7	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
LITTER 44 15MAR2019	1	M DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 1 (CONTROL)</b>			
	2 M	DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	7 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	8 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	9 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	10 F	DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	11 F	DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	12 F	DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	13 F	DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	14 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	15 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
LITTER 45 14MAR2019	1 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	6 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 1 (CONTROL)</b>			
LITTER 47 13MAR2019	1	M DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2	M DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3	M DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4	M DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	7	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
LITTER 48 16MAR2019	1	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	7	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8	F DAY 6 Missing	FLC No findings
	9	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	10	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	11	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	12	F DAY 4 Missing	FLC No findings
	13	F DAY 4 Culling	FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 1 (CONTROL)</b>			
	14 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 49 16MAR2019	1 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5 M	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	6 F	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	7 F	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	8 F	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	9 F	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	10 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	11 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	12 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	13 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 50 15MAR2019	1 M	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2 M	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3 M	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4 M	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5 M	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	6 M	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 1 (CONTROL)</b>			
	7	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	8	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	9	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	10	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	11	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC Wound nose
	12	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	13	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	14	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
<b>GROUP 2 (100 MG/KG)</b>			
LITTER 52 14MAR2019	1	M DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2	M DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3	M DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4	M DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	6	F DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	7	F DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	8	F DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	9	F DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	10	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	11	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 2 (100 MG/KG)</b>			
	12 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	13 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	14 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
LITTER 54 13MAR2019	1 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	2 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	3 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	4 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	5 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	6 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	7 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	8 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	9 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	10 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	11 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	12 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	13 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
LITTER 55 17MAR2019	1 M	DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	2 M	DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	3 M	DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	4 M	DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 2 (100 MG/KG)</b>			
	5	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	6	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	7	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	8	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	9	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	10	F DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	11	F DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	12	F DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	13	F DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
LITTER 56 15MAR2019	1	M DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	2	M DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	3	M DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	4	M DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	5	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	6	M DAY 4 Culling	MACRO No findings FLC Wound leg LLC No findings
	7	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	8	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	9	F DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	10	F DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	11	F DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 2 (100 MG/KG)</b>			
	12 F	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	13 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	14 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	15 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	16 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	17 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
LITTER 57 14MAR2019	1 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	2 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	3 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	4 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	5 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	6 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	7 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	8 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	9 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	10 F	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	11 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
LITTER 58 14MAR2019	1 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	2 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	3 M	DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 2 (100 MG/KG)</b>			
	4	M DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	5	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	6	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	7	F DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	8	F DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	9	F DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	10	F DAY 15 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	11	F DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	12	F DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	13	F DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	14	F DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
LITTER 59 16MAR2019	1	M DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	2	M DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	3	M DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	4	M DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	5	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	6	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	7	M DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	8	F DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	9	F DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 2 (100 MG/KG)</b>			
	10 F	DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	11 F	DAY 16 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	12 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	13 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	14 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	15 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	16 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
LITTER 60 15MAR2019	1 M	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	2 M	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	3 M	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	4 M	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	5 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	6 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	7 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	8 M	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	9 F	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	10 F	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	11 F	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	12 F	DAY 14 Planned Necropsy	MACRO No findings FLC No findings LLC No findings
	13 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 2 (100 MG/KG)</b>			
	14 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings
	15 F	DAY 4 Culling	MACRO No findings FLC No findings LLC No findings MACRO No findings
<b>GROUP 3 (300 MG/KG)</b>			
LITTER 61 17MAR2019	1 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 16 Spontaneous death	FLC No findings LLC Dead MACRO No findings
	3 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	6 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	7 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	10 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	11 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	12 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	13 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
LITTER 62 16MAR2019	1 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 3 (300 MG/KG)</b>			
	5 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	7 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	10 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	11 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	12 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	13 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
LITTER 64 14MAR2019	1 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	7 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	8 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	10 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	11 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 3 (300 MG/KG)</b>			
	12 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	13 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
LITTER 65 16MAR2019	1 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 M	DAY 1 Dead at FLC	FLC Dead LLC Dead MACRO Beginning autolysis
	6 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	7 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	8 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	10 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	11 F	DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	12 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	13 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
LITTER 66 14MAR2019	1 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 3 (300 MG/KG)</b>			
	6	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	7	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	8	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	10	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	11	F DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
LITTER 67 16MAR2019	1	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	7	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	8	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	9	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	10	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	11	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	12	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	13	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	14	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 3 (300 MG/KG)</b>			
LITTER 68 15MAR2019	1	M DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2	M DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3	M DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4	M DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6	M DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	7	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	10	F DAY 14 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
LITTER 69 16MAR2019	1	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3	M DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	6	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	7	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	8	F DAY 16 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	9	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	10	F DAY 4 Culling	FLC No findings LLC No findings MACRO No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 3 (300 MG/KG)</b>			
LITTER 70 14MAR2019	11 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	12 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	13 F	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	1 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
7 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings	
8 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings	
9 F	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings	
<b>GROUP 4 (1000 MG/KG)</b>			
LITTER 71 13MAR2019	1 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	2 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	3 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	4 M	DAY 15 Planned Necropsy	FLC No findings LLC No findings MACRO No findings
	5 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	6 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	7 M	DAY 4 Culling	FLC No findings LLC No findings MACRO No findings
	8 F	DAY 15 Planned Necropsy	FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS	
<b>GROUP 4 (1000 MG/KG)</b>				
LITTER 73 13MAR2019	9	F DAY 15 Planned Necropsy	LLC No findings	
			MACRO No findings	
	10	F DAY 15 Planned Necropsy	FLC No findings	
			LLC No findings	
	11	F DAY 15 Planned Necropsy	MACRO No findings	
			FLC No findings	
	LITTER 74 15MAR2019	1	M DAY 15 Planned Necropsy	LLC No findings
				MACRO No findings
		2	M DAY 15 Planned Necropsy	FLC No findings
				LLC No findings
		3	M DAY 15 Planned Necropsy	MACRO No findings
				FLC No findings
		4	M DAY 15 Planned Necropsy	LLC No findings
				MACRO No findings
5		M DAY 4 Culling	FLC No findings	
			LLC No findings	
6		F DAY 15 Planned Necropsy	MACRO No findings	
			FLC No findings	
7		F DAY 15 Planned Necropsy	LLC No findings	
			MACRO No findings	
8	F DAY 15 Planned Necropsy	FLC No findings		
		LLC No findings		
9	F DAY 15 Planned Necropsy	MACRO No findings		
		FLC No findings		
10	F DAY 4 Culling	LLC No findings		
		MACRO No findings		
11	F DAY 4 Culling	FLC No findings		
		LLC No findings		
12	F DAY 4 Culling	MACRO No findings		
		FLC No findings		
13	F DAY 4 Culling	LLC No findings		
		MACRO No findings		
14	F DAY 4 Culling	FLC No findings		
		LLC No findings		
1	M DAY 14 Planned Necropsy	MACRO No findings		
		FLC No findings		
		LLC No findings		
2	M DAY 14 Planned Necropsy	MACRO No findings		
		FLC No findings		
		LLC No findings		
3	M DAY 14 Planned Necropsy	MACRO No findings		
		FLC No findings		

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 4 (1000 MG/KG)</b>			
	4	M DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	6	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	7	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	8	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	9	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	10	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	11	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	12	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	13	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 75 15MAR2019	1	M DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2	M DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3	M DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4	M DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	6	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	7	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	8	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	9	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	10	F DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 4 (1000 MG/KG)</b>			
	11 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	12 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	13 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 76 15MAR2019	1 M	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2 M	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3 M	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4 M	DAY 14 Planned Necropsy	LLC Left hind leg bend at the hight of the knee MACRO Left hind leg bend at the hight of the knee FLC No findings
	5 F	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	6 F	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	7 F	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	8 F	DAY 14 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	9 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	10 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 77 16MAR2019	1 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4 M	DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5 M	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	6 M	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	7 M	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 4 (1000 MG/KG)</b>			
	8	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	9	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	10	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	11	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	12	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	13	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	14	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	15	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 78 16MAR2019	1	M DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2	M DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3	M DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4	M DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	6	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	7	M DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	8	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	9	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	10	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	11	F DAY 16 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	12	F DAY 4 Culling	LLC No findings MACRO No findings FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
<b>GROUP 4 (1000 MG/KG)</b>			
	13 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	14 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	15 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	16 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 79 13MAR2019	1 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	5 F	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	6 F	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	7 F	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	8 F	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	9 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	10 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	11 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
	12 F	DAY 4 Culling	LLC No findings MACRO No findings FLC No findings
LITTER 80 14MAR2019	1 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	2 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	3 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings
	4 M	DAY 15 Planned Necropsy	LLC No findings MACRO No findings FLC No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50



# Final Report

DIHEP AQUEOUS SOLUTION  
APPENDIX 2

Project 20172120

## 2.23 MORTALITY, CLINICAL SIGNS AND MACROSCOPY OF PUPS FEMALES

### F0-GENERATION - LACTATION

---

LITTER DELIVERY	PUP	END OF P.P. PHASE	FINDINGS
--------------------	-----	-------------------	----------

---

#### GROUP 4 (1000 MG/KG)

			LLC	No findings
			MACRO	No findings
5	M	DAY 4 Culling	FLC	No findings
			LLC	No findings
			MACRO	No findings
6	M	DAY 4 Culling	FLC	No findings
			LLC	No findings
			MACRO	No findings
7	M	DAY 4 Culling	FLC	No findings
			LLC	No findings
			MACRO	No findings
8	M	DAY 4 Culling	FLC	No findings
			LLC	No findings
			MACRO	No findings
9	F	DAY 15 Planned Necropsy	FLC	No findings
			LLC	No findings
			MACRO	No findings
10	F	DAY 15 Planned Necropsy	FLC	No findings
			LLC	No findings
			MACRO	No findings
11	F	DAY 15 Planned Necropsy	FLC	No findings
			LLC	No findings
			MACRO	No findings
12	F	DAY 15 Planned Necropsy	FLC	No findings
			LLC	No findings
			MACRO	No findings

FLC - FIRST LITTER CHECK, DAY P.P. - CLINICAL SIGNS ,  
LLC - LAST LITTER CHECK, MACRO - MACROSCOPIC FINDINGS

03Jun19 14h50

**2.24 KEY TO MISSING VALUES/REMARKS****Necropsy**

<b>Organ Weights:</b>				
Animal(s):	Organ	Weight (g)	Weight (g) aberrant side	Reason
35	Epididymis	---	0.736	Left side: Macroscopic finding
68	Brain	2.245	--	The brain was not weighed at necropsy but after fixation.

**End of Treatment**

<b>Haematology:</b>		
Animal(s):		
41, 44, 45, 50, 52, 56, 57, 58, 64, 66, 68, 70, 74, 80		Samples were stored at $\leq -75^{\circ}\text{C}$ prior to analysis on the STA Compact
50	---	= Citrate sample clotted
15, 66	---	= Value for PT could not be reproduced
1, 34		Differential leucocyte count was also performed manually because of an abnormal plot in the automated count and these results are reported
1, 34		Manual differential leucocyte count revealed that 5.6% and 5% of neutrophils were band cells, respectively.

<b>Clinical Biochemistry:</b>		
Animal(s):		
All animals		Samples were stored at $\leq -75^{\circ}\text{C}$ prior to (possible) analysis on the Immulite1000
62		Samples were stored at $\leq -75^{\circ}\text{C}$ prior to analysis on the AU400

**PND4**

<b>Clinical Biochemistry:</b>		
Animal(s):		
All animals		Samples were stored at $\leq -75^{\circ}\text{C}$ prior to analysis on the IMMULITE 1000
41, 43-45, 48-50, 52, 54-62, 64-70, 74-80		Lipemic serum sample; common occurrence during lactation
52, 57, 58, 61, 64, 66, 80		Hemolytic sample
47, 71, 73		Not Lipemic sample

**PND14-16**

<b>Clinical Biochemistry:</b>		
Animal(s):		
All animals		Samples were stored at $\leq -75^{\circ}\text{C}$ prior to analysis on the IMMULITE 1000
41, 43-45, 47-50, 52, 54-62, 64-68, 70-71, 73-76, 78-80 male + female & 77 female		Lipemic serum sample; common occurrence during lactation
47 male		Hemolytic serum sample
69 male + female, 77 male		Not lipemic serum sample

**Appendix 3**  
**Test Item Characterization**

**Certificate of analysis**

Page 1 of 1

**THE DOW CHEMICAL COMPANY**

Certificate of analysis		Quality order	
Product name	CANSOLV ABSORBANT DS	Customer Batch number	D2921BF000
Reference number		Batch number	
		Reference lot	

Test	Unit	Test result	Min	Max
% Water	%	51.14	51	54
Appearance		Pass	-	-
Gardner Color	Color	3	-	6
Foam Height	ml	75	-	100
Foam Break Time	Seconds	7.7	-	15
Gas Chromatography, HEP	%	9.01	-	14
Gas Chromatography, DIHEP	%	89.69	80	100
Gas Chromatograph Heavies GT DHEP	%	0.4	-	5
Gas Chromatography, Piperazine	%	0	-	2
Gas Chromatograph EG & DEG	%	0.2	-	0.6
Cansolv DS Alkalinity	meq/g	5.76	-	-

Notes: Tank 105

APPROVED BY: KENDRICK JUNIUS

**Appendix 4**  
**Histopathology Report**

**TABLE OF CONTENTS**

**LIST OF APPENDICES** .....2

1. SUMMARY .....3

2. INTRODUCTION .....4

3. STUDY DESIGN .....4

4. METHODS .....4

4.1. Macroscopic Examination .....4

4.2. Organ Weights .....7

4.3. Microscopic Examination .....7

4.4. Internal Peer Review .....8

5. RESULTS .....9

5.1. Mortality .....9

5.2. Clinical Pathology .....9

5.3. Macroscopic Findings .....9

5.4. Organ Weights .....9

5.5. Microscopic Findings .....9

5.6. Reproductive Performance .....10

6. DISCUSSION .....11

7. CONCLUSIONS .....11

8. REPORT AUTHENTICATION .....12

**LIST OF APPENDICES**

**APPENDIX 1 HISTOPATHOLOGY TABLES**

## 1. SUMMARY

Pathomorphologic examination was performed on 80 Wistar (Han) rats (40 males, 40 females) which had been subjected to a combined 28-Day oral (gavage) toxicity study with the reproduction/developmental toxicity screening test with the test item **DiHEP Aqueous Solution**.

The rats were assigned to four dose groups, each containing 10 males and 10 females. The test item was administered undiluted once daily by gavage at doses of 100, 300 and 1000 mg/kg/day (dose groups 2, 3 and 4 respectively) for a minimum of 28 days. The rats of the control group 1 received water (Elix), alone.

At the end of the treatment period all rats from all groups were killed and subjected to complete necropsies. Histopathologic examination was performed on an extensive list of organs and tissues from five selected group 1 and 4 animals as well as the adrenal glands and stomach from selected group 2 and 3 males and all organs with macroscopic findings from all rats. The reproductive organs were examined from all males that failed to sire and all females that failed to deliver healthy pups.

There were no unscheduled deaths.

There were no test item-related macroscopic findings.

A possible test item-related non-adverse lower thymus weight was observed in 300 and 1000 mg/kg/day males.

Test item-related microscopic findings were present in the **stomach** of 1000 mg/kg/day males, consisting of (multi)focal erosions of the mucosal layer of the glandular stomach. Based on the low degrees (up to slight) and absence of concomitant test item-related changes, this finding was regarded as non-adverse.

There were 2/10 couples of the Control group, 2/10 couples treated at 100 mg/kg/day, 1/10 couples treated at 300 mg/kg/day and 1/10 couples treated at 1000 mg/kg/day that failed to deliver healthy pups. Histopathology did not reveal any changes in the reproductive organs that could explain this, besides a necrotic placenta in control female no. 42.

In conclusion, non-adverse test item-related morphologic alterations following the oral (gavage) administration of DiHEP Aqueous Solution for at least 28 days to Wistar (Han) rats at doses up to 1000 mg/kg/day, were present in the stomach of 1000 mg/kg/day group males. Furthermore, possible test-item related lower thymus weights were present in 300 and 1000 mg/kg/day group males.

There were no morphologic alterations in males treated up to 300 mg/kg/day and in females up to 1000 mg/kg/day.

There were no morphological findings in the reproductive organs of either sex which could be attributed to the test item and stage aware evaluation of the testes did not show any indication for abnormal spermatogenesis.

## 2. INTRODUCTION

The objectives of this study were to determine the potential toxic effects of DiHEP Aqueous Solution when given orally by gavage for a minimum of 28 days to Wistar Han rats, and to evaluate the potential to affect male and female reproductive performance such as gonadal function, mating behavior, conception, parturition and early postnatal development. This pathology report addresses the anatomical pathology endpoints of the study. It is based on the study plan and any study plan amendment.

## 3. STUDY DESIGN

Wistar (Han) rats, males approximately 10-12 weeks of age and females approximately 12-14 weeks of age (for exact details see main study report) on treatment Day 1, were administered undiluted DiHEP Aqueous Solution via oral gavage daily from 2 weeks prior to mating, during mating, and up to the day prior to necropsy for at least 28 days as indicated in the following table.

Group Number	Dose level mg/kg bw /day	Number of animals		Animal numbers		Selected animals	
		Males	Females	Males	Females	Males	Females
1	0 <sup>a</sup>	10	10	01-10	41-50	01-05	41,44,45,47,50
2	100	10	10	11-20	51-60	11-15	52,54,56,57,58
3	300	10	10	21-30	61-70	21-25	62,64,66,68,70
4	1000	10	10	31-40	71-80	31-35	71,73,74,79,80

<sup>a</sup> The control group received only water (Elix).

## 4. METHODS

### 4.1. Macroscopic Examination

Complete postmortem examinations were performed on all animals. Animals were anesthetized using isoflurane and subsequently exsanguinated. At the time of necropsy, the following tissues and organs were collected and placed in 10% neutral-buffered formalin fixative unless otherwise noted:

Table 1: All selected animals:

Tissue	Weigh	Collect	Histology <sup>a</sup>	Microscopic Evaluation <sup>a</sup>	Comment
Animal identification	-	X	-	-	Location: ear and foot
Artery, aorta	-	X	-	-	-
Body cavity, nasopharynx	-	X	-	-	-
Bone marrow	-	X	X	X	Collected as part of the femur and sternum.
Bone, femur	-	X	X	X	Including joint.
Bone, sternum	-	X	X	X	-
Brain	X	X	X	X	Eight brain levels were examined including cerebellum, midbrain and cortex.
Cervix	X	X	X	X	Collected and weighed together with the uterus.
Epididymis	X	X	X	X	Paired examination. Initially preserved in modified Davidson's fixative.
Esophagus	-	X	-	-	-



Eye	-	X	X	X	Paired examination. Initially preserved in modified Davidson's fixative.
Gland, adrenal	X	X	X	X	Paired examination.
Gland, coagulation	X	X	X	X	Collected and weighed together with the seminal vesicles.
Gland, harderian	-	X	-	-	-
Gland, lacrimal	-	X	-	-	Collected exorbital.
Gland, mammary	-	X	X	X	Collected: inguinal region with skin. Examined for both males and females.
Gland, parathyroid	X	X	-	-	Examined only if present in the routine section of thyroid. Collected and weighed together with thyroid.
Gland, pituitary	-	X	X	X	-
Gland, prostate	X	X	X	X	-
Gland, salivary	-	X	-	-	Collected at mandibular, sublingual and parotid site.
Gland, seminal vesicle	X	X	X	X	Paired examination. Collected and weighed together with the coagulation gland.
Gland, thyroid	X	X	X	X	Paired examination. Collected and weighed together with the parathyroid.
Gross lesions/masses	-	X	X	X	-
Gut-associated lymphoid tissue	-	X	X	X	Examined only if present in routine section of intestine.
Heart	X	X	X	X	-
Kidney	X	X	X	X	Paired examination.
Large intestine, cecum	-	X	X	X	-
Large intestine, colon	-	X	X	X	-
Large intestine, rectum	-	X	X	X	-
Larynx	-	X	-	-	-
Liver	X	X	X	X	-
Lung	-	X	X	X	Infused with formalin.
Lymph node	-	X	X	X	Collected at mandibular and mesenteric site. Only 1 mandibular required for microscopic examination
Muscle, skeletal	-	X	X	X	-
Nerve, optic	-	X	-	-	Examined only if present in the routine section of the eye. Initially preserved in modified Davidson's fixative.
Nerve, sciatic	-	X	X	X	Only 1 required for microscopic examination
Ovaries	X	X	X	X	Paired examination.
Pancreas	-	X	-	-	-
Skin	-	X	-	-	-
Small intestine, duodenum	-	X	X	X	-
Small intestine, ileum	-	X	X	X	-

Small intestine, jejunum	-	X	X	X	-
Spinal cord	-	X	X	X	Examined one transverse and one longitudinal section from each of the following areas: cervical, mid-thoracic, lumbar.
Spleen	X	X	X	X	-
Stomach	-	X	X	X	-
Testes	X	X	X	X	Paired examination. Initially preserved in modified Davidson's fixative.
Thymus	X	X	X	X	-
Tongue	-	X	-	-	-
Trachea	-	X	X	X	-
Urinary bladder	-	X	X	X	-
Uterus	X	X	X	X	-
Vagina	-	X	X	X	-

X = Procedure conducted; - = Not applicable.

<sup>a</sup> Procedure conducted for selected group 1 and 4 animals, targets organs/tissues from group 2 and 3 animals and all gross lesions from all animals.

Table 2: All remaining animals (incl. males that failed to sire<sup>a</sup> and females that failed to deliver healthy pups):

Tissue	Weigh	Collect	Histology	Microscopic Evaluation	Comment
Animal identification	-	X	-	-	Location: ear and foot
Cervix	-	X	@	@	-
Epididymis	X	X	@	@	Paired examination. Initially preserved in modified Davidson's fixative.
Gland, coagulation	X	X	@	@	Collected and weighed together with the seminal vesicles.
Gland, mammary	-	X	-	-	Collected inguinal region with skin. Collected for both males and females.
Gland, parathyroid	X	X	-	-	Examined only if present in the routine section of thyroid. Collected and weighed together with thyroid.
Gland, pituitary	-	X	-	-	-
Gland, prostate	X	X	@	@	-
Gland, seminal vesicle	X	X	@	@	Paired examination. Collected and weighed together with the coagulation gland.
Gland, thyroid	X	X	-	-	Paired examination. Collected and weighed together with the parathyroid.
Gross lesions/masses	-	X	X	X	-
Ovaries	-	X	@	@	Paired examination.
Testes	X	X	@	@	Paired examination. Initially preserved in modified Davidson's fixative.
Uterus	-	X	@	@	-
Vagina	-	X	@	@	-

X = Procedure conducted for all remaining animals; - = Not applicable;

@ = Procedure conducted for males that failed to sire<sup>a</sup> and females that failed to deliver healthy pups only.

<sup>a</sup> Except for males that failed to sire which were also selected. These males were processed as noted in table 1.

## 4.2. Organ Weights

The organ weights (and terminal body weight) were recorded from all animals at the scheduled necropsy as indicated in the tables above.

Paired organs were weighed together. Absolute organ weights were reported and organ to terminal body weights were calculated and presented in the main study report.

## 4.3. Microscopic Examination

Microscopic examination of routinely prepared hematoxylin-eosin stained paraffin sections was performed:

Group	Dose level mg/kg bw/day	Selected animals		Non-Selected animals	
		Males	Females	Males	Females
1	0	All tissues*	All tissues*	Reproductive organs#: no. 6.	Reproductive organs#: nos. 42 and 46.
2	100	Stomach, Adrenals	Not applicable	Reproductive organs#: nos. 11 and 13.	Reproductive organs#: nos. 51 and 53.
3	300	Stomach, Adrenals	Not applicable	Reproductive organs#: no. 23.	Reproductive organs#: no. 63.
4	1000	All tissues*	All tissues*	Not applicable.	Reproductive organs#: no. 72.

\* All collected tissues, as indicated on the tissue list above.

# Selected reproductive organs, as indicated on the tissue list above.

A detailed qualitative evaluation of the testis was conducted on H&E stained sections from all control and high dose animals. Testes were evaluated to assess the progression of stages of the spermatogenic cycle, cell associations, and proportions expected to be present during spermatogenesis along with assessment of interstitial and supporting cell types (Leydig cells, macrophages, vasculature, and rete testis).

Gross lesions were examined from all animals and correlated to microscopic findings if possible.

The animal data and macroscopic findings were electronically transferred from the necropsy raw data files of ToxData system® into the computer system PathData®. Stained histologic sections were examined by light microscopy in the period 07 May – 07 June 2019 and the microscopic findings were recorded by the undersigned pathologist using on-line input under pathology number 31906 LAA.

Severity grades were assigned to non-neoplastic histopathologic diagnoses, as presented in the following table. Severity grades were assigned based on the severity of alterations in the examined histologic sections and may not reflect the overall severity of the pathologic process in the entire tissue, organ, or animal. The PathData® histopathology tables contain all of the recorded data and serve as the basis for this narrative report.

In the separate pathology tables file, all macroscopic and microscopic findings are given for each animal in text form under "Text of Gross and Microscopic Findings". The incidence of microscopic findings is also presented in tabular form: "Incidence table – Selected findings with grades" and "Incidence table - all microscopic findings". Incidence tables were created by computer.

Histopathological changes were described according to distribution, severity and morphological character. The International Harmonization of Nomenclature and Diagnostic Criteria for Lesions (INHAND) was used as guidance for the description of histopathological changes.

Severity scores were assigned as follows:

Present	Finding present, grading not scored.
Grade 1	Minimal/very few/very small.
Grade 2	Slight/few/small.
Grade 3	Moderate/moderate number/moderate size.
Grade 4	Marked/many/large.
Grade 5	Massive/extensive number/extensive size.
N.A.D.	No Abnormality Detected

#### **4.4. Internal Peer Review**

Pathology findings were subjected to an internal review conducted by Hetty van den Brink-Knol (Dutch CRP/TP Certified Toxicologic Pathologist). Following the peer review, a consensus was reached between the study pathologist and the peer review pathologist with regard to diagnoses and interpretation. Histopathology data entries in PathData® and pathology data presented in the pathology report reflect this consensus.

## 5. RESULTS

### 5.1. Mortality

There were no premature decedents in the study.

### 5.2. Clinical Pathology

Clinical pathology data were evaluated and discussed by the study pathologist and the study director. Clinical pathology results are presented in the main toxicology report.

### 5.3. Macroscopic Findings

There were no test item-related gross observations.

A macroscopic finding of note was recorded in two females of the 100 mg/kg/day group (nos. 52 and 57) and one female of the 300 mg/kg/day group (no. 70). This finding consisted of watery clear fluid of the thoracic cavity. Since this finding was absent in the high dose group, and since there were no other macroscopic findings of organs of the thoracic cavity, or related clinical findings, this finding was considered to be unrelated to the test item.

The remainder of the recorded macroscopic findings were within the range of background gross observations encountered in rats of this age and strain.

### 5.4. Organ Weights

Thymus weights of test item-treated males were lower in a dose-related manner. This organ weight change was not statistically significantly different from the concurrent controls and group mean values remained within background ranges. However, individual organ weight values of 1/5 males of the 300 mg/kg/day group and 2/5 males of the 1000 mg/kg/day group were at the lower range. Therefore a possible test item relationship at these dose levels could not be excluded.

Any other differences, including those that reached statistical significance (i.e. the absolute weight of adrenal glands and relative to body weight of the heart of 100 mg/kg/day males, and absolute weights of kidneys of 1000 mg/kg/day females and relative to body weight of thymus of 100 mg/kg/day females) were considered not to be DiHEP Aqueous Solution - related due to the direction of the change, lack of a dose-related pattern, and/or general overlap and variability in individual values.

### 5.5. Microscopic Findings

Test item-related microscopic findings after treatment with DiHEP Aqueous Solution were noted in the **stomach** of the 1000 mg/kg/day group males and are summarized in text table 1.

Text Table 1.  
Summary Test Item-Related Microscopic Findings – Scheduled Euthanasia Animals (Day 29)

Dose level (mg/kg/day):	Males			
	0	100	300	1000
STOMACH <sup>a</sup>	5	5	5	5
<i>Erosion glandular mucosa, focal</i>				
Minimal	-	-	-	2
Slight	-	-	-	1

<sup>a</sup> = Number of tissues examined from each group.

(Multi) focal erosions of the glandular mucosa of the **stomach** were noted in 1000 mg/kg/day males (up to slight degree). These erosions were all located at the pyloric area.

The remainder of the recorded microscopic findings, including the low degrees of vacuolation of the zona fasciculata of the adrenal glands of males, were within the range of background pathology encountered in rats of this age and strain. There was no test item-related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations.

## 5.6. Reproductive Performance

Text Table 2.

Correlation of Histopathology Findings with In-Life Reason for Males that Failed to Sire and Females that Failed to Deliver Healthy Pups.

Group	Dose level mg/kg bw/day	Female/Male nos.	In-Life Reason	Histopathology
1	0	42 / 2	Not pregnant	Implantation site and necrosis of placenta of the uterus (observed as nodule)
		46 / 6	Not pregnant	
2	100	51 / 11	Not pregnant	
		53 / 13	Not pregnant	
3	300	63 / 23	Not pregnant	
4	1000	72 / 32	Not pregnant	

There were 2/10 couples of the Control group (male no. 2 and female no. 42; male no. 6 and female no. 46), 2/10 couples of 100 mg/kg/day group (male no.11 and female no. 51; male no. 13 and female no. 53), 1/10 couples of the 300 mg/kg/day group (male no. 23 and female no. 63) and 1/10 couples) of the 1000 mg/kg/day group (male no. 32 and female no. 72) with no offspring. Female no. 42 showed evidence of a former pregnancy, in the form of the presence of a necrotic placenta. For the other couples, no abnormalities were seen in the reproductive organs, which could account for their lack of offspring.

There were no morphological findings in the reproductive organs of either sex which could be attributed to the test item and stage aware evaluation of the testes did not show any indication for abnormal spermatogenesis. The testis revealed normal progression of the spermatogenic cycle and the expected cell associations and proportions in the various stages of spermatogenesis were present.

## 6. DISCUSSION

Test item-related findings were present in the 1000 mg/kg/day group, consisting of (multi) focal erosions of the glandular mucosa of the **stomach** of males. Based on the low degrees (up to slight) and absence of concomitant test item-related microscopic changes, these findings were regarded as non-adverse.

The possible test item related lower thymus weights observed in 300 and 1000 mg/kg/day group males, was in absence of correlating microscopy or clinical pathology considered as non-adverse.

## 7. CONCLUSIONS

Non- adverse test item-related morphologic alterations following the oral (gavage) administration of **DiHEP Aqueous Solution** for at least 28 days to Wistar (Han) rats at doses up to 1000 mg/kg/day, were present in the stomach of 1000 mg/kg/day group males.

Furthermore, possible test-item related lower thymus weights were present in 300 and 1000 mg/kg/day group males.

There were no morphologic alterations in males treated up to 300 mg/kg/day and in females up to 1000 mg/kg/day.

There were no morphological findings in the reproductive organs of either sex which could be attributed to the test item and stage aware evaluation of the testes did not show any indication for abnormal spermatogenesis.

## 8. REPORT AUTHENTICATION

I, the undersigned, was responsible for the histopathology evaluation and reporting of the pathology data. The histopathology data in this report were compiled by me, and they reflect accurately the primary data records. Histopathology tables were created in PathData® under number 31906 LAA.

Final histopathology tables generated 11 September 2019

Project 20172120 Pathology Report

Report and Histopathology Tables Submitted By:



Ankie Lambregts, DVM

Dutch CRP/TP Certified Toxicologic Pathologist

Study Pathologist



**APPENDIX 1**  
**HISTOPATHOLOGY TABLES**

---

TEST ITEM	: DiHEP Aqueous Solution	PATHOL. NO.:	31906 LAA
TEST SYSTEM	: RAT, Combined 28-Day, Oral	FINALIZED	: 11-SEP-19
SPONSOR	: Shell International B.V.	PathData@System	V6.2e2

---

TABLE OF CONTENTS

---

PAGE :

EXPLANATION OF CODES AND SYMBOLS		1
SUMMARY TABLES		
SUMMARY INCIDENCE OF GRADINGS BY ORGAN/GROUP/SEX NECROPSY STATUS: TERMINAL SACRIFICE GROUP (K0) Incidence table - Selected findings with grades		2
NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX STATUS AT NECROPSY: K0 Incidence table - All microscopic findings	3 -	14
INDIVIDUAL ANIMAL DATA		
TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)	15 -	30
ANIMAL HEADING DATA DOSE GROUP 01		31
TEXT OF GROSS AND MICROSCOPIC FINDINGS DOSE GROUP 01	32 -	49
ANIMAL HEADING DATA DOSE GROUP 02		50
TEXT OF GROSS AND MICROSCOPIC FINDINGS DOSE GROUP 02	51 -	62
ANIMAL HEADING DATA DOSE GROUP 03		63
TEXT OF GROSS AND MICROSCOPIC FINDINGS DOSE GROUP 03	64 -	75
ANIMAL HEADING DATA DOSE GROUP 04		76
TEXT OF GROSS AND MICROSCOPIC FINDINGS DOSE GROUP 04	77 -	94

---

TEST ITEM	: DiHEP Aqueous Solution	PATHOL. NO.:	31906 LAA
TEST SYSTEM	: RAT, Combined 28-Day, Oral	FINALIZED	: 11-SEP-19
SPONSOR	: Shell International B.V.	PathData@System	V6.2e2

---

---

EXPLANATION OF CODES AND SYMBOLS

---

CODES AND SYMBOLS USED AT ANIMAL LEVEL:

---

M = Male animal  
F = Female animal  
K0 = Terminal sacrifice group

CODES AND SYMBOLS USED AT ORGAN LEVEL:

---

G = Gross observation checked off histologically  
! = Gross observat.not checked off histologically  
\* = Comment in text of individual animal data  
' = Histologic examination not required  
+ = Organ examined, findings present  
- = Organ examined, no pathologic findings noted (AOFT only)  
( = Only one of paired organs examined/present

CODES AND SYMBOLS USED AT FINDING LEVEL:

---

GRADE 1 = Minimal / very few / very small  
GRADE 2 = Slight / few / small  
GRADE 3 = Moderate / moderate number / moderate size  
GRADE 4 = Marked / many / large  
P = Finding present, severity not scored  
( = Finding unilateral in paired organs

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

SUMMARY INCIDENCE OF GRADINGS BY ORGAN/GROUP/SEX Necropsy Status: TERMINAL SACRIFICE GROUP (K0) Incidence table - Selected findings with grades				
Sex	Males			
Dose Group No. Animals per Dose Group	01 10	02 10	03 10	04 10
STOMACH No.Examined	5	5	5	5
- Erosion glandular stomach GRADE 1	-	-	-	2
GRADE 2	-	-	-	1
TOTAL AFFECTED	-	-	-	3
MEAN GRADE/TISS.AFFECTED	-	-	-	1.3

Group 01, CONTROL, males: DiHEP Aqueous Solution (0 MG/KG)  
 Group 02, 100 MG/KG, males: DiHEP Aqueous Solution (100 MG/KG)  
 Group 03, 300 MG/KG, males: DiHEP Aqueous Solution (300 MG/KG)  
 Group 04, 1000 MG/KG, males: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	MALE			
DOSE GROUP:	01	02	03	04
NO.ANIMALS:	10	10	10	10
BRAIN	5	-	-	5
N.A.D. :	5	-	-	5
SPINAL CORD, CERVIC. :	5	-	-	5
N.A.D. :	5	-	-	5
SPINAL CORD, THORAC. :	5	-	-	5
N.A.D. :	5	-	-	5
SPINAL CORD, LUMBAR :	5	-	-	5
N.A.D. :	5	-	-	5
PITUITARY GLAND :	5	-	-	5
N.A.D. :	5	-	-	5
SCIATIC NERVE, RIGHT :	5	-	-	5
N.A.D. :	5	-	-	5
EYES	5	-	-	5
N.A.D. :	5	-	-	5
THYMUS	5	-	-	5
N.A.D. :	4	-	-	5
- Cyst(s)	1	-	-	-
Grade 1:	1	-	-	-
MANDIB.LYMPH NODE :	5	-	-	5
N.A.D. :	5	-	-	4
- Erythrocytes, sinus :	-	-	-	1
Grade 1:	-	-	-	1
Grade 2:	-	-	-	-
Grade 3:	-	-	-	-

Group 01, CONTROL, males: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, males: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, males: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, males: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	MALE			
DOSE GROUP:	01	02	03	04
NO.ANIMALS:	10	10	10	10
ADRENAL GLANDS :	5	5	5	5
N.A.D. :	4	2	3	3
.....				
- Vacuol. fasciculata :	1	3	2	2
Grade 1:	1	3	2	2
.....				
THYROID GLAND :	5	-	-	5
N.A.D. :	4	-	-	4
.....				
- Hypertrophy foll. c.:	1	-	-	1
Grade 1:	1	-	-	1
.....				
MAMMARY GLAND :	5	-	-	5
N.A.D. :	5	-	-	5
.....				
LUNG :	5	-	-	5
N.A.D. :	1	-	-	3
.....				
- Inflamm. alv. acute :	2	-	-	-
Grade 1:	2	-	-	-
- Alveolar macrophages:	-	-	-	1
Grade 1:	-	-	-	1
- Inflamm. peribronch.:	3	-	-	2
Grade 1:	2	-	-	2
Grade 2:	1	-	-	-
.....				
TRACHEA :	5	-	-	5
N.A.D. :	5	-	-	5
.....				
SKELETAL MUSCLE :	5	-	-	5
N.A.D. :	3	-	-	3
.....				
- Infiltrate inflamm. :	2	-	-	2
Grade 1:	2	-	-	2

Group 01, CONTROL, males: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, males: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, males: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, males: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

	SEX :	MALE			
	DOSE GROUP:	01	02	03	04
	NO.ANIMALS:	10	10	10	10
<hr/>					
HEART	:	5	-	-	5
N.A.D.	:	5	-	-	4
.....					
- Infiltrate inflamm.	:	-	-	-	1
Grade 1:		-	-	-	1
<hr/>					
SPLEEN	:	5	-	-	5
- Hematopoiesis,	:	5	-	-	4
Grade 1:		4	-	-	4
Grade 2:		1	-	-	-
- Pigmentation, hemos.:		3	-	-	5
Grade 1:		3	-	-	5
Grade 2:		-	-	-	-
<hr/>					
LIVER	:	5	-	-	5
N.A.D.	:	1	-	-	3
.....					
- Infiltrate inflamm.	:	4	-	-	2
Grade 1:		4	-	-	2
<hr/>					
URINARY BLADDER	:	5	-	-	5
N.A.D.	:	5	-	-	5
<hr/>					
KIDNEYS	:	5	-	-	5
N.A.D.	:	1	-	-	2
.....					
- Infiltrate inflamm.	:	1	-	-	2
Grade 1:		1	-	-	2
- Hyaline droplet acc.:		1	-	-	-
Grade 1:		1	-	-	-
- Basophilia, tubule :		2	-	-	3
Grade 1:		2	-	-	3
- Cyst :		1	-	-	-
Grade 1:		1	-	-	-

Group 01, CONTROL, males: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, males: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, males: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, males: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	MALE				
DOSE GROUP:	01	02	03	04	
NO.ANIMALS:	10	10	10	10	
STOMACH	:	5	5	5	5
N.A.D.	:	4	2	5	1
.....					
- Inflammation, gland.:	1	2	-	-	
Grade 1:	1	2	-	-	
- Erosion glandular :	-	-	-	3	
Grade 1:	-	-	-	2	
Grade 2:	-	-	-	1	
- Hemorrhage :	-	1	-	-	
Grade 1:	-	1	-	-	
- Cyst(s) :	1	-	-	-	
Grade 1:	1	-	-	-	
- Vacuolation, lim. r.:	1	-	-	1	
Grade 1:	1	-	-	1	
.....					
DUODENUM	:	5	-	-	5
N.A.D.	:	5	-	-	5
.....					
JEJUNUM	:	5	-	-	5
N.A.D.	:	5	-	-	5
.....					
ILEUM	:	5	-	-	5
N.A.D.	:	5	-	-	5
.....					
GALT	:	5	-	-	5
N.A.D.	:	4	-	-	5
.....					
- Increas. vacuolation:	1	-	-	-	
Grade 1:	1	-	-	-	
.....					
CECUM	:	5	-	-	5
N.A.D.	:	5	-	-	5

Group 01, CONTROL, males: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, males: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, males: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, males: DiHEP Aqueous Solution (1000 MG/KG)



TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.  
PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	MALE			
DOSE GROUP:	01	02	03	04
NO. ANIMALS:	10	10	10	10
COLON	5	-	-	5
N.A.D. :	5	-	-	5
RECTUM	5	-	-	5
N.A.D. :	5	-	-	5
MESENT. LYMPH NODE	5	-	-	5
N.A.D. :	5	-	-	5
SEMINAL VESICLES	6	2	1	5
N.A.D. :	6	2	1	5
COAGULATING GLANDS	6	2	1	5
N.A.D. :	6	2	1	5
PROSTATE GLAND	6	2	1	5
N.A.D. :	4	2	1	5
.....				
- Infiltrate inflamm. :	2	-	-	-
Grade 1:	1	-	-	-
Grade 2:	1	-	-	-
TESTES	6	2	1	5
N.A.D. :	6	2	1	4
.....				
- Atrophy, tubular :	-	-	-	1
Grade 1:	-	-	-	1
EPIDIDYMIDES	6	2	1	5
N.A.D. :	6	2	1	4
.....				
- Sperm granuloma :	-	-	-	1
Grade 3:	-	-	-	1

Group 01, CONTROL, males: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, males: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, males: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, males: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

	SEX :					MALE
	DOSE GROUP:	01	02	03	04	
	NO.ANIMALS:	10	10	10	10	
BONE, STERNUM	:	5	-	-	5	
N.A.D.	:	5	-	-	5	
BONE MARROW, STERNUM	:	5	-	-	5	
N.A.D.	:	5	-	-	5	
BONE, FEMUR	:	5	-	-	5	
N.A.D.	:	5	-	-	5	
BONE MARROW, FEMUR	:	5	-	-	5	
N.A.D.	:	5	-	-	5	
JOINT, KNEE, LEFT	:	5	-	-	5	
N.A.D.	:	5	-	-	5	

Group 01, CONTROL, males: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, males: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, males: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, males: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

	SEX :	FEMALE			
	DOSE GROUP:	01	02	03	04
	NO.ANIMALS:	10	10	10	10
BRAIN	:	5	-	-	5
	N.A.D. :	5	-	-	5
SPINAL CORD, CERVIC.	:	5	-	-	5
	N.A.D. :	5	-	-	5
SPINAL CORD, THORAC.	:	5	-	-	5
	N.A.D. :	5	-	-	5
SPINAL CORD, LUMBAR	:	5	-	-	5
	N.A.D. :	5	-	-	5
PITUITARY GLAND	:	5	-	-	5
	N.A.D. :	5	-	-	5
SCIATIC NERVE, RIGHT	:	5	-	-	5
	N.A.D. :	5	-	-	5
EYES	:	6	-	1	6
	N.A.D. :	5	-	1	4
.....					
- Retinal dysplasia	:	-	-	-	1
	Grade 1:	-	-	-	1
- Hemorrhage	:	1	-	-	1
	Grade 3:	-	-	-	1
	Grade 4:	1	-	-	-

Group 01, CONTROL, females: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, females: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, females: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, females: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	FEMALE			
DOSE GROUP:	01	02	03	04
NO.ANIMALS:	10	10	10	10
THYMUS	6	-	1	5
N.A.D.	3	-	-	2
.....				
- Congestion	1	-	1	-
- Lymphoid atrophy,	1	-	-	2
Grade 1:	1	-	-	2
- Hyperplasia, epith.	2	-	-	1
Grade 1:	2	-	-	1
.....				
MANDIB.LYMPH NODE	6	-	-	6
N.A.D.	5	-	-	4
.....				
- Plasmacytosis	-	-	-	1
Grade 2:	-	-	-	1
- Erythrocytes, sinus	1	-	-	1
Grade 1:	-	-	-	-
Grade 2:	-	-	-	1
Grade 3:	1	-	-	-
.....				
ADRENAL GLANDS	5	-	-	5
N.A.D.	5	-	-	5
.....				
THYROID GLAND	5	-	1	5
N.A.D.	3	-	1	4
.....				
- Thymus, ectopic	1	-	-	-
- Hypertrophy foll. c.:	1	-	-	1
Grade 1:	1	-	-	1
.....				
MAMMARY GLAND	5	-	-	5
N.A.D.	-	-	-	-
.....				
- Lobuloalveolar dev.	5	-	-	5

Group 01, CONTROL, females: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, females: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, females: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, females: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.  
PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	FEMALE				
DOSE GROUP:	01	02	03	04	
NO.ANIMALS:	10	10	10	10	
LUNG	:	5	-	-	5
N.A.D.	:	4	-	-	2
.....					
- Alveolar macrophages:	:	1	-	-	2
Grade 1:	:	1	-	-	2
- Inflamm. peribronch.:	:	1	-	-	1
Grade 1:	:	1	-	-	1
Grade 2:	:	-	-	-	-
.....					
TRACHEA	:	5	-	-	5
N.A.D.	:	5	-	-	5
.....					
SKELETAL MUSCLE	:	5	-	-	5
N.A.D.	:	5	-	-	4
.....					
- Infiltrate inflamm.:	:	-	-	-	1
Grade 1:	:	-	-	-	1
.....					
HEART	:	5	-	-	5
N.A.D.	:	5	-	-	5
.....					
SPLEEN	:	5	-	-	5
- Hematopoiesis,	:	5	-	-	4
Grade 1:	:	5	-	-	4
Grade 2:	:	-	-	-	-
- Pigmentation, hemos.:	:	5	-	-	5
Grade 1:	:	3	-	-	2
Grade 2:	:	2	-	-	3
.....					
LIVER	:	5	-	-	5
N.A.D.	:	4	-	-	4
.....					
- Infiltrate inflamm.:	:	1	-	-	1
Grade 1:	:	1	-	-	1

Group 01, CONTROL, females: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, females: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, females: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, females: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.  
PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	FEMALE			
DOSE GROUP:	01	02	03	04
NO.ANIMALS:	10	10	10	10
URINARY BLADDER :	5	-	-	5
N.A.D. :	5	-	-	5
KIDNEYS :	5	-	-	5
N.A.D. :	4	-	-	3
- Basophilia, tubule :	1	-	-	2
Grade 1:	1	-	-	2
- Hyaline casts :	-	-	-	1
Grade 1:	-	-	-	1
STOMACH :	5	-	-	5
N.A.D. :	4	-	-	3
- Inflammation, gland.:	-	-	-	1
Grade 1:	-	-	-	1
- Glandular dilation, :	1	-	-	1
Grade 1:	-	-	-	1
Grade 2:	1	-	-	-
DUODENUM :	5	-	-	5
N.A.D. :	5	-	-	5
JEJUNUM :	5	-	1	5
N.A.D. :	5	-	-	5
- Congestion, :	-	-	1	-
Grade 1:	-	-	1	-
ILEUM :	5	-	-	5
N.A.D. :	5	-	-	5

Group 01, CONTROL, females: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, females: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, females: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, females: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.  
PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

	SEX :	FEMALE			
	DOSE GROUP:	01	02	03	04
	NO. ANIMALS:	10	10	10	10
GALT	:	5	-	-	5
	N.A.D. :	5	-	-	5
CECUM	:	5	-	-	5
	N.A.D. :	5	-	-	5
COLON	:	5	-	-	5
	N.A.D. :	5	-	-	5
RECTUM	:	5	-	-	5
	N.A.D. :	5	-	-	5
MESENT. LYMPH NODE	:	5	-	-	5
	N.A.D. :	5	-	-	5
OVARIES	:	7	2	1	6
	N.A.D. :	7	2	1	6
UTERUS	:	7	2	1	6
	N.A.D. :	2	2	-	-
.....					
- Cyclic dilation	:	-	-	1	1
- Implantation site(s)	:	5	-	-	5
- Placenta necrosis	:	1	-	-	-
Grade 3:	:	1	-	-	-
CERVIX	:	7	2	1	6
	N.A.D. :	7	2	1	6

Group 01, CONTROL, females: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, females: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, females: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, females: DiHEP Aqueous Solution (1000 MG/KG)

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX  
STATUS AT NECROPSY: K0  
Incidence table - All microscopic findings

SEX :	FEMALE			
DOSE GROUP:	01	02	03	04
NO.ANIMALS:	10	10	10	10
VAGINA	7	2	1	6
N.A.D. :	5	-	-	5
.....				
- Cycle: Proestrus :	-	-	1	1
- Cycle: Metestrus :	1	1	-	-
- Cycle: Diestrus :	-	1	-	-
- Incr. mucification :	1	-	-	-
Grade 3:	1	-	-	-
BONE, STERNUM	5	-	-	5
N.A.D. :	5	-	-	5
BONE MARROW, STERNUM	5	-	-	5
N.A.D. :	5	-	-	5
BONE, FEMUR	5	-	-	5
N.A.D. :	5	-	-	5
BONE MARROW, FEMUR	5	-	-	5
N.A.D. :	5	-	-	5
JOINT, KNEE, LEFT	5	-	-	5
N.A.D. :	5	-	-	5
BODY CAVITIES	-	-	1	-
- Fat necrosis,	-	-	1	-
Grade 1:	-	-	1	-
CLITORAL GLANDS	-	1	-	-
- Dilation,	-	1	-	-
Grade 2:	-	1	-	-

Group 01, CONTROL, females: DiHEP Aqueous Solution (0 MG/KG)  
Group 02, 100 MG/KG, females: DiHEP Aqueous Solution (100 MG/KG)  
Group 03, 300 MG/KG, females: DiHEP Aqueous Solution (300 MG/KG)  
Group 04, 1000 MG/KG, females: DiHEP Aqueous Solution (1000 MG/KG)



TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 01, CONTROL

ANIMAL NUMBER :

	1	2	3	4	5	6	7	8	9	10
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
BRAIN	-	-	-	-	-	†	†	†	†	†
.....										
SPINAL CORD, CERVIC.	-	-	-	-	-	†	†	†	†	†
.....										
SPINAL CORD, THORAC.	-	-	-	-	-	†	†	†	†	†
.....										
SPINAL CORD, LUMBAR	-	-	-	-	-	†	†	†	†	†
.....										
PITUITARY GLAND	-	-	-	-	-	†	†	†	†	†
.....										
SCIATIC NERVE, RIGHT	-	-	-	-	-	†	†	†	†	†
.....										
EYES	-	-	-	-	-	†	†	†	†	†
.....										
THYMUS	+	-	-	-	-	†	†	†	†	†
- Cyst(s)	1.	.	.	.	.					
.....										
MANDIB. LYMPH NODE	-	-	-	-	-	†	†	†	†	†
.....										
ADRENAL GLANDS	-	-	-	+	-	†	†	†	†	†
- Vacuol. fasciculata	.	.	.	1.	.					
.....										
THYROID GLAND	-	-	-	-	+	†	†	†	†	†
- Hypertrophy foll. c.	.	.	.	.	1.					
.....										
MAMMARY GLAND	-	-	-	-	-	†	†	†	†	†
.....										
LUNG	+	-	+	+	+	†	†	†	†	†
- Inflamm. alv. acute	1.	.	.	1.	.					
- Inflamm. peribronch.	.	.	1.	1.	2.					
.....										
TRACHEA	-	-	-	-	-	†	†	†	†	†
.....										
SKELETAL MUSCLE	-	-	-	+	+	†	†	†	†	†
- Infiltrate inflamm.	.	.	.	1.	1.					
.....										
HEART	-	-	-	-	-	†	†	†	†	†
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 01, CONTROL

ANIMAL NUMBER :

	1	2	3	4	5	6	7	8	9	10
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
SPLEEN	+	+	+	+	+	'	'	'	'	'
- Hematopoiesis,	1.	1.	2.	1.	1.					
- Pigmentation, hemos.	1.	.	1.	.	1.					
.....										
LIVER	+	+	+	+	-	'	'	'	'	'
- Infiltrate inflamm.	1.	1.	1.	1.	.					
.....										
URINARY BLADDER	-	-	-	-	-	'	'	'	'	'
.....										
KIDNEYS	+	+	-	+	+	'	'	'	'	'
- Infiltrate inflamm.	.	.	.	( 1.	.					
- Hyaline droplet acc.	1.	.	.	.	.					
- Basophilia, tubule	.	( 1.	.	.	( 1.					
- Cyst	.	( 1.	.	.	.					
.....										
STOMACH	-	-	-	-	+	'	'	'	'	'
- Inflammation, gland.	.	.	.	.	1.					
- Cyst(s)	.	.	.	.	1.					
- Vacuolation, lim. r.	.	.	.	.	1.					
.....										
DUODENUM	-	-	-	-	-	'	'	'	'	'
.....										
JEJUNUM	-	-	-	-	-	'	'	'	'	'
.....										
ILEUM	-	-	-	-	-	'	'	'	'	'
.....										
GALT	-	-	-	+	-	'	'	'	'	'
- Inceas. vacuolation	.	.	.	1.	.					
.....										
CECUM	-	-	-	-	-	'	'	'	'	'
.....										
COLON	-	-	-	-	-	'	'	'	'	'
.....										
RECTUM	-	-	-	-	-	'	'	'	'	'
.....										
MESENT. LYMPH NODE	-	-	-	-	-	'	'	'	'	'
.....										
SEMINAL VESICLES	-	-	-	-	-	'	'	'	'	'
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 01, CONTROL

ANIMAL NUMBER :

	1	2	3	4	5	6	7	8	9	10
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
COAGULATING GLANDS	-	-	-	-	-	-	'	'	'	'
PROSTATE GLAND	-	-	-	-	+	+	'	'	'	'
- Infiltrate inflamm.	.	.	.	.	2.	1.				
TESTES	-	-	-	-	-	-	'	'	'	'
EPIDIDYMIDES	-	-	-	-	-	-	'	'	'	'
BONE, STERNUM	-	-	-	-	-	'	'	'	'	'
BONE MARROW, STERNUM	-	-	-	-	-	'	'	'	'	'
BONE, FEMUR	-	-	-	-	-	'	'	'	'	'
BONE MARROW, FEMUR	-	-	-	-	-	'	'	'	'	'
JOINT, KNEE, LEFT	-	-	-	-	-	'	'	'	'	'

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 01, CONTROL

ANIMAL NUMBER :

	41	42	43	44	45	46	47	48	49	50
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
BRAIN	-	'	'	-	-	'	-	'	'	-
.....										
SPINAL CORD, CERVIC.	-	'	'	-	-	'	-	'	'	-
.....										
SPINAL CORD, THORAC.	-	'	'	-	-	'	-	'	'	-
.....										
SPINAL CORD, LUMBAR	-	'	'	-	-	'	-	'	'	-
.....										
PITUITARY GLAND	-	'	'	-	-	'	-	'	'	-
.....										
SCIATIC NERVE, RIGHT	-	'	'	-	-	'	-	'	'	-
.....										
EYES	-	( +G	'	-	-	'	-	'	'	-
- Hemorrhage	.	( 4.	.	.	.	.	.	.	.	.
.....										
THYMUS	+	+G	'	-	-	'	+	'	'	-
- Congestion	.	P.	.	.	.	.	.	.	.	.
- Lymphoid atrophy,	1.	.	.	.	.	.	.	.	.	.
- Hyperplasia, epith.	1.	.	.	.	.	1.	.	.	.	.
.....										
MANDIB. LYMPH NODE	-	+G	'	-	-	'	-	'	'	-
- Erythrocytes, sinus	.	3.	.	.	.	.	.	.	.	.
.....										
ADRENAL GLANDS	-	'	'	-	-	'	-	'	'	-
.....										
THYROID GLAND	-	'	'	-	+	'	+	'	'	-
- Thymus, ectopic	.	.	.	.	( P.	.	.	.	.	.
- Hypertrophy foll. c.	.	.	.	.	.	1.	.	.	.	.
.....										
MAMMARY GLAND	+	'	'	+	+	'	+	'	'	+
- Lobuloalveolar dev.	P.	.	.	P.	P.	.	P.	.	.	P.
.....										
LUNG	+	'	'	-	-	'	-	'	'	-
- Alveolar macrophages	1.	.	.	.	.	.	.	.	.	.
- Inflamm. peribronch.	1.	.	.	.	.	.	.	.	.	.
.....										
TRACHEA	-	'	'	-	-	'	-	'	'	-
.....										
SKELETAL MUSCLE	-	'	'	-	-	'	-	'	'	-
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 01, CONTROL

ANIMAL NUMBER :

	41	42	43	44	45	46	47	48	49	50
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
HEART	-	'	'	-	-	'	-	'	'	-
.....										
SPLEEN	+	'	'	+	+	'	+	'	'	+
- Hematopoiesis,	1.			1.	1.		1.			1.
- Pigmentation, hemos.	1.			1.	2.		2.			1.
.....										
LIVER	-	'	'	-	-	'	+	'	'	-
- Infiltrate inflamm.	.			.	.		1.			.
.....										
URINARY BLADDER	-	'	'	-	-	'	-	'	'	-
.....										
KIDNEYS	-	'	'	-	-	'	-	'	'	+
- Basophilia, tubule	.			.	.		.			( 1.
.....										
STOMACH	-	'	'	-	+	'	-	'	'	-
- Glandular dilation,	.			.	2.		.			.
.....										
DUODENUM	-	'	'	-	-	'	-	'	'	-
.....										
JEJUNUM	-	'	'	-	-	'	-	'	'	-
.....										
ILEUM	-	'	'	-	-	'	-	'	'	-
.....										
GALT	-	'	'	-	-	'	-	'	'	-
.....										
CECUM	-	'	'	-	-	'	-	'	'	-
.....										
COLON	-	'	'	-	-	'	-	'	'	-
.....										
RECTUM	-	'	'	-	-	'	-	'	'	-
.....										
MESENT. LYMPH NODE	-	'	'	-	-	'	-	'	'	-
.....										
OVARIES	-	-	'	-	-	-	-	'	'	-
.....										
UTERUS	+	+G	'	-	+	-*	+	'	'	+
- Implantation site(s)	P.	P.		.	P.	.	P.			P.
- Placenta necrosis	.	3.		.	.	.	.			.
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 01, CONTROL

ANIMAL NUMBER :

	41	42	43	44	45	46	47	48	49	50
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
CERVIX	-	-	'	-	-	-	-	'	'	-
VAGINA	-	+	'	-	-	+	-	'	'	-
- Cycle: Metestrus	.	.	.	.	.	P.	.	.	.	.
- Incr. mucification	.	3.	.	.	.	.	.	.	.	.
BONE, STERNUM	-	'	'	-	-	'	-	'	'	-
BONE MARROW, STERNUM	-	'	'	-	-	'	-	'	'	-
BONE, FEMUR	-	'	'	-	-	'	-	'	'	-
BONE MARROW, FEMUR	-	'	'	-	-	'	-	'	'	-
JOINT, KNEE, LEFT	-	'	'	-	-	'	-	'	'	-

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 02, 100 MG/KG

ANIMAL NUMBER :

	11	12	13	14	15	16	17	18	19	20
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
ADRENAL GLANDS	+	-	-	+	+	'	'	'	'	'
- Vacuol. fasciculata	1.	.	.	1.	1.					
.....										
STOMACH	+	+	-	+	-	'	'	'	'	'
- Inflammation, gland.	.	1.	.	1.	.					
- Hemorrhage	1.	.	.	.	.					
.....										
SEMINAL VESICLES	-	'	-	'	'	'	'	'	'	'
.....										
COAGULATING GLANDS	-	'	-	'	'	'	'	'	'	'
.....										
PROSTATE GLAND	-	'	-	'	'	'	'	'	'	'
.....										
TESTES	-	'	-	'	'	'	'	'	'	'
.....										
EPIDIDYMIDES	-	'	-	'	'	'	'	'	'	'
.....										
BONE	'	'	'	'	'	'	'	'	'	'
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 02, 100 MG/KG

ANIMAL NUMBER :

	51	52	53	54	55	56	57	58	59	60
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
OVARIES	-	'	-	'	'	'	'	'	'	'
UTERUS	-*	'	-*	'	'	'	'	'	'	'
CERVIX	-	'	-	'	'	'	'	'	'	'
VAGINA	+	'	+	'	'	'	'	'	'	'
- Cycle: Metestrus	P.		.							
- Cycle: Diestrus	.		P.							
BODY CAVITIES	'	'!	'	'	'	'	'!	'	'	'
CLITORAL GLANDS	'	'	'	'	'	'	+	'	'	'
- Dilation,							( 2.			



TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 03, 300 MG/KG

ANIMAL NUMBER :

	21	22	23	24	25	26	27	28	29	30
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
ADRENAL GLANDS	-	+	+	-	-	'	'	'	'	'
- Vacuol. fasciculata	.	1.	1.	.	.					
.....										
STOMACH	-	-	-	-	-	'	'	'	'	'
.....										
SEMINAL VESICLES	'	'	-	'	'	'	'	'	'	'
.....										
COAGULATING GLANDS	'	'	-	'	'	'	'	'	'	'
.....										
PROSTATE GLAND	'	'	-	'	'	'	'	'	'	'
.....										
TESTES	'	'	-	'	'	'	'	'	'	'
.....										
EPIDIDYMIDES	'	'	-	'	'	'	'	'	'	'
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 03, 300 MG/KG

ANIMAL NUMBER :

	61	62	63	64	65	66	67	68	69	70
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
EYES	'	'	'	'	'	( -G	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
THYMUS	'	'	'	'	'	'	+G	'	'	'
- Congestion	'	'	'	'	'	'	P.	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
THYROID GLAND	'	'	'	'	'	-G	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
JEJUNUM	'	'	'	+G	'	'	'	'	'	'
- Congestion,	'	'	'	1.	'	'	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
OVARIES	'	'	-	'	'	'	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
UTERUS	'	'	+G	'	'	'	'	'	'	'
- Cyclic dilation	'	'	P.	'	'	'	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
CERVIX	'	'	-	'	'	'	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
VAGINA	'	'	+	'	'	'	'	'	'	'
- Cycle: Proestrus	'	'	P.	'	'	'	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'
BODY CAVITIES	'	'	'	'	+G	'	'	'	'	!
- Fat necrosis,	'	'	'	'	1.	'	'	'	'	'
.....	'	'	'	'	'	'	'	'	'	'

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 04, 1000 MG/KG

ANIMAL NUMBER :

	31	32	33	34	35	36	37	38	39	40
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
BRAIN	-	-	-	-	-	+	+	+	+	+
.....										
SPINAL CORD, CERVIC.	-	-	-	-	-	+	+	+	+	+
.....										
SPINAL CORD, THORAC.	-	-	-	-	-	+	+	+	+	+
.....										
SPINAL CORD, LUMBAR	-	-	-	-	-	+	+	+	+	+
.....										
PITUITARY GLAND	-	-	-	-	-	+	+	+	+	+
.....										
SCIATIC NERVE, RIGHT	-	-	-	-	-	+	+	+	+	+
.....										
EYES	-	-	-	-	-	+	+	+	+	+
.....										
THYMUS	-	-	-	-	-	+	+	+	+	+
.....										
MANDIB. LYMPH NODE	-	-	-	+	-	+	+	+	+	+
- Erythrocytes, sinus	.	.	.	1.	.	.	.	.	.	.
.....										
ADRENAL GLANDS	-	+	-	-	+	+	+	+	+	+
- Vacuol. fasciculata	.	1.	.	.	1.	.	.	.	.	.
.....										
THYROID GLAND	-	-	-	-	+	+	+	+	+	+
- Hypertrophy foll. c.	.	.	.	.	1.	.	.	.	.	.
.....										
MAMMARY GLAND	-	-	-	-	-	+	+	+	+	+
.....										
LUNG	+	-	+	-	-	+	+	+	+	+
- Alveolar macrophages	.	.	1.	.	.	.	.	.	.	.
- Inflamm. peribronch.	1.	.	1.	.	.	.	.	.	.	.
.....										
TRACHEA	-	-	-	-	-	+	+	+	+	+
.....										
SKELETAL MUSCLE	+	-	+	-	-	+	+	+	+	+
- Infiltrate inflamm.	1.	.	1.	.	.	.	.	.	.	.
.....										
HEART	-	-	+	-	-	+	+	+	+	+
- Infiltrate inflamm.	.	.	1.	.	.	.	.	.	.	.
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 04, 1000 MG/KG

ANIMAL NUMBER :

	31	32	33	34	35	36	37	38	39	40
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
SPLEEN	+	+	+	+	+	'	'	'	'	'
- Hematopoiesis,	1.	1.	1.	1.	.					
- Pigmentation, hemos.	1.	1.	1.	1.	1.					
.....										
LIVER	+G	-	+	-	-	'	'	'	'	'
- Infiltrate inflamm.	1.	.	1.	.	.					
.....										
URINARY BLADDER	-	-	-	-	-	'	'	'	'	'
.....										
KIDNEYS	+	+G	-	-	+	'	'	'	'	'
- Infiltrate inflamm.	.	( 1.	.	.	( 1.					
- Basophilia, tubule	1.	1.	.	.	( 1.					
.....										
STOMACH	-	+	+	+	+	'	'	'	'	'
- Erosion glandular	.	2.	1.	1.	.					
- Vacuolation, lim. r.	.	.	.	.	1.					
.....										
DUODENUM	-	-	-	-	-	'	'	'	'	'
.....										
JEJUNUM	-	-	-	-	-	'	'	'	'	'
.....										
ILEUM	-	-	-	-	-	'	'	'	'	'
.....										
GALT	-	-	-	-	-	'	'	'	'	'
.....										
CECUM	-	-	-	-	-	'	'	'	'	'
.....										
COLON	-	-	-	-	-	'	'	'	'	'
.....										
RECTUM	-	-	-	-	-	'	'	'	'	'
.....										
MESENT. LYMPH NODE	-	-	-	-	-	'	'	'	'	'
.....										
SEMINAL VESICLES	-	-	-	-	-	'	'	'	'	'
.....										
COAGULATING GLANDS	-	-	-	-	-	'	'	'	'	'
.....										
PROSTATE GLAND	-	-	-	-	-	'	'	'	'	'
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 04, 1000 MG/KG

ANIMAL NUMBER :

	31	32	33	34	35	36	37	38	39	40
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
TESTES	-	+	-	-	-	'	'	'	'	'
- Atrophy, tubular	.	( 1.	.	.	.					
.....										
EPIDIDYMIDES	-	-	-	-	+G	'	'	'	'	'
- Sperm granuloma	.	.	.	.	( 3.					
.....										
BONE, STERNUM	-	-	-	-	-	'	'	'	'	'
.....										
BONE MARROW, STERNUM	-	-	-	-	-	'	'	'	'	'
.....										
BONE, FEMUR	-	-	-	-	-	'	'	'	'	'
.....										
BONE MARROW, FEMUR	-	-	-	-	-	'	'	'	'	'
.....										
JOINT, KNEE, LEFT	-	-	-	-	-	'	'	'	'	'
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 04, 1000 MG/KG

ANIMAL NUMBER :

	71	72	73	74	75	76	77	78	79	80
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
BRAIN	-	'	-	-	'	'	'	'	-	-
.....										
SPINAL CORD, CERVIC.	-	'	-	-	'	'	'	'	-	-
.....										
SPINAL CORD, THORAC.	-	'	-	-	'	'	'	'	-	-
.....										
SPINAL CORD, LUMBAR	-	'	-	-	'	'	'	'	-	-
.....										
PITUITARY GLAND	-	'	-	-	'	'	'	'	-	-
.....										
SCIATIC NERVE, RIGHT	-	'	-	-	'	'	'	'	-	-
.....										
EYES	-	( +G	-	+	'	'	'	'	-	-
- Retinal dysplasia	.	.	.	( 1.					.	.
- Hemorrhage	.	( 3.	.	.					.	.
.....										
THYMUS	+	'	-	+	'	'	'	'	-	+
- Lymphoid atrophy,	1.		.	1.					.	.
- Hyperplasia, epith.	.		.	.					.	1.
.....										
MANDIB. LYMPH NODE	-	+G	-	-	'	'	'	'	+	-
- Plasmacytosis	.	.	.	.					2.	.
- Erythrocytes, sinus	.	2.	.	.					.	.
.....										
ADRENAL GLANDS	-	'	-	-	'	'	'	'	-	-
.....										
THYROID GLAND	-	'	-	-G	'	'	'	'	+	-
- Hypertrophy foll. c.	.		.	.					1.	.
.....										
MAMMARY GLAND	+	'	+	+	'	'	'	'	+	+
- Lobuloalveolar dev.	P.		P.	P.					P.	P.
.....										
LUNG	-	'	-	+	'	'	'	'	+	+
- Alveolar macrophages	.		.	1.					.	1.
- Inflamm. peribronch.	.		.	.					1.	.
.....										
TRACHEA	-	'	-	-	'	'	'	'	-	-
.....										

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 04, 1000 MG/KG

ANIMAL NUMBER :

	71	72	73	74	75	76	77	78	79	80
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
SKELETAL MUSCLE	-	'	-	-	'	'	'	'	+	-
- Infiltrate inflamm.	.		.	.					1.	.
HEART	-	'	-	-	'	'	'	'	-	-
SPLEEN	+	'	+	+	'	'	'	'	+	+
- Hematopoiesis,	1.		1.	1.					1.	.
- Pigmentation, hemos.	2.		2.	1.					1.	2.
LIVER	-	'	-	-	'	'	'	'	-	+
- Infiltrate inflamm.	.		.	.					.	1.
URINARY BLADDER	-	'	-	-	'	'	'	'	-	-
KIDNEYS	-	'	-	-	'	'	'	'	+	+
- Basophilia, tubule	.		.	.					( 1.	( 1.
- Hyaline casts	.		.	.					.	( 1.
STOMACH	-	'	+	-	'	'	'	'	-	+
- Inflammation, gland.	.		1.	.					.	.
- Glandular dilation,	.		.	.					.	1.
DUODENUM	-	'	-	-	'	'	'	'	-	-
JEJUNUM	-	'	-	-	'	'	'	'	-	-
ILEUM	-	'	-	-	'	'	'	'	-	-
GALT	-	'	-	-	'	'	'	'	-	-
CECUM	-	'	-	-	'	'	'	'	-	-
COLON	-	'	-	-	'	'	'	'	-	-
RECTUM	-	'	-	-	'	'	'	'	-	-
MESENT. LYMPH NODE	-	'	-	-	'	'	'	'	-	-
OVARIES	-	-	-	-	'	'	'	'	-	-

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)  
 DOSE GROUP : 04, 1000 MG/KG

ANIMAL NUMBER :

	71	72	73	74	75	76	77	78	79	80
	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0	FK0
UTERUS	+	+G	+	+	'	'	'	'	+	+
- Cyclic dilation	.	P.	.	.					.	.
- Implantation site(s)	P.	.	P.	P.					P.	P.
.....										
CERVIX	-	-	-	-	'	'	'	'	-	-
.....										
VAGINA	-	+	-	-	'	'	'	'	-	-
- Cycle: Proestrus	.	P.	.	.					.	.
.....										
BONE, STERNUM	-	'	-	-	'	'	'	'	-	-
.....										
BONE MARROW, STERNUM	-	'	-	-	'	'	'	'	-	-
.....										
BONE, FEMUR	-	'	-	-	'	'	'	'	-	-
.....										
BONE MARROW, FEMUR	-	'	-	-	'	'	'	'	-	-
.....										
JOINT, KNEE, LEFT	-	'	-	-	'	'	'	'	-	-
.....										



TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

ANIMAL HEADING DATA  
 DOSE GROUP : 01, CONTROL

ANIMAL NUMBER	SEX M/F	DEFINED STATE	AND FINAL NECROPSY	TEST DAYS	FIRST DAY	AND LAST DAY UNDER TEST	DATE OF NECROPSY
1	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
2	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
3	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
4	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
5	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
6	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
7	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
8	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
9	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
10	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
41	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
42	F	K0	K0	42	05-FEB-19	18-MAR-19	19-MAR-19
43	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
44	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
45	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
46	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
47	F	K0	K0	51	05-FEB-19	27-MAR-19	28-MAR-19
48	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
49	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
50	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 1

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

THYMUS:

-Cyst(s), grade 1

LUNG:

-Inflammation bronchioalveolar, acute, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

LIVER:

-Infiltrate inflammatory cell, mononuclear, grade 1

KIDNEYS:

-Hyaline droplet accumulation, bilateral, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, TRACHEA, SKELETAL MUSCLE, HEART, URINARY BLADDER, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYIMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 2

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

LIVER:

-Infiltrate inflammatory cell, mononuclear, grade 1

KIDNEYS:

-Basophilia, tubule, unilateral, grade 1

-Cyst, single, unilateral, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, LUNG, TRACHEA, SKELETAL MUSCLE, HEART, URINARY BLADDER, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 3

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

LUNG:

-Inflammation peribronchial/perivascular, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 2

-Pigmentation, hemosiderin, grade 1

LIVER:

-Infiltrate inflammatory cell, mononuclear, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, TRACHEA, SKELETAL MUSCLE, HEART, URINARY BLADDER, KIDNEYS, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 4

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

-Vacuolation zona fasciculata, bilateral, grade 1

LUNG:

-Inflammation bronchioalveolar, acute, grade 1

-Inflammation peribronchial/perivascular, grade 1

SKELETAL MUSCLE:

-Infiltrate inflammatory cell, mononuclear, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

LIVER:

-Infiltrate inflammatory cell, mononuclear, grade 1

KIDNEYS:

-Infiltrate inflammatory cell, lymphocytic, unilateral, grade 1

GUT-ASSOCIATED LYMPHOID TISSUE:

-Increased vacuolation, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

CONT./FF. ANIMAL NO. : 4

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, TRACHEA, HEART, URINARY BLADDER, STOMACH, DUODENUM, JEJUNUM, ILEUM, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 5

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

THYROID GLAND (BOTH LOBES):

-Hypertrophy follicular cell, bilateral, grade 1

LUNG:

-Inflammation peribronchial/perivascular, grade 2

SKELETAL MUSCLE:

-Infiltrate inflammatory cell, mononuclear, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

KIDNEYS:

-Basophilia, tubule, unilateral, grade 1

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS

DOSE GROUP : 01, CONTROL MALE

CONT./FF. ANIMAL NO. : 5

STOMACH:

- Inflammation, glandular stomach, lymphogranulocytic, grade 1
- Cyst(s), squamous cell, grade 1
- Vacuolation, limiting ridge, grade 1

PROSTATE GLAND:

- Infiltrate inflammatory cell, mononuclear, grade 2

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, MAMMARY GLAND, TRACHEA, HEART, LIVER, URINARY BLADDER, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), TESTES, EPIDIDYMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 6

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 38/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

CONT./FF. ANIMAL NO. : 6

\* MICROSCOPIC FINDINGS

PROSTATE GLAND:

-Infiltrate inflammatory cell, mononuclear, grade 1  
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), TESTES,  
EPIDIDYMIDES.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 7

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.



PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 39/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 8

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 9

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 40/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 10

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 41

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

THYMUS:

- Lymphoid atrophy, grade 1
- Hyperplasia, epithelial tubules and cords, grade 1

MAMMARY GLAND:

- Lobuloalveolar development

LUNG:

- Alveolar macrophage aggregation, grade 1
- Inflammation peribronchial/perivascular, grade 1

SPLEEN:

- Hematopoiesis, extramedullary, grade 1
- Pigmentation, hemosiderin, grade 1

UTERUS:

- Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, KIDNEYS, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL FEMALE

\* STATE AT NECROPSY: K0  
DAYS ON TEST : 42 \* ANIMAL NO. : 42

\* NECROPSY FINDINGS

EYES:

01: LEFT SIDE: EXOPHTHALMUS.

THYMUS:

01: RIGHT SIDE: FOCUS/FOCI, ISOLATED, REDDISH.

MANDIBULAR LYMPH NODE:

01: BOTH SIDES: FOCUS/FOCI, ISOLATED, DARK RED.

UTERUS:

01: RIGHT HORN: CONTENTS: GREENISH, GELATINOUS.

02: RIGHT HORN: NODULE(S), D=8X7 MM, TAN, REDDISH, HARD.

NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

EYES:

Only one of paired organs examined/present

-Hemorrhage, peribulbar and intraocular, unilateral, grade 4

This finding corresponds to necropsy observation no: 01.

THYMUS:

-Congestion

This finding corresponds to necropsy observation no: 01.

MANDIBULAR LYMPH NODE:

One of the mandibular lymph nodes is not present/recognizable on the slide. Instead there is large hemmorrhaghe, with an area containing plant material.

-Erythrocytes, intrasinusoidal, grade 3

This finding corresponds to necropsy observation no: 01.

UTERUS:

Draining lymphnode without abnormalities.

-Implantation site(s)

This finding corresponds to necropsy observation no: 02.

-Placenta necrosis, grade 3

This finding corresponds to necropsy observations nos: 01,02.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 43/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

FEMALE

CONT./FF. ANIMAL NO. : 42

VAGINA:

-Increased mucification, grade 3

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- OVARIES, CERVIX.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 43

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

---

TEXT OF GROSS AND MICROSCOPIC FINDINGS

DOSE GROUP : 01, CONTROL FEMALE

---

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 44

.....

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

MAMMARY GLAND:

-Lobuloalveolar development

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, KIDNEYS, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, UTERUS, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

---

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 45

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

THYROID GLAND (BOTH LOBES):

-Thymus, ectopic, unilateral

MAMMARY GLAND:

-Lobuloalveolar development

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 2

STOMACH:

-Glandular dilation, grade 2

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, KIDNEYS, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 46/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 46

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

UTERUS:

Autolytic endometrium due to Salewski staining.

VAGINA:

-Cycle: metestrus

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- OVARIES, UTERUS, CERVIX.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 51

\* ANIMAL NO. : 47

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

THYMUS:

-Hyperplasia, epithelial tubules and cords, grade 1

THYROID GLAND (BOTH LOBES):

-Hypertrophy follicular cell, bilateral, grade 1



PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 47/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

FEMALE

CONT./FF. ANIMAL NO. : 47

MAMMARY GLAND:

-Lobuloalveolar development

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 2

LIVER:

-Infiltrate inflammatory cell, mononuclear, grade 1

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, LUNG, TRACHEA, SKELETAL MUSCLE, HEART, URINARY BLADDER, KIDNEYS, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 48

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 48/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

FEMALE

CONT./FF. ANIMAL NO. : 48

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 49

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 50

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 01, CONTROL

FEMALE

CONT./FF. ANIMAL NO. : 50

\* MICROSCOPIC FINDINGS

MAMMARY GLAND:

-Lobuloalveolar development

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

KIDNEYS:

-Basophilia, tubule, unilateral, grade 1

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

TEST ITEM : DiHEP Aqueous Solution  
 TEST SYSTEM : RAT, Combined 28-Day, Oral  
 SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
 FINALIZED : 11-SEP-19  
 PathData@System V6.2e2

ANIMAL HEADING DATA  
 DOSE GROUP : 02, 100 MG/KG

ANIMAL NUMBER	SEX M/F	DEFINED STATE	AND FINAL NECROPSY	TEST DAYS	FIRST DAY	AND LAST DAY UNDER TEST	DATE OF NECROPSY
11	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
12	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
13	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
14	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
15	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
16	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
17	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
18	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
19	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
20	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
51	F	K0	K0	42	05-FEB-19	18-MAR-19	19-MAR-19
52	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
53	F	K0	K0	42	05-FEB-19	18-MAR-19	19-MAR-19
54	F	K0	K0	51	05-FEB-19	27-MAR-19	28-MAR-19
55	F	K0	K0	56	05-FEB-19	01-APR-19	02-APR-19
56	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
57	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
58	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
59	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
60	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 11

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

-Vacuolation zona fasciculata, bilateral, grade 1

STOMACH:

-Hemorrhage, focal, glandular mucosa pylorus, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE),  
PROSTATE GLAND, TESTES, EPIDIDYMIDES.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 12

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 52/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

MALE

CONT./FF. ANIMAL NO. : 12

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

Organ examined, no pathologic findings noted

STOMACH:

-Inflammation, glandular stomach, lymphogranulocytic, grade 1

\* ORGANS WITHOUT ABNORMALITIES

- ADRENAL GLANDS.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 13

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO MICROSCOPIC FINDINGS NOTED.

\* ORGANS WITHOUT ABNORMALITIES

- ADRENAL GLANDS, STOMACH, SEMINAL VESICLES, COAGULATING GLANDS  
(ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYMIDES.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 14

\* NECROPSY FINDINGS

BONE:

01: TAIL, TAIL APEX: TAIL BENT.

NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

-Vacuolation zona fasciculata, bilateral, grade 1

STOMACH:

-Inflammation, glandular stomach, lymphogranulocytic, grade 1

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 15

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

-Vacuolation zona fasciculata, bilateral, grade 1

STOMACH:

Organ examined, no pathologic findings noted

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 54/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

MALE

CONT./FF. ANIMAL NO. : 15

\* ORGANS WITHOUT ABNORMALITIES

- STOMACH.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 16

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 17

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.



PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 55/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

MALE

CONT./FF. ANIMAL NO. : 17

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 18

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 19

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 56/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

MALE

CONT./FF. ANIMAL NO. : 19

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 20

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 57/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 42

\* ANIMAL NO. : 51

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

UTERUS:

Autolytic endometrium due to Salewski staining.

VAGINA:

-Cycle: metestrus

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- OVARIES, UTERUS, CERVIX.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 52

\* NECROPSY FINDINGS

BODY CAVITIES:

01: THORACIC CAVITY: CONTAINS FLUID, WATERY-CLEAR.

NO OTHER NECROPSY OBSERVATIONS NOTED

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 58/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

FEMALE

CONT./FF. ANIMAL NO. : 52

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 42

\* ANIMAL NO. : 53

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

UTERUS:

Autolytic endometrium due to Salewski staining.

VAGINA:

-Cycle: diestrus

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- OVARIES, UTERUS, CERVIX.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 59/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 51

\* ANIMAL NO. : 54

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 56

\* ANIMAL NO. : 55

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 60/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 56

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 57

\* NECROPSY FINDINGS

BODY CAVITIES:

01: THORACIC CAVITY: CONTAINS FLUID, WATERY-CLEAR.

CLITORAL GLANDS:

01: LEFT SIDE: FOCUS/FOCI, D=6X2 MM, TAN.

NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

CLITORAL GLANDS:

-Dilation, unilateral, grade 2

This finding corresponds to necropsy observation no: 01.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 61/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 58

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 59

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 62/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 02, 100 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 60

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.



TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

ANIMAL HEADING DATA  
DOSE GROUP : 03, 300 MG/KG

ANIMAL NUMBER	SEX M/F	DEFINED STATE	AND FINAL NECROPSY	TEST DAYS	FIRST DAY	AND LAST DAY UNDER TEST	DATE OF NECROPSY
21	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
22	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
23	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
24	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
25	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
26	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
27	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
28	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
29	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
30	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
61	F	K0	K0	56	05-FEB-19	01-APR-19	02-APR-19
62	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
63	F	K0	K0	42	05-FEB-19	18-MAR-19	19-MAR-19
64	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
65	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
66	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
67	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
68	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
69	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
70	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 64/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 21

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO MICROSCOPIC FINDINGS NOTED.

\* ORGANS WITHOUT ABNORMALITIES

- ADRENAL GLANDS, STOMACH.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 22

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

-Vacuolation zona fasciculata, bilateral, grade 1

STOMACH:

Organ examined, no pathologic findings noted

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 65/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

MALE

CONT./FF. ANIMAL NO. : 22

\* ORGANS WITHOUT ABNORMALITIES

- STOMACH.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 23

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

-Vacuolation zona fasciculata, bilateral, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- STOMACH, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR  
PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYMIDES.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 66/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 24

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO MICROSCOPIC FINDINGS NOTED.

\* ORGANS WITHOUT ABNORMALITIES

- ADRENAL GLANDS, STOMACH.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 25

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO MICROSCOPIC FINDINGS NOTED.

\* ORGANS WITHOUT ABNORMALITIES

- ADRENAL GLANDS, STOMACH.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 67/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 26

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 27

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 68/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 28

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 29

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 69/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 30

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 70/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 56

\* ANIMAL NO. : 61

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 62

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.



TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 42

\* ANIMAL NO. : 63

\* NECROPSY FINDINGS

UTERUS:

01: CONTAINS FLUID.

NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

UTERUS:

Autolytic endometrium due to Salewski staining.

For diagnosis of necropsy observation no. 01 see under: VAGINA.

-Cyclic dilation

This finding corresponds to necropsy observation no: 01.

VAGINA:

-Cycle: proestrus

This finding corresponds to necropsy observation no.: 01  
in the UTERUS.

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- OVARIES, CERVIX.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 64

\* NECROPSY FINDINGS

JEJUNUM:

01: FOCUS/FOCI, D=2X1 MM, REDDISH.  
NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

JEJUNUM:

-Congestion, grade 1

This finding corresponds to necropsy observation no: 01.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 65

\* NECROPSY FINDINGS

BODY CAVITIES:

01: ABDOMINAL CAVITY, UTERINE ADIPOSE TISSUE, RIGHT SIDE:  
NODULE(S), D=5X2 MM, TAN, HARD.  
NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

BODY CAVITIES:

Draining lymph nodes without abnormality.

-Fat necrosis, grade 1

This finding corresponds to necropsy observation no: 01.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 73/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 66

\* NECROPSY FINDINGS

EYES:

01: RIGHT SIDE: EXOPHTHALMUS.

THYROID GLAND (BOTH LOBES):

01: BOTH SIDES: DISCOLOURATION, PALE.

NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

EYES:

Only one of paired organs examined/present

No microscopic finding corresponding to necropsy observation no. 01.

THYROID GLAND (BOTH LOBES):

No microscopic finding corresponding to necropsy observation no. 01.

NO MICROSCOPIC FINDINGS NOTED.

\* ORGANS WITHOUT ABNORMALITIES

- EYES, THYROID GLAND (BOTH LOBES).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 67

\* NECROPSY FINDINGS

THYMUS:

01: RIGHT SIDE: FOCUS/FOCI, MANY, REDDISH.

NO OTHER NECROPSY OBSERVATIONS NOTED

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 74/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

FEMALE

CONT./FF. ANIMAL NO. : 67

\* MICROSCOPIC FINDINGS

THYMUS:

-Congestion

This finding corresponds to necropsy observation no: 01.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 68

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 69

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 75/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 03, 300 MG/KG

FEMALE

CONT./FF. ANIMAL NO. : 69

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 70

\* NECROPSY FINDINGS

BODY CAVITIES:

01: THORACIC CAVITY: CONTAINS FLUID, WATERY-CLEAR.  
NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

ANIMAL HEADING DATA  
DOSE GROUP : 04, 1000 MG/KG

ANIMAL NUMBER	SEX M/F	DEFINED STATE	AND FINAL NECROPSY	TEST DAYS	FIRST DAY	AND LAST DAY UNDER TEST	DATE OF NECROPSY
31	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
32	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
33	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
34	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
35	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
36	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
37	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
38	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
39	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
40	M	K0	K0	29	05-FEB-19	05-MAR-19	06-MAR-19
71	F	K0	K0	51	05-FEB-19	27-MAR-19	28-MAR-19
72	F	K0	K0	42	05-FEB-19	18-MAR-19	19-MAR-19
73	F	K0	K0	51	05-FEB-19	27-MAR-19	28-MAR-19
74	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
75	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
76	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19
77	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
78	F	K0	K0	55	05-FEB-19	31-MAR-19	01-APR-19
79	F	K0	K0	51	05-FEB-19	27-MAR-19	28-MAR-19
80	F	K0	K0	52	05-FEB-19	28-MAR-19	29-MAR-19

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 31

\* NECROPSY FINDINGS

LIVER:

01: DISCOLOURATION, RED-BROWN.  
NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

LUNG:

-Inflammation peribronchial/perivascular, grade 1

SKELETAL MUSCLE:

-Infiltrate inflammatory cell, mononuclear, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

LIVER:

No microscopic finding corresponding to necropsy observation no. 01.

-Infiltrate inflammatory cell, mononuclear, grade 1

KIDNEYS:

-Basophilia, tubule, bilateral, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG MALE

CONT./FF. ANIMAL NO. : 31

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, TRACHEA, HEART, URINARY BLADDER, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 32

\* NECROPSY FINDINGS

KIDNEYS:

01: CRANIAL POLE, RIGHT SIDE MIDDLE PART IRREGULAR SURFACE.  
NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:

-Vacuolation zona fasciculata, bilateral, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

KIDNEYS:

No microscopic finding corresponding to necropsy observation no. 01.

-Infiltrate inflammatory cell, lymphocytic, unilateral, grade 1

-Basophilia, tubule, bilateral, grade 1



TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

MALE

CONT./FF. ANIMAL NO. : 32

STOMACH:

-Erosion glandular stomach, multifocal, pylorus, grade 2

TESTES:

-Atrophy, tubular, unilateral, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, EPIDIDYMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 33

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG MALE

CONT./FF. ANIMAL NO. : 33

\* MICROSCOPIC FINDINGS

LUNG:

- Alveolar macrophage aggregation, grade 1
- Inflammation peribronchial/perivascular, grade 1

SKELETAL MUSCLE:

- Infiltrate inflammatory cell, mononuclear, grade 1

HEART:

- Infiltrate inflammatory cell, mononuclear, grade 1

SPLEEN:

- Hematopoiesis, extramedullary, grade 1
- Pigmentation, hemosiderin, grade 1

LIVER:

- Infiltrate inflammatory cell, mononuclear, grade 1

STOMACH:

- Erosion glandular stomach, multifocal, pylorus, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, TRACHEA, URINARY BLADDER, KIDNEYS, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYIMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 34

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

MANDIBULAR LYMPH NODE:

-Erythrocytes, intrasinusoidal, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

STOMACH:

-Erosion glandular stomach, focal, pylorus, grade 1

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), MAMMARY GLAND, LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, KIDNEYS, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, EPIDIDYMIDES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG MALE

\* STATE AT NECROPSY: K0  
DAYS ON TEST : 29 \* ANIMAL NO. : 35  
.....

\* NECROPSY FINDINGS

EPIDIDYMIDES:  
01: LEFT SIDE, TAIL: NODULE(S), D=10X6 MM, YELLOWISH, SOFT.  
NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

ADRENAL GLANDS:  
-Vacuolation zona fasciculata, bilateral, grade 1  
THYROID GLAND (BOTH LOBES):  
-Hypertrophy follicular cell, bilateral, grade 1  
SPLEEN:  
-Pigmentation, hemosiderin, grade 1  
KIDNEYS:  
-Infiltrate inflammatory cell, mononuclear, unilateral, grade 1  
-Basophilia, tubule, unilateral, grade 1  
STOMACH:  
-Vacuolation, limiting ridge, grade 1  
EPIDIDYMIDES:  
-Sperm granuloma, unilateral, grade 3  
This finding corresponds to necropsy observation no: 01.  
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 83/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

MALE

CONT./FF. ANIMAL NO. : 35

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, MAMMARY GLAND, LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, SEMINAL VESICLES, COAGULATING GLANDS (ANTERIOR PROSTATE), PROSTATE GLAND, TESTES, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 36

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 84/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

MALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 37

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 29

\* ANIMAL NO. : 38

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 85/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

MALE

\* STATE AT NECROPSY: K0  
DAYS ON TEST : 29

\* ANIMAL NO. : 39

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0  
DAYS ON TEST : 29

\* ANIMAL NO. : 40

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 51

\* ANIMAL NO. : 71

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

THYMUS:

-Lymphoid atrophy, grade 1

MAMMARY GLAND:

-Lobuloalveolar development

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 2

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, KIDNEYS, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).



---

TEST ITEM	: DiHEP Aqueous Solution	PATHOL. NO.:	31906 LAA
TEST SYSTEM	: RAT, Combined 28-Day, Oral	FINALIZED	: 11-SEP-19
SPONSOR	: Shell International B.V.	PathData@System	V6.2e2

---

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG FEMALE

---

\* STATE AT NECROPSY: K0  
DAYS ON TEST : 42 \* ANIMAL NO. : 72  
.....

\* NECROPSY FINDINGS

EYES:  
01: LEFT SIDE: EXOPHTHALMUS.  
MANDIBULAR LYMPH NODE:  
01: BOTH SIDES: FOCUS/FOCI, ISOLATED, DARK RED.  
UTERUS:  
01: CONTAINS FLUID.  
NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

EYES:  
Only one of paired organs examined/present  
-Hemorrhage, peribulbar, unilateral, grade 3  
This finding corresponds to necropsy observation no: 01.  
MANDIBULAR LYMPH NODE:  
-Erythrocytes, intrasinusoidal, bilateral, grade 2  
This finding corresponds to necropsy observation no: 01.  
UTERUS:  
For diagnosis of necropsy observation no. 01 see under: VAGINA.  
-Cyclic dilation  
This finding corresponds to necropsy observation no: 01.  
VAGINA:  
-Cycle: proestrus  
This finding corresponds to necropsy observation no.: 01  
in the UTERUS.  
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- OVARIES, CERVIX.

---

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 51

\* ANIMAL NO. : 73

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

MAMMARY GLAND:

-Lobuloalveolar development

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 2

STOMACH:

-Inflammation, glandular stomach, lymphogranulocytic, grade 1

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), LUNG, TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, KIDNEYS, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 89/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 74

\* NECROPSY FINDINGS

THYROID GLAND (BOTH LOBES):

01: BOTH SIDES: DISCOLOURATION, PALE.

NO OTHER NECROPSY OBSERVATIONS NOTED

\* MICROSCOPIC FINDINGS

EYES:

-Retinal dysplasia (rosettes), unilateral, grade 1

THYMUS:

-Lymphoid atrophy, grade 1

THYROID GLAND (BOTH LOBES):

No microscopic finding corresponding to necropsy observation no. 01.

MAMMARY GLAND:

-Lobuloalveolar development

LUNG:

-Alveolar macrophage aggregation, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 90/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

CONT./FF. ANIMAL NO. : 74

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), TRACHEA, SKELETAL MUSCLE, HEART, LIVER, URINARY BLADDER, KIDNEYS, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 75

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT (FINAL)  
INDIVIDUAL ANIMAL DATA

PAGE : 91/ 94  
PROJECT : 20172120

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 76

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 77

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

\* STATE AT NECROPSY: K0

DAYS ON TEST : 55

\* ANIMAL NO. : 78

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

\* STATE AT NECROPSY: K0

DAYS ON TEST : 51

\* ANIMAL NO. : 79

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

MANDIBULAR LYMPH NODE:

-Plasmacytosis, grade 2

THYROID GLAND (BOTH LOBES):

-Hypertrophy follicular cell, bilateral, grade 1

MAMMARY GLAND:

-Lobuloalveolar development

LUNG:

-Inflammation peribronchial/perivascular, grade 1

SKELETAL MUSCLE:

-Infiltrate inflammatory cell, mononuclear, grade 1

SPLEEN:

-Hematopoiesis, extramedullary, grade 1

-Pigmentation, hemosiderin, grade 1

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

CONT./FF. ANIMAL NO. : 79

KIDNEYS:

-Basophilia, tubule, unilateral, grade 1

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, THYMUS, ADRENAL GLANDS, TRACHEA, HEART, LIVER, URINARY BLADDER, STOMACH, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).

\* STATE AT NECROPSY: K0

DAYS ON TEST : 52

\* ANIMAL NO. : 80

\* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

\* MICROSCOPIC FINDINGS

THYMUS:

-Hyperplasia, epithelial tubules and cords, grade 1

MAMMARY GLAND:

-Lobuloalveolar development

LUNG:

-Alveolar macrophage aggregation, grade 1

TEST ITEM : DiHEP Aqueous Solution  
TEST SYSTEM : RAT, Combined 28-Day, Oral  
SPONSOR : Shell International B.V.

PATHOL. NO.: 31906 LAA  
FINALIZED : 11-SEP-19  
PathData@System V6.2e2

TEXT OF GROSS AND MICROSCOPIC FINDINGS  
DOSE GROUP : 04, 1000 MG/KG

FEMALE

CONT./FF. ANIMAL NO. : 80

SPLEEN:

-Pigmentation, hemosiderin, grade 2

LIVER:

-Infiltrate inflammatory cell, lymphocytic, grade 1

KIDNEYS:

-Basophilia, tubule, unilateral, grade 1

-Hyalin casts, unilateral, grade 1

STOMACH:

-Glandular dilation, grade 1

UTERUS:

-Implantation site(s)

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

\* ORGANS WITHOUT ABNORMALITIES

- BRAIN, SPINAL CORD (CERVICAL SEGMENT), SPINAL CORD (THORACIC SEGMENT), SPINAL CORD (LUMBAR SEGMENT), PITUITARY GLAND, SCIATIC NERVE (RIGHT), EYES, MANDIBULAR LYMPH NODE, ADRENAL GLANDS, THYROID GLAND (BOTH LOBES), TRACHEA, SKELETAL MUSCLE, HEART, URINARY BLADDER, DUODENUM, JEJUNUM, ILEUM, GUT-ASSOCIATED LYMPHOID TISSUE, CECUM, COLON, RECTUM, MESENTERIC LYMPH NODE, OVARIES, CERVIX, VAGINA, BONE (STERNUM), BONE MARROW (STERNUM), BONE (FEMUR), BONE MARROW (FEMUR), JOINT (KNEE, LEFT).



**Appendix 5**  
**Summary of the Dose Range Finder**

**Summary of the Dose Range Finder (DRF)**

A dose range finder (Test Facility Study No. 521627) was conducted to determine the tolerability of the test item when administered as received. No guidelines were applicable as this study was intended for dose level selection purposes only.

If not mentioned otherwise, test system, procedures and techniques were identical to those used during the main study.

For the DRF, all animal activities were performed at the 's-Hertogenbosch location.

Dosing of the DRF was initiated on 18 Jan 2019. The in-life phase of the DRF was completed on 23 Jan 2019.

**Dose Formulations**

As the test item was used as received from the Sponsor, samples for dose formulation analysis were not collected by the Test Facility.

**Test System**

On 12 Dec 2018, female Crl: WI(Han) rats were received from Charles River Deutschland, Sulzfeld, Germany. Females were 10 weeks old and weighed between 223 and 228 g at initiation of dosing.

On arrival, animals were group housed (up to 3 animals of the same dosing group together) in polycarbonate cages (Macrolon, MIV type, height 18 cm). At study assignment, each animal was identified using earmark and tailmark.

The actual daily mean temperature during the study period was 20-21°C with an actual daily mean relative humidity of 38 to 52% (see deviation in [Appendix 7](#)).

**Experimental Design**

Text Table 15  
Experimental Design DRF

Group No.	Test Item Id.	Dose Level (mg/kg/day) <sup>a</sup>	Dose Volume (mL/kg)	Dose Concentration (mg/mL)	Number of Females	Animal Numbers
1	DiHEP Aqueous Solution	1000	2.07	482.9	3	1-3

The test item was administered to the appropriate animals by once daily oral gavage for 5 consecutive days.

To determine the tolerability of the test item when administered as received, animals were dosed at the highest dose of the main study (1000 mg/kg) as the dose volume was the largest at this dose level.

**In-life procedures**

Mortality:	Twice daily throughout the study.
Clinical Observations:	At least daily from Days 1-10, at 0-15 minutes, 1 hour ( $\pm 15$ minutes) and 3 hours ( $\pm 30$ minutes) after dosing.
Body Weights:	On Day 1 prior to dosing and on Days 3 and 5.
Food Consumption	Over Days 1-3 and 3-5.

**Terminal Procedures**

All animals were subjected to an external, thoracic and abdominal examination on Day 6 (scheduled necropsy). Based on the high pH of the test item, special attention was paid to the esophagus and stomach at necropsy. Animals were not deprived of food prior to necropsy. No organs were fixed and histopathological examination was not performed.

**RESULTS**

No signs of toxicity were noted at 1000 mg/kg/day.

Parameter	1000 mg/kg/day
Mortality	No mortality.
Clinical appearance	No findings.
Body weight	Normal.
Food consumption	Normal.
Macroscopic examination	No abnormalities noted.

**CONCLUSION**

Based on the results of the dose range finder, 1000 mg/kg/day was considered tolerable and appropriate as high dose for the main study.

Since no clinical signs were observed in the dose range finder, clinical observations were conducted and functional observations were started in the main study after dosing at no specific time point, but within a similar time period after dosing for the respective animals.

**Appendix 6**  
**Study Numbers used for Data Collection**

Study Numbers which were used to collect online data are presented in the table below. All data was reported under Test Facility Study No. 20172120.

Text Table 16  
Data Collection Study Numbers

<b>Test Facility Study No.</b>	<b>Study Number</b>	<b>Online Data</b>
521627 (DRF)	521627	ToxData; all data of the Dose Range Finder (DRF)
20172120 (Main)	521417	ToxData Parental animals: Mortality Clinical signs (except arena observations) Functional tests (except motor activity) Body weights and food consumption pre-mating Body weights mating period Food consumption mating period (males only) Clinical laboratory investigations Macroscopic findings and organ weights Implantation sites
20172120 (Main)	521418	ToxData: Arena observations (F <sub>0</sub> - animals)
20172120 (Main)	521419	ToxData: Clinical pathology PND 14-16 pups
20172120 (Main)	521416	ToxData: All other data of the main study

**Appendix 7**  
**Study plan, Last Amended Study Plan, and Deviations**



**FINAL STUDY PLAN**

**Test Facility Study No. 20172120**

**Combined 28-Day Repeated Dose Toxicity Study with the  
Reproduction/Developmental Toxicity Screening Test of  
DiHEP Aqueous Solution by Oral Gavage in Rats**

**SPONSOR:**

Shell International B.V.  
Carel van Bylandtlaan 16  
2596 HR The Hague  
The Netherlands

**TEST FACILITY:**

Charles River Laboratories Den Bosch B.V.  
Hambakenwetering 7  
5231 DD 's-Hertogenbosch  
The Netherlands

**Page 1 of 41**

## TABLE OF CONTENTS

1.	OBJECTIVE(S).....	4
2.	PROPOSED STUDY SCHEDULE .....	4
3.	GUIDELINES FOR STUDY DESIGN.....	5
4.	REGULATORY COMPLIANCE .....	5
5.	QUALITY ASSURANCE.....	5
6.	SPONSOR .....	6
7.	RESPONSIBLE PERSONNEL.....	6
8.	DOSE RANGE FINDER (DRF) .....	7
9.	TEST ITEM.....	9
10.	SAFETY .....	10
11.	DOSE FORMULATION AND ANALYSIS .....	10
12.	TEST SYSTEM.....	11
13.	HUSBANDRY .....	12
14.	EXPERIMENTAL DESIGN.....	14
15.	IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F <sub>0</sub> - GENERATION .....	17
16.	IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F <sub>1</sub> - GENERATION .....	21
17.	LABORATORY EVALUATIONS.....	22
18.	TERMINAL PROCEDURES – F <sub>0</sub> -GENERATION.....	25
19.	HISTOLOGY AND HISTOPATHOLOGY .....	27
20.	TERMINAL PROCEDURES – F <sub>1</sub> -GENERATION.....	27
21.	CONSTRUCTED VARIABLES.....	28
22.	STATISTICAL ANALYSIS .....	29
23.	COMPUTERIZED SYSTEMS .....	30
24.	AMENDMENTS AND DEVIATIONS .....	30
25.	RETENTION OF RECORDS, SAMPLES, AND SPECIMENS .....	31
26.	REPORTING.....	32
27.	ANIMAL WELFARE .....	32



TEST FACILITY APPROVAL.....33  
SPONSOR APPROVAL .....34  
ATTACHMENT A .....35  
ATTACHMENT B .....36  
ATTACHMENT C .....37  
ATTACHMENT D .....41

## 1. OBJECTIVE(S)

The objectives of this study are to determine the potential toxic effects of DiHEP Aqueous Solution when given orally by gavage for a minimum of 28 days to Wistar Han rats, and to evaluate the potential to affect male and female reproductive performance such as gonadal function, mating behavior, conception, parturition and early postnatal development.

In addition, parental, reproduction (up to and including implantation) and developmental (from implantation onwards) No Observed Adverse Effect Levels (NOAELs) will be evaluated.

## 2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Experimental Start Date:	18 Jan 2019 (First date of study-specific data collection; randomization dose range finder)
Experimental Completion Date:	27 Jun 2019 (Last date data are collected from the study)
Animal Arrival:	Males: 30 Jan 2019 Females: 16 Jan 2019
Initiation of Estrous Cycle Determination (Pretest Period):	22 Jan 2019
Initiation of Dosing:	05 Feb 2019
Initiation of Mating:	19 Feb 2019
Necropsy Males incl. Blood Sampling:	06 Mar 2019 <sup>a</sup>
Delivery of Litters (PND 1):	≥ 13 Mar 2019
Measurement Anogenital Distance (PND 1):	≥ 13 Mar 2019
Culling of F <sub>1</sub> -pups incl. Blood Sampling (PND 4):	≥ 16 Mar 2019
Determination Areola/Nipple Retention (PND 13):	≥ 25 Mar 2019
Necropsy Females and Pups incl. Blood Sampling (PND 14-16):	≥ 26 Mar 2019
Completion of In-life:	12 Apr 2019 (Last date of necropsy)
Unaudited Draft Report:	28 Jun 2019

PND = postnatal day

<sup>a</sup> Necropsy and blood sampling dates of any males used for an extension of the mating phase will be specified and approved in the study files by the Study Director.

### 3. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective(s), the overall product development strategy for the test item, and the following study design guidelines:

- OECD Guideline 422. *Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*, July 2016.
- EPA Health Effects Test Guideline OPPTS 870.3650: *Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*, July 2000.

In addition, the procedures described in this study plan essentially conform to the following guidelines.

- OECD Guideline 421. *Reproduction/Developmental Toxicity Screening Test*, July 2016.
- EPA Health Effects Test Guideline OPPTS 870.3550: *Reproduction/Developmental Toxicity Screening Test*, July 2000.
- Council Regulation (EC) No 440/2008 Part B: *Methods for the Determination of Toxicity and other Health Effects; B.7: "Repeated Dose (28 days) Toxicity (oral)"*. Official Journal of the European Union No. L142, May 2008.
- OECD Guideline 407. *Repeated Dose 28-day Oral Toxicity Study in Rodents*, October 2008.
- EPA Health Effects Test Guideline OPPTS 870.3050: *Repeated dose 28-day oral toxicity study in rodents*, July 2000.

### 4. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

### 5. QUALITY ASSURANCE

#### 5.1 Test Facility

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the study plan, conduct study and/or process inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Test Facility QAU contact is indicated below:

C.J. Mitchell, BSc.  
Address as cited for Test Facility  
Tel: +31 73 640 6700  
E-mail: QADenBosch@crl.com

## 6. SPONSOR

### Sponsor Representative

M. Rooseboom, PhD, ERT  
Tel: +31.70.377.3463  
E-mail: Martijn.Rooseboom@shell.com

## 7. RESPONSIBLE PERSONNEL

### Study Director

D. van den Oetelaar, MSc  
address as cited for Test Facility  
Tel: +31 73 640 6700  
E-mail: daphne.vandenoetelaar@crl.com

### Management Contact

H.H. Emmen, MSc  
address as cited for Test Facility  
Tel: +31 73 640 6700  
E-mail: harry.emmen@crl.com

### Individual Scientist (IS) at the Test Facility

Histopathology	E.J.M. Lambregts, DVM address as cited for Test Facility Tel: +31 73 640 6700 E-mail: ankie.lambregts@crl.com
----------------	--

Each IS is required to report any deviations or other circumstances that could affect the quality or integrity of the study to the Study Director in a timely manner. Each IS will provide a report addressing their assigned phase of the study, which will be included as an appendix to the Final Report. The phase report will include the following:

- A listing of critical computerized systems used in the conduct and/or interpretation of the assigned study phase

## 8. Dose range finder (DRF)

A dose range finder (Test Facility Study No. 521627) will be conducted to determine the tolerability of the test item when administered as received. No testing guidelines are applicable as this dose range finder is intended for confirmation of tolerability only.

### 8.1 Proposed Study Schedule (DRF)

Initiation of Dosing:	Group 1	18 Jan 2019
Necropsy:	Group 1	23 Jan 2019

### 8.2 Test Item (DRF)

The information, procedures and safety instructions will be identical to those used during the main study. See sections 9 and 10 for details.

### 8.3 Dose Formulations and Analysis (DRF)

The preparation of test item will be identical as for the main study. See section 11 for details.

The test item will be used as received from the Sponsor; therefore, samples for dose formulation analysis will not be collected by the Test Facility.

### 8.4 Test System (DRF)

Species:	Rat.
Strain:	CrI: WI(Han).
Condition:	Outbred, SPF-Quality.
Source:	Charles River Deutschland, Sulzfeld, Germany or Charles River Laboratories France, L'Arbresle Cedex, France. Details will be documented in raw data and report.

Number of Females	3 (nulliparous and non-pregnant).
-------------------	-----------------------------------

Target Age at the Initiation of Dosing:	10-12 weeks.
---	--------------

Target Weight at the Initiation of Dosing:	200 to 250 g.
--	---------------

The actual age and weight of animals received will be listed in the Final report.

At study assignment, each animal will be identified using earmark and tailmark.

Animals will be assigned at random at the discretion of the biotechnician.

At least upon receipt of the animals, a health inspection will be performed and any assigned animals considered unsuitable for use in the study will be replaced by alternate animals maintained under the same environmental conditions.

The justification of test system and environmental acclimation will be identical as for the main study. See section 12 for details.

### 8.5 Husbandry (DRF)

On arrival and following randomization, animals will be group housed (up to 3 animals of the same dosing group together) in polycarbonate cages (Macrolon, MIV type, height 18 cm).

Other conditions will be identical as for the main study. See section 13 for details.

### 8.6 Experimental design (DRF)

Group No.	Test Item Id.	Dose Level (mg/kg)	Dose Volume (mL/kg) <sup>a</sup>	Dose Concentration (mg/mL) <sup>b</sup>	Number of Females	Animal Numbers
1	DiHEP Aqueous Solution	1000	2.07	482.9	3	1-3

Id.= identification.

<sup>a</sup> Dose volume will be calculated as dose level (mg/kg) / corrected test item concentration (mg/ml).

<sup>b</sup> The test item consists of 43.9% DiHEP, test item concentration will be corrected for % DiHEP and specific gravity (factor: 1.1). See section 11.1 for details.

The test item and vehicle will be administered to the appropriate animals by once daily oral gavage for a minimum of 5 days.

A dose control system (DCS) will be used as additional check to verify the dosing procedure according to Standard Operating Procedures (Study No. 521627 will be used for DCS).

The justification of the route of administration is identical as for the main study. See section 14.2 for details. To determine the tolerability of the test item when administered as received, animals will be dosed at the highest dose of the main study (1000 mg/kg) as the dose volume is the largest at this dose level.

### 8.7 In-life Procedures, Observations, and Measurements (DRF)

Only the following observations and measurements will be performed.

Mortality: Twice daily throughout the study.

Clinical Observations: At least once daily up to the day prior to necropsy, during the following observation intervals:

Actual time after dosing	Reported as hours after dosing	Nominal time <sup>a</sup>
0-15 min	0 hour	07:00
1 hr ± 15 min	1 hour	08:00
3 hr ± 30 min	3 hours	10:00

<sup>a</sup> Nominal times will be used for computer registration only.

Body Weights: On Day 1 prior to dosing and on Days 3 and 5.

In order to monitor the health status animals may be weighed more often. This will be documented in the study raw data.

Food Consumption Over Days 1-3 and 3-5.

### 8.8 Terminal Procedures (DRF)

All animals will be subjected to an external, thoracic and abdominal examination on Day 6 (scheduled necropsy) or sooner (decedents). Based on the high pH of the test item (see section 9.1), special attention will be paid to the esophagus and stomach at necropsy.

Animals will not be deprived of food prior to necropsy. Gross lesions will be recorded but will not be retained, no organs will be fixed and histopathological examination will not be performed.

## 8.9 Reporting (DRF)

The procedures of statistical analysis, computerized systems, amendments and deviations, and retention of records will be identical as for the main study, if applicable. See sections 22, 23, 24, and 25 for details.

A summary of results of the dose range finder will be included in report 20172120 as an appendix.

## 9. TEST ITEM

### 9.1 Test Item

Identification:	DiHEP Aqueous Solution <sup>1</sup>
Batch (Lot) Number:	D2921BF000
Expiry date:	31 August 2020 (expiry date)
Physical Description:	Yellow aqueous liquid
Purity/Composition:	See Certificate of Analysis <sup>2</sup>
Storage Conditions:	At room temperature protected from light

#### Additional information

Test Facility Test Item Number:	209813/A
Purity/Composition correction factor:	Correct for percentage solid matter and additionally for percentage DiHEP.
Test item handling:	No specific handling conditions required
Chemical name (IUPAC, synonym or trade name):	1,4-Bis(2-hydroxyethyl)piperazine Dihydroxyethylpiperazine
CAS number:	122-96-3
Molecular formula:	C <sub>8</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>
Molecular weight:	174.24
Irritant or corrosive:	Yes
pH:	13
Specific gravity / density:	1.1 kg/m <sup>3</sup> at 20°C

### 9.2 Test Item Characterization

The Sponsor will provide to the Test Facility documentation of the identity, purity, composition, and stability for the test item. If available, a Certificate of Analysis or equivalent documentation will be provided for inclusion in the Final Report. The Sponsor

<sup>1</sup> DiHEP stands for Dihydroxyethylpiperazine.

<sup>2</sup> For Certificate of Analysis see [ATTACHMENT D](#).

will also provide information concerning the regulatory standard that was followed for these evaluations.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test item, and this information is available to the appropriate regulatory agencies should it be requested.

### **9.3 Analysis of Test Item**

The stability of the bulk test item will not be determined during the course of this study. Information to support the stability of each lot of the bulk test item will be provided by the Sponsor.

### **9.4 Reserve Samples**

For each batch (lot) of test item, if practically possible a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility and destroyed after the expiry date.

### **9.5 Test Item and Vehicle Inventory and Disposition**

Records of the receipt, distribution, storage, and disposition of test item will be maintained. With the exception of reserve samples, all unused Sponsor-supplied bulk test item will be discarded.

## **10. SAFETY**

The following safety instructions apply to this study:

Standard safety precautions specified in Charles River Den Bosch procedures.

## **11. DOSE FORMULATION AND ANALYSIS**

### **11.1 Preparation of Test Item**

The test item, DiHEP Aqueous Solution will be administered as received. An adequate amount of the test item will be dispensed into daily aliquots, which will be stored in a controlled temperature area set to maintain 21°C until use.

Test item for dosing will be kept at room temperature until dosing. Adjustment will be made for specific gravity of the test item. A factor of 43.9 will be used to correct for the purity/composition of the test item (based on the 48.9% solid matter of which 89.7% is DiHEP).

Any residual volumes will be discarded.

### **11.2 Sample Collection and Analysis**

The test item will be used as received from the Sponsor; therefore, samples for dose formulation analysis will not be collected by the Test Facility.



## 12. TEST SYSTEM

Species:	Rat.
Strain:	CrI: WI(Han).
Condition:	Outbred, SPF-Quality.
Source:	Charles River Deutschland, Sulzfeld, Germany or Charles River Laboratories France, L'Arbresle Cedex, France. Details will be documented in raw data and report.
Number of Males:	40.
Number of Females:	48 (nulliparous and non-pregnant).
Number of Pups Expected:	Approximately 480 pups (40 litters x 12 pups).
Target Age at the Initiation of the Pretest Period:	Females: approximately 10-12 weeks.
Target Age at the Initiation of Dosing:	Males: approximately 10-12 weeks. Females: approximately 12-14 weeks.
Target Weight at the Initiation of Dosing:	Males: 250 to 350 g. Females: 200 to 250 g.

The actual age and weight of animals received will be listed in the Final report.

### 12.1 Justification of Test System and Number of Animals

The Wistar Han rat was chosen as the animal model for this study as it is an accepted rodent species for toxicity testing by regulatory agencies. Charles River Den Bosch has general and reproduction/developmental historical data in this species from the same strain and source. This animal model has been proven to be susceptible to the effects of reproductive toxicants.

The total number of animals to be used in this study is considered to be the minimum required to properly characterize the effects of the test item. This study has been designed such that it does not require an unnecessary number of animals to accomplish its objectives.

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

### 12.2 Animal Identification

Prior to start of the pretest period (females) or treatment period (males), each allocated animal will be identified using earmark and tattoo. Prior to start of the pretest period, reserve females will be numbered R1 through R8 at random by indelible marker. Any reserve female replacing an allocated female prior to treatment will receive identification by earmark and tattoo.

Pups will be identified on postnatal day (PND) 1. They will be randomized per litter and individually identified by means of subcutaneous injection of Indian ink. When general hair growth blurs the identification, the pups will be identified by tattoo on the feet.

### **12.3 Environmental Acclimation**

The animals will be allowed to acclimate to the Test Facility toxicology accommodation for at least 5 days prior to start of the pretest period (females) or at least 5 days before the commencement of dosing (males).

### **12.4 Selection, Assignment, Replacement, and Disposition of Animals**

A total of 40 females will be selected at randomization before initiation of the pretest phase. Each selected female classified as not having regular estrous cycles during the pretest phase will be replaced before initiation of dosing by one of the 8 additional females having regular estrous cycles, if feasible. A total of 40 females with regular estrous cycles will continue in the study. The supernumerary females will then be removed from the study, and their estrous cycle results will be kept in the raw data but will not be reported.

Animals will be assigned to groups by a computer-generated random algorithm according to body weights, with all animals within  $\pm 20\%$  of the sex mean. Males and females will be randomized separately. Animals in poor health or at extremes of body weight range will not be assigned to groups.

At least upon receipt of the animals, a health inspection will be performed and any assigned animals considered unsuitable for use in the study will be replaced by alternate animals obtained from the same shipment and maintained under the same environmental conditions.

After initiation of dosing, study animals may be replaced during the replacement period with alternate animals in the event of accidental injury, non-test item-related health issues, or similar circumstances. The alternate animals may be used as replacements on the study within 1 to 3 days.

The disposition of all animals will be documented in the study records.

## **13. HUSBANDRY**

### **13.1 Housing**

On arrival and following the pretest (females only) and pre-mating period, animals will be group housed (up to 5 animals of the same sex and same dosing group together) in polycarbonate cages (Macrolon, MIV type, height 18 cm).

During the mating phase, males and females will be cohabitated on a 1:1 basis in Macrolon plastic cages (MIII type, height 18 cm).

During the post-mating phase, males will be housed in their home cage (Macrolon plastic cages, MIV type, height 18 cm) with a maximum of 5 males/cage. Females will be individually housed in Macrolon plastic cages (MIII type, height 18 cm).

During the lactation phase, females will be housed in Macrolon plastic cages (MIII type, height 18 cm). Pups will be housed with the dam, except during locomotor activity monitoring of the dams, when the pups will be kept warm in their home cage using bottles filled with warm water. In order to avoid hypothermia of pups, pups should not be left without their dam or a bottle filled with warm water for longer than 30-40 minutes.

During locomotor activity monitoring, F<sub>0</sub>-animals will be housed individually in a Hi-temp polycarbonate cage (Ancare corp., USA; dimensions: 48.3 x 26.7 x 20.3 cm) without cage-enrichment, bedding material, food and water.

The cages will contain appropriate bedding (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) and will be equipped with water bottles. The housing conditions will be maintained unless deemed inappropriate by the Study Director and/or Clinical Veterinarian. The room(s) in which the animals will be kept will be documented in the study records.

Animals will be separated during designated procedures/activities. Each cage will be clearly labeled with a color-coded cage card indicating Test Facility Study No., group, animal number(s), and sex.

### **13.2 Environmental Conditions**

The target conditions for animal room environment will be as follows:

Temperature:	18 to 24°C.
Humidity:	40 to 70%.
Light Cycle:	12-hours light and 12-hours dark (may be interrupted for designated procedures).
Ventilation:	At least 10 air changes per hour.

Any variations to these conditions will be evaluated and maintained in the raw data.

### **13.3 Food**

Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) will be provided *ad libitum* throughout the study, except during designated procedures. During motor activity measurements, animals will not have access to food for a maximum of 2 hours.

The feed is analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis are provided by the supplier and are on file at the Test Facility.

It is considered that there are no known contaminants in the feed that would interfere with the objectives of the study.

### **13.4 Water**

Municipal tap water will be freely available to each animal via water bottles. During motor activity measurements, animals will not have access to water for a maximum of 2 hours.

Periodic analysis of the water is performed, and results of these analyses are on file at the Test Facility.

It is considered that there are no known contaminants in the water that would interfere with the objectives of the study.

### 13.5 Animal Enrichment

For psychological/environmental enrichment and nesting material, animals will be provided with paper (Enviro-dri, Wm. Lilico & Son (Wonham Mill Ltd), Surrey, United Kingdom), except when interrupted by study procedures/activities.

### 13.6 Veterinary Care

Veterinary care will be available throughout the course of the study and animals will be examined by the veterinary staff as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments, if any, will be documented in the study records.

In the event that animals show signs of illness or distress, the responsible veterinarian may make initial recommendations about treatment of the animal(s) and/or alteration of study procedures, which must be approved by the Study Director. All such actions will be properly documented in the study records and, when appropriate, by study plan amendment.

Treatment of the animal(s) for minor injuries or ailments may be approved without prior consultation with the Sponsor Representative when such treatment does not impact fulfillment of the study objectives. If the condition of the animal(s) warrants significant therapeutic intervention or alterations in study procedures, the Sponsor Representative will be contacted, when possible, to discuss appropriate action. If the condition of the animal(s) is such that emergency measures must be taken, the Study Director and/or attending veterinarian will attempt to consult with the Sponsor Representative prior to responding to the medical crisis, but the Study Director and/or veterinarian has authority to act immediately at his/her discretion to alleviate suffering. The Sponsor representative will be fully informed of any such events.

## 14. EXPERIMENTAL DESIGN

Group No.	Test Item Id.	Dose Level (mg/kg)	Dose Volume (mL/kg) <sup>a</sup>	Corrected Test Item Concentration (mg/mL) <sup>b</sup>	Number of Animals		Animal Numbers	
					Males	Females	Males	Females
1	-	0 (Control) <sup>c</sup>	2.07	-	10	10	01-10	41-50
2	DiHEP	100	0.21	482.9	10	10	11-20	51-60
3	Aqueous	300	0.62		10	10	21-30	61-70
4	Solution	1000	2.07		10	10	31-40	71-80

TBD. = To be determined. Id.= identification.

<sup>a</sup> Dose volume will be calculated as dose level (mg/kg) / corrected test item concentration (mg/ml).

<sup>b</sup> The test item consists of 43.9% DiHEP, test item concentration will be corrected for % DiHEP and specific gravity (factor: 1.1). See section 11.1 for details.

<sup>c</sup> Test-item treated animals will receive undiluted test item and consequently, no vehicle will be used. Control animals will be dosed with water (Elix) in the same dose volume as Group 4.

The following 5 animals/sex/group are selected for functional tests, clinical pathology, collection of full list of organs/tissues at macroscopic examination, organ weights (full list) and histopathology (full list), see also respective paragraphs:

Group No.	Animal numbers	
	Males	Females <sup>a</sup>
1	01-05	To be selected
2	11-15	To be selected
3	21-25	To be selected
4	31-35	To be selected

<sup>a</sup> Females with live pups, if feasible. These animals will be selected and approved by the Study Director in the study files. The selected female animal numbers will be specified in the report.

#### 14.1 Administration of Test item

The test item and vehicle will be administered to the appropriate animals by once daily oral gavage 7 days a week for a minimum of 28 days. Males will be treated for a minimum of 28 days, up to and including the day before scheduled necropsy. This includes a minimum of two weeks prior to mating and during the mating period. Females will be treated for at least 14 days prior to mating (with the objective of covering at least two complete estrous cycles), the variable time to conception, the duration of pregnancy and at least 13 days after delivery, up to and including the day before scheduled necropsy. Females will not be dosed during littering.

The dose volume for each animal will be based on the most recent body weight measurement. Dose volumes  $\leq 50 \mu\text{L}$  will be administered using a plastic feeding tube which is connected to a digital syringe and dose volumes  $> 50 \mu\text{L}$  will be given with a plastic feeding tube connected to an appropriately graded syringe. The accuracy of the digital syringe will be determined prior to start dosing at relevant dose levels for this study.

The first day of dosing will be designated as Day 1 (exception: alternate animals used for replacement after Day 1 will assume the day of the animal being replaced).

A dose control system (DCS) will be used as additional check to verify the dosing procedure according to Standard Operating Procedures (Study No. 521417 will be used for DCS).

Pups will not be treated directly but could potentially be exposed to the test item in utero, via maternal milk, or from exposure to maternal urine/feces.

#### 14.2 Justification of Route and Dose Levels

The oral route of administration was selected because this is the recommended route by OECD TG 422, REACH regulation and ECHA guidelines. In addition, the test material is a non-volatile aqueous solution.

The dose levels were selected based on information provided by the Sponsor (data on file at Sponsor site), and in an attempt to produce graded responses to the test item.

A previously performed acute toxicity study with DiHEP Aqueous Solution via oral gavage in Sprague Dawley (SD) rats indicated a low acute toxicity ( $\text{LD}_{50_{\text{males}}}$  was 20,093 mg/kg and  $\text{LD}_{50_{\text{females}}}$  was 18,738 mg/kg).

In addition, multiple studies were performed with several structural analogs:

- A dietary 7-day toxicity study was performed with a hydroxypiperazine solution (containing 12-20% piperazine, 38-47% hydroxypiperazine, 16-25% dihydroxypiperazine, 17-26% water). During this study Wistar han rats received 590, 1420, and 3720 mg/kg for males and 680, 1610 and 3970 mg/kg for female rats. A slight body weight decrease was observed in females fed 3970 mg/kg/day but was not observed in females fed lower doses or in males. This body weight decrease was statistically significant after days 1 and 4 but not day 7. Remaining parameters were considered unaffected by treatment.
- A dietary 90-day toxicity study was performed with analog piperazine. During this study, SD rats received 400, 1200 and 2394 mg/kg by dietary administration. A dose related decrease in body-weight gain (a decrease of 10% in high dose animals when compared with concurrent control) was noted. Remaining parameters were considered unaffected by treatment.
- A dietary 90-day toxicity study was performed with analog anhydrous piperazine. Rats received 1000, 3000 and 10000 ppm (corresponding to 50, 150 and 500 mg/kg/day piperazine base). At 10000 ppm histopathological degenerative changes were noted in the liver and kidney, at 3000 ppm similar changes were noted to a lesser extent and at 1000 ppm no adverse effects were noted. In addition, at 10000 ppm a decrease in body weight gain was noted (statistically significant in females only). Remaining parameters were considered unaffected by treatment.
- A developmental toxicity study was performed, in which pregnant SD females received 0, 105, 420 and 2100 mg/kg piperazine base during days 6-15 by oral gavage. In high-dose females, excessive salivation, lethargy and a reduction in body weight gain and food consumption were noted. Remaining parameters, including pre- and post-implantation loss, litter size and sex ratio, were considered unaffected by treatment.
- In a dietary two generation study with piperazine dihydrochloride, SD rats received 0, 5000, 12000 and 25000 ppm (corresponding to 0, 125, 300 and 625 mg/kg piperazine base). The mid-dose was considered as LOAEL, with effects mainly on fertility (i.e. reduced pregnancy index and decreased number of implantation sites). These effects were not observed in the developmental toxicity study, which is be considered to support that the effect on fertility are the main effect of piperazine on reproduction.

Based on the observed low acute toxicity of DiHEP Aqueous Solution and the effects noted in the studies performed with structural analogs, 0, 100, 300 and 1000 mg/kg were selected as dose levels for this study.

The high-dose level should produce some toxic effects, but not death nor obvious suffering. The mid-dose level is expected to produce minimal to moderate toxic effects. The low-dose level should produce no observable indications of toxicity.

## **15. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F<sub>0</sub>-GENERATION**

The in-life procedures, observations, and measurements listed below will be performed for parental animals.

### **15.1 Mortality/Moribundity Checks – F<sub>0</sub>-Generation**

Frequency: At least twice daily throughout the study.

Procedure: Animals will be observed for general health/mortality and moribundity. Animals will not be removed from cage during observation, unless necessary for identification or confirmation of possible findings.

### **15.2 Clinical Observations – F<sub>0</sub>-Generation**

Frequency: During treatment, animals will be observed at least once daily, up to the day prior to necropsy.

These clinical observations will at least be conducted after dosing at no specific time point, but within a similar time period after dosing for the respective animals.

Procedure: Animals will be observed for specific clinical signs. The time of onset, grade and duration of any observed signs will be recorded. Signs will be graded for severity and the maximum grade will be predefined at 3 or 4. Grades will be coded as slight (grade 1), moderate (grade 2), severe (grade 3) and very severe (grade 4). For certain signs, only its presence (grade 1) or absence (grade 0) will be scored. In the data tables, the scored grades will be reported, as well as the percentage of animals affected in summary tables.

#### **15.2.1 Arena Observations – F<sub>0</sub>-Generation**

Frequency: Once before the first administration of the test item and at weekly intervals during the treatment period.

Procedure: Animals will be observed for specific clinical signs in a standard arena. The time of onset, grade and duration of any observed signs will be recorded.

**15.3 Body Weights – F<sub>0</sub>-Generation**

Frequency: Males and females will be weighed on the first day of treatment (prior to dosing), and weekly thereafter. Mated females will be weighed on Days 0, 4, 7, 11, 14, 17, and 20 post-coitum and during lactation on PND 1, 4, 7, and 13.

In order to monitor the health status animals may be weighed more often. This will be documented in the study raw data.

Procedure: Animals will be individually weighed.

**15.4 Food Consumption – F<sub>0</sub>-Generation**

Frequency: Weekly, except for males and females which are housed together for mating and for females without evidence of mating. Food consumption of mated females will be measured on Days 0, 4, 7, 11, 14, 17, and 20 post-coitum and during lactation on PND 1, 4, 7, and 13.

Procedure: Food consumption will be quantitatively measured.

**15.4.1 Water Consumption – F<sub>0</sub>-Generation**

Frequency: Regular basis throughout the study.

Procedure: Water consumption will be monitored by visual inspection of the water bottles. If inter group differences are noted, consumption may be assessed by weight.



### 15.5 Functional Tests – F<sub>0</sub>-Generation

- Frequency: Once during the treatment period. The selected 5 males will be tested once during Week 4 of treatment and the selected 5 females will be tested once during the last week of lactation (i.e. PND 6-13). These tests will be performed after clinical observations and arena observation, if applicable.
- Procedure: The following tests will be performed :
- hearing ability, pupillary reflex and static righting reflex (score 0 = normal/present, score 1 = abnormal/absent).
  - fore- and hind-limb grip strength will be recorded as the mean of three measurements , using a grip strength meter.
  - locomotor activity (recording period: 1 hour under normal laboratory light conditions) will be tested using the Kinder Scientific Motor Monitor System. Total movements and ambulations will be reported. Ambulations represent movements characterized by a relocation of the entire body position like walking, whereas total movements represent all movements made by the animals, including ambulations but also smaller or finer movements like grooming, weaving or movements of the head.

### 15.6 Estrous Cycle Evaluations – F<sub>0</sub>-Generation

- Frequency: Daily vaginal lavage will be performed beginning 14 days prior to treatment (pretest period), the first 14 days of treatment and during mating until evidence of copulation is observed. Vaginal lavage will continue for those females with no evidence of copulation until termination of the mating period.
- On the day of necropsy, a vaginal lavage will also be taken to determine the stage of estrus. This will be done for all females, except for females that have to be euthanized in extremis or die spontaneously.
- Procedure: Estrous cycles will be evaluated by examining the vaginal cytology of samples obtained by serial vaginal lavage procedures.

### 15.7 Cohabitation/Mating Procedure – F<sub>0</sub>-Generation

- Frequency: Daily, after a minimum of 14 days of treatment. The mating period will consist of a maximum of 14 consecutive days.
- Procedure: Animals will be cohabitated on a 1:1 basis within the same treatment group, avoiding sibling mating. Detection of mating will be confirmed by evidence of sperm in the vaginal lavage or by the appearance of an intravaginal copulatory plug. This day will be designated Day 0 post-coitum. Once mating has occurred, the males and females will be separated.
- A maximum of 14 days will be allowed for mating, after which females who have not shown evidence of mating will be separated from their males. In case less than 9 females per group have shown evidence of mating, each non-mated female may be re-mated once with a male for a maximum of 7 days (if possible). A male of the same group having previously shown evidence of mating (non-selected male if possible, see section 14) will be used for re-mating.

### 15.8 General Reproduction Data – F<sub>0</sub>-Generation

- Frequency: Daily from the mating period onwards.
- Procedure: Male number paired with, mating date, confirmation of pregnancy, and delivery day will be recorded. Palpation may be used to aid in confirmation of pregnancy.
- The females will be allowed to litter normally. Postnatal day (PND) 1 is defined as the day when a litter is found completed (i.e. membranes and placentas cleaned up, nest built and/or feeding of pups started). The day prior to PND 1 is considered to be the day when the female started to deliver and is defined as PND 0 and used for recording of delivery. Females that are littering will be left undisturbed.
- Cage debris of pregnant females will be examined for evidence of premature delivery. Signs of difficult or prolonged parturition will be recorded, if applicable.
- Deficiencies in maternal care, such as inadequate construction or cleaning of the nest, pups left scattered and cold, physical abuse of pups or apparently inadequate lactation or feeding, will be recorded, if applicable.

## **16. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F<sub>1</sub>-GENERATION**

The in-life procedures, observations, and measurements listed below will be performed for the pups.

### **16.1 Mortality/Moribundity Checks – F<sub>1</sub>-Generation**

Frequency: The number of live and dead pups will be determined on PND 1 and daily thereafter.

Procedure: Pups will be observed for general health/mortality and moribundity. If possible, defects or cause of death will be evaluated. Pups will not be removed from the cage during observation, unless necessary for identification or confirmation of possible findings.

### **16.2 Clinical Observations – F<sub>1</sub>-Generation**

Frequency: At least once daily.

Procedure: Detailed clinical observations will be made for all pups. Only days on which clinical signs are present between the first and last litter check will be given in the respective report tables.

### **16.3 Body Weights – F<sub>1</sub>-Generation**

Frequency: On PND 1, 4, 7, and 13.

Procedure: Live pups will be individually weighed.

### **16.4 Sex – F<sub>1</sub>-Generation**

Frequency: On PND 1 and 4.

Procedure: Sex will be externally determined for all pups.

### **16.5 Anogenital Distance – F<sub>1</sub>-Generation**

Frequency: On PND 1.

Procedure: Anogenital distance (AGD) will be measured for all live pups. The AGD will be normalized to the cube root of body weight.

### **16.6 Areola/Nipple Retention – F<sub>1</sub>-Generation**

Frequency: On PND 13.

Procedure: All males in each litter will be examined for the number of areola/nipples.

## 16.7 Culling – F<sub>1</sub>-Generation

Frequency: On PND 4.

Procedure: To reduce variability among the litters, eight pups from each litter of equal sex distribution (if possible) will be selected. Blood samples will be collected from two of the surplus pups (if possible from one male and one female pup). Selective elimination of pups, e.g. based upon body weight or AGD, will not be done. Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is acceptable. See also sections [17.1.1](#) and [20.3](#).

## 17. LABORATORY EVALUATIONS

### 17.1 Clinical Pathology

#### 17.1.1 Sample Collection

Blood of F<sub>0</sub>-animals (except for animals which were sacrificed *in extremis* or found dead and females with total litter loss) will be collected on the day of scheduled necropsy. Samples will be collected, between 7.00 and 10.30 a.m., from the retro-orbital sinus under anesthesia using isoflurane in the animal facility. Additional blood samples may be obtained (e.g. due to clotting of non-serum samples) in both the animal facility and in the necropsy room if permissible sampling frequency and blood volume are not exceeded. After collection, samples will be transferred to the appropriate laboratory for processing.

F<sub>0</sub>-males (except for animals which were sacrificed *in extremis* or found dead) will be fasted overnight with a maximum of 24 hours before blood sampling, but water will be available. F<sub>0</sub>-females will not be fasted overnight.

Blood of F<sub>1</sub>-animals will be collected on PND 4 and PND 14-16, if possible. This will be performed in the necropsy room.

On PND 4 at culling, blood will be collected from two surplus pups per litter (if possible) by decapitation, between 7.00 and 10.30 a.m., and will be pooled to one sample per litter. If available, blood will be collected from one male and one female pup. If only one surplus pup per litter is available at culling, as much as possible blood will be collected from this single pup. If the target volume of 0.5 mL cannot be reached by pooling from two pups, blood from a third surplus pup of the same litter should be added, if available.

On PND 14-16, separate blood samples will be collected from two pups per litter (from one male and one female, if possible). If the target volume of 1.0 mL/pup cannot be reached, a separate blood sample should be collected from another pup of the same litter and sex (if possible). Any incomplete blood sample will be discarded. Blood will be drawn, between 7.00 and 10.30 a.m., by aorta puncture under anesthesia using isoflurane as part of the necropsy procedure.

Samples will be collected according to the table below.

## Samples for Clinical Pathology Evaluation

Animals	Time Point	Hematology	Coagulation	Clinical Chemistry	Thyroid Hormone
Selected F <sub>0</sub> -animals (5/sex/group) <sup>a, b</sup>	On the day of scheduled necropsy	X	X	X	X
Non-selected F <sub>0</sub> -animals (≤ 5/sex/group) <sup>a, b</sup>	On the day of scheduled necropsy	-	-	-	X
2 pups/litter	PND 4	-	-	-	X
2 pups/litter	PND 14-16	-	-	-	X

X = Sample to be collected; - = Not applicable.

<sup>a</sup> See section 14 for details of the selected F<sub>0</sub>-animals.

<sup>b</sup> Except for animals which were sacrificed *in extremis* or found dead, and females with total litter loss.

### 17.1.2 Hematology

Target Volume: 0.5 mL.

Anticoagulant: K<sub>3</sub>-EDTA (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).

#### Hematology Parameters

White blood cells (WBC)	Red Blood Cell Distribution Width (RDW)
Neutrophils (absolute)	Haemoglobin
Lymphocytes (absolute)	Haematocrit
Monocytes (absolute)	Mean corpuscular volume (MCV)
Eosinophils (absolute)	Mean corpuscular haemoglobin (MCH)
Basophils (absolute)	Mean corpuscular haemoglobin concentration (MCHC)
Red blood cells	Platelets
Reticulocyte (absolute)	

A blood smear will be prepared from each hematology sample. Blood smears will be labeled, stained, and stored. If additional examination of blood smears is deemed necessary, the smears may be subsequently evaluated and this evaluation will be described in a study plan amendment.

### 17.1.3 Coagulation

Target Volume: 0.45 mL.

Anticoagulant: Citrate (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).

#### Coagulation Parameters

Prothrombin Time (PT)	Activated Partial Thromboplastin Time (APTT)
-----------------------	--

**17.1.4 Clinical Chemistry**

Target Volume: 0.5 mL.  
 For bile acid measurement: 1.0 mL (same sample as for thyroid hormone measurement).

Anticoagulant: Li-Heparin (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).  
 Not applicable for serum tubes.

Processing: To serum (bile acids) or to plasma

Clinical Chemistry Parameters

Alanine aminotransferase (ALAT)	Creatinine
Aspartate aminotransferase (ASAT)	Glucose
Alkaline Phosphatase (ALP)	Cholesterol
Total protein	Sodium
Albumin	Potassium
Total Bilirubin	Chloride
Bile Acids	Calcium
Urea	Inorganic Phosphate (Inorg. Phos)

**17.1.5 Thyroid Hormone**

Target Volume: F<sub>0</sub>-animals: 1.0 mL (same sample as for bile acid measurement).  
 PND 4 pups: 0.5 mL in total (pooled).  
 PND 14-16 pups: 1.0 mL per pup.

Anticoagulant: Not applicable for serum. (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).

Thyroid Hormone Parameters

Thyroxine (T4)	Thyroid-Stimulating Hormone (TSH; only if required)
----------------	---

After clotting and centrifugation, serum will be used as listed below.

F<sub>0</sub>-Males: Serum from each sample will be divided into 2 aliquots: 150 µL serum for measurement of total T4 and the remaining volume of serum for possible future measurement of TSH (added by study plan amendment if applicable).

F<sub>0</sub>-Females: The serum will be used for possible future measurement of total T4 and/or thyroid-stimulating hormone TSH (added by study plan amendment if applicable).

PND 4 Pups: The pooled serum will be used for possible future measurement of total T4 (added by study plan amendment if applicable).

PND 14-16 Pups: Serum from each sample will be divided into 2 aliquots: 150 µL serum for measurement of total T4, and the remaining volume of serum for possible future measurement of TSH (added by study plan amendment if applicable).

Serum samples retained for possible future analysis will be maintained by the Test Facility in the freezer ( $\leq -75^{\circ}\text{C}$ ). Under these storage conditions, samples will be stable for 6 months. Any remaining sample will be discarded prior to finalization of the report

## 18. TERMINAL PROCEDURES – F<sub>0</sub>-GENERATION

Terminal procedures are summarized in the following table:

Terminal Procedures

Group No.	(Non) Selected Animals	No. of Animals		Scheduled Euthanasia	Necropsy Procedures			Histology and histopathology				
		M	F		Necropsy	Tissue Collection	Organ Weights					
1	Selected	5	5	<b>Males:</b> after a minimum of 28 days of administration  <b>Females:</b> PND 14-16	X	X <sup>a</sup>	X <sup>a</sup>	Full list				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
2	Selected	5	5					Gross lesions Target tissues <sup>c</sup> Reproductive tissues <sup>b</sup>				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
3	Selected	5	5					Gross lesions Target tissues <sup>c</sup> Reproductive tissues <sup>b</sup>				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
4	Selected	5	5					Full list				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
Unscheduled Deaths (sacrificed <i>in extremis</i> or found dead)								X	X	-	Full list	

X = Procedure to be conducted; - = Not applicable.

<sup>a</sup> See Tissue Collection and Preservation table in [ATTACHMENT C](#) for listing of tissues.

<sup>b</sup> Reproductive tissues are applicable for males that fail to sire and females that fail to deliver pups (i.e. non-pregnant females, implantation sites only or no offspring) and females with total litter loss. See [ATTACHMENT C](#) for listing of tissues.

<sup>c</sup> Target tissues are applicable in case of possible treatment-related changes in any of the tissues of any animal in the high dose group. Then, histological examination will be extended to that particular tissue of the selected 5 animals of Groups 2 and 3.

### 18.1 Unscheduled Deaths – F<sub>0</sub>-Generation

If an animal dies on study, a necropsy will be conducted and specified tissues will be saved, but not weighed. If necessary, the animal will be refrigerated to minimize autolysis.

Animals may be euthanized for humane reasons as per Test Facility SOPs. These animals will be deeply anaesthetized using isoflurane and subsequently exsanguinated. They will undergo necropsy, and specified tissues will be retained, but not weighed.

The specified tissues which will be retained are mentioned in [ATTACHMENT C](#).

### **18.2 Scheduled Euthanasia – F<sub>0</sub>-Generation**

Animals surviving until scheduled euthanasia will have a terminal body weight recorded and will be deeply anaesthetized using isoflurane and subsequently exsanguinated.

Scheduled necropsies are summarized below:

Males (which sire and fail to sire): Following completion of the mating period (a minimum of 28 days of administration).

Females which deliver: PND 14-16.

Females which fail to deliver: With evidence of mating: Post-coitum Days 25-27.  
Without evidence of mating: Approximately 24-26 days after the last day of the mating period.

Females with total litter loss: Dams with no surviving pups will be euthanized within 24 hours after the last pup is found dead or missing.

All males surviving to scheduled necropsy will be fasted overnight with a maximum of 24 hours before necropsy. Water will be available. F<sub>0</sub>- females will not be fasted overnight.

The specified tissues which will be retained are mentioned in [ATTACHMENT C](#).

### **18.3 Necropsy – F<sub>0</sub>-Generation**

All animals will be subjected to a full *post mortem* examination, with special attention being paid to the reproductive organs.

The numbers of former implantation sites will be recorded for all paired females.

In case no macroscopically visible implantation sites are present, nongravid uteri will be stained using the Salewski technique in order to detect any former implantation sites and the number of corpora lutea will be recorded in addition.

Necropsy procedures will be performed by qualified personnel with appropriate training and experience in animal anatomy and gross pathology. A veterinary pathologist, or other suitably qualified person, will be available.

### **18.4 Organ weights – F<sub>0</sub>-Generation**

The organs identified for weighing in the Tissue Collection and Preservation table in [ATTACHMENT C](#) will be weighed at necropsy for all scheduled euthanasia animals. Organ weights will not be recorded for animals found dead or euthanized in poor condition or *in extremis*. Paired organs will be weighed together. Organ weights as a percent of body weight (using the terminal body weight) will be calculated.



### **18.5 Tissue Collection and Preservation – F<sub>0</sub>-Generation**

Representative samples of the tissues identified in the Tissue Collection and Preservation table in [ATTACHMENT C](#) will be collected from all animals and preserved in 10% buffered formalin (neutral phosphate buffered 4% formaldehyde solution), unless otherwise indicated. Additional tissue samples may be collected to elucidate abnormal findings.

For females which fail to deliver a complete litter, uterine contents (i.e. any fetuses, placenta and implantation sites) will be fixed (if applicable), but will not be examined histopathologically in first instance.

## **19. HISTOLOGY AND HISTOPATHOLOGY**

### **19.1 Histology**

Tissues in the Tissue Collection and Preservation table in [ATTACHMENT C](#) from F<sub>0</sub>-animals identified in the Terminal Procedures table will be embedded in paraffin, sectioned at a thickness of 2-4 micrometers, mounted on glass slides, and stained with hematoxylin and eosin.

### **19.2 Histopathology**

All tissues as defined under Histology (section [19.1](#)) will be examined by a board-certified toxicological pathologist with training and experience in laboratory animal pathology. Target tissues identified by the study pathologist during microscopic evaluation will be communicated to the Study Director; tissues will be evaluated and reported.

Any additional stains or evaluations, if deemed necessary by the pathologist, will be added by study plan amendment following discussion with the Study Director and in consultation with the Sponsor.

At the discretion of the study pathologist and after acknowledgement by the Study Director, images may be captured for consultation purposes.

A peer review on the histopathology data will be performed by a second pathologist.

## **20. TERMINAL PROCEDURES – F<sub>1</sub>-GENERATION**

### **20.1 Method of Euthanasia – F<sub>1</sub>-Generation**

Pups, younger than 7 days will be euthanized by decapitation.

All remaining pups (PND 7-16), except the pups selected for blood collection, will be euthanized by an intraperitoneal injection of sodium pentobarbital (Euthasol® 20%).

The pups selected for blood collection on PND 14-16 will be anesthetized using isoflurane followed by exsanguination.

### **20.2 Unscheduled Deaths – F<sub>1</sub>-Generation**

Recognizable fetuses of females that die spontaneously or are euthanized *in extremis* will be examined externally and sexed (both externally and internally, if possible). Live fetuses will be euthanized by decapitation.

Pups that die or are euthanized before scheduled termination will also be examined externally and sexed (both externally and internally, if possible). Pups found dead during the weekend

can be fixed in identified containers containing 70% ethanol if not necropsied on the same day. The stomach of pups not surviving to the scheduled necropsy date will be examined for the presence of milk, if possible. If possible, defects or cause of death will be evaluated.

### 20.3 Scheduled Euthanasia – F<sub>1</sub>-Generation

On PND 4, the surplus pups (> 8 pups per litter) will be euthanized by decapitation. Sex will be determined both externally and internally. From two surplus pups per litter, blood will be collected, if possible. For details see also sections 16.7 and 17.1.1.

All remaining pups will be euthanized on PND 14-16. Sex will be determined both externally and internally. Descriptions of all external abnormalities will be recorded. Particular attention will be paid to the external reproductive genitals to examine signs of altered development. External abnormalities may be collected and fixed in 10% buffered formalin at discretion of the Study Director. In addition, the thyroid will be collected from two pups per litter (if possible, from one male and one female pup and preferably from the same pups as selected for (complete) blood collection, see also section 17.1.1), and will be preserved in 10% buffered formalin.

## 21. CONSTRUCTED VARIABLES

### 21.1 Parental Variables

Body Weight Gains: Calculated against the body weight on Day 1 (pre-mating, mating and lactation periods) or Day 0 (post-coitum period).

Relative Food Consumption: Calculated against the body weight for scheduled intervals.

Organ Weight Relative to Body Weight: Calculated against the terminal body weight.

### 21.2 Reproduction and Developmental Variables

For each group, the following calculations will be performed. Group mean values of precoital time and duration of gestation will be calculated from individual values of F<sub>0</sub>-females, the remaining group values will be calculated from the total number in each group. Additional calculations may be used, the methods and results will be described in the report.

Mating index (%): 
$$\frac{\text{Number of females mated}}{\text{Number of females paired}} \times 100$$

Precoital time: Number of days between initiation of cohabitation and confirmation of mating

Fertility index (%): 
$$\frac{\text{Number of pregnant females}}{\text{Number of females mated}} \times 100$$

Gestation index (%): 
$$\frac{\text{Number of females with living pups on Day 1}}{\text{Number of pregnant females}} \times 100$$

Duration of gestation:	Number of days between confirmation of mating and the beginning of parturition
Post-implantation survival index (%):	$\frac{\text{Total number of offspring born}}{\text{Total number of uterine implantation sites}} \times 100$ <p>Post-implantation survival index will be expressed as 100% when the number of offspring exceeds the number of implantation sites recorded.</p>
Live birth index (%):	$\frac{\text{Number of live offspring on Day 1 after littering}}{\text{Total number of offspring born}} \times 100$
Percentage live males at First Litter Check (%):	$\frac{\text{Number of live male pups at First Litter Check}}{\text{Number of live pups at First Litter Check}} \times 100$
Percentage live females at First Litter Check (%):	$\frac{\text{Number of live female pups at First Litter Check}}{\text{Number of live pups at First Litter Check}} \times 100$
Viability index (%):	$\frac{\text{Number of live offspring on Day 4 before culling}}{\text{Number live offspring on Day 1 after littering}} \times 100$
Lactation index (%):	$\frac{\text{Number of live offspring on Day 13 after littering}}{\text{Number live offspring on Day 4 (after culling)}} \times 100$

## 22. STATISTICAL ANALYSIS

All statistical tests will be conducted at the 5% significance level. All pairwise comparisons will be conducted using two sided tests and will be reported at the 1% and 5% levels.

Numerical data collected on scheduled occasions will be analyzed according to sex and occasion. Descriptive statistics number, mean and standard deviation will be reported whenever possible. Values may also be expressed as a percentage of predose or control values when deemed appropriate. Inferential statistics will be performed according to the comparison matrix below when possible, but will exclude semi-quantitative data, and any group with less than 3 observations.

The following pairwise comparisons will be made:

Group 2 vs. Group 1

Group 3 vs. Group 1

Group 4 vs. Group 1

### 22.1 Parametric

Datasets with at least 3 groups (the designated control group and 2 other groups) will be compared using Dunnett-test (many-to-one-t-test).

## 22.2 Non-Parametric

Datasets with at least 3 groups will be compared using a Steel-test (many-to-one rank test).

The motor activity data set (at least 3 groups) will be compared using an overall Kruskal-Wallis. Whenever, the overall test is significant, the Wilcoxon Rank-Sum test will be applied to compare the treated groups to the control group.

## 22.3 Incidence

An overall Fisher's exact test will be used to compare all groups. The above pairwise comparisons will be conducted using Fisher's exact test whenever the overall test is significant.

Additional methods of statistical analysis may be used at the discretion of the Study Director. The methods and the results will be described in the report.

## 23. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Data for parameters not required by study plan, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by study plan and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

Critical Computerized Systems

System Name	Description of Data Collected and/or Analyzed
ToxData <sup>a</sup>	In-life phase (Mortality; Clinical signs; Body weights; Food consumption; Functional tests; Organ weights; Reproduction parameters; Observations pups <sup>b</sup> ) data collection
REES Centron	Temperature and humidity (animal and laboratory facilities) data collection
MotorMonitor II	Motor activity measurement data collection
ADVIA® 2120i	Hematology data collection
STA Compact®	Clotting parameters data collection
AU400	Clinical biochemistry data collection
IMMULITE® 1000	Thyroid hormone data collection
Pathdata	Histopathology data collection

<sup>a</sup> For logistic reasons, data will be captured under separate Study numbers, see [ATTACHMENT B](#).

<sup>b</sup> Only at first and last litter check, and in case of clinical pup findings also on the respective days in between.

## 24. AMENDMENTS AND DEVIATIONS

Changes to the approved study plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary study plan changes in advance with the Sponsor.

All study plan and SOP deviations will be documented in the study records. Deviations from the study plan and/or SOP related to the phase(s) of the study conducted at a Test Site shall be documented, acknowledged by the PI/IS, and reported to the Study Director for

authorization/acknowledgement. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

## **25. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS**

All study-specific raw data, electronic data, documentation, study plan, retained samples and specimens and final reports will be archived by no later than the date of final report issue. All materials generated by Charles River from this study will be transferred to a Charles River archive.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study plan, study plan amendments, and deviations
- Study schedule
- Study-related correspondence
- Test system receipt, health, and husbandry
- Test item receipt, identification and preparation
- In-life measurements and observations
- Clinical pathology sample collection and evaluation
- Gross and microscopic observations and related data
- Organ weight measurements
- Statistical analysis results

After two years of archiving, all study-specific raw data, electronic data, documentation, study plan and final reports will be transferred to Iron Mountain Germany, Harpener Hellweg 31, D-44805 Bochum, Germany. They shall be indexed by using the Test Facility Project No. allowing unequivocal identification and providing the necessary information (e.g. test system, test item, date of Toxicology and Ecology for the time period set by the GLP regulations). Records of transfer will be retained by test facility. The Sponsor will be informed about the transfer and will receive a digital copy of the record of transfer. The sponsor will ensure that all study material is expediently returned to Charles River Den Bosch if requested by GLP monitoring authorities for audit.

## **26. REPORTING**

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable). The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. An original copy of the report with the Test Facility's signatures will be retained.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

## **27. ANIMAL WELFARE**

This study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of project license AVD2360020172866 (Appendix 2) approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

Animals showing pain, distress or discomfort, which is considered not transient in nature or is likely to become more severe, will be sacrificed for humane reasons based on OECD guidance document on humane endpoints (ENV/JM/MONO/ 2000/7).

By approving this study plan, the Sponsor affirms that this study is required by a relevant government regulatory agency and that it does not unnecessarily duplicate any previous experiments.

**TEST FACILITY APPROVAL**

The signature below acknowledges Test Facility Management's responsibility to the study as defined by the relevant GLP regulations.



Date: 16 January 2019

K.C.G. Hartman-Van Dycke, PhD  
Test Facility Management

The signature below indicates that the Study Director approves the study plan.

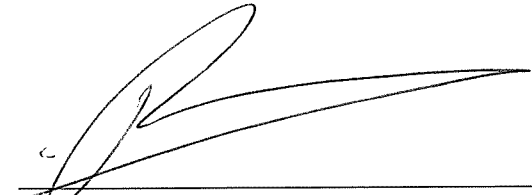


Date: 16 January 2019

D. van den Oetelaar, MSc.  
Study Director

**SPONSOR APPROVAL**

The signature of the Sponsor Representative below indicates approval of this study plan.



Date: 26 Jan 2015

M. Rooseboom, PhD, ERT  
Sponsor Representative



**ATTACHMENT A**

## Distribution List

Electronic copies will be supplied unless otherwise specified below.

<b>Version</b>	<b>Recipient</b>	
Original	Study Director	
1 Copy	Sponsor Representative / Study Monitor	
1 Copy	QAU / Management	Qaumailboxher;
1 Copy	Formulations	Tsfher;
1 Copy	Estrous Cycle Determination	Daoud, M; Hungs, S;
1 Copy	Clinical Pathology	Her/clinical pathology;
1 Copy	Necropsy	Her/necropsy;
1 Copy	Histotechnology	Her/histology
1 Copy	Study Assistants	Sagit;
1 Copy	Individual Scientist Histopathology	Lambregts, A;
1 Paper Copy	Coordinating Biotechnician DRF	Coppes, M;
1 Paper Copy	Coordinating Biotechnician Main	Van Beek, B;

**ATTACHMENT B**

Separate Study Numbers for online data collection will be used as indicated below. All data will be reported under Test Facility Study No. 20172120.

<b>Test Facility Study No.</b>	<b>Study Number</b>	<b>Online Data</b>
521627 (DRF)	521627	ToxData; all data of the Dose Range Finder (DRF)
20172120 (Main)	521417	ToxData Parental animals : Mortality Clinical signs (except arena observations) Functional tests (except motor activity) Body weights and food consumption pre-mating Body weights mating period Food consumption mating period (males only) Clinical laboratory investigations Macroscopic findings and organ weights Implantation sites
20172120 (Main)	521418	ToxData: Arena observations (F <sub>0</sub> - animals)
20172120 (Main)	521419	ToxData: Clinical pathology PND 14-16 pups
20172120 (Main)	521416	ToxData: All other data of the main study

## ATTACHMENT C

### Tissue Collection and Preservation

This attachment contains two tables describing the procedures of tissue weighing, collection, histology and microscopic evaluation. The first table is applicable for all selected animals and all animals that die spontaneously or are sacrificed *in extremis*. The second table is applicable for all remaining animals, including the males that fail to sire and females that fail to deliver pups (i.e. non-pregnant, implantation sites only or no offspring) and females with total litter loss.

Table 1: All selected animals and all animals that die spontaneously or are sacrificed *in extremis*:

Tissue	Weigh <sup>a</sup>	Collect	Histology	Microscopic Evaluation <sup>c</sup>	Comment
Animal identification	-	X	-	-	Location: ear and foot
Artery, aorta	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Body cavity, nasopharynx	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Bone marrow	-	X	X	X	Collect as part of the femur and sternum.
Bone, femur	-	X	X	X	Including joint.
Bone, sternum	-	X	X	X	-
Brain	X	X	X	X	Eight brain levels to be examined including cerebellum, midbrain and cortex.
Cervix	X	X	X	X	Collect and weigh together with the uterus.
Epididymis	X	X	X	X	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Esophagus	-	X	-	-	-
Eye	-	X	X	X	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Gland, adrenal	X	X	X	X	Paired examination.
Gland, coagulation	X	X	X	X	Collect and weigh together with the seminal vesicles.
Gland, harderian	-	X	-	-	Examine only if present in the routine section of the eye. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours. Only 1 required for microscopic examination.
Gland, lacrimal	-	X	-	-	Collect exorbital. Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Gland, mammary	-	X	X	X	Collect inguinal region with skin. Examine for both males and females.

Gland, parathyroid	X	X	-	-	Examine only if present in the routine section of thyroid. Collect and weigh together with thyroid.
Gland, pituitary	-	X	X	X	-
Gland, prostate	X	X	X	X	-
Gland, salivary	-	X	-	-	Collect at mandibular, sublingual and parotid site. Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Gland, seminal vesicle	X	X	X	X	Paired examination. Collect and weigh together with the coagulation gland.
Gland, thyroid	X	X	X	X	Paired examination. Collect and weigh together with the parathyroid.
Gross lesions/masses	-	X	X	X	Only at the discretion of the Study Director, this can be omitted.
Gut-associated lymphoid tissue	-	X	X	X	Examine only if present in routine section of intestine.
Heart	X	X	X	X	-
Kidney	X	X	X	X	Paired examination.
Large intestine, cecum	-	X	X	X	-
Large intestine, colon	-	X	X	X	-
Large intestine, rectum	-	X	X	X	-
Larynx	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Liver	X	X	X	X	-
Lung	-	X	X	X	Infused with formalin.
Lymph node	-	X	X	X	Collected at mandibular and mesentric site. Only 1 mandibular required for microscopic examination
Muscle, skeletal	-	X	X	X	-
Nerve, optic	-	X	-	-	Examine only if present in the routine section of the eye. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Nerve, sciatic	-	X	X	X	Only 1 required for microscopic examination
Ovaries	X	X	X	X	Paired examination.
Pancreas	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Skin	-	X	-	-	-
Small intestine, duodenum	-	X	X	X	-
Small intestine, ileum	-	X	X	X	-
Small intestine, jejunum	-	X	X	X	-

Spinal cord	-	X	X	X	Examine one transverse and one longitudinal section from each of the following areas: cervical, mid-thoracic, lumbar.
Spleen	X	X	X	X	-
Stomach	-	X	X	X	-
Testes	X	X	X <sup>b</sup>	X <sup>b</sup>	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Thymus	X	X	X	X	-
Tongue	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Trachea	-	X	X	X	-
Urinary bladder	-	X	X	X	-
Uterus	X	X	X	X	-
Vagina	-	X	X	X	-

X = Procedure to be conducted;

- = Not applicable.

<sup>a</sup> Organ weights will not be determined for animals which die spontaneously or are sacrificed *in extremis*.

<sup>b</sup> For the testes of all selected males of Groups 1 and 4, and all males that fail to sire or died before mating detailed qualitative examination will be made, taking into account the tubular stages of the spermatogenic cycle. The examination will be conducted in order to identify treatment related effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Any cell- or stage-specificity of testicular findings will be noted.

<sup>c</sup> Efforts will be made to evaluate all protocol-required tissues microscopically; however, it is not always feasible for every protocol-required tissue to be present on every slide. Protocol-required tissues that are not examined will be documented in the histopathology data and the impact of these missing tissues on the study will be documented in the pathology report.

Table 2: All remaining animals (incl. males that fail to sire<sup>a</sup>, females that fail to deliver pups and females with total litter loss):

Tissue	Weigh	Collect	Histology	Microscopic Evaluation <sup>c</sup>	Comment
Animal identification	-	X	-	-	Location: ear and foot
Cervix	-	X	@	@	-
Epididymis	X	X	@	@	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Gland, coagulation	X	X	@	@	Collect and weigh together with the seminal vesicles.
Gland, mammary	-	X	#	#	Collect inguinal region with skin. Collect for both males and females.
Gland, parathyroid	X	X	-	-	Examine only if present in the routine section of thyroid. Collect and weigh together with thyroid.
Gland, pituitary	-	X	-	-	-
Gland, prostate	X	X	@	@	-
Gland, seminal vesicle	X	X	@	@	Paired examination. Collect and weigh together with the coagulation gland.
Gland, thyroid	X	X	-	-	Paired examination. Collect and weigh together with the parathyroid.
Gross lesions/masses	-	X	X	X	-
Ovaries	-	X	@	@	Paired examination.
Testes	X	X	@ <sup>b</sup>	@ <sup>b</sup>	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Uterus	-	X	@	@	-
Vagina	-	X	@	@	-

X = Procedure to be conducted for all remaining animals;

- = Not applicable;

@ = Procedure to be conducted only for males that fail to sire<sup>a</sup>, females that fail to deliver pups and females with total litter loss.

# = Procedure to be conducted for females with total litter loss only.

<sup>a</sup> Except for males that fail to sire which are also selected. These males will be processed as noted in table 1 of [ATTACHMENT C](#).

<sup>b</sup> For the testes of all males that fail to sire or died before mating detailed qualitative examination will be made, taking into account the tubular stages of the spermatogenic cycle. The examination will be conducted in order to identify treatment related effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Any cell- or stage-specificity of testicular findings will be noted.

<sup>c</sup> Efforts will be made to evaluate all protocol-required tissues microscopically; however, it is not always feasible for every protocol-required tissue to be present on every slide. Protocol-required tissues that are not examined will be documented in the histopathology data and the impact of these missing tissues on the study will be documented in the pathology report.

**ATTACHMENT D**

Certificate of Analysis

**Certificate of analysis**

Page 1 of 1

**THE DOW CHEMICAL COMPANY**

Certificate of analysis		Quality order	
Product name	CANSOLV ABSORBANT DS	Customer Batch number	D2921BF000
Reference number		Batch number	
		Reference lot	

Test	Unit	Test result	Min	Max
% Water	%	51.14	51	54
Appearance		Pass	-	-
Gardner Color	Color	3	-	6
Foam Height	ml	75	-	100
Foam Break Time	Seconds	7.7	-	15
Gas Chromatography, HEP	%	9.01	-	14
Gas Chromatography, DIHEP	%	89.69	80	100
Gas Chromatograph Heavies GT DHEP	%	0.4	-	5
Gas Chromatography, Piperazine	%	0	-	2
Gas Chromatograph EG & DEG	%	0.2	-	0.6
Cansolv DS Alkalinity	meq/g	5.76	-	-

Notes: Tank 105

**APPROVED BY: KENDRICK JUNIUS**



**STUDY PLAN AMENDMENT NO. 1**

**Test Facility Study No. 20172120**

**Combined 28-Day Repeated Dose Toxicity Study with the  
Reproduction/Developmental Toxicity Screening Test of  
DiHEP Aqueous Solution by Oral Gavage in Rats**

**SPONSOR:**

Shell International B.V.  
Carel van Bylandtlaan 16  
2596 HR The Hague  
The Netherlands

**TEST FACILITY:**

Charles River Laboratories Den Bosch B.V.  
Hambakenwetering 7  
5231 DD 's-Hertogenbosch  
The Netherlands



**SUMMARY OF CHANGES AND JUSTIFICATIONS****Study Plan effective date: 16 Jan 2018**

Note: When applicable, additions are indicated in bold underlined text and deletions are indicated in bold strikethrough text in the affected sections of the document.

<b>Item or Section(s)</b>	<b>Justification</b>
<b>Amendment No. 1</b>	<b>Effective Date: 20 May 2019</b>
18. Terminal Procedures – F <sub>0</sub> -Generation	At request of the histopathologist. A treatment-related microscopic finding is suspected in these organs.

## TABLE OF CONTENTS

SUMMARY OF CHANGES AND JUSTIFICATIONS .....	2
1. OBJECTIVE(S) .....	5
2. PROPOSED STUDY SCHEDULE .....	5
3. GUIDELINES FOR STUDY DESIGN .....	6
4. REGULATORY COMPLIANCE .....	6
5. QUALITY ASSURANCE .....	6
6. SPONSOR .....	7
7. RESPONSIBLE PERSONNEL .....	7
8. DOSE RANGE FINDER (DRF) .....	8
9. TEST ITEM .....	10
10. SAFETY .....	11
11. DOSE FORMULATION AND ANALYSIS .....	11
12. TEST SYSTEM .....	12
13. HUSBANDRY .....	13
14. EXPERIMENTAL DESIGN .....	15
15. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F <sub>0</sub> - GENERATION .....	18
16. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F <sub>1</sub> - GENERATION .....	22
17. LABORATORY EVALUATIONS .....	23
18. TERMINAL PROCEDURES – F <sub>0</sub> -GENERATION .....	26
19. HISTOLOGY AND HISTOPATHOLOGY .....	28
20. TERMINAL PROCEDURES – F <sub>1</sub> -GENERATION .....	28
21. CONSTRUCTED VARIABLES .....	29
22. STATISTICAL ANALYSIS .....	30
23. COMPUTERIZED SYSTEMS .....	31
24. AMENDMENTS AND DEVIATIONS .....	31
25. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS .....	32
26. REPORTING .....	33
27. ANIMAL WELFARE .....	33

AMENDMENT APPROVAL .....34  
ATTACHMENT A .....35  
ATTACHMENT B .....36  
ATTACHMENT C .....37  
ATTACHMENT D .....41

## 1. OBJECTIVE(S)

The objectives of this study are to determine the potential toxic effects of DiHEP Aqueous Solution when given orally by gavage for a minimum of 28 days to Wistar Han rats, and to evaluate the potential to affect male and female reproductive performance such as gonadal function, mating behavior, conception, parturition and early postnatal development.

In addition, parental, reproduction (up to and including implantation) and developmental (from implantation onwards) No Observed Adverse Effect Levels (NOAELs) will be evaluated.

## 2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Experimental Start Date:	18 Jan 2019 (First date of study-specific data collection; randomization dose range finder)
Experimental Completion Date:	27 Jun 2019 (Last date data are collected from the study)
Animal Arrival:	Males: 30 Jan 2019 Females: 16 Jan 2019
Initiation of Estrous Cycle Determination (Pretest Period):	22 Jan 2019
Initiation of Dosing:	05 Feb 2019
Initiation of Mating:	19 Feb 2019
Necropsy Males incl. Blood Sampling:	06 Mar 2019 <sup>a</sup>
Delivery of Litters (PND 1):	≥ 13 Mar 2019
Measurement Anogenital Distance (PND 1):	≥ 13 Mar 2019
Culling of F <sub>1</sub> -pups incl. Blood Sampling (PND 4):	≥ 16 Mar 2019
Determination Areola/Nipple Retention (PND 13):	≥ 25 Mar 2019
Necropsy Females and Pups incl. Blood Sampling (PND 14-16):	≥ 26 Mar 2019
Completion of In-life:	12 Apr 2019 (Last date of necropsy)
Unaudited Draft Report:	28 Jun 2019

PND = postnatal day

<sup>a</sup> Necropsy and blood sampling dates of any males used for an extension of the mating phase will be specified and approved in the study files by the Study Director.

### 3. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective(s), the overall product development strategy for the test item, and the following study design guidelines:

- OECD Guideline 422. *Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*, July 2016.
- EPA Health Effects Test Guideline OPPTS 870.3650: *Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*, July 2000.

In addition, the procedures described in this study plan essentially conform to the following guidelines.

- OECD Guideline 421. *Reproduction/Developmental Toxicity Screening Test*, July 2016.
- EPA Health Effects Test Guideline OPPTS 870.3550: *Reproduction/Developmental Toxicity Screening Test*, July 2000.
- Council Regulation (EC) No 440/2008 Part B: *Methods for the Determination of Toxicity and other Health Effects; B.7: "Repeated Dose (28 days) Toxicity (oral)"*. Official Journal of the European Union No. L142, May 2008.
- OECD Guideline 407. *Repeated Dose 28-day Oral Toxicity Study in Rodents*, October 2008.
- EPA Health Effects Test Guideline OPPTS 870.3050: *Repeated dose 28-day oral toxicity study in rodents*, July 2000.

### 4. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

### 5. QUALITY ASSURANCE

#### 5.1 Test Facility

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the study plan, conduct study and/or process inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Test Facility QAU contact is indicated below:

C.J. Mitchell, BSc.  
Address as cited for Test Facility  
Tel: +31 73 640 6700  
E-mail: QADenBosch@crl.com

## 6. SPONSOR

### Sponsor Representative

M. Rooseboom, PhD, ERT

Tel: +31.70.377.3463

E-mail: Martijn.Rooseboom@shell.com

## 7. RESPONSIBLE PERSONNEL

### Study Director

D. van den Oetelaar, MSc

address as cited for Test Facility

Tel: +31 73 640 6700

E-mail: daphne.vandenoetelaar@crl.com

### Management Contact

H.H. Emmen, MSc

address as cited for Test Facility

Tel: +31 73 640 6700

E-mail: harry.emmen@crl.com

### Individual Scientist (IS) at the Test Facility

Histopathology

E.J.M. Lambregts, DVM

address as cited for Test Facility

Tel: +31 73 640 6700

E-mail: ankie.lambregts@crl.com

Each IS is required to report any deviations or other circumstances that could affect the quality or integrity of the study to the Study Director in a timely manner. Each IS will provide a report addressing their assigned phase of the study, which will be included as an appendix to the Final Report. The phase report will include the following:

- A listing of critical computerized systems used in the conduct and/or interpretation of the assigned study phase

## 8. Dose range finder (DRF)

A dose range finder (Test Facility Study No. 521627) will be conducted to determine the tolerability of the test item when administered as received. No testing guidelines are applicable as this dose range finder is intended for confirmation of tolerability only.

### 8.1 Proposed Study Schedule (DRF)

Initiation of Dosing:	Group 1	18 Jan 2019
Necropsy:	Group 1	23 Jan 2019

### 8.2 Test Item (DRF)

The information, procedures and safety instructions will be identical to those used during the main study. See sections 9 and 10 for details.

### 8.3 Dose Formulations and Analysis (DRF)

The preparation of test item will be identical as for the main study. See section 11 for details.

The test item will be used as received from the Sponsor; therefore, samples for dose formulation analysis will not be collected by the Test Facility.

### 8.4 Test System (DRF)

Species:	Rat.
Strain:	CrI: WI(Han).
Condition:	Outbred, SPF-Quality.
Source:	Charles River Deutschland, Sulzfeld, Germany or Charles River Laboratories France, L'Arbresle Cedex, France. Details will be documented in raw data and report.

Number of Females	3 (nulliparous and non-pregnant).
-------------------	-----------------------------------

Target Age at the Initiation of Dosing:	10-12 weeks.
---	--------------

Target Weight at the Initiation of Dosing:	200 to 250 g.
--	---------------

The actual age and weight of animals received will be listed in the Final report.

At study assignment, each animal will be identified using earmark and tailmark.

Animals will be assigned at random at the discretion of the biotechnician.

At least upon receipt of the animals, a health inspection will be performed and any assigned animals considered unsuitable for use in the study will be replaced by alternate animals maintained under the same environmental conditions.

The justification of test system and environmental acclimation will be identical as for the main study. See section 12 for details.

### 8.5 Husbandry (DRF)

On arrival and following randomization, animals will be group housed (up to 3 animals of the same dosing group together) in polycarbonate cages (Macrolon, MIV type, height 18 cm).

Other conditions will be identical as for the main study. See section 13 for details.

### 8.6 Experimental design (DRF)

Group No.	Test Item Id.	Dose Level (mg/kg)	Dose Volume (mL/kg) <sup>a</sup>	Dose Concentration (mg/mL) <sup>b</sup>	Number of Females	Animal Numbers
1	DiHEP Aqueous Solution	1000	2.07	482.9	3	1-3

Id.= identification.

<sup>a</sup> Dose volume will be calculated as dose level (mg/kg) / corrected test item concentration (mg/ml).

<sup>b</sup> The test item consists of 43.9% DiHEP, test item concentration will be corrected for % DiHEP and specific gravity (factor: 1.1). See section 11.1 for details.

The test item and vehicle will be administered to the appropriate animals by once daily oral gavage for a minimum of 5 days.

A dose control system (DCS) will be used as additional check to verify the dosing procedure according to Standard Operating Procedures (Study No. 521627 will be used for DCS).

The justification of the route of administration is identical as for the main study. See section 14.2 for details. To determine the tolerability of the test item when administered as received, animals will be dosed at the highest dose of the main study (1000 mg/kg) as the dose volume is the largest at this dose level.

### 8.7 In-life Procedures, Observations, and Measurements (DRF)

Only the following observations and measurements will be performed.

Mortality: Twice daily throughout the study.

Clinical Observations: At least once daily up to the day prior to necropsy, during the following observation intervals:

Actual time after dosing	Reported as hours after dosing	Nominal time <sup>a</sup>
0-15 min	0 hour	07:00
1 hr ± 15 min	1 hour	08:00
3 hr ± 30 min	3 hours	10:00

<sup>a</sup> Nominal times will be used for computer registration only.

Body Weights: On Day 1 prior to dosing and on Days 3 and 5.

In order to monitor the health status animals may be weighed more often. This will be documented in the study raw data.

Food Consumption Over Days 1-3 and 3-5.

### 8.8 Terminal Procedures (DRF)

All animals will be subjected to an external, thoracic and abdominal examination on Day 6 (scheduled necropsy) or sooner (decedents). Based on the high pH of the test item (see section 9.1), special attention will be paid to the esophagus and stomach at necropsy.

Animals will not be deprived of food prior to necropsy. Gross lesions will be recorded but will not be retained, no organs will be fixed and histopathological examination will not be performed.



## 8.9 Reporting (DRF)

The procedures of statistical analysis, computerized systems, amendments and deviations, and retention of records will be identical as for the main study, if applicable. See sections 22, 23, 24, and 25 for details.

A summary of results of the dose range finder will be included in report 20172120 as an appendix.

## 9. TEST ITEM

### 9.1 Test Item

Identification:	DiHEP Aqueous Solution <sup>1</sup>
Batch (Lot) Number:	D2921BF000
Expiry date:	31 August 2020 (expiry date)
Physical Description:	Yellow aqueous liquid
Purity/Composition:	See Certificate of Analysis <sup>2</sup>
Storage Conditions:	At room temperature protected from light

#### Additional information

Test Facility Test Item Number:	209813/A
Purity/Composition correction factor:	Correct for percentage solid matter and additionally for percentage DiHEP.
Test item handling:	No specific handling conditions required
Chemical name (IUPAC, synonym or trade name):	1,4-Bis(2-hydroxyethyl)piperazine Dihydroxyethylpiperazine
CAS number:	122-96-3
Molecular formula:	C <sub>8</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>
Molecular weight:	174.24
Irritant or corrosive:	Yes
pH:	13
Specific gravity / density:	1.1 kg/m <sup>3</sup> at 20°C

### 9.2 Test Item Characterization

The Sponsor will provide to the Test Facility documentation of the identity, purity, composition, and stability for the test item. If available, a Certificate of Analysis or equivalent documentation will be provided for inclusion in the Final Report. The Sponsor

<sup>1</sup> DiHEP stands for Dihydroxyethylpiperazine.

<sup>2</sup> For Certificate of Analysis see [ATTACHMENT D](#).

will also provide information concerning the regulatory standard that was followed for these evaluations.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test item, and this information is available to the appropriate regulatory agencies should it be requested.

### **9.3 Analysis of Test Item**

The stability of the bulk test item will not be determined during the course of this study. Information to support the stability of each lot of the bulk test item will be provided by the Sponsor.

### **9.4 Reserve Samples**

For each batch (lot) of test item, if practically possible a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility and destroyed after the expiry date.

### **9.5 Test Item and Vehicle Inventory and Disposition**

Records of the receipt, distribution, storage, and disposition of test item will be maintained. With the exception of reserve samples, all unused Sponsor-supplied bulk test item will be discarded.

## **10. SAFETY**

The following safety instructions apply to this study:

Standard safety precautions specified in Charles River Den Bosch procedures.

## **11. DOSE FORMULATION AND ANALYSIS**

### **11.1 Preparation of Test Item**

The test item, DiHEP Aqueous Solution will be administered as received. An adequate amount of the test item will be dispensed into daily aliquots, which will be stored in a controlled temperature area set to maintain 21°C until use.

Test item for dosing will be kept at room temperature until dosing. Adjustment will be made for specific gravity of the test item. A factor of 43.9 will be used to correct for the purity/composition of the test item (based on the 48.9% solid matter of which 89.7% is DiHEP).

Any residual volumes will be discarded.

### **11.2 Sample Collection and Analysis**

The test item will be used as received from the Sponsor; therefore, samples for dose formulation analysis will not be collected by the Test Facility.

## 12. TEST SYSTEM

Species:	Rat.
Strain:	CrI: WI(Han).
Condition:	Outbred, SPF-Quality.
Source:	Charles River Deutschland, Sulzfeld, Germany or Charles River Laboratories France, L'Arbresle Cedex, France. Details will be documented in raw data and report.
Number of Males:	40.
Number of Females:	48 (nulliparous and non-pregnant).
Number of Pups Expected:	Approximately 480 pups (40 litters x 12 pups).
Target Age at the Initiation of the Pretest Period:	Females: approximately 10-12 weeks.
Target Age at the Initiation of Dosing:	Males: approximately 10-12 weeks. Females: approximately 12-14 weeks.
Target Weight at the Initiation of Dosing:	Males: 250 to 350 g. Females: 200 to 250 g.

The actual age and weight of animals received will be listed in the Final report.

### 12.1 Justification of Test System and Number of Animals

The Wistar Han rat was chosen as the animal model for this study as it is an accepted rodent species for toxicity testing by regulatory agencies. Charles River Den Bosch has general and reproduction/developmental historical data in this species from the same strain and source. This animal model has been proven to be susceptible to the effects of reproductive toxicants.

The total number of animals to be used in this study is considered to be the minimum required to properly characterize the effects of the test item. This study has been designed such that it does not require an unnecessary number of animals to accomplish its objectives.

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

### 12.2 Animal Identification

Prior to start of the pretest period (females) or treatment period (males), each allocated animal will be identified using earmark and tattoo. Prior to start of the pretest period, reserve females will be numbered R1 through R8 at random by indelible marker. Any reserve female replacing an allocated female prior to treatment will receive identification by earmark and tattoo.

Pups will be identified on postnatal day (PND) 1. They will be randomized per litter and individually identified by means of subcutaneous injection of Indian ink. When general hair growth blurs the identification, the pups will be identified by tattoo on the feet.

### **12.3 Environmental Acclimation**

The animals will be allowed to acclimate to the Test Facility toxicology accommodation for at least 5 days prior to start of the pretest period (females) or at least 5 days before the commencement of dosing (males).

### **12.4 Selection, Assignment, Replacement, and Disposition of Animals**

A total of 40 females will be selected at randomization before initiation of the pretest phase. Each selected female classified as not having regular estrous cycles during the pretest phase will be replaced before initiation of dosing by one of the 8 additional females having regular estrous cycles, if feasible. A total of 40 females with regular estrous cycles will continue in the study. The supernumerary females will then be removed from the study, and their estrous cycle results will be kept in the raw data but will not be reported.

Animals will be assigned to groups by a computer-generated random algorithm according to body weights, with all animals within  $\pm 20\%$  of the sex mean. Males and females will be randomized separately. Animals in poor health or at extremes of body weight range will not be assigned to groups.

At least upon receipt of the animals, a health inspection will be performed and any assigned animals considered unsuitable for use in the study will be replaced by alternate animals obtained from the same shipment and maintained under the same environmental conditions.

After initiation of dosing, study animals may be replaced during the replacement period with alternate animals in the event of accidental injury, non-test item-related health issues, or similar circumstances. The alternate animals may be used as replacements on the study within 1 to 3 days.

The disposition of all animals will be documented in the study records.

## **13. HUSBANDRY**

### **13.1 Housing**

On arrival and following the pretest (females only) and pre-mating period, animals will be group housed (up to 5 animals of the same sex and same dosing group together) in polycarbonate cages (Macrolon, MIV type, height 18 cm).

During the mating phase, males and females will be cohabitated on a 1:1 basis in Macrolon plastic cages (MIII type, height 18 cm).

During the post-mating phase, males will be housed in their home cage (Macrolon plastic cages, MIV type, height 18 cm) with a maximum of 5 males/cage. Females will be individually housed in Macrolon plastic cages (MIII type, height 18 cm).

During the lactation phase, females will be housed in Macrolon plastic cages (MIII type, height 18 cm). Pups will be housed with the dam, except during locomotor activity monitoring of the dams, when the pups will be kept warm in their home cage using bottles filled with warm water. In order to avoid hypothermia of pups, pups should not be left without their dam or a bottle filled with warm water for longer than 30-40 minutes.

During locomotor activity monitoring, F<sub>0</sub>-animals will be housed individually in a Hi-temp polycarbonate cage (Ancare corp., USA; dimensions: 48.3 x 26.7 x 20.3 cm) without cage-enrichment, bedding material, food and water.

The cages will contain appropriate bedding (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) and will be equipped with water bottles. The housing conditions will be maintained unless deemed inappropriate by the Study Director and/or Clinical Veterinarian. The room(s) in which the animals will be kept will be documented in the study records.

Animals will be separated during designated procedures/activities. Each cage will be clearly labeled with a color-coded cage card indicating Test Facility Study No., group, animal number(s), and sex.

### **13.2 Environmental Conditions**

The target conditions for animal room environment will be as follows:

Temperature:	18 to 24°C.
Humidity:	40 to 70%.
Light Cycle:	12-hours light and 12-hours dark (may be interrupted for designated procedures).
Ventilation:	At least 10 air changes per hour.

Any variations to these conditions will be evaluated and maintained in the raw data.

### **13.3 Food**

Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) will be provided *ad libitum* throughout the study, except during designated procedures. During motor activity measurements, animals will not have access to food for a maximum of 2 hours.

The feed is analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis are provided by the supplier and are on file at the Test Facility.

It is considered that there are no known contaminants in the feed that would interfere with the objectives of the study.

### **13.4 Water**

Municipal tap water will be freely available to each animal via water bottles. During motor activity measurements, animals will not have access to water for a maximum of 2 hours.

Periodic analysis of the water is performed, and results of these analyses are on file at the Test Facility.

It is considered that there are no known contaminants in the water that would interfere with the objectives of the study.

### 13.5 Animal Enrichment

For psychological/environmental enrichment and nesting material, animals will be provided with paper (Enviro-dri, Wm. Lilico & Son (Wonham Mill Ltd), Surrey, United Kingdom), except when interrupted by study procedures/activities.

### 13.6 Veterinary Care

Veterinary care will be available throughout the course of the study and animals will be examined by the veterinary staff as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments, if any, will be documented in the study records.

In the event that animals show signs of illness or distress, the responsible veterinarian may make initial recommendations about treatment of the animal(s) and/or alteration of study procedures, which must be approved by the Study Director. All such actions will be properly documented in the study records and, when appropriate, by study plan amendment. Treatment of the animal(s) for minor injuries or ailments may be approved without prior consultation with the Sponsor Representative when such treatment does not impact fulfillment of the study objectives. If the condition of the animal(s) warrants significant therapeutic intervention or alterations in study procedures, the Sponsor Representative will be contacted, when possible, to discuss appropriate action. If the condition of the animal(s) is such that emergency measures must be taken, the Study Director and/or attending veterinarian will attempt to consult with the Sponsor Representative prior to responding to the medical crisis, but the Study Director and/or veterinarian has authority to act immediately at his/her discretion to alleviate suffering. The Sponsor representative will be fully informed of any such events.

## 14. EXPERIMENTAL DESIGN

Group No.	Test Item Id.	Dose Level (mg/kg)	Dose Volume (mL/kg) <sup>a</sup>	Corrected Test Item Concentration (mg/mL) <sup>b</sup>	Number of Animals		Animal Numbers	
					Males	Females	Males	Females
1	-	0 (Control) <sup>c</sup>	2.07	-	10	10	01-10	41-50
2	DiHEP	100	0.21	482.9	10	10	11-20	51-60
3	Aqueous	300	0.62		10	10	21-30	61-70
4	Solution	1000	2.07		10	10	31-40	71-80

TBD. = To be determined. Id.= identification.

<sup>a</sup> Dose volume will be calculated as dose level (mg/kg) / corrected test item concentration (mg/ml).

<sup>b</sup> The test item consists of 43.9% DiHEP, test item concentration will be corrected for % DiHEP and specific gravity (factor: 1.1). See section 11.1 for details.

<sup>c</sup> Test-item treated animals will receive undiluted test item and consequently, no vehicle will be used. Control animals will be dosed with water (Elix) in the same dose volume as Group 4.

The following 5 animals/sex/group are selected for functional tests, clinical pathology, collection of full list of organs/tissues at macroscopic examination, organ weights (full list) and histopathology (full list), see also respective paragraphs:

Group No.	Animal numbers	
	Males	Females <sup>a</sup>
1	01-05	To be selected
2	11-15	To be selected
3	21-25	To be selected
4	31-35	To be selected

<sup>a</sup> Females with live pups, if feasible. These animals will be selected and approved by the Study Director in the study files. The selected female animal numbers will be specified in the report.

#### 14.1 Administration of Test item

The test item and vehicle will be administered to the appropriate animals by once daily oral gavage 7 days a week for a minimum of 28 days. Males will be treated for a minimum of 28 days, up to and including the day before scheduled necropsy. This includes a minimum of two weeks prior to mating and during the mating period. Females will be treated for at least 14 days prior to mating (with the objective of covering at least two complete estrous cycles), the variable time to conception, the duration of pregnancy and at least 13 days after delivery, up to and including the day before scheduled necropsy. Females will not be dosed during littering.

The dose volume for each animal will be based on the most recent body weight measurement. Dose volumes  $\leq 50 \mu\text{L}$  will be administered using a plastic feeding tube which is connected to a digital syringe and dose volumes  $> 50 \mu\text{L}$  will be given with a plastic feeding tube connected to an appropriately graded syringe. The accuracy of the digital syringe will be determined prior to start dosing at relevant dose levels for this study.

The first day of dosing will be designated as Day 1 (exception: alternate animals used for replacement after Day 1 will assume the day of the animal being replaced).

A dose control system (DCS) will be used as additional check to verify the dosing procedure according to Standard Operating Procedures (Study No. 521417 will be used for DCS).

Pups will not be treated directly but could potentially be exposed to the test item in utero, via maternal milk, or from exposure to maternal urine/feces.

#### 14.2 Justification of Route and Dose Levels

The oral route of administration was selected because this is the recommended route by OECD TG 422, REACH regulation and ECHA guidelines. In addition, the test material is a non-volatile aqueous solution.

The dose levels were selected based on information provided by the Sponsor (data on file at Sponsor site), and in an attempt to produce graded responses to the test item.

A previously performed acute toxicity study with DiHEP Aqueous Solution via oral gavage in Sprague Dawley (SD) rats indicated a low acute toxicity ( $\text{LD50}_{\text{males}}$  was 20,093 mg/kg and  $\text{LD50}_{\text{females}}$  was 18,738 mg/kg).

In addition, multiple studies were performed with several structural analogs:

- A dietary 7-day toxicity study was performed with a hydroxypiperazine solution (containing 12-20% piperazine, 38-47% hydroxypiperazine, 16-25% dihydroxypiperazine, 17-26% water). During this study Wistar han rats received 590, 1420, and 3720 mg/kg for males and 680, 1610 and 3970 mg/kg for female rats. A slight body weight decrease was observed in females fed 3970 mg/kg/day but was not observed in females fed lower doses or in males. This body weight decrease was statistically significant after days 1 and 4 but not day 7. Remaining parameters were considered unaffected by treatment.
- A dietary 90-day toxicity study was performed with analog piperazine. During this study, SD rats received 400, 1200 and 2394 mg/kg by dietary administration. A dose related decrease in body-weight gain (a decrease of 10% in high dose animals when compared with concurrent control) was noted. Remaining parameters were considered unaffected by treatment.
- A dietary 90-day toxicity study was performed with analog anhydrous piperazine. Rats received 1000, 3000 and 10000 ppm (corresponding to 50, 150 and 500 mg/kg/day piperazine base). At 10000 ppm histopathological degenerative changes were noted in the liver and kidney, at 3000 ppm similar changes were noted to a lesser extent and at 1000 ppm no adverse effects were noted. In addition, at 10000 ppm a decrease in body weight gain was noted (statistically significant in females only). Remaining parameters were considered unaffected by treatment.
- A developmental toxicity study was performed, in which pregnant SD females received 0, 105, 420 and 2100 mg/kg piperazine base during days 6-15 by oral gavage. In high-dose females, excessive salivation, lethargy and a reduction in body weight gain and food consumption were noted. Remaining parameters, including pre- and post-implantation loss, litter size and sex ratio, were considered unaffected by treatment.
- In a dietary two generation study with piperazine dihydrochloride, SD rats received 0, 5000, 12000 and 25000 ppm (corresponding to 0, 125, 300 and 625 mg/kg piperazine base). The mid-dose was considered as LOAEL, with effects mainly on fertility (i.e. reduced pregnancy index and decreased number of implantation sites). These effects were not observed in the developmental toxicity study, which is be considered to support that the effect on fertility are the main effect of piperazine on reproduction.

Based on the observed low acute toxicity of DiHEP Aqueous Solution and the effects noted in the studies performed with structural analogs, 0, 100, 300 and 1000 mg/kg were selected as dose levels for this study.

The high-dose level should produce some toxic effects, but not death nor obvious suffering. The mid-dose level is expected to produce minimal to moderate toxic effects. The low-dose level should produce no observable indications of toxicity.



## **15. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F<sub>0</sub>-GENERATION**

The in-life procedures, observations, and measurements listed below will be performed for parental animals.

### **15.1 Mortality/Moribundity Checks – F<sub>0</sub>-Generation**

Frequency: At least twice daily throughout the study.

Procedure: Animals will be observed for general health/mortality and moribundity. Animals will not be removed from cage during observation, unless necessary for identification or confirmation of possible findings.

### **15.2 Clinical Observations – F<sub>0</sub>-Generation**

Frequency: During treatment, animals will be observed at least once daily, up to the day prior to necropsy.

These clinical observations will at least be conducted after dosing at no specific time point, but within a similar time period after dosing for the respective animals.

Procedure: Animals will be observed for specific clinical signs. The time of onset, grade and duration of any observed signs will be recorded. Signs will be graded for severity and the maximum grade will be predefined at 3 or 4. Grades will be coded as slight (grade 1), moderate (grade 2), severe (grade 3) and very severe (grade 4). For certain signs, only its presence (grade 1) or absence (grade 0) will be scored. In the data tables, the scored grades will be reported, as well as the percentage of animals affected in summary tables.

#### **15.2.1 Arena Observations – F<sub>0</sub>-Generation**

Frequency: Once before the first administration of the test item and at weekly intervals during the treatment period.

Procedure: Animals will be observed for specific clinical signs in a standard arena. The time of onset, grade and duration of any observed signs will be recorded.

**15.3 Body Weights – F<sub>0</sub>-Generation**

Frequency: Males and females will be weighed on the first day of treatment (prior to dosing), and weekly thereafter. Mated females will be weighed on Days 0, 4, 7, 11, 14, 17, and 20 post-coitum and during lactation on PND 1, 4, 7, and 13.

In order to monitor the health status animals may be weighed more often. This will be documented in the study raw data.

Procedure: Animals will be individually weighed.

**15.4 Food Consumption – F<sub>0</sub>-Generation**

Frequency: Weekly, except for males and females which are housed together for mating and for females without evidence of mating. Food consumption of mated females will be measured on Days 0, 4, 7, 11, 14, 17, and 20 post-coitum and during lactation on PND 1, 4, 7, and 13.

Procedure: Food consumption will be quantitatively measured.

**15.4.1 Water Consumption – F<sub>0</sub>-Generation**

Frequency: Regular basis throughout the study.

Procedure: Water consumption will be monitored by visual inspection of the water bottles. If inter group differences are noted, consumption may be assessed by weight.

### 15.5 Functional Tests – F<sub>0</sub>-Generation

- Frequency: Once during the treatment period. The selected 5 males will be tested once during Week 4 of treatment and the selected 5 females will be tested once during the last week of lactation (i.e. PND 6-13). These tests will be performed after clinical observations and arena observation, if applicable.
- Procedure: The following tests will be performed :
- hearing ability, pupillary reflex and static righting reflex (score 0 = normal/present, score 1 = abnormal/absent).
  - fore- and hind-limb grip strength will be recorded as the mean of three measurements , using a grip strength meter.
  - locomotor activity (recording period: 1 hour under normal laboratory light conditions) will be tested using the Kinder Scientific Motor Monitor System. Total movements and ambulations will be reported. Ambulations represent movements characterized by a relocation of the entire body position like walking, whereas total movements represent all movements made by the animals, including ambulations but also smaller or finer movements like grooming, weaving or movements of the head.

### 15.6 Estrous Cycle Evaluations – F<sub>0</sub>-Generation

- Frequency: Daily vaginal lavage will be performed beginning 14 days prior to treatment (pretest period), the first 14 days of treatment and during mating until evidence of copulation is observed. Vaginal lavage will continue for those females with no evidence of copulation until termination of the mating period.
- On the day of necropsy, a vaginal lavage will also be taken to determine the stage of estrus. This will be done for all females, except for females that have to be euthanized in extremis or die spontaneously.
- Procedure: Estrous cycles will be evaluated by examining the vaginal cytology of samples obtained by serial vaginal lavage procedures.

### 15.7 Cohabitation/Mating Procedure – F<sub>0</sub>-Generation

- Frequency: Daily, after a minimum of 14 days of treatment. The mating period will consist of a maximum of 14 consecutive days.
- Procedure: Animals will be cohabitated on a 1:1 basis within the same treatment group, avoiding sibling mating. Detection of mating will be confirmed by evidence of sperm in the vaginal lavage or by the appearance of an intravaginal copulatory plug. This day will be designated Day 0 post-coitum. Once mating has occurred, the males and females will be separated.
- A maximum of 14 days will be allowed for mating, after which females who have not shown evidence of mating will be separated from their males. In case less than 9 females per group have shown evidence of mating, each non-mated female may be re-mated once with a male for a maximum of 7 days (if possible). A male of the same group having previously shown evidence of mating (non-selected male if possible, see section 14) will be used for re-mating.

### 15.8 General Reproduction Data – F<sub>0</sub>-Generation

- Frequency: Daily from the mating period onwards.
- Procedure: Male number paired with, mating date, confirmation of pregnancy, and delivery day will be recorded. Palpation may be used to aid in confirmation of pregnancy.
- The females will be allowed to litter normally. Postnatal day (PND) 1 is defined as the day when a litter is found completed (i.e. membranes and placentas cleaned up, nest built and/or feeding of pups started). The day prior to PND 1 is considered to be the day when the female started to deliver and is defined as PND 0 and used for recording of delivery. Females that are littering will be left undisturbed.
- Cage debris of pregnant females will be examined for evidence of premature delivery. Signs of difficult or prolonged parturition will be recorded, if applicable.
- Deficiencies in maternal care, such as inadequate construction or cleaning of the nest, pups left scattered and cold, physical abuse of pups or apparently inadequate lactation or feeding, will be recorded, if applicable.

## **16. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS – F<sub>1</sub>-GENERATION**

The in-life procedures, observations, and measurements listed below will be performed for the pups.

### **16.1 Mortality/Moribundity Checks – F<sub>1</sub>-Generation**

Frequency: The number of live and dead pups will be determined on PND 1 and daily thereafter.

Procedure: Pups will be observed for general health/mortality and moribundity. If possible, defects or cause of death will be evaluated. Pups will not be removed from the cage during observation, unless necessary for identification or confirmation of possible findings.

### **16.2 Clinical Observations – F<sub>1</sub>-Generation**

Frequency: At least once daily.

Procedure: Detailed clinical observations will be made for all pups. Only days on which clinical signs are present between the first and last litter check will be given in the respective report tables.

### **16.3 Body Weights – F<sub>1</sub>-Generation**

Frequency: On PND 1, 4, 7, and 13.

Procedure: Live pups will be individually weighed.

### **16.4 Sex – F<sub>1</sub>-Generation**

Frequency: On PND 1 and 4.

Procedure: Sex will be externally determined for all pups.

### **16.5 Anogenital Distance – F<sub>1</sub>-Generation**

Frequency: On PND 1.

Procedure: Anogenital distance (AGD) will be measured for all live pups. The AGD will be normalized to the cube root of body weight.

### **16.6 Areola/Nipple Retention – F<sub>1</sub>-Generation**

Frequency: On PND 13.

Procedure: All males in each litter will be examined for the number of areola/nipples.

## 16.7 Culling – F<sub>1</sub>-Generation

Frequency: On PND 4.

Procedure: To reduce variability among the litters, eight pups from each litter of equal sex distribution (if possible) will be selected. Blood samples will be collected from two of the surplus pups (if possible from one male and one female pup). Selective elimination of pups, e.g. based upon body weight or AGD, will not be done. Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is acceptable. See also sections [17.1.1](#) and [20.3](#).

## 17. LABORATORY EVALUATIONS

### 17.1 Clinical Pathology

#### 17.1.1 Sample Collection

Blood of F<sub>0</sub>-animals (except for animals which were sacrificed *in extremis* or found dead and females with total litter loss) will be collected on the day of scheduled necropsy. Samples will be collected, between 7.00 and 10.30 a.m., from the retro-orbital sinus under anesthesia using isoflurane in the animal facility. Additional blood samples may be obtained (e.g. due to clotting of non-serum samples) in both the animal facility and in the necropsy room if permissible sampling frequency and blood volume are not exceeded. After collection, samples will be transferred to the appropriate laboratory for processing.

F<sub>0</sub>-males (except for animals which were sacrificed *in extremis* or found dead) will be fasted overnight with a maximum of 24 hours before blood sampling, but water will be available. F<sub>0</sub>-females will not be fasted overnight.

Blood of F<sub>1</sub>-animals will be collected on PND 4 and PND 14-16, if possible. This will be performed in the necropsy room.

On PND 4 at culling, blood will be collected from two surplus pups per litter (if possible) by decapitation, between 7.00 and 10.30 a.m., and will be pooled to one sample per litter. If available, blood will be collected from one male and one female pup. If only one surplus pup per litter is available at culling, as much as possible blood will be collected from this single pup. If the target volume of 0.5 mL cannot be reached by pooling from two pups, blood from a third surplus pup of the same litter should be added, if available.

On PND 14-16, separate blood samples will be collected from two pups per litter (from one male and one female, if possible). If the target volume of 1.0 mL/pup cannot be reached, a separate blood sample should be collected from another pup of the same litter and sex (if possible). Any incomplete blood sample will be discarded. Blood will be drawn, between 7.00 and 10.30 a.m., by aorta puncture under anesthesia using isoflurane as part of the necropsy procedure.

Samples will be collected according to the table below.

Samples for Clinical Pathology Evaluation

Animals	Time Point	Hematology	Coagulation	Clinical Chemistry	Thyroid Hormone
Selected F <sub>0</sub> -animals (5/sex/group) <sup>a, b</sup>	On the day of scheduled necropsy	X	X	X	X
Non-selected F <sub>0</sub> -animals (≤ 5/sex/group) <sup>a, b</sup>	On the day of scheduled necropsy	-	-	-	X
2 pups/litter	PND 4	-	-	-	X
2 pups/litter	PND 14-16	-	-	-	X

X = Sample to be collected; - = Not applicable.

<sup>a</sup> See section 14 for details of the selected F<sub>0</sub>-animals.

<sup>b</sup> Except for animals which were sacrificed *in extremis* or found dead, and females with total litter loss.

**17.1.2 Hematology**

Target Volume: 0.5 mL.

Anticoagulant: K<sub>3</sub>-EDTA (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).

Hematology Parameters

White blood cells (WBC)	Red Blood Cell Distribution Width (RDW)
Neutrophils (absolute)	Haemoglobin
Lymphocytes (absolute)	Haematocrit
Monocytes (absolute)	Mean corpuscular volume (MCV)
Eosinophils (absolute)	Mean corpuscular haemoglobin (MCH)
Basophils (absolute)	Mean corpuscular haemoglobin concentration (MCHC)
Red blood cells	Platelets
Reticulocyte (absolute)	

A blood smear will be prepared from each hematology sample. Blood smears will be labeled, stained, and stored. If additional examination of blood smears is deemed necessary, the smears may be subsequently evaluated and this evaluation will be described in a study plan amendment.

**17.1.3 Coagulation**

Target Volume: 0.45 mL.

Anticoagulant: Citrate (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).

Coagulation Parameters

Prothrombin Time (PT)	Activated Partial Thromboplastin Time (APTT)
-----------------------	--

**17.1.4 Clinical Chemistry**

Target Volume: 0.5 mL.  
 For bile acid measurement: 1.0 mL (same sample as for thyroid hormone measurement).

Anticoagulant: Li-Heparin (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).  
 Not applicable for serum tubes.

Processing: To serum (bile acids) or to plasma

Clinical Chemistry Parameters

Alanine aminotransferase (ALAT)	Creatinine
Aspartate aminotransferase (ASAT)	Glucose
Alkaline Phosphatase (ALP)	Cholesterol
Total protein	Sodium
Albumin	Potassium
Total Bilirubin	Chloride
Bile Acids	Calcium
Urea	Inorganic Phosphate (Inorg. Phos)

**17.1.5 Thyroid Hormone**

Target Volume: F<sub>0</sub>-animals: 1.0 mL (same sample as for bile acid measurement).  
 PND 4 pups: 0.5 mL in total (pooled).  
 PND 14-16 pups: 1.0 mL per pup.

Anticoagulant: Not applicable for serum. (tubes; Greiner Bio-One GmbH, Kremsmünster, Austria).

Thyroid Hormone Parameters

Thyroxine (T4)	Thyroid-Stimulating Hormone (TSH; only if required)
----------------	---

After clotting and centrifugation, serum will be used as listed below.

F<sub>0</sub>-Males: Serum from each sample will be divided into 2 aliquots: 150 µL serum for measurement of total T4 and the remaining volume of serum for possible future measurement of TSH (added by study plan amendment if applicable).

F<sub>0</sub>-Females: The serum will be used for possible future measurement of total T4 and/or thyroid-stimulating hormone TSH (added by study plan amendment if applicable).

PND 4 Pups: The pooled serum will be used for possible future measurement of total T4 (added by study plan amendment if applicable).

PND 14-16 Pups: Serum from each sample will be divided into 2 aliquots: 150 µL serum for measurement of total T4, and the remaining volume of serum for possible future measurement of TSH (added by study plan amendment if applicable).



Serum samples retained for possible future analysis will be maintained by the Test Facility in the freezer ( $\leq 75^{\circ}\text{C}$ ). Under these storage conditions, samples will be stable for 6 months. Any remaining sample will be discarded prior to finalization of the report

## 18. TERMINAL PROCEDURES – F<sub>0</sub>-GENERATION

Terminal procedures are summarized in the following table:

Terminal Procedures

Group No.	(Non) Selected Animals	No. of Animals		Scheduled Euthanasia	Necropsy Procedures			Histology and histopathology				
		M	F		Necropsy	Tissue Collection	Organ Weights					
1	Selected	5	5	Males: after a minimum of 28 days of administration  Females: PND 14-16	X	X <sup>a</sup>	X <sup>a</sup>	Full list				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
2	Selected	5	5					Gross lesions Target tissues <sup>c</sup> Reproductive tissues <sup>b</sup>				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
3	Selected	5	5					Gross lesions Target tissues <sup>c</sup> Reproductive tissues <sup>b</sup>				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
4	Selected	5	5					Full list				
	Non-selected	$\leq 5$	$\leq 5$					Gross lesions Reproductive tissues <sup>b</sup>				
Unscheduled Deaths (sacrificed <i>in extremis</i> or found dead)								X	X	-	Full list	

X = Procedure to be conducted; - = Not applicable.

<sup>a</sup> See Tissue Collection and Preservation table in [ATTACHMENT C](#) for listing of tissues.

<sup>b</sup> Reproductive tissues are applicable for males that fail to sire and females that fail to deliver pups (i.e. non-pregnant females, implantation sites only or no offspring) and females with total litter loss. See [ATTACHMENT C](#) for listing of tissues.

<sup>c</sup> **Target tissue: Adrenal glands and Stomach of selected males of Group 2 and 3, based on possible treatment-related changes in these tissues. Target tissues are applicable in case of possible treatment-related changes in any of the tissues of any animal in the high dose group. Then, histological examination will be extended to that particular tissue of the selected 5 animals of Groups 2 and 3.**

### 18.1 Unscheduled Deaths – F<sub>0</sub>-Generation

If an animal dies on study, a necropsy will be conducted and specified tissues will be saved, but not weighed. If necessary, the animal will be refrigerated to minimize autolysis.

Animals may be euthanized for humane reasons as per Test Facility SOPs. These animals will be deeply anaesthetized using isoflurane and subsequently exsanguinated. They will undergo necropsy, and specified tissues will be retained, but not weighed.

The specified tissues which will be retained are mentioned in [ATTACHMENT C](#).

### **18.2 Scheduled Euthanasia – F<sub>0</sub>-Generation**

Animals surviving until scheduled euthanasia will have a terminal body weight recorded and will be deeply anaesthetized using isoflurane and subsequently exsanguinated.

Scheduled necropsies are summarized below:

Males (which sire and fail to sire): Following completion of the mating period (a minimum of 28 days of administration).

Females which deliver: PND 14-16.

Females which fail to deliver: With evidence of mating: Post-coitum Days 25-27.  
Without evidence of mating: Approximately 24-26 days after the last day of the mating period.

Females with total litter loss: Dams with no surviving pups will be euthanized within 24 hours after the last pup is found dead or missing.

All males surviving to scheduled necropsy will be fasted overnight with a maximum of 24 hours before necropsy. Water will be available. F<sub>0</sub>- females will not be fasted overnight.

The specified tissues which will be retained are mentioned in [ATTACHMENT C](#).

### **18.3 Necropsy – F<sub>0</sub>-Generation**

All animals will be subjected to a full *post mortem* examination, with special attention being paid to the reproductive organs.

The numbers of former implantation sites will be recorded for all paired females.

In case no macroscopically visible implantation sites are present, nongravid uteri will be stained using the Salewski technique in order to detect any former implantation sites and the number of corpora lutea will be recorded in addition.

Necropsy procedures will be performed by qualified personnel with appropriate training and experience in animal anatomy and gross pathology. A veterinary pathologist, or other suitably qualified person, will be available.

### **18.4 Organ weights – F<sub>0</sub>-Generation**

The organs identified for weighing in the Tissue Collection and Preservation table in [ATTACHMENT C](#) will be weighed at necropsy for all scheduled euthanasia animals. Organ weights will not be recorded for animals found dead or euthanized in poor condition or *in extremis*. Paired organs will be weighed together. Organ weights as a percent of body weight (using the terminal body weight) will be calculated.

### **18.5 Tissue Collection and Preservation – F<sub>0</sub>-Generation**

Representative samples of the tissues identified in the Tissue Collection and Preservation table in [ATTACHMENT C](#) will be collected from all animals and preserved in 10% buffered formalin (neutral phosphate buffered 4% formaldehyde solution), unless otherwise indicated. Additional tissue samples may be collected to elucidate abnormal findings.

For females which fail to deliver a complete litter, uterine contents (i.e. any fetuses, placenta and implantation sites) will be fixed (if applicable), but will not be examined histopathologically in first instance.

## **19. HISTOLOGY AND HISTOPATHOLOGY**

### **19.1 Histology**

Tissues in the Tissue Collection and Preservation table in [ATTACHMENT C](#) from F<sub>0</sub>-animals identified in the Terminal Procedures table will be embedded in paraffin, sectioned at a thickness of 2-4 micrometers, mounted on glass slides, and stained with hematoxylin and eosin.

### **19.2 Histopathology**

All tissues as defined under Histology (section [19.1](#)) will be examined by a board-certified toxicological pathologist with training and experience in laboratory animal pathology. Target tissues identified by the study pathologist during microscopic evaluation will be communicated to the Study Director; tissues will be evaluated and reported.

Any additional stains or evaluations, if deemed necessary by the pathologist, will be added by study plan amendment following discussion with the Study Director and in consultation with the Sponsor.

At the discretion of the study pathologist and after acknowledgement by the Study Director, images may be captured for consultation purposes.

A peer review on the histopathology data will be performed by a second pathologist.

## **20. TERMINAL PROCEDURES – F<sub>1</sub>-GENERATION**

### **20.1 Method of Euthanasia – F<sub>1</sub>-Generation**

Pups, younger than 7 days will be euthanized by decapitation.

All remaining pups (PND 7-16), except the pups selected for blood collection, will be euthanized by an intraperitoneal injection of sodium pentobarbital (Euthasol® 20%).

The pups selected for blood collection on PND 14-16 will be anesthetized using isoflurane followed by exsanguination.

### **20.2 Unscheduled Deaths – F<sub>1</sub>-Generation**

Recognizable fetuses of females that die spontaneously or are euthanized *in extremis* will be examined externally and sexed (both externally and internally, if possible). Live fetuses will be euthanized by decapitation.

Pups that die or are euthanized before scheduled termination will also be examined externally and sexed (both externally and internally, if possible). Pups found dead during the weekend

can be fixed in identified containers containing 70% ethanol if not necropsied on the same day. The stomach of pups not surviving to the scheduled necropsy date will be examined for the presence of milk, if possible. If possible, defects or cause of death will be evaluated.

### 20.3 Scheduled Euthanasia – F<sub>1</sub>-Generation

On PND 4, the surplus pups (> 8 pups per litter) will be euthanized by decapitation. Sex will be determined both externally and internally. From two surplus pups per litter, blood will be collected, if possible. For details see also sections 16.7 and 17.1.1.

All remaining pups will be euthanized on PND 14-16. Sex will be determined both externally and internally. Descriptions of all external abnormalities will be recorded. Particular attention will be paid to the external reproductive genitals to examine signs of altered development. External abnormalities may be collected and fixed in 10% buffered formalin at discretion of the Study Director. In addition, the thyroid will be collected from two pups per litter (if possible, from one male and one female pup and preferably from the same pups as selected for (complete) blood collection, see also section 17.1.1), and will be preserved in 10% buffered formalin.

## 21. CONSTRUCTED VARIABLES

### 21.1 Parental Variables

Body Weight Gains: Calculated against the body weight on Day 1 (pre-mating, mating and lactation periods) or Day 0 (post-coitum period).

Relative Food Consumption: Calculated against the body weight for scheduled intervals.

Organ Weight Relative to Body Weight: Calculated against the terminal body weight.

### 21.2 Reproduction and Developmental Variables

For each group, the following calculations will be performed. Group mean values of precoital time and duration of gestation will be calculated from individual values of F<sub>0</sub>-females, the remaining group values will be calculated from the total number in each group. Additional calculations may be used, the methods and results will be described in the report.

Mating index (%): 
$$\frac{\text{Number of females mated}}{\text{Number of females paired}} \times 100$$

Precoital time: Number of days between initiation of cohabitation and confirmation of mating

Fertility index (%): 
$$\frac{\text{Number of pregnant females}}{\text{Number of females mated}} \times 100$$

Gestation index (%): 
$$\frac{\text{Number of females with living pups on Day 1}}{\text{Number of pregnant females}} \times 100$$

Duration of gestation:	Number of days between confirmation of mating and the beginning of parturition
Post-implantation survival index (%):	$\frac{\text{Total number of offspring born}}{\text{Total number of uterine implantation sites}} \times 100$ <p>Post-implantation survival index will be expressed as 100% when the number of offspring exceeds the number of implantation sites recorded.</p>
Live birth index (%):	$\frac{\text{Number of live offspring on Day 1 after littering}}{\text{Total number of offspring born}} \times 100$
Percentage live males at First Litter Check (%):	$\frac{\text{Number of live male pups at First Litter Check}}{\text{Number of live pups at First Litter Check}} \times 100$
Percentage live females at First Litter Check (%):	$\frac{\text{Number of live female pups at First Litter Check}}{\text{Number of live pups at First Litter Check}} \times 100$
Viability index (%):	$\frac{\text{Number of live offspring on Day 4 before culling}}{\text{Number live offspring on Day 1 after littering}} \times 100$
Lactation index (%):	$\frac{\text{Number of live offspring on Day 13 after littering}}{\text{Number live offspring on Day 4 (after culling)}} \times 100$

## 22. STATISTICAL ANALYSIS

All statistical tests will be conducted at the 5% significance level. All pairwise comparisons will be conducted using two sided tests and will be reported at the 1% and 5% levels.

Numerical data collected on scheduled occasions will be analyzed according to sex and occasion. Descriptive statistics number, mean and standard deviation will be reported whenever possible. Values may also be expressed as a percentage of predose or control values when deemed appropriate. Inferential statistics will be performed according to the comparison matrix below when possible, but will exclude semi-quantitative data, and any group with less than 3 observations.

The following pairwise comparisons will be made:

Group 2 vs. Group 1

Group 3 vs. Group 1

Group 4 vs. Group 1

### 22.1 Parametric

Datasets with at least 3 groups (the designated control group and 2 other groups) will be compared using Dunnett-test (many-to-one-t-test).

## 22.2 Non-Parametric

Datasets with at least 3 groups will be compared using a Steel-test (many-to-one rank test).

The motor activity data set (at least 3 groups) will be compared using an overall Kruskal-Wallis. Whenever, the overall test is significant, the Wilcoxon Rank-Sum test will be applied to compare the treated groups to the control group.

## 22.3 Incidence

An overall Fisher's exact test will be used to compare all groups. The above pairwise comparisons will be conducted using Fisher's exact test whenever the overall test is significant.

Additional methods of statistical analysis may be used at the discretion of the Study Director. The methods and the results will be described in the report.

## 23. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Data for parameters not required by study plan, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by study plan and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

Critical Computerized Systems

System Name	Description of Data Collected and/or Analyzed
ToxData <sup>a</sup>	In-life phase (Mortality; Clinical signs; Body weights; Food consumption; Functional tests; Organ weights; Reproduction parameters; Observations pups <sup>b</sup> ) data collection
REES Centron	Temperature and humidity (animal and laboratory facilities) data collection
MotorMonitor II	Motor activity measurement data collection
ADVIA® 2120i	Hematology data collection
STA Compact®	Clotting parameters data collection
AU400	Clinical biochemistry data collection
IMMULITE® 1000	Thyroid hormone data collection
Pathdata	Histopathology data collection

<sup>a</sup> For logistic reasons, data will be captured under separate Study numbers, see [ATTACHMENT B](#).

<sup>b</sup> Only at first and last litter check, and in case of clinical pup findings also on the respective days in between.

## 24. AMENDMENTS AND DEVIATIONS

Changes to the approved study plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary study plan changes in advance with the Sponsor.

All study plan and SOP deviations will be documented in the study records. Deviations from the study plan and/or SOP related to the phase(s) of the study conducted at a Test Site shall be documented, acknowledged by the PI/IS, and reported to the Study Director for

authorization/acknowledgement. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

## **25. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS**

All study-specific raw data, electronic data, documentation, study plan, retained samples and specimens and final reports will be archived by no later than the date of final report issue. All materials generated by Charles River from this study will be transferred to a Charles River archive.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study plan, study plan amendments, and deviations
- Study schedule
- Study-related correspondence
- Test system receipt, health, and husbandry
- Test item receipt, identification and preparation
- In-life measurements and observations
- Clinical pathology sample collection and evaluation
- Gross and microscopic observations and related data
- Organ weight measurements
- Statistical analysis results

After two years of archiving, all study-specific raw data, electronic data, documentation, study plan and final reports will be transferred to Iron Mountain Germany, Harpener Hellweg 31, D-44805 Bochum, Germany. They shall be indexed by using the Test Facility Project No. allowing unequivocal identification and providing the necessary information (e.g. test system, test item, date of Toxicology and Ecology for the time period set by the GLP regulations). Records of transfer will be retained by test facility. The Sponsor will be informed about the transfer and will receive a digital copy of the record of transfer. The sponsor will ensure that all study material is expediently returned to Charles River Den Bosch if requested by GLP monitoring authorities for audit.

## **26. REPORTING**

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable). The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. An original copy of the report with the Test Facility's signatures will be retained.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

## **27. ANIMAL WELFARE**

This study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of project license AVD2360020172866 (Appendix 2) approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

Animals showing pain, distress or discomfort, which is considered not transient in nature or is likely to become more severe, will be sacrificed for humane reasons based on OECD guidance document on humane endpoints (ENV/JM/MONO/ 2000/7).

By approving this study plan, the Sponsor affirms that this study is required by a relevant government regulatory agency and that it does not unnecessarily duplicate any previous experiments.



### AMENDMENT APPROVAL

DocuSigned by:  
*Daphne van den Oetelaar*

 Signer Name: Daphne van den Oetelaar  
Signing Reason: I approve this document  
Signing Time: 20-May-19 | 11:53 CEST  
9E3F61E303714817BCC0BE4CA117233E

D. van den Oetelaar, MSc  
Study Director

**ATTACHMENT A**

## Distribution List

Electronic copies will be supplied unless otherwise specified below.

<b>Version</b>	<b>Recipient</b>	
Original	Study Director	
1 Copy	Sponsor Representative / Study Monitor	
1 Copy	QAU / Management	Qaumailboxher;
1 Copy	Formulations	Tsfher;
1 Copy	Estrous Cycle Determination	Daoud, M; Hungs, S;
1 Copy	Clinical Pathology	Her/clinical pathology;
1 Copy	Necropsy	Her/necropsy;
1 Copy	Histotechnology	Her/histology
1 Copy	Study Assistants	Sagit;
1 Copy	Individual Scientist Histopathology	Lambregts, A;
1 Paper Copy	Coordinating Biotechnician DRF	Coppes, M;
1 Paper Copy	Coordinating Biotechnician Main	Van Beek, B;

**ATTACHMENT B**

Separate Study Numbers for online data collection will be used as indicated below. All data will be reported under Test Facility Study No. 20172120.

<b>Test Facility Study No.</b>	<b>Study Number</b>	<b>Online Data</b>
521627 (DRF)	521627	ToxData; all data of the Dose Range Finder (DRF)
20172120 (Main)	521417	ToxData Parental animals : Mortality Clinical signs (except arena observations) Functional tests (except motor activity) Body weights and food consumption pre-mating Body weights mating period Food consumption mating period (males only) Clinical laboratory investigations Macroscopic findings and organ weights Implantation sites
20172120 (Main)	521418	ToxData: Arena observations (F <sub>0</sub> - animals)
20172120 (Main)	521419	ToxData: Clinical pathology PND 14-16 pups
20172120 (Main)	521416	ToxData: All other data of the main study

## ATTACHMENT C

### Tissue Collection and Preservation

This attachment contains two tables describing the procedures of tissue weighing, collection, histology and microscopic evaluation. The first table is applicable for all selected animals and all animals that die spontaneously or are sacrificed *in extremis*. The second table is applicable for all remaining animals, including the males that fail to sire and females that fail to deliver pups (i.e. non-pregnant, implantation sites only or no offspring) and females with total litter loss.

Table 1: All selected animals and all animals that die spontaneously or are sacrificed *in extremis*:

Tissue	Weigh <sup>a</sup>	Collect	Histology	Microscopic Evaluation <sup>c</sup>	Comment
Animal identification	-	X	-	-	Location: ear and foot
Artery, aorta	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Body cavity, nasopharynx	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Bone marrow	-	X	X	X	Collect as part of the femur and sternum.
Bone, femur	-	X	X	X	Including joint.
Bone, sternum	-	X	X	X	-
Brain	X	X	X	X	Eight brain levels to be examined including cerebellum, midbrain and cortex.
Cervix	X	X	X	X	Collect and weigh together with the uterus.
Epididymis	X	X	X	X	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Esophagus	-	X	-	-	-
Eye	-	X	X	X	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Gland, adrenal	X	X	X	X	Paired examination.
Gland, coagulation	X	X	X	X	Collect and weigh together with the seminal vesicles.
Gland, harderian	-	X	-	-	Examine only if present in the routine section of the eye. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours. Only 1 required for microscopic examination.
Gland, lacrimal	-	X	-	-	Collect exorbital. Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Gland, mammary	-	X	X	X	Collect inguinal region with skin. Examine for both males and females.

Gland, parathyroid	X	X	-	-	Examine only if present in the routine section of thyroid. Collect and weigh together with thyroid.
Gland, pituitary	-	X	X	X	-
Gland, prostate	X	X	X	X	-
Gland, salivary	-	X	-	-	Collect at mandibular, sublingual and parotid site. Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Gland, seminal vesicle	X	X	X	X	Paired examination. Collect and weigh together with the coagulation gland.
Gland, thyroid	X	X	X	X	Paired examination. Collect and weigh together with the parathyroid.
Gross lesions/masses	-	X	X	X	Only at the discretion of the Study Director, this can be omitted.
Gut-associated lymphoid tissue	-	X	X	X	Examine only if present in routine section of intestine.
Heart	X	X	X	X	-
Kidney	X	X	X	X	Paired examination.
Large intestine, cecum	-	X	X	X	-
Large intestine, colon	-	X	X	X	-
Large intestine, rectum	-	X	X	X	-
Larynx	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Liver	X	X	X	X	-
Lung	-	X	X	X	Infused with formalin.
Lymph node	-	X	X	X	Collected at mandibular and mesentric site. Only 1 mandibular required for microscopic examination
Muscle, skeletal	-	X	X	X	-
Nerve, optic	-	X	-	-	Examine only if present in the routine section of the eye. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Nerve, sciatic	-	X	X	X	Only 1 required for microscopic examination
Ovaries	X	X	X	X	Paired examination.
Pancreas	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Skin	-	X	-	-	-
Small intestine, duodenum	-	X	X	X	-
Small intestine, ileum	-	X	X	X	-
Small intestine, jejunum	-	X	X	X	-

Spinal cord	-	X	X	X	Examine one transverse and one longitudinal section from each of the following areas: cervical, mid-thoracic, lumbar.
Spleen	X	X	X	X	-
Stomach	-	X	X	X	-
Testes	X	X	X <sup>b</sup>	X <sup>b</sup>	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Thymus	X	X	X	X	-
Tongue	-	X	-	-	Examine only if changes in macroscopic appearance are indicative of potential toxicity.
Trachea	-	X	X	X	-
Urinary bladder	-	X	X	X	-
Uterus	X	X	X	X	-
Vagina	-	X	X	X	-

X = Procedure to be conducted;

- = Not applicable.

<sup>a</sup> Organ weights will not be determined for animals which die spontaneously or are sacrificed *in extremis*.

<sup>b</sup> For the testes of all selected males of Groups 1 and 4, and all males that fail to sire or died before mating detailed qualitative examination will be made, taking into account the tubular stages of the spermatogenic cycle. The examination will be conducted in order to identify treatment related effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Any cell- or stage-specificity of testicular findings will be noted.

<sup>c</sup> Efforts will be made to evaluate all protocol-required tissues microscopically; however, it is not always feasible for every protocol-required tissue to be present on every slide. Protocol-required tissues that are not examined will be documented in the histopathology data and the impact of these missing tissues on the study will be documented in the pathology report.

Table 2: All remaining animals (incl. males that fail to sire<sup>a</sup>, females that fail to deliver pups and females with total litter loss):

Tissue	Weigh	Collect	Histology	Microscopic Evaluation <sup>c</sup>	Comment
Animal identification	-	X	-	-	Location: ear and foot
Cervix	-	X	@	@	-
Epididymis	X	X	@	@	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Gland, coagulation	X	X	@	@	Collect and weigh together with the seminal vesicles.
Gland, mammary	-	X	#	#	Collect inguinal region with skin. Collect for both males and females.
Gland, parathyroid	X	X	-	-	Examine only if present in the routine section of thyroid. Collect and weigh together with thyroid.
Gland, pituitary	-	X	-	-	-
Gland, prostate	X	X	@	@	-
Gland, seminal vesicle	X	X	@	@	Paired examination. Collect and weigh together with the coagulation gland.
Gland, thyroid	X	X	-	-	Paired examination. Collect and weigh together with the parathyroid.
Gross lesions/masses	-	X	X	X	-
Ovaries	-	X	@	@	Paired examination.
Testes	X	X	@ <sup>b</sup>	@ <sup>b</sup>	Paired examination. Preserve in modified Davidson's fixative. Tissues will be transferred to formalin after fixation for at least 24 hours.
Uterus	-	X	@	@	-
Vagina	-	X	@	@	-

X = Procedure to be conducted for all remaining animals;

- = Not applicable;

@ = Procedure to be conducted only for males that fail to sire<sup>a</sup>, females that fail to deliver pups and females with total litter loss.

# = Procedure to be conducted for females with total litter loss only.

<sup>a</sup> Except for males that fail to sire which are also selected. These males will be processed as noted in table 1 of [ATTACHMENT C](#).

<sup>b</sup> For the testes of all males that fail to sire or died before mating detailed qualitative examination will be made, taking into account the tubular stages of the spermatogenic cycle. The examination will be conducted in order to identify treatment related effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Any cell- or stage-specificity of testicular findings will be noted.

<sup>c</sup> Efforts will be made to evaluate all protocol-required tissues microscopically; however, it is not always feasible for every protocol-required tissue to be present on every slide. Protocol-required tissues that are not examined will be documented in the histopathology data and the impact of these missing tissues on the study will be documented in the pathology report.

**ATTACHMENT D**

Certificate of Analysis

**Certificate of analysis**

Page 1 of 1

**THE DOW CHEMICAL COMPANY**

Certificate of analysis		Quality order	
Product name	CANSOLV ABSORBANT DS	Customer Batch number	D2921BF000
Reference number		Batch number	
		Reference lot	

Test	Unit	Test result	Min	Max
% Water	%	51.14	51	54
Appearance		Pass	-	-
Gardner Color	Color	3	-	6
Foam Height	ml	75	-	100
Foam Break Time	Seconds	7.7	-	15
Gas Chromatography, HEP	%	9.01	-	14
Gas Chromatography, DIHEP	%	89.69	80	100
Gas Chromatograph Heavies GT DHEP	%	0.4	-	5
Gas Chromatography, Piperazine	%	0	-	2
Gas Chromatograph EG & DEG	%	0.2	-	0.6
Cansolv DS Alkalinity	meq/g	5.76	-	-

Notes: Tank 105

APPROVED BY: KENDRICK JUNIUS



## DEVIATIONS

All deviations that occurred during the study have been authorized/acknowledged by the Study Director, assessed for impact, and documented in the study records. All study plan deviations and those SOP deviations that could have impacted the quality or integrity of the study are listed below.

None of the deviations were considered to have impacted the overall integrity of the study or the interpretation of the study results and conclusions.

### Formulations and Dosing

- On Day 2 of treatment, all males of Group 2 received a dose volume corresponding with a dose level of 1000 mg/kg instead of 100 mg/kg.  
Evaluation: Based on the available results, a single dose at the same level as Group 4 animals did not impact the study outcome.

### In-life phase

- For the males of Litter No. 76 (1000 mg/kg/day), the nipple retention was not determined on PND 13.  
Evaluation: sufficient information from other litters at the same dose level was available for evaluation.

### Terminal procedures

- The brain from Female No. 68 (300 mg/kg/day) was not weighed on the day of necropsy.  
Evaluation: Brain weight of this female was determined after fixation but was considered affected by fixation and therefore excluded from the tables. Sufficient information was available from remaining Group 3 females for evaluation.
- For Female No. 42 (control), no visible implantation sites were noted in the uterus during necropsy. Due to a macroscopic finding (i.e. a nodule) and in consultation with the pathologist, the Salewski staining was not performed.  
Evaluation: As the Salewski staining could interfere with the evaluation of the uterus tissue, preservation of the gross finding for histopathological evaluation was preferred to the staining. This deviation was considered not to have a negative impact on the study outcome, as sufficient information was available for evaluation.

### Other

- Inadvertently, the new standard analysis method for the motor activity data set (i.e. parametric (ANOVA) tests on group means with Bonferroni correction for multiple testing) was used while this method was not included in the Study Plan.  
Evaluation: The new method (mixed model) is an extension of standard regression (or ANOVA) model for repeated data. The repeated measure (mixed models) is considered statistically the best approach for this type of dataset as multiple measurements are performed over an interval of one hour. Hence, the study outcome was not affected.

**Dose Range Finding Study**

- Temporary deviations from the minimum level of target humidity occurred on two consecutive days.  
Evaluation: This study plan deviation is considered not to have affected the integrity of the study because it did not noticeably affect the clinical condition of the animals or the outcome of the study.
- Dosing formulations were placed on a magnetic stirrer during dosing.  
Evaluation: As the dosing formulations consisted of water (control) or undiluted test item (Groups 2, 3 and 4) it was not required to place them on a magnetic stirrer. This deviation had no impact on the study outcome.

Technical Service Report on the Mutagenicity Analyses of  
Cansolv Nitrosamine Samples

Ref# K0309-P50RFQ001-R00-University of Illinois

SaskPower  
Boundary Dam BD3 Carbon Capture System

Submitted to

Cansolv Technologies Inc.  
400 boulevard de Maisonneuve Ouest, Suite 200  
Montreal, Quebec Canada H3A 1L4  
Attention of Tan-Trung Nguyen  
Project Manager

Submitted by

Michael J. Plewa, Ph.D. Professor of Genetics  
mplewa@illinois.edu  
Elizabeth D. Wagner, Ph.D. Principal Research Specialist  
Jennifer Osiol, M.S. Research Assistant

University of Illinois at Urbana-Champaign  
364 NSRC  
1101 W. Peabody Dr.  
Urbana, IL 61801 U.S.A.

April 11, 2013

CONFIDENTIAL: This communication is covered by a Secrecy and Restricted Use Agreement between the University of Illinois and Cansolv Technologies Inc. (signed December 19, 2012).

## Executive Summary: Non-Proprietary

The generation of electricity and steam from fossil fuel-fired power plants accounted for 41% of global carbon dioxide (CO<sub>2</sub>) emissions. Postcombustion carbon sequestration and storage will play a critical role in reducing global greenhouse gas emissions over the next several decades. Preeminent among CO<sub>2</sub> capture technologies are amine-based technologies. With this technology flue gases are passed through an “absorber” column with a countercurrent amine-based solvent. Following solvent partitioning, the CO<sub>2</sub> forms a carbamate complex with the amine. The amine- and carbamate-containing solution proceeds to a second “desorber” column, where high temperatures reverse this reaction, regenerating the amine sorbent and releasing the CO<sub>2</sub>, which is compressed and routed to underground storage. There is significant public health and environmental concerns regarding the potential for nitrosamine contamination of air and drinking water supplies downwind of amine-based CO<sub>2</sub> capture plants. As a chemical class, nitrosamines are potent mutagens and some are strong carcinogens. The primary objective of this project was to conduct quantitative analyses of the mutagenicity for selected nitrosamine agents using a *Salmonella typhimurium* (strain YG7108) suspension assay. The nitrosamines analyzed were, N-Nitrosodimethylamine, (NDMA), Cansolv Nitrosamine-1 (Cansolv-N1), Cansolv Nitrosamine-2 (Cansolv-N2) and Cansolv Nitrosamine-1 (Cansolv-N3),. The results of this project are the following. NDMA was an exceedingly potent mutagen after mammalian microsomal metabolism with a mutagenic potency of 47,695 revertants per μmol. Cansolv-N3 was a clear mutagen and the agent required mammalian microsomal metabolic activation with a mutagenic potency of 915 revertants per μmol. Cansolv N-1 was an exceedingly weak mutagenic agent that required S9 metabolic activation with a mutagenic potency of 23 revertants per μmol. Cansolv N-2 was a direct, albeit very weak mutagen (54 revertants per μmol); S9 metabolic activation detoxified the nitrosamine and reduced its mutagenic activity. Cansolv N-1 was 4.8×10<sup>-4</sup> times as mutagenic as NDMA; Cansolv N-2 was 1.1×10<sup>-3</sup> times as mutagenic as NDMA. When compared to environmental nitrosamines such as nitrosamine drinking water disinfection by-products, both Cansolv N-1 and Cansolv N-2 were considered very weak mutagens.

## Executive Summary: Proprietary (Confidential)

The generation of electricity and steam from fossil fuel-fired power plants accounted for 41% of global carbon dioxide (CO<sub>2</sub>) emissions. Postcombustion carbon sequestration and storage will play a critical role in reducing global greenhouse gas emissions over the next several decades. Preeminent among CO<sub>2</sub> capture technologies are amine-based technologies. With this technology flue gases are passed through an “absorber” column with a countercurrent amine-based solvent. Following solvent partitioning, the CO<sub>2</sub> forms a carbamate complex with the amine. The amine- and carbamate-containing solution proceeds to a second “desorber” column, where high temperatures reverse this reaction, regenerating the amine sorbent and releasing the CO<sub>2</sub>, which is compressed and routed to underground storage. There is significant public health and environmental concerns regarding the potential for nitrosamine contamination of air and drinking water supplies downwind of amine-based CO<sub>2</sub> capture plants. As a chemical class, nitrosamines are potent mutagens and some are strong carcinogens. The primary objective of this project was to conduct quantitative analyses of the mutagenicity for selected nitrosamine agents using a *Salmonella typhimurium* (strain YG7108) suspension assay. The nitrosamines analyzed were, N-Nitrosodimethylamine, (NDMA), 1-Nitroso-(4-hydroxyethyl)-piperazine (Nitroso-HEP, Cansolv-N1, Cansolv-N2 and 1-Nitrosopiperazine, (NPZ) Cansolv-N3. The results of this project are the following. NDMA was an exceedingly potent mutagen after mammalian microsomal metabolism with a mutagenic potency of 47,695 revertants per μmol. Nitrosopiperazine was a clear mutagen and the agent required mammalian microsomal metabolic activation with a mutagenic potency of 915 revertants per μmol. Nitroso-HEP (Cansolv N-1) was an exceedingly weak mutagenic agent that required S9 metabolic activation with a mutagenic potency of 23 revertants per μmol. Cansolv N-2 was a direct, albeit very weak mutagen (54 revertants per μmol); S9 metabolic activation detoxified the nitrosamine and reduced its mutagenic activity. Nitroso-HEP (Cansolv N-1) was  $4.8 \times 10^{-4}$  times as mutagenic as NDMA; Cansolv N-2 was  $1.1 \times 10^{-3}$  times as mutagenic as NDMA. When compared to environmental nitrosamines such as nitrosamine drinking water disinfection by-products, both Nitroso-HEP and Cansolv-N2 were considered very weak mutagens.

## Table of Contents

Section	Page
Executive Summary: Non-Proprietary	2
Executive Summary: Proprietary	3
1 Introduction and Background	5
2 Scope of Work	5
2.1 <i>In vitro</i> Mutagenicity Analyses of Nitrosamine Compounds	5
2.2 Study Plan	6
2.2.1 <i>Salmonella typhimurium</i> Cells	6
2.2.2 <i>S. typhimurium</i> Microsuspension Assay	7
2.2.2.1 Comparison of the <i>S. typhimurium</i> Microsuspension Mutagenicity Assay and the Plate Incorporation Test	9
2.3 Quality Assurance Quality Control	10
3 Communication and Review	10
3.1 Project Director	10
3.2 Project Manager	11
3.3 Shell's Study Monitors	11
3.3.1 Study Monitors	11
4 Results and Discussion	12
4.1 N-Nitrosodimethylamine	12
4.1.1 NDMA Mutagenicity and Cytotoxicity Data	12
4.1.2 NDMA Concentration-Response Curves	14
4.1.3 Statistical Analyses of the Mutagenicity Data for NDMA	15
4.1.4 Interpretation of NDMA Data	17
4.2 1-Nitroso-(4-hydroxyethyl)-piperazine	18
4.2.1 Nitroso-HEP Mutagenicity and Cytotoxicity Data	18
4.2.2 Nitroso-HEP Concentration-Response Curves	20
4.2.3 Statistical Analyses of the Mutagenicity Data for Nitroso-HEP	21
4.2.4 Interpretation of Nitroso-HEP Data	22
4.3 Cansolv-N2	24
4.3.1 Cansolv-N2 Mutagenicity and Cytotoxicity Data	24
4.3.2 Cansolv-N2 Concentration-Response Curves	26
4.3.3 Statistical Analyses of the Mutagenicity Data for Cansolv-N2	27
4.3.4 Interpretation of Cansolv-N2 Data	28
4.4. Nitrosopiperazine	30
4.4.1 Nitrosopiperazine Mutagenicity and Cytotoxicity Data	31
4.4.2 Nitrosopiperazine Concentration-Response Curves	32
4.4.3 Statistical Analyses of the Mutagenicity Data for Nitrosopiperazine	33
4.4.4 Interpretation of Nitrosopiperazine Data	34
5 Summary and Conclusions	35
6 References	37



## 1. Introduction and Background

Recent data demonstrate that the generation of electricity and steam from fossil fuel-fired power plants accounted for 41% of global carbon dioxide (CO<sub>2</sub>) emissions [1]. In the United States approximately 75% of power generation capacity is produced by fossil fuels. Postcombustion carbon sequestration and storage will play a critical role in reducing global greenhouse gas emissions over the next several decades. Preeminent among CO<sub>2</sub> capture technologies are amine-based technologies [2]. With this technology flue gases are passed through an “absorber” column with a countercurrent amine-based solvent. Following solvent partitioning, the CO<sub>2</sub> forms a carbamate complex with the amine. The amine- and carbamate-containing solution proceeds to a second “desorber” column, where high temperatures reverse this reaction, regenerating the amine sorbent and releasing the CO<sub>2</sub>, which is compressed and routed to underground storage. There is significant public health and environmental concerns regarding the potential for nitrosamine and nitramine contamination of air and drinking water supplies downwind of amine-based CO<sub>2</sub> capture plants [2]. Environmental contamination could arise when nitrosamines and nitramines formed within the amine based solvent are stripped into the washwater and thence transferred back into the exhaust gas. Also nitrosamines and nitramines could form downwind if amines released in the exhaust gas react with ambient NO<sub>x</sub> [3]. As a chemical class, nitrosamines are potent mutagens and some are strong carcinogens [4-6]. Recently, we characterized the formation conditions for the generation of nitrosamines and nitramines associated with amine based carbon capture technologies [7]. Of interest is the finding that N-nitroso derivatives of piperazine form at elevated pH in carbon capture solutions that contain piperazine [8].

## 2. Scope of Work

### 2.1 *In vitro* Mutagenicity Analyses of Nitrosamine Compounds

Our primary objective was to conduct a quantitative analyses of the mutagenic potential for selected nitrosamine agents using a *Salmonella typhimurium* suspension assay according to the procedures published by Wagner et al., 2012 [9]. The agents that were analyzed in this project are listed in Table 1.

Table 1. Nitrosamine chemicals analyzed in this project.

Chemical Name	Abbreviation	CASN	Purity	Source
N-Nitrosodimethylamine	NDMA	62-75-9	99.9%	Sigma-Aldrich, USA
1-Nitrosopiperazine	NPZ (Cansolv-N3)	5632-47-3	94.3%	Harlan Laboratories, UK
1-Nitroso-(4-hydroxyethyl)-piperazine	Nitroso-HEP (Cansolv-N1)	48121-20-6	97.9%	Harlan Laboratories, UK



Casolv-N2	-	N/A	97.3%	MATRIC, USA
	Casolv-N2			

Casolv-N1 (Nitroso-HEP) is generated in Casolv's absorbent No.1 under regular operation and was supplied by Casolv Technologies. The molecular weight for Nitroso-HEP as  $159 \text{ g mol}^{-1}$  was provided by Tan-Trung Nguyen, Project Manager, Casolv Technologies Inc. Casolv-N2 is generated in Casolv's absorbent No.2 under research development and was supplied by Casolv Technologies.

## 2.2 Study Plan

### 2.2.1 *Salmonella typhimurium* Cells

We employed *S. typhimurium* tester strain YG7108 (*hisG46*, *rfa*;  $\Delta(chl, uvrB, bio)$   $\Delta ada_{ST}$ ; *ogt\_{ST}*; *Cm^r*; *Amp^r*) with and without mammalian microsomal activation (S9 mix). Reverse mutation is measured at *hisG46* (Figure 1) [10, 11]. *S. typhimurium* strain YG7108 is highly sensitive to nitrosamines and other alkylating agents and was provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo, Japan [12].

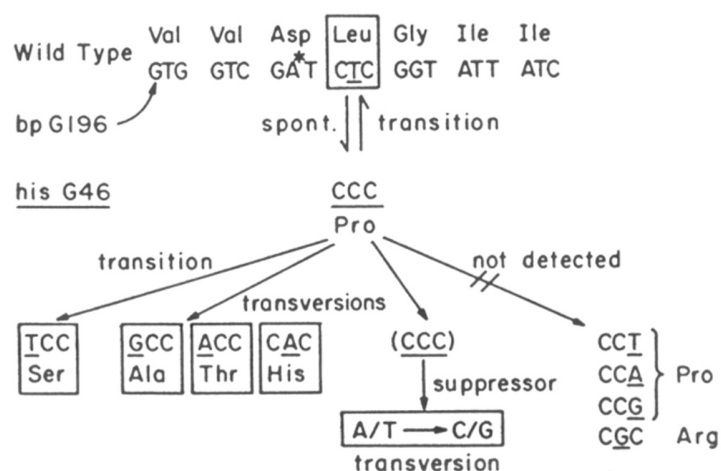


Figure 1. Reverse mutation at *hisG46* in *S. typhimurium* that leads to the growth of a visible colony on selective VB medium. The mutant colonies are referred to as revertants.

In our laboratory strain YG7108 was stored as a frozen culture in Luria-Bonner (LB) plus 10% dimethylsulfoxide (DMSO) at  $-80^{\circ}\text{C}$ . To construct a master plate, frozen log-phase *S. typhimurium* YG7108 cells were thawed and grown in 5 mL LB with kanamycin ( $25 \mu\text{g/mL}$ ) and

chloramphenicol (10 µg/mL) at 37°C overnight with shaking. The culture was streaked across an LB plate and incubated overnight at 37°C. The master plate was stored at 4°C [10]. We followed the published method for the mutagenicity and cytotoxicity (survivorship) experiments for the test agents [9]. The day before the experiment a single colony isolate of *S. typhimurium* YG7108 from the master plate was grown overnight in 100 mL LB medium with kanamycin (25 µg/mL) and chloramphenicol (10 µg/mL) at 37°C with shaking (200 rpm) [12]. The following day the culture was centrifuged for 5 min at 5000 rpm (4096 ×g) using the GSA rotor in a RC5B Superspeed Centrifuge (Sorvall). The supernatant was discarded, and the bacterial pellet suspended in 50 mL of 100 mM potassium-phosphate buffer, pH 7.4 (PPB). The suspension was centrifuged again for 5 min at 5000 rpm, and the supernatant fluid discarded. The pellet was suspended in 2.5-3 mL PPB. An aliquot (50 µL) of the bacterial suspension was added to 4.95 mL PPB and the optical density determined spectrophotometrically at 660 nm. The titer of the suspension was adjusted to  $6 \times 10^9$  cells/mL; the cells were kept on ice until used in the microsuspension mutagenicity assay [9, 13, 14].

Most nitrosamines require metabolic activation to exhibit mutagenicity. Metabolic activation of nitrosamines is primarily dependent on cytochrome P-450 which converts nitrosamines into DNA alkylating agents [15]. Oxidative transformation is an important route of metabolism of xenobiotics. These oxidative reactions are largely catalyzed by the monooxygenase system based on cytochrome P-450. The monooxygenase system is formed by the enzyme system of cytochrome P-450 and NADPH cytochrome P-450 reductase. The haemoprotein cytochrome P-450 functions as the terminal oxidase involved in the hydroxylation of xenobiotics such as nitrosamines. A description of the cytochrome P-450-mediated monooxygenation of dimethylnitrosamine (NDMA) is presented in Figure 2 [15].

### 2.2.2 *S. typhimurium* Microsuspension Assay

The *S. typhimurium* microsuspension assay was conducted in sterile round bottom 96-well microplates. Each reaction mixture (well) was composed of a known test chemical concentration,  $3 \times 10^8$  cells, and PPB in a total volume of 100 µL. When including a mammalian microsomal-mediated activation system, 35 µL of an S9 mix was added for a total volume of 100 µL. S9 (post

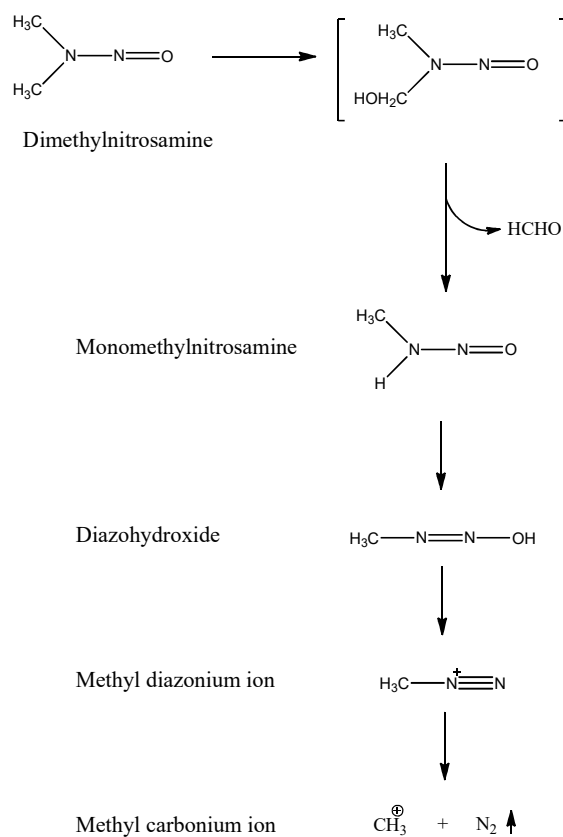


Figure 2. Metabolic activation of dimethylnitrosamine via cytochrome P-450 leading to the formation of a reactive methyl carbonium ion. This radical can alkylate DNA and induce mutation.

mitochondrial hepatic supernatant) from Aroclor 1254-induced male rats was purchased from Molecular Toxicology, Inc. (Moltox). The S9 mix consisted of 50 mM PPB pH 7.4, 10 mM MgCl<sub>2</sub>, 5 mM glucose-6-phosphate, 30 mM KCl, and 4 mM NADP plus 200  $\mu$ L/mL of Aroclor 1254-induced rat hepatic microsomal suspension (S9). The final S9 concentration in the treatment well was 7%. In general, for each experiment there were 3-6 microplate wells for each test agent concentration plus a concurrent negative control (buffer only) and concurrent positive controls (with S9 activation, NDMA, 50-1000  $\mu$ M; without S9 activation, ethylmethanesulfonate (EMS), 188  $\mu$ M). Each experiment was repeated. Each microwell is an independent clone and the number of repeated measurements was determined based on the power function of the ANOVA test statistic. We routinely require a power value (1- $\beta$ ) of  $\geq 0.8$  with  $\alpha \leq 0.05$ . This ensures that the statistical analyses have the sensitivity to reject the null hypothesis. The microplate wells were sealed with Alumnaseal to prevent any crossover volatilization or evaporation, and the microplate was placed in a plastic container and incubated at 37°C with shaking at 200 rpm for 60 min. After treatment, the entire contents of each well were added to 2 mL of molten histidine and biotin supplemented Vogel-Bonner (VB) top agar, mixed and poured onto selective VB plates. The VB selective plates were placed in a static 37°C incubator for 72 h. Histidine revertant colonies were counted by hand or with a New Brunswick Biotran III automatic colony counter. The data were saved as an Excel spreadsheet. The data were analyzed using the statistical and graphical functions of SigmaPlot 11.

Cell survivorship was measured by making serial dilutions from the treatment wells in PPB and plating on LB (complete media) plates. After incubation for 24 h the colonies were counted. The number of colonies from the negative control was set at 100% and the colonies from the treatment wells were compared and expressed as a percent of the negative control.

To confirm the genotype of the YG7108 cells, 100  $\mu$ L of the titered cell suspension was added to an LB plate and spread with a flamed glass rod. Flamed tweezers were used to place a crystal violet disk onto the center of the plate, and the disk was tapped lightly in place. Due to the presence of the *rfa* mutation [10], the large molecules of crystal violet are able to enter and kill the YG7108 cells. This is indicated by a clear zone around the crystal violet disk (Figure 3).



Figure 3. Diagnostic plates for the *S. typhimurium* microsuspension mutagenicity assay. A negative control is illustrated by the top plate, the left plate is the positive control (250  $\mu$ M NDMA +S9 activation) and the right plate is the crystal violet LB plate showing a clear zone of inhibition identifying the presence of the *rfa*<sup>-</sup> mutation.

### 2.2.2.1 Comparison of the *S. typhimurium* Microsuspension Mutagenicity Assay and the Plate Incorporation Test

We employed a quantitative microsuspension methodology for the *S. typhimurium* mutagenicity assay instead of the more commonly used plate incorporation Ames test method. An overall comparison is outlined in Table 2. In the plate incorporation and preincubation methods, the cell titer is not determined; an aliquot (usually 100  $\mu$ L) of an overnight culture in complete growth medium is used. For the plate incorporation assay the test agent is added to a 2-mL volume of supplemented molten top agar along with the cells and  $\pm$  S9. The top agar is poured upon a VB minimal plate. Thus the actual exposure concentration to the cells is difficult to determine. It is impossible to calculate a molar concentration and usually a unit of grams per plate is used to express the concentration. The volume during the exposure time for the cells is impossible to quantify. Cells are exposed in approximately 2 mL of molten top agar which is then poured onto a VB selective plate that consists of approximately 20 mL. The cells are retained in the top agar, but the test chemical diffuses throughout the approximately 22 mL volume of agar in the petri plate.

Table 2. Comparison between the plate incorporation method and the microsuspension method for the *S. typhimurium* mutagenicity assay.

Metric	Plate Incorporation Method	Microsuspension Method
Single colony isolate grown overnight in complete medium	Yes	Yes
Centrifugation and washing bacteria before treatment	No	Yes
Determination of titer before treatment (same number of cells treated for each experiment)	No	Yes. By treating a consistent number of cells, the results can be presented in revertants per 10 <sup>8</sup> cells plated. This provides a uniform level of mutagenicity response.
Treatment times can be adjusted	No	Yes
Precise control of concentration of test agents	No — With this method the test compound and bacteria are combined in 2 mL of molten agar and poured immediately onto a plate from which diffusion occurs throughout the plate. The concentration of agent can be expressed as the concentration in the treatment tube or as the concentration in the petri plate.	Yes — With this method a known number of bacteria are exposed to the agent while shaking for defined time periods. After treatment the mixture is added to molten agar and poured onto a petri plate. The concentration of agent is expressed as the concentration during treatment.
Determination of cytotoxicity	Not possible. A background lawn that is thin or sparse is only qualitative and not a quantitative measure of toxicity.	Yes, an aliquot of treated cells may be serially diluted and plated onto complete medium, allowing for determination of toxicity.

Another difficulty in the plate incorporation method is that the test agent is in contact with S9 monooxygenase enzymes and co-factors initially in the 2 mL overlay agar and then this volume changes when the contents are poured upon the VB plate. Finally, in the plate incorporation and preincubation methods no quantitative cytotoxicity measurement can be made. It is important that the *S. typhimurium* cells are not killed as the concentration of the test agent is increased throughout the concentration range. A qualitative visual inspection of the background lawn of cells is part of the plate incorporation and preincubation procedures, but this is not a true measurement of cytotoxicity.

In the microsuspension assay, *S. typhimurium* cells are washed, titered, and a specific number of cells are exposed to a test agent in a known volume of buffer with and without S9 for a specific time period. The concentrations of the test agent are expressed in molar units. An aliquot of the suspension is reserved after treatment, serial dilutions are prepared and known volumes are plated onto complete (LB) medium. All living bacterial cells will grow on LB medium and we can determine if we are conducting the assay at concentrations of test chemical that do not induce a cytotoxic response. Thus, the microsuspension procedure is a quantitative assay that provides control over cell titer, a precise measure of cytotoxicity and an exact determination of the concentration of test agent to which a specific number of cells are exposed for a specific time period. This level of precision provides high quality data on the relative mutagenic capacities and allows for a quantitative comparison amongst the test chemicals.

### **2.3 Quality Assurance Quality Control**

Weekly project team meetings were held and the results were discussed. At all times good laboratory practices associated with a toxicology laboratory were followed. All data were kept in data books and transferred to computer files. Ultimately, with the consent of all parties involved in this project the results will be published in a peer-reviewed scientific journal. Chemicals were of the highest purity available and were kept in a freezer or at room temperature as specified by the vendors in a locked room. Manipulations of toxic and mutagenic chemicals were conducted in certified biological/chemical safety hoods. All materials were handled using disposable papers and gloves. Liquid mutagens were dispensed with disposable pipet tips. All personnel were instructed in the correct handling of dangerous chemicals and the procedures were reviewed periodically. All items that were in contact with mutagens were disposed of in accordance with regulations of the Division of Research Safety of the University of Illinois at Urbana-Champaign. The quality assurance objectives for data obtained in this project were defined in terms of accuracy and precision. Our chemical balances are periodically calibrated and our automated pipettes are checked and calibrated by Rainin Co. (or other certified vendor) on an annual basis.

## **3. Communication and Review**

### **3.1 Project Director**

For this project the Study Director is Dr. Michael Plewa, University of Illinois at Urbana-Champaign. Dr. Plewa will be the focal point for all discussions on the scope of work.

### **3.2 Project Manager**

The Project Manager for this study is Tan-Trung Nguyen, Cansolv Technologies Inc., an affiliate of Shell Global Solutions International BV, 400 de Maisonneuve Ouest, Suite 200, Montréal, Québec, H3A 1L4 CANADA.

### **3.3 Shell's Study Monitors**

All study plans (protocols) from the laboratory were reviewed by one of the Shell's study monitors prior to test execution. The study followed the protocols as published by Wagner et al., 2012 [9].

#### **3.3.1 Study Monitors**

Chantal J.G.M. Smulders  
Shell International B.V. PO Box 162, 2501 AN The Hague, the Netherlands  
T. +31 70 3773642 C. +31 6 1136 6769  
Chantal.Smulders@shell.com

Marjoke Heneweer  
Shell International B.V. Carel van Bylandtlaan 16 2596 HR The Hague, The Netherlands  
T. +31 70 3774814 C. +31 611532046  
Marjoke.Heneweer@shell.com

## 4. Results and Discussion

### 4.1 N-Nitrosodimethylamine

#### 4.1.1 NDMA Mutagenicity and Cytotoxicity Data

The mutagenicity of NDMA was analyzed with and without S9 mammalian microsomal activation using *S. typhimurium* strain YG7108 in a concentration range from 5 to 1000  $\mu\text{M}$ . The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for NDMA are presented in Tables 3 and 4. These data were used to generate the concentration-response curves (Figure 4) and for the statistical analyses (Tables 5 and 6).

Table 3. Plate counts of NDMA (VB plates) and percent survivorship (LB plates) +S9.

NDMA +S9 *S. typhimurium* YG7108 Experiments 020813 and 022813

Conc $\mu\text{M}$	Revertants/Plate VB								
	0	5	10	25	50	100	250	500	1000
12	36	61	196	314	688	1026	1337	1375	
16	64	86	196	315	605	964	1324	1369	
15	51	90	164	292	547	981	1248	1453	
22	53	86	194	303	694	1119	1290	1506	
12	50	75	173	358	694	1027	1327	1579	
18	40	81	169	306	569	984	1056	1341	
23	57	71	163	248	540	794	1003	1439	
11	47	92	151	318	516	845	1189	1487	
35		69	152	288	628	811	1223	1434	
34				343	558	874			
33					663	848			
36					696				
22									
34									
26									
21									
20									
24									
19									
24									
22									
24									
48									
28									
14									
20									
17									

Table 3. Plate counts of NDMA (VB plates) and percent survivorship (LB plates) +S9.

NDMA +S9 *S. typhimurium* YG7108 Experiments 020813 and 022813

Conc $\mu$ M	Revertants/Plate VB									
	0	5	10	25	50	100	250	500	1000	
	26									
	25									
	30									
Number	30	8	9	9	10	12	11	9	9	Number
Average	23.7	49.8	79.0	173.1	308.5	616.5	933.9	1221.9	1442.6	Average
SE	1.5	3.2	3.6	6.0	9.5	19.9	31.7	40.2	25.0	SE
SD	8.4	8.9	10.9	18.1	30.2	69.0	105.2	120.7	75.1	SD

NDMA +S9 *S. typhimurium* YG7108 Experiments 022813

Conc. $\mu$ M	LB Survivorship					% Negative Control
	0	100	250	500	1000	
	314	346	315	355	309	
	331	355	336	303	312	
				331		
	97.4	107.3	97.7	110.1	95.8	
	102.6	110.1	104.2	94.0	96.7	
				102.6		
Number	2	2	2	3	2	Number
Average	100.0	108.7	100.9	102.2	96.3	Average
SE	2.6	1.4	3.3	4.7	0.5	SE
SD	3.7	2.0	4.6	8.1	0.7	SD



Table 4. Plate counts of NDMA (VB plates) and percent survivorship (LB plates) –S9.

NDMA –S9 *S. typhimurium* YG7108 Experiments 030713

Conc $\mu$ M	Revertants/Plate VB					EMS 554
	0	100	250	500	1000	
	21	25	26	26	36	
	19	26	29	20	28	
	25	25	30	32	25	
	26	39	38	32	22	
	30	32	29	22	35	
	17	26	26	26	26	
Number	6	6	6	6	6	Number
Average	23.0	28.8	29.7	26.3	28.7	Average
SE	2.0	2.3	1.8	2.0	2.3	SE
SD	4.9	5.6	4.4	5.0	5.6	SD

NDMA –S9 *S. typhimurium* YG7108 Experiments 022813

Conc. $\mu$ M	LB Survivorship					% Negative Control
	0	100	250	500	1000	
	384	349	339	315	358	
	360	344	327	378	389	
	355	394	357	371	362	
	104.8	95.2	92.5	85.9	97.7	
	98.2	93.9	89.2	103.1	106.1	
	96.9	107.5	97.4	101.2	98.8	
Number	3	3	3	3	3	Number
Average	100.0	98.9	93.1	96.8	100.9	Average
SE	2.4	4.3	2.4	5.4	2.7	SE
SD	4.2	7.5	4.1	9.4	4.6	SD

#### 4.1.2 NDMA Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of NDMA in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 4. The top panel illustrates the cytotoxicity (LB plate experiments) for NDMA with S9 metabolic activation (open squares) and without S9 activation (filled squares). The bottom panel represents the mutagenicity of NDMA with S9 activation (open circles) and without S9 activation (filled circles).

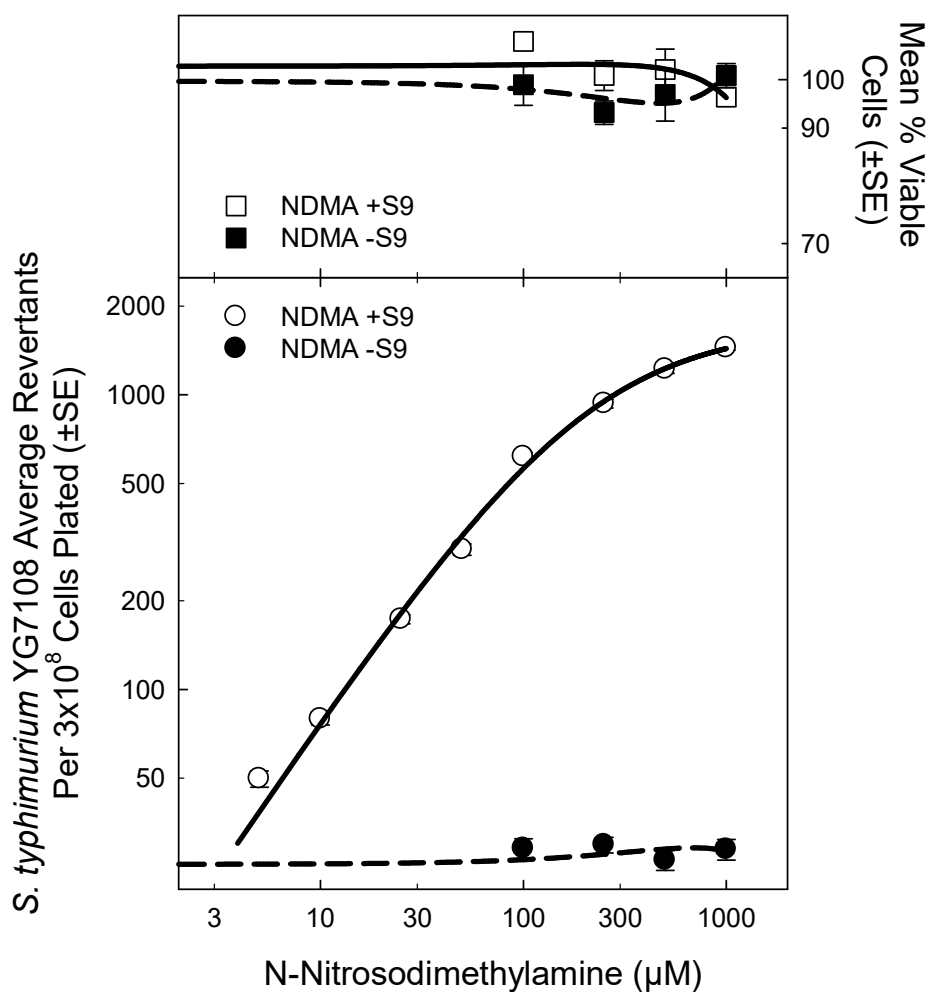


Figure 4. Log-log plot showing NDMA concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

#### 4.1.3 Statistical Analyses of the Mutagenicity Data for NDMA

An ANOVA test statistic for the mutagenicity data of NDMA with S9 metabolic activation was conducted and the results are presented in Table 5.

Table 5. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses NDMA +S9

**Data source:** Data 6 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NDMA+S9 0	30	0	23.700	8.400	1.534
NDMA+S9 5	8	0	49.750	8.940	3.161
NDMA+S9 10	9	0	79.333	10.874	3.625
NDMA+S9 25	9	0	173.111	18.100	6.033
NDMA+S9 50	10	0	298.500	42.022	13.288
NDMA+S9 100	12	0	616.500	68.975	19.911
NDMA+S9 250	11	0	933.909	105.163	31.708
NDMA+S9 500	9	0	1221.889	120.707	40.236
NDMA+S9 1000	9	0	1442.556	75.083	25.028

Source of Variation	DF	SS	MS	F	P
Between Groups	8	26026785	3253348	919.7	<0.001
Residual	98	346652	3537		
Total	106	26373437			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
NDMA+S9 0 vs. NDMA+S9 1000	1418.856	62.770	<0.001	0.006	Yes
NDMA+S9 0 vs. NDMA+S9 500	1198.189	53.008	<0.001	0.007	Yes
NDMA+S9 0 vs. NDMA+S9 250	910.209	43.418	<0.001	0.009	Yes
NDMA+S9 0 vs. NDMA+S9 100	592.800	29.181	<0.001	0.010	Yes
NDMA+S9 0 vs. NDMA+S9 50	274.800	12.654	<0.001	0.013	Yes
NDMA+S9 0 vs. NDMA+S9 25	149.411	6.610	<0.001	0.017	Yes
NDMA+S9 0 vs. NDMA+S9 10	55.633	2.461	0.016	0.025	Yes
NDMA+S9 0 vs. NDMA+S9 5	26.050	1.101	0.274	0.050	No

An ANOVA test statistic for the mutagenicity data of NDMA without S9 metabolic activation was conducted and the results are presented in Table 6.

Table 6. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses NDMA -S9

**Data source:** Data 6 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NDMA-S9 0	6	0	23.000	4.858	1.983
NDMA-S9 100	6	0	28.833	5.636	2.301
NDMA-S9 250	6	0	29.667	4.412	1.801
NDMA-S9 500	6	0	26.333	4.967	2.028
NDMA-S9 1000	6	0	28.667	5.645	2.305

Source of Variation	DF	SS	MS	F	P
Between Groups	4	175.46	43.86	1.670	0.188
Residual	25	656.83	26.27		
Total	29	832.30			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.188).

#### 4.1.4 Interpretation of NDMA Data

NDMA is a potent promutagen that requires mammalian microsomal activation (S9) to generate an ultimate alkylating mutagen that induced base-pair mutations in *S. typhimurium* strain YG7108. This result is clearly demonstrated in the comparison of the NDMA concentration-response curves of NDMA +S9 versus NDMA –S9 (Figure 4 lower panel and Table 5 versus Table 6). Mutagenic potency is a metric developed to compare different mutagens, for the purposes of this report to compare different nitrosamines. Note that throughout the concentration range of 5 to 1000  $\mu\text{M}$ , NDMA with or without S9 was not cytotoxic to *S. typhimurium* (Figure 4 upper panel and Tables 3 and 4, LB plate data). Thus we did not have to correct for cytotoxicity in the calculation of the mutagenic potency of NDMA. The NDMA potency values were derived from all concentrations analyzed in the concentration-response curve. This metric provides a measure of mutagenic activity as induced mutants per micromole ( $\mu\text{mol}$ ) of NDMA. The calculated mutagenic potency of NDMA with S9 metabolic activation was 47,694.7 revertants/ $\mu\text{mol}$ . The mutagenic potency of NDMA without S9 activation was 0.

## 4.2 1-Nitroso-(4-hydroxyethyl)-piperazine (Cansolv-N1)

### 4.2.1 Nitroso-HEP Mutagenicity and Cytotoxicity Data

Nitroso-HEP was analyzed with and without S9 mammalian microsomal activation in a concentration range from 1 to 20 mM. The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for Nitroso-HEP are presented in Tables 7 and 8. These data were used to generate the concentration-response curves (Figure 5).

Table 7. Plate counts of Nitroso-HEP (VB plates) and percent survivorship (LB plates) +S9.

Nitroso-HEP (Cansolv-N1) +S9 *S. typhimurium* YG7108 Experiments 021313 and 021913

Conc. mM	Revertants/Plate VB								
	0	1	2.5	5	7.5	10	15	20	NDMA + control
	18	23	31	26	37	32	36	35	0.25 mM NDMA 984
	23	34	28	38	36	32	40	43	0.1mM NDMA 569
	11	24	32	34	33	26	45	35	
	35	44	35	34	46	46	70	48	
	34	33	31	46	57	49	54	45	
	33	40	29	48	33	40	53	52	
	36	37	31	36	55	35	52	46	
	22	35	40	42	36	59	52	64	
	34	35	31	38	46	42	40	55	
Number	9	9	9	9	9	9	9	9	Number
Average	27.3	33.9	32.0	38.0	42.1	40.1	49.1	47.0	Average
SE	3.0	2.3	1.2	2.2	3.1	3.4	3.4	3.1	SE
SD	9.1	6.8	3.6	6.7	9.3	10.2	10.3	9.3	SD

Nitroso-HEP (Cansolv-N1) +S9 *S. typhimurium* YG7108 Experiment 021313

Conc. mM	LB Survivorship								
	0	1	2.5	5	7.5	10	15	20	% Negative Control
	349	390	333	310	300	347	255	278	
	317	335	311	280	328	287		306	
	334	336	324	279	265	251		288	
	104.7	117.0	99.9	93.0	90.0	104.1	76.5	83.4	% Negative Control
	95.1	100.5	93.3	84.0	98.4	86.1		91.8	
	100.2	100.8	97.2	83.7	79.5	75.3		86.4	
Number	3	3	3	3	3	3	1	3	Number
Average	100.0	106.1	96.8	86.9	89.3	88.5	76.5	87.2	Average
SE	2.8	5.5	1.9	3.1	5.5	8.4		2.5	SE
SD	4.8	9.4	3.3	5.3	9.5	14.5		4.3	SD

Table 8. Plate counts of Nitroso-HEP (VB plates) and percent survivorship (LB plates) –S9.

Nitroso-HEP (Cansolv-N1) –S9 *S. typhimurium* YG7108 Experiments 021313 and 021913

		Revertants/Plate VB								
Conc. mM	0	1	2.5	5	7.5	10	15	20		
	19	21	18	23	24	27	20	23	EMS = 943	
	26	18	20	30	34	15	22	34	EMS = 939	
	26	28	22	31	15	21	30	22		
	43	40	36	38	50	36	43	36		
	39	39	36	46	42	33	37	49		
	23	41	40	28	41	48	44	46		
	26	36	45	45	41	50	41	37		
	48	34	39	29	37	36	47	49		
	38	44	31	42	49	40		39		
Number	9	9	9	9	9	9	8	9	Number	
Average	32.0	33.4	31.9	34.7	37.0	34.0	35.5	37.2	Average	
SE	3.4	3.1	3.2	2.8	3.8	3.9	3.6	3.3	SE	
SD	10.1	9.2	9.7	8.3	11.4	11.6	10.3	10.0	SD	

Nitroso-HEP (Cansolv-N1) –S9 *S. typhimurium* YG7108 Experiments 021313

		LB Survivorship								
Conc. mM	0	1	2.5	5	7.5	10	15	20		
	291	287	323	339	277	236	265	319		
	334	283	287	313	289	239	316	310		
	178	313	296	324	300	229	266	257		
									% Negative Control	
	108.7	107.2	120.6	126.6	103.4	88.1	99.0	119.1		
	124.7	105.7	107.2	116.9	107.9	89.3	118.0	115.8		
	66.5	116.9	110.5	121.0	112.0	85.5	99.3	96.0		
Number	3	3	3	3	3	3	3	3	Number	
Average	100.0	109.9	112.8	121.5	107.8	87.6	105.4	110.3	Average	
SE	17.4	3.5	4.0	2.8	2.5	1.1	6.3	7.2	SE	
SD	30.1	6.1	7.0	4.9	4.3	1.9	10.9	12.5	SD	

#### 4.2.2 Nitroso-HEP Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of Nitroso-HEP in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 5. The top panel illustrates the cytotoxicity (LB plate experiments) for Nitroso-HEP with S9 metabolic activation (open squares) and without S9 activation (filled squares). The bottom panel represents the mutagenicity of Nitroso-HEP with S9 activation (open circles) and without S9 activation (filled circles).

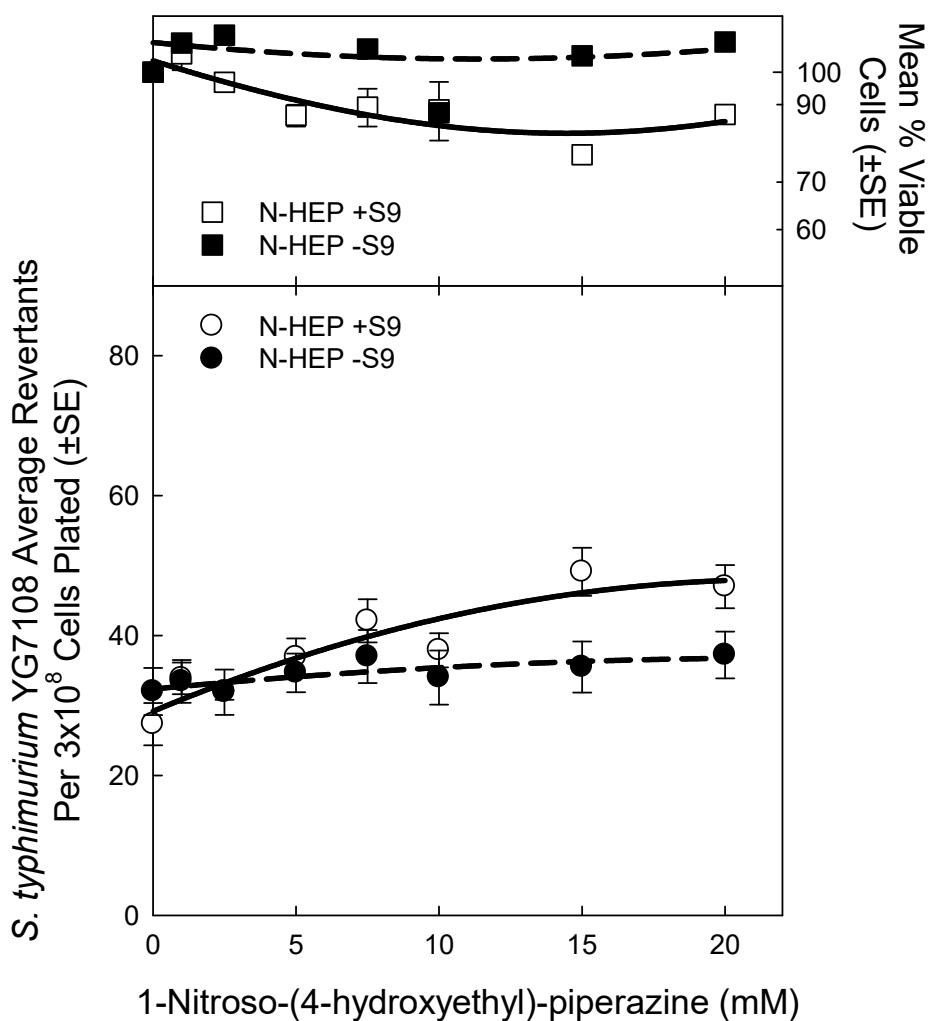


Figure 5. Nitroso-HEP concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

#### 4.2.3 Statistical Analyses of the Mutagenicity Data for Nitroso-HEP

An ANOVA test statistic for the mutagenicity data of Nitroso-HEP with S9 metabolic activation was conducted and the results are presented in Table 9.

Table 9. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitroso-HEP +S9

Data source: Data 2 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NHEP+S9 0.00	9	0	27.333	9.055	3.018
1.00 +S9	9	0	33.889	6.791	2.264
2.50 +S9	9	0	32.000	3.571	1.190
5.00 +S9	9	0	36.889	8.131	2.710
7.50 +S9	9	0	42.111	9.253	3.084
10.00 +S9	9	0	37.889	7.339	2.446
15.00	9	0	49.111	10.289	3.430
20.00 +S9	9	0	47.000	9.274	3.091

Source of Variation	DF	SS	MS	F	P
Between Groups	7	3498.000	499.714	7.426	<0.001
Residual	64	4306.444	67.288		
Total	71	7804.444			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):  
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
NHEP+S9 0.00 vs. 15.00 +S9	21.778	5.632	<0.001	0.007	Yes
NHEP+S9 0.00 vs. 20.00 +S9	19.667	5.086	<0.001	0.009	Yes
NHEP+S9 0.00 vs. 7.50 +S9	14.778	3.822	<0.001	0.010	Yes
NHEP+S9 0.00 vs. 10.00 +S9	10.556	2.730	0.008	0.013	Yes
NHEP+S9 0.00 vs. 5.00 +S9	9.556	2.471	0.016	0.017	Yes
NHEP+S9 0.00 vs. 1.00 +S9	6.556	1.695	0.095	0.025	No
NHEP+S9 0.00 vs. 2.50 +S9	4.667	1.207	0.232	0.050	No



An ANOVA test statistic for the mutagenicity data of Nitroso-HEP without S9 metabolic activation was conducted and the results are presented in Table 10.

Table 10. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitroso-HEP -S9

**Data source:** Data 2 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NHEP-S9 0	9	0	32.000	10.124	3.375
1.00 -S9	9	0	33.444	9.167	3.056
2.50 -S9	9	0	31.889	9.714	3.238
5.00 -S9	9	0	34.667	8.276	2.759
7.50 -S9	9	0	37.000	11.358	3.786
10.00 -S9	9	0	34.000	11.597	3.866
15.00 -S9	8	0	35.500	10.323	3.650
20.00 -S9	9	0	37.222	10.022	3.341

Source of Variation	DF	SS	MS	F	P
Between Groups	7	260.911	37.273	0.364	0.920
Residual	63	6452.667	102.423		
Total	70	6713.577			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.920).

#### 4.2.4 Interpretation of Nitroso-HEP Data

Nitroso-HEP is a weak promutagen that requires mammalian microsomal activation (S9) to generate an ultimate mutagen that induced base-pair mutations in *S. typhimurium* strain YG7108. This result is demonstrated in the comparison of the Nitroso-HEP concentration-response curves of Nitroso-HEP +S9 versus Nitroso-HEP -S9 (Figure 5 lower panel and Table 9 versus Table 10). With S9 activation the lowest concentration of Nitroso-HEP that expressed a mutagenic response was 5 mM (Table 9). Throughout the concentration range of 1 – 20 mM, Nitroso-HEP without S9 was not cytotoxic. However, Nitroso-HEP +S9 was weakly cytotoxic to *S. typhimurium* (Figure 5 upper panel and Tables 7 and 8, LB plate data). The Nitroso-HEP mutagenicity concentration-response curves based on revertants per  $1 \times 10^8$  surviving cells is presented in Figure 6. We adjusted for cytotoxicity in the calculation of the mutagenic potency of Nitroso-HEP +S9.

The Nitroso-HEP mutagenic potency values were derived from all concentrations analyzed in the concentration-response curve. The calculated mutagenic potency of Nitroso-HEP with S9 metabolic activation was 22.7 revertants/ $\mu\text{mol}$ . The mutagenic potency of Nitroso-HEP without S9 activation was 0.

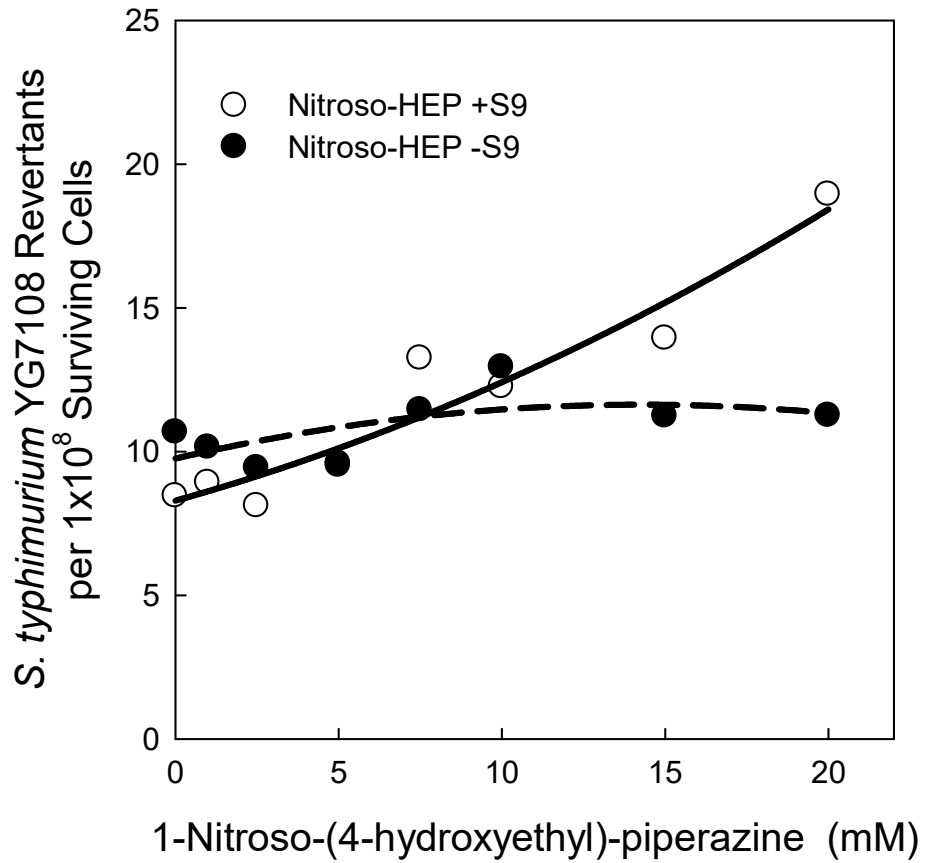


Figure 6. Nitroso-HEP mutagenicity concentration-response curves in *S. typhimurium* strain YG7108 with and without S9 metabolic activation. Data adjusted for cytotoxicity and expressed as revertants/ $1 \times 10^8$  surviving cells.

### 4.3 Cansolv-N2

#### 4.3.1 Cansolv-N2 Mutagenicity and Cytotoxicity Data

Cansolv-N2 was analyzed with and without S9 mammalian microsomal activation in a concentration range from 1 to 20 mM. The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for Cansolv-N2 are presented in Tables 11 and 12. These data were used to generate the Cansolv-N2 concentration-response curves (Figure 7).

Table 11. Plate counts of Cansolv-N2 (VB plates) and percent survivorship (LB plates) +S9.

Cansolv-N2 +S9 <i>S. typhimurium</i> YG7108 Experiments 021513 and 022113									
Revertants/Plate VB									
Conc. mM	0	1	2.5	5	7.5	10	15	20	
	26	20	21	26	30	35	34	43	250 $\mu$ M NDMA = 794
	21	19	23	24	34	29	40	35	100 $\mu$ M NDMA = 540
	20	13	15	30	29	19	32	44	
	24	46	24	33	43	49	42	50	
	19	44	21	32	35	37	31	75	
	24	14	15	29	40	47	45	50	
	22	24	22	32	36	29	32	56	
	24	31	23	23	41	29	39	40	
	48	19	20	25	40	26	53	50	
Number	9	9	9	9	9	9	9	9	Number
Average	25.3	25.6	20.4	28.2	36.4	33.3	38.7	49.2	Average
SE	2.9	4.1	1.1	1.3	1.6	3.3	2.4	3.8	SE
SD	8.8	12.2	3.3	3.8	4.9	9.8	7.3	11.5	SD

(Cansolv-N2 +S9 <i>S. typhimurium</i> YG7108 Experiment 021513)									
LB Survivorship									
Conc. mM	0	1	2.5	5	7.5	10	15	20	
	337	367	360	356	336	335	296	370	
	364	310	289	330	311	306	320	259	
		328	235	346	318	313	357	282	
	96.1	104.7	102.7	101.5	95.8	95.5	84.4	105.5	% Negative Control
	103.8	88.4	82.4	94.1	88.7	87.3	91.3	73.8	
		93.5	67.0	98.7	90.7	89.3	101.8	80.4	
Number	2	3	3	3	3	3	3	3	Number
Average	100.0	95.5	84.0	98.1	91.7	90.7	92.5	86.6	Average
SE	3.8	4.8	10.3	2.1	2.1	2.5	5.0	9.6	SE
SD	5.4	8.3	17.8	3.7	3.7	4.3	8.8	16.7	SD

Table 12. Plate counts of Cansolv-N2 (VB plates) and percent survivorship (LB plates) –S9.  
 Cansolv-N2 –S9 *S. typhimurium* YG7108 Experiments 021513 and 022113

		Revertants/Plate VB								
Conc. mM	0	1	2.5	5	7.5	10	15	20		
	17	37	52	51	48	83	65	91	EMS = 693	
	22	38	42	47	50	56	95	87	EMS = 592	
	20	27	43	51	39	76	66	52		
	20	32	33	54	34	48	60	64		
	53	23	39	46	43	61	56	64		
	23	21	29	42	30	56	50	60		
	21	18	37	34	35	59	71	51		
	29	36	20	45	38	48	55	49		
	13	28	53	37	36	48	52	65		
Number	9	9	9	9	9	9	9	9	Number	
Average	24.2	28.9	38.7	45.2	39.2	59.4	63.3	64.8	Average	
SE	3.9	2.4	3.5	2.2	2.2	4.2	4.6	5.0	SE	
SD	11.6	7.3	10.5	6.6	6.6	12.5	13.7	15.0	SD	

		Cansolv-N2 –S9 <i>S. typhimurium</i> YG7108 Expts. 021513								
		LB Survivorship								
Conc. mM	0	1	2.5	5	7.5	10	15	20		
	314	325	306	328	309	292	233	28		
	275	277	300	321	273	305	133	66		
	287	312	316	343	375	284	153	57		
	107.53	111.3	104.8	112.3	105.8	100.0	79.8	9.6	% Negative Control	
	94.2	94.9	102.7	109.9	93.5	104.5	45.6	22.6		
	98.3	106.8	108.2	117.5	128.4	97.3	52.4	19.5		
Number	3	3	3	3	3	3	3	3	Number	
Average	100.0	104.3	105.2	113.2	109.2	100.6	59.2	17.2	Average	
SE	3.9	4.9	1.6	2.2	10.2	2.1	10.5	3.9	SE	
SD	6.8	8.5	2.8	3.8	17.2	3.6	18.1	6.8	SD	

### 4.3.2 Cansolv-N2 Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of Cansolv-N2 in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 7. The top panel illustrates the cytotoxicity (LB plate experiments) for Cansolv-N2 with S9 metabolic activation (open squares) and without S9 activation (filled squares). It is clear that Cansolv-N2 is cytotoxic to *S. typhimurium* cells. The bottom panel represents the mutagenicity of Cansolv-N2 with S9 activation (open circles) and without S9 activation (filled circles).

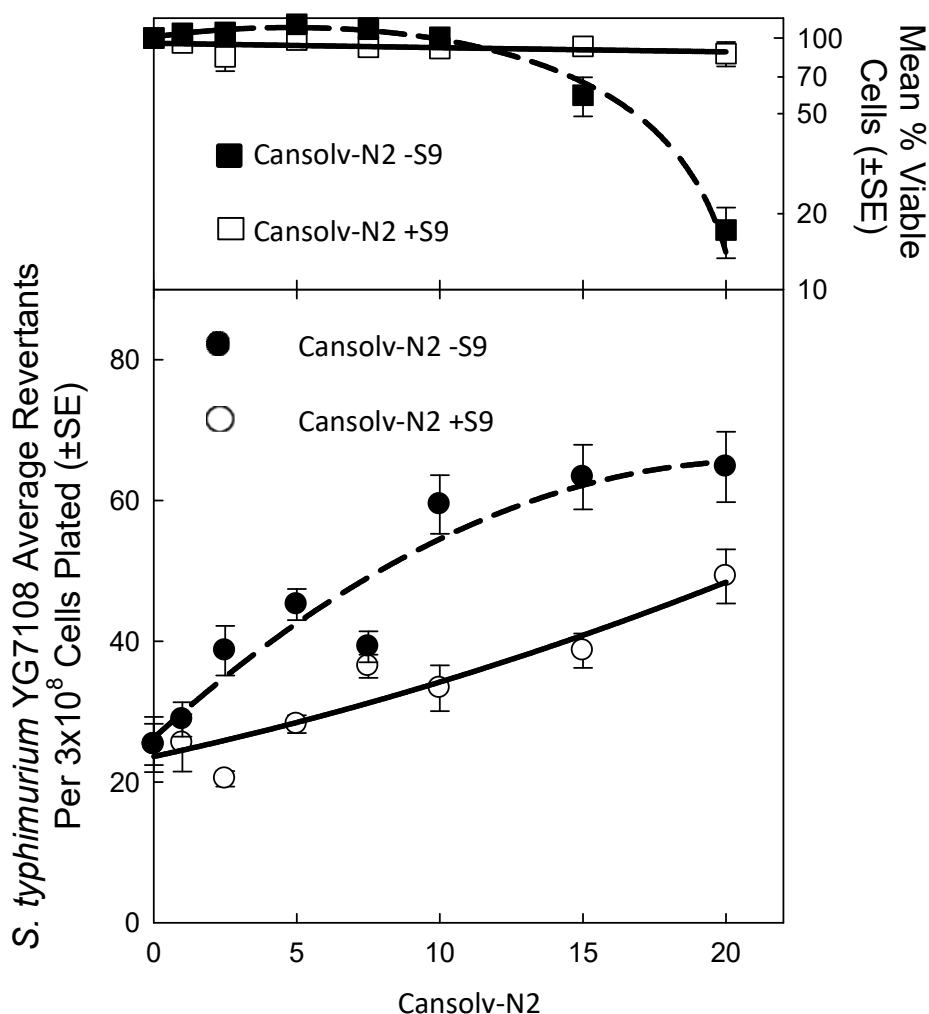


Figure 7. Cansolv-N2 concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

### 4.3.3 Statistical Analyses of the Mutagenicity Data for Cansolv-N2

An ANOVA test statistic for the mutagenicity data of Cansolv-N2 with S9 metabolic activation was conducted and the results are presented in Table 13.

Table 13. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Cansolv-N2 +S9

Data source: Data 4 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
0.00 Canv-N2 +S9	9	0	25.333	8.789	2.930
1.00 Can-N2+S9	9	0	25.556	12.238	4.079
2.50 Can-N2 +S9	9	0	20.444	3.321	1.107
5.00 Can-N2 +S9	9	0	28.222	3.801	1.267
7.50 Can-N2 +S9	9	0	36.444	4.927	1.642
10.00 Can-N2 +S9	9	0	33.333	9.772	3.257
15.00 Can-N2 +S9	9	0	38.667	7.314	2.438
20.00 Can-N2 +S9	9	0	49.222	11.541	3.847

Source of Variation	DF	SS	MS	F	P
Between Groups	7	5365.542	766.506	10.970	<0.001
Residual	64	4471.778	69.872		
Total	71	9837.319			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level
0.00 Can-N2 +S vs. 20.00 Can-N2 +S9	23.889	6.063	<0.001	0.007
0.00 Can-N2 +S vs. 15.00 Can-N2 +S9	13.333	3.384	0.001	0.009
0.00 Can-N2 +S vs. 7.50 Can-N2 +S9	11.111	2.820	0.006	0.010
0.00 Can-N2 +S vs. 10.00 Can-N2 +S9	8.000	2.030	0.046	0.013
0.00 Can-N2 +S vs. 2.50 Can-N2 +S9	4.889	1.241	0.219	0.017
0.00 Can-N2 +S vs. 5.00 Can-N2 +S9	2.889	0.733	0.466	0.025
0.00 Can-N2 +S vs. 1.00 Can-N2 +S9	0.222	0.0564	0.955	0.050

Comparison	Significant?
0.00 Can-N2 +S vs. 20.00 Can-N2+S9	Yes
0.00 Can-N2 +S vs. 15.00 Can-N2+S9	Yes
0.00 Can-N2 +S vs. 7.50 Can-N2+S9	Yes
0.00 Can-N2 +S vs. 10.00 Can-N2+S9	No
0.00 Can-N2 +S vs. 2.50 Can-N2+S9	No
0.00 Can-N2 +S vs. 5.00 Can-N2+S9	No
0.00 Can-N2 +S vs. 1.00 Can-N2+S9	No

An ANOVA test statistic for the mutagenicity data of Cansolv-N2 without S9 metabolic activation was conducted and the results are presented in Table 14.

Table 14. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Cansolv-N2--S9

Data source: Data 4 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
0.00 Can-N2 -S9	9	0	24.233	11.658	3.919
1.00 Can-N2 -S9	9	0	28.889	7.322	2.441
2.50 Can-N2 -S9	9	0	38.667	10.548	3.516
5.00 Can-N2 -S9	9	0	45.222	6.629	2.210
7.50 Can-N2 -S9	9	0	39.222	6.610	2.203
10.00 Can-N2 -S9	9	0	59.444	12.491	4.164
15.00 Can-N2 -S9	9	0	63.333	13.748	4.583
20.00 Can-N2 -S9	9	0	64.778	15.031	5.010

Source of Variation	DF	SS	MS	F	P
Between Groups	7	14875.333	2125.048	17.677	<0.001
Residual	64	7693.778	120.215		
Total	71	22569.111			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):  
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0.00 Can-N2-S vs. 20.00 Can-N2-S9	39.444	7.632	<0.001	0.007	Yes
0.00 Can-N2-S vs. 15.00 Can-N2-S9	38.000	7.352	<0.001	0.009	Yes
0.00 Can-N2-S vs. 10.00 Can-N2-S9	34.111	6.600	<0.001	0.010	Yes
0.00 Can-N2-S vs. 5.00 Can-N2-S9	19.889	3.848	<0.001	0.013	Yes
0.00 Can-N2-S vs. 7.50 Can-N2-S9	13.889	2.687	0.009	0.017	Yes
0.00 Can-N2-S vs. 2.50 Can-N2-S9	13.333	2.580	0.012	0.025	Yes
0.00 Can-N2-S vs. 1.00 Can-N2-S9	3.556	0.688	0.494	0.050	No

#### 4.3.4 Interpretation of Cansolv-N2 Data

Cansolv-N2 is a weak direct-acting mutagen that induced base-pair substitution mutations in *S. typhimurium* strain YG7108. The lowest Cansolv-N2 (-S9) concentration that induced a statistically significant increase in mutagenicity as compared to the negative control was 2.5 mM (Table 14). At concentrations above 10 mM Cansolv-N2 induced substantial cytotoxicity (Table 12, LB survivorship plates; Figure 7 upper panel). Thus this sample of Cansolv-N2 expresses direct mutagenic and cytotoxic activity. When Cansolv-N2 was analyzed with S9 activation we observed a reduction in mutagenicity (Figure 7 lower panel); the lowest concentration that induced a significant increase in mutagenic activity was 7.5 mM (Table 13). Throughout the concentration range from 1 – 20 mM Cansolv-N2 +S9 did not induce

cytotoxicity (Figure 7 upper panel). It appears that the mammalian microsomal monooxygenases detoxified the direct-acting activity of Cansolv-N2.

In order to calculate the mutagenic potency of Cansolv-N2 with and without S9 activation we normalized the data on a survivorship basis. The mutagenicity concentration-response curves of Cansolv-N2 +S9 and -S9 based on revertants per  $1 \times 10^8$  surviving cells is presented in Figure 8. The Cansolv-N2 mutagenic potency value was derived from all the concentrations analyzed in the concentration-response curve. The calculated mutagenic potency of Cansolv-N2 with S9 metabolic activation was 13.1 revertants/ $\mu\text{mol}$ . The mutagenic potency of Cansolv-N2 without S9 activation was 53.8 revertants/ $\mu\text{mol}$ .

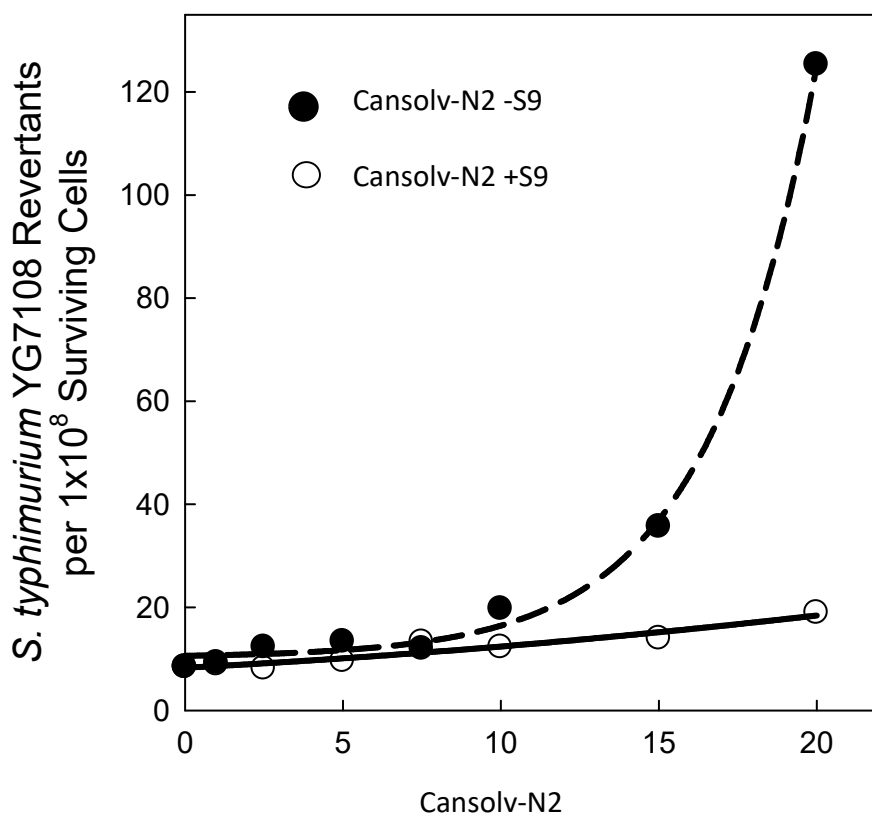


Figure 8. Cansolv-N2 mutagenicity concentration-response curves in *S. typhimurium* strain YG7108 with and without S9 metabolic activation. Data adjusted for cytotoxicity and expressed as revertants/ $1 \times 10^8$  surviving cells.



#### 4.4. Nitrosopiperazine Cansolv-N3

##### 4.4.1 Nitrosopiperazine Mutagenicity and Cytotoxicity Data

Nitrosopiperazine was analyzed with and without S9 mammalian microsomal activation in a concentration range from 1 to 20 mM. The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for Nitrosopiperazine are presented in Tables 15 and 16. These data were used to generate the Nitrosopiperazine concentration-response curves (Figure 9).

Table 15. Plate counts of Nitrosopiperazine (VB plates) and percent survivorship (LB plates) +S9.

Cansolv Nitrosopiperazine +S9 <i>S. typhimurium</i> YG7108 Experiments 022613, 030513, 030713									
<b>Revertants/Plate VB</b>									
Conc. mM	0	1	2.5	5	7.5	10	15	20	NDMA
	28	185	259	404	465	649	624	803	50 µM = 306
	14	168	289	467	480	549	626	821	1 mM = 1376
	20	205	256	428	549	567	816	859	0.1 mM=696
	18	261	326	406	716	641	689	749	
	21	306	367	477	618	692	696	836	
	12	236	357	449	700	711	787	794	
	26	267	361	444	512	666	764	796	
	20	275	419	470	594	664	685	683	
	20	252	393	432	473	591	786	841	
	30				538	502	663	822	
	21				545	720	747	715	
	29								
Number	12	9	9	9	11	11	11	11	Number
Average	21.6	239.4	336.3	441.9	562.7	632.0	716.6	792.6	Average
SE	1.6	15.1	19.3	8.9	26.0	21.3	20.2	16.6	SE
SD	5.7	45.2	57.9	26.7	86.3	70.6	66.8	55.1	SD
Cansolv Nitrosopiperazine +S9 <i>S. typhimurium</i> YG7108 Experiments 022613									
<b>LB Survivorship</b>									
Conc. mM	0	1	2.5	5	7.5	10	15	20	
	289	335	330	317	332	295	327	309	
	304	300	296	292	313	287	303	300	
	304	300	296	292	313	287	303	300	
									% Negative Control
	96.7	112.0	110.4	106.0	111.0	98.7	109.4	103.3	
	101.7	97.3	104.0	111.0	99.3	111.0	111.0	98.0	
	101.7	100.3	99.0	97.7	104.7	96.0	101.3	100.3	
Number	3	3	3	3	3	3	3	3	Number
Average	100.0	103.2	104.5	104.9	105.0	101.9	107.2	100.6	Average
SE	2.0	4.5	3.3	3.9	3.4	4.6	3.0	1.5	SE
SD	2.9	7.8	5.7	6.8	5.9	8.0	5.2	2.7	SD

The revertant plate count and the percent survivorship data for Nitrosopiperazine without S9 metabolic activation are presented in Table 16.

Table 16. Plate counts of Nitrosopiperazine (VB plates) and percent survivorship (LB plates) –S9.

Cansolv Nitrosopiperazine –S9 *S. typhimurium* YG7108 Experiments 022613 and 030513

		Revertants/Plate VB							
Conc. mM	0	1	2.5	5	7.5	10	15	20	EMS = 870
	27	29	25	32	33	33	22	28	EMS = 615
	25	30	22	38	27	32	24	24	
	31	25	25	32	32	25	27	31	
	29	18	23	25	26	24	25	30	
	23	24	24	27	29	20	22	34	
	24	26	23	26	30	28	19	25	
	23	28	20	24	19	25	18	24	
	26	29	19	22	28	23	26	28	
	27	23	26	24	28	30	31	23	
Number	9	9	9	9	9	9	9	9	Number
Average	26.1	25.8	23.0	27.8	28.0	26.7	23.8	27.4	Average
SE	0.9	1.3	0.8	1.7	1.4	1.5	1.4	1.2	SE
SD	2.7	3.8	2.3	5.2	4.1	4.4	4.1	3.7	SD

Cansolv Nitrosopiperazine –S9 *S. typhimurium* YG7108 Experiments 022613

		LB Survivorship							
Conc. mM	0	1	2.5	5	7.5	10	15	20	
	286	300	324	317	308	299	232	295	
	320	349	292	362	304	314	298	328	
	345	335	364	325	337	296	273	343	
	90.2	94.6	102.2	100.0	97.2	94.3	73.2	93.1	% Negative Control
	100.9	110.1	92.1	114.2	95.9	99.1	94.0	103.5	
	108.8	105.7	114.8	102.5	106.3	93.4	86.1	108.2	
Number	3	3	3	3	3	3	3	3	Number
Average	100.0	103.5	103.0	105.6	99.8	95.6	84.4	101.6	Average
SE	6.6	4.6	6.6	4.4	3.3	1.8	6.1	4.5	SE
SD	9.3	8.0	11.4	7.6	5.7	3.0	10.5	7.7	SD

#### 4.4.2 Nitrosopiperazine Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of Nitrosopiperazine in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 9. The top panel illustrates the cytotoxicity (LB plate experiments) for Nitrosopiperazine with S9 metabolic activation (open squares) and without S9 activation (filled squares). The bottom panel represents the mutagenicity of Nitrosopiperazine with S9 activation (open circles) and without S9 activation (filled circles).

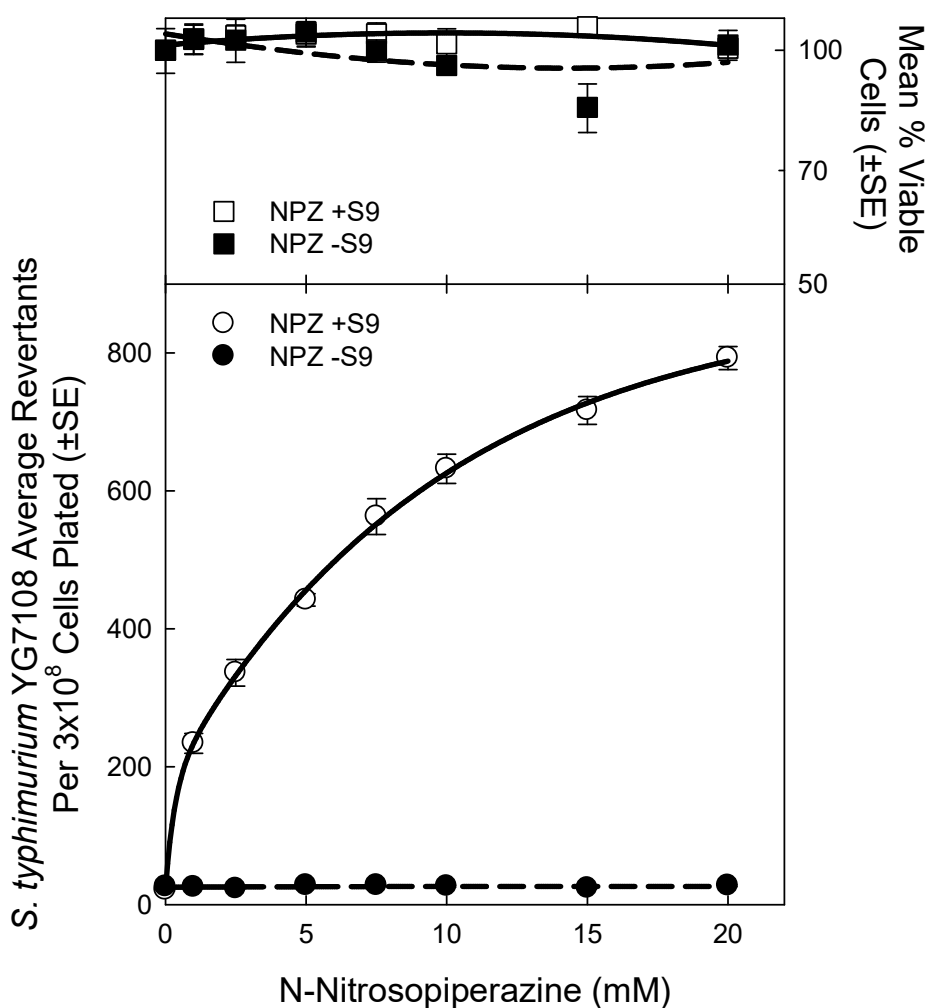


Figure 9. Nitrosopiperazine concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

#### 4.4.3 Statistical Analyses of the Mutagenicity Data for Nitrosopiperazine

An ANOVA test statistic for the mutagenicity data of Nitrosopiperazine with S9 metabolic activation was conducted and the results are presented in Table 17.

Table 17. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitrosopiperazine +S9

Data source: Data 7 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NPZ +S9 0	12	0	21.583	5.696	1.644
+S9 1	9	0	239.48	45.214	15.138
+S9 2.5	9	0	336.33	57.881	19.294
+S9 5	9	0	441.88	26.746	8.915
+S9 7.5	11	0	562.72	86.315	26.025
+S9 10	11	0	632.00	70.590	21.284
+S9 15	11	0	716.63	66.832	20.150
+S9 20	11	0	792.63	55.102	16.614

Source of Variation	DF	SS	MS	F	P
Between Groups	7	5279880.6	754268.6	228.7	<0.001
Residual	75	247249.96	3296.6		
Total	82	5527130.6			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ( $P = <0.001$ ).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):  
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
NPZ +S9 0 vs. +S9 20	771.053	32.171	<0.001	0.007	Yes
NPZ +S9 0 vs. +S9 15	695.053	29.000	<0.001	0.009	Yes
NPZ +S9 0 vs. +S9 10	610.417	25.469	<0.001	0.010	Yes
NPZ +S9 0 vs. +S9 7.5	541.144	22.579	<0.001	0.013	Yes
NPZ +S9 0 vs. +S9 5	420.306	16.601	<0.001	0.017	Yes
NPZ +S9 0 vs. +S9 2.5	314.750	12.432	<0.001	0.025	Yes
NPZ +S9 0 vs. +S9 1	212.306	8.385	<0.001	0.050	Yes

An ANOVA test statistic for the mutagenicity data of Nitrosopiperazine without S9 metabolic activation was conducted and the results are presented in Table 18.

Table 18. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitrosopiperazine -S9

Data source: Data 7 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
0 -S9	9	0	26.111	2.713	0.904
1 -S9	9	0	25.822	3.801	1.267
2.5 -S9	9	0	23.000	2.345	0.782
5 -S9	9	0	27.778	5.167	1.722
7.5 -S9	9	0	28.000	4.062	1.354
10 -S9	9	0	26.667	4.359	1.453
15 -S9	9	0	23.778	4.055	1.352
20 -S9	9	0	27.444	3.745	1.248

Source of Variation	DF	SS	MS	F	P
Between Groups	7	218.2	31.1	2.079	0.059
Residual	64	959.7	14.9		
Total	71	1178.0			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.059).

#### 4.4.4 Interpretation of Nitrosopiperazine Data

Nitrosopiperazine is a promutagen that requires mammalian microsomal activation (S9) to generate an ultimate alkylating mutagen that induced base-pair substitution mutations in *S. typhimurium* strain YG7108. This result is clearly demonstrated in the comparison of the Nitrosopiperazine concentration-response curves of Nitrosopiperazine +S9 versus Nitrosopiperazine -S9 (Figure 9 lower panel and Table 17 versus Table 18). Note that throughout the concentration range of 1 to 20 mM, Nitrosopiperazine with or without S9 was not cytotoxic to *S. typhimurium* (Figure 9 upper panel and Tables 15 and 16, LB plate data). Thus we did not have to adjust for cytotoxicity in the calculation of the mutagenic potency of Nitrosopiperazine. The Nitrosopiperazine mutagenic potency values were derived from all concentrations analyzed in the concentration-response curve. This metric provides a measure of mutagenic activity as induced mutants per micromole ( $\mu\text{mol}$ ) of Nitrosopiperazine. The calculated mutagenic potency of Nitrosopiperazine with S9 metabolic activation was 914.7 revertants/ $\mu\text{mol}$ . The mutagenic potency of Nitrosopiperazine without S9 activation was 0.

## 5. Summary and Conclusions

In this project we employed a highly sensitive *S. typhimurium* strain, YG7108, that was developed to evaluate the mutagenicity of alkylating agents such as nitrosamines to study the mutagenicity of two carcinogenic nitrosamines, Nitrosodimethylamine and Nitrosopiperazine (Cansolv-N3). The U.S. EPA risk level for increased cancer of 1/1000000 via oral consumption of NDMA is  $7.0 \times 10^{-4}$   $\mu\text{g/L}$  for drinking water [16]. The Carcinogenic Potency Database (CPDB) was developed at the University of California, Berkeley, and Lawrence Berkeley Laboratory and is a standardized analysis of animal cancer tests. The primary CPDB metric is the TD<sub>50</sub> value which is the “dose-rate in mg/kg body wt/day which, if administered chronically for the standard lifespan of the species, will halve the probability of remaining tumorless throughout that period” [17]. Both NDMA and Nitrosopiperazine (Cansolv-N3) are listed in the Carcinogenic Potency Database with TD<sub>50</sub> values as 0.0959 and 8.78, respectively. Based on the TD<sub>50</sub> values, NDMA is approximately 91.6 times more carcinogenic than Nitrosopiperazine (Cansolv-N3). NDMA is a potent promutagenic nitrosamine (Figure 4) while Nitrosopiperazine is also a promutagen that requires metabolic activation; it expressed less mutagenic activity than NDMA (Figure 9). Comparing the mutagenic potencies for NDMA and Nitrosopiperazine (Cansolv-N3) derived from this study, the former nitrosamine is approximately 52× more mutagenic (Table 19).

Table 19. Comparative mutagenicity of Cansolv nitrosamines in *S. typhimurium* strain YG7108 with and without S9 metabolic activation

Compound	CASN <sup>a</sup>	<i>Salmonella typhimurium</i> YG7108 Reversion Assay			
		Concentration Range	S9 <sup>b</sup>	Lowest Mutagenic Conc. <sup>c</sup>	Mutagenic Potency (rev/ $\mu\text{mol}$ ) <sup>d</sup>
NDMA	62-75-9	100-1000 $\mu\text{M}$	-S9	Negative	Negative
NDMA	62-75-9	5-1000 $\mu\text{M}$	+S9	10 $\mu\text{M}$	47,694.7
Nitrosopiperazine	5632-47-3	1-20 mM	-S9	Negative	Negative
Nitrosopiperazine	5632-47-3	1-20 mM	+S9	1 mM	914.7
Nitroso-HEP	48121-20-6	1-20 mM	-S9	Negative	Negative
Nitroso-HEP	48121-20-6	1-20 mM	+S9	5 mM	22.7
Cansolv-N2	-	1-20 mM	-S9	2.5 mM	53.8
Cansolv-N2	-	1-20 mM	+S9	7.5 mM	13.1

<sup>a</sup> American Chemical Society chemical abstract service number. <sup>b</sup> Aroclor-induced hepatic microsomal mixture (MolTox Inc.). <sup>c</sup> The lowest nitrosamine concentration that induced a statistical increase over the concurrent negative control as defined by an ANOVA test statistic. <sup>d</sup> The mutagenic potency was the average induced revertants per  $\mu\text{mol}$  calculated from the concentration-response curve. Higher mutagenic potency values denote greater mutagenicity.

For the Cansolv nitrosamines, Nitroso-HEP (Cansolv-N1) was  $4.8 \times 10^{-4}$  times as mutagenic as NDMA while Cansolv-N2 with and without S9 activation were  $2.7 \times 10^{-4}$  and  $1.1 \times 10^{-3}$  times as mutagenic as NDMA, respectively (Table 19). With respect to the mutagenicity of

Nitrosopiperazine (Cansolv-N3), Nitroso-HEP and Cansolv-N2 were 0.02 and 0.06 times as mutagenic.

In comparison to other environmental nitrosamines, such as those found in drinking water, we found that Nitroso-HEP was 0.003×, 0.002×, or 0.02× as mutagenic as Nitrosomorpholine, Nitrosopiperidine or Nitrosopyrrolidine, respectively. Similarly, for Cansolv-N2 its mutagenicity was 0.008×, 0.006× or 0.05× that of Nitrosomorpholine, Nitrosopiperidine or Nitrosopyrrolidine, respectively (Table 20). We conclude that Nitroso-HEP and Cansolv-N2 are very weak mutagens.

Table 20. Comparison of the mutagenic potency of project nitrosamines with selected nitrosamines found in drinking water

Nitrosamine	CASN	Lowest Mutagenic Concentration (mM)	Mutagenic Potency (rev/μmol)	Reference
N-Nitrosodimethylamine	62-75-9	0.010	47,694.7	this study
1-Nitrosopiperazine	5632-47-3	1.0	914.7	this study
1-Nitroso-(4-hydroxyethyl)-piperazine	48121-20-6	5.0	22.7	this study
Cansolv-N2	-	2.5	53.8	this study
N-Nitrosomorpholine	59-89-2	0.5	6724	[9]
N-Nitrosopiperidine	100-75-4	0.1	9588	[9]
N-Nitrosopyrrolidine	930-55-2	0.75	997	[9]

The results from this study are the following.

1. NDMA is an exceedingly potent mutagen after mammalian microsomal metabolism.
2. The Nitrosopiperazine (Cansolv-N3) sample provided was mutagenic and the agent required mammalian microsomal metabolic activation. Without S9 activation Nitrosopiperazine was not mutagenic.
3. Nitroso-HEP (Cansolv N-1) was an exceedingly weak mutagenic agent that required S9 metabolic activation. Nitroso-HEP was not directly mutagenic.
4. Cansolv-N3 was a direct, albeit very weak mutagen and S9 metabolic activation detoxified the nitrosamine and reduced its mutagenic activity.
5. When compared to environmental nitrosamines such as nitrosamine drinking water disinfection by-products, both Nitroso-HEP and Cansolv-N2 were considered very weak mutagens.

## References

1. International Energy Agency *Tracking Progress in Carbon Capture and Storage: International Energy Agency/Global CCS Institute report to the third Clean Energy Ministerial*; OECD/International Energy Agency and Global CCS Institute: 2012; p 20.
2. Reynolds, A. J.; Verheyen, T. V.; Adeloju, S. B.; Meuleman, E.; Feron, P., Towards commercial scale postcombustion capture of CO<sub>2</sub> with monoethanolamine solvent: key considerations for solvent management and environmental impacts. *Environ. Sci. Technol.* **2012**, *46*, (7), 3643-3654.
3. Fostas, B.; Gangstad, A.; Nienseter, B.; Pedersen, S.; Sjøvoll, M.; Sørensen, A. L., Effects of NO<sub>x</sub> in the flue gas degradation of MEA. *Energy Procedia* **2011**, *4*, 1566-1573.
4. Scherf, H. R.; Frei, E.; Wiessler, M., Carcinogenic properties of N-nitrodimethylamine and N-nitromethylamine in the rat. *Carcinogenesis* **1989**, *10*, (11), 1977-1981.
5. Magee, P. N., Toxicity of nitrosamines: their possible human health hazards. *Food Cosmet. Toxicol.* **1971**, *9*, (2), 207-218.
6. Hassel, M.; Frei, E.; Streeter, A. J.; Wiessler, M., Pharmacokinetics of N-nitrodimethylamine and N-nitromethylamine in the rat. *Carcinogenesis* **1990**, *11*, (3), 357-360.
7. Dai, N.; Shah, A. D.; Hu, L.; Plewa, M. J.; McKague, B.; Mitch, W. A., Measurement of nitrosamine and nitramine formation from NO<sub>x</sub> reactions with amines during amine-based carbon dioxide capture for postcombustion carbon sequestration. *Environ. Sci. Technol.* **2012**, *46*, (17), 9793-9801.
8. Goldman, M. J.; Fine, N. A.; Rochelle, G. T., Kinetics of N-nitrosopiperazine formation from nitrite and piperazine in CO<sub>2</sub> capture. *Environ. Sci. Technol.* **2013**, *In Press*.
9. Wagner, E. D.; Hsu, K. M.; Lagunas, A.; Mitch, W. A.; Plewa, M. J., Comparative genotoxicity of nitrosamine drinking water disinfection byproducts in *Salmonella* and mammalian cells. *Mutat.Res.* **2012**, *741*, 109-115.
10. Maron, D. M.; Ames, B. N., Revised methods for the Salmonella mutagenicity test. *Mutat.Res.* **1983**, *113*, (3-4), 173-215.
11. Claxton, L. D.; Umbuzeiro, G. d.; DeMarini, D. M., The Salmonella mutagenicity assay: The stethoscope of genetic toxicology for the 21st century. *Environ. Health Perspect.* **2010**, *118*, (11), 1515-1522.
12. Yamada, M.; Matsui, K.; Sofuni, T.; Nohmi, T., New tester strains of *Salmonella typhimurium* lacking O<sup>6</sup>-methylguanine DNA methyltransferases and highly sensitive to mutagenic alkylating agents. *Mutat.Res.* **1997**, *381*, 15-24.
13. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in *Salmonella typhimurium*. *Teratogen. Carcinogen. Mutagen.* **2002**, *22*, (2), 113-128.
14. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., A new assessment of the cytotoxicity and genotoxicity of drinking water disinfection by-products. In *Natural Organic Matter and Disinfection By-Products: Characterization and Control in Drinking Water*, Barrett, S. E.; Krasner, S. W.; Amy, G. L., Eds. American Chemical Society: Washington, D.C., 2000; pp 16-27.



15. Timbrell, J. A., *Principles of Biochemical Toxicology*. Taylor and Francis Ltd.: London, 1987; p 248.
16. U. S. Environmental Protection Agency, Integrated Risk Information System; United States Environmental Protection Agency *Office of Research and Development (ORD), National Center for Environmental Assessment* **2005**.
17. The Carcinogenic Potency Database (CPDB) <http://potency.berkeley.edu/>