Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50100
1.2	Provide the name of the licenced establishment.	TNO
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 Type of animal procedure: Characterization of drug responses, including pharmacokinetics

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The aim of these studies is to characterize drug responses, including pharmacokinetics, in the specific species used for the validated exposure to toxic chemicals. In this phase, dose range finding and possible side effects of medical (pharmacological) interventions will be characterized in healthy animals. Critical physiological parameters and/ or pharmacokinetics will be determined to estimate best practices for intervention in a field situation.
Linearity of kinetics can be determined, and additional physiological information (EEG, ECG) could be used to assess the adequacy of and relevance to the efficacy of compounds, including dose response (pharmacodynamics). In particular, these studies will be aimed at providing information useful for addressing bioavailability, changes in pharmacokinetics and pharmacodynamics depending on the route of administration. This will allow the determination of optimal timing of dosing in relation to the clinical signs of toxicity as determined in phase I, to optimize the design of studies in the framework of appendix 3.4.4.3 (determination of therapeutic efficacy in an animal model).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

The experiments within the framework of this appendix consist of 2 phases, starting with a preparation phase, mostly surgically, and followed by an exposure phase, to monitor pharmacokinetics and/ or physiology. Depending on the route of administration, and study aim, animals can undergo one of the procedures described below.

1. Animal preparation (optional)

The initial phase consists of preparation of the selected animal species for dosing and selected necessary read out parameters to enable real time monitoring of pharmacokinetics and -dynamics.
All surgical procedures will be carried out under species- and procedure appropriate anesthesia. Specifics, will be specified in the individual research plan. Appropriate peri-operative care will be provided (such as analgesia). Animals will be surgically equipped with one or more of the following, as described in appendix 3.4.4.1: **Jugular vein and/ or carotid artery cannula; EEG/ ECG electrode (Telemetry, externally, possibly implant), Implantation of osmotic minipump.** Instead of with a toxicant, animals will be dosed with a pharmaceutical.

2. **Administration of drugs**

Dosing will be performed in awake animals, after an appropriate recovery period of minimal 3 days up to 2 weeks, depending on the instrumentation and prechallenge surgery. In case of serial sampling within a few hours, an indwelling cannula allows low-invasive blood sampling. In case longer intervals (hours to days) and smaller volumes are required, alternate blood sampling strategies, such as tail vein puncture (depending on species), could be employed. However, the majority of studies within this framework will be aimed at short term efficacy, and thus acute pharmacokinetics.
Animals can be exposed to agents via various routes, depending on the nature of the research question. Animals can be exposed according to the routes, described below. Dose and route depend on the nature of the research question and will follow be in line with Diehl *et al*. Journal of Appl. Tox 2001; "A good practice…"

Routes envisaged are:
subcutaneous injection (all species)
intravenous injection
Under restraint via the tail vein (rat, mouse), under anesthesia in the penile vein (guinea pig) or via indwelling permanent catheter (small animals) (or temporary via butterfly access (minipigs)
intramuscular injection (rat, guinea pigs, minipigs)
intraperitoneal injection (mice, rats, guinea pigs)
intraosseus injection: a needle is rapidly drilled into the bone (all species except mice)

3 Monitoring

Blood sampling

Generally from the tail (rat, mouse) or ear (guinea pig) under restraint (relatively small volumes with long intervals), via an indwelling cannula when larger volumes and short intervals are needed. Temporary vein access with e.g. butterfly needles will be applied in minipigs). Maximum volumes will be determined according to Diehl *et al*. Journal of Appl. Tox 2001; "A good practice…"

EEG, ECG monitoring (Telemetry) (all species)

The electrical signals (ECG/EEG) will be wirelessly transferred, from the small wireless transmitter attached to the head stage. The animals will be housed individually during assessment to prevent interference of the signals of the different animals.

At the end of the monitoring phase, tissues will be harvested after appropriate euthanasia.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The administration route, dose, etc. will be determined *a priori* based on literature and required parameters for follow-up studies described in appendix 3.4.4.3. In cases in which only very limited information of a drug is available, a pilot experiment with a few (1-2) animals per dosing group may be performed to obtain initial pharmacokinetic and distribution information. A study design could consist of minimal 4 animals per dosing group, and 1-3 doses. When more doses are applied, less animals may be needed per group, as dose response information can be obtained. In most cases the number of animals

per group will be higher, in particular when physiological data is to be obtained (cardiovascular, respiration, EEG). Such designs, with more readout parameters with possibly more variability and more comparisons, require correction for such multiple comparisons. Alternate dosing routes than intravenous generally show higher variability.Additionally, for drugs that require extensive workup for analysis, possibly increasing variability in analytical outcome, numbers may be increased up to 6-8 animals per group.
In this phase, drug levels (maximum plasma levels, or other PK parameters) could be an outcome, or a certain increase or decrease of physiological parameters. On the other hand, in certain cases it might be an objective to find a dose without physiological adverse effects influencing the number of animals to be used. A clinical adverse effect will be determined, defined in a percentage increase or decrease, based on which a statistical power analysis will be conducted to estimate the number of animals required per dosing group.

Karl-Heinz Diehl, Robin Hull, David Morton, Rudolf Pfister, Yvon Rabemampianina, David Smith,*, Jean-Marc Vidal and Cor van de Vorstenbosch: A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. J. Appl. Toxicol. 21, 15–23 (2001)

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Based on the research question the animal model as described in appendix 3..4.4.1 will be used, ranging from small animals (mice, rats, guinea pigs), to larger animals (minipigs). For foundation: see appendix 3.4.4.1.
Depending on the target population for the treatment, juvenile, adult or aged animals will be used. The choice of gender will be based on literature, but also in line with the gender to be used in the efficacy phase. Initial phases will most likely be performed in one gender only, in line with results obtained in studies performed in the framework of Appendix 3.4.4.1., taking into account whether the response to pharmaceutical is gender specific or not. However, to minimize possible variations, initial studies will preferably be performed in one gender at a time, and in follow up stages critical challenges and doses might be transferred to the opposite sex. (as described in appendix 1).
Estimated number over 5 year period per species:

Annex II	Mice	Rats	Guinea Pigs	Minipigs
Total estimated 5-year	40	100	80	60
Nr of studies	1-2	4-5	2-3	1-2
1 Terminal	60%	10%	10%	20%
2 Mild	0%	0%	0%	0%
3 Moderate	40%	90%	90%	80%
4 Severe	0%	0%	0%	0%

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement
In general, the ADME/PK/TK studies are obligatory for registration of a test substance or in support of clinical trials, or for use in Phase III or replacement thereof as described in appendix 3.4.4.1. If the guideline suggests to use alternatives that do not involve animals, then these types of tests will be used for registration purposes. However, in the most recent guidelines, no authority approved-alternatives for ADME/PK/TK studies. For Drug approval for the general population in the USA, FDA approval is required. In Europe, EMA regulates approval of new drugs.
Reduction
The number of animals will be reduced by well-designed studies (e.g. appropriate control groups, dose(s) and route(s) etc). In addition, the use of telemetric readout parameters allows gathering of pharmacodynamic information, and can help to reduce the number of animals used for establishing the dose.
Refinement
See below.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Pre-dosing surgery will be performed under anaesthesia and appropriate peri-operative care such as analgesia. Animals are housed according to EU regulations and provided appropriate cage space, bedding material and species specific environmental enrichment. Furthermore, research designs will be optimized to obtain as much information as (technically) possible, taking into account possibly increasing discomfort. Obtaining physiological data by telemetry requires one surgery, but after that less handling and discomfort is required. Similarly, placement of a cannula induces discomfort once, but does prevent repeated skin puncturing or restraint.
If necessary, for any animal showing signs of discomfort, measures will be taken to relieve this discomfort and, if necessary, treatment of the animals will be altered. If an animal shows signs of severe discomfort (Humane endpoints), they will be humanely killed after consultation with the Animal Welfare Body.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Before any intervention, animals will be housed according to the guidelines, or whenever possible. In cases in which animals are instrumented by surgery, animals will be housed individually to prevent damage to cannulas or electrodes, always taking into account that the animals cannot hurt themselves.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Not applicable

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Surgical procedures will be carried out under appropriate anesthesia, such as isoflurane (4-5% induction, 1-2% maintenance) anesthesia, depending on surgical procedure and animal species. If deviant, this will be specified in the individual study plan.
Appropriate peri-operative care will be provided with analgesics and/ or antibiotics up to 48 hours. Animals will be monitored frequently immediately after surgery (until full recovery, based on voluntary movement and consciousness, then lowered to twice daily, or once at later stage (after ~1 week). See humane endpoints for observation criteria.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

The aim of the study is to determine a pharmacokinetic/ pharmacodynamic profile of a prospective drug, depending on route of administration and dosing. Adverse effects, could occur during these studies, which are expected to be possibly mild toxic effects due to the test substance and/or treatment of the animals. In the unlikely event a surgery would be not successful, this could lead to compromised recovery, measures to be taken in such a case are described in paragraph (J).

Intraosseus: Intraosseus administration is a fairly new fieldable easy to achieve administration route directly in the bone, already used in human beings (see: https://www.youtube.com/watch?v=oIVJdEwW1Bg). For classification of discomfort, see H).

Explain why these effects may emerge.

Although surgeries are always performed by well-trained technicians or scientists, certain procedures are not without risk. In certain cases, drugs might be relatively new, or alternate administration routes may affect pharmacokinetics and dynamics. Intraosseus is a fairly new technique with which we have no experience yet.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Surgery will be performed using appropriate analgesia and anaesthesia, and animals will be monitored closely for adverse signs during recovery from a procedure. Furthermore, animals will be observed closely after dosing, and humane endpoints are defined.

Intraosseus injection is a fairly new technique to rapidly access the blood stream. In case this technique will be applied in animals, a stepwise training protocol will be designed in consultation with the Animal Welfare Body, and will at least include performing the technique on dead animals, followed by animals under terminal anaesthesia. Eventually, the procedure will be setup in awake animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

A failure from the animals to recover from surgery, i.e. has not returned to initial body weight at the day of exposure after surgery. A normal short body weight dip of around 10% can occur, but normally the animals rapidly return to their pre-operative weight within a 1-5 day recovery period. After nerve agent exposure, the body weight loss is similar, and lasts for 1-3 days. If the animals fail to recover to their pre-injection weight within 5 days, or show progressive weight loss during 3-4 days, euthanasia will be considered.
Furthermore a drop in heart rate under ~35% of baseline level is an indication for early termination of the experiment. Specific end points will be specified per individual study plan.
Scoring for overall condition after a treatment/ surgery:
0: Good (no clinical signs, normal weight gain, smooth fur)
1: Adequate (normal effects of low invasive surgery, normal feeding and drinking, stable weight, no weight gain)
2: Average (average signs of low invasive surgery, slightly decreased eating and drinking, bad appearance of fur, slight weight decrease (<20%), recovering weight)
3: Poor (bad appearance of fur, weight decrease <30%, lethargy, poor coordination, no eating and drinking)
4: Bad (no eating, drinking, weight decrease >30%, progressive weight loss >2 days, Painful, hunched posture, or worse)

Conditions 0-2 are expected to recover, progression to 3 could occur for a maximum of 1 day, but should return to 2 the next day. Progression to score 4, or score 3 for 2 days indicates a very bad recovery, and will indicate removal of the animal of the study by i.p. injection of pentobarbital for euthanasia as indicated, in consultation with the Animal Welfare Body.

Indicate the likely incidence.

The incidence is estimated as minimal (~5%) and depends on the complexity of prechallenge surgery.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

	Annex II	Species				Discomfort			
	Procedure	Mice	Rats	Guinea pigs	Minipigs	1 Non recovery	2 Mild	3 Moderate	4 Severe
Preparation	Cannulation		X	x	x			x	
	Butterfly access				x		x		
	EEG electrode placement		X	x	x			x	

	ECG electrode placement		X	x	x			x	
	Skin microdialysis probe placement		X	x	x	x		X*	
	Brain Microdialysis guide placement		X	x				x	
	Implantation of osmotic minipump	x	X	x			x		
Drug administration	subcutaneous injection	x	X	x			x		
	intravenous injection	x	X	x	x		x		
	intraperitoneal injection	x	X	x			x		
	intramuscular injection		X	x	x		x		
	Intraosseus injection		X	x	x			x	
Monitoring	Anesthetized model		X	x	x	x			
	Blood sampling	x	X	x	x		x		
	Telemetry	x	X	x	x		x		
	Euthanasia	x	X	x	x	x			

The cumulative discomfort estimated to be a maximally 'moderate', based on the animal procedures. Surgical procedures are classified as moderate; blood sampling and most administration techniques are classified as mild. Intraosseus administration: In humans, classification of discomfort can be estimated as mild. Classification in animal models needs to be determined as yet. To give the animal benefit of doubt, classification will a priori be set at moderate, until proven mild.
*:moderate when non recovery

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Tissues are required for analysis of organ distribution, or for example tissue reactions (organ toxicity). This will be at a fixed time point following challenge, or in rare cases after reaching HEP.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

x☐ Yes