B.43. NEUROTOXICITY STUDY IN RODENTS

1. METHOD

This method is equivalent of OECD TG 424 (1997).

This Test Method has been designed to obtain the information necessary to confirm or to further characterise the potential neurotoxicity of chemicals in adult animals. It can either be combined with existing Test Methods for repeated dose toxicity studies or to be carried out as a separate study. It is recommended that the OECD Guidance Document on Neurotoxicity Testing Strategies and Methods (1) be consulted to assist in the design of studies based on this Test Method. This is particularly important when modifications of the observations and test procedures as recommended for routine use of this Method are considered. The Guidance Document has been prepared to facilitate the selection of other test procedures for use in specific circumstances.
The assessment of developmental neurotoxicity is not the subject of this Method.

1.1 INTRODUCTION

In the assessment and evaluation of the toxic characteristics of chemicals, it is important to consider the potential for neurotoxic effects. Already the Test Method for repeated dose systemic toxicity includes observations that screen for potential neurotoxicity. This Test Method can be used to design a study to obtain further information on, or to confirm, the neurotoxic effects observed in the repeated dose systemic toxicity studies. However, consideration of the potential neurotoxicity of certain classes of chemicals may suggest that they may be more appropriately evaluated using this Method without prior indications of the potential neurotoxicity from repeated dose systemic toxicity studies. Such considerations include, for example:

- observation of neurological signs or neuropathological lesions in toxicity studies other than repeated dose systemic toxicity studies, or
- structural relationship or other information linking them to known neurotoxicants.

In addition there may be other instances when use of this Test Method is appropriate; for further details see (1).

This Method has been developed so that it can be tailored to meet particular needs to confirm the specific histopathological and behavioural neurotoxicity of a chemical as well as provide a characterization and quantification of the neurotoxic responses.

In the past, neurotoxicity was equated with neuropathy involving neuropathological lesions or neurological dysfunctions, such as seizure, paralysis or tremor. Although neuropathy is an important manifestation of neurotoxicity, it is now clear that there are many other signs of nervous system toxicity (e.g. loss of motor co-ordination, sensory deficits, learning and memory dysfunctions) that may not be reflected in neuropathy or other types of studies.

This neurotoxicity Test Method is designed to detect major neurobehavioural and neuropathological effects in adult rodents. While behavioural effects, even in the absence of morphological changes, can reflect an adverse impact on the organism, not all behavioural changes are specific to the nervous system. Therefore, any changes observed should be evaluated in conjunction with correlative histopathological, haematological or biochemical data as well as data on other types of systemic toxicity. The testing called for in this Method to provide a characterization and quantification of the neurotoxic responses includes specific histopathological and behavioural procedures that may be further supported by electrophysiological and/or biochemical investigations (1)(2)(3)(4).

Neurotoxicants may act on a number of targets within the nervous system and by a variety of mechanisms. Since no single array of tests is capable of thoroughly assessing the neurotoxic potential of all substances, it may be necessary to utilize other *in vivo* or *in vitro* tests specific to the type of neurotoxicity observed or anticipated.

This Test Method can also be used, in conjunction with the guidance set out in the OECD Guidance Document on Neurotoxicity Testing Strategies and Methods (1) to design studies intended to further characterize or increase the sensitivity of the dose-response quantification in order or better estimate a no-observed-adverse effect level or to substantiate known or suspected hazards of the chemical. For example, studies may be designed to identify and evaluate the neurotoxic mechanism(s) or supplement the data already available from the use of basic neurobehavioural and neuropathological observation procedures. Such studies need not replicate data that would be generated from the use of the standard procedures recommended in this Method, if such data are already available and are not considered necessary for the interpretation of the results of the study.

This neurotoxicity study, when used alone or in combination, provides information that can:

- identify whether the nervous system is permanently or reversibly affected by the chemical tested;
- contribute to the characterization of the nervous system alterations associated with exposure to the chemical, and to understanding the underlying mechanism.
- determine dose-and time-response relationships in order to estimate a no-observed-adverse-effect level (which can be used to establish safety criteria for the chemical).

This Test Method uses oral administration of the test substance. Other routes of administration (e.g. dermal or inhalation) may be more appropriate, and may require modification of the procedures recommended. Considerations of the choice of the route of administration depend on the human exposure profile and available toxicological or kinetic information.

1.2 DEFINITIONS

Adverse effect: is any treatment-related alteration from baseline that diminishes an organism's ability to survive, reproduce or adapt to the environment.

Dose: is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of the test animal (e.g. mg/Kg), or as constant dietary concentrations (ppm).

Dosage: is a general term comprising of dose, its frequency and the duration of dosing.

Neurotoxicity: is an adverse change in the structure or function of the nervous system that results from exposure to a chemical, biological or physical agent.

Neurotoxicant: is any chemical, biological or physical agent having the potential to cause neurotoxicity.

NOAEL: is the abbreviation for no-observed-adverse effect level and is the highest dose level where no adverse treatment-related findings are observed.

1.3 PRINCIPLE OF THE TEST METHOD

The test chemical is administered by the oral route across a range of doses to several groups of laboratory rodents. Repeated doses are normally required, and the dosing regimen may be 28 days, subchronic (90 days) or chronic (1 year or longer). The procedures set out in this Test Method may also be used for an acute neurotoxicity study. The animals are tested to allow the detection or the characterization of behavioural and/or neurological abnormalities. A range of behaviours that could be affected by neurotoxicants is assessed during each observation period. At the end of the test, a subset of animals of each sex from each group are perfused *in situ* and sections of the brain, spinal cord, and peripheral nerves are prepared and examined.

When the study is conducted as a stand-alone study to screen for neurotoxicity or to characterize neurotoxic effects, the animals in each group not used for perfusion and subsequent histopathology (see Table 1) can be used for specific neurobehavioural, neuropathological, neurochemical or electrophysiological procedures that may supplement the data obtained from the standard examinations required by this Method (1). These supplemental procedures can be particularly useful when empirical observations or anticipated effects indicate a specific type or target of a chemical's neurotoxicity. Alternatively, the remaining animals can be used for evaluations such as those called for in Test Methods for repeated dose toxicity studies in rodents.

When the procedures of this Test Method are combined with those of other Test Methods, a sufficient number of animals is needed to satisfy the requirements for the observations of both studies.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 Selection of animal species

The preferred rodent species is the rat, although other rodent species, with justification, may be used. Commonly used laboratory strains of young adult healthy animals should be employed. The females should be nulliparous and non-pregnant. Dosing should normally begin as soon as possible after weaning, preferably not later than when animals are six weeks, and, in any case, before the animals are nine weeks age. However, when this study is combined with other studies this age requirement may need adjustment. At the commencement of the study the weight variation of animals used should not exceed ± 20 % of the mean weight of each sex. Where a repeated dose study of short duration is conducted as a preliminary to a long term study, animals from the same strain and source should be used in both studies.

1.4.2 Housing and feeding conditions

The temperature in the experimental animal room should be 22 ^{o}C (± 3 ^{o}C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Loud intermittent noise should be kept to a minimum. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex.

1.4.3 Preparation of animals

Healthy young animals are randomly assigned to the treatment and control groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely and kept in their cages for at least (5) five days prior the start of the study to allow for acclimatization to the laboratory conditions.

1.4.4 Route of administration and preparation of doses

This Test Method specifically addresses the oral administration of the test substance. Oral administration may be by gavage, in the diet, in drinking water or by capsules. Other routes of administration (e.g. dermal or inhalation) can be used but may require modification of the procedures recommended. Considerations of the choice of the route of administration depend on the human exposure profile and available toxicological or kinetic information. The rationale for choosing the route of administration as well as resulting modifications to the procedures of this Test Method should be indicated.

Where necessary, the test substance may be dissolved or suspended in a suitable vehicle. It is recommended that the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/suspension in oil (e.g., corn oil) and then by possible solution/suspension in other vehicle. The toxic characteristics of the vehicle must be known. In addition, consideration should be given to the following characteristics of the vehicle: effects of the vehicle on absorption, distribution, metabolism, or retention of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

1.5 PROCEDURES

1.5.1 **Number and sex animals**

When the study is conducted as a separate study, at least 20 animals (10 females and 10 males) should be used in each dose and control group for the evaluation of detailed clinical and functional observations. At least five males and five females, selected from these 10 males and 10 females, should be perfused *in situ* and used for detailed neurohistopathology at the end of the study. In cases where only a limited number of animals in a given dose group are observed for signs of neurotoxic effects, consideration should be given to the inclusion of these animals in those selected for perfusion. When the study is conducted in combination with a repeated dose toxicity study, adequate numbers of animals should be used to meet the objectives of both studies. The minimum numbers of animals per group for various combinations of studies are given in Table 1. If interim kills or recovery groups for observation of reversibility, persistence or delayed occurrence of toxic effects post treatment are planned or when supplemental observations are considered, then the number of animals should be increased to ensure that the number of animals required for observation and histopathology are available.

1.5.2 **Treatment and control group**

At least three dose groups and a control group should generally be used, but if from the assessment of other data, no effects would be expected at a repeated dose of 1000 mg/kg body weight/day, a limit test may be performed. If there are no suitable data available, a range finding study may be performed to aid in the determination of the doses to be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test substance, the control group should receive the vehicle at the highest volume used.

1.5.3 **Reliability check**

The laboratory performing the study should present data demonstrating its capability to carry out the study and the sensitivity of the procedures used. Such data should provide evidence of the ability to detect and quantify, as appropriate, changes in the different end points recommended for observation, such as autonomic signs, sensory reactivity, limb grip strength and motor activity. Information on chemicals that cause different types of neurotoxic responses and could be used as positive control substances can be found in references 2 to 9. Historical data may be used if the essential aspects of the experimental procedures remain the same. Periodic updating of historical data is recommended. New data that demonstrate the continuing sensitivity of the procedures should be developed when some essential element of the conduct of the test or procedures has been changed by the performing laboratory.

1.5.4 **Dose selection**

Dose levels should be selected by taking into account any previously observed toxicity and kinetic data available for the test compound or related materials. The highest dose level should be chosen with the aim of inducing neurotoxic effects or clear systemic toxic effects. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dose-related response and no-observed-adverse effect (NOAEL) at the lowest dose level. In principle, dose levels should be set so that primary toxic effects on the nervous system can be distinguished from effects related to systemic toxicity. Two to three intervals are frequently optimum and addition of a fourth test group is often preferable to using very large intervals (e.g., more than a factor of 10) between dosages. Where there is a reasonable estimation of human exposure this should also be taken into account.

1.5.5 **Limit test**

If a study at one dose level of at least 1000 mg/kg body weight/day, using the procedures described, produces no observable neurotoxic effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher oral dose level to be used in the limit test. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test substance often may dictate the maximum attainable level of exposure. For the conduct of an oral acute study, the dose for a limit test should be at least 2000 mg/kg.

1.5.6 **Administration of doses**

The animals are dosed with the test substance daily, seven days each week, for a period at least 28 days; use of a five-day dosing regime or a shorter exposure period needs to be justified. When the test substance is administered by gavage, this should be done in a single dose using a stomach tube or a suitable intubation cannula. The maximum volume of a liquid that can be administered at one time depends on the size of the test animals. The volume should not exceed 1 ml/100 g body weight. However in the case of aqueous solutions, the use of up to 2 ml/100 g body weight can be considered. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

For substances administered via the diet or drinking water, it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight. Where a repeat dose study is used as a preliminary to a long term study, a similar diet should be used in both studies. For acute studies, if a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

1.6 OBSERVATION

1.6.1 **Frequency of observations and tests**

In repeated dose studies, the observation period should cover the dosage period. In acute studies, 14-day post-treatment period should be observed. For animals in satellite groups which are kept without exposure during a post-treatment period, observations should cover this period as well.

Observations should be made with sufficient frequency to maximize the probability of detection of any behavioural and/or neurological abnormalities. Observations should be made preferably at the same times each day with consideration given to the peak period of anticipated effects after dosing. The frequency of clinical observations and functional tests is summarized in Table 2. If kinetic or other data generated from previous studies indicates the need to use different time points for observations, tests or post-observation periods, an alternative schedule should be adopted in order to achieve maximum information. The rationale for changes to the schedule should be provided.

1.6.1.1 *Observations of general health condition and mortality/morbidity*

All animals should be carefully observed at least once daily with respect to their health condition as well as at least twice daily for morbidity and mortality.

1.6.1.2 *Detailed clinical observations*

Detailed clinical observations should be made on all animals selected for this purpose (see Table 1) once before the first exposure (to allow for within-subject comparisons) and at different intervals thereafter, dependant on the duration of the study (see Table 2). Detailed clinical observations on satellite recovery groups should be made at the end of the recovery period. Detailed clinical observations should be made outside the home cage in a standard arena. They should be carefully recorded using scoring systems that include criteria or scoring scales for each measurement in the observations. The criteria or scales used should be explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the test conditions are minimal (not systematically related to treatment) and that observations are conducted by trained observers unaware of the actual treatment.

It is recommended that the observations be carried out in a structured fashion in which well-defined criteria (including the definition of the normal "range") are systematically applied to each animal at each observation time. The "normal range" should be adequately documented. All observed signs should be recorded. Whenever feasible, the magnitude of the observed signs should also be recorded. Clinical observations should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern and/or mouth breathing, any unusual signs of urination or defecation, and discoloured urine).

Any unusual responses with respect to body position, activity level (e.g., decreased or increased exploration of the standard arena) and co-ordination of movement should also be noted. Changes in gait (e.g., waddling, ataxia), posture (e.g., hunched-back) and reactivity to handling, placing or other environmental stimuli, as well as the presence of clonic or tonic movements, convulsions or tremors, stereotypes (e.g., excessive grooming, unusual head movements, repetitive circling) or bizarre behaviour (e.g., biting or excessive licking, self mutilation, walking backwards, vocalization) or aggression should be recorded.

1.6.1.3 *Functional tests*

Similar to the detailed clinical observations, functional tests should also be conducted once prior to exposure and frequently thereafter in all animals selected for this purpose (see Table 1). The frequency of functional testing is also dependent on the study duration (see Table 2). In addition to the observation periods as set out in Table 2, functional observations on satellite recovery groups should also be made as close as possible to the terminal kill. Functional tests should include sensory reactivity to stimuli of different modalities [e.g., auditory, visual and proprioceptive stimuli (5)(6)(7)], assessment of limb grip strength (8) and assessment of motor activity (9). Motor activity should be measured with an automated device capable of detecting both decreases and increases in activity. If another defined system is used it should be quantitative and its sensitivity and reliability should be demonstrated. Each device should be tested to ensure reliability across time and consistency between devices. Further details of the procedures that can be followed are given in the respective references. If there are no data (e.g. structure-activity, epidemiological data, other toxicology studies) to indicate the potential neurotoxic effects, the inclusion of more specialized tests of sensory and motor function or learning and memory to examine these possible effects in greater details should be considered. More information on more specialized tests and their use is provided in (1).

Exceptionally, animals that reveal signs of toxicity to an extent that would significantly interfere with the functional test may be omitted from that test. Justification for the elimination of animals from a functional test should be provided.

1.6.2 **Body weight and food/water consumption**

For studies up to 90 days duration, all animals should be weighed at least once a week and measurements should be made of food consumption (water consumption, when the test substance is administered by that medium) at least weekly. For long term studies, all animals should be weighed at least once at week for the first 13 weeks and at least once every 4 weeks thereafter. Measurements should be made of food consumption (water consumption, when the test substance is administered by that medium) at least weekly for the first 13 weeks and then at approximately three-month intervals unless the health status or body weight changes dictate otherwise.

1.6.3 **Ophthalmology**

For studies longer than 28 days duration, ophthalmologic examination, using an ophthalmoscope or an equivalent suitable instrument, should be made prior to the administration of the test substance and at the termination of the study, preferably on all animals, but at least on animals in the high dose and control groups. If changes in the eyes are detected or, if clinical signs indicate the need, all animals should be examined. For long term studies, an ophthalmologic examination should also be carried out at 13 weeks. Ophthalmologic examinations need not to be conducted if this data is already available from others studies of similar duration and at similar dose levels.

1.6.4 **Haematology and clinical biochemistry**

When the neurotoxicity study is carried out in combination with a repeated dose systemic toxicity study, haematological examinations and clinical biochemistry determinations should be carried out as set out in the respective Method of the systemic toxicity study. Collection of samples should be carried out in such a way that any potential effects on neurobehaviour are minimized.

1.6.5 **Histopathology**

The neuropathological examination should be designed to complement and extend the observations made during the *in vivo* phase of the study. Tissues from at least 5 animals/sex/group (see Table 1 and next paragraph) should be fixed *in situ*, using generally recognized perfusion and fixation techniques (see reference 3, chapter 5 and reference 4, chapter 50). Any observable gross changes should be recorded. When the study is conducted as a stand-alone study screen for neurotoxicity or to characterize neurotoxic effects, the remainder of the animals may be used either for specific neurobehavioural (10)(11), neuropathological (10)(11)(12)(13), neurochemical (10)(11)(14)(15) or electrophysiological (10)(11)(16)(17) procedures that may supplement the procedures and examinations described here, or to increase the number of subjects examined for histophatology. These supplementary procedures are of particular use when empirical observations or anticipated effects indicate a specific type or target of neurotoxicity (2)(3). Alternatively, the remainder of the animals can also be used for routine pathological evaluations as described in Method for repeated dose studies.

A general staining procedure, such as haematoxylin and eosin (H&E), should be performed on all tissue specimens embedded in paraffin and microscopic examination should be carried out. If signs of peripheral neuropathy are observed or suspected, plastic-embedded samples of peripheral nerve tissue should be examined. Clinical signs may also suggest additional sites for examination or the use of special staining procedures. Guidance on additional sites to be examined can be found in (3)(4). Appropriate special stains to demonstrate specific types of pathological change may also be helpful (18).

Representative sections of the central and peripheral nervous system should be examined histologically (see reference 3, chapter 5 and reference 4, chapter 50). The areas examined should normally include: the forebrain, the centre of the cerebrum, including a section through the hippocampus, the midbrain, the cerebellum, the pons, the medulla oblongata, the eye with optic nerve and retina, the spinal cord at the cervical and lumbar swellings, the dorsal root ganglia, the dorsal and ventral root fibres, the proximal sciatic nerve, the proximal tibial nerve (at the knee) and the tibial nerve calf muscle branches. The spinal cord and peripheral nerve sections should include both cross or transverse and longitudinal sections. Attention should be given to the vasculature of the nervous system. A sample of skeletal muscle, particularly calf muscle, should also be examined. Special attention should be paid to sites with cellular and fibre structure and pattern in the CNS and PNS known to be particularly affected by neurotoxicants.

Guidance on neurophatological alterations that typically result from toxicant exposure can be found in the references (3)(4). A stepwise examination of tissue samples is recommended in which sections from the high dose group are first compared with those of the control group. If no neurophatological alterations are observed in the samples from these groups, subsequent analysis is not required. If neuropathological alterations are observed in the high dose group, sample from each of the potentially affected tissues from the intermediate and low dose groups should then be coded and examined sequentially.

If any evidence of neuropathological alterations is found in the qualitative examination, then a second examination should be performed on all regions of the nervous system showing these alterations. Sections from all dose groups from each of the potentially affected regions should be coded and examined at random without knowledge of the code. The frequency and severity of each lesion should be recorded. After all regions from all dose groups have been rated, the code can be broken and statistical analysis performed to evaluate dose-response relationships. Examples of different degrees of severity of each lesion should be described.

The neuropathological findings should be evaluated in the context of behavioural observations and measurements, as well as other data from preceding and concurrent systemic toxicity studies of the test substance.

2 DATA

2.1 TREATMENT OF RESULTS

Individual data should be provided. Additionally, all data should be summarized in tabular form showing for each test or control group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, type and severity of any toxic effects, the number of animals showing lesions, including the type and severity of the lesion(s).

2.2 EVALUATION AND INTERPRETATION OF RESULTS

The findings of the study should be evaluated in terms of the incidence, severity and correlation of neurobehavioural and neuropathological effects (neurochemical or electrophysiological effects as well if supplementary examinations are included) and any other adverse effects observed. When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

3 REPORTING

TEST REPORT

The test report must include the following information:

Test substance:

- physical nature (including isomerism, purity and physicochemical properties);
- identification data.

Vehicle (if appropriate):

- justification for choice of vehicle.

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, acclimatization, diet, etc;
- individual weights of animals at the start of the test.

Test conditions:

- details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:
- specification of the doses administered, including details of the vehicle, volume and physical form of the material administered;
- details of the administration of the test substance;
- rationale for dose levels selected;
- rationale for the route and duration of the exposure;
- conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of the food and water quality.

Observation and Test Procedures:

- details of the assignment of animals in each group to the perfusion subgroups;
- details of scoring systems, including criteria and scoring scales for each measurement in the detailed clinical observations;
- details on the functional tests for sensory reactivity to stimuli of different modalities (e.g., auditory, visual and proprioceptive); for assessment of limb grip strength; for motor activity assessment (including details of automated devices for detecting activity); and other procedures used;
- details of ophthalmologic examinations and, if appropriate, haematological examinations and clinical biochemistry tests with relevant base-line values;
- details for specific neurobehavioural, neuropathological, neurochemical or electrophysiological procedures.

Results:

- body weight/body weight changes including body weight at kill;
- food consumption and water consumption, as appropriate;
- toxic response data by sex and dose level, including signs of toxicity or mortality;
- nature, severity and duration (time of onset and subsequent course) of the detailed clinical observations (whether reversible or not);
- a detailed description of all functional test results;
- necropsy findings;
- a detailed description of all neurobehavioural, neuropathological, and neurochemical or electrophysiological findings, if available;
- absorption and metabolism data, if available;
- statistical treatment of results, where appropriate.

Discussion of results;

- dose response information;
- relationship of any other toxic effects to a conclusion about the neurotoxic potential of the test chemical;
- no-observed-adverse effect level.

Conclusions:

- a specific statement of the overall neurotoxicity of the test chemical is encouraged.

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Table 1:

Minimum numbers of animals needed per group when the neurotoxicity study is conducted separately or in combination whit studies

	NEUROTOXICITY STUDY CONDUCTED AS :			
	Separate study	**Combined study with the 28-day study**	**Combined study with the 90-day study**	**Combined study with the chronic toxicity study**
Total number of animals per group	10 males and 10 females	10 males and 10 females	15 males and 15 females	25 males and 25 females
Number of animals selected for functional testing including detailed clinical observations	10 males and 10 females	10 males and 10 females	10 males and 10 females	10 males and 10 females
Number of animals selected per perfusion *in situ* and neurohistopathology	5 males and 5 females	5 males and 5 females	5 males and 5 females	5 males and 5 females
Number of animals selected for repeated dose/subchronic/chronic toxicity observations, haematology, clinical biochemistry, histopathology, etc. as indicate in the respective *Guidelines*		5 males and 5 females	10 males † and 10 females †	20 males † and 20 females †
Supplemental observations, as appropriate	5 males and 5 females			

† - Includes five animals selected for functional testing and detailed clinical observations as part of the neurotoxicity study

Table 2 :

Frequency of clinical observation and functional tests

Type of observations		Study duration			
		Acute	**28-day**	**90-day**	**Chronic**
In all animals	**General health condition**	daily	daily	daily	daily
	Mortality/morbidity	Twice daily	Twice daily	Twice daily	Twice daily
In animals selected for functional observations	**Detailed clinical observations**	- prior to first exposure - within 8 hours of dosing at estimate time of peak effect - at day 7 and 14 after dosing	- prior to first exposure - once weekly thereafter	- prior to first exposure - once during the first or second week of exposure - monthly thereafter	- prior to first exposure - once at the end of the first month of exposure - every three months thereafter
	Functional tests	- prior to first exposure - within 8 hours of dosing at estimate time of peak effect - at day 7 and 14 after dosing	- prior to first exposure - during the fourth week of treatment as close as possible to the end of the exposure period	- prior to first exposure - once during the first or second week of exposure - monthly thereafter	- prior to first exposure - once at the end of the first month of exposure - every three months thereafter