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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Chronic Toxicity Studies

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals (TGs) are periodically reviewed in the light of scientific progress, changing assessment practices and animal welfare considerations. The original Guideline 452 was adopted in 1981. Development of a revised TG 452 was considered necessary in order to reflect recent developments in the field of animal welfare and regulatory requirements (1) (2) (3) (4). The updating of TG 452 has been carried out in parallel with revisions of the Test Guidelines 451, Carcinogenicity Studies and 453, Combined Chronic Toxicity/Carcinogenicity studies, with the objective of obtaining additional information from the animals used in the study and providing further detail on dose selection. This Test Guideline is designed to be used in the testing of a broad range of chemicals, including pesticides and industrial chemicals.

2. The majority of chronic toxicity studies are carried out in rodent species, and this Test Guideline is intended therefore to apply primarily to studies carried out in these species. Should such studies be required in non-rodent species, the principles and procedures outlined in this Guideline, together with those outlined in OECD TG 409, Repeated Dose 90 day Oral Toxicity Study in Non Rodents (5) may also be applied, with appropriate modifications, as outlined in the OECD Guidance Document No. 116 on the Design and Conduct of Chronic Toxicity and Carcinogenicity Studies (6).

3. The three main routes of administration used in chronic toxicity studies are oral, dermal and inhalation. The choice of the route of administration depends on the physical and chemical characteristics of the test chemical and the predominant route of exposure of humans. Additional information on choice of route of exposure is provided in the OECD Guidance Document No. 116 (6).

4. This Guideline focuses on exposure via the oral route, the route most commonly used in chronic toxicity studies. While long–term chronic toxicity studies involving exposure via the dermal or inhalation routes may also be necessary for human health risk assessment and/or may be required under certain regulatory regimes, both routes of exposure involve considerable technical complexity. Such studies will need to be designed on a case-by-case basis, although the Guideline outlined here for the assessment and evaluation of chronic toxicity by oral administration could form the basis of a protocol for inhalation and/or dermal studies, with respect to recommendations for

In accordance with the Decision of the Council on a Delegation of Authority to amend Annex I of the Decision of the Council on the Mutual Acceptance of Data in the Assessment of Chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.

treatment periods, clinical and pathology parameters, etc. OECD Guidance is available on the administration of test chemicals by the inhalation (6) (7) and dermal routes (6). TG 412 (8) and TG 413 (9), together with the associated OECD Guidance Document on acute inhalation testing (7), should be specifically consulted in the design of longer term studies involving exposure via the inhalation route. TG 410 (10) should be consulted in the case of testing carried out by the dermal route.

5. The chronic toxicity study provides information on the possible health hazards likely to arise from repeated exposure over a considerable part of the lifespan of the species used. The study will provide information on the toxic effects of the substance, indicate target organs and the possibility of accumulation. It can also provide an estimate of the no-observed-adverse effect level which can be used for establishing safety criteria for human exposure. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed.

6. The objectives of studies covered by this test guideline include:

- The identification of the chronic toxicity of a chemical;
- The identification of target organs;
- Characterisation of the dose-response relationship;
- Identification of a no-observed-adverse-effect level (NOAEL) or point of departure for establishment of a Benchmark Dose (BMD);
- The prediction of chronic toxicity effects at human exposure levels;
- Provision of data to test hypotheses regarding mode of action (6).

INITIAL CONSIDERATIONS

7. In the assessment and evaluation of the toxicological characteristics of a chemical, all available information on the test chemical should be considered by the testing laboratory prior to conducting the study, in order to focus the design of the study to more efficiently test for chronic toxicity potential and to minimize animal usage. Information that will assist in the study design includes the identity, chemical structure, and physico-chemical properties of the test chemical; any information on the mode of action; results of any in vitro or in vivo toxicity tests; anticipated use(s) and potential for human exposure; available (Q)SAR data and toxicological data on structurally-related substances; available toxicokinetic data (single dose and also repeat dose kinetics where available) and data derived from other repeated exposure studies. The determination of chronic toxicity should only be carried out after initial information on toxicity has been obtained from repeated dose 28-day and/or 90-day toxicity tests. A phased testing approach to chronic toxicity testing should be considered as part of the overall assessment of the potential adverse health effects of a particular chemical (11) (12) (13) (14).

8. The statistical methods most appropriate for the analysis of results, given the experimental design and objectives, should be established before commencing the study. Issues to consider include whether the statistics should include adjustment for survival and analysis in the event of premature termination of one or more groups. Guidance on the appropriate statistical analyses and key references to internationally accepted statistical methods are given in Guidance Document No. 116 (6), and also in Guidance Document No.35 on the analysis and evaluation of chronic toxicity and carcinogenicity studies (15).

9. In conducting a chronic toxicity study, the guiding principles and considerations outlined in the OECD Guidance Document No. 19 on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (16), in particular paragraph 62 thereof, should always be followed. This paragraph states that "In studies involving repeated dosing, when an animal shows clinical signs that are progressive, leading to further deterioration in condition, an informed decision as to whether or not to humanely kill the animal should be made. The decision should include consideration as to the value of the information to be gained from the continued maintenance of that animal on study relative to its overall condition. If a decision is made to leave the animal on test, the frequency of observations should be increased, as needed. It may also be possible, without adversely affecting the purpose of the test, to temporarily stop dosing if it will relieve the pain or distress, or reduce the test dose."

10. Detailed guidance on and discussion of the principles of dose selection for chronic toxicity and carcinogenicity studies can be found in Guidance Document No. 116 (6), as well as two International Life Sciences Institute publications (17) (18). The core dose selection strategy is dependent on the primary objective or objectives of the study (paragraph 6). In selecting appropriate dose levels, a balance should be achieved between hazard screening on the one hand and characterisation of low-dose responses and their relevance on the other. This is particularly relevant in the situation where a combined chronic toxicity and carcinogenicity study (TG 453) is to be carried out (paragraph 11).

11. Consideration should be given to carrying out a combined chronic toxicity and carcinogenicity study (TG 453), rather than separate execution of a chronic toxicity study (TG 452) and carcinogenicity study (TG 451). The combined test provides greater efficiency in terms of time and cost compared to conducting two separate studies, without compromising the quality of the data in either the chronic phase or the carcinogenicity phase. Careful consideration should however be given to the principles of dose selection (paragraphs 9 and 20-25) when undertaking a combined chronic toxicity and carcinogenicity study (TG 453), and it is also recognised that separate studies may be required under certain regulatory frameworks.

12. Definitions used in the context of this Test Guideline can be found in Guidance Document No. 116.

PRINCIPLE OF THE TEST

13. The test chemical is administered daily in graduated doses to several groups of experimental animals, normally for a period of 12 months, although longer or shorter durations may also be chosen depending on regulatory requirements (see paragraph 33). This duration is chosen to be sufficiently long to allow any effects of cumulative toxicity to become manifest, without the confounding effects of geriatric changes. Deviations from exposure duration of 12 months should be justified, particularly in the case of shorter durations. The test chemical is normally administered by the oral route although testing by the inhalation or dermal route may also be appropriate. The study design may also include one or more interim kills, e.g. at 3 and 6 months, and additional groups of animals may be included to accommodate this (see paragraph 19). During the period of administration the animals are observed closely for signs of toxicity. Animals which die or are killed during the test are necropsied and, at the conclusion of the test, surviving animals are killed and necropsied.

DESCRIPTION OF THE METHOD

Selection of animal species

14. This Guideline primarily covers assessment and evaluation of chronic toxicity in rodents (see paragraph 2) although it is recognised that similar studies in non-rodents may be required under certain regulatory regimes. The choice of species should be justified. The design and conduct of chronic toxicity studies in non-rodent species, when required, should be based on the principles outlined in this Guideline together with those in OECD TG 409, Repeated Dose 90-day Oral Toxicity Study in Non-Rodents (5). Additional information on choice of species and strain is provided in Guidance Document No. 116 (6).

15. In this Guideline, the preferred rodent species is the rat, although other rodent species, e.g. the mouse, may be used. Rats and mice have been preferred experimental models because of their relatively short life span, their widespread use in pharmacological and toxicological studies, their susceptibility to tumour induction, and the availability of sufficiently characterised strains. As a consequence of these characteristics, a large amount of information is available on their physiology and pathology. Young healthy adult animals of commonly used laboratory strains should be employed. The chronic toxicity study should be carried out in animals from the same strain and source as those used in preliminary toxicity study(ies) of shorter duration. The females should be nulliparous and non-pregnant.

Housing and feeding conditions

16. Animals may be housed individually, or be caged in small groups of the same sex; individual housing should be considered only if scientifically justified (19) (20) (21). Cages should be arranged in such a way that possible effects due to cage placement are minimised. The temperature in the experimental animal room should be 22°C (± 3°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. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The diet should meet all the nutritional requirements of the species tested and the content of dietary contaminants including but not limited to pesticide residues, persistent organic pollutants, phytoestrogens, heavy metals and mycotoxins, that might influence the outcome of the test, should be as low as possible. Analytical information on the nutrient and dietary contaminant levels should be generated periodically, at least at the beginning of the study and when there is a change in the batch used, and should be included in the final report. Analytical information on the drinking water used in the study should similarly be provided. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical and to meet the nutritional requirements of the animals when the test chemical is administered by the dietary route.

Preparation of animals

17. Healthy animals, which have been acclimated to laboratory conditions for at least 7 days and have not been subjected to previous experimental procedures, should be used. In the case of rodents, dosing of the animals should begin as soon as possible after weaning and acclimatisation and preferably before the animals are 8 weeks old. The test animals should be characterised as to species, strain, source, sex, weight and age. At the

commencement of the study, the weight variation for each sex of animals used should be minimal and not exceed ± 20 % of the mean weight of all the animals within the study, separately for each sex. Animals should be randomly assigned to the control and treatment groups. After randomisation, there should be no significant differences in mean body weights between groups within each sex. If there are statistically significant differences, then the randomisation step should be repeated, if possible. Each animal should be assigned a unique identification number, and permanently marked with this number by tattooing, microchip implant, or other suitable method.

PROCEDURE

Number and sex of animals

18. Both sexes should be used. A sufficient number of animals should be used so that at the end of the study enough animals in every group are available for thorough biological and statistical evaluation. For rodents, at least 20 animals per sex per group should normally be used at each dose level, while for non-rodents a minimum of 4 per sex per group is recommended. In studies involving mice, additional animals may be needed in each dose group to conduct all required haematological determinations.

Provision for interim kills, satellite groups and sentinel animals

19. The study may make provision for interim kills (at least 10 animals/sex/group), e.g. at 6 months, to provide information on progression of toxicological changes and mechanistic information, if scientifically justified. Where such information is already available from previous repeat dose toxicity studies on the substance, interim kills may not be scientifically justified. Satellite groups may also be included to monitor the reversibility of any toxicological changes induced by the chemical under investigation; these will normally be restricted to the highest dose level of the study plus control. An additional group of sentinel animals (typically 5 animals per sex) may also be included for monitoring of disease status, if necessary, during the study (22). If interim kills or inclusion of satellite or sentinel groups are planned, the number of animals included in the study design should be increased by the number of animals scheduled to be killed before the completion of the study. These animals should normally undergo the same observations, including body weight, food/water consumption, haematological and clinical biochemistry measurements and pathological investigations as the animals in the chronic toxicity phase of the main study, although provision may also be made (in the interim kill groups) for measurements to be restricted to specific, key measures such as neurotoxicity or immunotoxicity.

Dose groups and dosage

20. Guidance on all aspects of dose selection and dose level spacing is provided in Guidance Document No.116 (6). At least three dose levels and a concurrent control should be used, except where a limit test is conducted (see paragraph 27). Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test chemical or related materials.

21. In the dose selection the investigator should also consider and ensure that data generated is adequate to fulfil the regulatory requirements across OECD countries as

appropriate (e.g., hazard and risk assessment, classification and labelling, ED assessment, etc.)

22. Unless limited by the physical-chemical nature or biological effects of the test chemical, the highest dose level should normally be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death. While taking into account the factors outlined in paragraph 22 below, the highest dose level should be chosen to elicit evidence of toxicity, as evidenced by, for example, depression of body weight gain (approximately 10%).

22. However, dependent on the objectives of the study (see paragraph 6), a top dose lower than the dose providing evidence of toxicity may be chosen, e.g., if a dose elicits an adverse effect of concern that nonetheless has little impact on lifespan or body weight. The top dose should not exceed 1000 mg/kg body weight/day (limit dose, see paragraph 27).

23. Dose levels and dose level spacing may be selected to establish a dose-response and a NOAEL or other intended outcome of the study, e.g a BMD (see paragraph 25) at the lowest dose level. Factors that should be considered in the placement of lower doses include the expected slope of the dose–response curve, the doses at which important changes may occur in metabolism or mode of toxic action, where a threshold is expected, or where a point of departure for low-dose extrapolation is expected.

24. The dose level spacing selected will depend on the characteristics of the test chemical, and cannot be prescribed in this Guideline, but two to four fold intervals frequently provide good test performance when used for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g., more than a factor of about 6-10) between dosages. In general the use of factors greater than 10 should be avoided, and should be justified if used.

25. As outlined further in Guidance Document No.116 (6), points to be considered in dose selection include:

- Known or suspected nonlinearities or inflection points in the dose–response;

- Toxicokinetics, and dose ranges where metabolic induction, saturation, or nonlinearity between external and internal doses does or does not occur;

- Precursor lesions, markers of effect, or indicators of the operation of key underlying biological processes;

- Key (or suspected) aspects of mode of action, such as doses at which cytotoxicity begins to arise, hormone levels are perturbed, homeostatic mechanisms are overwhelmed, etc.;

- Regions of the dose–response curve where particularly robust estimation is needed, e.g., in the range of the anticipated BMD or a suspected threshold;

- Consideration of anticipated human exposure levels.

26. The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test chemical. Except for treatment with the test chemical, animals in the control group should be handled in an identical manner to those in the test groups. If a vehicle is used, the control group shall receive the vehicle in the highest volume used among the dose groups. If a test chemical is administered in the diet, and causes significantly reduced dietary intake due to the reduced palatability of the diet, an additional pair-fed control group may be useful, to serve as a more suitable control.

27. If it can be anticipated, based on information from preliminary studies, that a test at one dose level, equivalent to at least 1000 mg/kg body weight/day, using the procedures described for this study, is unlikely to produce adverse effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. A limit of 1000 mg/kg body weight/day may apply except when human exposure indicates the need for a higher dose level to be used.

Preparation of doses and administration of test chemical

28. The test chemical is normally administered orally, via the diet or drinking water, or by gavage. Additional information on routes and methods of administration is provided in Guidance Document No. 116 (6). The route and method of administration is dependent on the purpose of the study, the physical/chemical properties of the test chemical, its bioavailability and the predominant route and method of exposure of humans. A rationale should be provided for the chosen route and method of administration. In the interests of animal welfare, oral gavage should normally be selected only for those agents for which this route and method of administration reasonably represent potential human exposure (e.g., pharmaceuticals). For dietary or environmental chemicals including pesticides, administration is typically via the diet or drinking water. However, for some scenarios, e.g., occupational exposure, administration via other routes may be more appropriate

29. Where necessary, the test chemical is dissolved or suspended in a suitable vehicle. Consideration should be given to the following characteristics of the vehicle and other additives, as appropriate: effects on the absorption, distribution, metabolism, or retention of the test chemical; effects on the chemical properties of the test chemical which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/ emulsion in oil (e.g., corn oil) and then by possible solution in other vehicles. For vehicles other than water, the toxic characteristics of the vehicle should be known. Information should be available on the stability of the test chemical and the homogeneity of dosing solutions or diets (as appropriate) under the conditions of administration (e.g. diet).

30. For substances administered via the diet or drinking water it is important to ensure that the quantities of the test chemical involved do not interfere with normal nutrition or water balance. In long-term toxicity studies using dietary administration, the concentration of the chemical in the feed should not normally exceed an upper limit of 5% of the total diet, in order to avoid nutritional imbalances. When the test chemical is administered in the diet, either a constant dietary concentration (mg/kg diet or ppm) or a

constant dose level in terms of the animal's body weight (mg/kg body weight), calculated on a weekly basis, may be used. The alternative used should be specified.

31. In the case of oral administration, the animals are dosed with the test chemical daily (seven days each week), normally for a period of 12 months (see also paragraph 33), although a longer duration may be required depending on regulatory requirements. Any other dosing regime, e.g., five days per week, needs to be justified. In the case of dermal administration, animals are normally treated with the test chemical for at least 6 hours per day, 7 days per week, as specified in TG 410 (11), for a period of 12 months. Exposure by the inhalation route is carried out for 6 hours per day, 7 days per week, but exposure for 5 days per week may also be used, if justified. The period of exposure will normally be for a period of 12 months. If rodent species other than rats are exposed nose-only, maximum exposure durations may be adjusted to minimise species-specific distress. A rationale should be provided when using an exposure duration of less than 6 hours per day. See also TG 412 (8).

32. When the test chemical is administered by gavage to the animals this should be done using a stomach tube or a suitable intubation cannula, at similar times each day. Normally a single dose will be administered once daily, where for example a compound is a local irritant, it may be possible to maintain the daily dose-rate by administering it as a split dose (twice a day). The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should be kept as low as practical, and should not normally exceed 1 ml/100g body weight for rodents (23). Variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels. Potentially corrosive or irritant substances are the exception, and need to be diluted to avoid severe local effects. Testing at concentrations that are likely to be corrosive or irritant to the gastrointestinal tract should be avoided.

Duration of study

33. While this Test Guideline primarily is designed as a 12 month chronic toxicity study, the study design also allows for and can be applied to either shorter (e.g. 6 or 9 months) or longer (e.g. 18 or 24 months) duration studies, depending on the requirements of particular regulatory regimes or for specific mechanistic purposes. Deviations from an exposure duration of 12 months should be justified, particularly in the case of shorter durations. Satellite groups included to monitor the reversibility of any toxicological changes induced by the chemical under investigation should be maintained without dosing for a period not less than 4 weeks and not more than one third of the total study duration after cessation of exposure. Further guidance, including consideration of survival in the study, is provided in Guidance Document No. 116 (6).

OBSERVATIONS

34. All animals should be checked for morbidity or mortality, usually at the beginning and end of each day, including at weekends and holidays. General clinical observations should be made at least once a day, preferably at the same time(s) each day, taking into consideration the peak period of anticipated effects after dosing in the case of gavage administration.

35. Detailed clinical observations should be made on all animals at least once prior to the first exposure (to allow for within-subject comparisons), at the end of the first week of the study and monthly thereafter. The protocol for observations should be arranged such

that variations between individual observers are minimised and independent of test group. These observations should be made outside the home cage, preferably in a standard arena and at similar times on each occasion. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Efforts should be made to ensure that variations in the observation conditions are minimal. Signs noted 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, and unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling) or bizarre behaviour (e.g., self-mutilation, walking backwards) should also be recorded (24).

36. Ophthalmological examination, using an ophthalmoscope or other suitable equipment, should be carried out on all animals prior to the first administration of the test chemical. At the termination of the study, this examination should be preferably conducted in all animals but at least in the high dose and control groups. If treatment-related changes in the eyes are detected, all animals should be examined. If structural analysis or other information suggests ocular toxicity, then the frequency of ocular examination should be increased.

37. For chemicals where previous repeated dose 28-day and/or 90-day toxicity tests indicated the potential to cause neurotoxic effects, sensory reactivity to stimuli of different types (24) (e.g., auditory, visual and proprioceptive stimuli) (25), (26), (27), assessment of grip strength (28) and motor activity assessment (29) may optionally be conducted before commencement of the study and at 3 month periods after study initiation up to and including 12 months, as well as at study termination (if longer than 12 months). Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could also be used.

38. For chemicals where previous repeated dose 28-day and/or 90-day toxicity tests indicated the potential to cause immunotoxic effects, further investigations of this endpoint may optionally be conducted at termination.

Body weight, food/water consumption and food efficiency

39. All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks, and at least monthly thereafter. Measurements of food consumption and food efficiency should be made at least weekly for the first 13 weeks and at least monthly thereafter. Water consumption should be measured at least weekly for the first 13 weeks and at least monthly thereafter when the substance is administered in drinking water. Water consumption measurements should also be considered for studies in which drinking activity is altered.

Haematology and clinical biochemistry

40. In studies involving rodents, haematological examinations should be carried out in at least 10 male and 10 female animals per group, at 3, 6, and 12 months, as well as at study termination (if longer than 12 months), using the same animals throughout. In mice, satellite animals may be needed in order to conduct all required haematological determinations (see paragraph 18). In non-rodent studies, samples will be taken from smaller numbers of animals (e.g. 4 animals per sex and per group in dog studies), at interim sampling times and at termination as described for rodents. Measurements at 3

months, either in rodents or non-rodents, need not be conducted if no effect was seen on haematological parameters in a previous 90 day study carried out at comparable dose levels. Blood samples should be taken from a named site, for example by cardiac puncture or from the retro-orbital sinus, under anaesthesia.

41. The following list of parameters should be investigated (30): Total and differential leukocyte count, erythrocyte count, platelet count, haemoglobin concentration, haematocrit (packed cell volume), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), prothrombin time, and activated partial thromboplastin time. Other hematology parameters such as Heinz bodies or other atypical erythrocyte morphology or methaemoglobin may be measured as appropriate depending on the toxicity of the substance. Overall, a flexible approach should be adopted, depending on the observed and/or expected effect from a given substance. If the chemical has an effect on the haematopoietic system, reticulocyte counts and bone marrow cytology may also be indicated, although these need not be routinely conducted.

42. Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from at least 10 male and 10 female animals per group at the same time intervals as specified for the haematological investigations, using the same animals throughout. In mice, satellite animals may be needed in order to conduct all required clinical biochemistry determinations. In non-rodent studies, samples will be taken from smaller numbers of animals (e.g. 4 animals per sex and per group in dog studies), at interim sampling times and at termination as described for rodents. Measurements at 3 months, either in rodents or non-rodents, need not be conducted if no effect was seen on clinical biochemistry parameters in a previous 90 day study carried out at comparable dose levels. Overnight fasting of the animals (with the exception of mice) prior to blood sampling is recommended1. The following list of parameters should be investigated (30): glucose, urea (urea nitrogen), creatinine, total protein, albumin, calcium, sodium, potassium, total cholesterol, at least two appropriate tests for hepatocellular evaluation (alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase, total bile acids)(31), and at least two appropriate tests for hepatobiliary evaluation (alkaline phosphatase, gamma glutamyl transferase, 5'-nucleotidase, total bilirubin, total bile acids)(31). Other clinical chemistry parameters such as fasting triglycerides, specific hormones and cholinesterase may be measured as appropriate, depending on the toxicity of the substance. Overall, there is a need for a flexible approach, depending on the observed and/or expected effect from a given substance.

43. Urinalysis determinations should be performed on at least 10 male and 10 female animals per group on samples collected at the same intervals as for haematology and clinical chemistry. Measurements at 3 months need not be conducted if no effect was seen on urinalysis in a previous 90 day study carried out at comparable dose levels. The following list of parameters was included in an expert recommendation on clinical pathology studies (30): appearance, volume, osmolality or specific gravity, pH, total protein, and glucose. Other determinations include ketone, urobilinogen, bilirubin, and occult blood. Further parameters may be employed where necessary to extend the investigation of observed effect(s).

44. It is generally considered that baseline haematological and clinical biochemistry variables are needed before treatment for dog studies, but need not be determined in

rodent studies (30). However, if historical baseline data (see paragraph 50) are inadequate, consideration should be given to generating such data.

Pathology

Gross necropsy

45. All animals in the study shall normally be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. However provision may also be made (in the interim kill or satellite groups) for measurements to be restricted to specific, key measures such as neurotoxicity or immunotoxicity (see paragraph 19). These animals need not be subjected to necropsy and the subsequent procedures described in the following paragraphs. Sentinel animals may require necropsy on a case-by-case basis, at the discretion of the study director.

46. Organ weights should be collected from all animals, other than those excluded by the latter part of paragraph 45. The adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thyroid (weighed post-fixation, with parathyroids), and uterus of all animals (apart from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to prevent drying. In a study using mice, weighing of the adrenal glands is optional.

47. The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination (32) (tissues in square brackets are optional):

all gross lesions	heart	pancreas	stomach (forestomach, glandular stomach)
adrenal gland	ileum	parathyroid gland	[teeth]
aorta	jejunum	peripheral nerve	testis
brain (including sections of cerebrum, cerebellum, and medulla/pons)	kidney	pituitary	thymus
caecum	lacrimal gland (exorbital)	prostate	thyroid
cervix	liver	rectum	[tongue]
coagulating gland	lung	salivary gland	trachea
colon	lymph nodes (both superficial and deep)	seminal vesicle	urinary bladder
duodenum	mammary gland (obligatory for females and, if visibly dissectable, from males)	skeletal muscle	uterus (including cervix)
epididymis	[upper respiratory tract, including nose, turbinates, and paranasal sinuses]	skin	[ureter]
eye (including retina)	oesophagus	spinal cord (at three levels: cervical, mid-thoracic, and lumbar)	[urethra]
[femur with joint]	[olfactory bulb]	spleen	vagina
gall bladder (for species other than rat)	ovary	[sternum],	section of bone marrow and/or a fresh bone marrow aspirate
Harderian gland			

In the case of paired organs, e.g., kidney, adrenal, both organs should be preserved. The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test chemical should be preserved. In studies involving the dermal route of administration, the list of organs as set out for the oral route should be preserved, and specific sampling and preservation of the skin from the site of application is essential. In inhalation studies, the list of preserved and examined tissues from the respiratory tract should follow the recommendations of TG 412 (8) and TG 413 (9). For other organs/tissues (and in addition to the specifically preserved tissues from the respiratory tract) the list of organs as set out for the oral route should be examined.

Histopathology

48. Guidance is available on best practices in the conduct of toxicological pathology studies (32). The minimum histopathological examinations should be:

- all tissues from the high dose and control groups;
- all tissues from animals dying or killed during the study;
- all tissues showing macroscopic abnormalities;
- target tissues, or tissues which showed treatment-related changes in the high dose group, from all animals in all other dose groups;
- in the case of paired organs, e.g., kidney, adrenal, both organs should be examined.

DATA AND REPORTING

Data

49. Individual animal data should be provided for all parameters evaluated. Additionally, all data should be summarised in tabular form showing for each test 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, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. Summary data tables should provide the means and standard deviations (for continuous test data) of animals showing toxic effects or lesions, in addition to the grading of lesions.

50. Historical control data may be valuable in the interpretation of the results of the study, e.g. in the case when there are indications that the data provided by the concurrent controls are substantially out of line when compared to recent data from control animals from the same test facility/colony. Historical control data, if evaluated, should be submitted from the same laboratory and relate to animals of the same age and strain generated during the five years preceding the study in question.

51. When applicable, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods and the data to be analysed should be selected during the design of the study (paragraph 8). Selection should make provision for survival adjustments, if needed.

TEST REPORT

52. The test report should include the following information:

Test chemical:

- physical nature, purity, and physicochemical properties;
- identification data;
- source of substance;

- batch number;
- certificate of chemical analysis

Vehicle (if appropriate):

- justification for choice of vehicle (if other than water).

Test animals:

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

Test conditions:

- rationale for route of administration and dose selection;
- when applicable, the statistical methods used to analyse the data;
- details of test chemical formulation/diet preparation;
- analytical data on achieved concentration, stability and homogeneity of the preparation;
- route of administration and details of the administration of the test chemical;
- for inhalation studies, whether nose only or whole body;
- actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water test chemical concentration (mg/kg or ppm) to the actual dose, if applicable;
- details of food and water quality.

Results:(summary tabulated data and individual animal data should be presented):

- survival data;
- body weight/body weight changes;
- food consumption, calculations of food efficiency, if made, and water consumption if applicable;
- toxic response data by sex and dose level, including signs of toxicity;
- nature, incidence (and, if scored, severity), and duration of clinical observations ((whether transitory or permanent);
- ophthalmological examination;
- haematological tests;
- clinical biochemistry tests;
- urinalysis tests;
- outcome of any investigations of neurotoxicity or immunotoxicity

- terminal body weight;
- organ weights (and their ratios, if applicable);
- necropsy findings;
- a detailed description of all treatment-related histopathological findings;
- absorption data if available;

Statistical treatment of results, as appropriate

Discussion of results including:

- Dose:response relationships
- Consideration of any mode of action information
- Discussion of any modelling approaches
- BMD, NOAEL or LOAEL determination
- Historical control data
- Relevance for humans

Conclusions

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