

PRELIMINARY DRAFT UPDATED TEST GUIDELINE 407:

**REPEATED DOSE 28-DAY ORAL TOXICITY STUDY IN RODENTS; UPDATED WITH
PARAMETERS FOR ENDOCRINE EFFECTS**

This document was initially prepared by Peter Gelbke, as a consultant for the OECD Secretariat. It includes the comments made at the VMG-mammalian meeting (4-5 April 2006), and at the EDTA Task Force meeting (26-27 April 2006) in particular the revised Paragraph 4a. This Draft Test Guideline will provide useful information for the finalisation of the validation of the test method.

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Repeated Dose 28-Day Oral Toxicity Study in Rodents; Updated with Parameters for Endocrine Effects

INTRODUCTION

1a The OECD initiated a high-priority activity in 1998 to revise existing guidelines and to develop new guidelines for the screening and testing of potential endocrine disruptors (1). One element of the activity was to update the existing OECD guideline for “repeated dose 28-day oral toxicity study in rodents” (TG 407) by parameters suitable to detect endocrine activity of test substances. This procedure underwent an extensive international program to test for the relevance and practicability of the additional parameters, the performance of these parameters for chemicals with (anti)oestrogenic, (anti)androgenic, and (anti)thyroid activity, the intra- and interlaboratory reproducibility, and the interference of the new parameters with those required by the prior TG 407. The chemicals used in this international program were ethinylestradiol, genistein, nonylphenol, tamoxifen, CGS 18320 B, methyl testosterone, flutamide, p,p'-DDE, propylthiouracil, and l-thyroxine. The large amount of data thereby obtained has been compiled and evaluated in detail in a comprehensive OECD report (2). This updated Test Guideline 407 is the outcome of the experience and results gained during the international test program. This TG 407 now allows to put endocrine mediated effects into context to other toxicological effects.

1b The rationale for enhancing existing test guidelines to detect endocrine mediated effects is contained in the OECD monograph 21 (3). A list of potential updates is given in this monograph and the suitability of most of them was tested in phase I of this international program with flutamide and propylthiouracil. Further compounds were investigated in phase-I as well, but these studies did not include all endpoints required by the prior TG 407 such as FOB or motor activity assessment, some of the studies were not reported adequately, and some used too low dose levels in comparison to the requirements of the prior TG 407. The experience thereby gained with regard to practicability, reproducibility and sensitivity of the updated parameters led to the selection of those to be added to the prior TG 407 for the international phase II testing. The results obtained by this phase II testing are the basis of the current TG 407.

1c This assay serves as an *in vivo* method providing data about multiple endocrine mechanisms and effects and should be seen in the context of the “OECD Conceptual Framework of the Testing and Assessment of Endocrine Disrupting Chemicals” (ANNEX 2). In this Conceptual Framework this TG 407 is contained in level 4. It provides data on and gives an indication of the dose response relationship of multiple endocrine mechanisms and effects in addition to the information provided by the prior TG 407 (4).

1d In the course of the international evaluation of this TG 407 test procedure, strongly acting endocrine active chemicals with (anti) oestrogenic, (anti) androgenic or (anti) thyroidal activity were clearly identified with appropriate sensitivity. There also was a good indication by the data obtained with methyl testosterone and p,p'-DDE that this TG 407 will detect chemicals exerting a weak activity on the thyroid. Regarding genistein (phytoestrogen), nonylphenol (weak estrogenic potential), and p,p'-DDE

(weak anti-androgenic potential) the hormonal activity was too low compared to other effects to clear a clear response in hormone related parameters up to the highest dose tested or results were equivocal (genistein) across the laboratories.

1e A detailed analyses of the results obtained by the international testing program led to the conclusion that some further updates to the prior TG 407 might increase the sensitivity especially for weak oestrogens and (anti) androgens (2). It is recommended to perform histopathology of the female as well as of the male mammary gland and to evaluate the histopathology of the female reproductive tract not only for clear pathological changes but especially also for synchronisation with the oestrous cycle as determined by vaginal smears. These investigations were not contained in the protocol provided to the laboratories participating in the international program.

1f The results obtained by the international program could not give clear guidance whether the determination of thyroid hormones should be included in the TG 407(2). There was a good indication that T4, T3 and TSH should be added as updated parameters.

1g The international program showed that the quality of data obtained by this TG 407 will depend much on the experience of the test laboratory. This relates specifically to the histopathological determination of cyclic changes in the female reproductive organs and to the weight determination of the small hormone dependent organs which are difficult to dissect. Thus, appropriate quality controls are recommended for laboratories carrying out TG 407 tests.

2a This TG 407 is the outcome of a meeting of the Validation Management Group on mammalian tests (VMG-mammalian) of the Endocrine Disruptor Testing and Assessment Task Force (EDTA), held in Washington in April 2006 (5) and of the EDTA, held in Stockholm in April 2006 (6). It is based on the data obtained by the international program during development of this method.

2b Taking account of the large number of animals, the high costs, and the long time required for performing an investigation according to the TG 407 it was and it is not appropriate to carry out a full validation program as required for example for in vitro tests. Neither animal welfare considerations nor economic aspects would justify such a complete validation effort. It is recognized that some of the parameters to be investigated within this TG 407 need further validation and possibly refinements. Such additional experience will be gained in the course of routine applications of this new test procedure.

2c It is proposed that this TG 407 should be used in the years to come to test for effects arising from repeated exposure including those related to the endocrine system. After further experience has been gained with this test procedure worldwide a group of experts should be called together in order to modify, refine or endorse the TG 407. For future modifications, if necessary, specific emphasis should be placed on the value of some of the endpoints that at present cannot be judged definitively, e.g. histopathology of the mammary gland, synchronisation of cyclicity of the female reproductive organs, and thyroid related hormones.

2d This TG 407 should be read in conjunction with the prior "Repeated Dose 28-Day Oral Toxicity Study in Rodents" TG 407 as adapted 27. 07. 1995 (4). If not stated otherwise the requirements for the prior TG 407 also apply to this guideline. Therefore, the structure and enumeration of this TG 407 relate to those of the prior TG 407.

INITIAL CONSIDERATIONS AND LIMITATIONS

3 In the assessment and evaluation of the toxic characteristics of a chemical, the determination of oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained

by acute toxicity testing. This study provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time including effects on the endocrine system. The method comprises the basic repeated dose toxicity study (4) that maybe used for chemicals on which a 90-day study is not warranted (e.g. when the production volume does not exceed certain limits) or as a preliminary to a long-term study. The duration of exposure should be 28 days.

4a The TG 407 has been modified to include endpoints to identify chemicals that interfere with thyroid physiology and affect the male and/or female reproductive organs in young adult animals, while still investigating all other toxicological parameters required under the prior TG 407. On the basis of data generated in the validation process, it must be emphasized that the sensitivity of this assay is not sufficient to identify all substances with (anti)androgenic or (anti)estrogenic modes of action. Consequently, the lack of effects related to these modes of action can not be taken as evidence for the lack of effects on the endocrine system.

4b The TG 407 is designed to identify possible chemical hazards from the toxic effects observed in the study, including effects related to the endocrine system. That is, the results from the TG 407 should be used for hazard identification and risk assessment. The results obtained by the endocrine related parameters should be seen in the context of the “OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals” (Attachment 1).

4c It is acknowledged that all animal-based procedures will conform to local standards of animal care; the descriptions of care and treatment set forth below are minimal performance standards, and will be superseded by local regulations. Further guidance of the humane treatment of animals is given by the OECD (7).

5. Definitions used are given in the annex.

PRINCIPLE OF THE TEST

6. c.f. prior TG 407

DESCRIPTION OF THE METHOD

Selection of animal species

7. This guideline specifically relates to the rat, since in the international testing program the rat was the only species used. If the parameters specified within this TG 407 are investigated in another rodent species a detailed justification should be given and it should be demonstrated that the species selected will respond to the updated parameters with a sensitivity comparable to the rat. Commonly used laboratory strains of young healthy adult animals should be employed. Females should be nulliparous and non pregnant. Dosing should begin as soon as possible after weaning when the animals are 7 weeks old. In any case, at the start of the study the animals must not be older than 9 weeks. At the commencement of the study the weight variation of animals used should be minimal and not exceed $\pm 20\%$ of the mean weight of each sex. When a repeated oral dose is conducted as a preliminary to a longer-term study, preferably animals from the same strain and source should be used in both studies.

Housing and feeding

8. c.f. prior TG 407

8a The feed should be regularly analysed for contaminants. A sample of the diet should be retained until finalisation of the report. In case of unexpected results an analysis of the diet for oestrogenic compounds may be considered.

Preparation of animals

9. c.f. prior TG 407

Preparation of doses

10. c.f. prior TG 407

11. c.f. prior TG 407

PROCEDURE

Number and sex of animals

12. c.f. prior TG 407

Dosage

13. c.f. prior TG 407

14. c.f. prior TG 407

14a Endocrine-mediated effects may be a secondary consequence of toxicity. In order to correctly identify such interference or confounding there should be at least 2 doses below the overtly toxic dose.

Limit test

15. c.f. prior TG 407

Administration of doses

16. The animals are dosed with test substance daily 7 days each week for a period of 28 days for males; females are treated for a period of 28 to 32 days depending on the day they are found in dioestrus. When the test substance is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100g body weight except in the case of aqueous solutions where 2 ml/100 g body weight may be used. 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.

17. c.f. prior TG 407

Observations

18. The observation period should be 28 days for males and for individual females until completion of an oestrus cycle, maximally 32 days (see paragraph 16). Animals in a satellite group scheduled for follow-

up observations should be kept for at least 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.

19. c.f. prior TG 407

20. c.f. prior TG 407

21. c.f. prior TG 407

22. c.f. prior TG 407

23. c.f. prior TG 407

23a At the end of the 4th week, the oestrus cycle of all females shall be determined daily by taking vaginal smears for at least 5 consecutive days starting on day 24 at the latest. Vaginal smears should be evaluated blind to the treatment groups, although with some knowledge of the previous days' identity.

23b. It should be understood that the limited number of days that the oestrus cycle is evaluated will not permit a reliable determination of cycle irregularities, but will allow females to be sacrificed on a comparable day. This will be during dioestrus following 28-32 days chemical administration. Female not found in dioestrus during this time period are necropsied on day 32.

Body weight and food/water consumption

24. c.f. prior TG 407

Haematology

25. c.f. prior TG 407

26. Blood samples should be taken from a named site just prior to or as part of the procedure for killing the animals, and stored under appropriate conditions. Animals will not be fasted prior to killing.

Clinical biochemistry

27. Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained of all animals just prior to or as part of the procedure for killing of the animals (apart from those found moribund and/or intercurrently killed). Blood shall be collected from females at the same stage of the oestrus cycle. Investigations of plasma and serum shall include sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanin aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ -glutamyl trans-peptidase and sorbitol dehydrogenase). Measurements of additional enzymes (of hepatic or other origin) and bile acids may provide useful information under certain circumstances.

28. c.f. prior TG 407

29. In addition, studies to investigate serum markers of general tissue damage should be considered. Other determinations that should be carried out, if the known properties of the test substance may, or are suspected to, affect related metabolic profiles include calcium, phosphate, triglycerides, specific hormones,

methaemoglobin, and cholinesterase. These need to be identified for chemicals in certain classes or on a case-by-case basis.

29a Although in the international evaluation of the endocrine related endpoints a clear advantage for the determination of thyroid hormones (T₃, T₄) and TSH could not be demonstrated, it may be helpful to retain blood samples to measure T₃, T₄ and TSH if there is an indication for an effect on the pituitary-thyroid axis. The following factors may influence the variability and the absolute concentrations of the hormone determinations:

- time of sacrifice because of diurnal variation of hormone concentrations
- method of sacrifice to avoid undue stress to the animals that may affect hormone concentrations
- test kits for hormone determinations that may differ by their standard curves.

The benefit of these determinations should be re-evaluated after more experience has been gained with the application of this guideline. However, widespread and routine analyses appear to be redundant to the histopathology for identification of thyroid-active chemicals.

29b. Plasma samples specifically intended for hormone determination should be obtained at a comparable time of the day, preferably during a period between late morning and early afternoon, when TSH is highest. It is recommended that consideration should be given to T₃, T₄ and TSH determinations triggered based upon alterations of thyroid histopathology. The numerical values obtained when analysing hormone concentrations differ with various commercial assay kits. Consequently, it may not be possible to provide performance criteria based upon uniform historical data. Alternatively, laboratories should strive to keep control coefficients of variation below 25 for T₃ and T₄ and below 35 for TSH. All concentrations are to be recorded in ng/ml.

30. c.f. prior TG 407

31. c.f. prior TG 407

PATHOLOGY

Gross necropsy

32. c.f. prior TG 407

32a In addition, the wet weight should be determined for the following organs as soon as possible after dissection to avoid drying: paired ovaries, uterus, seminal vesicles (including coagulating glands), and prostate (dorsolateral and ventral part combined). Alternatively, seminal vesicles and prostate may be trimmed and weighed after fixation. Clamp or ligature should be present during fixation as leakage of fluid provokes damage to fine structures in seminal vesicles.

32b The following organ weights should be determined after fixation: Thyroid (trimming should also be done after fixation to avoid tissue damage) and dorsolateral and ventral part of the prostate separately after separation.

33. c.f. prior TG 407

33a The following tissues may give valuable indication for endocrine-related effects: Gonads (ovaries and testes), accessory sex organs (uterus, cervix, vagina, epididymides, seminal vesicles with coagulation gland, dorsolateral and ventral prostate), pituitary, male and female mammary gland and thyroid.

33b In the international test program some evidence was obtained that subtle endocrine effects by chemicals with a low potency for affecting sex hormone homeostasis may be identified by disturbance of the synchronisation of the oestrus cycle in different tissues and not so much by frank histopathological alterations in female sex organs. Although no definitive proof was obtained for such effects, it is recommended that specific emphasis should be given by histopathology on synchronisation of cyclic alterations in ovaries (follicular, thecal, and granulosa cells), uterus, cervix, vagina, pituitary, and mammary gland in comparison to the stage of cycle as determined by vaginal smears. The validity of such detailed examinations should be evaluated at a later stage when more experience has been gained with the TG 407 .

Histopathology

- 34. c.f. prior TG 407
- 35. c.f. prior TG 407
- 36. c.f. prior TG 407

DATA AND REPORTING

Data

- 37. c.f. prior TG 407
- 38. c.f. prior TG 407

38.a For quality control it is proposed that historical control data are collected and that for numerical data coefficients of variation are calculated, especially for the updated parameters. These data can be used for comparison purposes when actual studies are evaluated.

Test report

- 39. c.f. prior TG 407

LITERATURE

- (1) OECD. (1998). Report of the First Meeting of the OECD Endocrine Disrupter Testing and Assessment (EDTA) Task Force, 10th-11th March 1998, ENV/MC/CHEM/RA(98)5.
- (2) OECD. (2006). Draft Final Report of the Validation of the Updated Test Guideline 407 Repeat Dose 28-day Oral Toxicity Study in Laboratory Rats.
- (3) OECD. (1998). Detailed Review Paper on the Appraisal of Test Methods for Sex Hormone Disrupting Chemicals. OECD Monograph No 21.
- (4) OECD. (1995). Repeated Dose 28-day Oral Toxicity Study in Rodents. OECD Guideline for the Testing of Chemicals. Adopted 27.07.1995.

(5) Include: Report on VMG Meeting in Washington, April 2006.

(6) Include: Report on EDTA Meeting in Stockholm, April 2006.

(7) OECD (2000) Guidance document on the recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No 19. ENV/JM/MONO(2000)7.

ANNEX

DEFINITIONS

Dose is the amount of test substance administered. The dose is expressed as weight of test substance per unit body weight of test animal per day (e.g. mg/kg body weight/day), or as a constant dietary concentration.

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

Evident toxicity is a general term describing clear signs of toxicity following administration of test substance. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

NOEL is the abbreviation for no-observed-effect level. This is the highest dose level where no treatment related-findings in any parameter are observed due to treatment.

NOAEL is the abbreviation for no-observed-adverse-effect level. This is the highest dose level where no adverse treatment-related findings are observed due to treatment.

Oestrogenicity is the capability of a chemical to act like a natural oestrogenic hormone (e.g. oestradiol 17 β) in a mammalian organism.

Androgenicity is the capability of a chemical to act like a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Thyroid activity is the capability of a chemical to act like a natural thyroid hormone (e.g. T₃) in a mammalian organism.

Antioestrogenicity is the capability of a chemical to suppress the action of a natural oestrogenic hormone (e.g. oestradiol 17 β) in a mammalian organism.

Antiandrogenicity is the capability of a chemical to suppress the action of a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antithyroid activity is the capability of a chemical to suppress the action of a natural thyroid hormone (e.g. T₃) in a mammalian organism.

Validation is a scientific process designed to characterise the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose.