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GUIDANCE DOCUMENT ON THE UTEROTROPHIC BIOASSAY - PROCEDURE TO TEST FOR ANTIOESTROGENICITY

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GUIDANCE DOCUMENT ON THE UTEROTROPHIC BIOASSAY - PROCEDURE TO TEST FOR ANTIOESTROGENICITY

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides and Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and the Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (http://www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The participating organisations are FAO, ILO, OECD, UNEP, UNIDO, UNITAR and WHO. The World Bank and UNDP are observers. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

FOREWORD

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 disrupters. One element of the activity was to develop a Test Guideline (TG) for the rodent Uterotrophic Bioassay. The rodent Uterotrophic Bioassay then underwent an extensive validation programme including the compilation of a detailed background document and the conduct of extensive intra- and interlaboratory studies to show the relevance and reproducibility of the bioassay with a potent reference oestrogen, weak oestrogen receptor agonists, a strong oestrogen receptor antagonist, and a negative reference chemical.

The draft TG on Uterotrophic Bioassay that was initially developed, included the oestrogenic and the antioestrogenic protocols of the uterotrophic bioassay. However, comments received from the National Coordinators during the circulation of the draft TG were in favour of the separation of the two protocols, due to their different level of validation. As the ability of the uterotrophic assay to detect anti-estrogens had not been sufficiently characterized in the validation studies, the Secretariat also considered that the antioestrogenic protocol should not be in a Test Guideline (i.e. part of Mutual Acceptance of Data) and proposed to include it in a Guidance Document (GD).

The first draft Guidance Document was approved by the Validation Management Group for Mammalian Testing and the Task Force for Endocrine Disrupters Testing and Assessment (EDTA) at their meetings in January and March 2007 respectively, provided that changes already agreed for the Test Guideline on the Uterotrophic Bioassay also become integrated into this GD. The GD has been modified accordingly. This final Guidance Document was adopted by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) at its meeting, in March 2007.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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INTRODUCTION

1. 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 disrupters (1). One element of the activity was to develop a Test Guideline for the rodent Uterotrophic Bioassay. The rodent Uterotrophic Bioassay then underwent an extensive validation programme including the compilation of a detailed background document (2)(3) and the conduct of extensive intra- and interlaboratory studies to show the relevance and reproducibility of the bioassay with a potent reference oestrogen, weak oestrogen receptor agonists, a strong oestrogen receptor antagonist, and a negative reference chemical (4)(5)(6)(7)(8)(9). A Test Guideline (TG 440) has been developed for the screening of oestrogenic properties. This Guidance document focuses on the antioestrogenic protocol; it is the outcome of the experience gained during the validation test programme and the results obtained thereby with the use of a strong oestrogen receptor antagonist. Due to insufficient validation of this antioestrogenic protocol, this Guidance Document is provided for experimental purpose only. The test will provide supporting evidence in relation to antioestrogenic activity (see paragraph 7).

2. The Uterotrophic Bioassay is a short-term screening test that originated in the 1930's (27)(28) and was first standardized for screening by an expert committee in 1962 (32)(35). It is based on the increase in uterine weight or uterotrophic response (29). It evaluates the ability of a chemical to elicit biological activities consistent with agonists or antagonists of natural oestrogens (e.g. 17ß-estradiol), however, its use for antagonist detection is much less common than for agonists. The uterus responds to oestrogens in two ways. An initial response is an increase in weight due to water imbibition. This response is followed by a weight gain due to tissue growth (30). The uterus responses in rats and mice qualitatively are comparable.

3. When better validated, the application of this bioassay as an in vivo screening assay for oestrogenic antagonists could be seen in the context of the "OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals" (Annex2). As for the oestrogenic part of the Uterotrophic Bioassay, the antioestrogenic protocol of the Uterotrophic Bioassay would also be contained in Level 3 of this Conceptual Framework as an *in vivo* assay providing data about a single endocrine mechanism, i.e. antioestrogenicity.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Oestrogen agonists and antagonists act as ligands for oestrogen receptors α and β and may activate or inhibit, respectively, the transcriptional action of the receptors. This may have the potential to lead to adverse health hazards, including reproductive and developmental effects. Therefore, the need exists to rapidly assess and evaluate a chemical as a possible oestrogen agonist or antagonist. While informative, the affinity of a ligand for an oestrogen receptor or transcriptional activation of reporter genes *in vitro* is only one of several determinants of possible hazard. Other determinants can include metabolic activation and deactivation upon entering the body, distribution to target tissues, and clearance from the body, depending at least in part on the route of administration and the chemical being tested. This leads to the need to screen the possible activity of a chemical *in vivo* under relevant conditions, unless the chemical's characteristics regarding Absorption – Distribution – Metabolism – Elimination (ADME) already provide appropriate information. Uterine tissues respond with rapid and vigorous growth to stimulation by oestrogens, particularly in laboratory rodents, where the oestrous cycle lasts approximately 4 days. Rodent species, particularly the rat, are also widely used in toxicity studies for hazard characterization. Therefore, the rodent uterus is an appropriate target organ for the *in vivo* screening of oestrogen agonists and antagonists.

5. This Guidance Document is based on those protocols employed in the OECD validation study of Phase 1 (4)(5). Currently two methods, namely, the ovariectomised adult female method (ovx-adult method) and the immature non-ovariectomised method (immature method) are available. It was shown in the OECD validation test program that both methods have comparable sensitivity and reproducibility. However, the immature, as it has an intact hypothalamic-pituitary-gonadal (HPG) axis, is somewhat less specific but covers a larger scope of investigation than the ovariectomized animal because it can respond to substances that interact with the HPG axis rather than just the oestrogen receptor. The HGP axis of the rat is functional at about 15 days of age. Prior to that, puberty cannot be accelerated with treatments like GnRH. As the females begin to reach puberty, prior to vaginal opening, the female will have several silent cycles that do not result in vaginal opening or ovulation, but there are some hormonal fluctuations. If a chemical stimulates the HGP axis directly or indirectly, precocious puberty, early ovulation and accelerated vaginal opening result. Not only chemicals that act on the HPG axis do this but some diets with higher metabolizable energy levels than others will stimulate growth and accelerate vaginal opening without being estrogenic. Such substances would not induce an uterotrophic response in ovx-adult animals as their HPG axis doesn't work.

6. For animal welfare reasons preference should be given to the method using immature rodents, avoiding surgical pre-treatment of the animals.

7. Taking into account that the Uterotrophic Bioassay serves as an in vivo screening assay, the validation approach taken, served both animal welfare considerations and a tiered testing strategy. To this end, effort was directed at rigorously validating reproducibility and sensitivity for oestrogenicity - the main concern for many chemicals -, while little effort was directed at the antioestrogenicity component of the assay. Only one antioestrogen with strong activity was tested since the number of substances with a clear antioestrogenic profile is very limited. Thus the protocol for the antagonist mode of the assay was not included in the Test Guideline developed for the oestrogenic protocol, but it is described in this Guidance Document as a suggested experimental approach. The reproducibility and sensitivity of the assay for substances with anti-oestrogenic activity will be more clearly defined later on, after the test procedure has been in routine use for some time and more substances with this modality of action are identified.

8. 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 (25).

9. As with all assays using live animals, it is essential to ensure that the data are truly necessary prior to the start of the assay. For example, two conditions where the data may be required are:

- high exposure potential (Level 1 of the Conceptual Framework, Annex 2) or indications for antioestrogenicity (Level 2) to investigate whether such effects may occur in vivo

- effects indicating antioestrogenicity in Level 4 or 5 in vivo tests to substantiate that the effects were related to an antioestrogenic mechanism that cannot be elucidated using an *in vitro* test.

10. Definitions used in this Guidance Document are given in Annex 1.

PRINCIPLE OF THE TEST

11. The Uterotrophic Bioassay relies for its sensitivity on an animal test system in which the hypothalamic-pituitary-ovarian axis is not functional, leading to low homeostatic regulation against exogenous oestrogenic stimuli. This will ensure a high sensitivity to alteration in estrogenicity of the treatment at any estrogenic base levels. Two oestrogen sensitive states in the female rodent meet this requirement:

i) immature females after weaning and prior to puberty and

ii) young adult females after ovariectomy with adequate time for uterine tissues to regress.

12. The test substance is administered daily by oral gavage or subcutaneous injection. Graduated test substance doses are administered to a minimum of three treatment groups of experimental animals using one dose level per group and a minimum administration period of three consecutive days. The animals are necropsied approximately 24 hours after the last dose. For oestrogen antagonists, a potent reference oestrogen in a submaximal response dose is co-administered with the test substance and the response is compared to a reference oestrogen-only control group. The mean uterine weight of the test substance groups relative to the reference oestrogen-only group is assessed for a statistically significant alteration. A statistically significant decrease in uterine weight indicates that the test substance has an ability to reduce or block the action of natural oestrogens, and, therefore, is considered to be a potential anti-oestrogen.

DESCRIPTION OF THE METHOD

Selection of animal species

13. Commonly used laboratory rodent strains may be used. As an example, Sprague-Dawley and Wistar strains of rats were used during the validation. Strains with uteri known or suspected to be less responsive should not be used. The laboratory should demonstrate the sensitivity of the strain used, as described in paragraph 22. The validation study carried out on mice (16) contains data on the antioestrogenicity of bisphenol A and genistein.

Housing and feeding conditions

14. All procedures should conform to local standards of laboratory animal care. These descriptions of care and treatment are minimum standards and will be superseded by local regulations, when present. The temperature in the experimental animal room should be 22°C (with an approximate range ± 3°C). The relative humidity should be a minimum of 30% and preferably should not exceed a maximum 70%, other than during room cleaning. The aim should be relative humidity of 50-60%. Lighting should be artificial. The daily lighting sequence should be 12 hours light, 12 hours dark.

15. Laboratory diet and drinking water should be provided *ad libitum*. Young adult animals may be housed individually or be caged in groups of up to three animals. Due to the young age of the immature animals, social group housing is recommended.

16. High levels of phytooestrogens in laboratory diets have been known to increase uterine weights in rodents to a degree enough as to interfere with the Uterotrophic Bioassay (13)(14)(15). High levels of phytooestrogens and of metabolizable energy in laboratory diets may also result in early puberty, if immature animals are used. The presence of phytooestrogens results primarily from the inclusion of soy and alfalfa products in the laboratory diets and concentrations of phtooestrogens have been shown to vary from batch-to-batch of standard laboratory diets (23). Body weight is an important variable, as the quantity

of food consumed is related to body weight. Therefore, the actual phytooestrogen dose consumed from the same diet may vary among species and by age (9). For immature female rats, food consumption on a body weight basis may be approximately double that of ovariectomised young adult females. For young adult mice, food consumption on a body weight basis may be approximately quadruple that of ovariectomised young adult female rats.

17. Uterotrophic Bioassay results (9)(17)(18)(19), however, show that limited quantities of dietary phytooestrogens are acceptable and do not reduce the sensitivity of the bioassay. As a guide, dietary levels of phytooestrogens should not exceed 350 μg of genistein equivalents/gram of laboratory diet for immature female Sprague Dawley and Wistar rats (6)(9). Such diets should also be appropriate when testing in young adult ovariectomised rats because food consumption on a body weight basis is less in young adult as compared to immature animals. If adult ovariectomised mice or more phytooestrogen-sensitive rats are to be used, proportional reduction in dietary phytooestrogen levels must be considered (20). In addition, the differences in available metabolic energy from different diets may lead to time shifts for the onset of puberty (21)(22).

18. Prior to the study, careful selection is required of a diet without an elevated level of phytooestrogens (for guidance see (6)(9)) or metabolizable energy, that can confound the results (15)(17)(19)(22)(36). Ensuring the proper performance of the test system used by the laboratory is an important check on both of these factors. As a safeguard consistent with GLP, representative sampling of each batch of diet administered during the study should be conducted for possible analysis of phytooestrogen content (e.g. in the case of an inadequate response to the reference oestrogen, 17 alpha ethinyl estradiol). Aliquots should be analyzed as part of the study or frozen at -20°C or in such a way as to prevent the sample from decomposing prior to analysis.

19. Some bedding materials may contain naturally occurring oestrogenic or antioestrogenic substances (e.g. corn cob is known to affect the cyclicity of rats and appears to be antioestrogenic). Thus, the selected bedding material should contain a minimum level of phytooestrogens.

Preparation of animals

20. Experimental animals without evidence of any disease or physical abnormalities are randomly assigned to the control and treatment groups and cages. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals should be identified uniquely. Preferably, immature animals should be caged with dams or foster dams until weaning during acclimatization. The acclimatization period prior to the start of the study should be about 5 days for young adult animals and for the immature animals delivered with dams or foster dams. If immature animals are obtained as weanlings without dams a shorter duration of the acclimatization period may become necessary as dosing should start immediately after weaning (see paragraph 25).

PROCEDURE

Verification of Laboratory Proficiency

21. The assay should be conducted in conformity with the OECD Good Laboratory Practice and Quality Assurance Procedures (31).

22. **Baseline Oestrogenic Control Study -** On a regular basis or prior to the study, laboratory proficiency should be demonstrated by testing the responsiveness of the animal model, with the uterotrophic dose-response of a reference oestrogen: 17α-ethinyl estradiol (CAS No. 57-63-6) (EE), and

results compared with established historical data (see reference (5)). This will enable to select the dose of the reference oestrogen agonist to be used (see paragraphs 30, 31). If this baseline oestrogenic control study does not yield the anticipated results the experimental conditions should be examined and modified.

Number and condition of animals

23. Each treated and control group should include at least 6 animals (for both immature and ovx-adult protocols).

Age of immature animals

24. For the Uterotrophic Bioassay with immature animals the day of birth must be specified. Dosing should begin early enough to ensure that, at the end of test substance administration, the physiological rise of endogenous oestrogens associated with puberty has not yet taken place. On the other hand, there is evidence that very young animals may be less sensitive. For defining the optimal age each laboratory should take its own background data on maturation into consideration.

25. As a general guide, dosing in rats may begin immediately after early weaning on postnatal day 18 (with the day of birth being postnatal day 0). Dosing in rats preferably should be completed on postnatal day 21 but in any case prior to postnatal day 25, because, after this age, the hypothalamic-pituitary-ovarian axis becomes functional and endogenous oestrogen levels may begin to rise with a concomitant increase in baseline uterine weight means and an increase in the group standard deviations (2)(3)(10)(11)(12).

26. If the immature female mouse is used (not validated for agonist assay), treatment should be carried out at an earlier age, i.e. dosing may begin immediately after early weaning on postnatal day 16 (with the day of birth being postnatal day 0). Dosing in mice should be completed prior to postnatal day 21, after which, as for rats the hypothalamic-pituitary-ovarian axis may become functional, thus an increase in baseline uterine weight means and an increase in the group standard deviations may occur (23).

Procedure for ovariectomy

27. For the ovariectomised female rat and mouse (treatment and control groups), ovariectomy should occur between 6 and 8 weeks of age. For rats, a minimum of 14 days should elapse between ovariectomy and the first day of administration in order to allow the uterus to regress to a minimum, stable baseline. For mice, at least 7 days should elapse between ovariectomy and the first day of administration. As small amounts of ovarian tissue are sufficient to produce significant circulating levels of oestrogens (3), the animals should be tested prior to use by observing epithelial cells swabbed from the vagina on at least five consecutive days (e.g., days 10-14 after ovariectomy). If the animals indicate any evidence entering oestrous, the animals should not be used. Further, at necropsy, the ovarian stubs should be examined for any evidence that ovarian tissue is present. If so, the animal should not be used in the calculations (3).

28. The ovariectomy procedure begins with the animal in ventral recumbency after the animal has been properly anesthetized. The incision opening the dorso-lateral abdominal wall should be approximately 1 cm lengthways at the mid point between the costal inferior border and the iliac crest, and a few millimetres lateral to the lateral margin of the lumbar muscle. The ovary should be removed from the abdominal cavity onto an aseptic field. The ovary should be disconnected at the junction of the oviduct and the uterine body. After confirming that no massive bleeding is occurring, the abdominal wall should be closed by a suture and the skin closed by autoclips or appropriate suture. The ligation points are shown schematically in **Figure 1**. Appropriate post operative analgesia should be used as recommended by a veterinarian experienced in rodent care.

Body weight

29. In the ovx-adult method, body weight and uterine weight are not correlated because uterine weight is affected by hormones like oestrogens but not by the growth factors that regulate body size. On the contrary, body weight is related to uterine weight in the immature model, while it is maturing (34). Thus, at the commencement of the study the weight variation of animals used, in the immature model, should be minimal and not exceed ± 20 % of the mean weight. This means that the litter size should be standardized by the breeder, to assure that offspring of different mother animals will be fed approximately the same. Animals should be assigned to groups (both control and treatment) by randomized weight distribution, so that mean body weight of each group is not statistically different from any other group. Consideration should be given to avoid assignment of littermates to the same treatment group as far as practicable without increasing the number of litters to be used for the investigation.

Dosage

30. To test for oestrogen antagonists the test chemical is administered to immature or ovariectomised female rats together with the reference oestrogen agonist, 17α-ethinyl estradiol (CAS No. 57-63-6). A minimum of 3 test groups with different doses of the test chemical (putative antioestrogen) and a constant dose of the reference oestrogen agonist should be used. The oestrogenic control group will receive the reference agonist at the same dose as in the test groups. The oestrogenic control group must show a clear increase in uterus weight above the base line. An antioestrogen will either abolish or significantly decrease the increase in uterine weight in the test groups. Half log unit intervals (factor 3.2) may be an appropriate spacing between the dose groups. A vehicle control group will only be dosed with the vehicle to establish the base line for the uterus weight to demonstrate the stimulatory potency of the reference oestrogen.

31. When testing for antioestrogenicity 17α-ethinyl estradiol is the reference oestrogen agonist. It must not be dosed so high as to reach the saturation plateau of the dose response curve as this could mask the antiestrogenic effects. Thus, the dose of the reference oestrogen agonist should lead to an increase in uterine weight near the top of the ascending dose response curve but before the maximum has been achieved. For 17α-ethinyl estradiol this could be in the range of 1 µg/kg body weight/day by subcutaneous administration (5)(16) (see paragraph 39). In this respect the historical data with the animal strain used and the experience of the laboratory are decisive for the final dose selection.

32. The objective in the case of the Uterotrophic Bioassay is to select doses that ensure animal survival and that are without significant toxicity or distress to the animals after three to seven consecutive days of chemical administration up to a maximum dose of 1000 mg/kg/d. All dose levels of the tested chemical should be proposed and selected taking into account any existing toxicity and (toxico-) kinetic data available for the test compound or related materials. The highest dose level should first take into consideration the LD50 and/or acute toxicity information in order to avoid death, severe suffering or distress in the animals (24)(25)(26). The highest dose should represent the maximum tolerated dose (MTD).

33. If the antioestrogenic potency of an antagonist can be estimated by in vitro (or in silico) data, these may be taken into consideration for dose selection. However, care must be exercised to avoid using doses in excess of the MTD.

Considerations for range finding

34. If necessary, a preliminary range finding study can be carried out with few animals. In this respect, OECD Guidance Document n°19 (25) may be used defining clinical signs indicative of toxicity or distress to the animals.

Administration of doses

35. The test compound is administered by oral gavage or subcutaneous injection. Animal welfare considerations as well as toxicological aspects like the relevance to the human route of exposure to the chemical (e.g. oral gavage to model ingestion, subcutaneous injection to model inhalation or dermal adsorption), the physical/chemical properties of the test material and especially existing toxicological information and data on metabolism and kinetics (e.g. need to avoid first pass metabolism, better efficiency via a particular route) have to be taken into account when choosing the route of administration.

36. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first. But as most oestrogen ligands or their metabolic precursors tend to be hydrophobic, the most common approach is to use a solution/suspension in oil (e.g. corn, peanut, sesame or olive oil). However, these oils have different caloric and fat content, thus the vehicle might affect total metabolizable energy (ME) intake, thereby potentially altering measured endpoints such as the uterine weight especially in the immature method (32). Thus, prior to the study, any vehicle to be used should be tested against controls without vehicles. Test substances can be dissolved in a minimal amount of 95% ethanol or other appropriate solvents and diluted to final working concentrations in the test vehicle. The toxic characteristics of the solvent must be known, and should be tested in a separate solvent-only control group. If the test substance is considered stable, gentle heating and vigorous mechanical action can be used to assist in dissolving the test substance. The stability of the test substance in the vehicle should be determined. If the test substance is stable for the duration of the study, then one starting aliquot of the test substance may be prepared, and the specified dosage dilutions prepared daily.

37. Dosage timing will depend of the model used (refer to paragraphs 24 to 26 for the immature model and to paragraph 27 for ovx-adult model). Immature female are dosed with the test substance daily for three consecutive days. For the ovariectomised female, data suggest that using a seven-day dosing regimen may have a sensitivity advantage over the three-day exposure (4). The dose should be given at similar times each day. They should be adjusted as necessary to maintain a constant dose level in terms of animal body weight (e.g., mg of test substance per kg of body weight per day). Regarding the test volume, its variability, on a body weight basis, should be minimized by adjusting the concentration of the dosing solution to ensure a constant volume on a body weight basis at all dose levels and for any route of administration.

38. As an appropriate reference antioestrogen is not available, this protocol does not require positive control for antagonistic activity. The antagonistic effect will be monitored as decrease in uterine weight from the reference oestrogen group.

39. In assays for antioestrogenicity the test substance and the reference oestrogen are normally given within 15 minutes. For oral route, to avoid direct mixing of the test substance with the reference estrogen in the body, the reference estrogen is given by subcutaneous injection. For subcutaneous route, the test substance will be injected on one side of the dorsum and the reference estrogen on the other side of the dorsum of the animal. The application volumes for both should not exceed those used when testing for oestrogenicity i.e. 5 ml/kg body weight for subcutaneous injection (except in the case of aqueous solutions where 10 ml/kg body weight may be used) divided into 2 injection sites and 5 ml/kg body weight for oral application with the exception of aqueous solutions where 10 ml/kg body weight may be used.

Observations

General and clinical observations

41. General clinical observations should be made at least once a day and more frequently when signs of toxicity are observed. Observations should be carried out preferably at the same time(s) each day and considering the period of anticipated peak effects after dosing. All animals are to be observed for mortality, morbidity and general clinical signs such as changes in behaviour, skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern).

Body weight and food consumption

42. All animals should be weighed daily to the nearest 0.1 g, starting just prior to initiation of treatment i.e., when the animals are allocated into groups. As an optional measurement, the amount of food consumed during the treatment period may be measured per cage by weighing the feeders. The food consumption results should be expressed in grams per rat per day.

Dissection and measurement of uterus weight

43. Twenty-four hours after the last treatment, the rats will be humanely killed. Ideally, the necropsy order will be randomized across groups to avoid progression directly up or down dose groups that could subtly affect the data. The bioassay objective is to measure both the wet and blotted uterus weights. The wet weight includes the uterus and the luminal fluid contents. The blotted weight is measured after the luminal contents of the uterus have been expressed and removed.

44. Before dissection the vagina will be examined for opening status in immature animals. The dissection procedure begins by opening the abdominal wall starting at the pubic symphysis. Then, uterine horn and ovaries, if present, are detached from the dorsal abdominal wall. The urinary bladder and ureters are removed from the ventral and lateral side of uterus and vagina. Fibrous adhesion between the rectum and the vagina is detached until the junction of vaginal orifice and perineal skin can be identified. The uterus and vagina are detached from the body by incising the vaginal wall just above the junction between perineal skin as shown in **Figure 2**. The uterus should be detached from the body wall by gently cutting the uterine mesentery at the point of its attachment along the full length of the dorsolateral aspect of each uterine horn. Once removed from the body, uterine handling should be sufficiently rapid to avoid desiccation of the tissues. Loss of weight due to desiccation becomes more important with small tissues such as the uterus (23). If ovaries are present, the ovaries are removed at the oviduct avoiding loss of luminal fluid from the uterine horn. If the animal has been ovariectomised, the stubs should be examined for the presence of any ovarian tissue. Excess fat and connective tissue should be trimmed away. The vagina is removed from the uterus just below the cervix so that the cervix remains with the uterine body as shown in **Figure 2**.

45. Each uterus should be transferred to a uniquely marked and weighed container (e.g. a petri-dish or plastic weight boat) with continuing care to avoid desiccation before weighing (e.g. filter paper slightly dampened with saline may be placed in the container). The uterus with luminal fluid will be weighed to the nearest 0.1 mg (wet uterine weight).

46. Each uterus will then be individually processed to remove the luminal fluid. Both uterine horns will be pierced or cut longitudinally. The uterus will be placed on lightly moistened filter paper (e.g. Whatman No. 3) and gently pressed with a second piece of lightly moistened filter paper to completely remove the luminal fluid. The uterus without the luminal contents will be weighed to the nearest 0.1 mg (blotted uterine weight).

47. The uterus weight from the vehicle control group at termination can be used to assure that the appropriate age in the immature intact rat was not exceeded. As a guide the mean blotted uterus weight should be around 30 mg at postnatal day 23. However, the historical data of the rat strain used by the laboratory are decisive in this respect.

Dissection and measurement of liver weight

48. Liver should be dissected and weighted to look for hypertrophy, which may indicate induction of xenobiotic metabolizing enzymes, which could secondarily reduce the effective dose of estrogen. This would cause reduced stimulation of the uterus and, hence, cause a positive response without the substance necessarily interacting with the estrogen receptor.

49. A liver sample can be taken and kept deep-frozen for further biochemical analysis of liver enzymes. This may be helpful for equivocal cases and further mechanistic work. It should be noted that increased liver weight is not necessarily an indication of induction of oestrogen-metabolizing enzymes, although large increases in liver weight are usually associated with induction of a broad range of liver enzymes.

Optional investigations

50. After weighing, the uterus may be fixed in 10% neutral buffered formalin to be examined histopathologically after Haematoxylin & Eosin (HE)-staining. The vagina may be investigated accordingly. In addition, morphometric measurement of endometrial epithelium may be done for quantitative comparison.

DATA AND REPORTING

Data

51. Study data should include:

- the number of animals at the start of the assay,
- the number and identity of animals found dead during the assay or killed for humane reasons and the date and time of any death or humane kill,
- the number and identity of animals showing signs of toxicity, and a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, and
- the number and identity of animals showing any lesions and a description of the type of lesions.

52. Individual animal data should be recorded for the body weights, the wet uterine weight, the blotted uterine weight and the liver weight. One-tailed statistical analyses for antagonists should be used to determine whether the administration of a test substance resulted in a statistically significant decrease ($p < 0.05$) in the uterine weight as compared to that of the control group treated with the reference oestrogen agonist. The statistical analyses are in principle the same as those used for oestrogen agonists. Appropriate statistical analyses should be carried out to test for treatment related changes in blotted and wet uterine weight. For example, the data may be evaluated by an analysis of covariance (ANCOVA) approach with body weight at necropsy as the co-variable. A variance-stabilizing logarithmic transformation may be carried out on the uterine data prior to the data analysis. Dunnett and Hsu's test are appropriate for making pair wise comparisons of each dosed group to oestrogen controls and to calculate the confidence intervals.

Studentised residual plots can be used to detect possible outliers and to assess homogeneity of variances. These procedures were applied in the OECD validation program using the PROC GLM in the Statistical Analysis System (SAS Institute, Cary, NC), version 8 (6)(7).

53. A final report shall include:

Testing facility:

- Responsible personnel and their study responsibilities
- Data from the Baseline Oestrogenic Control Test (see paragraphs 22)

Test Substance:

- Characterization of test substances
- Physical nature and where relevant physicochemical properties
- Method and frequency of preparation of dilutions
- Any data generated on stability
- Any analyses of dosing solutions

Vehicle:

- Characterization of test vehicle (nature, supplier and lot)
- Justification of choice of vehicle (if other than water)

Reference Oestrogen:

- Source and lot number
- Method of dissolution
- Method and frequency of dilution
- Route and timing (relative to test substance dosing) of administration

Test animals:

- Species and strain
- Supplier and specific supplier facility
- Age on supply with birth date
- If immature animals, whether or not supplied with dam or foster dam and date of weaning
- Details of animal acclimatization procedure
- Number of animals per cage
- Detail and method of individual animal and group identification

Assay Conditions:

- Details of randomization process (i.e., method used)
- Rationale for dose selection
- Details of test substance formulation, its achieved concentrations, stability and homogeneity
- Details of test substance administration
- Diet (name, type, supplier, content, and, if known, phytooestrogen levels)
- Water source (e.g., tap water or filtered water) and supply (by tubing from a large container, in bottles, etc.)

- Bedding (name, type, supplier, content)
- Record of caging conditions, lighting interval, room temperature and humidity, room cleaning
- Detailed description of necropsy and uterine weighing procedures
- Description of statistical procedures

Results

For individual animals:

- All daily individual body weights (from allocation into groups through necropsy) (to the nearest 0.1 g)
- Age of each animal (in days counting day of birth as day 0) when administration of test compound begins
- Date and time of each dose administration
- Calculated volume and dosage administered and observations of any dosage losses during or after administration
- Daily record of status of animal, including relevant symptoms and observations
- Suspected cause of death (if found during study in moribund state or dead)
- Date and time of humane killing with time interval to last dosing
- Wet uterine weight (to the nearest 0.1 mg) and any observations of luminal fluid losses during dissection and preparation for weighing
- Blotted uterine weight (to the nearest 0.1 mg)
- Liver weight

For each group of animals:

- Mean daily body weights (to the nearest 0.1 g) and standard deviations (from allocation into groups through necropsy)
- Mean wet uterine weights and mean blotted uterine weights (to the nearest 0.1 mg) and standard deviations
- If measured, daily food consumption (calculated as grams of food consumed per animal)
- The results of statistical analyses comparing both the wet and blotted uterine weights of treated groups relative to the same measures in the reference oestrogen agonist control groups.
- The results of statistical analysis comparing the total body weight and the body weight gain of treated groups relative to the same measures in the reference oestrogen agonist control groups.

GUIDANCE FOR THE INTERPRETATION

54. In general, a test for antioestrogenicity should be considered positive if there is a statistically significant decrease in uterine weight ($p<0.05$) as compared to the referent oestrogen control group. The referent oestrogen control group must show increased uterine weights in relation to the solvent control group as expected for such treatment conditions. A positive result is further supported by the demonstration of biologically plausible relationship between the dose and magnitude of response. However, weak antagonists, particularly those that show partial agonist/antagonist activity, may display atypical (e.g. U-shaped) dose-response relationships. The ED70 dose response of the reference oestrogen has its own inherent variability, including from group to group with a study. Thus, the control group response can be sufficiently high or low relative to test substance groups to result in infrequent false positives or false negatives, respectively. Therefore, consideration of historical reference oestrogen response to ethinyl estradiol may be necessary (Kanno et al., unpublished data).

55. Care must be taken in order not to exceed the maximum tolerated dose to allow a meaningful interpretation of the data. Reduction of body weight, clinical signs, and other findings should be thoroughly assessed in this respect.

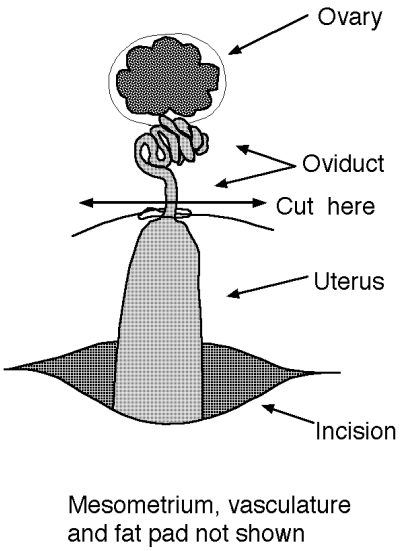
56. A major source of variability identified in the course of the OECD validation study was the expertise and care within a laboratory (6). It is recommended to verify the performance of the test by comparing with the historical data. For laboratories carrying out this assay on a routine basis it is recommended to periodically verify the responsiveness of the animal model to a reference oestrogen: 17α-ethinyl estradiol (CAS No. 57-63-6) (see paragraph 22).

57. Historical data for vehicle control groups and for responses to the reference oestrogen should be maintained in the laboratory. These data can help to ensure that the laboratory's methods yield sufficient sensitivity.

58. The blotted uterine weights showed less variability in the course of the OECD validation study than the wet uterine weights (6)(7). However, a significant response in either measure would indicate that the test substance is positive for estrogenic activity.

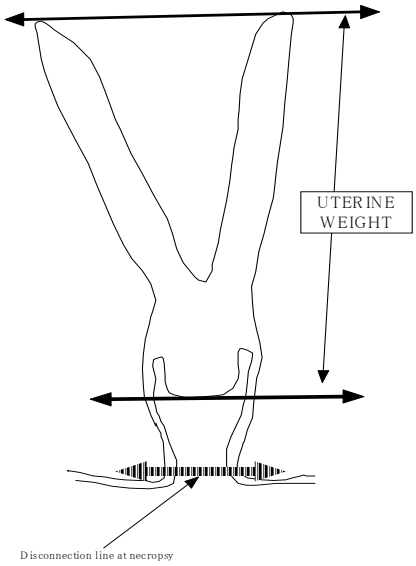
59. Detection of anti-estrogenicity in the uterotrophic assay requires co-administration of test compound and estrogen, typically 17α-ethinyl estradiol. When evaluating the outcome of such studies, potential kinetic interactions between the co-administered compounds as well as the induction of estrogen metabolizing enzymes by the test compound should be considered (see paragraphs 48 and 49).

Figure 1: Schematic diagram showing the surgical removal of the ovaries



The procedure begins by opening dorso-lateral abdominal wall at the mid point between the costal inferior border and the iliac crest, and a few millimetres lateral to the lateral margin of the lumbar muscle. Within the abdominal cavity, the ovaries should be located. On an aseptic field, the ovaries are then physically removed from the abdominal cavity, a ligature placed between the ovary and uterus to control bleeding, and the ovary detached by incision above the ligature at the junction of the oviduct and each uterine horn. After confirming that no significant bleeding persists, the abdominal wall should be closed by suture, and the skin closed, e.g., by autoclips or suture. The animals should be allowed to recover and the uterus weight to regress for a minimum of 14 days before use.

Figure 2: The removal and preparation of the uterine tissues for weight measurement.



The procedure begins by opening the abdominal wall at the pubic symphysis. Then, each ovary, if present and uterine horn is detached from the dorsal abdominal wall. Urinary bladder and ureters are removed from the ventral and lateral side of uterus and vagina. Fibrous adhesion between the rectum and the vagina are detached until the junction of vaginal orifice and perineal skin can be identified. The uterus and vagina are detached from the body by incising the vaginal wall just above the junction between perineal skin as shown in the figure. The uterus should be detached from the body wall by gently cutting the uterine mesentery at the point of its attachment along the full length of the dorsolateral aspect of each uterine horn. After removal from the body, the excess fat and connective tissue is trimmed away. If ovaries are present, the ovaries are removed at the oviduct avoiding loss of luminal fluid from the uterine horn. If the animal has been ovarectomised, the stubs should be examined for the presence of any ovarian tissue. The vagina is removed from the uterus just below the cervix so that the cervix remains with the uterine body as shown in the figure. The uterus can then be weighed.

ANNEX 1

DEFINITIONS

Antioestrogenicity is the capability of a chemical to suppress the action of estradiol 17ß in a mammalian organism.

Date of birth is postnatal day 0.

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

Dose is the amount of test substance administered. For the Uterotrophic Bioassay, 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).

Maximum Tolerable Dose (MTD) is the highest amount of a substance that, when introduced into the body does not kill test animals (denoted by DL_0) (IUPAC, 1993)

Oestrogenicity is the capability of a chemical to act like estradiol 17ß in a mammalian organism.

Postnatal day X is the Xth day of life after the day of birth.

Sensitivity is the proportion of all positive/active substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method.

Specificity is the proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method.

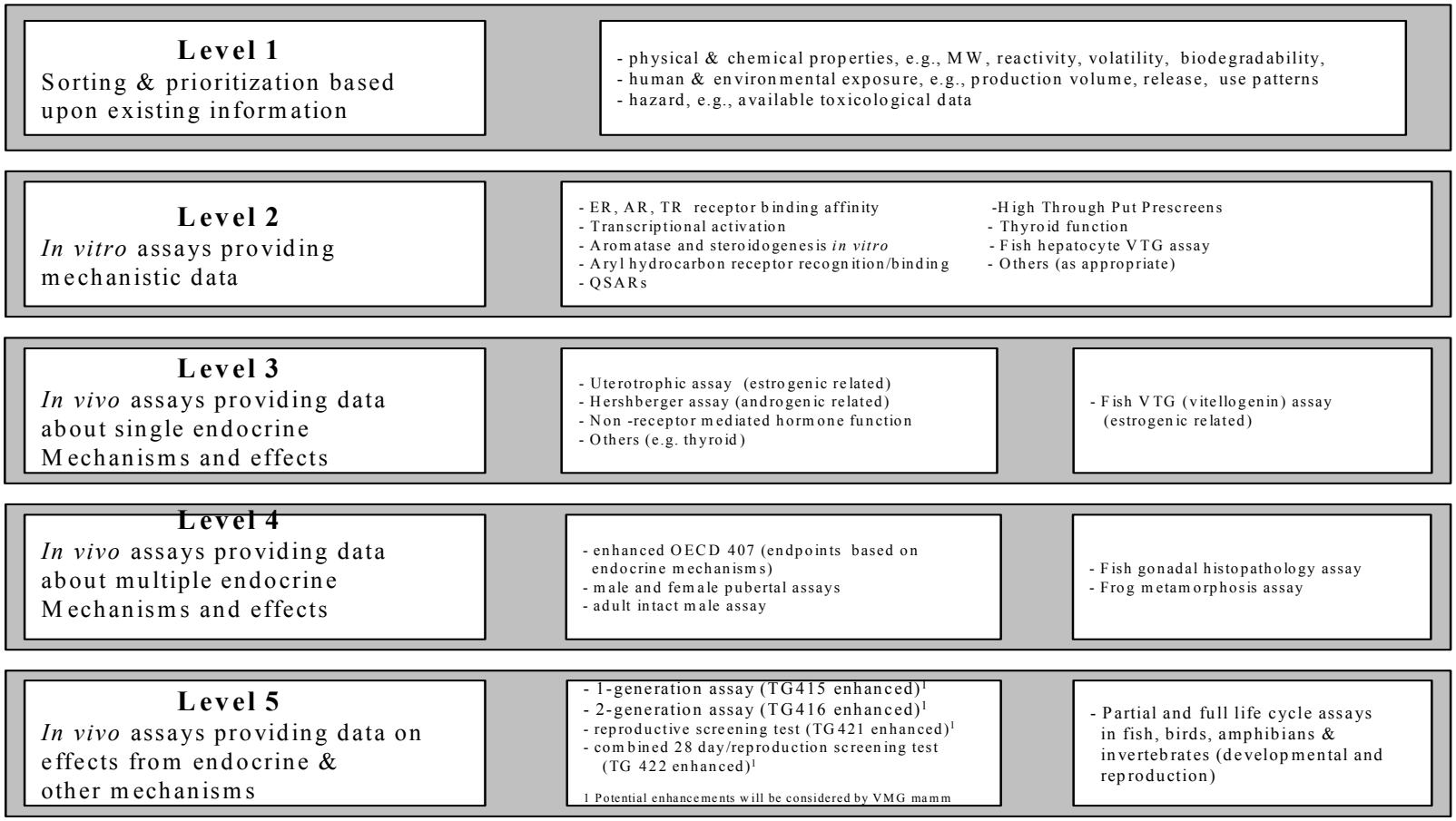
Uterotrophic is a term used to describe a positive influence on the growth of uterine tissues.

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

ANNEX 2

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals



VMG mamm: Validation Management Group on Mammalian Testing and Assessment

Notes to the Framework

Note 1: Entering at all levels and exiting at all levels is possible and depends upon the nature of existing information needs for hazard and risk assessment purposes

Note 2: In level 5,ecotoxicology should include endpoints that indicate mechanisms of adverse effects, and potential population damage

Note 3: When a multimodal model covers several of the single endpoint assays, that model would replace the use of those single endpoint assays

Note 4: The assessment of each chemical should be based on a case by case basis, taking into account all available information, bearing in mind the function of the framework levels.

Note 5: The framework should not be considered as all inclusive at the present time. At levels 3,4 and 5 it includes assays that are either available or for which validation is under way. With respect to the latter, these are provisionally included. Once developed and validated, they will be formally added to the framework.

Note 6: Level 5 should not be considered as including definitive tests only. Tests included at that level are considered to contribute to general hazard and risk assessment.

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. (2003). Detailed Background Review of the Uterotrophic Bioassay: Summary of the Available Literature in Support of the Project of the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) to Standardise and Validate the Uterotrophic Bioassay. OECD Environmental Health and Safety Publication Series on Testing and Assessment No. 38. ENV/JM/MONO(2003)1.

(3) Owens JW, Ashby J. (2002). Critical Review and Evaluation of the Uterotrophic Bioassay for the Identification of Possible Estrogen Agonists and Antagonists: In Support of the Validation of the OECD Uterotrophic Protocols for the Laboratory Rodent. Crit. Rev. Toxicol. 32:445-520.

(4) OECD. (2001). Final Report of the Phase 1 of the Validation Study of the Uterotrophic Assay. [ENV/JM/TG/EDTA (2001)1/REV1].

(5) Kanno, J, Onyon L, Haseman J, Fenner-Crisp P, Ashby J, Owens W. (2001). The OECD program to validate the rat uterotrophic bioassay to screen compounds for in vivo estrogenic responses: Phase 1. Environ Health Perspect. 109:785-94.

(6) OECD (2003). OECD Draft Report of the Validation of the Rat Uterotrophic Bioassay. Phase 2. Testing of Potent and Weak Oestrogen Agonists by Multiple Laboratories. [ENV/JM/TG/EDTA (2003)1].

(7) Kanno J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W. (2003). The OECD program to validate the rat uterotrophic bioassay: Phase Two - Dose Response Studies. Environ. Health Persp. 111:1530-1549

(8) Kanno J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W. (2003). The OECD program to validate the rat uterotrophic bioassay: Phase Two – Coded Single Dose Studies. Environ. Health Persp. 111:1550-1558.

(9) Owens W, Ashby J, Odum J, Onyon L. (2003). The OECD program to validate the rat uterotrophic bioassay: Phase Two – Dietary phytoestrogen analyses. Environ. Health Persp. 111:1559-1567.

(10) Ogasawara Y, Okamoto S, Kitamura Y, Matsumoto K. (1983). Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or adrenalectomized mice assayed by incorporation of [I^{125}]iododeoxyuridine. Endocrinology 113:582-587.

(11) Branham WS, Sheehan DM, Zehr DR, Ridlon E, Nelson CJ. (1985). The postnatal ontogeny of rat uterine glands and age-related effects of 17β-estradiol. Endocrinology 117:2229-2237.

(12) Schlumpf M, Berger L, Cotton B, Conscience-Egli M, Durrer S, Fleischmann I, Haller V, Maerkel K, Lichtensteiger W. (2001). Estrogen active UV screens. SÖFW-J. 127:10-15.

(13) Zarrow MX, Lazo-Wasem EA, Shoger RL. (1953). Estrogenic activity in a commercial animal ration. Science 118:650-651.

(14) Drane HM, Patterson DSP, Roberts BA , Saba N. (1975). The chance discovery of oestrogenic activity in laboratory rat cake. Fd. Cosmet. Toxicol. 13:425-427.

(15) Boettger-Tong H, Murphy L, Chiappetta C, Kirkland JL, Goodwin B, Adlercreutz H, Stancel GM, Makela S. (1998). A case of a laboratory animal feed with high estrogenic activity and its impact on *in vivo* responses to exogenously administered estrogens. Environ. Health Perspec. 106:369-373.

(16) OECD (2006) Validation of the Uterotrophic Bioassay in mice by bridging data to rats

(17) Degen GH, Janning P, Diel P, Bolt HM. (2002). Estrogenic isoflavones in rodent diets. Toxicol. Lett. 128:145-157.

(18) Wade MG, Lee A, McMahon A, Cooke G, Curran I. (2003). The influence of dietary isoflavone on the uterotrophic response in juvenile rats. Food Chem. Toxicol. 41:1517-1525.

(19) Yamasaki K, Sawaki M, Noda S, Wada T, Hara T, Takatsuki M. (2002). Immature uterotrophic assay of estrogenic compounds in rats given different phytoestrogen content diets and the ovarian changes in the immature rat uterotrophic of estrogenic compounds with ICI 182,780 or antide. Arch. Toxicol. 76:613-620.

(20) Thigpen JE, Haseman JK, Saunders HE, Setchell KDR, Grant MF, Forsythe D. (2003). Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. Comp. Med. 53:477-485.

(21) Ashby J, Tinwell H, Odum J, Kimber I, Brooks AN, Pate I, Boyle CC. (2000). Diet and the aetiology of temporal advances in human and rodent sexual development. J. Appl. Toxicol. 20:343-347.

(22) Thigpen JE, Lockear J, Haseman J, Saunders HE, Caviness G, Grant MF, Forsythe DB. (2002). Dietary factors affecting uterine weights of immature CD-1 mice used in uterotrophic bioassays. Cancer Detect. Prev. 26:381-393.

(23) Thigpen JE, Li L-A, Richter CB, Lebetkin EH, Jameson CW. (1987). The mouse bioassay for the detection of estrogenic activity in rodent diets: I. A standardized method for conducting the mouse bioassay. Lab. Anim. Sci. 37:596-601.

(24) OECD (2001). Acute oral toxicity – up-and-down procedure. OECD Guideline for the testing of chemicals 425.

(25) 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.

(26) OECD (2001) Guidance document on acute oral toxicity. Environmental Health and Safety Monograph Series on Testing and Assessment No 24. ENV/JM/MONO(2001)4.

(27) Bulbring, E., and Burn, J.H. (1935). The estimation of oestrin and of male hormone in oily solution. J. Physiol. 85: 320 - 333.

(28) Dorfman, R.I., Gallagher, T.F. and Koch, F.C (1936). The nature of the estrogenic substance in human male urine and bull testis. Endocrinology 19: 33 - 41.

(29) Reel, J.R., Lamb IV, J.C. and Neal, B.H. (1996). Survey and assessment of mammalian estrogen biological assays for hazard characterization. Fundam. Appl. Toxicol. 34: 288 - 305.

(30) Jones, R.C. and Edgren, R.A. (1973). The effects of various steroid on the vaginal histology in the rat. Fertil. Steril. 24: 284 – 291.

(31) OECD (1982). Organization for Economic Co-operation and Development - Principles of Good Laboratory Practice, ISBN 92-64-12367-9, Paris.

(32) R.I. Dorfman. Methods in Hormone Research, Vol. II, Part IV: Standard Methods Adopted by Official Organization. New York, Academic Press (1962).

(33) J. E. Thigpen et al. Selecting the appropriate rodent diet for endocrine disruptor research and testing studies. ILAR J 45(4): 401-416 (2004)

(34) L.E. Gray and J. Ostby. Effects of pesticides and toxic substances on behavioral and morphological reproductive development: endocrine versus non-endocrine mechanism. Toixicol Ind Health. 14 (1-2): 159-184 (1998)

(35) Booth AN, Bickoff EM and Kohler GO. 1960. Estrogen-like activity in vegetable oils and mill by-products. Science 131:1807-1808.

(36) Kato H, Iwata T, Katsu Y, Watanabe H, Ohta Y, Iguchi T (2004). Evaluation of estrogenic activity in diets for experimental animals using in vitro assay. J. Agric Food Chem. 52, 1410-1414.