

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Repeated Dose 28-Day Oral Toxicity Study in Rodents

INTRODUCTION

1 OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress. The original Test Guideline 407 was adopted in 1981. In 1995 a revised version was adopted, to obtain additional information from the animal used in the study, in particular on neurotoxicity and immunotoxicity.

2 In 1998, the OECD initiated a high-priority activity, to revise existing Test Guidelines and to develop new Test Guidelines for the screening and testing of potential endocrine disruptors (8). 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 large amount of data thereby obtained has been compiled and evaluated in detail in a comprehensive OECD report (9). This updated Test Guideline 407 is the outcome of the experience and results gained during the international test program. This TG 407 allows certain endocrine mediated effects to be put into context with other toxicological effects.

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 TG is intended to investigate effects on a very broad variety of potential targets of toxicity. It provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time, including effects on the nervous, immune and endocrine systems. Regarding these particular endpoints, it should identify chemicals with neurotoxic potential, which may warrant further in-depth investigation of this aspect, and chemicals that interfere with thyroid physiology. It may also provide data on chemicals that affect the male and/or female reproductive organs in young adult animals and may give an indication of immunological effects.

4 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” (11). The method comprises the basic repeated dose toxicity study that may be 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.

5 The international program conducted on the validation of parameters suitable to potentially detect endocrine activity of test substance 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

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small hormone dependent organs which are difficult to dissect. A guidance on histopathology has been developed (19). It is available on the OECD public website on Test Guidelines. It is intended to assist pathologists in their examinations and help increase the sensitivity of the assay. A variety of parameters were found to be indicative of endocrine-related toxicity and have been incorporated in the TG. Parameters for which insufficient data were available to prove usefulness or which showed only weak evidence in the validation programme of their ability to help in detection of endocrine disrupters are proposed as optional endpoints (see Annex 2).

6 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)oestrogenic modes of action (9). The TG is not performed in a life-stage that is most sensitive to endocrine disruption. The TG nevertheless, during the validation process identified compounds weakly and strongly affecting thyroid function, and strong and moderate endocrine active substances acting through oestrogen or androgen receptors, but in most cases failed to identify endocrine active substances that weakly affect oestrogen or androgen receptors. Thus it can't be described as a screening assay for endocrine activity.

7 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. Regarding endocrine mediated effects, compound characterization should not therefore be based on the results of this TG alone but should be used in a weight of evidence approach incorporating all existing data on a chemical to characterise potential endocrine activity. For this reason, regulatory decision making on endocrine activity (compound characterisation) should be a broadly based approach, not solely reliant on results from application of this TG.

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 where more stringent. Further guidance of the humane treatment of animals is given by the OECD (14).

9 Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

10 The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 28 days. During the period of administration the animals are observed closely, each day for signs of toxicity. Animals that die or are euthanised during the test are necropsied and at the conclusion of the test surviving animals are euthanised and necropsied. A 28 day study provides information on the effects of repeated oral exposure and can indicate the need for further longer term studies. It can also provide information on the selection of concentrations for longer term studies. The data derived from using the TG should allow for the characterization of the test substance toxicity, for an indication of the dose response relationship and the determination of the No-Observed Adverse Effect Level (NOAEL).

DESCRIPTION OF THE METHOD

Selection of animal species

11 The preferred rodent species is the rat, although other rodent species may be used. If the parameters specified within this TG 407 are investigated in another rodent species a detailed justification should be given. Although it is biologically plausible that other species should respond to toxicants in a similar manner to the rat, the use of smaller species may result in increased variability due to technical

challenges of dissecting smaller organs. In the international validation program for the detection of endocrine disrupters, the rat was the only species used. Young healthy adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non pregnant. Dosing should begin as soon as feasible after weaning, and, in any case, before the animals are nine weeks old. 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, it is preferable that animals from the same strain and source should be used in both studies.

Housing and feeding

12 All procedures should conform to local standards of laboratory animal care. The temperature in the experimental animal room should be 22°C ($\pm 3^{\circ}\text{C}$). Although the relative humidity should be at least 30% and preferably not to exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the photoperiod being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals should be group housed in small groups of the same sex; animals may be housed individually if scientifically justified. For group caging, no more than five animals should be housed per cage.

13 The feed should be regularly analysed for contaminants. A sample of the diet should be retained until finalisation of the report.

Preparation of animals

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

Preparation of doses

15 The test compound is administered by gavage or via the diet or drinking water. The method of oral administration is dependent on the purpose of the study, and the physical/chemical/toxico-kinetic properties of the test material.

16 Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/suspension in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known. The stability of the test substance in the vehicle should be determined.

PROCEDURE

Number and sex of animals

17 At least 10 animals (five female and five male) should be used at each dose level. If interim euthanasia are planned, the number should be increased by the number of animals scheduled to be euthanised before the completion of the study. Consideration should be given to an additional satellite group of ten animals (five per sex) in the control and in the top dose group for observation of reversibility, persistence, or delayed occurrence of toxic effects, for at least 14 days post treatment.

Dosage

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

19 Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available for the test compound or related materials. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no-observed-adverse effects at the lowest dose level (NOAEL). Two to four fold intervals are frequently optimal 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 10) between dosages.

20 In the presence of observed general toxicity (e.g. reduced body weight, liver, heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g. reduced food intake, liver enlargement), observed effects on immune, neurological or endocrine sensitive endpoints should be interpreted with caution.

Limit test

21 If a test at one dose level of at least 1000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet, or drinking water (based upon body weight determinations), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

Administration of doses

22 The animals are dosed with test substance daily 7 days each week for a period of 28 days. 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.

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

Observations

24 The observation period should be 28 days. 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.

25 General clinical observations should be made at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily, all animals are observed for morbidity and mortality.

26 Once before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made outside the home cage in a standard arena and preferably at the same time of day on each occasion. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the test conditions are minimal and that observations are preferably conducted by observers unaware of the treatment. 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, 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 (2).

27 In the fourth exposure week sensory reactivity to stimuli of different types (2) (e.g. auditory, visual and proprioceptive stimuli) (3)(4)(5), assessment of grip strength (6) and motor activity assessment (7) should be conducted. Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could be used.

28 Functional observations conducted in the fourth exposure week may be omitted when the study is conducted as a preliminary study to a subsequent subchronic (90-day) study. In that case, the functional observations should be included in this follow-up study. On the other hand, the availability of data on functional observations from the repeated dose study may enhance the ability to select dose levels for a subsequent subchronic study.

29 As an exception, functional observations may also be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with the functional test performance.

30 At necropsy, the oestrus cycle of all females could be determined (optional) by taking vaginal smears. These observations will provide information regarding the stage of oestrus cycle at the time of sacrifice and assist in histological evaluation of estrogen sensitive tissues (see guidance on histopathology (19)).

Body weight and food/water consumption

31 All animals should be weighed at least once a week. Measurements of food consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly.

Haematology

32 The following haematological examinations should be made at the end of the test period: haematocrit, haemoglobin concentrations, erythrocyte count, reticulocytes, total and differential leucocyte count, platelet count and a measure of blood clotting time/potential. Other determinations that should be carried out, if the test substance or its putative metabolites have or are suspected to have oxidising properties include methaemoglobin concentration and Heinz bodies.

33 Blood samples should be taken from a named site just prior to or as part of the procedure for euthanasia of the animals, and stored under appropriate conditions. Animals should be fasted overnight prior to euthanasia ¹.

Clinical biochemistry

34 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 euthanasia of the animals (apart from those found moribund and/or euthanised prior to the termination of the study). Investigations of plasma or 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 glutamate dehydrogenase), and bile acids. Measurements of additional enzymes (of hepatic or other origin) and bilirubin may provide useful information under certain circumstances.

35 Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collection; appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

36 In addition, studies to investigate plasma or 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, and cholinesterase. These need to be identified for chemicals in certain classes or on a case-by-case basis.

37 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 plasma or serum samples to measure T3, T4 and TSH (optional) if there is an indication for an effect on the pituitary-thyroid axis. These samples may be frozen at -20° for storage. 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.

¹ For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable. The major reason for this preference is that the increased variability which would inevitably result from non-fasting, would tend to mask more subtle effects and make interpretation difficult. On the other hand, however, overnight fasting may interfere with the general metabolism of the animals and, particularly in feeding studies, may disturb the daily exposure to the test substance. If overnight fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations in week 4 of the study.

Definitive identification of thyroid-active chemicals is more reliable by histopathological analysis rather than hormone levels.

38 Plasma samples specifically intended for hormone determination should be obtained at a comparable time of the day. 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.

39 If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry variables before dosing commences or preferably in a set of animals not included in the experimental groups.

PATHOLOGY

Gross necropsy

40 All animals in the study shall 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. The liver, kidneys, adrenals, testes, epididymides, prostate + seminal vesicles with coagulating glands as a whole, thymus, spleen, brain and heart of all animals (apart from those found moribund and/or euthanised prior to the termination of the study) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. Care must be exercised when trimming the prostate complex to avoid puncture of the fluid filled seminal vesicles. Alternatively, seminal vesicles and prostate may be trimmed and weighed after fixation.

41 In addition, two other tissues could be optionally weighed as soon as possible after dissection, to avoid drying: paired ovaries (wet weight) and uterus, including cervix (guidance on removal and preparation of the uterine tissues for weight measurement is provided in TG 440 (18)).

42 The thyroid weight (optional) could be determined after fixation. Trimming should also be done very carefully and only after fixation to avoid tissue damage. Tissue damage could compromise histopathology analysis.

43 The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination (see paragraph 47): all gross lesions, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, eye, stomach, small and large intestines (including Peyer's patches), liver, kidneys, adrenals, spleen, heart, thymus, thyroid, trachea and lungs (preserved by inflation with fixative and then immersion), gonads (testis and ovaries), accessory sex organs (uterus and cervix, epididymides, prostate + seminal vesicles with coagulating glands), vagina, urinary bladder, lymph nodes (besides the most proximal draining node another lymph node should be taken according to the laboratory's experience (15)), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, skeletal muscle and bone, with bone marrow (section or, alternatively, a fresh mounted bone marrow aspirate). It is recommended that testes be fixed by immersion in Bouin's or modified Davidson's fixative (16) (17). The tunica albuginea must be gently and shallowly punctured at the both poles of the organ with a needle to permit rapid penetration of the fixative. 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 substance should be preserved.

44 The following tissues may give valuable indication for endocrine-related effects: Gonads (ovaries and testes), accessory sex organs (uterus including cervix, epididymides, seminal vesicles with coagulation glands, dorsolateral and ventral prostate), vagina, pituitary, male mammary gland, the thyroid and adrenal gland. Changes in male mammary glands have not been sufficiently documented but this parameter may be very sensitive to substances with estrogenic action. Observation of organs/tissues that are not listed in paragraph 43 is optional (see Annex 2).

45 The Guidance on histopathology (19) details extra information on dissection, fixation, sectioning and histopathology of endocrine tissues.

46 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 evidence of possible asynchrony of the oestrus cycle should be taken into account in interpretation of the histopathology of the ovaries (follicular, thecal, and granulosa cells), uterus, cervix and vagina. If assessed, the stage of cycle as determined by vaginal smears could be included in this comparison as well.

Histopathology

47 Full histopathology should be carried out on the preserved organs and tissues of all animals in the control and high dose groups. These examinations should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group.

48 All gross lesions shall be examined.

49 When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

DATA AND REPORTING

Data

50 Individual data should be provided. 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 euthanised for humane reasons and the time of any death or euthanasia, 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, their severity and the percentage of animals displaying each type of lesion.

51 When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. Comparisons of the effect along a dose range should avoid the use of multiple t-tests. The statistical methods should be selected during the design of the study.

52 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 parameters linked with endocrine disrupter detection. These data can be used for comparison purposes when actual studies are evaluated.

Test report

53 The test report must include the following information:

Test substance:

- physical nature, purity and physicochemical properties;
- identification data.

Vehicle (if appropriate):

- justification for choice of vehicle, if other than water.

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.
- justification for species if not rat

Test conditions:

- rationale for dose level selection;
- details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the test substance;
- conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of food and water quality.

Optional endpoints investigated

- list of optional endpoints investigated

Results:

- body weight/body weight changes;
- food consumption, and water consumption, if applicable;
- toxic response data by sex and dose level, including signs of toxicity;
- nature, severity and duration of clinical observations (whether reversible or not);
- sensory activity, grip strength and motor activity assessments;
- haematological tests with relevant base-line values;
- clinical biochemistry tests with relevant base-line values;
- body weight at euthanasia and organ weight data;
- necropsy findings;
- a detailed description of all histopathological findings;
- absorption data if available;
- statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

ANNEX 1

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.

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.

ANNEX 2

Endpoints recommended for the detection of endocrine disruptors (EDs) in TG 407

Mandatory endpoints	Optional endpoints
Weight	
<ul style="list-style-type: none"> - Testes - Epididymides - Adrenals - Prostate + seminal vesicles with coagulating glands 	<ul style="list-style-type: none"> - Ovaries - Uterus, including cervix - Thyroid
Histopathology	
<ul style="list-style-type: none"> - Gonads: <ul style="list-style-type: none"> - Testes and - Ovaries - Accessory sex organs : <ul style="list-style-type: none"> - Epididymides, - Prostate + seminal vesicle with coagulating glands - Uterus, including cervix - Adrenal - Thyroid - Vagina 	<ul style="list-style-type: none"> - Vaginal smears - Male mammary glands - Pituitary
Hormones measurement	
	<ul style="list-style-type: none"> - Circulating levels of T3, T4 - Circulating levels of TSH

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