B.33 COMBINED CHRONIC TOXICITY /CARCINOGENICITY TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The objective of a combined chronic toxicity carcinogenicity test is to determine the chronic and carcinogenic effects of a substance in a mammalian species following prolonged exposure.

To this end a carcinogenicity test is supplemented with a least one treated satellite group and a control satellite group. The dose used for the high-dose satellite group may be higher than that used for the high-dose group in the carcinogenicity test. The animals in the carcinogenicity test are examined for general toxicity as well as for carcinogenic response. The animals in the treated satellite group are examined for general toxicity.

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their lifespan. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity and the development of tumours.

1.5. Quality criteria

None.

1.6. Description of the test method

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

Experimental animals

The preferred species is the rat. Based upon the results of previously conducted tests other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencment of the test the weight variation in the animals used should not exceed ± 20% of the mean value. Where a sub-chronic oral test is conducted as a preliminary to a long-term test, the same species and breed/strain should be used in both studies.

Number and sex

For rodents, at least 100 animals (50 female and 50 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

The treated satellite group(s) for the evaluation of pathology other than tumours should contain 20 animals of each sex, while the satellite control group should contain 10 animals of each sex.

Dose levels and frequency of exposure

For carcinogenicity testing purposes, at least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit signs of minimal toxicity, such as a slight depression of body weight gain (less than 10%), without substantially altering the normal lifespan due to effects other than tumours.

The lowest dose level should not interfere with normal growth, development and longevity of the animal or produce any indication of toxicity. In general, this should not be lower than 10% of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

For chronic toxicity testing purposes, additional treated groups and a concurrent control satellite group are included in the test. The high dose for treated satellite animals should produce definite signs of toxicity.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet, it should be continuously available.

Controls

A concurrent group which is identical in every respect to the treated groups, except for exposure to the test substance; should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, an additional control group which is not exposed to the vehicle should be utilized.

Route of administration

The three main routes of administration are oral, dermal and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

Oral tests

Where the test substance is absorbed from the gastro-intestinal tract and the ingestion route is one by which humans may be exposed, the oral route of administration is preferred, unless there are contra-indications. The animals may receive the test substance in their diet, dissolved in drinking water or given by capsule. Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

Dermal tests

Cutaneous exposure by skin painting may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions.

Inhalation tests

Because inhalation tests present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chambers. In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that, with the former, there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure, with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the test and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure and by the need for more complicated (and reliable) aerosol and vapour generation and monitoring techniques.

Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5% of the volume of the chamber.

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should perferably be monitored continuously;

(ii) Concentration: during the daily exposure period the concentration should not vary more than ± 15% of the mean value. During the total duration of this study, the day-to-day concentrations should be held as constant as practicable;

(iii) Temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2 °C, and the humidity within the chamber at 30 to 70%, except when water is used to suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;

(iv) Particle size measurements: particle size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

Duration of test

The duration of the carcinogenicity part of the test comprises the major portion of the normal life span of the test animals. The termination of the test should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumour rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. Alternatively, termination of such an extended test is acceptable when the number of survivors in the lowest dose or control group reaches 25%. When terminating a test in which there is an apparent sex difference in response, each sex should be considered separately. Where only the high-dose group dies prematurely for obvious reasons of toxicity, this need not trigger termination providing toxic manifestations are not causing problems in the other groups. For a negative test result to be acceptable not more than 10% of any group may be lost from the experiment due to autolysis, cannibalism or management problems, and the survival of all groups is not less than 50 at 18 months fotmice an hamsters and at 24 months for rats.

The satellite groups of 20 dosed animals per sex and 10 associated control animals per sex used for chronic toxicity testing should be retained in the test for at least 12 months. These animals should be scheduled for sacrifice for an examination of test-substance-related pathology uncomplicated by gerontological changes.

Procedure

Observations

Daily cageside observations should be made and should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern.

Clinical examination should be performed at appropriate intervals on animals in the treated satellite group(s).

Regular observations of the animals is necessary to ensure, as far as possible, that animals are not lost from the test due to causes such as cannibalism, autolysis of tissues or misplacment. Moribund animals should be removed and necropsied when noticed.

Clinical signs, including neurological and ocular changes as well as mortality should be recorded for all animals. Special attention must be paid to tumour development: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded; the time of onset and progression of toxic conditions should be recorded.

Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) weekly during the first 13 weeks of the study and then at approximately three-month intervals unless health status or body weight changes dictate otherwise.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

Clinical examinations

Haematology

Haematological examination (e.g. haemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at three months, six months and at approximately six-month intervals thereafter, and at termination on blood samples collected from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval.

If cageside observations suggest a deterioration in the health of the animals during the study, a differential blood count of the affected animals should be performed.

A differential blood count is performed on samples of those animals in the highest dose group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls, or if indicated by pathological findings.

Urinalysis

Urine samples from 10 rats/sex of all groups, if possible from the same rats at the same intervals as haematological examination, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group of rodents:

-appearance: volume and density for individual animals,
-protein, glucose, ketones, occult blood (semi-quantitatively),
-microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals, and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

-total protein concentration,
-albumin concentration,
-liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase ([1]) activity and glutamic oxaloacetic transaminase ([2]) activity), gamma glutamyl transpeptidase, ornithine decarboxylase,

([1]) Now known as serum alanine aminotransferase.
([2]) Now known as serum aspartate aminotransferase.

-carbohydrate metabolism such as fasting blood glucose,
-kidney function tests such as blood urea nitrogen.

Gross necropsy

Full gross necropsy should be performed in all animals, including those which died during the experiment or were sacrificed having been found in a moribund condition. Prior to sacrifice, samples of blood should be collected from all animals for differential blood counts. All grossly visible tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microsopic findings.

All organs and tissues should be preserved for histopathological examination. This usually concerns the following organs and tissues: brain ([3]) (medulla/pons, cerebellar cortex, cerebral cortex); pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver ([1]), spleen, kidneys ([1]), adrenals ([1]), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, lymph nodes, pancreas, gonads ([1]), accessory genital organs; female mammary gland, skin, musculature, peripheral nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes.

Although inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues, inflation of the lungs in inhalation studies is a necessary requirement for appropriate histopathological examination. In special studies such as inhalation studies, the entire respiratory tract should be studied, including nose, pharynx and larynx.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microsopic examination, because it may give significant guidance to the pathologist.

Histopathology

For the chronic toxicity testing portion:

Detailed examination should be made of all preserved organs of all animals of the satellite high-dose and control groups. Where test-substance-related pathology is found in the high-dose satellite group, target organs of all other animals in any other treated satellite group should be subjected to full and detailed histological examination as well as those of the heated groups in the carcinogenicity testing portion of the study at its termination.

For the carcinogenicity testing portion:

(a) Full histopathology should be carried out on the organs and tissues of all animals that died or were sacrificed during the test, and of all animals in the control and high-dose groups;

(b) All grossly visible tumours or lesions suspected of being tumours in all groups occurring in any organ should be examined microscopically;

(c) If there is a significant difference in the incidence of neoplastic lesions in the high-dose and control groups, histopathology should be carried out on that particular organ or tissue in the orther groups;

(d) If the survival of the high-dose group is substantially less than the control then the next-lower dose group should be examined fully;

(e) If there is evidence in the high-dose group of the induction of toxic or other effects that might affect a neoplastic response, the next-lower dose level should be examined fully.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing tumours or toxic effects detected during the test, the time of detection and the number of animals found to have tumours following sacrifice. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

([3]) These organs, from 10 animals per sex per groups for rodents, should be weighed.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

-species, strain source, environmental conditions, diet,

-test conditions:

Description of exposure apparatus:
including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

Exposure data:
These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation), and should include:

(a) air flow rates through the inhalation equipment;
(b) temperature and humidity of air;
(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);
(d) nature of vehicle, if used;
(e) actual concentrations in test breathing zone;
(f) median particle sizes (where appropriate),

-dose levels (including vehicle, if used) and concentrations,

-tumour incidence data by sex, dose and tumour type;

-time of death during the study or whether animals survived to termination, including satellite group,

-toxic response data by sex and dose,

-description of toxic or other effects,

-the time of observation of each abnormal sign and its subsequent course,

-ophthalmological findings,

-food and bodyweight data,

-haematological tests employed and all results,

-clinical biochemistry test employed and all results (including any urinalysis),

-necropsy findings,

-a detailed description of all histopathological findings,

-statistical treatment of results with a description of the methods used,

-discussion of the results,

-interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.