ACUTE ORAL SAFETY LIMIT STUDY IN RATS AND MICE

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1 INTRODUCTION

Since the genetic material introduced into recombinant DNA plants may be derived from organisms (including microorganisms) that have not previously been present in the human diet to any great extent, the corresponding gene products are considered to be novel with respect to human consumption. Therefore, the principal focus of the toxicological assessment of foods derived from recombinant DNA plants is on assessing the potential toxicity of the protein expression product(s) of the inserted gene(s). For protein products that have a history of significant human dietary exposure, acute toxicity testing is not warranted.

Because proteins exhibiting toxicity generally exert their effect at low dosages and in a short time frame, acute toxicity tests have been considered adequate for evaluating potential toxicity (Jones and Maryanski, 1991; EPA, 2000; NRC, 2000). As indicated by Sjoblad et al. (1992), “if toxicity testing of a protein is considered necessary then acute exposure studies in laboratory animals should be sufficient, since – if toxic – proteins are known to act via acute mechanisms.” Therefore, when a protein demonstrates no acute oral toxicity in high-dose testing using a standard laboratory mammalian test species, this supports the determination that the protein will be nontoxic to humans and other mammals, and will not present a hazard under any realistic exposure scenario, including long-term exposure.

Among the various routes of exposure to potential toxicants, the oral route is the most relevant for food safety assessment. Oral exposure is usually accomplished by gastric gavage, wherein a tube is inserted through the oral cavity and the esophagus of the test animal and the test substance is injected directly into the stomach. An evaluation of acute toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

In the assessment of the toxic characteristics of a protein, the determination of oral toxicity is routinely carried out by acute testing. Such studies provide information on the possible health hazards likely to arise from dietary exposure to a novel protein. This method comprises the basic single dose toxicity study that is commonly used for proteins for which low toxicity is expected due to prior knowledge of the source and previous exposure. The duration of post exposure observation is 14 days. Lack of mortality, moribundity or evident toxicity is generally interpreted as a lack of oral toxicity associated with the test substance.

The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physiochemical properties; the results of any other in vitro or in vivo toxicity tests on the substance; toxicological data on structurally related substances; the anticipated use(s) of the substance; and the likely dietary exposure to the substance. This information is necessary to satisfy all concerned that the test is relevant for the protection of human health, and will help in the selection of an appropriate starting dose.
In experimental studies that involve procedures that could cause clinical symptoms or morbidity in animals, consideration must be given to the selection of the most appropriate endpoint(s). This requires careful consideration of the scientific requirements of the study, the expected and possible adverse effects the research animals may experience (pain, distress, illness, etc.), the most likely time course and progression of those adverse effects, and the earliest most predictive indicators of present or impending adverse effects. The effective use of endpoints requires that properly qualified individuals perform both general and study-specific observations of the research animals at appropriate time points. Optimally, studies are terminated when animals begin to exhibit clinical signs of toxicity if this endpoint is compatible with meeting the research objectives. Such endpoints are preferable to death or morbidity since they minimize pain and distress. Efforts must be made to minimize pain and distress experienced by animals used in research.

Unlike the situation for chemicals, there are no internationally recognized protocols that deal specifically with assessing the potential oral toxicity of proteinaceous substances. In preparing this test protocol, general guidance was drawn from existing practice internationally with respect to acute oral toxicity testing of isolated proteins and other guidance for toxicity testing of chemical substances, including OECD Test Guideline 420 Acute Oral Toxicity – Fixed Dose procedure and the US-EPA OPTTS 870.1100 Health Effects Test Guideline. This test protocol is intended for use in the testing of novel proteins expressed in recombinant DNA plants and foods/products derived from these, and the development of test data that must be submitted to RCGM and/or GEAC as the case may be, for seeking approval under Rules, 1989, of the Environmental Protection Act, 1986.

Following is one of a series of test protocols for use in the testing of novel proteins expressed in recombinant DNA plants and foods derived from these, and the development of test data that must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under Rules, 1989, of the Environmental Protection Act, 1986.

The source materials used in developing this protocol include the OECD Test Guideline 420 Acute Oral Toxicity – Fixed Dose procedure and the US-EPA OPTTS 870.1100 Health Effects Test Guideline.

2 DEFINITIONS

Relevant definitions to this test protocol are as follows:

2.1 ACUTE ORAL TOXICITY

Refers to those adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours.
2.2 **DELAYED DEATH**

Means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.

2.3 **DOSE**

Is the amount of test substance administered and is expressed as weight of test substance per unit weight of test animal (*e.g.*, mg/kg).

2.4 **EVIDENT TOXICITY**

Is a general term describing clear signs of toxicity following the administration of test substance, such that at the next highest fixed dose either severe pain and enduring signs of severe distress, moribund status (criteria are presented in the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation), or probable mortality in most animals can be expected.

Clinical signs that may be indicative of toxicity include, but are not limited to: rapid weight loss; diarrhea (if debilitating); progressive dermatitis; rough hair coat; hunched posture; lethargy or persistent recumbency; labored breathing; nasal discharge; jaundice or anemia; neurological signs; bleeding from any orifice; self-induced trauma; any condition interfering with eating or drinking (*e.g.*, difficulty moving); or excessive or prolonged hyperthermia or hypothermia.

2.5 **GENETICALLY ENGINEERED (GE) PLANT**

A plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant-deoxyribonucleic acid (r-DNA) and direct injection of nucleic acid into cells or organelles.

2.6 **LD50**

Median lethal oral dose is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

2.7 **LIMIT DOSE**

Refers to a dose at an upper limitation on testing (*e.g.*, limit dose of 2000 mg/kg body weight or when this cannot be achieved in the recommended volume for administration, the dose used should be the maximum possible based on the solubility of the protein).
2.8 **Moribund Status**

Being in a state of dying or inability to survive, even if treated.

3 **Principle of the Test**

This test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, *i.e.*, having toxicity only above a regulatory limit dose.

Information about the toxicity of a protein can be gained from knowledge about similar tested proteins, taking into consideration the source organism and history of prior use. When prior data suggest the protein has low toxicity, a limit test using a single dose equivalent to at least 10X the estimated dietary exposure of the test protein may be administered to a single group of five male and/or five female animals using the procedures described under section 4 of this guideline.

In situations where data on plant-expressed protein concentration and/or potential consumption are inadequate to predict a realistic estimated dietary exposure for the target protein, a limit dose of 2000 mg/kg should be employed. On a case-by-case basis, a limit dose less than 2000 mg/kg may be justified either on the basis of valid scientific rationale or on the basis of practical considerations, such as limits of solubility of the test protein. Where there is limited solubility of the test material, the highest dose that can be practically administered in the maximum recommended volume is used. If treatment-related mortality, morbidity or clinical symptoms result, then further study may have to be considered for ascertaining the cause of toxicity.

4 **Description of the Method**

4.1 **Selection of Animal Species**

The preferred rodent species include the rat and the mouse. Commonly used laboratory strains of young healthy adult animals, male and female in equal numbers (*e.g.*, five per sex), should be employed. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are 9 weeks old. At the commencement of the study the weight variation of animals used should be minimal and not exceed ±20% of the mean weight of each sex.

4.2 **Accommodation and Husbandry**

The temperature of the experimental animal room should be 22°C (±3°C). Relative humidity should be maintained between 50–60%, and in any event should be at least 30% and not greater than 70%, except during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with drinking water supplied *ad libitum*. Animals may be housed individually, or be caged in
small groups of the same sex; for group caging, no more than five animals should be housed per cage.

4.3 Preparation of Animals

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 (i.e., via ear punch) and acclimatized for at least 5 days in their cages prior to the start of the study.

4.4 Test Protein Dose Preparation

It is recommended that, wherever possible, the use of an aqueous solution/suspension of the test protein be considered first, followed by consideration of a solution/emulsion in oil (e.g., corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known.

The maximum volume of liquid that can be administered at one time depends on the size of the test animal and must not be exceeded. In rodents, this volume should not normally exceed 10 ml/kg of body weight: however in the case of aqueous solutions 20–25 ml/kg body weight can be considered.

Where the limit dose of 2000 mg/kg body weight cannot be achieved in the recommended volume for administration, the dose used should be the maximum possible based on the solubility of the protein.

Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

4.5 Test Protein Dose Administration

The test protein is administered in a single dose by gavage using a stomach tube or a suitable intubation canula.

In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a maximum period of 12 hours normally and in any case not exceeding 24 hours.

Animals should be fasted prior to dosing (e.g. with the rat, food but not water should be withheld overnight; with the mouse, food but not water should be withheld for 3-4 hours). Following the period of fasting, the animals should be weighed and the test substance administered. After the substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice.
Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period.

### 4.6 OBSERVATIONS

#### 4.6.1 Visual

Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and euthanized for animal welfare reasons or are found dead. All observations are systematically recorded, with individual records being maintained for each animal.

Additional observations will be necessary if animals display signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be euthanized. When animals are euthanized for humane reasons or found dead, the time of death should be recorded as precisely as possible.

#### 4.6.2 Body Weight and Feed Consumption

Individual weights of animals should be determined shortly before the test substance is administered (Day 0) and on Days 7 and 14. Measurements of feed consumption should be made at least weekly.

#### 4.6.3 Pathology

All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross morphological changes should be recorded for each animal. In the event that gross morphological changes are observed, the relevant tissues should be subject to histopathological examination.

### 4.7 DATA AND REPORTING

Individual animal data should be provided. Additionally, all data should be summarized in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or euthanized for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings. When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

The test report must include the following information, as appropriate:
4.7.1 Test protein

Physical state, purity, concentration, source, batch/lot reference number, and storage conditions. When the test protein has been isolated from a source other than the GE plant, a characterization of the test protein and demonstration of equivalence with the plant-expressed form of the protein is required (normally as a separate study and report).

4.7.2 Control substance and vehicle

Identification of the vehicle (e.g., water, 10% aqueous carboxymethyl cellulose, etc), and justification for choice of vehicle substance if other than water.

4.7.3 Test animals

Species and strain used, including: source of animals; number; age and sex (including, where appropriate, a rationale for use of males instead of females); accommodation conditions; and diet.

4.7.4 Test conditions

- Details of test substance formulation, including details of the physical form of the material administered.
- Details of the administration of the test substance including dosing volumes and time of dosing;
- Details of food and water quality (including diet type/source, water source); and
- The rationale for the selection of the starting dose.

4.7.5 Results

- Tabulation of response data and dose level for each animal (i.e. animals showing signs of toxicity including mortality, nature, severity and duration of effects);
- Tabulation of body weight and body weight changes;
- Individual weights of animals at the day of dosing, in weekly intervals thereafter, and at time of death or sacrifice;
- Date and time of death if prior to scheduled sacrifice;
- Time course of onset of signs of toxicity and whether these were reversible for each animal; and
- Necropsy findings and histopathological findings for each animal, if available.

4.7.6 Discussion and Interpretation of Results.

The significance and likely impacts of any abnormal findings should be discussed. Where there are statistically significant differences in parameters (e.g., body weight) between test and control groups, these should be discussed in terms of their biological significance and impact on safety. The need, or not, of any additional or follow up studies should be discussed.
5 LITERATURE


