B. 37. DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES FOLLOWING ACUTE EXPOSURE

1. METHOD

1.1 Introduction

In the assessment and evaluation of the toxic effects of substances, it is important to consider the potential of certain classes of substances to cause specific types of neurotoxicity that might not be detected in other toxicity studies. Certain organophosphorus substances have been observed to cause delayed neurotoxicity and should be considered as candidates for evaluation.

In vitro screening tests could be employed to identify those substances which may cause delayed polyneuropathy; however, negative findings from *in vitro* studies do not provide evidence that the test substance is not a neurotoxicant.

See General Introduction Part B.

1.2 Definitions

Organophopsphorus substances include uncharged organophosphorus esters, thioesters or anhydrides of organophosphoric, organophosphonic or organophosphoramidic acids or of related phosphorothioic, phosphonothioic or phosphorothioamidic acids, or other substances that may cause the delayed neurotoxicity sometimes seen in this class of substances.

Delayed neurotoxicity is a syndrome associated with prolonged delayed onset of ataxia, distal axonopathies in spinal cord and peripheral nerve, and inhibition and aging of neuropathy target esterase (NTE) in neural tissue.

1.3 Reference substances

A reference substance may be tested with a positive control group as a means of demonstrating that under the laboratory test conditions, the response of the tested species has not changed significantly.

An example of a widely used neurotoxicant is tri–o–tolyl phosphate (CAS 78-30-8, EINECS 201-103-5, CAS nomenclature : phosphoric acid, tris(2–methylphenyl)ester), also known as tris–o–cresylphosphate.

1.4 Principle of the test method

The test substance is administered orally in a single dose to domestic hens which have been protected from acute cholinergic effects, when appropriate. The animals are observed for 21 days for behavioural abnormalities, ataxia, and paralysis. Biochemical measurements, in particular neuropathy target esterase inhibition (NTE), are undertaken on hens randomly selected from each group, normally 24 and 48 hours after dosing. Twenty-one days after exposure, the remainder of the hens are killed and histopathological examination of selected neural tissues is undertaken.

1.5 Description of the test method

1.5.1 *Preparations*

Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be randomized and assigned to treatment and control groups and acclimatized to the laboratory conditions for at least 5 days prior to the start of the study.

Cages or enclosures which are large enough to permit free mobility of the hens, and easy observation of gait should be used.

Dosing with the test substance should normally be by the oral route using gavage, gelatine capsules, or a comparable method. Liquids may be given undiluted or dissolved in an appropriate vehicle such as corn oil; solids should be dissolved if possible since large doses of solids in gelatine capsules may not be absorbed efficiently. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

1.5.2 ***Test conditions***

1.5.2.1 Test animals

The young adult domestic laying hen (*Gallus gallus domestícus*), aged 8 to 12 months, is recommended. Standard size breeds and strains should be employed and the hens normally should have been reared under conditions which permitted free mobility.

1.5.2.2 Number and sex

In addition to the treatment group, both a vehicle control group and a positive control group should be used. The vehicle control group should be treated in a manner identical to the treatment group, except that administration of the test substance is omitted.

Sufficient number of hens should be utilized in each group of birds so that at least six birds can be killed for biochemical determination (three at each of two time points) and six can survive the 21 day observation period for pathology.

The positive control group may be run concurrently or be a recent historical control group. It should contain at least six hens, treated with a known delayed neurotoxicant, three hens for biochemistry and three hens for pathology. Periodic updating of historical data is recommended. New positive control data should be developed when some essential element (e.g. strain, feed, housing conditions) of the conduct of the test has been changed by the performing laboratory.

1.5.2.3 Dose levels

A preliminary study using an appropriate number of hens and dose levels groups should be performed to establish the level to be used in the main study. Some lethality is typically necessary in this preliminary study to define an adequate main study dose. However, to prevent death due to acute cholinergic effects, atropine or another protective agent, known to not interfere with delayed neurotoxic responses, may be used. A variety of test methods may be used to estimate the maximum non-lethal dose of test substances (See method B.1bis). Historical data in the hen or other toxicological information may also be helpful in dose selection.

The dose level of the test substance in the main study should be as high as possible taking into account the results of the preliminary dose selection study and the upper limit dose of 2,000 mg/kg body weight. Any mortality which might occur should not interfere with the survival of sufficient animals for biochemistry (six) and histology (six) at 21 days. Atropine or another protective agent, known to not interfere with delayed neurotoxic responses, should be used to prevent death due to acute cholinergic effects.

1.5.2.4 Limit test

If a test at a dose level of at least 2,000 mg/kg body weight/day, 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 substances, then a study using a higher dose may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

1.5.2.5 Observation period

Observation period should be 21 days.

1.5.3 *Procedure*

After administration of a protective agent to prevent death due to acute cholinergic effect, the test substance is administered in a single dose.

1.5.3.1 General observation

Observations should start immediately after exposure. All hens should be carefully observed several times during the first 2 days and thereafter at least once daily for a period of 21 days or until scheduled kill. All signs of toxicity should be recorded, including the time of onset, type, severity and duration of behavioural abnormalities. Ataxia should be measured on an ordinal grading scale consisting of at least four levels, and paralysis should be noted. At least twice a week the hens selected for pathology should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to facilitate the observation of minimal toxic effects. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

1.5.3.2 Body weight

All hens should be weighed just prior to administration of the test substance and at least once a week thereafter.

1.5.3.3 Biochemistry

Six hens randomly selected from each of the treatment and vehicle control groups, and three hens from the positive control group (when this group is run concurrently), should be killed within a few days after dosing, and the brain and lumbar spinal cord prepared and assayed for neuropathy target esterase inhibition activity. In addition, it may also be useful to prepare and assay sciatic nerve tissue for neuropathy target esterase inhibition activity. Normally, three birds of the control and each treatment group are killed after 24 hours and three at 48 hours, whereas the three hens of the positive controls should be killed at 24 hours. If observation of clinical signs of intoxication (this can often be assessed by observation of the time of onset of cholinergic signs) indicates that the toxic agent may be disposed of very slowly then it may be preferable to sample tissue from three birds at each of two times between 24 and as late as 72 hours after dosing.

Analyses of acetylcholinesterase (AChE) may also be performed on these samples, if deemed appropriate. However, spontaneous reactivation of AChE may occur *in vivo*, and so lead to underestimation of the potency of the substance as an AChE inhibitor.

1.5.3.4 Gross necropsy

Gross necropsy of all animals (scheduled killed and killed when moribund) should include observation of the appearance of the brain and spinal cord.

1.5.3.5 Histopathological examination

Neural tissue from animals surviving the observation period and not used for biochemical studies should be subjected to microscopic examination. Tissues should be fixed *in situ*, using perfusion techniques. Sections should include cerebellum (mid-longitudinal level), medulla oblongata, spinal cord, and peripheral nerves. The spinal cord sections should be taken from the upper cervical segment, the mid-thoracic and the lumbo-sacral regions. Sections of the distal region of the tibial nerve and its branches to the gastrocnemial muscle and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains.

2. DATA

Negative results on the endpoints selected in this method (biochemistry, histopathology and behavioural observation) would not normally require further testing for delayed neurotoxicity. Equivocal or inconclusive results for these endpoints may require further evaluation.

Individual data should be provided. Additionally, all 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 lesions, behavioural or biochemical effects, the types and severity of these lesions or effects, and the percentage of animals displaying each type and severity of lesion or effect.

The findings of this study should be evaluated in terms of the incidence, severity, and correlation of behavioural, biochemical and histopathological effects and any other observed effects in the treated and control groups.

Numerical results should be evaluated by appropriate and generally acceptable statistical methods. The statistical methods used should be selected during the design of the study.

3. REPORTING

Test report

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

Test animals :

— strain used;

— number and age of animals;

— source, housing conditions, etc.;

— individual weights of animals at the start of the test.

Test conditions :

— details of test substance preparation, stability and homogeneity, where appropriate;

— justification for choice of vehicle;

— details of the administration of the test substance;

— details of food and water quality;

— rationale for dose selection;

— specification of doses administered, including details of the vehicle, volume and physical form of the material administered;

— identity and details of the administration of any protective agent.

Results :

— body weight data;

— toxic response data by group, including mortality;

— nature, severity and duration of clinic observations (whether reversible or not);

— a detailed description of biochemical methods and findings;

— necropsy findings;

— a detailed description of all histopathological findings;

— statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

4. REFERENCES

This method is analogius to OECD TG 418.