B. 38. DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES 28 DAY REPEATED DOSE STUDY

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.

This 28-day delayed neurotoxicity test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information on dose response and can provide an estimate of a no-observed-adverse effect level which can be of use for establishing safety criteria for exposure.

See also General Introduction Part B.

1.2 Definitions

Organophosphorus 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 ageing of neuropathy target esterase (NTE) in neural tissue.

1.3 Principle of the test method

Daily doses of the test substance are administered orally to domestic hens for 28 days. The animals are observed at least daily for behavioural abnormalities, ataxia and paralysis until 14 days after the last dose. Biochemical measurements, in particular neuropathy target esterase inhibition (NTE), are undertaken, on hens randomly selected from each group, normally 24 and 48 hours after the last dose. Two weeks after the last dose, the remainder of the hens are killed and histopathological examination of selected neural tissues is undertaken.

1.4 Description of the test method

1.4.1 *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.

Oral dosing each day, 7 days per week, should be carried out, preferably by gavage or administration of gelatine capsules. 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.

1.4.2 *Test conditions*

1.4.2.1 Test animals

The young adult domestic laying hen (Gallus gallus domesticus), 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.

1.4.2.2 Number and sex

Generally at least three treatment groups and a vehicle 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 determinations (three at each of two timepoints) and six birds can survive the 14-day post-treatment observation period for pathology.

1.4.2.3 Dose levels

Dose levels should be selected taking into account the results from an acute test on delayed neurotoxicity and any other existing toxicity or kinetic data available for the test compound. The highest dose level should be chosen with the aim of inducing toxic effects, preferably delayed neurotoxicity, but not death nor obvious suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrate any dose-related response and no-observed-adverse effects at the lowest dose level.

1.4.2.4 Limit test

If a test at a dose level of at least 1000 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 expected human exposure indicates the need for a higher dose level to be used.

1.4.2.5 Observation period

All the animals should be observed at least daily during the exposure period and 14 days after, unless scheduled necropsy.

1.4.3 *Procedure*

Animals are dosed with the test substance on seven days per week for a period of 28 days.

1.4.3.1 General observations

Observations should start immediately after treatment begins. All hens should be carefully observed at least once daily on each of the 28 days of treatment, and for 14 days after dosing or until scheduled kill. All signs of toxicity should be recorded including their time of onset, type, severity and duration. Observations should include, but not be limited to, 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 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 in severe distress or pain should be removed when noticed, humanely killed and necropsied.

1.4.3.2 Body weight

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

1.4.3.3 Biochemistry

Six hens randomly selected from each of the treatment and vehicle control groups should be killed within a few days after the last dose, and the brain and lumbar spinal cord prepared and assayed for neuropathy target esterase (NTE) inhibition activity. In addition, it may also be useful to prepare and assay sciatic nerve tissue for neuropathy target esterase (NTE) inhibition activity. Normally, three birds of the control and each treatment group are killed after 24 hours and three at 48 hours after the last dose. If data from the acute study or other studies (e.g. toxicokinetics) indicate that other times of killing after final dosing are preferable then these times should be used and the rationale documented.

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.

1.4.3.4 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.

1.4.3.5 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. Initially, microscopic examination should be carried out on the preserved tissues of all animals in the control and high dose group. When there is evidence of effects in the high dose group, microscopic examination should also be carried out in hens from the intermediate and low dose groups.

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 each of the treated and control groups.

Numerical results should be evaluated by appropriate and generally acceptable statistical methods. The statistical methods 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;

— rationale for choosing other times for biochemical determination, if other than 24 and 48 h.

Results :

— body weight data;

— toxic response data by dose level, including mortality;

— no-observed adverse effect level;

— 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 analogous to OECD TG 419.