OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 27th July 1995

Delayed Neurotoxicity of Organophosphorus Substances: 28-day Repeated Dose Study

INTRODUCTION

1. OECD Guidelines are periodically reviewed in light of scientific progress and in consideration of animal welfare. This updated guideline uses revised methods including measurement of neuropathy target esterase inhibition (NTE; formerly neurotoxic esterase) for determination of the effects of the test substance (1)(2)(3). Inhibition of NTE in brain and spinal cord within 24-48 hours after dosing correlates well with the clinical and morphological effects of delayed neurotoxicity. The NTE test model was found to be valid for all organophosphorus esters known to cause delayed neuropathy in man (4). Therefore, quantitative data on NTE inhibition will significantly improve the ability to determine whether ambiguous results sometimes seen in behavioral or histopathological data should be considered to indicate a potential delayed neurotoxicant as well as its no-observed-adverse effect level (1)(2)(3)(4). This updated guideline now requires at least 28 days of exposure in comparison to the previous version which required 90 days of exposure. This duration should generally be sufficient to characterise the repeated dose exposure potency of these organophosphorus substances (4)(5). For many pesticides, 28 days may also be closer to the actual exposure duration of applicators than 90 days.

2. This updated version of Guideline 419 resulted from a Consultation Meeting of the *ad hoc* Working Group of Experts on Systemic Short-term and (Delayed) Neurotoxicity, held in Paris in February 1992 (6). It is further based on an earlier proposal, discussed at the OECD ad hoc Meeting on Neurotoxicity Testing, held outside Washington in March 1990 (7), and the comments received on that proposal from Member countries.

INITIAL CONSIDERATIONS

3. This Guideline is used in the assessment and evaluation of the toxic effects of organophosphorus substances. Usually the delayed neuropathic potential of repeated exposures is determined after acute tests (Guideline 418). Information from acute tests for delayed neurotoxicity and other tests may be useful in planning repeated exposure studies (8)(9)(10).

4. 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 (4).

5. Based on the use patterns of some substances (e.g. plasticizers), longer periods of exposure, i.e. 90 days, may be needed. In some cases, however, further study may be required to resolve data that may be difficult to interpret, or to more clearly characterize the use pattern of a particular substance.

6. Definitions used are given in the Annex.

PRINCIPLE OF THE TEST

7. Daily doses of the test substance are administered orally to domestic hens for 28 days. The animals are observed at least daily for behavioral abnormalities, ataxia, and paralysis, until 14 days after the last dose. Biochemical measurements, in particular NTE (1)(2)(3), 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.

DESCRIPTION OF THE METHOD

Selection of animal species

8. 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.

Housing and feeding conditions

9. Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be provided along with an unlimited supply of drinking water.

Preparation of animals

10. 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.

Route of administration and preparation of doses

11. Oral dosing each day, 7 days per week, should be carried out, preferably by gavage or administration of gelatin capsules. Liquids may be given undiluted or dissolved in an appropriate vehicle such as corn oil; solids should be dissolved if at all possible since large doses of solids in gelatin 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.

PROCEDURE

Number of animals and treatment groups

12. A sufficient number of hens should be utilized in each group of birds so that six birds be killed for biochemical determinations (three at each of two timepoints) and six birds survive the 14-day post-treatment observation period.

13. Generally, at least three treatment groups and a vehicle control group should be used, but if from assessment of other data [i.e. from the single dose study (Guideline 418), from *in vitro*

screening tests (10)(11)(12) or from structure-activity relationships (8)(9)], no effects would be expected at a dose of 1000 mg/kg body weight/day, a limit test may be performed.

14. The vehicle control group and each of the treatment groups should contain at least six hens for biochemistry and six hens for pathology. The vehicle control group should be treated in a manner identical to the treatment groups, except that administration of the test substance is omitted.

Dose selection

15. Dose levels should be selected taking into account the results from an acute test on delayed neurotoxicity (e.g. Guideline 418) 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.

Limit test

16. 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 compounds, 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.

Observations

17. 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 behavioral abnormalities. Ataxia should be measured on an ordinal grading scale consisting of at least four levels, and paralysis should be noted (13). 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. Any moribund hens should be removed and killed and subjected to gross necropsy.

Body weight

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

Biochemistry

19. 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 NTE activity (1)(14)(15)(16). In addition, it may also be useful to prepare and assay sciatic nerve tissue for NTE activity (17)(18)(19). 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 (Guideline 418) 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.

20. Analyses of acetylcholinesterase (AChE) may also be performed on these samples, if deemed appropriate (20)(21). However, spontaneous reactivation of AChE may occur *in vivo*, and so lead to underestimation of the potency of the substance as an AChE inhibitor.

Pathology

Gross necropsy

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

Histopathology

22. 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.

DATA AND REPORTING

Data

23. 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, behavioral, 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.

Evaluation of Results

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

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

Test report

26. The test report must include the following information:

Test substance:

- physical nature (including isomerization, purity and physicochemical properties);
- identification data.

Vehicle (if appropriate):

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

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;
- 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.

Results:

- body weight data;
- toxic response data by dose level, including mortality
- no-observed-adverse effect level;
- nature, severity and duration of clinical 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.

LITERATURE

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ANNEX

DEFINITIONS

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 in neural tissue.

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