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Single dose neurotoxicity screening studies of insecticide combination (Cypermethrin and Profenofos) in Wistar rats

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Abstract

The present investigation has been taken up to determine the toxic effects of combination insecticide (Profenofos 40% + Cypermethrin 4% EC) on nervous system of rats. A single oral dose of test substance was administered to five rats per sex at a concentration of 25, 75 and 225mg/kg body weight to experimental animals. The animals were observed under carefully standardized conditions with sufficient frequency to ensure the detection and quantification of behavioral and/ or neurologic abnormalities. In addition, serum cholinesterase was estimated after 24 hours of dosing. Motor activity of individual animal was measured using an automated device. Perfused (*in-situ*) nervous tissues were examined microscopically. The test article at the dose level of 75 and 225 mg/kg induced treatment related and dose dependent neurobehavioural alterations in rats of either sex. However, observed alterations were reversible, further no structural/ histopathological alterations were apparent. Single dose of test article at 25 mg/kg did not reveal any toxic signs or neurobehavioral alterations hence, it is considered to be the No Observed Adverse Effect Level (NOAEL).

Keywords: Cypermethrin, Profenofos, FOB, Neurotoxicity, Neurobehaviour, Motor activity

Introduction

Organophosphates and synthetic pyrethroids are widely used in agriculture to control crop pests and in livestock to control ectoparasites (Nolan *et al.,* 1979). The pyrethroids constitute a major class of highly active synthetic insecticides derived from the natural pyrethrins. Insecticide combination of cypermethrin together with profenofos is used in a variety of agriculture and non-agriculture application. The combination of two pesticides may have additive, synergistic, potentiative or antagonistic effect (Ballantyne *et al.,* 1995). Profenofos is an organo-thiophosphate insecticide that is mainly applied to control cotton bollworm and tobacco budworm. Profenofos is a broad spectrum organophosphate insecticide and acaricide. Its mode of action is by inhibiting acetylcholinesterase. Wing *et al.* (1983) observed that profenofos is stereo specifically converted to a more potent inhibitor of acetylcholinesterase by mouse liver microsomal mixed-function oxidase system.

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Organophosphorous compounds are very well established to cause neurotoxicity to organisms through inhibition of acetylcholinesterase and/or neurotoxic esterase (NTE) (De Bleecker, 1993). Pyrethroids are also known as neuropoisons acting on the axons in the peripheral and central nervous system by interacting with sodium channels in mammals (Bloomquist, 1993). Considerable body of literature has been found on toxicity related studies of organophosphorous and synthetic pyrethroid insecticides independently but very limited information is available on the toxic manifestations of combination of these classes of insecticides. Particularly, there is an apparent lack of data regarding neurotoxicity aspects in rats. Hence, the present investigation has been taken up to determine the toxic effects of combination insecticide (Profenofos 40% + Cypermethrin 4% EC) on nervous system of rats when exposed at single sub lethal dose.

Before the assessment sub-chronic neurotoxicity, it is necessary to screen the neurotoxic potential of combination insecticide following sub-lethal acute doses. Hence, this neurotoxicity screening battery study was undertaken. Neurotoxicity screening battery study

consists of a functional observational battery (FOB), motor activity and neuropathology. Functional observational battery is a type of neurological examination in which a wide range of neurological functions is assessed including measures of sensory, motor and autonomic functions. The functional observational battery consists of non-invasive procedures designed to detect gross functional deficits in rats (Moser, 1996).

Material and Methods

Test chemical and treatments

The test article, Polytrine C 44 EC which is a combination of cypermethrin (4%) and profenofos (40%) was obtained from Novertis India Limited, 14, J. Tata Road, Mumbai-400020. The test chemical was administered orally by gavage in distilled water (vehicle). The dose levels were selected based on the results of a range finding study. In the main study, five Wistar rats per sex per dose were stratified by weight and then randomly assigned to a dose group using a computer program. The numerous observations in a short time period made it necessary to stagger- start the study over 2 days. Therefore, males and females were treated on two separate days. Five rats per sex per dose group were used for the FOB and motor activity assays pre exposure, on day of treatment (day 1), and on days 7 and 14. After 24 hr of treatment, serum cholinesterase was assayed for all the animals. At the end of the study neuropathology of central and peripheral nervous tissues (*in-situ* perfused) was conducted on all the rats.

Laboratory animals and care

Male and female Wistar rats of 5 week old were obtained from Breeding Facility of Jai Research Foundation, Valvada-396 108, Gujarat, India. Animals were allowed to acclimatize to the experimental conditions for a period of 5 days. The animals were housed individually in polypropylene rat cages. A 12 h light/dark cycle was maintained in the room, and food (Amrut Brand, manufactured by Pranav Agro Industries Ltd., Pune, India) and water were available *ad libitum.* Protocols for the present experiments were approved by IAEC (Institutional Animal Ethics Committee) according to CPCSEA, India (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Animal identification and in life observation: Individual animals in the experiment was identified with picric acid marking on the head region representing group number and on body coat representing animal number and colored cage label showing study No, Group No & Sex, Study, Cage No. and Animal No. Animals were observed for overt toxicity, morbidity and mortality at least twice daily. Body weights were taken on days 1, 7 and 14. FOB and motor activity testing was performed as per the methods described by Moser *et al.,* (1988) and the intervals were day -1 (Pre exposure), on the day of dosing (day1) at the time of peak clinical effect (approximately 4-5 h), and then on days 7 and 14 of the study period.

Functional observational battery (FOB): Functional observational battery was conducted during following times:

1. In home cage
2. While hand held
3. In open field
4. During manipulative tests

Observed were recorded using proper descriptions, counts or ranks. Ranked observations were graded from least/lowest intensity to greatest intensity.

Grip performance

Hind limb and forelimb grip performance were tested using a grip strength meter (Columbus Instruments, Ohio, USA) as per the procedure described by Mattson *et al.* (1986). The grip strength of each animal was measured for 3 consecutive times; the results were averaged separately for the forelimbs and hind limbs.

Landing hind limb foot splay

Landing foot splay was evaluated according to a procedure similar to that published by Edwards and Parker (1997). The tarsal joint of each hind foot of each rat was marked with non-permanent ink just prior to testing. The animal was suspended in

Table 1: FOB Parameters and type of data point

Measurement	Recorded as
Body weight	Grams
Home Cage Observations	
Posture	Description
Convulsions	Presence/ absence
Hand held observations	
Ease of Removal from Home Cage	Description
Handling reactions	Description
Palpebral closure	Rank
Lacrimation	Rank
Eye examination	Description
Piloerection	Presence/ absence
Skin examination	Description
Salivation	Rank
Open Field Observations	
Gait	Rank
Mobility score	Rank
Arousal level	Rank
Vocalization	Count
Rearing	Count
Respiration	Description
Clonic or tonic movements	Description
Urination and defecation	Count
Stereotype	Description
Bizzare behaviour	Description

Table 2: Sensory motor measurements

Measurement	Recorded as
Approach Response	Rank
Touch Response	Rank
Click Response	Rank
Tail pinch response	Rank
Pupil response	Rank
Air righting reflex	Description
Landing hind limb foot splay	Centimeter
Hind limb grip performance	Gram force
Fore limb grip performance	Gram force
Motor activity	Stereotypic, ambulatory & total count

a prone position and then dropped from a height of approximately 30 cm on to a recording sheet. This procedure was repeated three times for each animal. The distance between two foot prints was measured and average of the three of the three foot splay values was calculated. A clean recording sheet was used for each animal.

Motor activity

The motor activity was measured using an automated animal activity measuring system (Columbus Instruments, Ohio, USA) equipped with a computer analyzer. Each animal was tested individually. Rats were monitored for 3 consecutive ten minutes intervals allowing for examination of both exploratory and acclimatization activity levels. During this period total and ambulatory activity of the rat was evaluated. Streotypic activity was calculated by subtracting ambulatory activity from total activity.

Cholinesterase estimation

Blood samples were collected from all animals of control and treatment groups after 24 of dosing. Serum was separated and cholinesterase activity was estimated as described in the manufacturer's manual (Hitachi-902).

Neuropathology

At the end of the study period i.e. on 15th day all the rats were perfused *in- situ* with 10 % neutral formalin. Rats were examined for gross pathological alterations. Brain, spinal cord, sciatic nerve and skeletal muscle were collected and evaluated for histopathology.

Statistical Analysis

Raw data were processed and analyzed to give group means and standard error with significance, wherever, the difference occurs between the controls and treated groups using validated in-house developed statistical software. All the parameters characterized by continuous data such as body weight, food consumption, grip strength, motor activity, organ weight, acetyl cholinesterase data were subjected to relevant statistical method (*viz.*, Bartlett's test, ANOVA, Dunnett's test or Student's t–test) using GraphPad Prism version 3.0 for

Windows, GraphPad Software, San Diego California USA (Motulsky, 1999).

Results and Discussion

Animals administered with insecticide combination (test article) at the dose rate of 75 and 225 mg/kg showed acute cholinergic symptoms *viz*., tremors, salivation, writhing, hyperactivity to sound/touch, and abnormal gait immediately after dosing and these symptoms were persisted for 8 hours. Gradually all the symptoms diminished and totally disappeared by the third day post treatment. Righi and Palermo (2003) reported that rats treated with cyhalothrin, a synthetic pyrethroid revealed signs and symptoms of intoxication that included salivation, tremors, and liquid feces. Stevens and Breckenridge (2001) stated that organophosphorus insecticides react with acetlycholinesterase at the serine hydroxyl group within the active site of enzyme. In this reaction, serine hydroxyl group is phosphorylated, yielding a leaving group. The phosphorylated acetlycholinesterase is inactivated, blocking acetacholine degradation in the synapse. Carey (1999) stated that all OP anticholinesterases potentially have a mechanism of toxicity in common *i.e.,* the phosphorylation of AChE causing accumulation of ACh. This buildup of neurotransmitter at the nerve ending results in signs of intoxication includes restlessness, hyper excitability, tremors, convulsions and paralysis (Stevens *et al*., 2001).

Group mean body weights of the entire test article treated group animals during pre exposure, on the day of commencement of treatment, and on day 7 and 14 were comparable to control group. Since the animals recovered from cholinergic symptoms, mean body weight of animals from treatment groups were unaffected during the experiment.

Serum cholinesterase activity was significantly low (143.6 IU/L, 154.2 IU/L and 70.4 IU/L) in males treated with test article at 25, 75 and 225 mg/kg body weight dose level as compared to control group (246.2 IU/L). Similar to males, cholinesterase activity was significantly reduced (146.8 IU/L and 55.8 IU/L) in females treated with test article at 75 and 225 mg/kg body weight dose level as compared to control group (289.8 IU/L). The observed reduction in cholinesterase was consistent and also dose-dependent and considered to be due to the organophosphorus entity of the test article *i.e.,* profenofos. Observations of McDaniel and Moser (2004) support our current notion as they reported significant reduction in cholinesterase due to profenofos treatment in rats.

Functional observational battery tests

During pre exposure test no functional or behavioral abnormalities were noted in any of the groups. Whereas, FOB test performed on day 1 of treatment at peak time of effect *i.e.,* 4 hours after dosing, following observations were made:

Home cage: Animal from control group revealed normal postures and did not reveal any clonic or tonic convulsions. Whereas, all the males from 225 mg/kg dose group and females from 75 and 225 mg/kg dose groups exhibited vertical jumping, writhing, clonic and tonic convulsions. Severity of symptoms was more pronounced in females.

Handling and open-field observations: All the males and females from control, 25 and 75 mg/kg dose groups did not reveal any abnormality during hand-held observations. Majority of males from 225 mg/kg dose group and majority of females from 75 and 225 mg/kg dose groups exhibited hyperactivity to handling. They also showed slight to severe piloerection and salivation. Observations performed in open field revealed normal gait, mobility and high arousal in all the control and 25 mg/kg dose group animals of either sex. Whereas, few animals from 75 mg/kg dose group and all the animals from 225 mg/kg dose group exhibited slight to severely abnormal gait, slight to totally impaired mobility, heightened arousal level and mild clonic tremors of whole body and limbs. These symptoms are characteristic of the exaggerated stimulation of parasympathetic nervous system. Such symptoms are observed in rats administered with both phosphorothioates and phosphorodithioates (DuBois, 1971).

Sensory Reactivity Measurements: Approach response was slight or energetic in almost all the animals from control and test article

treated groups, whereas, majority of animals from 75 and 225 mg/kg body weight revealed exaggerated response to touch and click stimulus. No constriction of pupil was observed in majority of animals from 225 mg/kg body weight dose group. All the males from 225 mg/kg landed on side in air righting reflex test, whereas, 2 out of 5 females of the same dose group landed on side and rest of the females landed on back. Majority of males and females from 225 mg/kg body weight dose group did not respond to tail pinch and few responded slightly to tail pinch. These effects were thought to be due to treatment. FOB test performed on day 7 and 14 of experiment did not reveal any treatment related abnormalities in any of the parameters studied.

Grip strength: No statistically significant variation was observed in the forelimb grip strength of males and females from treatment groups as compared to respective controls. On day 1 of treatment *i.e.,* during peak time of effect, hind limb grip strength was significantly reduced in males (301.2 gram force) and females (296.6 gram force) treated at 225 mg/kg dose group as compared to respective control group values. Females treated at 25 (296.6 gram force) and 75 (334.4 gram force) mg/kg dose level revealed statistically significant lower hind limb grip strength on day 7 post dosing as compared to control group values *viz.,* 421.4 gram force values. On day 14 of post dosing hind limb grip strength in treated animals of either sex were comparable to control.

Hind limb foot splay: Hind limb foot splay of both males (99.0 mm) and females (110.5 mm) measured on the day of treatment at peak time of effect revealed statistically significant increase in animals treated at 225 mg/kg body weight as compared to control values 73.8 mm and 76.3 mm respectively. Hind limb foot splays on pre exposure and on days 7 and 14 post doing were comparable to control group. The above neurobehavioural symptoms in treated animals were primarily due to organophosphorous poisoning (WHO, 1986; Brar *et al*., 2000). The mode of action of the organophosphate poisoning is inhibition of the acetylcholinesterase (Casida, 1963; O'Brien, 1967) Phosphorylation of acetylcholinesterase enzyme allows accumulation of acetylcholine (ACh) in the PNS at cholinergic neuroeffector junctions (muscarinic effects), skeletal nerve-muscle junctions, and autonomic ganglia (nicotinic effects), as well as in the CNS. At cholinergic nerve junctions with smooth muscle and gland cells, high acetylcholine (ACh) concentration causes muscle contraction and secretion, respectively. At skeletal muscle junctions, excess ACh may be excitatory (cause muscle twitching), but may also weaken or paralyze the cell by depolarizing end plate. In the CNS, high ACh concentrations cause sensory and behavioural disturbances, incoordination, depressed motor function, and respiratory depression. Increased pulmonary secretions coupled with respiratory failure are the usual causes of death from organophosphate poisoning. Recovery depends ultimately on generation of new enzyme in all critical tissues (Taylor, 1985).

The observed neurobehavioural symptoms in 75 and 225 mg/kg body weight dose groups revealed very clear evidence of dose dependent severity of the effect of the test article. Effects were most evident on the day of exposure and disappeared over the next few days. No signs of treatment were apparent after 7 days of exposure. Histopathology showed no evidence of treatment–related changes in either the nervous system or muscle.

Motor activity: Motor activity measured on the day of treatment at peak time of effect revealed significantly reduced total activity and ambulatory activity in animals treated at 225 mg/kg body weight as compared to control group. Mattsson *et al.,* (1986) reported similar observations for chlorpyrifos an OP insecticide. During pre exposure, on days 7 and 14 of post dosing, motor activity was comparable to respective control groups.

Necropsy and Histopathology: Test article had no morphological effect on muscle or the nervous system under the conditions of the study. Histopathologic observations from the five rats/sex/dose from control and high dose groups fixed by perfusion revealed no treatment–related effects. Similar observations were reported for chlorpyrifos, an OP insecticide by Mattsson *et al.,* (1996) in a single dose neurotoxicity screening study

in rats. Hence it is logical to conclude that acute dosage of the test article does not evoke structural alterations.

To summarize, the test article at the dose level of 75 and 225 mg/kg body weight induced treatment related and dose dependent neurobehavioural alterations in both male and female rats. However, observed alterations were reversible, further no structural/histopathological alterations were apparent. As Wistar rats of either sex treated at single dose of 25 mg/kg body weight did not reveal any toxic signs or neurobehavioral alterations, it is considered to be the No Observed Adverse Effect Level (NOAEL).

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