ETHEPHON (addendum)

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Explanation 77
Evaluation for acute reference dose 77
Acute neurotoxicity 77
Short-term studies of neurotoxicity 79
Mechanism of cholinesterase inhibition 81
Observations in humans 81
Comments 82
References 83

Explanation

Ethephon (2-chloroethylphosphonic acid) was evaluated by the Joint Meeting in 1977, 1978, 1993, 1995 and 1997 (Annex 1, references 28, 30, 68, 74 and 80). An ADI of 0–0.05 mg/kg bw was allocated in 1993 on the basis of a NOAEL of 0.5 mg/kg bw per day in a 16-day study in humans treated orally and a safety factor of 10. This ADI was maintained by the 1995 JMPR. The 1995 Meeting recommended re-evaluation of ethephon in 1997 to take into account the results of a study that was under way of the effects of the compound on rat plasma and erythrocyte cholinesterase activity in vitro. The 1997 Meeting was informed that ethephon had not inhibited cholinesterase activity in this study and that further research was being undertaken. The Meeting recommended that a re-evaluation be scheduled when those data became available.

Evaluation for acute reference dose

1. Acute neurotoxicity

Rats

A range-finding study was conducted to determine the appropriate time (within 8 h of dosing) at which to assess the peak behavioural effects of ethephon in a subsequent study of acute neurotoxicity and to evaluate the time course of the effects on cholinesterase activity. Ethephon was administered by gavage to groups of 18 Sprague-Dawley rats of each sex at a single dose of 250, 500, 1000 or 2000 mg/kg bw. Three animals of each sex per group were selected for an abbreviated battery of functional observational tests (FOB), which was administered before dosing and 0.5, 1, 2, 4, 6, 8 and 24 h after dosing. The FOB, applied to individual rats in an open field and while being handled, included observations of locomotor activity, arousal, gait, tremors, twitches, convulsions, bizarre or stereotypic behaviour, respiratory rate and pattern, lachrymation, salivation, urinary staining and diarrhoea. In addition, cholinesterase activity was determined in whole blood, plasma and brain from these animals on day 7 before termination. The remaining 15 animals of each sex per dose were assigned for cholinesterase testing by the Ellman method (Ellman et al., 1961). Body weights were recorded during the acclimation period and on days 0, 1 and 7 of treatment.

No treatment-related deaths occurred. Moderate to large losses of body weight were observed in all males and one female at 2000 mg/kg bw and in one male at 1000 mg/kg bw between day 0 and day 1. These animals also tended to gain more weight between day 1 and day 7 than the controls. Cholinesterase activity in whole blood and brain was unaffected by treatment, although there was some variation in the data. Plasma cholinesterase activity was decreased in males and females in all treated groups in a dose-related manner, with maximum suppression 4–8 h after treatment. On the basis of the findings of this study, the times selected as appropriate for application of the FOB in the main study on acute neurotoxicity were 5–5.5 h after dosing, and those for testing of motor activity were 5.5–6 h after dosing (Beyrouty, 1996a).

The main study to assess the potential acute neurotoxicity of ethephon was performed according to the Environmental Protection Agency (USA; FIFRA) section 81-8 guidelines and GLP. Groups of 12 Sprague-Dawley Crl:CD(SD)BR rats of each sex were given a single oral dose of 5 ml/kg of ethephon in deionized water by gavage at a dose of 0, 500, 1000 or 2000 mg/kg bw. The animals were then observed for up to 15 days. Body weights and food consumption were measured at least weekly, and animals were observed daily for any abnormal clinical signs. A FOB and motor activity tests were performed before dosing and on days 0 (after dosing at the time of peak effect), 7 and 14. At completion of the study on day 15, six rats of each sex per group were perfused, and central and peripheral nervous tissues from animals receiving 0 or 2000 mg/kg bw were examined histologically. The remaining animals were necropsied.

Treatment-associated deaths occurred in 2/12 females at 2000 mg/kg bw and 1/12 females at 1000 mg/kg bw on day 1 or 2 after dosing. The body weights of males at 2000 mg/kg bw were significantly ($p < 0.05$) decreased on day 7, and the food intake of females at 1000 or 2000 mg/kg bw was significantly ($p < 0.01$) reduced in the week following treatment. Males at 2000 mg/kg bw showed a slight, nonsignificant decrease in food consumption during the same period. No significant differences in grip strength or hind-limb splay were apparent between control and treated groups.

Significant decreases in total motor activity counts were observed in males and females at 2000 mg/kg bw and males at 1000 mg/kg bw on day 0 (first day of treatment), but no differences were noted on day 7 or 14. The FOB showed a significant ($p < 0.001$) increase in the incidence of pinpoint pupils in females at 2000 mg/kg bw, several females at 500 and 1000 mg/kg bw, several males at 2000 mg/kg bw and some males at 500 and 1000 mg/kg bw on day 0, and also in one male and one female in the control group. A few animals also showed miosis on days 7 and 14. Males at 2000 mg/kg bw showed a significant ($p < 0.001$) increase in urination in the open field on day 0, and one of these males had abnormal breathing on day 7. Females at this dose had significantly decreased body temperature on day 0, but no other differences were found on day 0, 7 or 14. One female had ptosis, lachrymation, urinary staining and decreased locomotor activity and arousal levels from day 0. On day 7, this animal showed piloerection, an abnormal body tone, a thin appearance and an altered visual placing response. Most of these effects were still present on day 14. Another female at 2000 mg/kg bw had abnormal respiration accompanied by abnormal breathing sounds on day 0 and an abnormal response to the visual placing test. Altered visual placing was also observed in the group given 2000 mg/kg bw on day 0. One female at 1000 mg/kg bw also had abnormal respiration with abnormal breathing sounds on day 0. A significant ($p < 0.05$) increase in adoption of the supine position and a decrease in sitting or standing in the home cage seen in females at 1000 mg/kg bw on day 0 was attributed to inter-group variation, as no such effect was observed at 2000 mg/kg bw. A significant ($p < 0.05$) decrease in the number of vocalizations on day 7 was attributed to the high value for the control group.

Gross necropsy of one female at 2000 mg/kg bw found dead before termination revealed effects on the stomach (dark, discoloured ingesta and multiple, dark, raised areas of the gastric mucosa). There were no other gross or neuropathological findings that were attributable to

treatment, and no significant differences were detected in brain weight, length or width between control and treated groups.

Thus, treatment of rats with a single dose of ethephon resulted in death (females only) and transitory effects including pupillary constriction, increased urination (males only), reduced food consumption and body-weight gain, decreased body temperature (females only) and reduced motor activity at the highest dose. Death and reduced food consumption were also observed in females at 1000 mg/kg bw; motor activity was decreased in males at 1000 mg/kg bw and females at 2000 mg/kg bw, and constricted pupils were seen in some animals at all lower doses. No neuropathological lesions were seen that were attributable to treatment (Beyrouty, 1996b).

2. Short-term studies of neurotoxicity

Rats

In a range-finding study to assess the potential toxicity of ethephon and to determine the doses to be uses in a subsequent 13-week study of neurotoxicity, ethephon was administered by gavage to six male and six female Sprague-Dawley rats at a dose of 100, 300, 600 or 1000 mg/kg bw for 14 days. Body weights were recorded twice weekly, food consumption was measured weekly, the animals were observed for abnormal clinical signs daily, and a FOB was performed before treatment, before dosing on days 2 and 8 and on day 15. Blood samples were collected from the tail vein of all animals before treatment, before dosing on days 2 and 8 and on day 15 for determination of erythrocyte and plasma cholinesterase activity. All animals were subjected to a gross pathological examination at study termination on day 15, or earlier for those that did not survive.

All males and five females at 1000 mg/kg bw per day died or were killed because of poor condition between days 3 and 10. Decreased body weights (by up to 35%) and decreased food consumption (by up to 48%) were observed from day 2 in animals at this dose. Two males and four females at 600 mg/kg bw per day died between days 2 and 9; the body weights of animals at this dose were decreased by up to 18% from day 5, and the food intake was reduced in males during the entire treatment period and in females during the first week of treatment. Abnormal clinical signs seen in animals shortly before death and in most animals at 600 or 1000 mg/kg bw per day that survived to study completion included fur staining, skin pallor, abnormal breathing and respiratory sounds, dehydration, cold to touch, decreased activity, weak appearance and abdominal distension. No treatment-related clinical signs were observed at the lower doses.

The effects found in the FOB on days 2 and/or 8 included abnormal respiratory sounds in some animals at doses ž 300 mg/kg bw per day and in one male at 100 mg/kg bw per day. These were sometimes accompanied by abnormal respiration in animals at the two higher doses. A few animals at these doses had pinpoint pupils, which were also seen in one male and one female at 300 mg/kg bw per day and in one control female. Snout staining was seen in a small number of animals at 1000 mg/kg bw per day and in one male and one female at 600 mg/kg bw per day; diarrhoea was observed in some animals at 1000 mg/kg bw per day and in one male at 600 mg/kg bw per day. On day 8, impaired gait was observed in one male and two females at 1000 mg/kg bw per day. On day 15, the findings were limited to abnormal respiratory sounds in surviving females at 1000 mg/kg bw per day and in a small number of animals at 600 mg/kg bw per day and pinpoint pupils in one male and one female at 300 mg/kg bw per day.

No significant differences in erythrocyte cholinesterase activity were found between control and treated groups. Significant decreases in plasma cholinesterase activity on days 2, 8 and 15 were noted for males in all treated groups and for females at doses ž 300 mg/kg bw per day; a slight reduction was recorded for females at 100 mg/kg bw per day.

Gross pathological changes, such as dilatation of the stomach and/or intestine, dark, raised and/or depressed areas in the stomach, dark areas on the thymus and small spleen or thymus, were

seen. No gross pathological changes indicative of treatment-related effects were evident in animals that survived to study termination (Beyrouty, 1997a).

The main study to assess the potential acute neurotoxicity of ethephon was performed according to the Environmental Protection Agency (USA; FIFRA) section 82-7 guidelines and according to GLP. Ethephon was administered by gavage to groups of 22 male and 22 female Sprague-Dawley rats for 13 weeks at doses, determined in the above 2-week study, of 75, 150 and 400 mg/kg bw per day, in a volume of 5 ml. Neurotoxicity was assessed in behavioural (FOB and tests for motor activity), neurochemical (brain cholinesterase activity) and neuropathological evaluations. Because of deaths in the group at the highest dose, this dose was reduced to 300 mg/kg bw per day during weeks 10–11. Control animals received the vehicle (deionized water) alone. The main study group consisted of four animals of each sex per dose, and an additional three or four (highest dose) were added for evaluation of cholinesterase activity. Body weights and food consumption were recorded weekly, and animals were observed daily for abnormal clinical signs. FOB and motor activity test were performed on 12 rats of each sex per group before treatment and once before dosing during weeks 4, 8 and 13 of treatment. On completion of treatment, six of these rats of each sex were perfused, and central and peripheral nervous tissues were taken from animals in the control and high-dose groups and examined histopathologically. Blood samples for measurement of erythrocyte and plasma cholinesterase activity were collected from 10 rats of each sex per group before treatment and then before dosing in weeks 4 and 8. At study completion, blood and brain samples were collected for determination of cholinesterase activity.

Three males and three females at the highest dose died during the study, one male and one female during week 5 and the other animals during week 10. No other deaths occurred. Many animals at 300–400 mg/kg bw per day had abnormal respiratory sounds and/or abnormal breathing during the study. Other findings at this dose included reduced body temperature and generally poor condition (weak, thin, dehydrated). The body weights of animals at the highest dose were slightly lower than those of control animals from week 3 of treatment (day 15) and for the duration of the study. Food consumption was slightly lower than control values from the second week of treatment and remained slightly lower throughout treatment. There were no remarkable findings in animals at lower doses.

Erythrocyte and plasma cholinesterase activities were reduced at all doses when compared with control values, and the reductions appeared to be dose-related. At the end of the study, the depression in erythrocyte activity at the lowest dose was 8.1% in males ($p < 0.05$; Dunnett test) and 10% in females (not significant); and the depression in plasma cholinestrase activity was 20% in males ($p < 0.001$; Dunnett test) and 10% in females (not significant). However, there were no toxicologically significant effects on brain cholinesterase activity. A slight depression in brain cholinesterase activity (8.5%) was seen in females at the highest dose, which was significantly different from the control value. This finding was not considered to be of toxicological significance because the individual values were generally within the range of those of other controls in the same laboratory, there were no cholinergic signs, the effect was seen only in females, and the decrease was < 10%.

A reduced willingness to emerge from the home cage was observed for males at 300–400 mg/kg bw per day throughout the study, and this effect attained statistical significance ($p < 0.05$) at week 13. This was accompanied by a significant ($p < 0.01$) increase in vocalization when the males were removed from their home cage at week 8. These changes were associated with poor condition of the animals and were considered not to represent a direct neurotoxic effect. No significant differences were detected in grip strength, hind-limb splay or body temperature. No significant differences in motor activity were detected between control and treated groups on any occasion.

The absolute weight of the brain of males at 300–400 mg/kg bw per day was significantly ($p < 0.05$) decreased when compared with the control group, but the relative brain weight was unaffected, indicating that the difference was probably related to the lower body weights of the treated group. Brain length and width were not significantly affected. No gross or histological lesions of nervous tissue were observed that were attributable to treatment.

Ethephon thus showed no evidence of neurotoxicity when administered for 13 weeks. The NOAEL was 75 mg/kg bw per day on the basis of significant inhibition of erythrocyte cholinesterase activity by > 20% at 150 mg/kg bw per day (Beyrouty, 1997b).

3. Mechanism of cholinesterase inhibition

Ethephon has been shown to inhibit cholinesterases, and particularly plasma butyrylcholinesterase, in rats, mice, dogs and humans in vivo. A series of experiments was performed to assess the potential of ethephon (analytical standard; purity, 98.4%) and technical-grade ethephon concentrate (active ingredient, 72.4% by weight) to inhibit acetylcholinesterase activity in vitro. Enzyme activity was determined spectrophotometrically by the Ellman method (Ellman et al., 1961), and type VI-S acetylcholinesterase from electric eels was used.

In the initial experiments, the pH of the solution containing the test article was adjusted to approximate that of the phosphate buffer (~8.0) used in the reaction solution for the spectroscopic measurement. This adjustment was required because preliminary experiments had shown inhibition of acetylcholinesterase with decreasing pH. A second series of experiments was performed with a different approach to pH neutralization, which involved increasing the buffering capacity of the reaction solution by increasing the molarity of the buffer. In both series of experiments, ethephon was tested at a concentration of 50, 500 or 5000 µg/ml

As the maximum inhibition of acetylcholinesterase activity observed in these experiments was < 50%, IC_{50} values for the two formulations of ethephon could not be defined accurately. At a dose of 50 µg/ml, analytical-grade ethephon inhibited activity by 9%. Maximum inhibition (37%) was observed at 500 µg/ml, with less inhibition at 5000 µg/l (22%). With technical-grade ethephon, maximum inhibition (25%) was also observed at 500 µg/ml, whereas at 5000 µg/ml the activity was inhibited by only 23%. At 50 µg/ml, activity was inhibited by 8.2%.

These results indicate that ethephon is a very weak inhibitor of acetylcholinesterase. However, in view of the acidic properties of the test material and the possible hydrolysis, decomposition and ionization of ethephon at a pH of ~8.0, the relevance of this study is unclear (Van Miller, 1986 a,b,c,d; Haux, 2000).

The mechanistic basis for inhibition of butyrylcholinesterase was investigated in a series of published studies. In vitro, dog plasma butyrylcholinesterase was most sensitive to ethephon-induced inhibition, human and mouse butyrylcholinesterase were of intermediate sensitivity, and rat butyrylcholinesterase was the least sensitive. Ethephon was about 10-fold more potent as an inhibitor of butyrylcholinesterase than of acetylcholinesterase.

Ethephon is unique among inhibitors of butyrylcholinesterase in that the phosphorylation appears to be due to the dianionic form of the compound. This species phosphorylates the esteratic site of the enzyme, apparently at serine-198 of the human enzyme, to produce the inactive phosphoenzyme. There is evidence that the phosphorylation is slowly reversible (Haux, 2000).

4. Observations in humans

When volunteers were given ethephon orally at a dose of 0.17 or 0.33 mg/kg bw per day (in three divided doses), the only significant finding was inhibition of plasma cholinesterase activity. No gross symptoms or changes in haematological, clinical chemical or urine end-points were associated with treatment (Weir, 1977a).

In another study, 10 male and 10 female volunteers received ethephon orally by capsule at 0.5 mg/kg bw per day for 16 days. Plasma cholinesterase activity was significantly inhibited, but no gross symptoms or changes in haematological, clinical chemical or urine end-points were associated with treatment (Weir, 1977b).

In a third study, five male volunteers received a dose of 1.5 mg/kg bw per day and five women received 2.2 mg/kg bw per day for 28 days, followed by a 14-day dose-free period. Neither plasma nor erythrocyte cholinesterase activity was inhibited, but transient symptoms consistent with inhibition of acetylcholinesterase activity, including urinary urgency and gastrointestinal effects, were reported. The overall NOAEL for short-term effects of ethephon in humans was 0.5 mg/kg bw per day (Reese, 1971, 1972).

Comments

The results of studies of acute and short-term neurotoxicity in rats, including evaluation of the time-course of the effects of ethephon on cholinesterase activity and a study of the potential of ethephon to inhibit cholinesterase activity in vitro, were available for consideration by the present Meeting. In addition, the results of a Magnusson and Kligman skin sensitization test in guinea-pigs were available, in which ethephon did not induce delayed contact hypersensitivity.

Ethephon is a dibasic phosphonic acid and hence does not behave like a typical organophosphorus compound towards cholinesterase enzymes. However, the phosphonic acid dianion form can phosphorylate serine residues in the active site of cholinesterases. Plasma cholinesterase is more susceptible than acetylcholinesterase to the effects of ethephon.

The oral LD_{50} in rats was > 2000 mg/kg bw. WHO has classified ethephon as 'unlikely to present an acute hazard in normal use' (WHO, 2000).

In preliminary studies, peak effects were observed in rats 5–6 h after a single oral dose. There was no effect on acetylcholinesterase activity. In a study of acute neurotoxicity, rats were given ethephon by gavage at a single dose of 250, 500, 1000 or 2000 mg/kg bw. Cholinesterase activity was not determined. One or two animals at the two higher doses died, and abnormal clinical signs and some changes in a battery of functional tests were observed at these doses on the day of treatment, which persisted for a few days in one or two animals. Pinpoint pupils were seen at 500, 1000 and 2000 mg/kg bw, although not in all animals at the lower doses, and this effect persisted for several days in a few animals. Pinpoint pupils occurred predominantly in moribund animals. The NOAEL was 250 mg/kg bw on the basis of an increased incidence of miosis.

After preliminary studies to establish a suitable dose range, a 90-day study of neurotoxicity was performed in rats in which ethephon was administered by gavage at a dose of 75, 150 or 400 mg/kg bw per day. The highest dose was reduced to 300 mg/kg bw per day at week 10–11 because of excessive mortality. These were the only deaths that occurred. Abnormal clinical signs were observed at the highest dose. Erythrocyte cholinesterase activity was significantly inhibited by > 20% at the higher doses. Brain cholinesterase activity was inhibited by < 10% at the highest dose. The NOAEL was 75 mg/kg bw per day on the basis of > 20% inhibition of erythrocyte cholinesterase activity at 150 mg/kg bw per day.

In studies previously evaluated by the JMPR, the short-term effects of ethephon were evaluated in volunteers. In three studies, no inhibition of erythrocyte cholinesterase activity was observed at doses up to 1.5 mg/kg bw per day for 28 days, while plasma cholinesterase activity was inhibited. Symptoms consistent with inhibition of acetylcholinesterase activity were reported at the highest dose (1.5 mg/kg bw per day for men, 2.2 mg/kg bw per day for women), including effects on the gastrointestinal tract and urinary urgency. On the basis of these symptoms, the

overall NOAEL was 0.5 mg/kg bw per day for the effects of ethephon in humans exposed for at least 2 weeks.

After considering the previous evaluation of ethephon, the new data submitted and recent publications in the open literature, the Meeting established an acute RfD of 0.05 mg/kg bw on the basis of the NOAEL of 0.5 mg/kg bw in studies in humans given repeated doses and a 10-fold safety factor.

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