The Role of Brain Methamidophos in Acephate Poisoning in Mice

Toshiko Tanaka*, Hiroaki Sato, Kosho Yoshida and Kentaro Kasai

Department of Forensic Medicine, School of Medicine, University of Occupational and Environmental Health, Japan. Yahatanishi-ku, Kitakyushu 807-8555, Japan

Abstract: We gave mice a 540 mg/kg dose of LD50 acephate, followed by an assessment of acephate, methamidophos (MP), and choline esterase (ChE) activity for up to 4 hours (hr) in order to investigate the time course of acephate intoxication. At 1 hr, the blood acephate and MP levels were $428 \pm 90 \mu g/ml$ (mean $\pm$ SEM) and $4.2 \pm 0.4 \mu g/ml$, respectively. The liver acephate levels were similar to those in the blood, but the liver MP levels were approximately 3.5 times that of the blood at 1 hr. The brain MP level tended to be higher than the blood MP at 1 hr. These levels decreased gradually over 4 hr, but the brain acephate and MP levels surpassed the blood levels significantly at 4 hr, and after 2 hr, respectively. Serum, liver, cerebrum, cerebellum, and brainstem cholinesterase activity (ChE) were inhibited at 1 hr, and remained inhibited in all but the cerebellum until the end of the experiment. The obtained data were applied to previously reported autopsy cases of acephate intake. Experimental data suggest that brain MP is involved in acute acephate-induced poisoning, even after a reduction in blood acephate. In autopsy cases with suspected acephate poisoning, the MP level in the brain should be considered in addition to the ChE activity to diagnose the cause of death.

Keywords: organophosphate, methamidophos, intoxication, acetylcholine esterase, autopsy.
In a recent autopsy case, high levels of MP in the brain were linked to the cause of death, despite the acephate concentration being low [7]. The time course of ChE and MP in the brain of mammals after acephate administration has not been fully elucidated. Clarifying these changes in mammals may allow for a more accurate estimation of the cause of death in autopsy cases where acephate has been taken. Unlike common organophosphorus compounds, acephate and MP have hydrophilicity and a short dipole structure [14], which might lead to different pharmacokinetics in mammals and make analysis difficult.

This study tracked the activity of acephate, MP, and ChE in the blood, liver, and brain of mice for up to 4 hr after administration of acephate, and we discuss their involvement in reported autopsy cases.

**Materials and methods**

**Administration of acephate to mice**

We used ddY strain mice weighing 30–50 g, obtained from Seiwa Experimental Animal Co. (Oita, Japan). The mice fasted overnight but were allowed to drink water ad libitum before the experiment. They were administered acephate intraperitoneally in a dose of 540 mg/kg (LD50 in 24 hr [1]), a 13.5% aqueous solution at 4 ml per kg. The mice were anesthetized at 1, 2, and 4 hr after the treatment, followed by a sampling of heart blood, the liver after thoracotomy, and the brain after craniotomy. The brain was divided into three parts for ChE assay: the cerebrum, cerebellum, and brain stem. The cerebrum was used for the analysis of acephate and MP in the brain.

The Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan, approved all requests for animals and intended procedures of the present study (Approval number AE 05-001) according to the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No.85-23, revised 1996).

**Extraction methods**

Acephate, MP, and trichlorfon (internal standard, IS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The extraction was performed by the modified method of Maroni et al [15]. The samples were analyzed in duplicate, and the average values were used as the results. A 100-ppm aqueous solution of IS was added to the blood in an amount of 20 μg/ml, diluted to more than 5 times its quantity by adding distilled water, saturated with NaCl, and centrifuged at 3,000 rpm for 5 min. For the liver and brain, two hundred mg of each sample was homogenized with 1.8 ml of water after adding IS in a quantity of 20 μg per g, and was saturated with NaCl. The homogenate of the liver and the brain was centrifuged for 1 hr and 2 hr at 10,000 g at 4°C, respectively. The supernatant was applied to an extraction column (Chem Elut 12198002, Agilent, LA) in a quantity of less than 0.8 ml. After standing at room temperature for 30 min, acephate, MP, and IS were eluted from the column by twice adding 3 ml of dichloromethane. The aliquot was collected and dried under nitrogen gas.

### Table 1. Acephate, Methamidophos, and ChE in the autopsy cases of references

| Case | Age/ Sex | Sample | Acephate (μg/ml or g) | MP (μg/ml or g) | ChE*1 (IU/l) | Cause of death | Reference |
|------|----------|--------|----------------------|----------------|--------------|----------------|-----------|
| Case 1 | 70/M | blood | 149 | 3.0 | 78 | poisoning | [5] |
| Case 2 | 60/M | blood | 46 | not detected | 3,539 | hemorrhage | [5] |
| Case 3*2 | 30s/F | serum | 558 | 11 | - | poisoning | [6] |
| Case 4 | 60s/M | blood | 44 | 2.26 | 66 | poisoning | [7] |
| | | frontal lobe | 27.1 | 9.17 | | | |
| | | occipital lobe | 53.1 | 8.91 | | | |
| | | temporal lobe | 55.4 | 9.14 | | | |

*1 : Serum was used for the assay. Normal range is 3,500–8,000 IU/l.
*2 : Organophosphorus fenitrothion 15 μg/ml was also detected in serum.
The Role of Brain Methamidophos in Acephate Poisoning in Mice

GC/MS Apparatus and its conditions

The residue was diluted by acetone and injected into a GC/MS apparatus (Automass 150, JEOL, Tokyo, Japan) equipped with a capillary column (DB-1, 0.32 mm i.d. x 15 m length, 1.0 µm film thickness) (Agilent, LA). The column temperature was programmed to hold steady at 80°C for 2 min, elevate to 230°C at 20°C/min and stay at the final temperature for 6 min. The injection port and ion source were held at 200°C. The scan mode was used for the qualitative analysis, and single ion-monitoring (SIM) mode for the quantitative analysis, and the objective peak was identified by the ion ratios of the characteristic ions in scan mode, comparing with those of each authentic standard.

The calibration curves of spiked authentic acephate at the concentrations of 0, 100, 200, 300 and 400 mg/ml or g in the blood, brain and liver obtained from the control mice showed good linearity (for blood, y = 0.2222x, R² = 0.9978; for liver, y = 0.058x, R² = 0.9996; for brain, y = 0.0859x, R² = 0.9975). The calibration curves of spiked authentic MP at the concentrations of 0, 5, 10, 15 and 20 mg/ml or g in the samples also showed good linearity (for blood, y = 0.0905x, R² = 0.9958; for liver, y = 0.1306x, R² = 0.9967; for brain, y = 0.0853x, R² = 0.9902). The recoveries in these procedures of blood acephate and MP were both 34%. The detection limit of acephate in the blood was less than 0.5 µg/ml, and that of MP was less than 0.6 µg/ml.

ChE assay

Ten times of 100 mM phosphate buffer (pH7.4) was added to each brain section and liver, and then they were homogenized. After centrifugation for 10 min at 3,000 rpm, the supernatant of the homogenates and serum of heart blood were collected to determine the ChE activity by the spectrometric method based on the method of Ellman et al [16, 17]. Acetylthiocholine iodide was used as a substrate in the assay, so both the pseudo-ChE (mainly observed in the serum) and true-ChE (mainly observed in the cerebral nerve membrane, muscle and red blood cells) were determined at the same time in the assay. The ChE activity of the homogenate was corrected by the protein contents obtained from a protein assay (Bio-Rad, CA).

Statistics

Data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using Student’s t-test for unpaired data. A p-value of less than 0.1 denoted a tendency to be significant, and less than 0.05 denoted the presence of a statistically significant difference.

Results

Time course of acephate and MP in mice

The mass chromatograms of the blood, liver and brain at 1 hr after the administration of acephate are shown in Figure 1. There were no misleading peaks in the chromatogram that could be confused with the objects. The mass spectra of acephate, MP and IS in the blood are shown in Figure 2.

At 1 hr after the administration, the blood acephate was 428 ± 90 µg/ml (mean ± SEM) (Figure 3), which peaked at 1 hr and decreased rapidly with time. The acephate in the liver showed a similar concentration to that in the blood throughout the experimental period. The acephate in the brain was slightly lower than that in the blood at 1 hr, decreased slowly, and was significantly higher than in the blood at 4 hr post-administration. The MP in the blood at 1 hr was 4.2 ± 0.4 µg/ml (Figure 4) and the MP in the liver was approximately 3.5 times that in the blood. The MP in the brain was 7.5 ± 1.5 µg/ml at 1 hr, which showed a tendency to be more than in the blood (p<0.0518), and was 6.0 ± 1.4 µg/ml at 2 hr, and 3.4 ± 0.9 µg/ml at 4 hr, showing a significantly higher level at 2 hr and 4 hr than in the blood (Figure 4). The ratio of MP to acephate in the blood was 0.012 ± 0.001 (mean ± SEM) at 1 hr, 0.025 ± 0.003 at 2 hr and 0.028 ± 0.006 at 4 hr after the administration (Figure 5). The ratios in the liver and brain were significantly higher than that in the blood at 1 hr (Figure 5).

ChE assay

ChE activity before and after the administration of acephate is shown in Figure 6. The serum ChE activity at 0 h was rapidly reduced to 19% at 1 hr, 9% at 2 hr, and 21% at 4 hr. The liver ChE activity was similar to that in the serum. The ChE activity in each section of the brain was significantly lower at 1 hr.
Figure 1. Mass Chromatograms obtained from the acephate-administered mice. Each panel shows the mass chromatogram obtained from the blood, brain, and liver of the mouse at 1 hr after the administration of acephate at a dose of 540 mg/kg. The value of $m/z$ is mass-to-charge ratio (mass number) of chromatogram. The peaks directly below the arrows were identified as MP (methamidophos), IS (internal standard, trichlorfon), and acephate, respectively, by comparing the retention time and mass spectrum with the standard products.

Figure 2. Mass Spectra of the blood obtained from the acephate-administered mice. The mass spectra were gotten from the heart blood, the chromatograms of which are shown in the upper panel of Figure 1. Each spectrum was obtained at the arrow point on the upper panel in Figure 1. The value of $m/z$ on the horizontal axis is the unified atomic mass unit (mass number). The vertical axis represents the ionic strength with a maximum fragment intensity of 100%. MP: methamidophos, IS: internal standard, trichlorfon.

Figure 3. Time Course of Acephate in Mice. The data of the blood are shown with solid circles and a solid line; the liver is expressed with open triangles and a hatched line; the brain is shown with open circles and a solid line, respectively. Values are expressed as mean ± SEM, n = 5–6, * $p < 0.05$ versus blood.

Figure 4. Time Course of MP in Mice. The data of the blood are shown with solid circles and a solid line; the liver is expressed with open triangles and a hatched line; the brain is shown with open circles and a solid line, respectively. Values are expressed as mean ± SEM, n = 5–6, * $p < 0.1$, * $p < 0.05$ and ** $p < 0.01$ versus blood.

Figure 5. Ratio of MP to Acephate. The data of blood are shown with solid circles and a solid line; the liver is expressed with open triangles and a hatched line; the brain is shown with open circles and a solid line, respectively. Values are expressed as mean ± SEM, n = 5–6, * $p < 0.1$, * $p < 0.05$ versus blood. MP: methamidophos.
The ChE activity in the cerebellum recovered at 4 hr, while maintaining significantly decreased activity in the cerebrum and brainstem, at 4 hr at 52% and 36%, respectively, compared with the pre-administration values. Inhibition of the ChE activity in the brainstem was most prominent in the brain sections at 4 hr after administration of acephate.

Discussion

IC50 of ChE in References

Inhibition of 50% of ChE activity (IC50) has been investigated to estimate the acute toxicity of acephate [1, 2, 4, 9, 18], which varies by enzyme sources. The IC50 of acephate in vitro was determined in the brain, red blood cells (RBC), and plasma in humans and rats (Bennett & Morimoto quoted in FAO 1985 [9]); they were between 1 mM and 5 mM for all enzyme sources. In humans, the IC50 of acephate and MP were described as 5.6 mM and 0.00184 mM, respectively [8]. Autopsies generally do not check for true-ChE activity in the membrane of the erythrocyte or brain, but assay serum pseudo-ChE (Table 1).

Time course of acephate, methamidophos and ChE inhibition

In the present study, both the acephate and MP levels in the mouse blood and organs peaked at 1 hr after the administration of acephate, the first observation time point (Figure 3, 4). These levels decreased with time, and the substances tended to remain in the brain rather than in the blood and the liver. Acephate conversion to MP was confirmed both in the liver and brain of rats in vitro, where the production in liver microsomes after 60 min of incubation at 37°C was 19 times higher than in brain microsomes [11]. This finding can explain the higher MP levels and MP-acephate ratios in both the brain and liver than in the blood at 1 hr in the present study, but does not explain the maintenance of surpassing levels in the brain MP at 2 hr and 4 hr post-dose. There may be factors other than metabolism that allow MP to remain selectively in the brain.

The ChE inhibition in the brain, especially in the brainstem, was maximal, even at 4 hr after the administration, when the concentration of these compounds had decreased. In a previous study, organophosphate sarin (isopropylmethyl phosphonofluoridate) decreased AChE activity in the four brain parts of mice (the pons with medulla oblongata, mesencephalon, diencephalon, and basal ganglia) at 2 hr, where AChE activity was lowest in the pontomedullary part and highest in the basal ganglia [19]. A correlation was shown between AChE activity in the pontomedullary part and mortality in sarin-treated mice in the same study [19]. The effects of sarin are similar to the present localization of ChE inhibition in brain sections after acephate administration.

The toxic levels of acephate and MP in mice cannot be directly applied to those in human organs, but the time course of acephate metabolism and ChE inhibition could be used as a reference.

On the causes of death in autopsy cases

The blood acephate level 44 µg/g in Case 4 of Table 1 is estimated as 0.24 mM, which is less than in other poisoning cases or the human IC50 of ChE 1.8–5.6 mM in plasma and RBC [8, 9]. The blood MP 2.26 µg/ml in Case 4 and 3.0 µg/ml in Case 1 were estimated to be over 0.016 mM, which is more than the IC50 of ChE 0.00184 mM in human plasma [8]. Cerebrum MP was as high as 8.91–9.17 µg/g in Case 4, which is estimated to be more than 0.06 mM, and serum ChE was suppressed. It was speculated that the high MP in the brain was responsible for the death due to acephate poisoning in Case 4 [7]. In the present study, the MP levels in the brain exceeded those in the blood at least 2 hr after acephate administration, and maintained in-
hibition of ChE in the cerebrum and brainstem even after the acephate decreased. The distribution of MP and ChE inhibition in the late phase were similar to those in Case 4.

The true-ChE inhibitory effect on the brain was described to reflect a matter of life and death caused by acephate in housefly and mouse [1]. MP was an at least 100 times more potent inhibitor of AChE and ChE than was acephate, and brain AChE to MP was relatively more sensitive than red blood cell AChE in dogs [18]. Cholinergic circuits in the brain are integral to many aspects of the central control of respiration [12]. The presence of acetylcholine nuclei in the basal forebrain [20] and in the pons of the brainstem is known [12]. The degree of acephate poisoning appears to be reflected by brain levels of MP rather than blood levels after a period of time. These findings might explain the mechanism and time course of death in Case 4 and perhaps in Case 1.

Typical poisoning cases with organophosphate, such as malathion and diazinon, have been reported to have higher levels in the blood than in the brain [21], but that was reversed in the MP levels in Case 4 and in mice at more than 2 hr post-dose in this study. Unlike other organophosphorus compounds, the hydrophilicity and the short dipole structure of acephate and MP described above [14] might relate to this phenomenon, but the details are unknown and were not investigated in this study.

Conclusions

This study suggests that mouse brain MP is involved in acute acephate-induced poisoning even after blood acephate levels have decreased. In autopsy cases with suspected acephate poisoning, it may be necessary to assess MP levels in the brain as well as ChE to diagnose the cause of death.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Mahajna M, Quistad GB & Casida JE (1997): Acephate insecticide toxicity: safety conferred by inhibition of the bioactivating carboxyamidase by the metabolite methamidophos. Chem Res Toxicol 10(1): 64–69
2. Chukwudebe AC, Hussain MA & Olofís PC (1984): Hydrolytic and metabolic products of acephate in water and mouse liver. J Environ Sci Health B 19(6): 501–522
3. Singh AK (1990): Molecular properties and inhibition kinetics of acetylcholinesterase obtained from rat brain and cockroach ganglion. Toxicol Ind Health 6(6): 551–570
4. Kao T-S & Fukuto TR (1977): Metabolism of O,S-dimethyl propionyl- and hexanoylphosphoramidothioate in the house fly and white mouse. Pesticide Biochemistry and Physiology 7(1): 83–95
5. Tanaka T, Tanaka N, Kita T, Kasai K & Sato H (2005): Acephate in biological fluids of two autopsy cases after ingestion of the chemical. J Forensic Sci 50(4): 933–936
6. Adachi N, Kinoshita H, Nishiguchi M et al (2008): Simultaneous analysis of acephate and methamidophos in human serum by improved extraction and GC-MS. Forensic Toxicology 26(2): 76–79
7. Takayasu T, Yamamoto H, Ishida Y et al (2019): Post-mortem distribution of acephate and its metabolite methamidophos in body fluids and organ tissues of an intoxication case. Forensic Sci Int 300: e38–e43
8. Dowla HA, Panemangalore M & Byers ME (1996): Comparative inhibition of enzymes of human erythrocytes and plasma in vitro by agricultural chemicals. Arch Environ Contam Toxicol 31(1): 107–114
9. Bennett EL & Morimoto H (1982): The comparative in vitro activity of acephate technical on brain, erythrocyte and plasma cholinesterase from the human, monkey, and rat. Unpublished report quoted in FAO, 1985.
10. Adachi N, Kinoshita H, Nishiguchi M et al (2011): Determination of acephate and methamidophos in tissues: Appearance of matrix effect in gas chromatography-mass spectrometry. Forensic Toxicology 29(2): 159–162
11. Spassova D, White T & Singh AK (2000): Acute effects of acephate and methamidophos on acetylcholinesterase activity, endocrine system and amino acid concentrations in rats. Comp Biochem Physiol C Toxicol Pharmacol 126(1): 79–89

12. Carey JL, Dunn C & Gaspari RJ (2013): Central respiratory failure during acute organophosphate poisoning. Respir Physiol Neurobiol 189(2): 403–410

13. Houze P, Pronzola L, Kayouka M, Villa A, Debray M & Baud FJ (2008): Ventilatory effects of low-dose paraoxon result from central muscarinic effects. Toxicol Appl Pharmacol 233(2): 186–192

14. Singh AK, White T, Spassova D & Jiang Y (1998): Physicochemical, molecular-orbital and electronic properties of acephate and methamidophos. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 119(1): 107–117

15. Maroni M, Catenacci G, Galli D, Cavallo D & Ravazzani G (1990): Biological monitoring of human exposure to acephate. Arch Environ Contam Toxicol 19(5): 782–788

16. Ellman GL, Courtney KD, Andres V, Jr. & Featherstone RM (1961): A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7: 88–95

17. Cometa MF, Lorenzini P, Fortuna S, Volpe MT, Meneguz A & Palmer M (2005): In vitro inhibitory effect of aflatoxin B1 on acetylcholinesterase activity in mouse brain. Toxicology 206(1): 125–135

18. Singh AK (1985): Kinetic analysis of inhibition of brain and red blood cell acetylcholinesterase and plasma cholinesterase by acephate or methamidophos. Toxicol Appl Pharmacol 81(2): 302–309

19. Baigari J, Jakl A & Hrdina V (1972): The influence of obidoxime on acetylcholinesterase activity in different parts of the mouse brain following isopropylmethyl phosphonofluoridate intoxication. Eur J Pharmacol 19(2): 199–202

20. Conner JM, Culberson A, Packowski C, Chiba AA & Tuszynski MH (2003): Lesions of the Basal forebrain cholinergic system impair task acquisition and abolish cortical plasticity associated with motor skill learning. Neuron 38(5): 819–829

21. Baselt RC (2017): Malathion, Disposition of Toxic Drugs and Chemicals in Man 11th ed. Biomedical Publications Seal Beach, CA. pp 1241–1243