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Development of a urinary biomarker for exposure to the organophosphate propetamphos: data from an oral and dermal human volunteer study

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Propetamphos is a member of the vinyl phosphate group of insecticides and is mainly used for sheep dipping. There have been no published metabolic studies on the effect of propetamphos in man to date, although the present authors have published the identification of a metabolite. The present paper presents data from a human volunteer study investigating the toxicokinetics of the organophosphorus pesticide propetamphos following oral and dermal exposure. Five volunteers ingested a propetamphos dose of 10 μg kg^{-1} (35 nmol kg^{-1}) body weight. Following a washout of 4 weeks, a 100 mg (356 μmol) dermal dose of propetamphos was applied, occluded to 80 cm^2 of the inner forearm, for 8 h to the same five volunteers. In a pilot study (several weeks before the main study), one volunteer also received an occluded dermal dose of 50 mg (178 μmol) propetamphos. Unabsorbed propetamphos on the skin was washed off after 8 h and collected. Blood and urine samples were collected over 30 and 54 h for the oral and dermal exposures respectively. Blood samples were analysed for plasma and erythrocyte cholinesterase. Urine samples were analysed for a urinary metabolite of propetamphos: methylethylphosphoramidothioate (MEPT). Following oral and dermal exposure, peak urinary MEPT levels occurred at 1 and 10–12 h respectively. The apparent urinary elimination half-lives of MEPT had means of 1.7 h (oral exposure) and 3.8 h (dermal exposure). Approximately 40% of the oral dose and 1% of the dermal dose were recovered as urinary MEPT or metabolites, which could be hydrolysed to MEPT. Approximately 90% of the dermal dose was recovered from the skin washings. Data from a volunteer showed that a doubling of the dermal dose resulted in approximately double the concentration of total MEPT. Alkaline hydrolysis of urine samples increased the level of MEPT detected after both oral and dermal doses. The increase was greater and statistically significant ($p < 0.001$, paired t-test) for the dermal dose. This increase in MEPT suggests the presence of other MEPT-containing metabolites or conjugates. The difference in the increase between oral and dermal doses raises the question of a difference in metabolism between the two routes. No individual showed a significant depression compared with their pre-exposure levels of erythrocyte acetyl cholinesterase or plasma cholinesterase activity for either dosing route. However, on a group basis, there was a statistically significant mean depression in plasma cholinesterase activity at 8 and 24 h for oral exposure, with a maximum mean depression of 7% from pre-exposure levels at 8 h. Hydrolysis of urine samples had the effect of reducing the interindividual coefficient of variation (CV) for total excretion of MEPT following both oral (CV reduced from 36 to 8%) and dermal (CV reduced from 40 to 17%) exposure. The ability to detect and follow the elimination of low doses of propetamphos by measurement of 'total' (after hydrolysis) urinary MEPT suggests it is a suitable biomarker of propetamphos exposure. The comparatively short elimination half-lives suggest a strategy for biological monitoring of occupational exposure based on samples collected at the end of the shift.

Keywords: biological monitoring, urine, organophosphorus pesticide, toxicokinetics.

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Introduction

The organophosphate pesticide propetamphos ((E)-*O*-2-isopropylcarbonyl-1-methylvinyl-*O*-methylethylphosphoramidothioate) is widely used internationally as an indoor insecticide and in the treatment of animals such as sheep and cattle for ectoparasites. In the UK, there has been considerable concern about the effects that organophosphate-based sheep dips, including propetamphos, may have on the health of exposed people. The development and validation of a non-invasive biomarker for propetamphos exposure would aid in monitoring the efficiency of occupational exposure control measures, in defining those work practices that give rise to unacceptable exposures or in investigating reported incidents where exposure has led to reports of ill-health.

The inhibition of blood cholinesterase activity is used as a measure of organophosphate exposure as well as health risk (HSE 2000). Owing to the large interindividual variability in cholinesterase activity, this involves the collection of both a post-exposure and a baseline sample from an individual. Blood sampling is invasive and for occupational groups such as farmers, it may be logistically difficult. Alternatively, the measurement of urinary metabolites offers a non-invasive monitoring strategy that detects exposures at levels below those which depress cholinesterase activity (Nutley and Cocker 1993, Griffin *et al*. 1999).

We identified and developed an assay for a human urinary metabolite of propetamphos: methylethylphosphoramidothioate (MEPT) (Jones *et al*. 1999). MEPT is formed by hydrolytic cleavage of the enol-vinyl phosphate bond and retains the phosphate part of the propetamphos molecule (figure 1). We could not find any other data on the absorption and elimination of propetamphos or MEPT in humans, which would aid the development of a biological monitoring strategy and the interpretation of results.

The purpose of the work was to develop a urine sampling strategy for MEPT as a biomarker of propetamphos exposure using toxicokinetic data from oral and dermal exposure in human volunteers.

Materials and methods

Human study

Approval for the study was obtained from the Health and Safety Executive Research Ethics Committee (ETHCOM/REG/98/05). Five volunteers, four men and a woman (age range 29–49 years, weight range 74–90 kg) took part in the study. All volunteers were assessed by a physician before, during and after the study and were healthy and fit to volunteer.

The oral and dermal dosing strategies were based on a previous volunteer study using chlorpyrifos (Griffin *et al*. 1999). A pilot dermal dose study involving one volunteer was conducted before the main study using 50 mg propetamphos. This showed low levels of absorption and subsequently the dermal

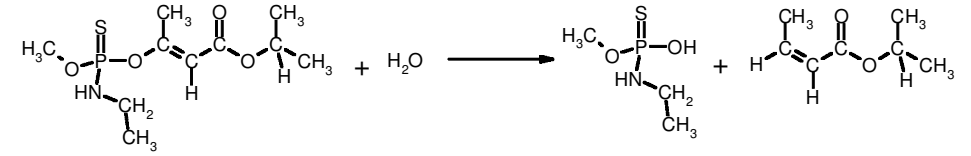

Figure 1. Proposed metabolic pathway (by hydrolysis) of propetamphos.

dose was increased to 100 mg for the main study in order to follow the elimination of propetamphos for 48 h.

Oral exposure

An oral dose of propetamphos (certified reference material, Promochem) equivalent to 10 µg kg^{-1} body weight was administered to each volunteer by dilution of an ethanolic solution (1000 mg l^{-1} propetamphos) in 200 ml water.

Blood samples were taken from each volunteer predose and at 2-h intervals for 8 h by means of an indwelling venous catheter (Veneflon). The catheter was maintained patent with heparinized saline and the first few millilitres of each collection were discarded. An additional blood sample was collected the following day (24 h post-exposure) by venepuncture.

Urine samples were collected as total voidings (fluid intake was not controlled) before the dose and at 1, 2, 3, 4, 6, 8, 10, 12, 22, 26 and 30 h. A 25-ml aliquot of each sample was taken and frozen at or below −20°C until analysed.

Dermal exposure

Four weeks after the oral study, a 100-mg occluded dermal dose of propetamphos (certified reference material, Promochem) was administered to each volunteer. A dermal application of 400 µl 250 g l^{-1} propetamphos solution in ethanol was spread evenly over a marked area of 80 cm^2 on the inner forearm, the ethanol allowed to evaporate and then the area covered using a raised (15 mm) impermeable plastic container which was left on the skin for 8 h. The residual dose was washed off with 20 ml ethanol and cotton wool swabs. The eluate was allowed to evaporate to dryness and reconstituted in 100 ml acetonitrile prior to analysis. Any residual propetamphos on the inside of the covering container was also recovered by extraction into 100 ml acetonitrile.

Urine samples were collected as for the oral dose, but collection was extended to 54 h, with samples taken at 4-h intervals between 30 and 54 h including one 8-h overnight collection. Blood samples were collected predose and at 4, 8 and 24 h after dose application.

Analysis of cholinesterase activity

Plasma and erythrocyte cholinesterase activities were measured for each blood sample on the day of collection using a UKAS accredited assay (Mason and Lewis 1989).

In vitro investigation of gastric propetamphos degradation

The stability of propetamphos under acid conditions representative of those in the stomach was investigated by monitoring the decrease in propetamphos levels over time. A solution of propetamphos (250 mg l^{-1}) was prepared in 1 M hydrochloric acid (pH 1) and incubated at 37°C for up to 4 h. Aliquots of the solution were analysed at regular intervals. Analysis was by HPLC-diode array detection at the selected wavelengths 210 and 215 nm with a Sphereclone 3µ ODS 2 column with dimensions 100 × 4.6 mm (Phenomenex), and a gradient elution with acetonitrile and water using a flow rate of 1 ml min^{-1}.

Analysis of propetamphos by GC-FPD

The acetonitrile extracts of the dermal washings and the residue on the container used to cover the skin were analysed directly by GC-FPD using splitless injection. The injection port was at 250°C. The oven temperature was initially 180°C (held for 1 min), then increased at 5°C min^{-1} to 200°C (held for 3 min) and then ramped at 20°C min^{-1} to a final 260°C (held for 2.5 min). The detector was at 200°C and the column used was a BP10, 25 m × 0.32 mm i.d., 1 µm film (Anachem). The total unabsorbed dermal dose recovered was calculated by summation of the two results from the dermal washings and the container, corrected for sample stability, and expressed as a percentage of the administered dose.

The concentration of propetamphos in urine was measured using a method that involved the addition of 500 µl distilled water and 250 µl 3 M sodium chloride solution to 1 ml urine, extraction with 5 ml hexane, followed by evaporation to dryness and reconstitution in 100 µl hexane. Samples were subjected to GC-FPD analysis under the same conditions as previously detailed for the acetonitrile extracts of both the dermal washings and the residue on the container. The detection limit of the analytical method was 1 µmol/mol creatinine.

Analysis of MEPT

The concentrations of urinary MEPT were measured using a slight modification of the method of Jones *et al.* (1999). Briefly, the method involved azeotropic distillation of the metabolite with acetonitrile, derivatization using pentafluorobenzylbromide and GC-FPD analysis. All samples were analysed with and without hydrolysis. Alkaline hydrolysis was achieved by the inclusion of a

preliminary step involving the addition of 250 µl 1 M potassium hydroxide to the 1-ml urine sample, placement in a boiling water bath for 4 h and neutralization with 250 µl 1 M hydrochloric acid. An improvement in method sensitivity was achieved by increasing the sample volume analysed to 2 ml for dermal exposure samples. Urine samples spiked with MEPT were included for quality assurance with each batch of samples analysed.

Analysis of creatinine

All urine samples were analysed for creatinine using alkaline picrate methodology on a COBAS MIRA analyser.

Software

Data manipulation and analysis was performed by the use of Excel and C-Stat for Windows™.

Results

All five volunteers completed the protocol for oral dosing and sampling. However, only four volunteers completed the protocol for dermal dosing and sampling. Data from one volunteer were incomplete, omitting a number of samples, and have been removed from the final results for the dermal part of the study. There were no reported signs or symptoms of ill health or anticholinergic-associated effects.

Cholinesterase activity

Enzyme activity levels for individuals were expressed as percentages of their pre-exposure baseline measurements. The percentage changes in enzyme activity for individuals were compared against quoted significant levels of inhibition (Mason and Lewis 1989, ACP 2000, HSE 2000) and also statistically analysed on a group basis using repeated measures analysis of variance.

No individuals' samples showed any inhibition > 20% of baseline values, but three plasma levels after oral dosing showed > 10% inhibition. On a group basis after the oral dose, there was no significant change ($p > 0.05$) in the erythrocyte enzyme over time, but the plasma enzyme showed consistent lower mean levels post-exposure, and at 8 and 24 h this reduction was statistically significantly lower (7 and 5% inhibition respectively, $p < 0.05$). For the dermal dose, repeated measures analysis of variance showed no significant change ($p > 0.05$) in the plasma or the erythrocyte enzyme activity.

Stability of propetamphos and MEPT

Under acid conditions representative of those in the stomach (pH 1, 37°C), a steady decrease in propetamphos levels was observed over 4 h (approximate half-life of 1 h). The results (figure 2) suggest some hydrolysis of propetamphos under the acidic conditions likely to be present in the stomach during the absorption of the oral dose (about 30 min). However, it may be assumed that the majority of the oral dose (> 75%) was absorbed as intact propetamphos.

The recovery of propetamphos from swabs spiked directly with the dermal dosing solution and stored under the same conditions as the swabs taken from the volunteers was determined as 77%. The propetamphos on the skin swabs was adjusted for this and the total dose recovered from the skin was $92 \pm 10\%$.

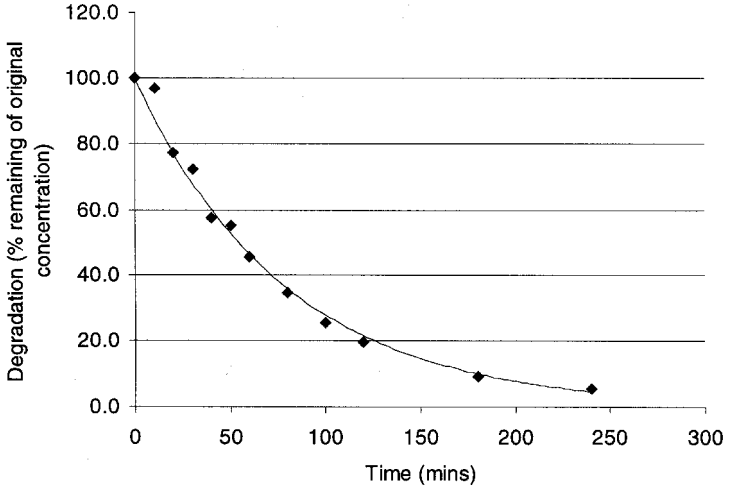

Figure 2. Degradation of propetamphos in 1 M HCl at 37°C over time.

Sample stability of MEPT in urine at room (20°C), fridge (< 5°C) and freezer (< −20°C) storage temperatures has previously been established: no decline in MEPT levels was observed during 43 days under all three temperature conditions described (unpublished data).

Propetamphos and the excretion of the urinary metabolite MEPT

A summary of the data is shown in table 1. Hydrolysis increased (except for the oral dose in volunteer C) the level of MEPT detected after both oral and dermal doses (figure 3). The increase was greater and statistically significant ($p < 0.001$,

Table 1. Human volunteer study data following an oral propetamphos dose of 10 μg kg^{-1} (35 nmol kg^{-1}) body weight ($n = 5$); a 100 mg (356 μmol) occluded dermal dose of propetamphos ($n = 4$) and a 50 mg (178 μmol) occluded dermal dose of propetamphos ($n = 1$).

	Oral dose (10 μg kg^{-1} body weight) (five volunteers)		Dermal dose (100 mg) (four volunteers)		Dermal dose (50 mg) (one volunteer)	
	Unhydrolysed	Hydrolysed	Unhydrolysed	Hydrolysed	Unhydrolysed	Hydrolysed
Apparent half-lives (h)						
Mean	2.22	1.74	4.5	3.83	5.7	5.36
SD	0.38	0.41	1.11	0.46	N/A	N/A
Range	(1.63–2.82)	(1.38–2.21)	(3.37–6.28)	(3.30–4.51)	N/A	N/A
Proportion of dose excreted as urinary MEPT (%)						
Mean	28	41	0.19	0.68	0.13	0.52
SD	8	6	0.07	0.12	N/A	N/A
Range	(19–44)	(31–46)	(0.09–0.27)	(0.56–0.82)	N/A	N/A
Total urinary excretion of MEPT metabolites (nmol)						
Mean	802	1145	666	2404	235	920
SD	292	88	264	412	N/A	N/A
Range	(534–1295)	(990–1198)	(319–960)	(1996–2934)	N/A	N/A

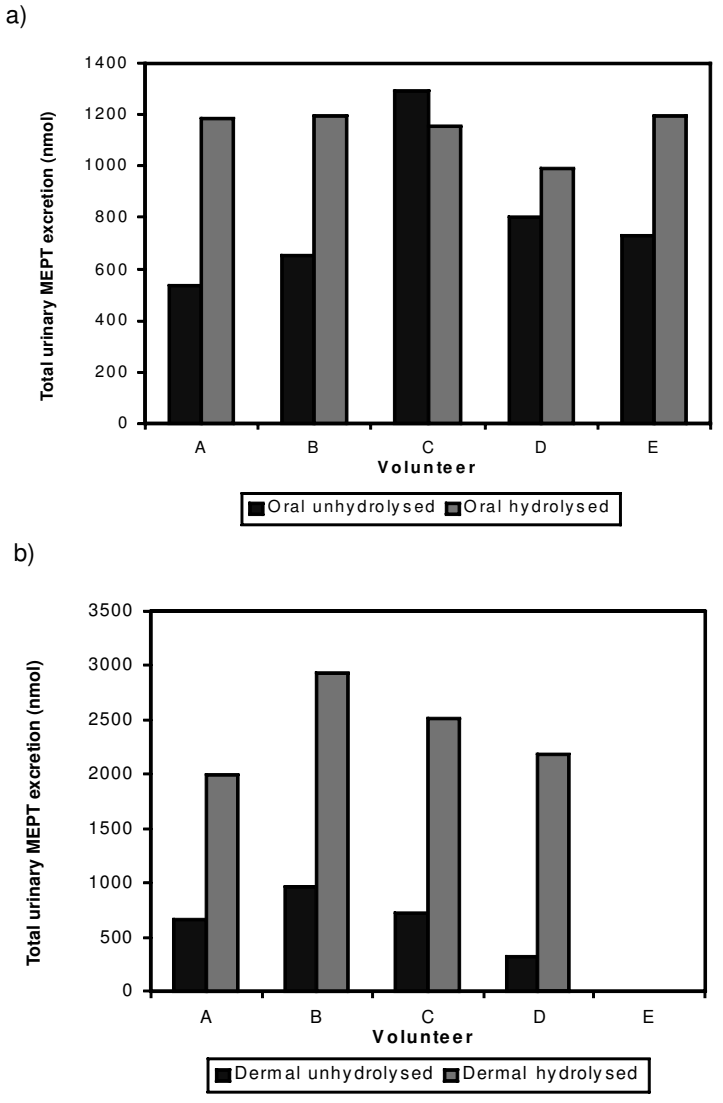

Figure 3. Total MEPT metabolites (nmol) excreted (a) over 30 h following a 10 μg kg^{-1} body weight dose of propetamphos and (b) over 54 h following a 100 mg occluded dermal dose of propetamphos.

paired *t*-test) for the dermal dose. Furthermore, hydrolysis had the effect of reducing the interindividual coefficient of variation (CV) for total excretion following both oral (CV reduced from 36 to 8%) and dermal (CV reduced from 40 to 17%) exposure.

Urinary MEPT results expressed as creatinine-corrected values (μmol/mol creatinine) showed similar elimination profiles to those expressed as the rate of excretion over time (nmol h^{-1}). Furthermore, regression analysis between excretion expressed as creatinine corrected and per hour gave a regression coefficient of 0.96. The excretion profiles after oral and dermal doses of propetamphos are shown in figure 4.

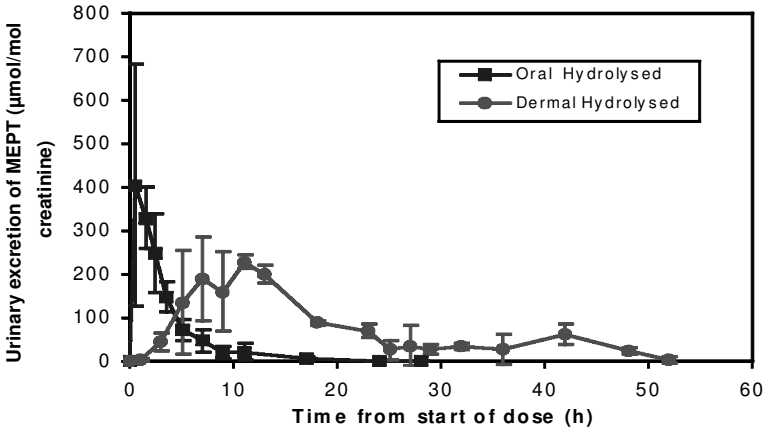

Figure 4. Urinary excretion of MEPT following an oral dose of 10 µg kg^{-1} body weight and an occluded dermal dose of 100 mg propetamphos adjusted for creatinine (µmol/mol creatinine). Values are the mean ± SD for five volunteers (oral) and four volunteers (dermal).

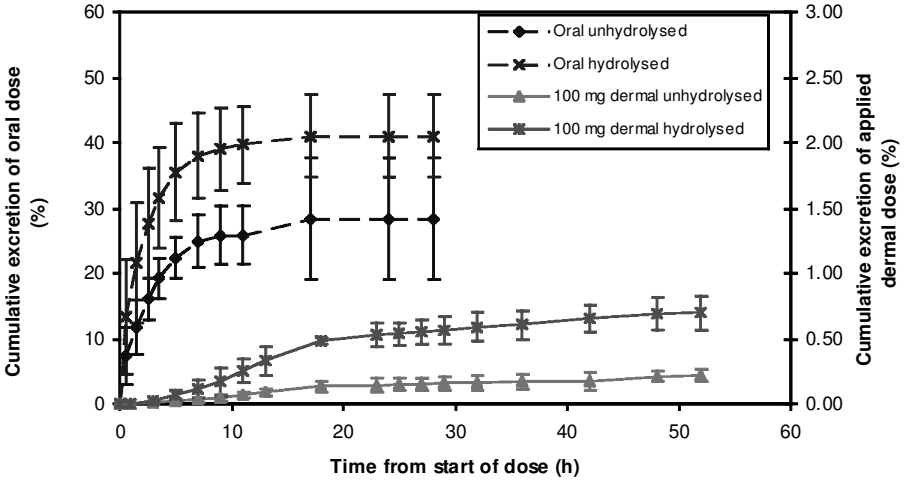

Figure 5. Cumulative urinary excretion of MEPT following a single oral dose of 10 µg kg^{-1} body weight (five volunteers) and 100 mg occluded dermal dose (8 h, four volunteers). Values are the mean ± SD.

Oral exposure to propetamphos (10 µg kg^{-1} body weight, five volunteers) resulted in rapid absorption, metabolism and excretion. The elimination of propetamphos followed first-order kinetics ($r > 0.93$). The mean peak rates of urinary MEPT excretion were 201 nmol h^{-1}, 210 µmol/mol creatinine (unhydrolysed) and 362 nmol h^{-1}, 404 µmol/mol creatinine (hydrolysed) and occurred during the first hour post-exposure. The majority (> 90% for hydrolysed and unhydrolysed samples) of the excreted oral dose was recovered within 12 h (figure 5). The calculated (by regression analysis of log-transformed data) half-lives and percentage oral dose recovered as urinary MEPT are shown in table 1.

Following a 100-mg occluded dermal dose of propetamphos to four volunteers, mean peak excretion rates of urinary MEPT were 38 nmol h^{-1}, 46 µmol/mol

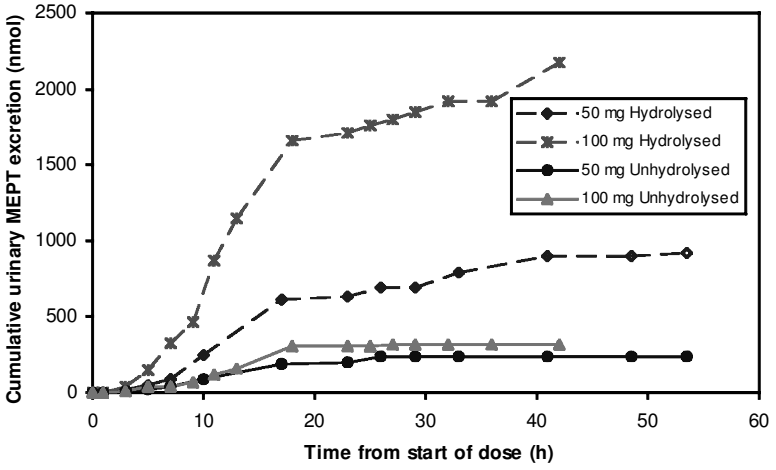

Figure 6. Cumulative urinary excretion of MEPT following an occluded dermal dose (8 h) of 50 or 100 mg propetamphos in a single volunteer.

creatinine (unhydrolysed) and 142 nmol h^{-1}, 188 µmol/mol creatinine (hydrolysed) and occurred at 10–12 h after the start of exposure (2–4 h after the dose was washed off). The majority (60% for both hydrolysed and unhydrolysed) of the excreted dermal dose was recovered between 8 and 24 h, with a return to pre-exposure levels between 26 and 40 h after the start of the dose exposure (figure 4). The calculated half-lives and percentage dermal dose recovered as MEPT are shown in table 1. The mean (range) percentage dermal dose recovered from the skin washings as propetamphos was 92 (83–104)%.

This dermal absorption resulted in a mean (range) excretion of 2404 (1996–2934) nmol of 'total' MEPT metabolites. In the case of the oral exposure study, 40% of the dose appeared in the urine as total MEPT metabolites. If bioavailability from the oral dose was assumed as 100%, then the rate of dermal absorption was estimated as 9.4 nmol cm^{-2} h^{-1}.

Following a 50-mg occluded dermal dose of propetamphos only to one volunteer, the amount of MEPT recovered after hydrolysis was about half that seen with the 100-mg dermal dose. However, there was no dose-related increase in unhydrolysed MEPT levels (figure 6).

Unmetabolized propetamphos was not detected in urine following oral or dermal exposure in this study.

Discussion

The results following an oral dose of 10 µg kg^{-1} suggest a small depression in mean plasma cholinesterase of around 7 and 5% at approximately 8 and 24 h after exposure, but without any evidence of a similar change in acetyl cholinesterase, considered the more clinically and toxicologically relevant measurement (Heath and Vale 1992). There was no detectable effect on the plasma or erythrocyte enzyme after dermal dosing. Thus, these data confirm the greater sensitivity of urinary metabolites of organophosphorus pesticides in detecting exposure compared with blood cholinesterase measurements.

Hydrolysis increased the urinary levels of MEPT detected and hence the sensitivity of the method to detect exposure. Furthermore, hydrolysis had the effect of reducing the interindividual CV of total MEPT excretion following both oral and dermal exposure, probably by reducing the effects of interindividual variability in metabolism. Sample hydrolysis was particularly beneficial in the case of dermal exposure, where urinary MEPT levels were close to the detection limit of the biological monitoring method and where the effects of metabolism were more significant (ratio of hydrolysed to unhydrolysed mean MEPT levels 4:1 compared with the 1.3:1 following oral exposure).

The increase in urinary MEPT following hydrolysis could not be due to the hydrolysis of propetamphos itself since intact propetamphos was not detected in urine, but rather the hydrolysis of other metabolites of propetamphos retaining the MEPT moiety or their conjugates. An analogy would be the increase in urinary dialkyl phosphate metabolites released by hydrolysis from malathion mono- and di-acid metabolites which also contain a carboxyl ester group as does propetamphos (Fenske and Leffingwell 1989).

The difference between the ratios of hydrolysed to unhydrolysed levels following oral and dermal exposure (figure 3) suggests a difference in the types of urinary metabolites excreted following exposure to propetamphos by the two routes. This could be due to extensive hydrolysis of propetamphos in the stomach leading to absorption of MEPT, however, this is unlikely as only 25% of propetamphos was hydrolysed after 30 min; a difference in oral route to dermal route metabolism; or saturation of the metabolic pathway leading to MEPT via the dermal route. Although the percentage of the dermal dose absorbed was small, the overall systemic dose by the dermal route was 1.7 times larger than the oral dose. Some support for saturation comes from the single volunteer with separate 50 and 100 mg dermal doses where the levels of MEPT (unhydrolysed) were similar, but there was the expected twofold difference after hydrolysis.

The oral dose exhibited a shorter apparent half-life than those seen for the two different dermal studies. Oral exposure to propetamphos resulted in rapid absorption, metabolism and excretion, whereas dermal exposure resulted in both lengthier absorption and elimination phases. The different elimination profiles and half-lives reflect both the different dosing regimes employed and the different toxicokinetics involved. Dermal exposure to propetamphos was prolonged with a duration of 8 h compared with a rapid oral and uptake via the skin can lead to a reservoir effect in the skin which prolongs exposure, with the effect of a longer elimination phase and an increased half-life. The elimination half-life of MEPT and metabolites capable of hydrolysis to MEPT was much shorter than that for the parallel dialkyl phosphate metabolites found in other volunteer studies such as chlorpyrifos, where the half-lives were approximately 15 h (oral) and 30 h (dermal) (Griffin *et al.* 1999). The overall extent of dermal absorption of propetamphos seen in the present study is 0.68% or 9.4 nmol cm^{-2} h^{-1}. This is similar to that seen in other volunteer studies of organophosphorus pesticides: Nolan *et al.* (1984) and Griffin *et al.* (1999) reported dermal absorption of chlorpyrifos of 1.3% and 1% respectively.

Collection of total urine voidings at timed intervals in field studies can pose difficulties. The pragmatic approach is to collect part of a voiding at a convenient time, e.g. the end of shift. The close correlation ($r = 0.96$) between urinary excretion of MEPT expressed as a rate in nmol h^{-1} and as creatinine corrected

values in μmol mol^{-1} shows that creatinine and MEPT are excreted in a similar way and that adjustment of MEPT concentration by creatinine is a valid procedure for correcting for urine dilution. Single, spot urine sampling with creatinine correction of urinary MEPT levels may be used for monitoring occupational exposure to propetamphos.

Although similar results were obtained for both the 50- and 100-mg occluded dermal exposures when expressed as free MEPT, there was an apparent dose–response relationship in a single volunteer if the results were expressed as total urinary MEPT. The increase in total MEPT from 1000 to 2000 nmol after a doubling of dose gives further confidence in the suitability of urinary total MEPT as a biomarker of propetamphos exposure.

These results could help in the development of biological-monitoring strategies and the interpretation of results of field studies. If we use the Droz model (Droz and Fiserova-Bergerova 1992), then a dermal half-life of 4–5 h would suggest that a urine measurement at the end of a typical 8-h shift reflects around 15% of exposure in the previous hour and 80% exposure over the working day. For an oral exposure with a half-life of around 2 h, the last prior hour to sampling explains 25% of the urinary measurement from the rest of the working day. This would suggest that if monitoring is to be based on single urine samples they should be collected at the end of a work shift. If future field studies collect multiple samples from exposed workers these might produce elimination profiles which might allow the apparent half-lives to be compared with those in this study to investigate the possibility of determining whether occupational exposure was dermal or oral in origin.

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