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Identification of a biomarker for propetamphos and development of a biological monitoring assay

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This paper describes the identification of a human metabolite of propetamphos ((*E*-*O*-2-isopropylcarbonyl-1-methylvinyl-*O*-methylethylphosphoramidothioate), formed by the hydrolytic cleavage of the enol-vinyl–phosphate bond, and the development of an analytical method suitable for biological monitoring of propetamphos exposure. The metabolite has been detected in the urine of exposed workers but not in that of control subjects. The analytical method involves azeotropic distillation of the urine with acetonitrile, followed by derivatization with pentafluorobenzyl bromide and analysis using gas chromatography with flame photometric detection.

Keywords: biological monitoring, urine, propetamphos, organophosphorous pesticide.

Introduction

Propetamphos is a member of the vinyl phosphate group of insecticides and is mainly used for sheep dipping. Historically, biological monitoring of occupational exposure to organophosphate pesticides has been based on detecting the depression of acetyl cholinesterase activity in blood (erythrocyte and plasma enzymes). Although this approach (strictly, biological effect monitoring) is effective in monitoring workers and helping to avoid and identify acute poisoning, it requires baseline samples (i.e. pre-exposure) to be taken and is not suitable for low-level exposures. It also requires the taking of a blood sample which must be analysed on the same day as it is received by the laboratory. Current UK guidelines on biological monitoring are in favour of non-invasive sampling where viable alternatives to blood samples exist. Previously, we have developed a urine assay for determining the dialkyl phosphate metabolites of a number of organophosphate pesticides (Nutley and Cocker 1993). This has the advantages of being able to assess low-level exposure and is non-invasive and, if required, samples can be frozen for several months prior to analysis. Unfortunately this assay is not currently able to assess propetamphos exposure as the human metabolism of propetamphos has not been studied and no suitable metabolites for biological monitoring have been determined. The structure of propetamphos is shown in figure 1(a).

There have been no published metabolic studies of propetamphos in man to date. The only available data are from the studies by the company Sandoz, now Novartis (1981, personal communication) on metabolism in rats, and by Wells (1985) in houseflies. The Sandoz study used single oral doses of ^{14}C-labelled propetamphos fed to rats (doses 0.6, 6 and 16 mg kg^{-1} body weight). The radiolabelled carbons were at positions 1 and 3 on the butenoic acid moiety of the compound which left the phosphate part of the compound unlabelled. Their proposed metabolic pathway of propetamphos in rats concentrated on the

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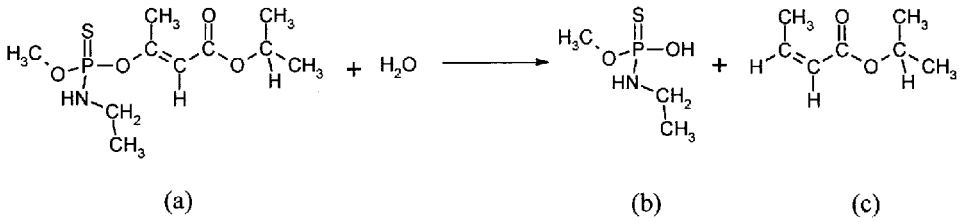


Figure 1. Proposed metabolic pathway (by hydrolysis) of propetamphos (a).

hydrolytic split to form isopropylacetoacetate (compound c, figure 1): they have omitted the remaining fraction, i.e. the thiophosphate fragment (compound b, figure 1). This fragment is thus excluded from all subsequently proposed metabolic pathways. The broad conclusions of this study were that propetamphos is completely metabolized and rapidly excreted via urine and exhaled air. They claimed that the major excretion product was exhaled $^{14}CO_2$. The main non-conjugated urinary metabolites in their study were desmethyl-, desisopropyl- and desmethyldesisopropyl propetamphos (some conjugated forms were also detected). Isopropylacetoacetate and acetone (which occurs endogenously) were also detected. Many of these metabolites are unlikely to be suitable for a biological monitoring strategy due to their similarity to, or presence as, endogenous compounds.

The Wells' study showed that houseflies, cockroaches and mouse liver preparations were all capable of hydrolysing the phosphoric anhydride bond of propetamphos *in vitro*. This was also the case in houseflies *in vivo*. Glutathione conjugation at the vinyl bond of propetamphos was also noted as a possible detoxification reaction in less susceptible houseflies.

By analogy with other organophosphate pesticides, it was possible that a biological monitoring strategy might be based on the analysis of the phosphate-containing part of propetamphos. In many organophosphate pesticides, hydrolysis of the phosphate–ester bond yields dialkylphosphate metabolites which have been used for biological monitoring (Griffin *et al.* 1999). Therefore the most likely metabolite of propetamphos, suitable for biological monitoring, would be that obtained after cleavage of the enol-vinyl–phosphate bond during hydrolysis. Figure 1 shows a proposed pathway. Evidence for the process has been shown in the previously published studies (Sandoz 1981; Wells 1985), although Sandoz only looked at the other half of the products.

The hypothesis of the study reported here was that the hydrolysis product is a metabolite of propetamphos and could form the basis of a biological monitoring strategy. The approach was to chemically hydrolyse propetamphos and produce the putative metabolite (identified using mass spectrometry), followed by analysis of rat and human urine after exposure to propetamphos. The metabolite was then synthesized and the analytical method fully developed and validated.

Methods

Chemical hydrolysis of propetamphos and identification of the metabolite

A standard solution of propetamphos (7 mg ml^{-1}) was prepared in methanol (HPLC grade, Rathburns, UK) using certified reference material (Laboratory of the Government Chemist, UK). An aliquot (100 µl) of this solution was then mixed with 500 µl of 5 M NaOH (Fisher Scientific, UK) and hydrolysed in a waterbath at 90 °C overnight. After cooling, the water was removed in an azeotropic

distillation with acetonitrite (7 ml, HPLC grade, Rathburns, UK) at 90 °C under a stream of nitrogen. The residue was resuspended in 500 µl acetonitrile, 25 mg anhydrous potassium carbonate (Fisher Scientific, UK) was added and the sample was then derivatized at 50 °C for 16 h using 50 µl pentafluorobenzyl bromide (Aldrich, UK). The sample was analysed by fullscan GC–MS (El+) and GC–MS (Cl–) (Hewlett Packard 5890 gas chromatograph with 5989A mass spectrometer).

Using a BPX-3 25 m × 0.32 mm i.d. fused silica column with a 0.5 µm film, samples were injected (splitless) at 280 °C. The oven temperature was held at 100 °C for 1 min, then ramped at 6 °C min^{-1} to 230 °C. A second ramp (10 °C min^{-1}) was then used to take the oven to 280 °C, where it was held for 10 min. For EI^+ mass spectrometry, the source and quadrapole temperatures were 250 °C and 100 °C respectively. Scanning was from *m/z* 50 to 550 with 0.8 scans per second. For Cl^- mass spectrometry, the source and quadrapole temperatures were 200 °C and 100 °C respectively with methane as the reagent gas. Scanning was from *m/z* 50 to 550 with 0.8 scans per second.

A second chemical hydrolysis of propetamphos standard solution was undertaken as before. The reaction mixture was extracted with ethyl acetate (HPLC grade, Rathburns, UK) to remove any unreacted propetamphos. The sample was then acidified to pH 2–3 and extracted with ethyl acetate. The organic layer was removed and evaporated under a stream of nitrogen. The residue was analysed by solid probe mass spectrometry (EI^+) using a VG 12-250 (VG Masslab, UK). The sample was scanned from *m/z* 40 to 650 at 1 scan per second.

Animal study

A study was conducted to collect urinary metabolites from five male and five female rats following a single oral dose of propetamphos (3.58 mg g^{-1} in polyethylene glycol 400) by gavage. The study was conducted by a contract laboratory following Good Laboratory Practice. Wistar-derived rats weighing 170–215 g were given a single oral dose of 16 mg propetamphos per kg body weight. During the study, excreta were collected and removed immediately prior to dosing and at 24 and 48 h after dosing.

Water from the urine samples was then removed in an azeotrophic distillation with acetonitrile and the residue was derivatized using pentafluorobenzyl bromide as described above. Analysis was by GC–FPD (flame photometric detection), according to the method of Nutley and Cocker (1993). Samples were also analysed by GC–MS, both electron impact and negative ion chemical ionization, using the conditions described above.

Human urine samples from exposed workers

Urine samples from a worker exposed to propetamphos were prepared as for the samples from the animal study and analysed by GC–FPD.

Additionally, samples from five exposed workers and 20 occupationally unexposed people were also analysed for the propetamphos metabolite.

Synthesis of the putative metabolite

Once the suggested structure of the putative metabolite had been elucidated (see figure 1(b), a synthesis of the compound was commissioned from Ultrafine Chemicals, Manchester, UK. The preparation was adapted from a procedure to synthesize propetamphos using stepwise substitution of thiophosphoryl chloride (US Patent 3758645). Ultrafine Chemicals determined structure and purity using proton NMR and HPLC–UV detection. The integrity of the synthesis was verified in-house by GC–MS (EI^+ and GC–FPD.

Analytical method development

The framework for the analytical method was based on the dialkylphosphate metabolite method (Nutley and Cocker 1993). Modifications were made to the method in terms of optimal conditions for derivatization time and GC injection and detection temperatures. Briefly, 1 ml of urine, with dibutylphosphate added as internal standard, was diluted with 8 ml acetonitrile. After mixing and centrifugation, 7 ml of acetonitrile was removed (avoiding any sediment) and transferred to a clean tube. After addition of anti-bumping granules, water was removed from a sample by azeotropic distillation at 90 °C under a stream of nitrogen. Once dry, a further 4 ml of acetonitrile was added and evaporated. When the water had been removed the sample was reconstituted in acetonitrile (500 µl), 25 mg anhydrous potassium carbonate was added and the sample was derivatized at 50 °C for 90 min using 50 µl pentafluorobenzyl bromide. Samples were analysed by GC–FPD using splitless injection at 250 °C. The oven temperature was initially 180 °C (held for 1 min) then ramped at 5 °C min^{-1} to 200 °C (held for 3 min) then ramped at 20 °C min^{-1} to 260 °C (held for 2.5 min). The column used was a BP10, 25 m × 0.32 min i.d., 1 µm film. Detector temperature was 200 °C.

Linearity and detection limit studies were conducted over the range 100–6400 nmol l^{-1}. Quality control samples, spiked at a nominal 320 nmol l^{-1}, were stored frozen and used to determine intra- and inter-assay coefficients of variation.

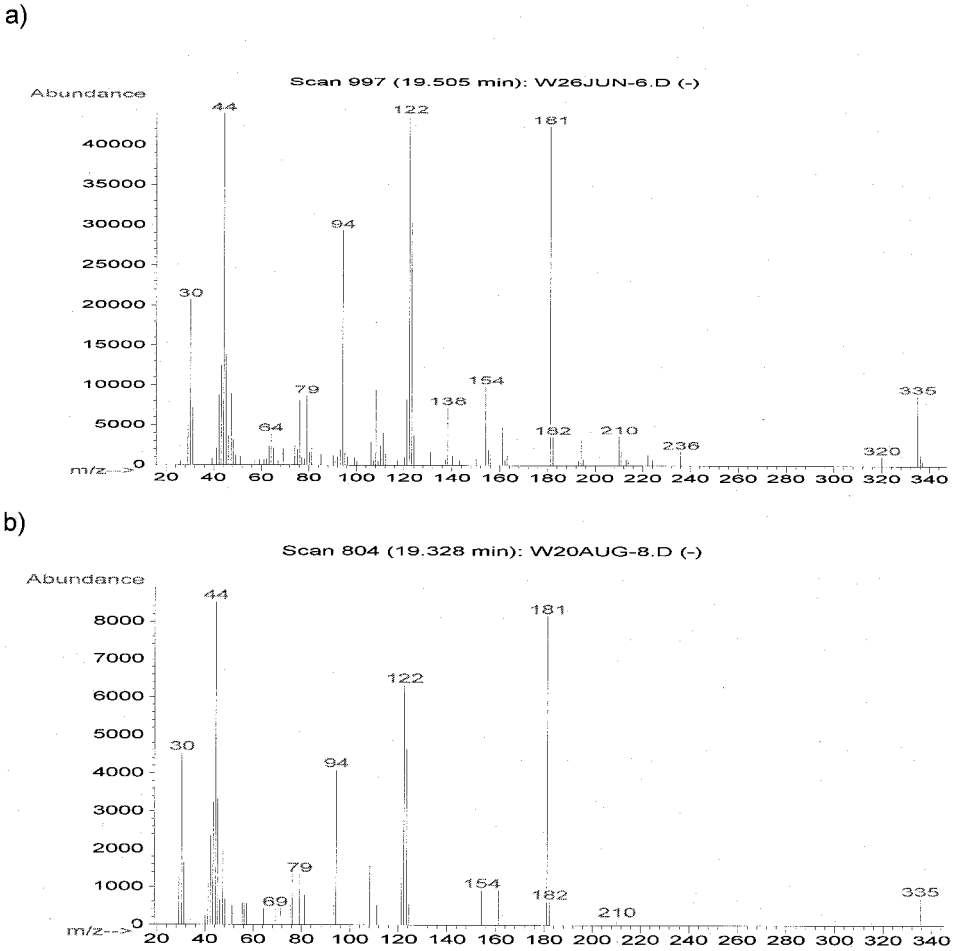


Figure 2. El^+ mass spectrum of PFBBr derivative of the putative metabolite (a) obtained by alkaline hydrolysis of propetamphos and (b) present in urine of rats exposed to propetamphos.

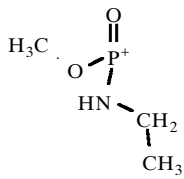
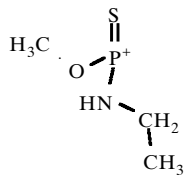
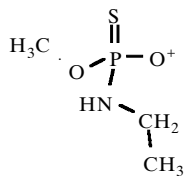
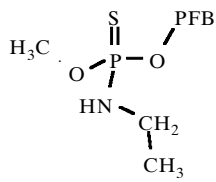
Results

Chemical hydrolysis of propetamphos and identification of the metabolite

GC–MS analysis of the chemically hydrolysed propetamphos detected a peak eluting at ~19.5 min. The fullscan mass spectrum of this peak is shown in figure 2(a). From this spectrum, a proposed structure of the compound is given in table 1. This is the pentafluorobenzyl derivative of the putative metabolite as formed by the hydrolytic splitting of the enol-vinyl–phosphate bond shown in figure 1. The sample was also analysed by GC–FPD, using the GC conditions stated by Nutley and Cocker (1993). A number of peaks were present with the dominant peak eluting at ~13 min (see figure 3(a)).

From the solid probe mass spectrometric analysis, the real-time spectra obtained by scanning the sample were combined (figure 4) and corresponded to the

Table 1. Proposed structural breakdown of the putative metabolite from its spectrum (figure 2(a)).

Mass (anu)	Proposed fragment structure
122	
138	
154	
181	PFB+
335 (molecular ion)	

structure of the underivatized putative metabolite with a molecular ion of *m/z* 155, as shown in figure 1(b).

Animal study

Figure 3 (c and d) shows GC–FPD chromatograms obtained from rat urine before and 24 h after dosing respectively. The peak at ~13 min in figure 3(d) corresponds to that seen in the hydrolysed propetamphos sample.

The exposed rat urine samples were also analysed by fullscan GC–MS in both El^+ and Cl^- modes. The spectra for the putative metabolite peak compared well with those for the hydrolysed propetamphos standard (see figures 2 and 5).

Human urine samples from exposed workers

Figure 3 (e and f) shows chromatograms from a control and an exposed worker analysed by GC–FPD. The putative metabolite is seen with a retention time of ~13 min as for the exposed rat sample and the hydrolysed propetamphos standard 3(d)

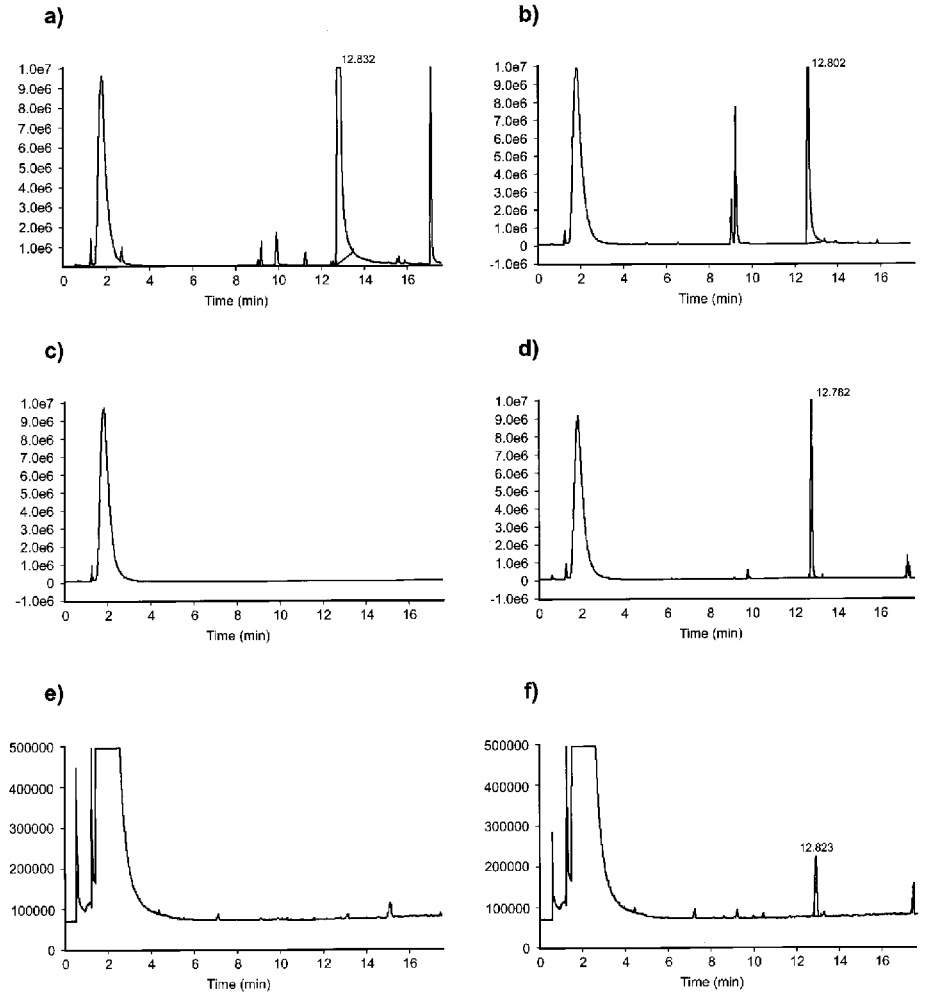


Figure 3. GC–FPD traces of (a) alkaline-hydrolysed propetamphos standard, (b) custom synthesized metabolite, (c) control rat urine, (d) exposed rat urine, (e) control human urine and (f) exposed human urine.

and 3(a) respectively). The exposed worker showed metabolite levels of 3200 (illustrated in figure 3(f) and 1280 nmol l^{-1} in successive samples.

Analysis was also carried out on archived samples from other workers exposed to propetamphos and control samples from occupationally unexposed people. The samples from workers ($n = 5$) showed some low-level exposure as indicated by the presence of the metabolite. All 20 samples from unexposed people were 'none detected' for the presence of the metabolite.

Synthesis of the putative metabolite

Analysis by Ultrafine Chemicals determined the purity of the standard to be 64% (w/w). Impurities included dimethylated and trimethylated compounds. Figure 3(b) shows the GC–FPD trace of the derivatized synthesized compound.

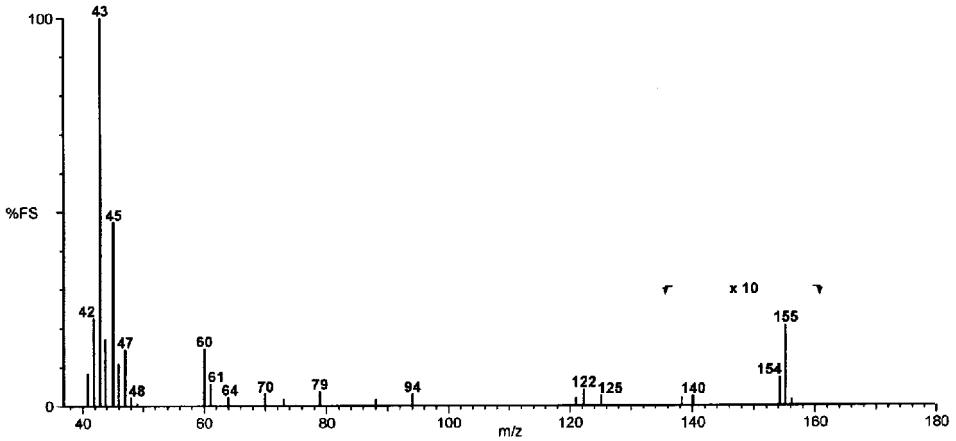


Figure 4. Combined real time spectra from solid probe analysis of the putative metabolite.

The peak seen at ~13 min corresponds to those seen in the hydrolysed propetamphos sample and the exposed rat and human urine samples.

The synthesized compound was also analysed by GC–MS. The mass spectrum obtained agreed well with that obtained for the alkaline-hydrolysed propetamphos standard, both in fragment ions detected and their relative abundance (figure 6).

Analytical method development

Sample preparation was as described for the dialkyl phosphate metabolites by Nutley and Cocker (1993). Derivatization time was optimized at 90 min at 50 °C. Repeated analysis of quality control material gave coefficients of variation of 6 % (intra-assay, $n = 10$) and 20 % (inter-assay, $n = 7$). The method was shown to be linear (defined as a least squares regression coefficient of > 0.99) over the concentration range 0–6400 nmol l^{-1} with a detection limit of 250 nmol l^{-1} (signal : noise ratio of 3).

Discussion

A metabolite of propetamphos, suitable for biological monitoring, has been identified. The metabolite is formed by hydrolysis of the enol-vinyl–phosphate bond and retains the phosphate part of the molecule. The metabolite has been synthesized and the proposed structure is consistent with the results of solid probe mass spectrometry. NMR and HPLC analysis conducted by Ultrafine Chemicals was interpreted as mostly the metabolite of interest (64 % w/w) with some di- and tri-methylated compounds also present. In addition, the proposed structure is consistent with the results seen after GC–MS analysis in both electron impact (EI^+) and chemical (Cl^-) ionization modes of a pentafluorobenzyl derivative.

The metabolite has been determined in urine from rats given a single oral dose of propetamphos (this was confirmed by mass spectrometry), and in urine from workers exposed to propetamphos.

An analytical method for this metabolite has been developed, based on the

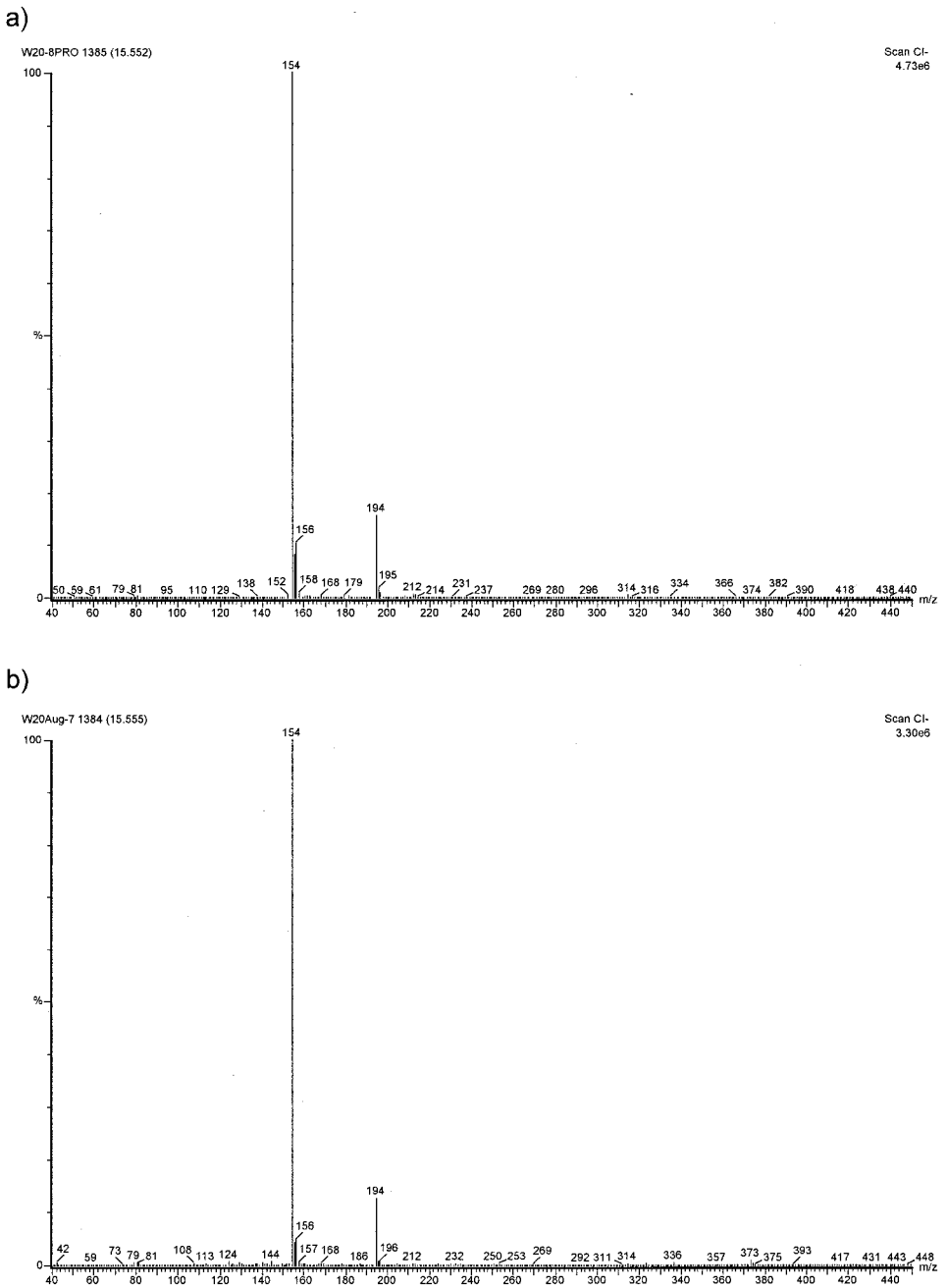


Figure 5. Cl^- mass spectrum of PFBBr derivative of the putative metabolite (a) obtained by alkaline hydrolysis of propetamphos and (b) present in urine of rats exposed to propetamphos.

method for analysis of dialkylphosphate metabolites of organophosphate pesticides by Nutley and Cocker (1993). The method involves azeotropic distillation followed by derivatization with pentafluorobenzyl bromide. Analysis is by gas chromatography–flame photometric detection. The method has been shown to be linear and reproducible with suitable sensitivity and specificity for determining

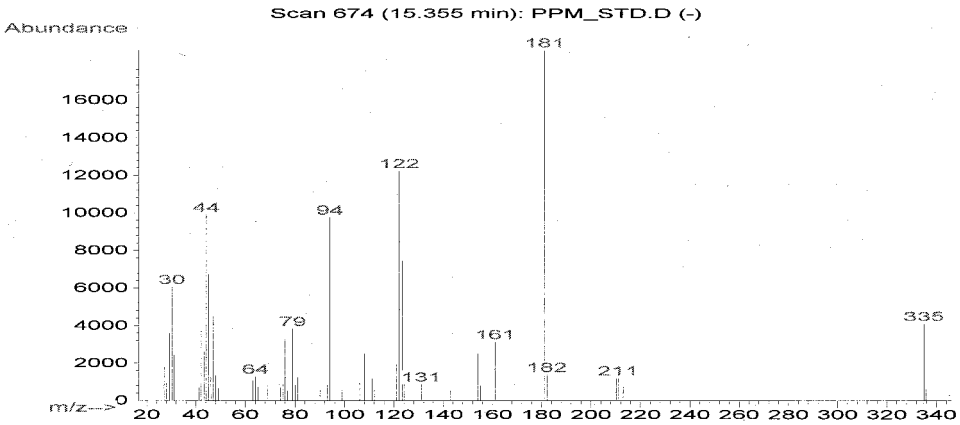


Figure 6. EI^+ mass spectrum of PFBBr derivative of the custom synthesized putative metabolite of propetamphos.

propetamphos exposure (exposed samples were positive, control samples were negative).

This metabolite and analytical method will be used to further study the human toxicokinetics of propetamphos in a controlled, human volunteer study investigating absorption by both oral and dermal exposure. These data will be published separately at a later date.

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