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The trace analysis of alkyl alkylphosphonic acids in urine using gas chromatography–ion trap negative ion tandem mass spectrometry

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Abstract

A sensitive method has been developed for the trace analysis of alkyl alkylphosphonic acids, metabolites of nerve agents, in urine using a benchtop ion trap mass spectrometer. The acids were isolated from urine by simple solid phase extraction and converted to their pentafluorobenzyl esters. An ion trap mass spectrometer in selected reaction monitoring mode provided limits of detection of 0.1 ng/ml for isopropyl, isobutyl, pinacolyl and cyclohexyl methylphosphonic acids and for ethyl ethylphosphonic acid. The detection limit for ethyl methylphosphonic acid was higher (0.5 ng/ml) due to a lower recovery.

Keywords: Nerve agents; Biomarkers; Alkyl alkylphosphonic acids

1. Introduction

1.1. Background

In cases of allegations of use of chemical warfare (CW) agents, environmental [1] or biomedical samples [2] may be collected for forensic analysis. Unequivocal identification of agents, degradation products, metabolites or covalently bound residues, provides key supporting evidence of non-compliance with the Chemical Weapons Convention. The perceived increasing threat of terrorist use of CW agents has further stimulated the requirement for forensic methods of analysis. The analysis of biomedical samples was extensively reported following two releases of the nerve agent sarin by terrorists in Japan in 1994 and 1995, e.g. [3–8], and following the use of VX for an assassination [9,10]. In addition to forensic applications, the analysis of biomedical samples may be used for diagnostic purposes, to ensure that casualties receive appropriate therapy, and for monitoring exposure to CW agents, e.g. in workers engaged in demilitarisation activities.

Urinary metabolites offer one of the simplest means of confirming an exposure to a CW agent. Urine has several advantages over other biological matrices. It is a simpler matrix than blood or tissues, and does not require invasive collection. Excretion in urine is generally the major route of elimination of an absorbed dose of a CW agent, much lesser amounts being excreted in the faeces or covalently bound to macromolecules such as protein or DNA. Disadvantages of urine are its inherent variability and the relatively rapid excretion of metabolites. Excretion is characterised by a rapid rise to maximum rate, usually within hours of exposure, and a period of 2–3 days when excretion levels are relatively high. Up to ∼90% of the total amount excreted may be eliminated in the first 48–72 h following exposure. The detection of urinary metabolites during this initial phase of elimination may not be too demanding in terms of limits of detection, as was illustrated by samples collected following the release of sarin in the Tokyo subway [5,6]. After this initial elimination phase, a more prolonged excretion of low concentrations of metabolites usually occurs [11,12], with detectable levels up to about 2 weeks after the exposure [13], depending on the severity of the exposure and the limit of detection of the analytical method. This prolonged elimination phase may arise from

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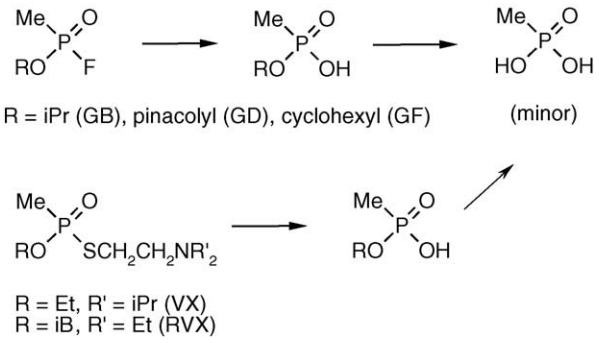

Fig. 1. The major metabolites of nerve agents derived from hydrolysis.

agent being slowly released from depots or metabolism of covalently bound residues. In cases of allegations of CW use, particularly in remote conflicts, experience has shown that samples are more likely to be collected several days or even weeks after the alleged event, and much lower detection limits are required [13]. In order to analyse samples collected from such scenarios, our laboratory generally aims to achieve detection limits for metabolites in the range 0.1–1 ng/ml. Covalently bound residues on proteins are more appropriate as longer-lived biological markers of poisoning but analysis is generally more demanding in comparison to urinary metabolites and requires the collection of blood or other tissue [2].

The primary metabolites of organophosphorus nerve agents are their simple hydrolysis products [14]. Nerve agents of the alkyl alkylphosphonofluoridate class (G agents) and *O*-alkyl *S*-dialkylaminoethyl alkylphosphonothioate class (V agents) hydrolyse predominantly to alkyl alkylphosphonic acids. This is illustrated in Fig. 1 for the most important nerve agents GB (sarin), GD (soman), GF, VX and Russian 'VX'.

1.2. Analytical methods

Alkyl alkylphosphonic acids can be analysed directly by LC–MS and CE–MS [15–19] but these techniques have generally shown modest sensitivity other than with specialised equipment [7]. The latest generation of more sensitive LC–MS instruments should continue to improve detection limits, and LC–MS–MS may eventually become the method of choice. In order to analyse low to sub-nanogram levels of phosphonic acids, using commonly available instrumentation, the preferred methods in most laboratories involve conversion to less polar, more volatile derivatives and analysis by GC–MS or GC–MS–MS. More than a dozen GC–MS and GC–MS–MS methods have been reported for the trace analysis of alkyl alkylphosphonic acids in environmental and biomedical samples [20]. A number of factors determine the limits of detection obtainable; the major ones are the derivative chosen, its method of detection, sample preparation, and the instrumentation available.

1.2.1. Choice of derivative

Trimethylsilyl (TMS), *tert*-butyldimethylsilyl (TBDMS) and methyl esters are commonly used for GC–MS analysis in environmental samples [20]. Both types of silyl derivative were successfully used in the analysis of biomedical samples from Japanese terrorist incidents [4–6,8,9], although most samples were collected within a few hours of the exposure. A simple method using methyl esters has been reported for urine analysis but aimed at rapid throughput of samples collected soon after the event rather than low limits of detection [21]. The most sensitive methods reported for urine analysis convert the acids to their pentafluorobenzyl esters to facilitate detection using negative ion chemical ionisation (NICI) [22–24]. Lowest limits of detection, at least for standards, have been reported using tandem mass spectrometry (MS–MS) in the multiple reaction monitoring mode [23]. In contrast to methylation and silylation, pentafluorobenzylation of phosphonic acids using pentafluorobenzyl bromide (PFBBr) is a relatively slow reaction. It requires more complex conditions, and a number of different procedures have been reported [22–26]. Pentafluorobenzyl esters have been used for this current method.

1.2.2. Sample preparation

A number of solid phase extraction (SPE) procedures have been used for phosphonic acids in biomedical and environmental matrices. These include extraction of the non-ionised acid on hydrophobic cartridges [22] or capture of the ionised acid on anion exchange cartridges [23,27,28]. An alternative to selective extraction is to remove extraneous materials from urine, e.g. inorganic cations can be removed from urine by cation exchange [6,24,29]. Extraneous hydrophobic materials can be removed from urine at around neutral pH by elution through a C_{18} [6] or polymeric cartridge, although some losses of the more hydrophobic acids occur [30]. Our experience has been that few of these procedures are robust, probably due to variations in SPE cartridges combined with the inherent variability of urine.

Several sample preparation procedures have been reported for use with derivatisation to pentafluorobenzyl esters. Shih et al. [22] isolated isopropyl methylphosphonic acid (iPrMPA) and the more hydrophobic pinacolyl MPA (PinMPA) and cyclohexyl MPA (CMPA) from urine using reverse phase silica based cartridges, C_{18} for iPrMPA and C_2 for PinMPA and CMPA. The use of different SPE cartridges for different analytes illustrates the problems of developing a generic procedure for analytes with broadly different hydrophobic properties. Isolated residues were derivatised with pentafluorobenzyl bromide in the presence of a crown ether. Detection limits using a quadrupole instrument were in the range 1–10 ng/ml. Fredriksson et al. [23] isolated the acids from urine using anion exchange SPE; derivatisation was achieved under modified conditions with an additional Florisil clean-up of the derivatised product. Detection limits similar to those reported by Shih et al. [22] were obtained using a benchtop quadrupole instrument. Detection of low attogram

amounts of standards was reported using a hybrid magnetic sector-quadrupole tandem mass spectrometer but detection limits were not quoted for biomedical samples using this instrument [23]. Miki et al. [24] reported an alternative clean-up of urine based on removal of cations by elution through a Sep IC-Ag cation exchange cartridge followed by phase transfer catalysed derivatisation into toluene using tri-*n*-butylmethylphosphonium bromide. Detection limits for NICI analysis on a benchtop quadrupole instrument were 0.06 ng/ml, however, chromatograms displayed for the 1 ng/ml level showed a number of extraneous peaks. This may hamper the unequivocal identification of these compounds at levels close to the detection limit. No doubt tandem mass spectrometric detection would improve the selectivity.

1.2.3. Instrumentation

One of the drawbacks to some of the methods reported is that they have been developed using expensive research grade mass spectrometers. These instruments have generally been more sensitive than benchtop systems with a greater capability for selective detection. We initially developed a method based on that of Fredriksson et al. [23], but with a modified clean-up procedure, using a hybrid magnetic sector quadrupole mass spectrometer [30]. Following decommissioning of this instrument we needed to adapt the method for use on a more basic benchtop instrument. Ion trap instruments are particularly attractive because they offer the high selectivity of tandem mass spectrometry at moderate cost.

This paper describes the development of a method suitable for screening urine samples for a range of phosphonic acids. It is based on the analysis of the acids as their pentafluorobenzyl derivatives using NICI and tandem ion trap mass spectrometry in the selected reaction monitoring mode. The method is intended to provide a high degree of confidence in the detection of these compounds at sub-nanogram levels rather than high throughput.

2. Experimental

2.1. Materials

Isopropyl methylphosphonic acid (iPrMPA), isobutyl methylphosphonic acid (iBMPA), cyclohexyl methylphosphonic acid (CMPA), ethyl ethylphosphonic acid (EEPA), and deuterated internal standards [$^2H_3$]-iPrMPA, [$^2H_3$]-iBMPA and [$^2H_3$]-pinacolyl methylphosphonic acid (PinMPA) (C[^{2}H]$_3$ linked to phosphorus) were synthesized by the Organic Chemistry Group, Dstl, Porton Down. Purities were estimated as $\geq$95% by ^{31}P NMR. Ethyl methylphosphonic acid (EMPA) and PMPA (purity 98%) were obtained from Sigma–Aldrich (Gillingham, UK). The pentafluorobenzyl esters of EMPA and PinMPA were synthesised by reaction of the acids with pentafluorobenzyl bromide and were 97% pure by ^{31}P NMR. HPLC grade acetonitrile and methanol, and pentafluorobenzyl bromide 98% pure, were obtained from Sigma–Aldrich (Gillingham, UK). Distol grade dichloromethane was obtained from Fisher Ltd., Loughborough, UK, and AnalaR grade potassium carbonate was obtained from VWR International Ltd. (Lutterworth, UK). Trifluoroacetic acid (TFA) (Pierce, Rockford, USA) was dissolved in water from a Millipore Milli-Q water system.

Stock solutions of the phosphonic acids ($\sim$0.5 mg/ml) were prepared in Milli-Q water and stored in a refrigerator. For spiking urine samples, a mixture of the acids was prepared at a concentration of 1.0 μg/ml in Milli-Q water and diluted to provide concentrations of 0.1 and 0.01 μg/ml. A stock solution of [$^2H_3$]-iBMPA was prepared in acetonitrile and diluted to 0.1 μg/ml with acetonitrile for use as a derivatisation control. Aliquots (10 μl) were added to each sample prior to derivatisation.

2.2. Sample preparation

An Oasis HLB SPE cartridge (Waters Ltd., Elstree, UK) was conditioned with acetonitrile (1 ml) followed by 1% TFA in water (1 ml). Urine (1 ml) was acidified by addition of 10% TFA in water (150 μl) and loaded onto the SPE cartridge. The cartridge was washed with 1% TFA in water (1 ml) and the analytes eluted with acetonitrile (2 × 0.75 ml). The two eluent fractions were then combined and concentrated to dryness at 50 °C under a gentle stream of nitrogen. The residue was redissolved in acetonitrile (500 μl), spiked with [$^2H_3$]-iBMPA (derivatisation control), and derivatised with pentafluorobenzyl bromide (10 μl) in the presence of potassium carbonate (20 mg), at 90 °C for 45 min. The reaction mixture was carefully evaporated to dryness under a stream of nitrogen at 50 °C and re-dissolved in dichloromethane (300 μl). The derivatised sample was added to a Varian Ltd. (Walton-on Thames, UK) 100 mg Bond-Elut Florisil SPE cartridge preconditioned with 1 ml dichloromethane. The cartridge was washed with dichloromethane to remove excess pentafluorobenzyl bromide, and the analytes eluted with 10% methanol in dichloromethane (2 × 0.75 ml) [23]. This eluent was evaporated to dryness under a stream of nitrogen and re-dissolved in dichloromethane (100 μl). For more rapid screening, the derivatised sample can be analysed directly, however, Florisil clean-up prolongs injector liner lifetime.

To assess the recovery of the acids from the SPE cartridge, urine samples (1 ml) were spiked with 10 μl of a 1 μg/ml solution of phosphonic acids either before or after the SPE extraction process. The recovery, expressed as a percentage, was calculated as the peak area ratio for the sample spiked before SPE extraction with that after extraction. Apparent reaction efficiency where quoted relates to the peak area for a urine sample compared to a control in which the same quantity of acid was spiked directly into acetonitrile, derivatised, and processed through the remaining method.

2.3. Gas chromatography–mass spectrometry

A Thermo-Finnigan GCQ ion trap GC–MS system was used for the analysis. The GC was fitted with a 30 m Restek

Table 1
Ion trap parameters used for the analysis of pentafluororbenzyl esters of alkyl alkylphosphonic acids; for all product ions excitation voltage was 1.0 V, excitation time 15 ms and *q* value Med (0.3)

Analyte	Time (min)	Number of micro scans	Multiplier offset	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)
EMPA	3.0–6.8	3	100	123	95
iPrMPA	3.0–6.8	3	100	137	95
EEPA	6.8–7.5	3	200	137	109
iBMPA	6.8–7.5	3	200	151	95
[$^2H_3$]-iBMPA	6.8–7.5	3	200	154	98
PinMPA	7.5–9.0	7	200	179	95
CMPA	9.0–17.0	7	300	177	95

Rtx-5MS capillary column (0.25 mm i.d., 0.25 μm film thickness). A 0.5 m Restek fused silica IP deactivated guard column was used before the analytical column. The injector was fitted with a 4 mm internal diameter single gooseneck injector liner deactivated with dichlorodimethylsilane. The injector was operated in splitless injection mode at a temperature of 250 °C (purge delay 1.0 min). Auto-Sep T (SGE Ltd.) low bleed septa were used in the injector. The carrier gas was helium (constant velocity 40 cm/s). The oven temperature was held at 60 °C for 1 min, programmed from 60 to 180 °C at 30 °C/min, from 180 to 280 °C at 10 °C/min and held at 280 °C for 2 min. An injection volume of 1 μl was used.

The mass spectrometer was operated using negative ion chemical ionization with methane as reagent gas. The ion trap was configured to operate in selected reaction monitoring (SRM) mode. In this mode a precursor ion is isolated in the trap and fragmented; only ions from a characteristic fragmentation are recorded. The ion trap parameters could not be optimised automatically. Each parameter was optimised using three increments over its range. A similar response was obtained for each analyte and no fine-tuning of the parameters was carried out. Scanning parameters used are summarised in Table 1. The maximum ion time for each scan was 50 ms and the ion source temperature was 180 °C. An increase in response with ion source temperature was noted, however, the instrument used has an upper limit for continuous use of 180 °C. Excitation voltage was 1.0 V, excitation time 15 ms and *q* value Med (0.3).

3. Results and discussion

3.1. Sample preparation

Derivatisation using pentafluorobenzyl bromide is preferably carried out in the absence of water and the phosphonic acids must therefore be transferred to an organic solvent. This can be achieved by solvent extraction [7], co-distillation [21], various solid phase extraction techniques [22,23,27,28] or by phase transfer processes [24]. In this work, isolation by anion exchange SPE was evaluated but found to require excessive time for concentrating the eluate (aqueous sodium bromide) containing the acids to dryness. The procedure also resulted in a relatively large inorganic residue. Extraction of the acids on a hydrophobic cartridge was selected for ease of use. A polymeric adsorbent gave better overall recoveries than C_{18}. Good recoveries (≥85%) were obtained for all the analytes except EMPA (24%). We also explored removal of extraneous hydrophobic material from urine by an initial elution through a polymeric cartridge at neutral pH, followed by extraction of the acids at low pH. Cleaner extracts were obtained but some loss of the more hydrophobic acids, PinMPA and CMPA, was observed due to extraction onto the first cartridge. The derivatisation efficiency was in the approximate range 47–73% for all the acids. Some variation was observed dependent on the urine sample. Recovery and derivatisation efficiency data for the method is shown in Table 2.

3.2. GC–MS–MS analysis

The pentafluorobenzyl derivatives give sharp, symmetrical GC peaks. They are usually intended specifically for use with NICI-MS. With positive EI, much of the ion current is concentrated in the non-characteristic ion *m*/*z* 181 $[C_6F_5CH_2]^+$. Tandem NICI mass spectrometry of pentafluorobenzyl derivatives of alkyl alkylphosphonic acids were reported and discussed by Fredriksson et al. [23]. The esters ionise by dissociative electron capture to produce a characteristic alkyl alkylphosphonate anion $[M-181]^-$. This ion, which retains the alkoxy group of the original nerve agent, is structurally characteristic of the acid and therefore useful for identification. With virtually all of the ion current concentrated in this ion, very high sensitivity can be obtained in single MS mode. CID of the alkyl alkylphosphonate anion results in a characteristic rearrangement with neutral loss of alkene from the alkoxy group, giving the corresponding

Table 2
Recoveries (Oasis HLB SPE cartridge) and derivatisation efficiencies ($n = 3$) for alkyl alkylphosphonic acids from urine

Compound	Recovery (%)	Derivatisation efficiency (%)	Overall method efficiency (%)
EMPA	24 ± 1	68 ± 8	17 ± 2
iPrMPA	85 ± 12	73 ± 18	60 ± 5
EEPA	87 ± 6	70 ± 11	61 ± 7
iBMPA	90 ± 8	56 ± 14	50 ± 8
PinMPA	98 ± 8	47 ± 9	45 ± 3
CMPA	95 ± 4	57 ± 7	55 ± 8

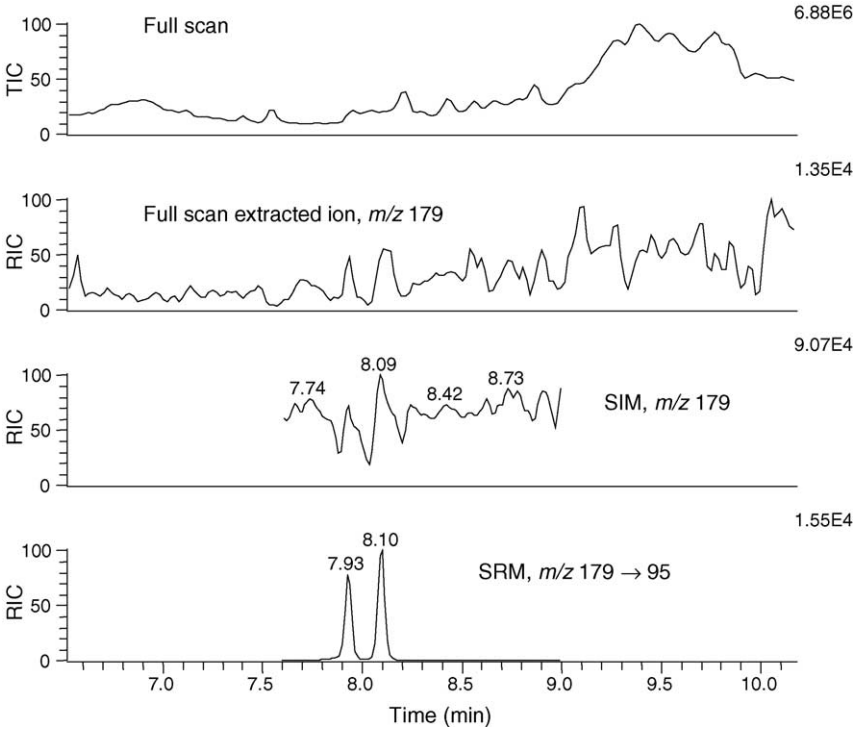

Fig. 2. Comparison of full scan, full scan (extracted ion), SIM and SRM chromatograms derived from human urine spiked with PinMPA at 5 ng/ml.

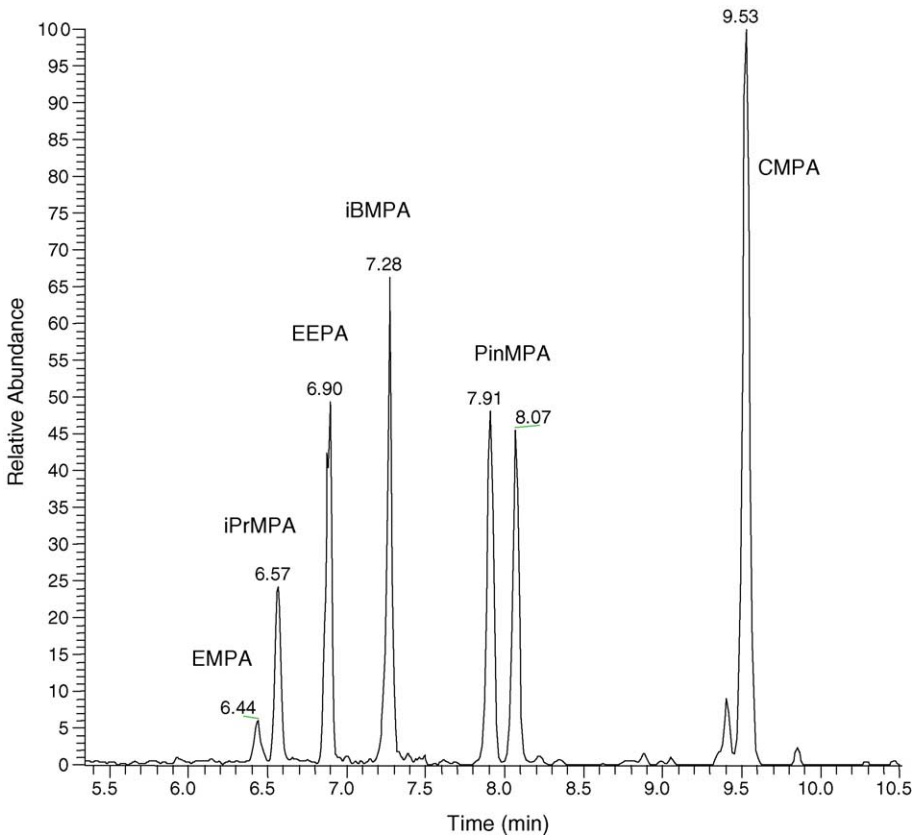

Fig. 3. Total ion chromatogram derived from SRM data showing the relative signal-to-noise ratios obtained for alkyl alkylphosphonic acids spiked into human urine at 1 ng/ml.

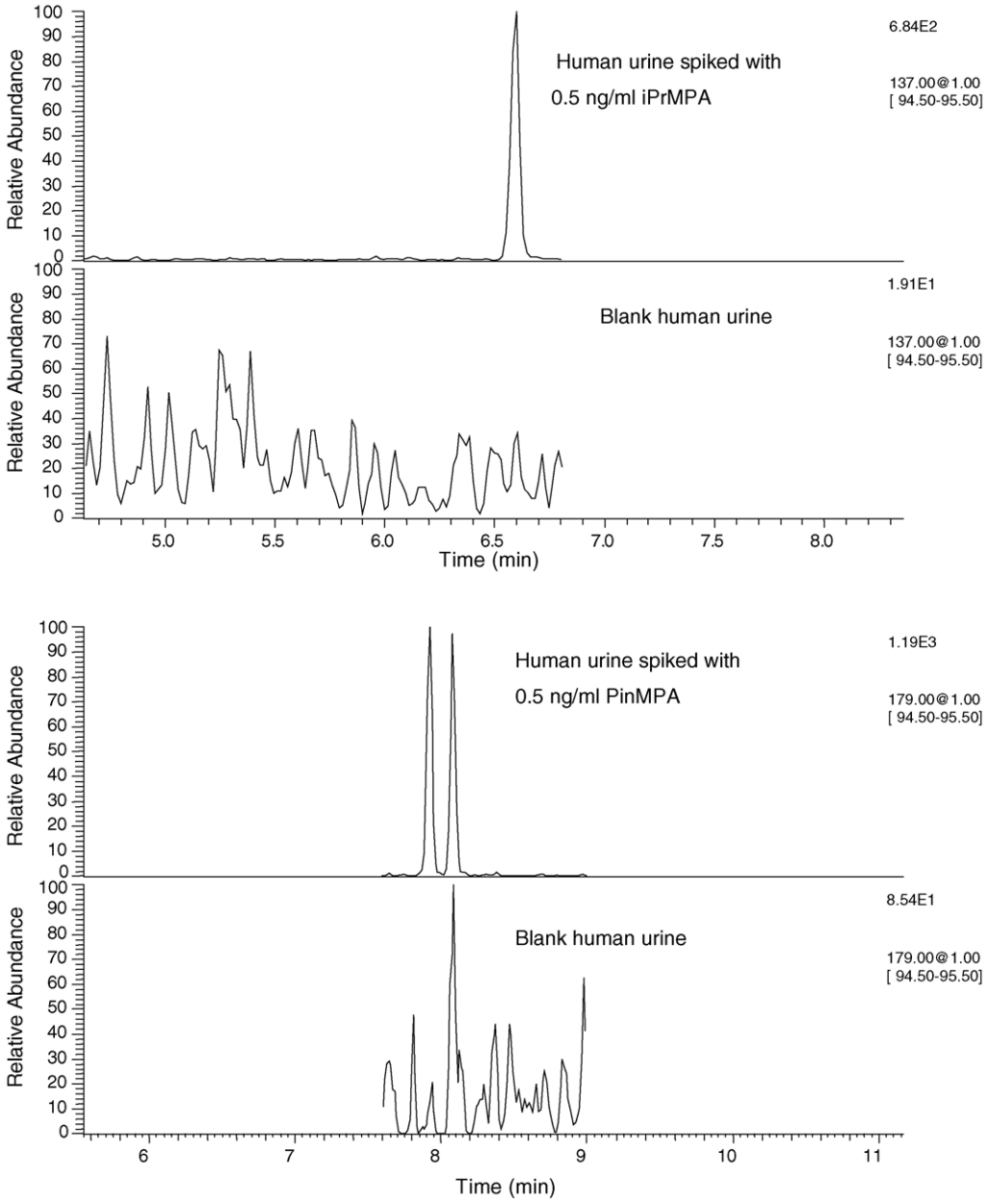

Fig. 4. SRM chromatograms derived from human urine a spiked with alkyl alkylphosphonic acids at 0.5 ng/ml and the corresponding chromatograms from a blank urine sample.

methyl ($m/z = 95$) or ethyl ($m/z = 109$) phosphonate anions. At the collision energies available on the ion trap spectrometer, this was the only fragmentation that gave product ions of significant intensity, and was used for all the analytes. Additional fragmentations can be observed at the higher collision energies available with a tandem magnetic sector instrument [23].

In full scan NICI mode, the chromatograms from urine extracts were extremely complex and the analytes of interest were only just detectable in extracted ion chromatograms at a concentration of 5 ng/ml; no improvement was observed in SIM mode. In sharp contrast, an extremely strong signal for the analytes was observed in SRM mode and chromatograms showed no extraneous peaks across a broad retention window. A comparison of full scan, SIM and SRM data for a urine sample spiked with 5 ng/ml of PinMPA is shown in Fig. 2. The double peak is the result of chromatographic separation of the two diastereoisomer pairs of Soman.

A typical chromatogram from a human urine sample spiked with alkyl alkylphosphonic acids at 1 ng/ml is shown in Fig. 3. The absence of interfering peaks using this method is demonstrated in Fig. 4 in which SRM chromatograms for the iPrMPA and PinMPA derivatives (spiked at 0.5 ng/ml) are shown alongside an unspiked urine sample.

3.3. Quantitative and quality control aspects

Although the method was intended to be primarily qualitative, linear calibrations were obtained. A calibration over the range 0.5–10 ng/ml was carried out prior to a blind spiking exercise (see Section 3.4). Two samples were processed at each calibration level. All the analytes showed acceptable linearity ($y = 23949x + 3488.9$; $R^2 = 0.9927$).

The limits of detection for iPrMPA, EEPA, iBMPA, PinMPA and CMPA were approximately 0.1 ng/ml. The detection limit for EMPA was higher (0.5 ng/ml) because of the lower recovery. As referred to above, some variability was observed due to the inherent variability of urine.

A factor affecting the sensitivity of the method is the cleanliness of the analytical system. Immediately after cleaning the ion volume and changing the injector liner and guard column, a drop in sensitivity of about 50% was observed over the first 10 injections for urine extracts. The system then became more stable and could be used for several weeks without major changes in response. Quantitative analysis was more successful when the system had been conditioned with several urine samples. The low concentration spiked samples reported here were obtained on a system that had been used for several days.

An important aspect in the confirmation of phosphonic acids in urine is obtaining a true blank signal from an unspiked sample. Contamination problems were encountered in the present work and communications from other workers in the field have indicated similar experiences. This problem has also been eluded to by other authors [23,24]. It appears that the acids bind strongly to some surfaces. There are a number of likely sources of contamination in a method requiring substantial sample preparation such as this. Underivatised phosphonic acids contaminating the GC injector have been suggested as one possible source, although the clean-up after derivatization minimises this possibility [23]. An exhaustive study of possible sources of contamination has not been carried out in the current work but experience suggests that a likely source is the solid phase extraction vacuum manifold used for processing up to twelve SPE cartridges. The taps of this equipment inevitably become contaminated and may be very difficult to clean thoroughly. Likewise, re-usable glassware may be difficult to clean to the level of providing blanks at the sub 1 ng/ml level. Our most persistent contaminant was EMPA, which is the analyte most poorly retained by the SPE cartridge. It seems likely that the standard washing procedures did not remove traces of this analyte from the taps before the manifold was used for the later Florisil clean-up part of the method. True blank signals were reliably achieved in the present work only when samples were processed individually without the vacuum manifold and no re-usable glassware was used in the method preparation. This clearly lengthens sample preparation time for multiple samples, but without going to these lengths it may be difficult to

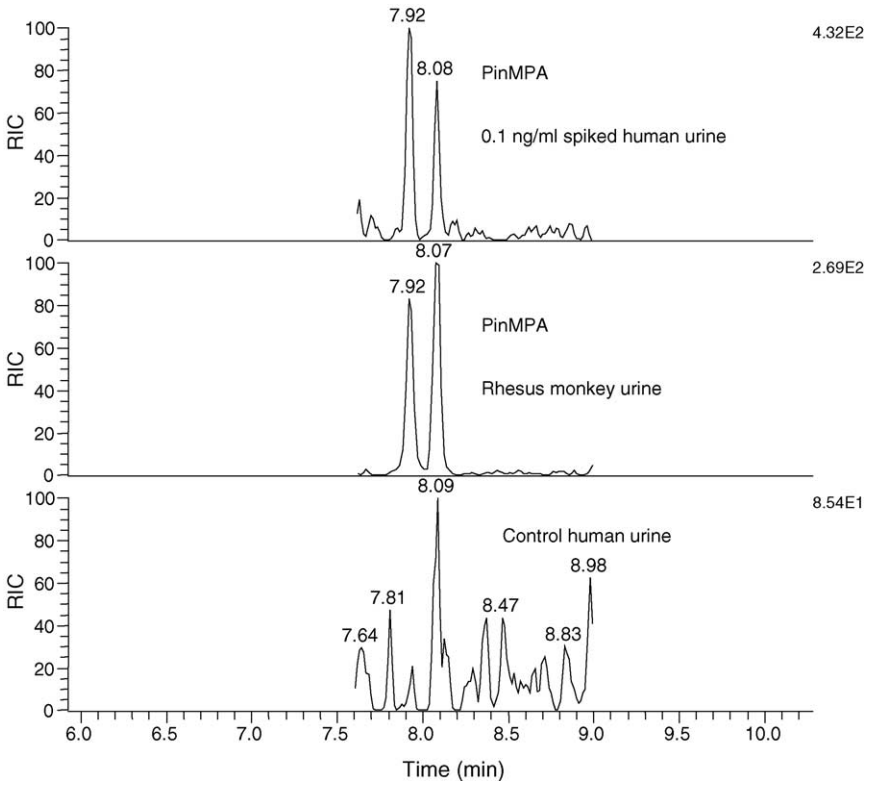

Fig. 5. SRM chromatograms demonstrating the detection of PinMPA (~0.2 ng/ml) in the urine of a Rhesus monkey, 11 days following intramuscular administration of soman ($0.5 \times LD_{50}$) as part of a medical countermeasures study.

Table 3
Analytical results from a blind spiking exercise

Sample	Analytes spiked	Concentration (ng/ml)	Analytes identified	Concentration (ng/ml)
A	iPrMPA	1.0	iPrMPA	1.0
	CMPA	2.0	CMPA	2.9
B	EMPA	2.0	EMPA	1.5
	iPrMPA	0.5	iPrMPA	0.5
	EEPA	0.5	EEPA	0.7
C	Blank	–	Blank	–
D	iBMPA	1.0	iBMPA	1.0
	PinMPA	1.0	PinMPA	0.8

unequivocally identify sub-nanogram levels of phosphonic acids.

3.4. Blind spiking exercise

The method was tested in a blind spiking exercise, in which four urine samples were spiked with alkyl alkylphosphonic acids in the concentration range 0.5–10 ng/ml. A calibration graph was plotted over this range and estimates of the concentration of the analytes given. The results are given in Table 3. All the analytes were correctly identified.

3.5. Application to animal study

The low limits of detection of the method, and its potential for retrospective identification of nerve agent poisoning in samples collected several days after an exposure was demonstrated by the detection of PinMPA in the urine of a Rhesus monkey being used in a study of medical countermeasures. The urine sample was collected 11 days after intramuscular administration of 0.5× an estimated LD_{50} dose of soman. A chromatogram, plus a control and spiked sample of human urine, are shown in Fig. 5. An estimate of the concentration was approximately 0.2 ng/ml.

4. Conclusions

The method described provides very low limits of detection for urinary metabolites of nerve agents on a relatively inexpensive benchtop GC–ion trap MS instrument. The use of tandem mass spectrometry with multiple reaction monitoring provides a high degree of specificity. A disadvantage is the relatively poor recovery for the least hydrophobic analyte EMPA. Although a sub-nanogram detection limit is readily achieved for this analyte an alternative sample preparation procedure would be required to achieve the 0.1 ng/ml detection limit obtained for the other analytes. The method should be applicable to human urine samples collected several days after a suspected exposure in cases of allegations of CW use.

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