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Negative ion chemical ionization GC/MS-MS analysis of dialkylphosphate metabolites of organophosphate pesticides in urine of non-occupationally exposed subjects

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Low level exposure to organophosphate (OP) pesticides can be determined by the measurement of dialkylphosphate (DAP) metabolites in urine. An analytical method is presented here which can measure the metabolites dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP) at low levels. This was achieved by lyophilization of the urine, derivatization with pentafluorobenzyl bromide (PFBBr) and quantification by negative ion chemical ionization GC/MS-MS. The detection limits for the metabolites were 0.5 μg L^{-1} DMP, 0.1 μg L^{-1} DEP, 0.1 μg L^{-1} DMTP, 0.04 μg L^{-1} DMDTP, 0.04 μg L^{-1} DETP and 0.02 μg L^{-1} DEDTP. The RSD for the analytical method was 4–14% for the six metabolites. The method was used to monitor a group of non-occupationally exposed individuals in Sydney, Australia. The metabolites DMP, DEP, DMTP, DMDTP, DETP and DEDTP occurred in 73, 77, 96, 48, 100 and 2% of the samples with median values of 13, 3, 12, < 1, 1 and 1 μg L^{-1} respectively. The method is simple to use, sensitive and suitable for routine analysis of non-occupational exposure levels. These detection limits are between one and two orders of magnitude lower than those previously reported in the literature.

Introduction

Currently, there are approximately 700 different products containing organophosphate (OP) pesticides commercially available in Australia. Since the 1970s the gradual phasing out of organochlorine pesticides in most developed countries has caused an increase in the manufacture, sale and application of OP pesticides. Consequently, there has been an increasing level of concern with exposure to these pesticides by the general community. Therefore, it is timely that analytical methods such as the one presented here be developed to monitor low level exposures.

Exposure to OP pesticides is usually determined by measuring a reduction of the cholinesterase enzyme activity in blood.[1] However, this method lacks sensitivity to low level exposure, it has a need for the establishment of a subject's unexposed baseline activity level, and it has a wide reference range for unexposed subjects.

Other approaches in the past have been directed towards the detection and quantification of the OP pesticide itself or one of its phenolic metabolites in blood, plasma or urine. Examples are the analysis of trichloropyridinol in urine to monitor the exposure to chlorpyriphos,[2] and the analysis of *p*-nitrophenol in urine for the exposure to parathion.[3–5] These assays have all employed the conventional technique of solvent extraction. This type of technique is not easily applied to DAP metabolite analysis, as the metabolites are ionic in nature. The structures of the DAP metabolites studied in this paper are shown in Fig. 1.

The DAP metabolites are strongly acidic, and have their pK_a values in water or aqueous alcohol in the range of pH 1 to 2.[6–8] Since the pH of urine is normally in the range of pH 4 to 8,[9] the metabolites are usually present in the ionised form. This makes the extraction of the metabolites into an organic phase difficult. However, extraction of the DAP metabolites from blood, serum and urine was achieved using phase transfer extraction by Miki *et al.*[10] and with sodium chloride saturation by Drevenkar *et al.*[11] Nutley and Cocker[12] reported extraction by using azeotropic distillation of water with acetonitrile. However, this technique is very labour intensive. A more convenient technique has been employed by Peterson[13] who lyophilized the urine samples.

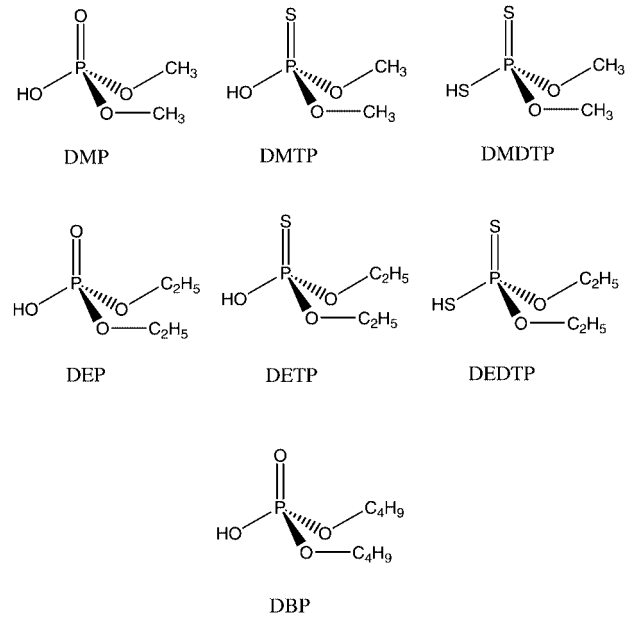

Fig. 1 Dimethyl and diethyl phosphate metabolites of OP pesticides and the internal standard dibutyl phosphate.

Several derivatization procedures have been developed for gas chromatography analysis of the urinary DAP metabolites. These have included the use of diazoalkanes such as diazomethane by Drevenkar *et al.*,[11] diazopentane by Bradway *et al.*[14] and Knaak *et al.*[15] The diazoalkane derivatization can suffer from the occurrence of possible side reactions. Pentafluorobenzyl bromide derivatization gives good results and has been employed by Jauhiainen *et al.*,[16] Nutley and Cocker,[12] Lin *et al.*,[17] Moate *et al.*,[18] Aprea *et al.*[19] and Miki *et al.*[10] Different researchers have used many different detection methods. These have included nitrogen/phosphorus or flame ionisation detection by Bradway and Moseman[20] and Vasilic *et al.*,[21] flame photometric detection by Nutley and Cocker[12] and Shafik *et al.*,[22] and electron capture detection by Miki *et al.*,[10] Bradway and Moseman[20] and Lin *et al.*[17] GC/MS has been employed by Miki *et al.*,[10] Lin *et al.*,[17] Park *et al.*[23] and Hardt and Angerer.[24] At present, there have been no reports in the literature of any other researchers using GC/MS-MS to detect DAP metabolites in urine.

Direct determination of DAP metabolites in urine by HPLC using UV/VIS detection is not possible due to the absence of a chromophore in the molecules. However, after separation using an ion pairing reagent and post-column derivatisation, detection limits in the low mg L^{-1} levels were achieved by Priebe and Howell.[25] Techniques, such as negative ion thermospray LCMS and LC/MS-MS, have also been used for mg L^{-1} levels of the metabolites in aqueous solutions.[26]

In this paper we report a method of analysis for six DAP metabolites of OP pesticides in urine with significantly improved limits of detection. This is achieved by lyophilization of urine, derivatization with pentafluorobenzyl bromide (PFBBr) and quantification by negative ion chemical ionization GC/MS-MS.

Experimental

Reagents and chemicals

Reference materials of DMP, DMTP, DETP, DMDTP and DEDTP were obtained from the US EPA, (Research Triangle Park, NC, USA); DEP and dibutylphosphate (DBP) were from ChemService, (West Chester, PA, USA); pentafluorobenzyl bromide (PFBBr) and anhydrous potassium carbonate were obtained from Merck (Darmstadt, Germany); acetonitrile with 0.006% water content was from EM Science (Gibbstown, NJ, USA).

Apparatus

Samples were analysed on a Finnigan/MAT TSQ 46 GC/MS-MS (San Jose, CA, USA) in negative ion chemical ionization mode with a 7673A Hewlett Packard autosampler (Palo Alto, CA, USA). The capillary column, AT-5 (30 m length × 0.32 mm id × 0.25 μm film thickness), was obtained from Alltech Associates Inc. (Deerfield, IL, USA) and was inserted directly into the ion source of the mass spectrometer. Ultra high purity (UHP) helium was used as the carrier gas at a flow rate of 2 mL min^{-1} and UHP methane was used as the chemical ionization (CI) reagent gas at a total ion source pressure of 0.9 Torr (120 Pa). Both gases were obtained from BOC HiTech Gases (Sydney, Australia). The injection port liner was replaced after 75 samples as sensitivity decreased slowly beyond this number of injections.

Instrumental conditions

The experimental parameters were evaluated after initial MS/MS experiments were conducted to determine the product ions of interest. The optimal ion source temperature for the most abundant product ion formation was 140 °C. The optimal collision pressure and collision energy were 2.0 mTorr (0.2 Pa) and 10.0–15.0 eV, respectively. UHP Argon from BOC, HiTech Gases (Sydney, Australia) was used for the collision experiment.

Gas chromatography

The temperature program for this analysis had an initial temperature of 80 °C held for 0.5 min, then increased at 8 °C min^{-1} to 240 °C then at 25 °C min^{-1} to 300 °C. The injection port temperature was held at 220 °C. The injection volume of 1 μL was made in the splitless mode with the split valve only being opened 1 min after the injection. The sweep valve was left open continually to minimise contamination of the septum and injector. Using this temperature program, the pentafluorobenzyl (PFB) derivatives of the DAP metabolites had retention times of DMP 9.20 min, DEP 11.10 min, DMTP 12.00 min, DMDTP 13.20 min, DETP 13.34 min, DEDTP 14.42 min and the internal standard DBP had a retention time of 16.06 min.

Freeze-drying

Lyophilization was performed using a Dura-Top™ freeze-dryer (FTS Systems, New York, NY, USA) in 14–16 mL capacity vials. The freeze-dryer was operated in the program mode. For a run of up to 100 samples, the program was as follows: 60 min initial freezing at −50 °C; 30 min at 20 mTorr and −40 °C; 360 min at 20 mTorr and −30 °C; 300 min at 20 mTorr and 0 °C; and finally for 180 min at 10 mTorr and +16 °C. Such an extended program was necessary to achieve complete dryness of the urine samples. This lyophilization procedure took approximately 16–24 h and was normally performed overnight.

Derivatization procedure

A 2 mL aliquot of each urine sample and standard was spiked with an internal standard of 100 μL of 10 mg L^{-1} DBP solution in acetonitrile. This was then subjected to the lyophilization procedure. The derivatizing reagent PFBBr was prepared by mixing 5 mL of pure reagent with 15 mL of acetonitrile. After the urine samples had been lyophilized, 2 mL of acetonitrile, 50 mg of anhydrous potassium carbonate and 100 μL of PFBBr reagent solution were added to the dried urine extract in each vial. Vials were then capped and heated to 60 °C while being stirred on a magnetic stirring block for 4 h. The samples were then cooled and a portion was transferred to a GC vial ready for chromatographic analysis.

Standard preparation and reporting

Standards were prepared by spiking a urine at three concentration levels in the range 40–500 μg L^{-1} with the six DAP metabolites. The standard curves were prepared by using the spiked and blank urines. The linear range of each metabolite was determined with 9 calibration standards in the range 10–700 μg L^{-1} and can be seen with regression statistics in Table 1. The calibration curves were blank corrected as background amounts of some of these metabolites can be found in urine. Standards must be made in the urine matrix as other matrices give different recoveries. All samples were analysed in duplicate and the means reported.

Results and discussion

The high sensitivity and ease of use of this method was achieved by the lyophilization of the urine, the optimization of the derivatization reaction conditions, and by the careful selection of the mass spectrometer conditions.

The lyophilization of urine was used because it was the simplest way to extract the DAP metabolites from an aqueous phase without lengthy liquid/liquid or liquid/solid extraction techniques. It lends itself to routine analysis and does not require a highly skilled analyst to perform the task.

The PFBBr reagent was used for the derivatization of the DAP metabolites because it formed only one reaction product with each metabolite. The previously reported use of diazoalkane reagents gave multiple reaction products.[11,14,15] Specifically, diazomethane and diazoethane derivatized inorganic phosphate to DMP and DEP, respectively, and gave false positives or larger positives of these metabolites than should have been reported. The reaction time and temperature were studied in more detail. As previously reported,[18,19] it was observed in this study that the reaction rates of sulfur containing metabolites and those of non-sulfur containing metabolites differed markedly. The sulfur containing metabolites reacted easily at ambient temperature, but DMP, DEP and DBP did not, and required a temperature of 90 °C to react in a reasonable time. However, at elevated temperatures the sulfur containing metabolites were observed to break down and possibly lose the sulfur atom to form DMP or DEP reaction products. Therefore, the reaction conditions of 60 °C for 4 h were the optimum to yield the highest amounts of each metabolite in one derivatization step. These conditions gave the following derivatization recoveries of 39% DMP, 58% DEP, 82% DMTP, 100% DETP, 100% DMDTP, 87% DEDTP, and 82% DBP.

These conditions gave the best compromise between the full reaction of DMP, DEP and DBP and the loss of the sulfur containing compounds. The conditions also allowed the reaction to be performed in a reasonable amount of time without having to separate the sulfur metabolites by extraction before they degraded.

If a particular study only required the quantification of the metabolites DMP and DEP then the reaction time could be lengthened to about 18–24 h at 60 °C to allow full reaction of these metabolites and hence improve recoveries. However, these results will be confounded if any of the other sulfur containing metabolites are present because prolonged heating can lead to the formation of DMP and DEP. Hence, this modification would not yield valid results as all urine samples in our study contained small amounts of these sulfur containing metabolites. This problem was addressed by Miki *et al.*,[10] Moate *et al.*,[18] and Aprea *et al.*[19] who first reacted the sulfur containing metabolites with the PFBBr at a lower temperature, then extracted these metabolites and then followed with a further reaction at 90 °C to react the DMP and DEP to completion. This type of procedure was deemed to be too labour intensive and not amenable for routine analysis.

The high sensitivity of our method was achieved by the use of negative ion chemical ionization GC/MS-MS. The tuning of the mass spectrometer was conducted to give the formation of optimum amounts of precursor and product ions. These conditions are listed earlier in this paper. The precursor and product ions monitored for each derivatized DAP metabolite are shown in Table 2. The unequivocal identification of the metabolites was obtained from the retention times and the MS fragmentation pattern of precursor ions [M-PFB]. From the fragmentation pattern the two most specific product ions were selected, the ratio of these ions was compared to standards, and quantification was achieved to low levels.

The limits of detection and the upper limits of the linear concentration ranges can be seen in Table 1 for each DAP metabolite. The limits of detection were based on the level of the noise plus three standard deviations of that noise. The method shows good linearity for each metabolite to its limit value with a squared correlation coefficient (r^2) of 0.97 or better. The linearity range of the method for all analytes can be extended 10-fold by diluting the final solution with acetonitrile 1 : 10 prior to GC/MS-MS analysis. The limits of detection ranging from 0.5 μg L^{-1} for DMP down to 0.02 μg L^{-1} for DEDTP are sufficient to measure environmental exposure to OP pesticides. These detection limits are between one and two orders of magnitude lower than those previously reported by the GC/MS technique.[10,23,24] Miki *et al.*[10] reported detection limits ranging from 250–500 μg L^{-1}, Park *et al.*[23] reported detection limits ranging from 50–100 μg L^{-1} and Hardt and Angerer[24] reported detection limits ranging from 1–5 μg L^{-1}. The relative standard deviations (RSD) of the calibration curves for this method can also be seen in Table 1 and range from 4–14% for the metabolites.

Stability of the analytes was determined using pooled male urine spiked with 100 μg L^{-1} of each DAP metabolite. After storage of urine at −18 °C, there was no statistically significant loss of any of the six DAP metabolites over 21 days.

Table 1 Limits of detection, limits of linearity, squared correlation coefficients and relative standard deviations of negative ion chemical ionization GC/MS-MS analysis of DAP metabolites as their PFB derivatives

Metabolite	Limit of detection/μg L^{-1}	Upper limit of linearity/μg L^{-1}	r^2	RSD (%) (n = 8)
DMP	0.5	500	0.9980	12
DEP	0.1	250	0.9886	11
DMTP	0.1	100	0.9698	14
DMDTP	0.04	100	0.9968	5
DETP	0.04	100	0.9820	9
DEDTP	0.02	100	0.9939	4

Non-occupationally exposed population study

Urinary DAP data for 48 non-occupationally exposed subjects, determined by the current method, are compared with those of two published studies in Table 3 using summarised analytical results. The study presented in this paper is quite comparable to

Table 2 Ions used for detection and quantification of DAP metabolites as PFB derivatives

			Product ions			
Metabolite	Precursor ion	m/z	Ion 1	m/z	Ion 2	m/z
DMP	$[(CH_3O)_2PO_2]^-$	125	$[(CH_3O)PO_3]^-$	110	$[PO_3]^-$	79
DEP	$[(C_2H_5O)_2PO_2]^-$	153	$[(C_2H_5O)HPO_3]^-$	125	$[PO_3]^-$	79
DMTP	$[(CH_3O)_2PSO]^-$	141	$[(CH_3O)PO_2S]^-$	126	$[PO_2S]^-$	95
DETP	$[(C_2H_5O)_2PSO]^-$	169	$[(C_2H_5O)HPO_2S]^-$	141	$[PO_2S]^-$	95
DMDTP	$[(CH_3O)_2PS_2]^-$	157	$[(CH_3O)POS_2]^-$	142	$[POS_2]^-$	111
DEDTP	$[(C_2H_5O)_2PS_2]^-$	185	$[(C_2H_5O)HPOS_2]^-$	157	$[POS_2]^-$	111

Table 3 Comparison of urinary DAP metabolites of non-occupationally exposed subjects found in the current study (n = 48) with data from previous studies by Hardt and Angerer[24] (n = 54) and Aprea *et al.*[27] (n = 124). Summary statistics were calculated using logarithmic transformation and a value of half the detection limit was used for samples where the results were below the detection limit

Metabolite	Mean/μg L^{-1}	Median/μg L^{-1}	Maximum/μg L^{-1}	Standard deviation/μg L^{-1}	Found (%)	Reference
	9	13	134	5	73	Current study
DMP	NA	30	322	54	96	Hardt and Angerer
	12	9	91	13	87	Aprea *et al.*
	3	3	362	5	77	Current study
DEP	NA	4	46	11	94	Hardt and Angerer
	6	4	82	9	82	Aprea *et al.*
	10	2	70	4	96	Current study
DMTP	NA	22	324	89	100	Hardt and Angerer
	16	10	91	16	99	Aprea *et al.*
	1	< 1	141	8	48	Current study
DMDTP	NA	1	51	4	89	Hardt and Angerer
	5	4	21	4	48	Aprea *et al.*
	2	1	202	4	100	Current study
DETP	NA	< 1	55	8	46	Hardt and Angerer
	5	4	17	4	73	Aprea *et al.*
	1	1	1	NA[a]	2	Current study
DEDTP	NA	< 1	19	< 1	2	Hardt and Angerer
	3	2	10	3	7	Aprea *et al.*

NA – not available. [a] Insufficient positive results to calculate a standard deviation.

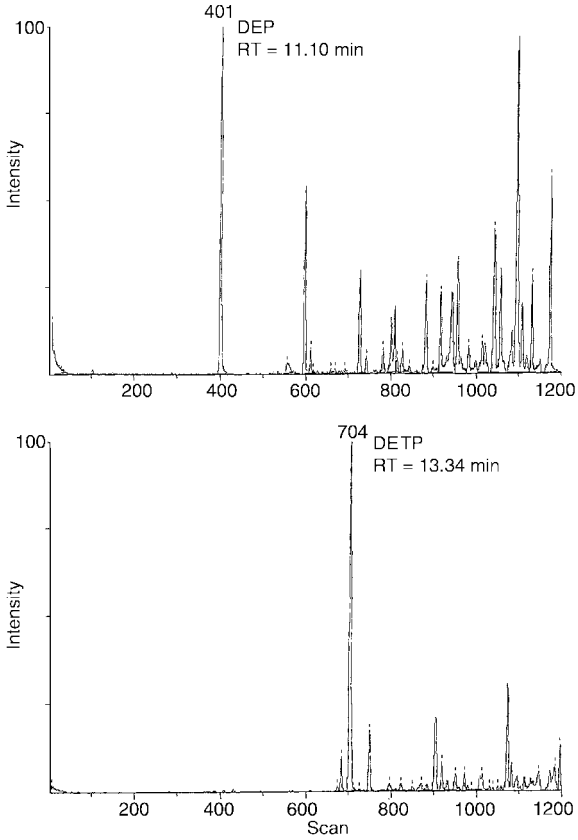

Fig. 2 GC/MS-MS extracted ion chromatograms of a urine sample from a non-occupationally exposed subject. The dialkylphosphate metabolites found were DEP (scan no. 401, RT = 11.10 min) and DETP (scan no. 704, RT = 13.34 min) at the levels of 227 and 114 μg L^{-1}, respectively. The other peaks on the chromatograms were due to other compounds found in the urine matrix which were not identified.

an Italian study by Aprea *et al.*[27] and a recent German study by Hardt and Angerer.[24] These studies all had high percentage positives > 73% for DMP and > 77% for DEP and similar median values of 9 to 30 μg L^{-1} DMP and 3 to 4 μg L^{-1} DEP. Another metabolite DMTP occurred in all three studies in nearly all samples with a median value from 10 to 22 μg L^{-1}. The current study found all samples to contain the metabolite DETP with a median value of 1μg L^{-1}. Surprisingly one sample in the study contained all six metabolites. Examples of extracted ion chromatograms from a urine sample in this study are presented in Fig. 2. The DAP metabolites found were DEP and DETP at the levels of 227 and 114 μg L^{-1}, respectively.

Conclusion

The analytical method described is simple, sensitive and suitable for routine analysis. It can be used to monitor environmental background levels of these metabolites in urine. It represents an improvement on previously published methods in its ease of use and its low detection limits.

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