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Simultaneous determination of six dialkylphosphates in urine by liquid chromatography tandem mass spectrometry

S. Dulaurent [a], F. Saint-Marcoux [a], P. Marquet [a,b], G. Lachâtre [a,c,*]

[a] *Department of Pharmacology and Toxicology, University Hospital, 2 Avenue Martin Luther King, 87042 Limoges, France*
[b] *Laboratory of Pharmacology, Faculty of Medicine, 2 Rue du Docteur Marcland, 87000 Limoges, France*
[c] *Laboratory of Toxicology, Faculty of Pharmacy, 2 Rue du Docteur Marcland, 87000 Limoges, France*

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Abstract

Dialkylphosphates (DAP) are urinary markers of the exposure to organophosphates pesticides. The aim of this study was to develop a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantitative determination of the following DAP: dimethylphosphate (DMP), dimethythiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP). Dibutylphosphate (DBP) was used as internal standard. This method was based on a liquid–liquid extraction procedure, a chromatographic separation using an Inertsil ODS3 C18 column and mass spectrometric detection in the negative ion, multiple reaction monitoring (MRM) mode, following two ion transitions per compound. It yielded a limit of quantification of 2 μg/L for the six compounds and intra-assay coefficients of variation (CV%) lower than 20%. This method was applied to the analysis of urines samples from a small cohort of non-exposed volunteers. At least one of the six DAP was detected in each sample. This result confirmed the feasibility of a LC–MS/MS procedure for monitoring the general population exposure to some frequently employed organophosphate pesticides.

Keywords: Dialkylphosphates; Organophosphorus pesticides; Urine; Liquid chromatography; Tandem mass spectrometry

1. Introduction

Organophosphates form an old family of pesticides still widely used today. In France, about 30 organophosphates are still commercialised in 2005 for agricultural [1] or domestic purposes. Most of them are used for their insecticide properties, such as chlorpyriphos-ethyl and methyl, malathion or dimethoate [1]. As organophosphates inhibit the activity of cholinesterases and can be very injurious for human health [2], the monitoring of the exposure to organophosphates has been classically performed using the determination of plasma cholinesterase activity in plasma and acetylcholinesterase activity in erythrocytes [2,3]. Overexposure to organophosphates is suspected when human plasma or erythrocyte enzymic activity is less than 85% of the population mean value. However, considering the great inter-individual variability observed in large populations, it would require knowing the physiological cholinesterase activity before any exposure in each individual, for it to be really an accurate biomarker of the exposure to organophosphates [3].

Organophosphates are rapidly metabolised in blood (e.g., dichlorvos half-life is 0.29 h in vitro at 37 °C [4]), suggesting that the monitoring of parent compounds in this matrix is inappropriate in most cases. On the contrary, concentrations of organophosphates and/or their metabolites, the dialkylphosphates (DAP), are usually much higher and detectable for a longer period of time in urine [5]. Moreover, it has been reported that approximately 75% of organophosphates yield the following DAP (Fig. 1) [6,7]: dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMTP), diethylphosphate (DEP), diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP). Therefore, another approach to the monitoring of organophosphates is based on the determination of these DAP in urine [6–31]. Numerous methods have been proposed in this field [6–25]. Most of them are gas chromatography methods requiring derivatization prior to the analysis. Classically, pentafluorobenzylbromide (PFBBr) has been employed as

* Corresponding author. Present address: Service de Pharmacologie et Toxicologie, CHU Dupuytren, 87042 LIMOGES Cedex, France.
E-mail address: lachatre@unilim.fr (G. Lachâtre).

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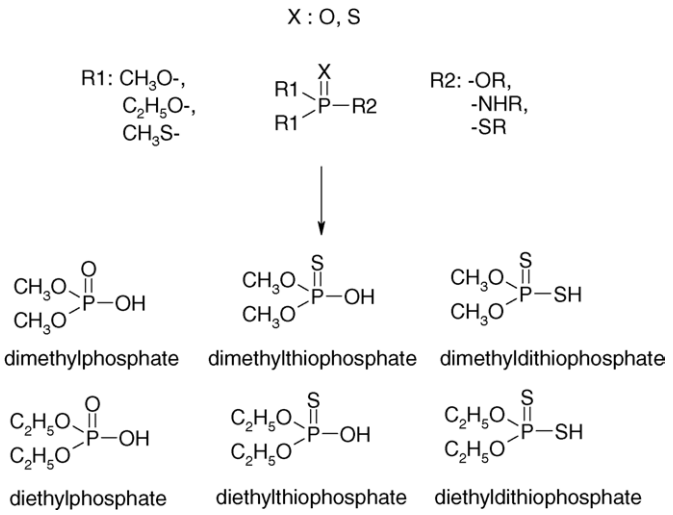

Fig. 1. General metabolism of organophosphate pesticides.

derivatization agent [7–12,23]. However, two different temperature conditions are required for the derivatization of DAP using this product: DAP without a sulphur atom require a higher temperature (90 °C) than that of DAP with sulphur atoms, which must be employed at ambient temperature to prevent the loss of the sulphur atoms [7–10,12]. Some authors [9,10,12] have proposed to perform two derivatization steps: one at ambient temperature and one at 90 °C. Intermediate temperatures (40 and 50 °C) have also been proposed to derivatize simultaneously the DAP [7,8]. Diazoalkanes (such as diazomethane and diazopentane) were used in a few methods as derivatization reagents [13–15,18]. However, they are explosive and carcinogenic, they are not specific and gives mixed products with alkylating agents [9]. These multiple steps procedures are obviously cumbersome and time-consuming.

To the best of our knowledge, only three liquid chromatography techniques have been published for the determination of DAP [16,24,25]. Bardarov et al. [16] proposed a study using an anion-exchange column coupled to either electrochemical or spectrophotometric detection. However, this study reported a moderate molar absorbance of DMTP and DMDTP, and a negligible molar absorbance of DMP in the non-specific region of the UV spectrum (200–220 nm), that decreased dramatically both the sensitivity and selectivity of this detection mode [16]. Additionally, electrochemical detection did not seem to be appropriate because DMP and DEP are electrochemically inactive. Recently, Hernandez et al. proposed liquid chromatography–tandem mass spectrometry (LC–MS/MS) procedures for the determination of four DAP on the one hand [24], and two DAP in association with some specific metabolites on the other hand [25]. In a recent review article, Hernandez et al. also illustrated the advantages of LC–MS/MS methods for the identification, the quantification and the confirmation of pesticides and their metabolites in biological samples [32].

In the present study, a simple and fast LC–MS/MS procedure was developed for the simultaneous determination of six DAP. To evaluate its performance, the whole analytical procedure was applied to urine samples collected from a group of volunteers.

2. Experimental

2.1. Chemicals

DMP (98%), DEP (75%), and dibutylphosphate (DBP) (97%) were obtained from ACROS (Noisy-le-grand, France). DMTP (95%) and DMDTP (85%) as their sodium salts were supplied by Applichem (Darmstadt, Germany). DETP (98%) as potassium salt and DEDTP (90%) were purchased from ALDRICH (St Quentin Fallavier, France). Methanol was purchased from Carlo Erba Reagenti (Rodano, Italy), diethylether (Normapur), ethyl acetate (Suprasolv, MERCK) and acetonitrile (Pestinorm) from Prolabo (Fontenay-sous-bois, France), and ammonium formate from Fluka (St-Quentin-Fallavier, France). Water was purified with a Millipore Direct Q purification system (St-Quentin-en-Yvelines, France).

A stock standard solution of each alkylphosphate was prepared at 1 g/L in methanol. The internal standard (I.S.) was DBP, prepared at 1 g/L in methanol.

All the working solutions at 1 and 10 mg/L were prepared by appropriate dilution of stock solutions in a mixture of acetonitrile and 2 mM ammonium formate buffer pH 3.0 (50/50, v/v). The I.S. solution was prepared at 1 mg/L using the same procedure. All standards and stock solutions were stored at a temperature less than +9 °C for a maximum of 3 months.

2.2. Extraction procedure

After homogenisation of urine sample, 5 mL were pipetted into a 15 mL screw top vial, to which were sequentially added 4 g sodium chloride, 25 μL of I.S. solution (1 mg/L) and 1 mL hydrochloric acid (6 M). The mixture was extracted with 5 mL diethylether by shaking for 15 min and centrifuging at 3000 rpm for 5 min. The organic phase was then collected in another vial. The extraction was repeated with 5 mL ethyl acetate. The two extracts were pooled and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The dry residue was reconstituted with 80 μL of 2 mM ammonium formate (pH 3.0)/methanol (50/50, v/v). Finally, 2 μL of this solution were injected into the LC–MS/MS system.

2.3. HPLC conditions

The chromatographic system consisted of a Series 200LC micro-flow rate, high pressure gradient pumping system, and a Series 200 Auto-sampler (Perkin-Elmer Instruments, Les Ulis, France) including a Rheodyne model 8125 injection valve equipped with a 5 μL external loop. Chromatographic separation was performed on an Inertsil ODS3 C18, 5 μm (150 mm × 1 mm I.D.) column (GL Science, Tokyo, Japan), using a linear gradient of acetonitrile (ACN) in 2 mM, pH 3.0 ammonium formate as mobile phase (constant flow-rate 50 μL/min), programmed as follows: 0–1 min, 30% ACN; 1–6 min, 30–50% ACN; 6–8 min, 50–70% ACN; 8–9 min, 70–90% ACN; 9–11 min, 90% ACN;

Table 1
Retention times and optimised mass spectrometry parameters for the determination of dialkylphosphates

	Retention time (min)	Precursor ion (*m/z*)	Declustering potential (V)	Type of transition	Collision energy (V)	Product ion (*m/z*)	*Q*/*C* ratio
DMP	2.97	124.9	−51	Q	−28	79.0	2.10
				C	−22	63.1	
DMTP	3.86	140.9	−36	*Q*	−20	125.8	1.86
				C	−26	96.0	
DMDTP	6.48	156.9	−41	*Q*	−20	141.9	1.55
				C	−26	111.9	
DEP	3.43	152.9	−41	*Q*	−24	79.2	1.22
				C	−14	125.0	
DETP	6.02	168.9	−31	*Q*	−22	94.8	2.28
				C	−16	140.8	
DEDTP	11.85	185.0	−51	*Q*	−24	110.9	23.60
				C	−42	95.1	
DBP (I.S.)	10.96	209.0	−36		−26	79.0	

Q: quantification; *C*: confirmatory.

11–12 min decrease from 90–30% ACN; 12–22 min, column equilibration with 30% ACN. All chromatographic solvents were degassed with helium beforehand.

2.4. Mass spectrometry

Mass spectrometric analyses were conducted using a Sciex (Toronto, ON, Canada) API-2000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray source. The full-scan mass spectra of the six DAP were first obtained by infusing working solutions at 1 mg/L in acetonitrile/2 mM, pH 3.0 ammonium formate (50/50, v/v). Due to their inherent negative charge, all compounds were analysed in the negative mode. The main parameter settings of the quantitative technique were: declustering potential between −51 and −31 V adjusted for each compound as shown in Table 1; ionspray voltage −4200 V. Acquisition was performed in the multiple reaction monitoring (MRM) mode, monitoring two transitions per compounds (one for quantification and the other for confirmation) and one for the internal standard (see Table 1), with dwell times between 100 and 300 ms (the lower the ion intensity the higher the dwell time). Detection of the confirmatory transition with ion abundance ratios within ±20% with respect to those of reference standards was necessary to consider a component as positive.

Additionally, ion suppression phenomenon was studied following the experimental system previously proposed by Antignac et al. [33]. Briefly, a standard solution containing the six DAP (at 100 μg/L) was continuously and directly infused into the mass spectrometer interface. A simultaneous LC flow containing either a pure mobile phase or a blank biological extract (urines from five non-exposed patients were collected) was introduced through a "T" coupling system. Evolution of the signal of the transitions at the retention times of the corresponding DAP and the I.S. was studied to evaluate the intensity of ion suppression.

2.5. Validation

All validation procedures were performed using each day a fresh sample of DAP-free human urine. Calibration standards were prepared by adding appropriate working standard solutions to 5 mL of DAP-free urine prior to extraction, in order to obtain concentrations ranging from 2 to 200 μg/L. Recoveries were determined in triplicate at four concentration levels (2, 50, 100 and 200 μg/L) by comparing the analyte/I.S. peak area ratios obtained after extraction of spiked samples with those of DAP-free urine extracts further spiked with the DAP.

The intra-assay precision and accuracy were assessed at 2, 50, 100 and 200 μg/L after extraction and analysis of five different fortified urine samples for each level. For assessing the inter-assay precision and accuracy, a set of calibrating samples was analysed each day for 5 days. The detection limit (LOD) was defined as the lowest concentration of DAP giving a response of at least three-times the average of the baseline noise determined from three unfortified urine samples (Table 2). The limit of quantification (LOQ) was initially defined as the lowest concentration of DAP that could be measured with an intra-assay and inter-assay precision CV% less than 20%. For the accuracy at the LOQ a CV% less than 20% was set.

Calibration graphs of the DAP-to-internal standard peak-area ratios of the quantification transition versus expected DAP concentration were constructed using a linear through zero with no weighting regression analysis.

2.6. Specimen donors

To test the efficiency of the method, 19 urine samples were collected from 8 women and 11 men, aged 21 to 59. These donors lived in Limousin, which is an agricultural region, but recent pesticide application and occupational contact with pesticides were excluded by questionnaire in all cases.

Table 2
Summary of the method specifications

Analyte	LOD[a] (μg/L)	Recovery (%)				Intra-assay precision (CV %)				Inter-assay precision (CV %)			
		2 μg/L	50 μg/L	100 μg/L	200 μg/L	2μg/L	50 μg/L	100 μg/L	200 μg/L	2μg/L	50 μg/L	100 μg/L	200μg/L
DMP	1.3	13.2	14.7	14.7	19.4	13.0	6.3	13.1	4.6	19.4	13.1	5.8	18.5
DMTP	1.1	25.2	96.2	60.3	46.2	9.3	4.1	6.5	1.9	21.8	12.9	9.7	8.3
DMDTP	1.1	70.0	91.8	79.8	67.5	15.0	3.2	10.4	4.7	16.0	20.9	17.8	6.7
DEP	0.7	88.5	70.9	59.0	50.2	9.3	5.1	9.5	6.5	23.1	12.0	6.7	9.0
DETP	0.5	54.9	99.8	67.0	49.5	6.8	4.0	4.9	3.0	21.9	9.0	5.3	4.6
DEDTP	0.9	59.2	80.8	90.6	58.5	15.8	3.5	15.1	6.4	23.7	8.4	10.5	11.2

[a] LOD: limit of detection.

3. Results and discussion

3.1. Sample preparation

A liquid–liquid extraction procedure (LLE) in acidic conditions using polar solvents was developed for the analysis of the six DAP. Similar LLE procedures were previously reported [8,13–15,16,18]. Additionally, 4 g of sodium chloride were added to urine in order to increase water density (which improves the separation between the two phases), and to improve the extraction of DAP from the aqueous phase (salt effect). This and the double extraction with diethylether and then ethylacetate led to better recoveries than those previously obtained with diethylether–acetonitrile mixtures [8,13,15]. Indeed, in the present study recovery values were >50%, except for DMP (approx. 15%) (Table 2), and they were not consistent as a function of the concentration tested. In fact, DMP is characterized by a low pK_a and was probably hardly extracted from the aqueous phase, despite acidification and addition of sodium chloride. These results hamper the applicability of the procedure for quantitative analysis. The sample extraction might be the reason of poor recoveries in some cases, and this step should be improved or even eliminated. Thus, the direct injection of urine samples in the LC–MS/MS allowed to obtain satisfactory precision and recoveries (above 80%), using tetrabutylammonium as an ion-pairing reagent [24].

Several other procedures were previously published to extract DAP from urine: azeotropic distillation with urine/acetonitrile mixtures [6,7,9,10,12,16,22], solid-phase extraction (SPE) [11], lyophilization [21,23] and polymeric phase transfer catalyst [17]. Azeotropic distillation using acetonitrile presents the advantage of avoiding losses during extraction, as it is equivalent to evaporate urine to dryness. However, the dry extracts obtained were described as gummy residues [12], which can hinder DAP derivatization by their encapsulation. Indeed, this technique led to low recovery yields [6]. Only one study, focused on mevinphos, employed a SPE procedure that gave a recovery of 86% for DMP [11]. Lyophilization of urine offers the best recovery values [21,23]: using GC/MS–MS, Bravo et al. [21] reported extraction recoveries between 75 and 100% for six DAP. However, lyophilization is time-consuming (12 h to 19.5 h) [21,23] and is hardly compatible with a routine activity.

Previously published recoveries are very heterogeneous: Moate et al. [12] reported values between 58 and 119% using azeotropic distillation (urine concentrations from 2 to 200 μg/L), whereas Bravo et al. [6] reported values between 17 and 65% (urine concentrations 10 and 50 μg/L) using the same extraction technique.

3.2. LC–MS conditions

The linear gradient of acetonitrile (ACN) employed allowed the six DAP to be eluted with convenient retention times, even the most polar DMP, and well separated from each other. A typical chromatogram is shown in Fig. 2.

As previously described by Hernandez et al. [24], we observed that the transition 124.9/79 was not specific of DMP, as DEP (*m*/*z* 152.9) also produced a fragment at *m*/*z* 124.9 in the electrospray source that decomposed further in the collision cell to generate a product ion at *m*/*z* 79 (Table 1). However, contrary to Hernandez et al. who reported a poor retention of DMP [24], in the present study, the chromatographic conditions allowed a correct elution of DMP and the separation of the two compounds (retention times were 2.97 and 3.43 min for DMP and DEP, respectively). Moreover, the method proposed by Hernandez et al. [24] included DMTP, DEP, DETP and DEDTP, but that did not allow the determination of DMP and DMDTP. As a consequence, this method could not detect an exposure to mevinphos nor to dichlorvos that are specifically and rapidly (i.e. dichlorvos blood half-life is 0.29 h in vitro at 37 °C [4]) metabolized into DMP.

Ion suppression phenomenon can influence the analytical results. Classically, it reduces the detection capability and leads to a modification (decrease or increase) in the analyte signal. Interestingly, it affects the ion ratio and the quantification, as well as the repeatability because the degree of ion suppression may vary from a sample to another. Fig. 2 illustrates the results of the ion suppression investigations performed in this study in accordance with a previously published system [33]. When analysing fresh urines obtained from five volunteers without occupational exposure, no major influence of interfering compounds on the signal of the analytes were observed. In fact, when looking at the retention times windows of the six DAP and the I.S. no significant signal loss or increase was observed.

3.3. Validation

For the six DAP, the intra-assay precision CV% were acceptable (less than 20%) (Table 2), although recoveries values were

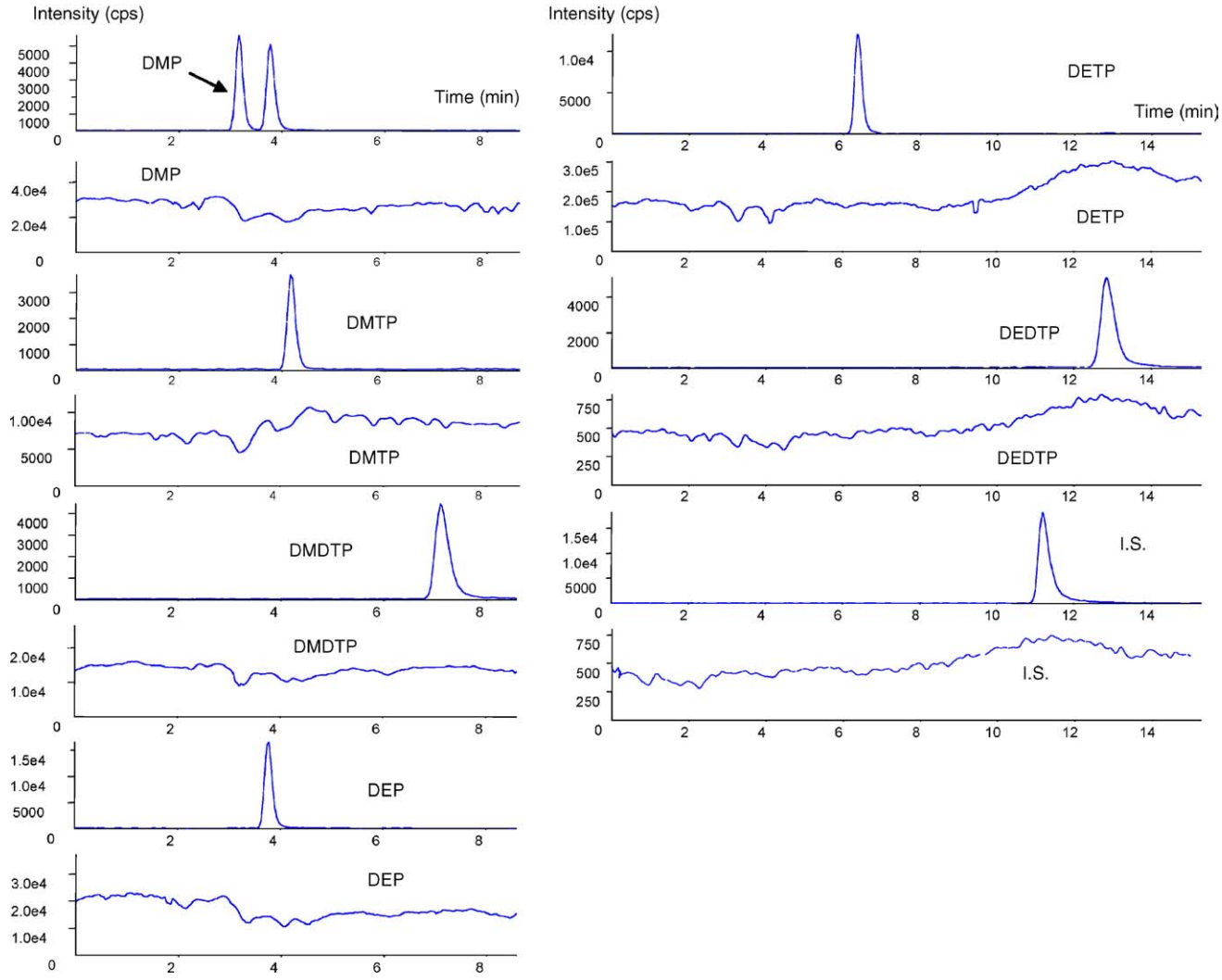

Fig. 2. Chromatograms of the six DAP and the internal standard (top) and their corresponding experiment authorizing the observation of ion suppression (bottom).

in many cases below 70%, and were not consistent with the concentration level tested. When considering samples spiked at 2 μg/L (LOQ), inter-assay precision ranged from 16 to 24%. During this validation procedure, a different, "free" urine sample was used each day, which might explain this poor result owing to the difficulty in obtaining urine samples really free of DAP. Moreover, the I.S. seemed not to correct the losses during extraction and/or the matrix effect. However, the LOQ was arbitrarily fixed at 2 μg/L for the six DAP, as such precision was found acceptable for urine measurements. Of note, very few of the previous studies mentioned inter-assay CV% at the LOQ [12,19]. Hardt et al. [8] tried to use deionised water instead of urine for the calibration standards, but concluded that it was absolutely necessary to use urinary calibration standards with creatinine content of at least 0.3 g/L in order to ensure reproducible results. For method validation near the LOQ, the use of synthetic urine (i.e. distilled and deionised H_2O fortified with major urine constituents) was also proposed [12]. With this procedure, the calculated inter-assay precision CV% were less than 15% at levels close to LOQ, but about 45% for DEP at 50 μg/L.

The use of labelled internal standards (^2H or ^{13}C) was also proposed [6,21]. As the native and labelled analytes behave almost identically they would be expected to have the same recoveries, and the ratio of the native to labelled species could be used to compensate for variable or poor extraction recovery, as well as large precision CV% values. However, these labelled internal standards can only be synthesized on-demand and are still very expensive.

To the best of our knowledge, only two LC–MS/MS methods were reported for the determination of DAP in urine [24,25]. Interestingly, Hernandez et al. reported a procedure with direct injection of urine samples in the LC–MS/MS system. Their procedure led to good recoveries and low detection limits for four DAP and obviously shorten the time of analysis [24]. However, our method is the first to propose the determination of the six metabolites simultaneously, with good chromatographic separation of DMP and DEP on the one hand, DMTP and DETP on the other (Fig. 2). In the present study, the limit of quantification obtained for the six DAP was 2 μg/L, that correspond to those reported in one of the best GC–MS/MS technique [6] and in the study of Hernandez et al. [24]. However, among other advan-

Table 3
Concentrations of dialkylphosphates in urine samples of 19 individuals with no occupational exposure to organophosphates

Metabolites	Results >LOD[a] (%)	Median (μg/L)	95th percentile (μg/L)	Maximum value (μg/L)
DMP	84	20.1	42.1	50.0
DMTP	79	4.6	76.0	139.2
DMDTP	5	<LOD	<LOD	3.6
DEP	100	4.5	16.9	85.4
DETP	26	<LOD	3.2	3.7
DEDTP	26	<LOD	4.4	7.0

[a] LOD: limit of detection.

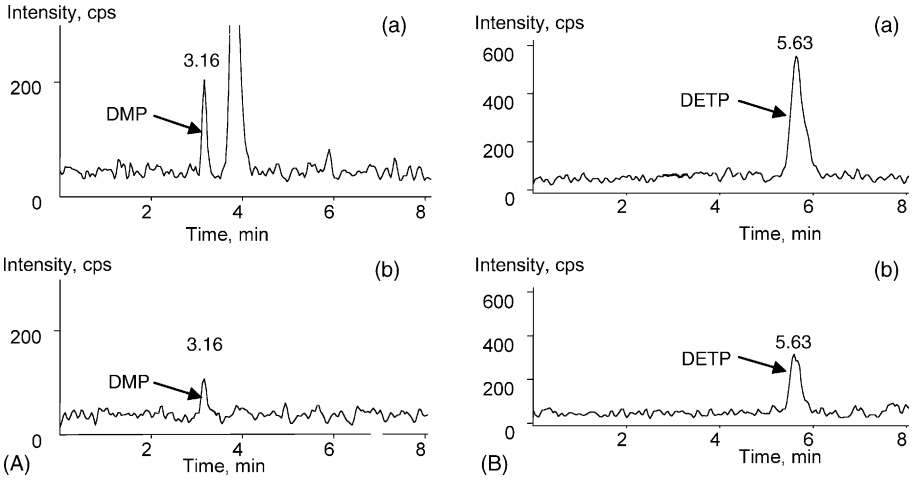

Fig. 3. (A) Chromatograms of DMP following the transitions 124.9/79.0 (a) and 124.9/63.1 (b) in a urine sample from a volunteer with no occupational exposure. The calculated concentration was 2.0 μg/L. The calculated *Q*/*C* ratio of the two transitions was 2.22. (B) Chromatograms of DETP following the transitions 168.9/94.8 (a) and 168.9/140.8 and (b) in a urine sample from a volunteer with no occupational exposure. The calculated concentration was 3.1 μg/L. The calculated *Q*/*C* ratio of the two transitions was 2.04.

tages in terms of turn-over time and workload, our LC–MS/MS procedure only requires cleaning the orifice plate of the mass spectrometer after each 250 injections instead of cleaning the ion source and the glass injection port liner, and cutting 25 cm of the pre-column after each 50 injections [6].

3.4. Application to urine samples of non-exposed volunteers

The results of the population study performed using the present LC–MS/MS technique are summarized in Table 3. As an illustration, chromatograms obtained from two volunteers are depicted in Fig. 3. In this study, at least one of the DAP was detected in each sample. DEP was present in all patients and almost every samples contained DMP and DMTP. DMDTP, DETP and DEDTP were found in lower concentrations. These preliminary results confirmed that the method developed could be applied to the monitoring of DAP in urine.

Although the population studied herein was small, our results are comparable to those previously reported in larger populations without occupational exposure [6–8,10–15,26–31].

The source of DAP in urine in such populations is still debated. The Food and Drug Administration suggests that the general population can be exposed by application of pesticides in private gardens or by pesticides residues in the diet [34,35]. However, the hypothesis of organophosphates ingestion might only partially explain this phenomenon. Other hypotheses are: the direct ingestion of metabolites instead of organophosphate pesticides in food matrices [27,28]; and the methylation of urinary inorganic phosphates [8].

4. Conclusion

This paper reports the first LC–MS/MS method for the simultaneous determination of six DAP in urine. This method should be useful for monitoring human exposure to organophosphate pesticides registered for agricultural use, as most are metabolised to at least one of these six DAP. Contrary to GC-based methods, the present technique does not require time-consuming derivatization of DAP. Its sensitivity has enabled the finding of DAP in urine from volunteers with no occupational exposure and confirms the suitability of LC–MS/MS in this field. Although this method is useful for the screening and identification of DAP in urine, it should be improved for a quantitative application in epidemiological prospective studies. Recoveries reported here were not sufficiently consistent and were normally below 70%, the value typically accepted in most of analytical guidelines for pesticide residue analysis. This result indicates that the sample pre-treatment step was sub-optimal and may be replaced by a direct injection in the LC–MS/MS system.

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