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RESEARCH ARTICLE

Analysis of dialkyl phosphate metabolites in hair using gas chromatography-mass spectrometry: A biomarker of chronic exposure to organophosphate pesticides

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Abstract

The aim of our study was to develop and validate an analytical approach for the quantitative determination of three dialkyl phosphate (DAP) metabolites, dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP) and diethyl phosphate (DEP), of organophosphate pesticides (OPs) in hair samples. The proposed methodology comprises a decontamination step, solid–liquid extraction, followed by liquid–liquid extraction, pentafluorobenzyl bromide derivatization, clean-up on Florisil/PSA column and analysis by gas chromatography–mass spectrometry (GC-MS). Extraction recovery, obtained from 50 mg hair samples spiked at two concentration levels, ranged from 56.1 to 107.9% and the within-day precision ranged from 13.5 to 17.5%. Limits of detection (LODs) ranged from 0.02 to 0.10 ng mg^{-1}. The results obtained from the analysis of hair samples of 30 agricultural workers show the suitability of the proposed method for monitoring people occupationally exposed to OPs. The most frequently detected compound was DEP followed by DMP. This is the first report on the detection of dialkyl phosphates in human hair which reflects the ability of hair testing to assess chronic exposure to OPs.

Keywords: *Hair; organophosphate pesticides; dialkyl phosphates; gas chromatography; mass spectrometry*

Introduction

The majority of papers on hair analysis have dealt with drugs of abuse (Kikura et al. 2000), doping agents (Thieme et al. 2003), pharmaceuticals (Psillakis et al. 1999, Tsatsakis et al. 2000) and heavy metals (Kazi et al. 2008). Recent studies on hair analysis (Tsatsakis & Tutudaki 2004, Ostrea et al. 2006) have gradually been changing to other chemical species, such as organochlorine pesticides, pyrethroids, carbamates, as well as organophosphate pesticides (OPs) and chloroacetanilides.

Assessment of pesticide exposure is generally based on blood and urine analysis (Barr & Needham 2002, Margariti et al. 2007), and sometimes on liver or kidney tissue in postmortem cases. However, blood and urine analyses provide information about recent pesticide exposures. Also, measurements of pesticides in a cleaner matrix, saliva, (instead of whole blood) have lately been proposed for the biomonitoring of very recent exposures to pesticides as OPs (Lu et al. 2003). The use of biosensors for detecting the OP exposures is often practised as well (Wang et al. 2008). At the same time, interest in the improvement of pesticide detection in specimens other than the conventional ones to estimate a much longer record of pesticide exposure continues unabated. This is especially true for OPs (Liu & Pleil 2002, Tutudaki et al. 2003), which are cleared rapidly from these body fluids rendering them less useful for long-term pesticide detection.

The interest in the development and optimization of analytical techniques to detect pesticides in hair is explained by the several advantages hair has over other biological matrices, such as short-term indicators, e.g. blood or urine. Pesticides are incorporated into the

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hair shaft and retained in the relatively inert matrix for long periods of time (Tsatsakis et al. 1998). Thus, hair has the widest window of detection of all biological matrices. Moreover, hair is collected in an easy, non-invasive way without requiring special apparatus and qualified personnel, and it can be stored and transported without special conditions thanks to its stability.

So, recently, researchers showed that hair provides a useful matrix for demonstrating exposure to OPs. Organophosphate pesticides, which are widely used in agriculture, in the home and in the garden, inhibit the cholinesterase enzymes in the nervous system, including acetylcholinesterase, the enzyme responsible for catalyzing the decomposition of acetylcholine, resulting in overstimulation at cholinergic synapses (Ballantyne & Marrs 1992) and can be very harmful to human health. Moreover, it has been reported (Costa et al. 2005) that children may be especially vulnerable to the adverse health effects of OPs due to their lower metabolic abilities. As a consequence, the biological monitoring of OP exposures is an absolute essential to evaluate the toxic risks associated with this exposure.

As researchers demonstrated, OPs are deposited in hair and can be analyzed from this complex matrix mainly by solid–liquid extraction, sometimes followed by liquid–liquid extraction, and gas chromatography–mass spectrometry (GC-MS) (Liu & Pleil 2002, Tutudaki et al. 2003, Tutudaki & Tsatsakis 2005, Ostrea et al. 2006, Tsatsakis et al. 2008). However, all these procedures were able to test for a limited number of OPs (from one to four OPs only), and therefore do not permit a quantitative estimate for cumulative exposure to OPs.

OPs are rapidly metabolized in the human body (Gallo & Lawryk 1991). Through hydrolysis and oxidative desulfuration, nearly all OPs are metabolized into any of the following non-specific dialkyl phosphate (DAP) metabolites: dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP), diethyl thiophosphate (DETP), dimethyl dithiophosphate (DMDTP) and diethyl dithiophosphate (DEDTP). These metabolites are highly polar, acidic and water soluble compounds and are frequently analyzed as biomarkers of OP exposure in human population. The analytical determination of dialkyl phosphates has been mainly performed in the past on blood (Drevenkar et al. 1994, Tarbah et al. 2004), urine (Dulaurent et al. 2006, Ueyama et al. 2006), tissues (Richardson & Seiber 1993), meconium (Whyatt & Barr 2001) and amniotic fluid samples (Bradman et al. 2003).

In this paper, we report hair as a potential biomonitor for assessing DAP metabolites. Hair matrix is increasingly being used for analysis of selected pesticide metabolites and/or the parent compounds (Ostrea et al. 2006, Tsatsakis 2006, Posecion et al. 2006) and two recent attempts (Ostrea et al. 2006, Posecion et al. 2006) at determining specific OP metabolites in maternal hair show the utility, but also the limitations of this matrix. The first of those studies (Ostrea et al. 2006) determined the acidic malathion metabolite (malathion monocarboxylic acid) in hair. The advantage of this approach is the high specificity (pinpoints exposure to a certain OP); however, it is limited to measuring only two specific OP metabolites and therefore is not useful for detecting and monitoring the range of OPs that people are exposed to. As yet, no research has reported detection of DAPs in this specimen.

The mechanism of incorporation and retention of DAPs into hair is an unexplored field. Drugs may be incorporated into the hair interior through the bloodstream or by diffusion from body sweat or sebum (Balíková & Habrdová 2003). Also, lipophilicity and basicity of a drug are considered to be essential characteristics for its deposition in hair from blood, whereas acidic pesticide metabolites seem to enter hair to a lesser extent (Pragst & Balikova 2006). Metabolism studies indicated that DAPs are mainly excreted in urine but it is also reported (Duggan et al. 2003) that these urinary metabolites may also be excreted in the sweat. This presence of the water soluble DAPs in sweat is likely to contribute to their incorporation into hair via sweat absorption outside the hair shaft through the cuticle or incorporation from sweat into the hair shaft.

The advantage of this approach is that in detecting in a single assay the major metabolites of a wide range of OPs rather than the individual parent compounds or specific metabolites in hair, there exists a far greater opportunity for detecting and monitoring chronic occupational and environmental human total OP exposure than by using methods that assay for particular parent pesticides or specific metabolites; however without the ability to identify which specific OP has caused exposure. But, it is possible to estimate the class of OPs (dimethyl OPs (e.g. malathion, methidathion) and diethyl OPs (e.g. diazinon, chlorpyrifos)) causing exposure from the levels of the dimethyl phosphate (DMP, DMTP, DMDTP) and diethyl phosphate (DEP, DETP, DEDTP) metabolites. This is especially important because the cumulative assessment of OP exposure level based on concentrations in human hair of DAPs could allow development of a comprehensive evaluation of the harmful effects of DAPs and OPs on human health, particularly for vulnerable populations such as young children and infants, and help to reduce exposure.

So, the purpose of the present contribution was to develop and validate a method for the simultaneous analysis of three DAPs in human hair using GC-MS. In our study the target compounds that we used were DMP, DEP and DMTP, and our test samples were head hair specimens of 30 growers occupationally exposed to OPs who live in areas of intensive agriculture. This work is part of our investigations about the possibility of

incorporation of DAPs into the hair of animals undergoing a long-term dimethoate treatment which will be published in due course. Therefore, the method was initially developed for determining two of the major breakdown products of dimethoate (DMP, DMTP) using DEP as the internal standard. However, in the present study DEP was also used as target metabolite due to the possibility of detecting it in the test samples.

Materials and methods

Materials and standards

DMP (purity, 98%), DMTP (97%) and the derivatization reagent pentafluorobenzyl bromide (PFBBr) were purchased from Acros Organics (Geel, Belgium). DEP (99.3%) was obtained from Chem Service (West Chester, PA, USA). Diethyl ether, acetonitrile, *n*-hexane and toluene, which are HPLC-grade, and analytical-grade hydrochloric acid (37%), sodium sulfate anhydrous, sodium chloride (NaCl), sodium disulfite ($Na_2S_2O_5$) and potassium carbonate (K_2CO_3) were obtained from Merck (Darmstadt, Germany). HPLC-grade methanol was purchased from Scharlau (Barcelona, Spain), and HPLC-grade acetone and water from Sigma-Aldrich (Buchs, Switzerland). Florisil (60–100 mesh) was bought from Fluka (Buchs, Switzerland) and Bondesil-Primary/Secondary Amine (PSA) 40 μm from Varian, Inc. (Palo Alto, CA, USA). Helium (99.999% pure) and nitrogen (99.99% pure) gases were from Messer Hellas (Athens, Greece).

A stock solution of each metabolite was prepared at 1 mg ml^{-1} in methanol with aliquots taken and diluted in methanol to compose a standard stock mixture of three dialkyl phosphates. Serial dilutions of this stock solution using methanol produced a range of working solutions with metabolite concentrations ranging between 0.1 and 100 ng μl^{-1}. All stock solutions were stored frozen (−20°C) in the dark for a maximum of 1 month and equilibrated at room temperature prior to use. All the working solutions were made fresh for each day of analysis or validation.

Sample collection

Thirty head hair samples with potential metabolite content were collected from agricultural workers occupationally exposed to OPs living in two regions of intensive agriculture of Greece. Hair specimens (about 500 strands each), approximately 4.0–6.0 cm in length, were cut from the nape of the neck, as close as possible to the scalp, and were stored in paper envelopes at room temperature and in a dry place until analysis. They were of different colours, and some samples were dyed or bleached. Information on pesticide exposure was obtained by evaluation of a questionnaire and personal interview. The study was approved by the Ethics Committee of the University of Crete and the University Hospital. All the specimens were collected at site by physicians of the University Hospital and all subjects gave written informed consent.

Instrumentation

Gas chromatographic conditions

A Shimadzu GCMS-QP2010 system was utilized for the determination of the target metabolites equipped with an autosampler (AOC-5000, Shimadzu, Kyoto, Japan), with the volume injection set to 1 μl. The injector was set at a temperature of 250°C and used in the splitless mode with the split flow path opening after 1.10 min. Helium was used as the carrier gas with a linear velocity of 36.7 cm s^{-1}. The analytes were separated on an Equity®-5 capillary column (poly(5% diphenyl/95% dimethylsiloxane); 30 m × 0.20 mm i.d. × 0.20 μm) from Supelco Company (Bellefonte, PA, USA). The initial oven temperature of 70°C was held for 1 min, then raised at a rate of 5°C min^{-1} to 220°C, and finally raised at 15°C min^{-1} to 280°C, remaining at this temperature for 5 min. The total run time per sample was 40 min.

Mass spectrometric conditions

An auto-tune of the mass spectrometer using perfluorotributylamine (PFTBA, tuning standard) was performed before analysis of every set of samples. Ionization was performed in the electron impact (EI) mode at an electron energy of 70 eV and an emission current of 60 μA. The ion source and interface (transfer line) temperatures were 250°C and 300°C, respectively. The solvent cut time was 14.20 min, the detector off at 25.00 min. Full scan GC-MS chromatograms were obtained by scanning from *m/z* 40 to 600 with a scan time of 0.5 s.

Quantitative analysis was accomplished in selected ion monitoring (SIM) mode with a scan time of 0.2 s, using the most abundant or characteristic (target) ion for quantification and when possible two fragment (qualifier) ions for confirmation for each compound. Derivatized DAP standards and spiked hair samples were analyzed using full scan in order to obtain the mass spectral data, from which background and abundant ions were identified, and characteristic ions were carefully chosen for the individual compounds of interest for analysis in the SIM mode. Furthermore, we selected target and qualifier ions with relative abundances that were consistent at low concentrations and stable over time. Chosen target and qualifier ions were then cross-referenced with existing methodologies.

The chromatographic acquisition time was divided into three different MS segments. The first segment was from 14.20 to 17.25 min, the second from 17.26 to 19.25 min and the third from 19.26 to 25.00 min.

To ensure specificity as a quality assurance measure, identification of metabolites in hair samples was based on the comparison of both the retention times (±0.20 min) and relative abundances of monitored ions (the target and at least one of the qualifier ions to exhibit the correct relative abundance for each analyte) with those of the standard analytes and positive control samples (see quality control materials section for analyte concentration) (with a maximum acceptable error of ±20%).

Sample preparation

Decontamination of hair

Hair samples were submitted to an initial step of decontamination by washing a 300-mg sample sequentially with 15 ml water and 15 ml methanol, to eliminate possible external deposition of compounds. Then, the sample was dried at room temperature, finely cut with scissors into approximately 1-mm sections and a subsample (50 mg) was taken into a screw-top glass vial.

Solid-liquid extraction

Five millilitres of water was added into the vial containing the hair sample. Then, the sample was sonicated in an ultrasonic water bath for 1 h, followed by horizontal shaking at room temperature for 12 h using an orbital shaker to liberate the metabolites from the matrix.

Isolation of metabolites, derivatization, clean-up

After solid–liquid extraction, the water extract was separated by centrifugation at 2600 *g* for 15 min, transferred to a clean 15-ml screw-top glass vial and processed according to a previous procedure used for measuring DAPs in urine (Ueyama et al. 2006). Briefly, 5 g of NaCl, 1 ml of HCl (6 mol l^{-1}), 50 mg of $Na_2S_2O_5$ and 5 ml of diethyl ether:acetonitrile (1:1, v/v) were sequentially added into the vial. After vigorous mechanical shaking for 5 min, the vial was centrifuged (2000 *g* for 5 min at 4°C). The supernatant was then collected in another screw-top glass vial containing 15 mg of K_2CO_3. The extraction was repeated with 5 ml of diethyl ether:acetonitrile (1:1, v/v). The two extracts were pooled and evaporated to dryness under a mild nitrogen stream at 45°C.

To the resulting residue, 15 mg of K_2CO_3, 1 ml of acetonitrile and 50 μl of PFBBr were added. The samples were then vortexed and incubated in a water bath at 80°C for 30 min with mild swirling. After the derivatization was completed, 4.5 ml of water and 4.5 ml of *n*-hexane were added, and the content was shaken vigorously for 5 min and centrifuged for 5 min at 2000 *g*. The supernatant was then carefully removed and collected in a screw-top glass test tube. The extraction was repeated with 4.5 ml of *n*-hexane.

The two extracts were pooled and passed through a three-layer column, packed (into a Pasteur pipette) from the bottom with 0.3 g of Florisil, 0.1 g of Bondesil-PSA and 0.5 g of anhydrous sodium sulfate, under gravity. The column was then washed with 5 ml of acetone:*n*-hexane (2:98, v/v) in order to remove the unreacted PFBBr (Ueyama et al. 2006). Finally, the column was eluted by gravity with 5 ml of acetone: *n*-hexane (15:85, v/v), and the eluate was evaporated at 45°C to dryness under a mild stream of nitrogen, reconstituted in 200 μl of toluene and then injected into the GC-MS system described above.

Quality control materials

DAP-free human head hair was provided by unexposed subjects (babies), and this hair was used as blank sample for quality control purposes. Prior to spiking experiments, babies' hair was analyzed for the selected metabolites by the proposed method described in the sample preparation section, to ensure that it was free of the analytes of interest. For quality assurance, quality control samples, composed of unspiked blank hair (negative control) and spiked blank hair at 1 and 10 ng mg^{-1} of each analyte (positive controls) were included in every batch of samples analyzed. Positive control samples were used to monitor several parameters such as retention times, the target to qualifier ion relative abundances, recovery, within- and between-day precision for the analytes assayed.

Quantification

A five-point calibration curve for the quantification was constructed for every analytical run by analyzing blank hair samples spiked with the target metabolites. The concentrations of the five calibration samples ranged from 0.1 to 5 ng mg^{-1}. To obtain spiked hair samples, the proper volume of the appropriate methanol working solution was added to 50 mg of blank hair prior to solid–liquid extraction. The total concentration of methanol in each sample throughout the study was kept constant thus not affecting the extraction performance. The spiked samples were shaken thoroughly, then, allowed to equilibrate at room temperature and darkness for 1 h before solid–liquid extraction so as to achieve the metabolite distribution in the hair, and then were processed as described above (sample preparation section). At each concentration level three separate samples were extracted and analyzed.

Validation study

The proposed method was validated using DAP-free babies' hair by evaluating limits of detection and quantification (LODs and LOQs), extraction recovery, within- and between-day precision for the analytes assayed.

The limits of detection were calculated from the chromatogram of the lowest spiked sample (0.1 ng mg^{-1}) of

the calibration curves, by considering a response equivalent to three times the background noise for the selected quantification ions. In one instance (DEP), the calculated detection limit was lower than the lowest spiked concentration of the calibration curves; for this analyte the calculated LOD was verified as a reasonable estimate by analyzing samples spiked at the LOD level. At the LOD, at least two of the registered ions of each analyte should still be detected and the GC-MS data of the detected ions should still meet the requirements for the identification of compounds described in the section on mass spectrometric conditions. The limits of quantification were considered the concentration of analytes at which the signal-to-noise ratio of the quantification ions was at least 10.

The extraction recovery was determined by spiking with the target metabolites decontaminated finely cut blank hair samples (50 mg) before solid–liquid extraction in triplicate at each concentration level of 1 and 10 ng mg^{-1}, in the same way as the calibration samples, and analyzing according to the method. Six additional blank hair samples (unspiked) were analyzed concurrently. The samples that were not spiked before preparation were spiked in triplicate at the 1 and 10 ng mg^{-1} level of each metabolite on the derivatization step, to serve as control samples representative of 100% recovery. The sample preparation after the derivatization step was completed according to the method and the samples were analyzed. The recovery was determined as the ratio of the analyte peak area of the recovery samples to that of the control samples. The precision of the method was calculated in terms of relative standard deviation, RSD, as a percentage. Three replicates of the positive control sample (1 ng mg^{-1}) were prepared and analyzed for each validation day to test the within-day precision of the overall analytical procedure. Between-day precision of the method was evaluated by a triplicate assay of the positive control sample (1 ng mg^{-1}) for three different days.

With the aim of testing the instrumental precision, the within-day RSD was determined by injecting the same final toluene extraction solution from the positive control sample (1 ng mg^{-1}) five times during one working day into the GC-MS system under the selected conditions. The between-day RSD of the instrument was also evaluated by injecting the same positive control sample (1 ng mg^{-1}) each day for four consecutive days.

Finally, to test the efficiency of the method, a 30 samples of hair from agricultural farmers were analyzed (in triplicate) for quantification of DMP, DEP and DMTP.

Results

Method optimization

The method described in this paper proved to be suitable for quantitative analysis of three DAP metabolites in hair. Quantification of residues from hair is a difficult task because of the complexity of the matrix. A difficulty to overcome in the development of such a quantitative analysis was the extraction of metabolites from hair, the relation between solvent, studied compounds and analyzed matrix. The target analytes are highly water soluble and thus hard to efficiently extract from hair with common organic solvents. Furthermore, acidic and alkaline hydrolysis could not be applied for the extraction of ester compounds like OPs (Tutudaki et al. 2003) and DAP metabolites from hair, due to the instability of these compounds in such harsh chemical conditions. Therefore, for our study, a proper pretreatment method to extract efficiently the compounds of interest from the hair matrix without destroying them was developed based on a solid–liquid extraction procedure (as described in the solid–liquid extraction section) using water (5 ml) as the extraction solvent, simplifying thus, the sample preparation procedure compared with the conventional methods for hair analysis (extraction with organic solvents, acid or base hydrolysis, or enzymatic digestion) (Nakahara 1999). Ultrasonic treatment was used to provide good contact between the solvent and the solid and to accelerate liberation of the compounds from the matrix, and a sonication time of 1 h was found to contribute to the improvement of extraction efficiency. The extraction period was considered a critical parameter in the development of the sample preparation method. After trying several extraction times (4, 6 and 12 h) most efficient extraction with greatest recovery was obtained when samples were extracted for 12 h.

After the pretreatment stage, we applied the isolation, derivatization and clean-up procedures proposed for urine by Ueyama et al. (2006) with minor modifications, and our experience with dialkyl phosphates in urine and hair indicated high extraction efficiency. Another part of the sample preparation method which had a strong effect on the performance of the extraction, was the liquid–liquid extraction step carried out in a solvent mixture of diethyl ether and acetonitrile. This solvent mixture proved to be efficient for isolation of metabolites from water as most of them move from the aqueous phase to the diethyl ether phase. We found the derivatization of extracted DAPs using PFBBr to be satisfactory and reproducible in both standard solutions and in spiked hair samples, whereas the reaction conditions during derivatization procedure are severe. Clean-up is a crucial step in the analytical procedure as excess amounts of PFBBr can harm columns and detectors (Ueyama et al. 2006) and is also needed to remove lipids and other interfering compounds coming from the matrix.

Initial experiments to optimize the GC and MS detection conditions were carried out by injection of the individual derivatized analyte standards and spiked hair using full scan mode, and the appropriate ions were selected for

the SIM mode. The chromatographic acquisition time was divided into three different MS segments (one segment per each analyte) in order to include the lowest number of selected ions in each, thus maximizing sensitivity.

Method performance

From the full scan mass spectra of the three individual derivatized standards in the experimental conditions proposed identified and used for the identification and quantification purpose the following fragment ions (*m/z*) (quantification ions underlined): DMP (<u>110</u>/194/306), DEP (197/<u>258</u>/334) and DMTP (211/<u>322</u>) (Figure 1), in accordance with previous literature (Ueyama et al. 2006). Typical chromatograms of derivatized metabolite standards are also presented in Figure 1.

The performance of this approach is illustrated in Figure 2A–C showing the GC-MS analyses of head hair samples (blank sample spiked with 10 ng mg^{-1} of each metabolite, a blank sample, and a positive sample of a person occupationally exposed to OPs). In the latter sample, the analysis revealed the presence of DMP at a concentration of 0.46 ng mg^{-1}, DEP (0.37 ng mg^{-1}) and DMTP (0.41 ng mg^{-1}). As can be seen, the GC oven temperature program used for the analysis yielded a satisfactory chromatographic separation of all the compounds (except for DEP) over a reasonable analysis time. The retention times for the metabolites in these chromatographic conditions are 16.062 min (DMP), 18.409 min (DEP) and 19.834 min (DMTP) (Figure 2A). However, we observed an interfering peak in the region of DEP (eluting about 0.5 min later than DEP) that was not baseline-separated in the spiked sample mainly due to the DEP tailing. Nevertheless, this low peak resolution did not constitute a difficulty as the matrix interference has the same identification ion (*m/z* 197) as the analyte and was completely removed (Figure 3) when the quantification ion (*m/z* 258) of DEP was selected. The peak performance was satisfactory for the studied metabolites under the chromatographic conditions used, in both, spiked and unknown samples (Figure 2A and C), although we noted that their peak shapes exhibited some tailing. The analysis

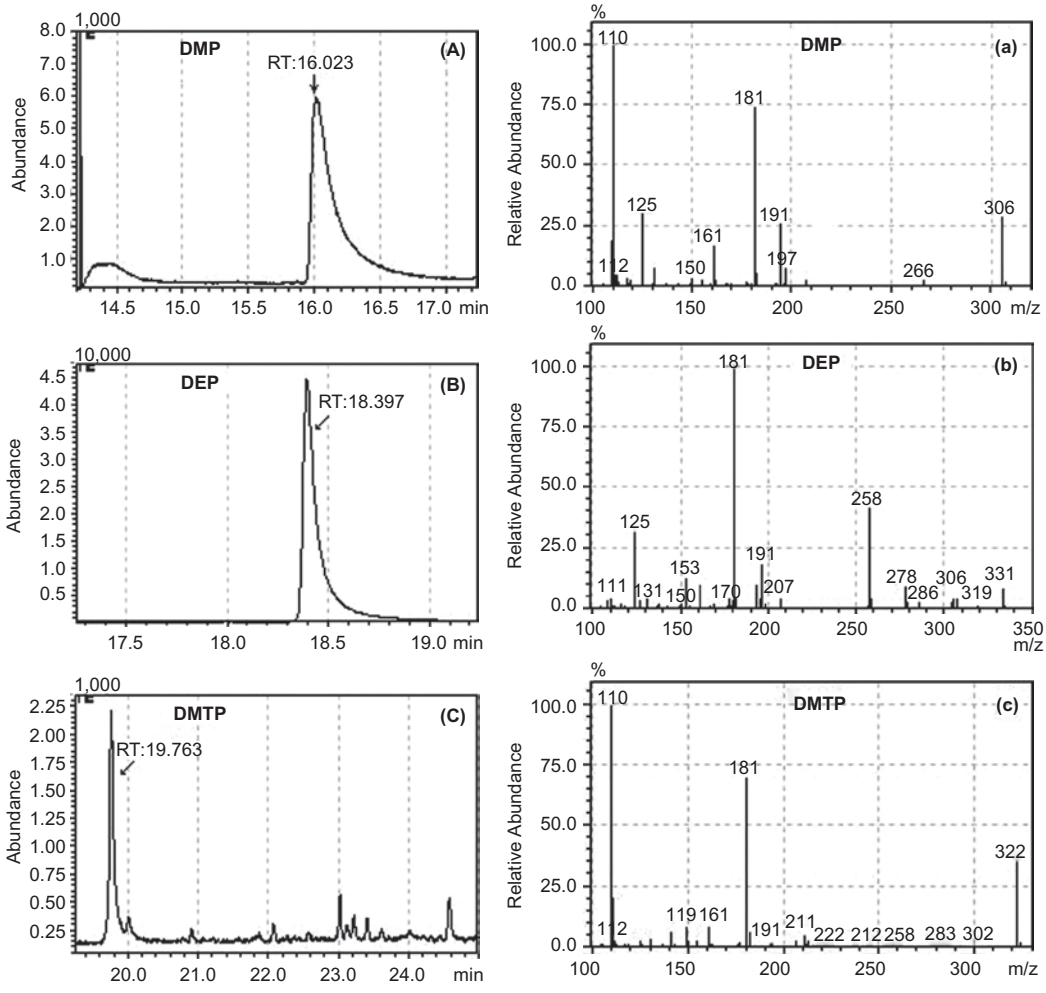

Figure 1. GC-MS total ion chromatograms (TICs) obtained in SIM mode (A, B, C) and full scan mass spectra (a, b, c) of derivatized DAP standards (1500 ng in methanol): dimethyl phosphate (DMP); diethyl phosphate (DEP); dimethyl thiophosphate (DMTP).

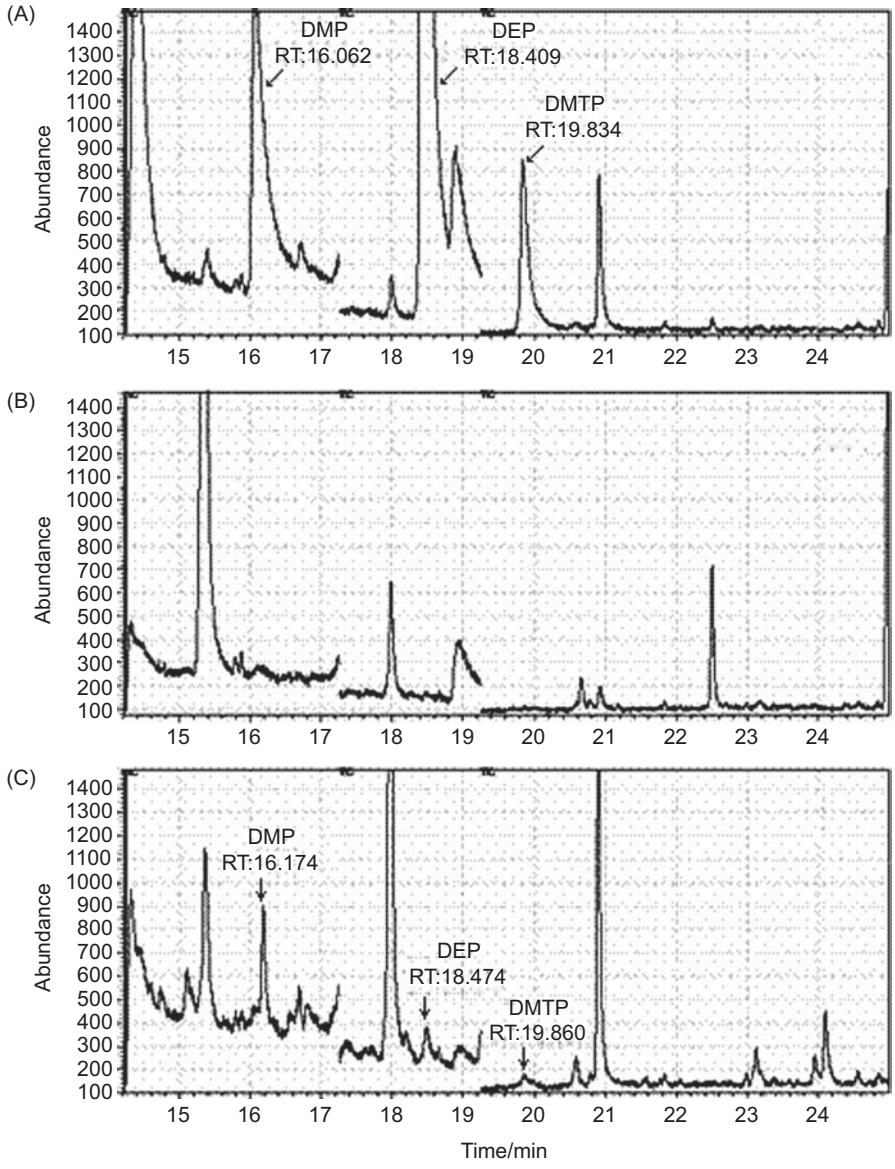

Figure 2. Representative GC-MS total ion chromatograms (TICs) of (A) a blank hair sample spiked with 10 ng mg^{-1} of each metabolite (dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP)); (B) hair sample blank; (C) hair sample found positive on DMP (0.46 ng mg^{-1}), DEP (0.37 ng mg^{-1}) and DMTP (0.41 ng mg^{-1}).

of unspiked blank samples (negative controls) showed that there were no matrix compounds that might give a false-positive signal at the expected retention times for each compound (Figure 2B). The background obtained from chromatograms of real samples was relatively low, indicative of an efficient removing of co-extracted matrix components (efficient clean-up procedure) present in the very complex hair matrix (Figure 2A–C). Although a few matrix peaks exist they do not hamper identification and quantification of metabolites.

The calibration curves were linear in the range of 0.1–5 ng mg^{-1} with correlation coefficients (R^2) of 0.9956 for DMP, 0.9995 for DEP and 0.9987 for DMTP (Table 1). The error in the slopes of calibration curves was <5%. As Table 1 shows, the LOD of spiked blank hair samples was 0.10 ng mg^{-1} for DMP and DMTP, and 0.02 ng mg^{-1} for DEP. LOQs were also determined in ng mg^{-1} and were 0.33 for DMP, 0.06 for DEP and 0.34 for DMTP.

Precision and recoveries of the method are reported in Table 1. As it can be seen in the Table, good extraction recoveries were obtained for all the target metabolites, ranging from 56.1 to 107.9%. Although not yet investigated in detail, we assume that the relatively poor recovery obtained for the DMP compared with the other compounds was probably due to its higher polarity and its much higher affinity for the aqueous phase. Within-day precision of the overall analytical procedure ranged from 13.5 to 17.5% at a 1 ng mg^{-1} spiking level while the between-day precision of the assay ranged from 11.1 to 16.2%. The instrument within-day RSD was in the range

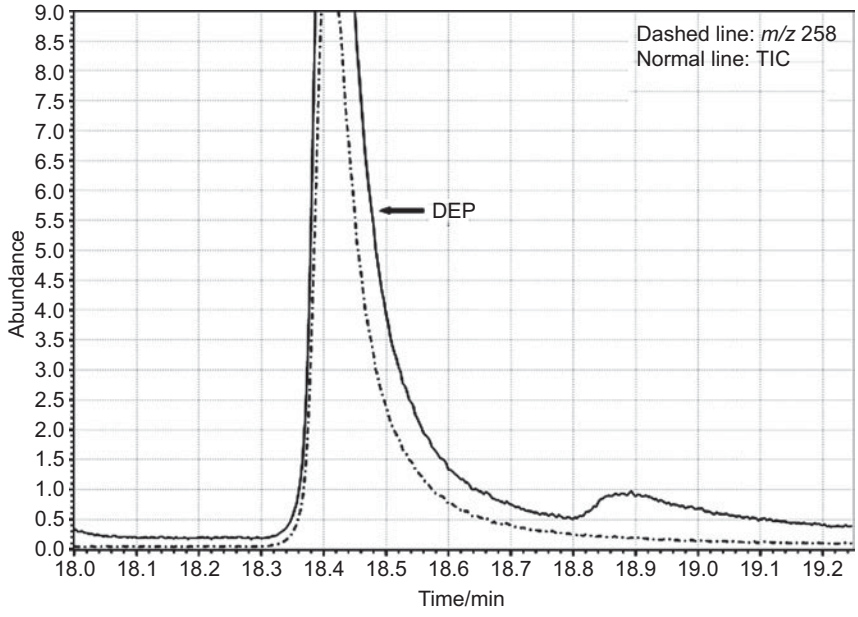

Figure 3. GC-MS chromatograms of diethyl phosphate (DEP) in a blank hair sample spiked at the 10 ng mg^{-1} level. Normal line: total ion chromatogram (TIC); dashed line: extracted single ion chromatogram selecting the quantification ion (*m/z* 258) of DEP.

Table 1. Linearity data, limits of detection (LODs), limits of quantification (LOQs), extraction recoveries and relative standard deviations (RSDs) for the analysis of dialkyl phosphates (dimethyl phosphate (DMP); diethyl phosphate (DEP); dimethyl thiophosphate (DMTP)) in hair samples.

					Extraction recovery (%)		Method precision, RSD (%)		Instrument precision, RSD (%)	
Compound	Correlation coefficient	Error of slope (%)	LOD (ng mg^{-1})	LOQ (ng mg^{-1})	1 ng mg^{-1}	10 ng mg^{-1}	Within-day (n=3)	Between-day (n=3)	Within-day (n=5)	Between-day (n=4)
DMP	0.9956	4.7	0.10	0.33	56.1	66.5	13.5	11.2	3.9	8.7
DEP	0.9995	0.8	0.02	0.06	61.1	98.9	15.4	11.1	4.9	12.9
DMTP	0.9987	2.6	0.10	0.34	97.7	107.9	17.5	16.2	2.0	11.3

Table 2. Mean value and concentration range of dialkyl phosphates (dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP)) in hair samples of 30 agricultural workers.

Compound	Mean value (ng mg^{-1})	Concentration range (ng mg^{-1})	Number of positive samples	Frequency of detection (%)
DMP	0.24	0.10−0.46	12	40
DEP	0.36	0.32−0.44	21	70
DMTP	0.36	0.32-0.41	6	20

of 2.0–4.9%, whereas between-day RSD ranged from 8.7 to 12.9%.

Application of method

The validated method was successfully applied to evaluate the exposure to OPs in a group of 30 agricultural workers occupationally exposed to OPs, as part of an extensive monitoring programme. The summary results of the DAP determination in the hair samples are presented in Table 2. Twenty four (80%) samples had detectable levels of at least one of the target metabolites, and six of 30 samples (20%) were positive for all of the examined analytes. On the other hand, 20% of the samples contained no detectable residues of the target metabolites. DEP and DMTP residues were detected in 70% and 20% of samples, respectively, at concentrations ranging from 0.32 to 0.44 ng mg^{-1} and from 0.32 to 0.41 ng mg^{-1}, respectively. DMP was detected in 40% of samples at concentrations ranging from 0.10 to 0.46 ng mg^{-1}.

Discussion

Although it is difficult to compare with other biological matrices, it is of note that the levels of the metabolites detected in our study in hair samples were generally lower than previously reported levels in meconium and higher than those of amniotic fluid and urine in occupational and environmental exposure studies (Table 3). From this Table, it can be observed that the concentrations of the three target dialkyl phosphates varied between sample matrices and among examined populations (with or without occupational exposure and poisoning cases). For example, concentrations of 16.00 ng mg^{-1} (16000 ng ml^{-1}) (DMP) and 0.80–3.20 ng mg^{-1} (800–3200 ng ml^{-1}) (DEP) were measured in meconium (Whyatt & Barr 2001),

Table 3. Concentrations of dialkyl phosphates (dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP)) in hair, urine, meconium, amniotic fluid, blood and plasma samples from occupationally (Hernández et al. 2002, 2004, Ueyama et al. 2006) and non-occupationally (Hardt & Angerer 2000, Whyatt & Barr 2001, Oglobline et al. 2001, Bradman et al. 2003, Dulaurent et al. 2006, Ueyama et al. 2006) exposed populations, and from poisoning cases (Drevenkar et al. 1994, Tarbah et al. 2004).

	Concentrations of dialkyl phosphates, range (mean) in ppb						
	Exposed population		General population			Poisoning cases	
Compound	Urine (ng ml^{-1})	Hair (pg mg^{-1})	Urine (ng ml^{-1})	Meconium (pg mg^{-1})	Amniotic fluid (ng ml^{-1})	Blood Plasma[a] (ng ml^{-1})	Urine (ng ml^{-1})
DMP	22.7–52	100–460 (240)	13–30	16 000	0.30–0.34 (0.32)	3900–4900 (4400)	33 500–50 400 (41 950)
DEP	0.4–100	320–440 (360)	0.7–4.5	800–3200 (1540)	0.26–0.36 (0.31)	200–8530 (2244)[a]	
DMTP	4.1–47	320–410 (360)	1.1–22	n.d.	0.43		

Results in hair and meconium are expressed as concentrations in pg mg^{-1} so as to be in the same concentration range as those of urine, blood, plasma and amniotic fluid (ng ml^{-1}).
n.d., not detected.

which are significantly higher than those in urine and hair of occupationally or non-occupationally exposed subjects, and can probably be explained by the high residential use of OPs in the study area. As for amniotic fluid, concentrations of 0.30–0.34 ng ml^{-1} (DMP), 0.26–0.36 ng ml^{-1} (DEP) and 0.43 ng ml^{-1} (DMTP) were obtained (Bradman et al. 2003).

In the case of urine, DAP concentrations in occupationally exposed persons are generally higher than those in non-occupationally exposed groups (Table 3). One recent study (Ueyama et al. 2006) reported the detection of DMP, DEP and DMTP with median concentrations of 22.7, 0.4 and 4.1 ng ml^{-1}, respectively, in persons occupationally exposed to OPs. In another study (Hernández et al. 2002), DEP was detected at concentrations ranging from 30 to 100 ng ml^{-1} in the urine of farmers exposed to chlorpyrifos. The same group has recently detected DMP and DMTP at concentration levels of 52 and 47 ng ml^{-1}, respectively, in urine samples of a grower after application of methyl parathion (Hernández et al. 2004). As regards non-occupationally exposed subjects, the results of urinary DAPs varied insignificantly between studies. Concisely, average urinary concentrations of 13–30 ng ml^{-1} (DMP), 0.7–4.5 ng ml^{-1} (DEP) and 1.1–22 ng ml^{-1} (DMTP) have been observed (Hardt & Angerer 2000, Oglobline et al. 2001, Dulaurent et al. 2006, Ueyama et al. 2006).

Finally, DAPs have also been measured in cases of OP poisonings. Urine levels of DAPs in poisoning cases (Tarbah et al. 2004) are significantly higher than those in exposed and non-exposed groups (Table 3). Blood levels are also much higher than levels of DMP in hair of occupationally exposed subjects. In particular, in a phosphamidon poisoning case, DMP was detected at concentrations as high as 3900 and 4900 ng ml^{-1} in the patient's blood samples (Tarbah et al. 2004). Drevenkar et al. (1994) reported cases in which the concentrations of DEP ranged from 200 to 8530 ng ml^{-1} in plasma samples of persons poisoned by OPs.

Our results showed that DEP was the most frequently detected metabolite (70%), which confirms the high agricultural use that has been reported for diethyl OPs (e.g. diazinon, chlorpyrifos) in the study regions compared with dimethyl OPs (e.g. methyl parathion, dimethoate), which are used less frequently. Interestingly, 50% of the 12 samples found positive for DMP, also contained detectable levels of DMTP. This may confirm the reported agricultutal application of OPs which produce only DMP, such as dichlorvos or dicrotophos. However, it must be noted that because DAPs originate from more than one OP, the metabolite concentrations found in hair represent chronic exposure to multiple OPs. On the other hand, it is possible that the presence of metabolite residues in hair is associated not only with occupational exposure but also with dietary and drinking water exposure to the intact OPs or to the DAP residues themselves.

The findings of the present study corroborate the presence and the co-occurrence of residues of the three metabolites in this small sample of hair specimens taken from agricultural workers, demonstrating that it is appropriate to include their determination in routine hair analysis. Our study is the first to propose the detection and quantification of DAP metabolites in hair. This work is particularly important because it proves that hair provides a valuable alternative to a wide range of biological specimens such as blood, urine, etc. for the analysis of the target compounds. Certainly, it is a first step toward validating measurements of DAPs in human hair as an exposure biomarker.

Its application to the analysis of hair samples from agricultural workers occupationally exposed to organophosphate pesticides proved the method's practical use and usefulness, as well as its robustness. The high sensitivity of the proposed procedure allows the monitoring not only of occupationally exposed people but also of the general population, whose exposure occurs mainly through the ingestion of contaminated food and water. Consequently, it would be very interesting to attempt measuring metabolite levels in hair samples collected

from the general population, using the present method, and to compare the metabolite content of hair samples from occupationally and non-occupationally exposed subjects.

The methodology presented showed good linearity, satisfactory precision, high sensitivity and good recoveries for such a complicated analysis as a metabolite residue analysis in the hair matrix is and without the use of any internal standard. Although the absence of an internal standard resulted in adequate quantification, we plan to investigate in future studies if the method can be further improved by using a suitable internal standard. The LODs of the target analytes were lower than those reported in the meconium method (0.51, 0.2 and 0.18 ng mg^{-1} for DMP, DEP and DMTP, respectively) (Whyatt & Barr 2001). Moreover, our method is more sensitive than previously published methods (Ostrea et al. 2006, Posecion et al. 2006) measuring pesticide metabolites in hair (LODs, 0.18–5.88 ng mg^{-1}).

The procedure is relatively easy and rapid. One trained person can process a minimum of 50 samples within 4–5 working days. Other important advantages of this method are the simple extraction of dialkyl phosphates from hair (water extraction), the safe operations, the low sample amount (50 mg) and the relatively small quantities of expensive and hazardous solvents needed. Our methodology requires relatively simple laboratory equipment and modest GC-MS instrumentation that are easily available in most routine laboratories. Consequently, it has the requirements of a routinely applicable method. The use of GC-MS in the SIM mode has shown the advantage of eliminating the effect of the complex matrix analyzed in this paper. Specificity is observed by using at least two ions for each compound.

Moreover, the capability of measuring DAP metabolites in hair samples allows assessment of the long-term cumulative exposure to a wide range of OPs in one analytical run, which increases analytical throughput and saves analysis time and costs. The results obtained in this study allow us to apply the developed method to further investigations and clinical studies, as well as to large-scale epidemiological studies of non-occupationally exposed persons and for the biological monitoring of high-level occupational exposure to OPs. In such situations, hair testing could be used as an alternative or complementary approach to conventional blood and urine analysis as it enlarges the window of detection and offers a series of highly interesting advantages over other biological matrices (stability of specimen, easy and non-invasive sampling, simple handling, easy storage).

The developed procedure could be also potentially applied to other DAP metabolites, such as DETP and dialkyl dithiophosphates (DMDTP, DEDTP) for confirming exposure to organophosphate pesticides. These studies are currently ongoing.

However, despite the valuable results, the main drawback of the method presented is the derivatization and clean-up procedures required which were time-consuming. This analytical approach may be further improved in the future in terms of time and labour by eliminating the derivatization step by using liquid chromatography (LC)-MS system instead of GC-MS. Work in this direction is currently in progress.

In conclusion, it should be noted that notwithstanding the difficulty (Zhang et al. 2007, Covaci et al. 2008, Schramm 2008) involved in the real interpretation of the observed metabolite concentrations in hair, our results clearly demonstrate the ability to assess chronic human past pesticide exposure which offers valuable information to epidemiological and other clinical studies, and open up great potential applications of hair testing in research concerning identification and quantification of pesticide metabolites.

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