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RESEARCH ARTICLE

Dialkyl phosphates in meconium as a biomarker of prenatal exposure to organophosphate pesticides: A study on pregnant women of rural areas in Crete, Greece

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Abstract

1. The authors developed a sensitive analytical method for the determination of dialkyl phosphates (DAPs) in meconium. This method was applied to determine the DAPs, which are non-specific metabolites of the organophosphate pesticides (OPs), in meconium of newborns by mothers who live in rural areas in Crete, Greece. DAPs are considered as biomarkers of exposure to OPs. Meconium is produced in the foetus at approximately 16 weeks of gestation and it acts as a repository of many xenobiotics. The determined organophosphate metabolites were dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP).
2. The DAPs were extracted from meconium by liquid–solid extraction, derivatized, and analysed by gas chromatography–mass spectrometry (GC–MS). The mean percentile recoveries were 76.9%, 65.2%, 94.1%, 109.4%, and 107.2% for DMP, DEP, DMTP, DETP, and DEDTP, respectively. The percentage of positive samples was 92.1% for DMP, 36.8% for DEP, 60.5% for DMTP, 63.2% for DETP, and 57.9% for DEDTP. Mean (\pm standard deviation) and the range concentrations of the positive samples (ng g^{-1}) were 126.74 ± 142.73 (10.64–739.45), 11.46 ± 20.43 (1.50–79.14), 215.05 ± 187.34 (8.54–662.16), 4.92 ± 5.09 (1.25–19.04), and 1.84 ± 2.07 (0.5–8.04) for DMP, DEP, DMTP, DETP, and DEDTP, respectively.
3. Statistical analysis revealed no significant difference in meconium levels between high- and low-risk groups of exposure of pregnant women. However, the results of this study demonstrate that DAPs in meconium may be considered as a potential biomarker for the assessment of foetal exposure to organophosphate pesticides.

Keywords: Meconium; dialkyl phosphates; prenatal exposure; gas chromatography–mass spectrometry (GC–MS); pesticides; pregnancy

Introduction

The majority of the insecticides are neurotoxicants so the exposure of pregnant women and the foetus to organophosphate pesticides (OPs) is a topic of major public concern (Eriksson 1997; Bruckner 2000; Ostrea et al. 2008). Aberrations in neuronal proliferation, migration, differentiation, synaptogenesis, myelination and apoptosis in the foetus have been described in animals and humans antenatally exposed to these compounds (Eriksson 1997; Barone et al. 2000).

An important source of income on the island of Crete, Greece, is agriculture and the most abundant agricultural products are olive oil, vegetables and fruits. The use of OPs in these types of cultivations is widespread. Intoxication of pesticides sprayers due to organophosphates has been extensively reported (Tsatsakis et al. 1998). The potential exposure of pregnant women to these pesticides is under investigation. Many OPs are lipophilic and cross the placenta (Richardson 1995). They are rapidly metabolized in the human body by hydrolysis or oxidative desulfuration, giving the

non-specific metabolites dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP), diethyl thiophosphate (DETP), dimethyl dithiophosphate (DMDTP) and diethyl dithiophosphate (DEDTP), referred as dialkyl phosphate metabolites (DAPs) (Table 1). These metabolites are polar water-soluble compounds and are used as biomarkers of OPs exposure in human population (Margariti et al. 2007; Margariti & Tsatsakis forthcoming 2009).

Blood, urine and post-mortem tissues samples have been used for the assessment of pesticide exposure in the past. These samples provide information for a short period of pesticide exposure and are usually analysed in cases of acute intoxications. The parent pesticides concentrations in various biological samples in cases of poisoning have been extensively reported (Bertsias et al. 2004). Other biological tissues like hair or meconium can provide a wider window of detection (Covaci et al. 2002; Tsatsakis & Tutudaki 2004; Margariti et al. 2007).

Meconium is a complex matrix, consisting mainly of water, but also containing mucopolysaccharides, lipids, proteins, bile acids and salts, epithelial cells, cholesterol and sterol precursors, blood group substances,

squamous cells, residual amniotic fluid, and enzymes. It begins to accumulate in the foetus bowels at approximately 16 weeks' gestation and is not excreted until after delivery (Moriya et al. 1994; Moore et al. 1998; Whyatt & Barr 2001). Meconium is also mentioned as a repository of endogenous compounds and many xenobiotics like licit and illicit drugs (Ostrea et al. 2002; Zhao et al. 2007), food additives, heavy metals, nicotine, alcohol (Wessels et al. 2003), and pesticides (Barr et al. 1999; Bielawski et al. 2005; Posecion et al. 2008) that are transplacentally transferred from the mother to the foetus over the last two trimesters of pregnancy. Hence, meconium can provide a longer historical record for prenatal exposure to pollutant residues. Furthermore, meconium provides several advantages over other biological samples such as wider window of foetal exposure detection, non-invasive analysis, easier sample collection and large amount of sample available for analysis (Hong et al. 2002; Ostrea et al. 2008).

The aim of this study was the development of a sensitive analytical method for the determination of OP non-specific metabolites (DAPs) in meconium and the assessment of foetus exposure to OPs in the island of Crete. This is in continuation and advancement of the authors' studies hitherto focused on the determination of the parent compounds in various matrices (Tutudaki & Tsatsakis 2005).

Table 1. Non-specific metabolites and the parent organophosphate pesticides.

Dialkyl phosphate metabolites	Parent pesticide
Dimethylphosphate (DMP)	Azinphos-methyl, dichlorvos, dicrotophos, dimethoate, fenitrothion, fenthion, malathion, methyl parathion, trichlorfon, chlorpyrifos-methyl, methidathion, mevinphos, oxydemeton-methyl, phosmet, primiphos-methyl, temephos, tetrachlorvinphos, trichlorfon, isazofos-methyl, naled, dicrotophos
Diethylphosphate (DEP)	Chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, sulfotepp, terbufos, tribufos
Dimethylthiophosphate (DMTP)	Azinphos-methyl, dimethoate, fenchlorphos, fenitrothion, fenthion, malathion, methyl parathion, chlorpyrifos-methyl, methidathion, oxydemeton-methyl, phosmet, pirimiphos-methyl, temephos, isazofos-methyl
Diethylthiophosphate (DETP)	Chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, ethyl parathion, phorate, phosalone, sulfotepp, terbufos, tribufos
Diethyldithiophosphate (DEDTP)	Disulfoton, ethion, phorate, phosalone, terbufos,
Dimethyldithiophosphate (DMDTP)	Azinphos-methyl, dimethoate, malathion, methidathion, phosmet

Materials and methods

Sample collection

Thirty-eight meconium samples were collected over 4 months (January–April 2008) by the staff of the Fetal–Maternal Unit, Department of Ob/Gyn, University Hospital of Heraklion, Crete. Each meconium sample was frozen at -20°C immediately after collection. At the end of collection period, meconium samples were transferred to the laboratory of Toxicology, Medical School, University of Crete for analysis.

Data regarding the maternal level of potential exposure to pesticides via occupational and residential environment was collected. The participants in this study were called on to fill a questionnaire and answer the following questions:

- Agricultural work before pregnancy.
- Agricultural work during pregnancy.
- Spouses' occupation (pesticide applicator as farmer or other).
- Involvement in usage of pesticides (spraying, etc.).

The answers were classified in a yes/no form. A non-weighted index of high risk of exposure was estimated

when more than two questions were answered positively. In any other case, the participant was classified in the low risk of exposure group.

Materials

DMP (98%) and dimethyl chlorothiophosphate (DMCITP) (97%) were purchased from Acros Organics (Geel, Belgium); DEP (98.9%) was from Chem Service (West Chester, PA, USA); and *O,O*-diethylthiophosphate potassium salt (98%) (DETP) and diethylthiophosphate salt (95%) (DEDTP) were from Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether (95.5%), toluene (99.5%) and potassium carbonate (K_2CO_3) were obtained from Merck (Darmstadt, Germany). HPLC-grade methanol and acetonitrile were purchased from Roth (Karlsruhe, Germany); and water (HPLC grade) was from Sigma-Aldrich (Buchs, Switzerland). The derivative agent 2,3,4,5,6-pentafluorobenzylbromide (PFBBBr) (99%) was from Sigma-Aldrich (Steinheim, Germany).

DMTP synthesis

The synthesis of DMTP was achieved by hydrolysis of DMCITP as previously described by Hernandez et al. (2002). Briefly, a solution of triethylamine (3 ml) in 40 ml of HPLC-grade water and acetonitrile (10:30 v/v) was prepared and stirred at 0°C. Five grams of DMCITP were added to the above solution. Acetonitrile was added in order to achieve a final volume 200 ml.

Stock solutions

DAPs stock solutions at a concentration of 1 mg ml⁻¹ were prepared in methanol, while dilutions in methanol (working solutions) were prepared weekly in order to cover the entire range of organophosphate metabolite concentrations expected in biological samples. All stock solutions were stored at -20°C, while working solutions were stored at 0°C in the dark.

Statistical evaluation

Levels of metabolites in meconium samples were expressed as the mean and standard deviation (SD). Differences between low risk of exposure and high risk of exposure groups were examined by independent samples *t*-test. Package SPSS 16.0 was used for statistical analysis.

Dialkyl phosphate extraction

Before analysis, meconium samples were brought to room temperature and dried under vacuum and nitrogen stream at room temperature (approximately 26°C)

for 12 h to remove residual water. The dried meconium was homogenized and an amount of 0.5 g was transferred to 6 ml screw top vials. A total of 25 µl of 100 µg ml⁻¹ dibutylphosphate (DBP) were added as an internal standard (IS). After the addition of 2 ml of methanol, the suspension was incubated in an ultrasonic bath for 1 h at room temperature with occasional vortexing, and solid-liquid extraction with mechanical shaking for 30 min followed. Methanol was separated from the solid phase by centrifugation at 4000 rpm for 5 min and transferred to a clean vial which contained 15 mg of K_2CO_3 (Ueyama et al. 2006). A total of 2 ml of acetonitrile-diethylether (1:1 v/v) were added to the meconium (solid phase) and the above procedure was repeated (ultrasonic for 1 h, solid-liquid extraction for 30 min). The organic phase of acetonitrile-diethylether was removed by centrifugation (at 4000 rpm for 5 min) and transferred to the vial containing the initial methanol phase. The combined organic phases were evaporated to dryness under a stream of nitrogen at 35°C or in a vacuum evaporator at room temperature.

A total of 15 mg of K_2CO_3 was added to the residue, reconstituted in 1 ml of acetonitrile and 0.1 ml solution of pentafluorobenzylbromide (PFBBBr) in acetonitrile (1:3 v/v) and incubated in a bath at 80°C for 30 min with occasional swirling (Ueyama et al. 2006). After incubation, the mixture was brought at room temperature and acetonitrile was evaporated to dryness under a stream of nitrogen at 35°C. The residue was dissolved in 100 µl of toluene and 1 µl was injected to GC-MS.

GC-MS analysis

Electron ionization mass spectrometric analysis was performed on a GC-2010 Shimadzu system equipped with a BPX5 (30 m × 0.25 mm × 0.25 µm) capillary column (SGE). Pure helium (99.999%) with a column flow of 1 ml min⁻¹ was used as a carrier gas. A total of 1 µl of the solution was injected into the system in the splitless mode and analysed under the following conditions. The column temperature was initially held at 60°C for 1 min, raised to 180°C at 20°C min⁻¹, held for 1 min, raised to 250°C at 4°C min⁻¹, held for 1 min and was finally raised to 300°C at 25°C min⁻¹, where it remained stable for 2 min. The injector temperature was 270°C. The interface temperature was set at 310°C. The ion source temperature was 230°C. The *m/z* ions, target *m/z* ions and the retention time of each DAP are shown in the Table 2.

Quantitative analysis was achieved in selected ion-monitoring (SIM) mode with a scan time of 0.2 s, using one target ion for quantification and one qualifier ion for confirmation for each compound. Table 2 shows the target ions for each DAP (*m/z*=306 for DMP, 258 for DEP, 322 for DMTP, 350 for DETP and 366 for DEDTP) and the

Table 2. Analytical parameters of the non-specific organophosphate metabolites.

	Retention time (RT) (min)	Target ion (<i>m/z</i>)	Fragments (<i>m/z</i>)	Recoveries (%)	Limit of detection (LOD) (ng ml ⁻¹)	Limit of determination (LOQ) (ng g ⁻¹)	R ² of standard calibration curves	R ² of spiked calibration curves
DMP	17.34	306	306, 110	76.9	5.0	2.50	0.9997	0.9888
DEP	19.84	258	258, 334	65.2	5.0	1.50	0.9989	0.9945
DMTP	21.37	322	322, 211	94.1	10.0	5.00	0.9936	0.9864
DETP	23.40	350	350, 274	109.4	5.0	1.25	0.9989	0.9816
DEDTP	24.97	366	366, 185	107.2	2.5	0.50	0.9977	0.9930
DBP (IS)	26.98	335	335		-	-		

qualifier ions (*m/z*=110 for DMP, 334 for DEP, 211 for DMTP, 274 for DETP and 185 for DEDTP).

Results

Linearity

A nine-point standard curve at concentrations of 0, 5, 10, 25, 50, 100, 250, 500 and 1000 ng ml⁻¹ in methanol was prepared for each of the DAPs. The standard curves of DAPs were linear between the concentrations of zero and 1000 ng ml⁻¹ with coefficients of linearity greater than 0.99 (Table 2). Eight-point calibration curves were prepared using blank meconium samples spiked with known concentrations of DAPs with concentration from zero to 1000 ng g⁻¹ (0, 10, 25, 50, 100, 250, 500 and 1000 ng g⁻¹). Peak area ratios (DAPs response/internal standard response) were used for quantification. The results indicated a good linearity in the concentration range between zero and 1000 ng g⁻¹. The spiked sample curve was linear given $r^2=0.9888$ for DMP; and $r^2=0.9945$ for DEP; 0.9864 for DMTP; 0.9816 for DETP; 0.9930 for DEDTP (Table 2).

Limit of detection and determination

The limit of detection (LOD) for methanolic standards solutions and the limit for quantification (LOQ) were determined empirically by injecting decreasing concentrations of standard and spiked solutions and we defined as our limits the peaks that gave a signal-to-noise ratio of 3 for the LOD and of 10 for LOQ. The LOD was 5.0 ng ml⁻¹ for DMP, DEP and DETP, 10.0 ng ml⁻¹ for DMTP, and 2.5 ng ml⁻¹ for DEDTP. The LOQ was 2.50, 1.50, 5.00, 1.25 and 0.50 ng g⁻¹ for DMP, DEP, DMTP, DETP and DEDTP, respectively (Table 2).

Method validation

The recovery evaluation of the extraction method was performed on spiked meconium. A mix of DAPs was added to 0.5 g of meconium to a final concentrations of 25, 100 and 200 ng g⁻¹. Each sample was measured

in triplicate. Extraction recovery was determined by comparing the ratio of DAPs peak areas/IS peak areas of extracted meconium with the ratio of methanolic standards at the same concentration. The mean recovery of the target compounds with the employed method was estimated from 65.2% (DEP) to 109.4% (DETP) (Table 2).

Methanolic standard solutions at concentration 100 and 500 ng ml⁻¹ of each analyte were included in every batch of samples analysed. Spiked solutions with concentration from 10 to 1000 ng g were used to monitor any possible change to analytical parameters.

Relative standard deviation (% RSD) was used for the determination of the precision of the method. Positive control samples, at a concentration of 100 ng g⁻¹ (for each analyte), were prepared and analysed. The within-day RSD was determined by injecting the positive control sample solutions four times during one working day into the GC-MS system. The between-day RSD of the procedure was also evaluated by injecting positive control samples (100 ng g⁻¹) prepared in three sequential working days. Within-day precision ranged from 9.1% to 14.7% while the between-day precision ranged from 11.2% to 18.1%.

Biological monitoring

Table 3 shows the concentration (ng g⁻¹) of DMP, DEP, DMTP, DETP and DEDTP of 38 meconium samples collected over 4 months. The mean values (\pm standard deviation, SD) of DMP, DEP, DMTP, DETP and DEDTP were 126.74 \pm 142.73, 11.46 \pm 20.43, 215.05 \pm 187.34, 4.92 \pm 5.09, and 1.84 \pm 2.07 ng g⁻¹, respectively. A total of 92.1% of meconium samples were positive for DMP, 36.8% for DEP, 60.5% for DMTP, 63.2% for DETP, and 57.9% for DEDTP. The concentration range (ng g⁻¹) of positive meconium samples for DMP was 10.64–739.45, for DEP was 1.50–79.14, for DMTP was 8.54–662.16, for DETP was 1.25–19.04, and for DEDTP was 0.50–8.04 (Table 3).

The sum of the concentration values of dimethylphosphate metabolites (DMPs), diethylphosphate metabolites (DEPs) and DAPs are also given in Table 3. The 97.4%

Table 3. Concentrations (ng g⁻¹), mean values, sum values, standard deviation, range, and positive samples (in centile) of non-specific organophosphate metabolites from 38 meconium samples collected from newborns whose mothers live in rural areas in Crete.

Sample number	Concentration (ng g ⁻¹)						Sum DMPs ^a	Sum DEPs ^b	Sum DAPs
	DMP	DEP	DMTP	DETP	DEDTP				
1	80.32	8.08	297.73	2.75	0.73	378.05	11.56	389.61	
2	82.76	2.22	n.d.	1.85	0.56	82.76	4.63	87.39	
3	98.79	8.97	48.41	1.53	0.50	147.20	10.95	158.14	
4	87.31	n.d.	20.53	1.96	n.d.	107.84	1.96	109.79	
5	69.59	n.d.	58.52	2.01	0.50	128.11	2.46	130.57	
6	180.41	3.91	n.d.	3.38	0.80	180.41	8.09	188.50	
7	67.80	n.d.	339.12	7.09	1.04	406.93	8.13	415.06	
8	136.25	n.d.	457.61	19.04	2.42	593.86	21.46	615.31	
9	143.96	n.d.	34.72	2.75	0.78	178.67	3.53	182.20	
10	42.40	n.d.	89.12	13.26	2.78	131.53	16.04	147.57	
11	88.90	n.d.	n.d.	1.36	0.52	88.90	1.88	90.78	
12	57.40	25.17	293.17	2.65	n.d.	350.57	27.82	378.39	
13	74.53	n.d.	n.d.	1.56	0.57	74.53	2.13	76.66	
14	90.65	1.50	181.51	4.31	n.d.	272.16	5.81	277.97	
15	71.91	3.38	19.88	n.d.	0.65	91.79	4.02	95.81	
16	82.16	n.d.	114.19	n.d.	n.d.	196.35		196.35	
17	118.80	n.d.	423.71	n.d.	8.04	542.50	8.04	550.54	
18	739.45	79.14	n.d.	9.18	4.50	739.45	92.83	832.28	
19	339.99	n.d.	474.38	18.10	4.33	814.37	22.43	836.80	
20	468.71	1.61	n.d.	5.30	0.98	468.71	7.89	476.60	
21	65.14	6.16	411.60	n.d.	n.d.	476.74	6.16	482.90	
22	58.29	n.d.	53.87	1.90	0.58	112.16	2.48	114.64	
23	190.74	4.19	n.d.	5.10	1.61	190.74	10.90	201.64	
24	106.35	n.d.	n.d.	5.67	0.72	106.35	6.39	112.74	
25	61.25	n.d.	n.d.	1.28	0.50	61.25	1.77	63.02	
26	291.91	3.46	n.d.	n.d.	1.35	291.91	4.81	296.72	
27	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
28	13.24	n.d.	n.d.	n.d.	n.d.	13.24	n.d.	13.24	
29	n.d.	n.d.	93.76	n.d.	n.d.	93.76	n.d.	93.76	
30	189.67	n.d.	n.d.	n.d.	n.d.	189.67	n.d.	189.67	
31	55.44	n.d.	662.16	n.d.	n.d.	717.60	n.d.	717.60	
32	21.89	n.d.	55.01	1.25	n.d.	76.90	1.25	78.15	
33	141.19	n.d.	n.d.	n.d.	n.d.	141.19	n.d.	141.19	
34	41.67	10.68	336.52	n.d.	n.d.	378.18	10.68	388.86	
35	10.64	n.d.	128.48	n.d.	n.d.	139.11	n.d.	139.11	
36	n.d.	n.d.	343.62	3.32	n.d.	343.62	3.32	346.94	
37	26.87	n.d.	n.d.	1.43	6.00	26.87	7.43	34.30	
38	39.64	1.89	8.54	n.d.	n.d.	48.18	1.89	50.07	
Mean values	126.74	11.46	215.05	4.92	1.84				
± standard deviation (SD)	142.73	20.43	187.34	5.09	2.07				
Range	10.64–739.45	1.50–79.14	8.54–662.16	1.25–19.04	0.50–8.04	13.24–814.37	1.25–92.83	13.24–836.80	
Percentage positive sample	92.1	36.8	60.5	63.2	57.9	97.4	78.9	97.4	

^aDMPs, methyl phosphate metabolites.^bDEPs, ethyl phosphate metabolites.

n.d., Not detected.

of the meconium was positive for at least one DAP with concentration range from 13.24 to 836.80 ng g⁻¹, and only one meconium sample was negative (below the LOQ) for all the analysed DAPs (Table 3). As is obvious, DMPs

are the metabolites with the main contribution to the detected concentration levels compared with those of DEPs. The 5.3% of samples were positive for all analysed DAPs, the 55.3% were positive for both DMP and DMTP,

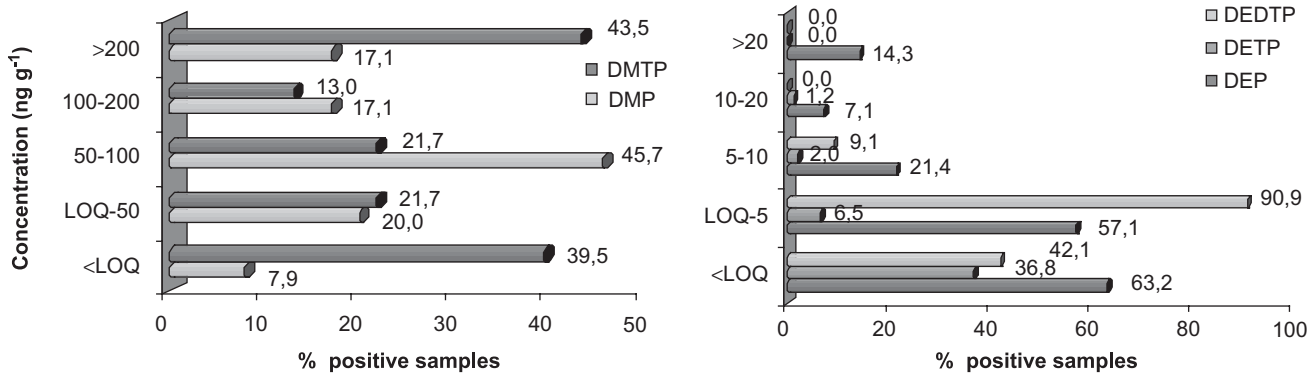


Figure 1. Percentage of positive meconium samples with concentrations falling within the specified concentration scale.

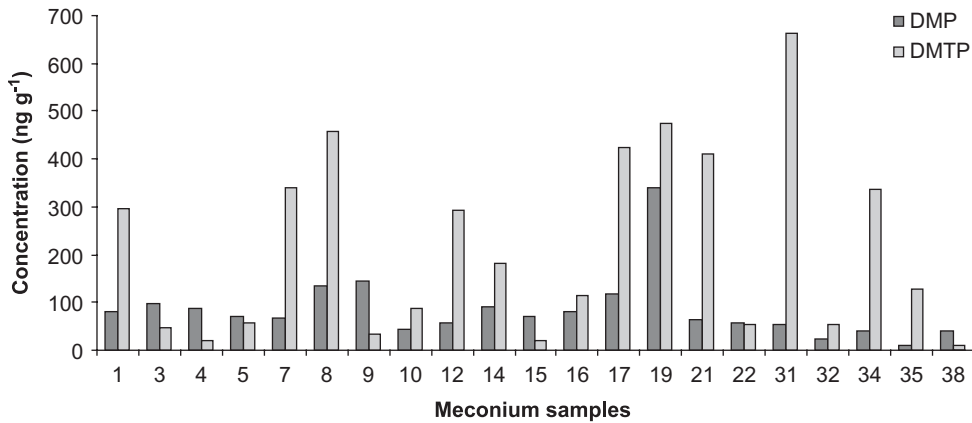


Figure 2. Differences in concentration values between DMP and DMTP in selected positive samples.

50.0% for both DETP and DEDTP, and the 18.4% were positive for DEP, DETP and DEDTP.

The majority (57.1%, 6.5% and 90.9%) of the positive meconium samples for DEP, DETP and DEDTP, respectively, were in the concentration range from LOQ to 5 ng g⁻¹, 21.4%, 2.0% and 9.1% were in the concentration range from 5 to 10 pg mg⁻¹, and only 7.1%, 1.2% and 0% were in the concentration range from 10 to 20 ng g⁻¹. There was no positive sample for DEDTP at a concentration above 10 ng g⁻¹ and for a DETP concentration above 20 ng g⁻¹. On the other hand, the majority of the positive samples (45.7%) for DMP was in a concentration range from 50 to 100 ng g⁻¹ and the 43.5% of the positive for DMTP samples were in a range above 200 ng g⁻¹ (Figure 1).

A total of 21 of 38 samples were positive for both DMP and DMTP. As can be seen in Figure 2, most of them (14 of 21) gave higher values for DMTP than DMP. Similarly, seven of 38 sample were positive for DEP, DETP and DEDTP. In five of seven samples, DEP concentrations were higher than the values of DETP and DEDTP and only on two of seven DETP given higher values than DEP. The concentration values of DEDTP in all cases were lower than these of DEP and DETP (Figure 3). Generally,

97.4% of the analysed samples were positive for DMP or DMTP (DMPs, methyl phosphate metabolites) and 78.9% were positive for DEP or DETP or DEDTP (DEPs, ethyl metabolites) (Table 3).

Table 4 presents statistical analysis of DAP (DMP, DEP, DMTP, DETP, DEDTP) levels in meconium-positive samples derived from the low risk of exposure and the high risk of exposure groups of pregnant women living in rural areas in Crete. The relation of the samples to groups was effected according to the index described in the second section. No statistically significant difference in mean levels between the low risk and high risk of exposure group is observed ($p > 0.05$).

Discussion and conclusion

The aim of this study was the development of a sensitive analytical method for the determination of OP non-specific metabolites in meconium and the assessment of foetus exposure to OPs in the island of Crete, Greece.

DAP metabolites are considered as biomarkers for a great number of OP pesticides. DAPs can be detected

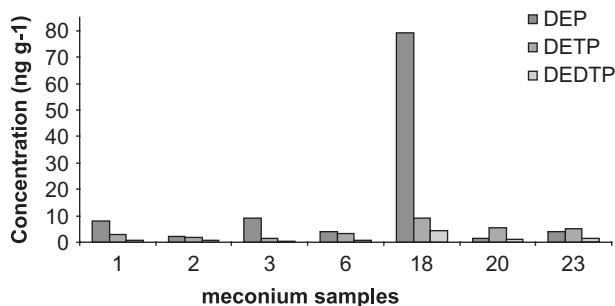


Figure 3. Differences in concentration values for DEP, DETP and DEDTP in selected positive samples.

Table 4. Statistical data of DAP (DMP, DEP, DMTP, DETP, DEDTP) levels (ng g⁻¹) in meconium-positive samples derived from low risk of exposure and high risk of exposure groups of pregnant women living in rural areas in Crete.

	Low risk of exposure			High risk of exposure			P
	n	Mean (ng g ⁻¹)	± SD	n	Mean (ng g ⁻¹)	± SD	
DMP	25	149.5	159.9	10	67.8	58.9	0.128
DEP	9	12.0	25.3	5	9.2	9.4	0.818
DMTP	15	210.3	200.6	8	222.6	172.4	0.884
DETP	19	4.0	4.7	5	6.4	7.3	0.374
DEDTP	19	1.3	2.3	3	1.0	1.0	0.819
SumDMPs	26	265.4	224.3	11	223.8	193.9	0.596
SumDEPs	22	10.0	19.0	8	10.2	9.3	0.977
SumDAPs	26	274.3	234.2	11	231.7	200.7	0.602

n, Number of pregnant women; SD, standard deviation.

in urine at exposure levels below those levels that affect cholinesterase activity. Because the parent OPs differ significantly in toxicity, the measurement of DAP levels does not provide direct measures of toxicity potential (Coye et al. 1986; Wessels et al. 2003).

Meconium was used for the assessment of pregnant women to OPs only in one study (Whyatt & Barr 2001), in which authors used methanol for the extraction of DAPs from dry meconium. Authors mentioned good results for DEP and DETP but they detected DMP in only one of 20 meconium samples. DMTP and DMDTP were not detected in any meconium sample. Other researchers used a mixture of acetonitrile–diethylether for the extraction of DAPs from urine. Hardt & Angerer (2000) used the above mixture for the determination of DAP in acidified human urine. Bravo et al. (2002) analysed DAPs using azeotropic co-distillation with acetonitrile and also determined (Bravo et al. 2004) six DAP metabolites (DMP, DEP, DMTP, DETP, DEDTP and DMDTP) in lyophilizate urine samples. The lyophilizate urine samples were extracted with acetonitrile and ethyl ether with DAPs recoveries greater than 80%. The acetonitrile–diethylether solvent mixture proved to be efficient for the isolation of metabolites from biological samples. Our

experiments based on acetonitrile–diethylether solvent gave satisfactory analyte recovery yields.

Meconium has also been used in the past to determine foetal exposure to heavy metals and organophosphate or organochlorine pesticides. Ostrea et al. (2002) analysed 426 meconium samples (Manila, Philippines) for lead, mercury, cadmium and arsenic as well for chlorpyrifos, DDT, chlordane, diazinon, lindane, malathion, parathion and pentachlorophenol. The concentration range for the organophosphate diazinon was 0.24–6027.00 µg ml⁻¹ (34.3% positive samples) with a mean value of 226.46 µg ml⁻¹, for malathion was 0.04–771.8 µg ml⁻¹ (53% positive, mean = 71.69 µg ml⁻¹), for parathion 0.04–2116.36 µg ml⁻¹ (32% positive, mean = 86.03 µg ml⁻¹) and for chlorpyrifos 0.40–458.04 µg ml⁻¹ (11% positive, mean = 53.46 µg ml⁻¹).

The same group of investigators analysed cord blood, infant hair and meconium samples simultaneously to determine the most sensitive matrix to detect antenatal pesticide exposure (Ostrea et al. 2008). In this comparison, analysis of infant hair, cord blood and meconium the authors reached the conclusion that meconium is the best matrix for this purpose. The pesticides under investigation were propoxur, diazinon, lindane, transfluthrin, malathion, chlorpyrifos, bioallethrin, pretilachlor, DDT, cyfluthrin and cypermethrin. Eight of these pesticides were detected in meconium with a frequency between 0.2% for diazinon and 23.8% for propoxur. Cord blood and infant hair were positive, each for a single pesticide, propoxur and chlorpyrifos, respectively. In general, the low incidence of parent pesticide detection success in the above study signifies either no exposure or low sensitivity of the applied analytical methodology for the concrete analysis. This indicates the competent usage of DAPs as biomarkers of organophosphorous exposure.

Hong et al. (2002) also reported an analytical method for the determination of foetal exposure to organochlorine pesticides by GC-MS using meconium. They analysed 60 meconium samples collected in a period of two years, three of which were positive for *p,p*-DDE, a metabolite of DDT (5% positive samples). This paper also notes that the sensitivity of the analytical methodology is probably non adequate for low-level exposure detection.

Whyatt & Barr (2001) showed that DAPs (DMP, DEP and DETP) can be detected in meconium samples (without knowledge of prenatal pesticides use). They also showed that DEP, DETP and DMP are stable in pooled meconium samples over 12 h at room temperature. In their study, the concentration range of DEP was from 0.8 to 3.2 µg g⁻¹ (95% positive samples) and from 2.0 to 5.6 µg g⁻¹ for DETP (100% positive samples). DMP and DEDTP were detected only in one of 20 samples with concentrations of 16.0 and 1.8 µg g⁻¹, respectively.

DMTP and DMDTP were not detected in any meconium sample. The level of DMP given in that study ($16 \mu\text{g g}^{-1}$) is comparable with blood samples levels ($3.9\text{--}4.9 \mu\text{g ml}^{-1}$) or urine samples ($33.5\text{--}50.4 \mu\text{g ml}^{-1}$) from cases of poisonings (Tarbah et al. 2004).

As can be observed in Table 5, the urine DAP concentrations in general population are generally lower than those in exposed population. The mentioned urinary concentration levels of DAPs in general population varied from 5.1 to 20.1 ng ml^{-1} for DMP, from 0.7 to 8.6 ng ml^{-1} for DEP, from 1.1 to 21.2 ng ml^{-1} for DMTP, from 1.0 to 1.4 ng ml^{-1} for DETP, and from 0.06 to 1 ng ml^{-1} for DEDTP (Oglobline et al. 2001; Arcury et al. 2006; Dulaurent et al. 2006; Ueyama et al. 2006). Hernandez et al. (2004) detected 52 ng ml^{-1} DMP and 47 ng ml^{-1} DMTP in urine samples of a grower after application of methyl parathion (exposed population) (Table 5). Bradman et al. (2003) reported a low concentration level and low detection frequency of DMP, DEP and DMTP (0.32 , 0.31 and 0.43 ng ml^{-1} , respectively) in amniotic fluid collected from women living in agricultural area.

In the present study, the detected levels of DAPs in meconium are higher than those reported in urine from non-occupationally exposed population (general population) and similar to, or higher than those in urine from occupationally exposed population (Table 5). DAPs concentrations are also lower (for all the dialkyl phosphate metabolites) than those previously reported (Whyatt & Barr 2001). Furthermore, a great number of meconium samples were found positive for DMP, DMTP and DEDTP

and their detection frequency was higher than in previous studies (Whyatt & Barr 2001). This could probably be attributed to the high sensitivity of the presently developed method. The determination limits ranged from 0.5 ng g^{-1} for DEDTP to 5.0 ng g^{-1} for DMTP.

The observed high incidence of DAPs detection in meconium samples is probably due to the wide use of pesticides in the region of Crete (Tsatsakis et al. 2008). The most frequently used pesticides in the region of Crete are azinphos methyl, dichlorvos, malathion, dimethoate, chloropyrifos methyl which produce the non specific dimethylphosphate metabolites (DMP and DMTP) and chloropyrifos which produces the diethylphosphate metabolites (DEP and DETP) (Table 1). Especially dimethoate is widely used in the cultivation of olive trees which is the most prevalent cultivation in Crete. Olive oil is one of the most important constituents of the Mediterranean diet and contains residual levels (below maximum residue limits (MRLs)) of dimethoate and fenthion if derived from conventional cultivations (Tsatsakis et al. 2003).

Our methodology and derived results indicate that DAPs can be detected in meconium samples, suggesting that there is a cumulative transplacental exposure of the foetus to organophosphate pesticides, probably through the diet of the mothers or via occupational exposure. The detected values though, do not suggest that this exposure may be attributed to heavy exposure of organophosphate pesticides according to the existing knowledge of the scientific literature.

Table 5. Reported concentrations (median, mean, and range) of dialkyl phosphates (DAPs) in urine, meconium, and amniotic fluid from mothers and newborns of the general and exposed population and from meconium of newborns born by mothers who live in rural areas in Crete.

	Concentration (ng ml^{-1} or ng g^{-1})					Values	Sample	References
	DMP	DEP	DMTP	DETP	DEDTP			
General population	20.1	4.5	4.6	< LOD	< LOD ^c	Median	Urine	Dulaurent et al. (2006)
	5.1	8.6	21.2	1.4	0.06	Mean	Urine	Arcury et al. (2006)
	13	3	2	1	1	Median	Urine	Oglobline et al. (2001)
	17.7	0.7	1.1	1.0		Median	Urine	Ueyama et al. (2006)
	16 000 ^a	800–3200	n.d.	2000–5600	1800 ^a	Range	Meconium	Whyatt & Barr (2001)
Exposed population	22.7	0.4	4.1	1.5		Median	Urine	Ueyama et al. (2006) ^b
	52	-	47	-	-	^a	Urine	Hernandez et al. (2004) ^b
	0.30–0.340.32	0.26–0.360.31	0.43	-	-	RangeMean	Amniotic fluid	Bradman et al. (2003)
Present study ^d	126.74	11.46	215.05	4.92	1.83	Mean	Meconium	
	10.64–739.45	1.50–79.14	8.54–662.16	1.25–19.04	0.50–8.04	Range	Meconium	

^aOne positive value.

^bSprayers.

^cLimit of detection (LOD) = $0.5 \mu\text{g l}^{-1}$ for DETP and $0.9 \mu\text{g l}^{-1}$ for DEDTP.

^dGeneral population from rural areas with potential occupational exposure.

Clinical data regarding the outcome of pregnancy in our studied population is available, and includes presence/absence of pregnancy complications, gestational week of delivery, and presence/absence of foetal and neonatal congenital abnormalities, birth weight and neonatal somatometric measurements. However, as the pregnancy and neonatal outcome are influenced by a large number of contributing factors, statistical correlations with multiple regression analysis will be performed in a larger sample group in our ongoing study.

The present method allows the detection and determination of five non-specific metabolites of organophosphates in dry meconium. The extraction procedure of dialkyl phosphates from meconium (ultrasonic and liquid–solid extraction) is simple and fast and no special equipment or technique is required for the preparation stage. The described methodology enables high sensitivity and allows the detection of DAPs at concentration levels of ng g^{-1} meconium. The results indicate that the measurement of non-specific organophosphate metabolites in meconium may be used as a valuable biomarker of prenatal exposure. Further investigations will evaluate potential correlation of DAPs levels with foetal growth parameters, congenital abnormalities, and pregnancy complications or adverse pregnancy outcome.

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