The Analysis of Common Metabolites of Organophosphorus Pesticides in Urine by Gas Chromatography/Mass Spectrometry

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Most organophosphorus pesticides may be metabolized to yield some common phosphates in human or in animals, and these metabolites may be used as the exposure biomarkers to pesticides. In this study, we developed the extraction method of four phosphate metabolites from the spiked human urine in high recovery by the solid phase extraction with a reverse-phase cartridge (cyclohexyl silica) followed by the elution with methanol. The extracted urinary metabolites were derivatized with hexamethyldisilazane/trimethyl-chlorosilane/pyridine (2 : 1 : 10, v/v/v) and identified by gas chromatography/mass spectrometry. Calibration curve obtained from each metabolite standard using by GC/MS/SIM has shown good linearity and detection limits of metabolites were the range of 0.05-0.1 μg/ml in urine. Phenthoate, one of the organophosphorus pesticides, was orally administrated to rats. Four metabolites were detected in the rat urine. The results of this study may be applied to development of exposure biomarkers for monitoring of environmental pollutants.

Introduction

There has been a long-standing concern in the estimation of human exposure to organophosphorus pesticides, particularly since these compounds are widely used and are the most frequent cause of pesticide related illnesses. On entering the body, most organophosphorus pesticides may be metabolized to yield one or more of the four common dialkyl phosphates shown in Table 1. Quantities of metabolites excreted in the urine have been found to correspond to the pesticide dose.[1,2]

The result obtained from measurement of these urinary metabolites could be applied to monitor the exposure to organophosphorus pesticides. The dialkyl phosphates in Figure 1 represent possible metabolites of about 75% of the organophosphate pesticides in the EPA's Pesticides and Industrial Chemicals Repository. Actually, the primary disadvantage of measurement of urinary dialkyl phosphates lies in the analytical difficulties. These compounds are highly water soluble, ionized in urine, and need to be derivatized to be sufficiently volatile for gas chromatographic analysis.

Although there are many published procedures for extraction and analysis of urinary dialkyl phosphates,[2-7] the methods remain tedious and cumbersome. The majority of the published methods relies on liquid-liquid extraction or solid phase extraction (SPE) of the metabolites, derivatization with diazoalkane reagents or trimethylanilinium hydroxide (TMAH), and analysis by gas chromatography (GC). Diazoalkanes have frequently been used for alkylation of dialkyl phosphates and thiophosphates prior to GC analysis. Diazoalkane reagents such as diazoethane and diazopentane give a isomer mixture of thionate and thiolate esters, formed in irregular proportions, when the metabolite *O,O*-diethylphosphorothionate (DETP) is derivatized.[7] Due to the possible hazards in the preparation and use of diazoalkanes, it is better to devise a safer alternative method for derivatization of these compounds.[4] TMAH is a methylating reagent frequently used in clinical tests for barbiturates. At a high temperature, provided in this case by the GC injector block, TMAH may methylate dialkyl phosphates.[8] The disadvantage of this reagent is that it requires a column dedicated to its use, since it leaves a residue on the column that will methylate compounds injected subsequently, whether required or not.[9]

In addition, these metabolites could not be confirmed, because in conventional approaches to urinary organophosphate metabolite analysis, they used a GC/Flame Photometric Detector (FPD). A mass spectrometer(MS) is better than a FPD due to the ability of identifying these metabolites. Metabolites can be identified by a GC detector by comparing the retention times of the sample with those of the standard. In GC-MS, metabolites are identified on the basis of their mass spectra.

In this study, solid phase extraction was used for extraction of organophosphate metabolites from urine, and the extract was derivatized before the GC/MS run was made.

As an application of this method, phenthoate, one of the organophosphorus pesticide, was orally administered to rats.

Experimental

Materials. Dialkyl phosphates were purchased from Ultra Scientific Co (N.kingstown, RI, USA) and triphenylphosphate, the internal standard, was purchased from Sigma Co. (St Louis, MO, U.S.A.).

The cyclohexyl cartridge (40 μm, 60 Å) for extraction was obtained from J.T. Baker (Phillsberg, NJ, U.S.A.). The silylating agents, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from Sigma Co. and other organic solvents were purchased from J.T.Baker. Sodium chloride, ammonium sulfate, ammonium acetate, acetic acid, and hydrochloric acid were of analytical reagent grade and obtained from Kanto Chemical Co (Chuo-Ku,

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Tokyo, Japan).

Instruments. A Hewlett-Packard model 5890 Series II Plus gas chromatograph directly interfaced to a HP 5972 mass selective detector was used. For the data analysis, a HPG 1034C MS chemstation was used. The extraction of biological sample were done by a J. T. Baker SPE-24G Extraction Kit.

A HP fused-silica capillary column (length 25 m, i.d. 0.2 mm, film thickness 0.33 μm) was coupled to the ion source. Helium at a flow rate of 0.8 ml/min was employed as a carrier gas. Samples were injected in split at a ratio of 10 : 1. The injector and the transfer line in GC/MS were at 280 °C and 300 °C, respectively.

The oven temperature was set at 70 °C initially, subsequently increased by 4 °C per min to 150 °C, held there for 1 min, subsequently increased by 20 °C per min to 310 °C, and held there for 1 min. A 2 μl aliquot of a derivatized sample was injected. The mass spectrometer was operated at 70 eV in the electon impact (EI) mode using SCAN or SIM (selected ion monitoring). The operating conditions of GC/MS and the selected ion groups for the identification of 4 metabolites in SIM mode were listed in Table 1.

Preparation of standard solutions. All Standards of four metabolites in Figure 1 were prepared in methanol at a concentration of 1 mg/ml and stored at 4 °C in the dark. Any analytical mixed standard of all four metabolites was prepared in acetone.

Derivatization of standards. The dry residue (containing 100 μg of each metabolite) was dissolved in 100 μl of a mixture of HMDS-TMCS-pyridine (2 : 1 : 10, v/v/v). The mixture was agitated on vortex.

Extraction procedure for recovery test. The cyclohexyl extraction cartridge was used to extract dialkyl phosphate metabolites from urine. The effect of various salts and pH on recovery of dialkyl phosphate metabolites were studied. A 3 ml aliquot of urine was pipetted into a 15 ml test tube. A 30 μl aliquot of the metabolite standard solution (10 μg/ml) was spiked in the urine sample (3 ml). Sodium chloride, ammonium sulfate, ammonium acetate were added as salts, and acetic acid, or hydrochloric acid was added as a pH modifier, and the mixture was vortexed for 1 min.

The cyclohexyl extraction cartridge was pre-washed with one column volumn (3 ml) of methanol, water, and salt-saturated water. The urine sample was applied to the cartridge. The cartidge was washed with 2 ml 20% methanol in hexane. Under a negative pressure (30 mmHg), the cartri dge was then placed into a 15 mL graduated centrifuge tube and eluted with 3 ml methanol. Triphenylphosphate (10 μg/ml, 25 μl) was added as an internal standard and 0.4 g powdered anhydrous sodium sulfate was added to the methanol eluate, and the solution was vortex-mixed.

Table 1. GC/MS operating parameters

- Column : Ultra-2 (25 m × 0.2 mm I.D., 0.33 um film thickness)
- Carrier gas: He at 0.8 ml/min
- Split ratio: 1/10
- Injection part temp.: 280 °C
- Transferline temp.: 300 °C
- Oven temp. program:

Initial temp. (°C)	Initial time (min)	Rate (°C/min)	Final temp. (°C)	Final time (min)
70	0	4	150	1
		20	310	1

- SIM mode (Solvent delay: 3.0 min)

Group	Start time (min)	Selected ions, m/z
1	3.0	12 (183, 153, 105, 172, 125 109, 226, 211, 155, 242 199, 171)
2	25.0	3 (326, 233, 170)

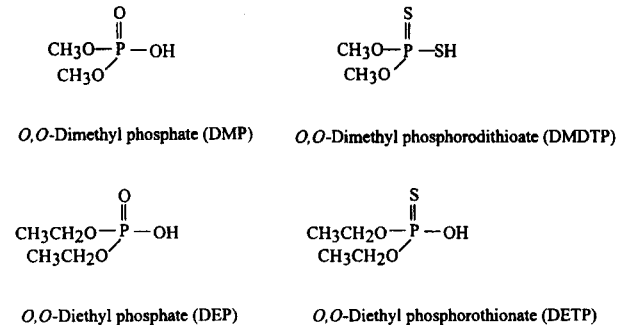
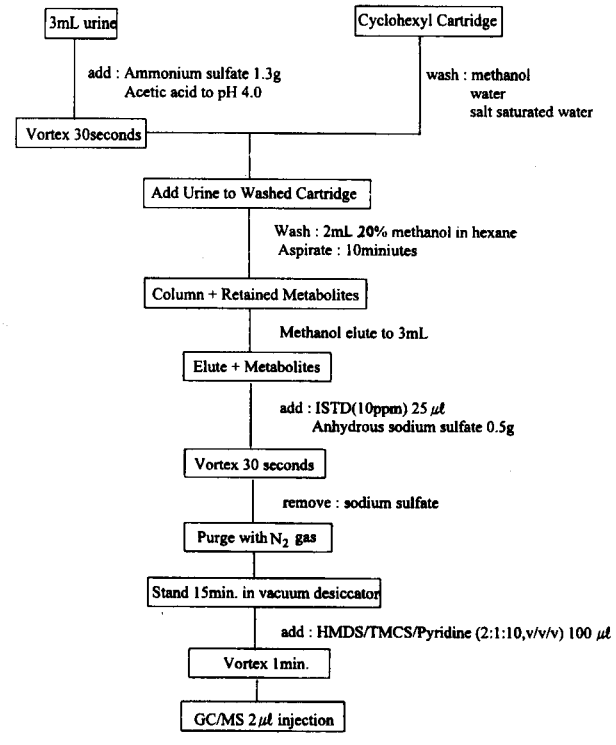


Figure 1. The structures of metabolites of organophosphorous pesticides.

After removing sodium sulfate, methanol was evaporated under nitrogen stream. The centrifuge tube was dried in a vacuum desiccator with P_2O_5/KOH for 15 min. An 100 μl portion of the derivatization solution (HMDS/TMCS/pyridine, 2 : 1 : 10, v/v/v) was added, and the mixture was vortex-mixed for 1 min. The extraction procedure of urinary metabolites was the same as Scheme 1.

Oral administration of phenthoate to rats. Phenthoate (Figure 2) was orally administered to the two male



Scheme 1. Plow diagram for analysis of common metabolites of organophosphorous pesticides in uribne.

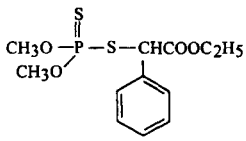


Figure 2. The structures of phenthoate (MW=320).

Sprague-Dawley rats (200 mg/kg, in 0.5% Carboxymethylcellulose solution). Urine samples were collected for 24 hr in metabolic cages. A blank urine sample was collected before the administration of phenthoate to rats.

Results and Discussion

This procedure was capable of analyzing *O,O*-dimethyl phosphate (DMP), *O,O*-diethylphosphate (DEP), *O,O*-dimethyl phosphorodithioate (DMDTP), and *O,O*-diethyl phosphorothionate (DETP).

The efficiency of HMDS/TMCS/pyridine as a silylation agent was examined by using various reaction temperatures (room temperature, 60 °C, and 80 °C) and reaction times (1, 5, 30 min, 1, 2, 4, 8, 12, and 24 hr) The reaction was completed only by vortex-mixing for 1 min at room temperature. The structure of derivatization reagent and the reaction mechanism are shown in Figure 3. and Figure 4, respectively. The mass spectra of all derivatized metabolites are shown in Figure 5. Unusually methylation reaction occured in DMDTP instead of silylation reaction. The retention times and specific mass fragment ions of the derivatized metabolites are shown in Table 2. The calibration curve of each standard metabolite has shown good linearity and the detection limits of four metabolites from urine were the range of 0.05-0.1 μg/ml (Table 3).

The total ion chromatogram (TIC) and the screening profile of those metabolites were shown in Figure 6. The ef-

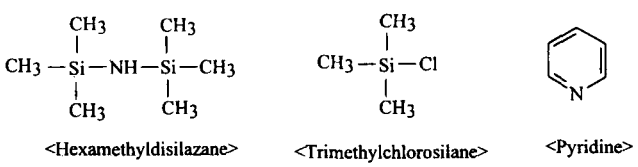


Figure 3. The structures of derivative reagents.

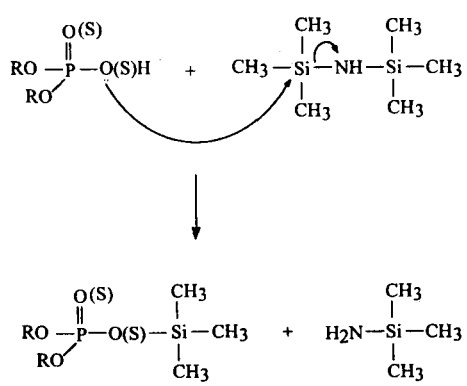


Figure 4. The reaction mechanism between metabolites and derivatizing reagent.

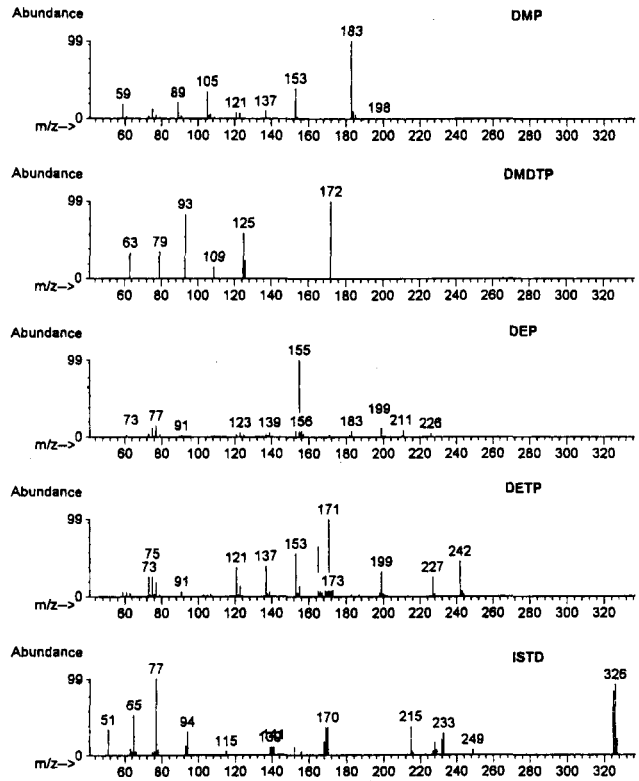


Figure 5. The mass spectra of four metabolites and ISTD.

fects of various salts on the recovery of dialkyl phosphate metabolites were studied in developing this procedure. Salts were added to urine at amounts slightly in excess of that needed to produce a saturated solution. Salt addition usually improved the extraction recovery of metabolites from spiked urine samples, sulfate salts giving better results than either acetate or chloride salts (Table 4).

A saturated solution of ammonium sulfate was found to be the best of those tested. The pH of the urine imposed a great effect on recoveries (Table 5). Recoveries were the best when acetic acid was used to adjust pH to ap-

Table 2. Retentions and characteristic mass fragment ions of derivatized metabolites

Peak no.	Metabolites	Retention		MW	Mass fragment ions m/z
		T_R, min	RT_R		
1	DMP	7.73	0.27	198	198 183 153 105
2	DMDTP	10.87	0.38	172	172 125 109 93
3	DEP	11.08	0.39	226	226 211 199 155
4	DETP	12.47	0.44	242	242 199 171 153
ISTD	TPP	28.43	1.00	326	326 233 170 94

Table 3. *Calibration curves and detection limits of metabolites*

Metabolites	Ion	Conc. range (μg/ml)	*Y=ax+b*			PQL (μg/ml)
			a	*b*	*r*	
DMP	183	0.1-10	1.61	+0.0041	0.998	0.05
DMDTP	172	0.1-10	1.64	+0.00165	0.999	0.1
DEP	155	0.1-10	2.54	+0.0331	0.996	0.05
DETP	171	0.1-10	1.35	+0.0153	0.997	0.05

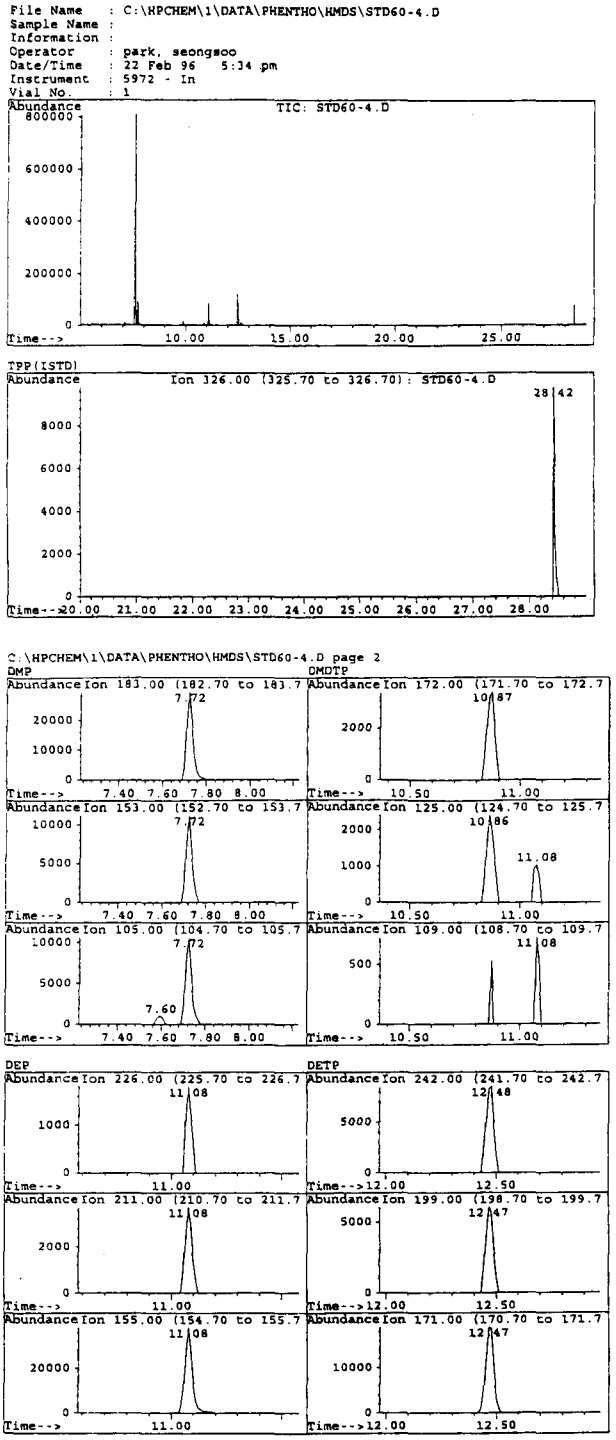


Figure 6. GC/MS/SIM screening profile of the standard mixture.

proximately 4. This may be due to the suppression of ionic form of phosphate metabolites. The recovery data in Table 6 was obtained by the analysis of urine samples spiked at two levels. The low recovery of DMDTP was the result of incomplete retention on the extraction cartridges.

Phenthoate is one of the organophosphorus pesticides, widely used as insecticide in Korea. Because phenthoate has two methoxy groups next to phosphorous, it is possible to detect DMP and DMDTP of four metabolites in urine after oral dose. Both DMP and DMDTP were detected after

Table 4. Effect of salt addition on metabolite recovery normalized for ammonium sulfate

Salt	DMP	DMDTP	DEP	DETP
None	0.2	0.1	0.3	0.2
Sodium chloride	0.4	0.5	0.7	0.7
Ammonium sulfate	1.0	1.0	1.0	1.0
Ammonium acetate	0.3	0.1	0.0	0.3

Table 5. Effect of pH modification on metabolite recovery normalized for acetic acid

Modifier	PH	DMP	DMDTP	DEP	DETP
None	7.0	0.4	0.5	0.4	0.5
Acetic acid	4.0	1.0	1.0	1.0	1.0
Hydrochloric acid	3.0	0.5	0.7	0.3	0.7

Table 6. Recovery of dialkyl phosphates from fortified urine samples

Concentration	% Recovery (RSD) (n=4)			
ppm	DMP	DMDTP	DEP	DETP
0.3	87.0 (4.2)	35.7 (9.5)	102 (2.3)	97.3 (5.1)
0.1	89.3 (6.1)	34.1 (9.2)	81.0 (4.6)	85.9 (2.2)

oral administration to rats. Their TIC and mass spectral data are shown in Figure 7 and 8, respectively. In addition, monomethyl phosphate and dimethylthiophosphate which

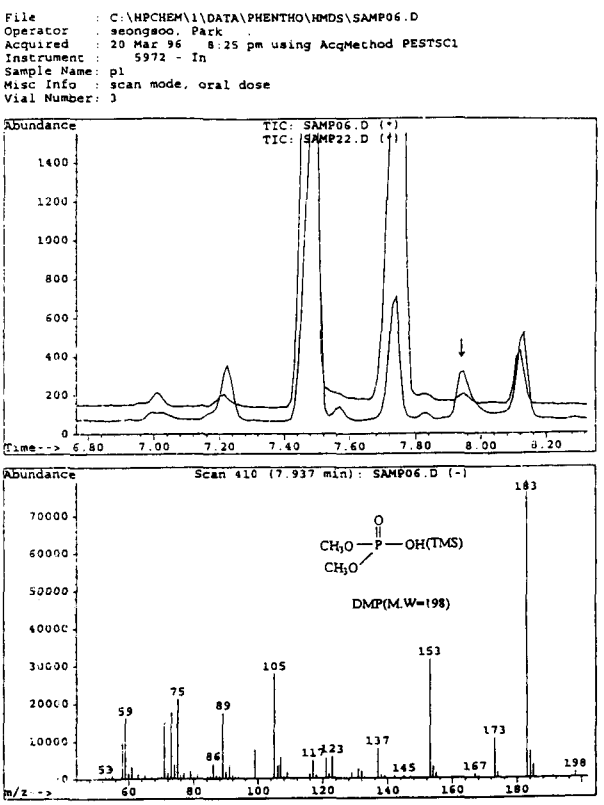


Figure 7. GC/MS total ion chromatogram and mass spectrum of urinary metabolite, DMP. Molecular ion at m/z 198, and major fragment at m/z 183 (due to the loss of a methyl group).

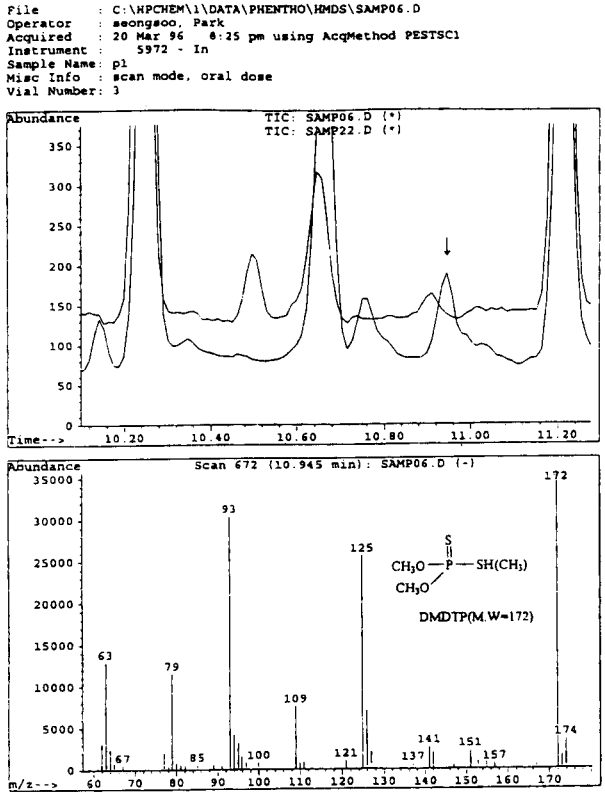


Figure 8. GC/MS total ion chromatogram and mass spectrum of urinary metabolite, DMDTP. Molecular ion at m/z 172, and major fragment at m/z 172.

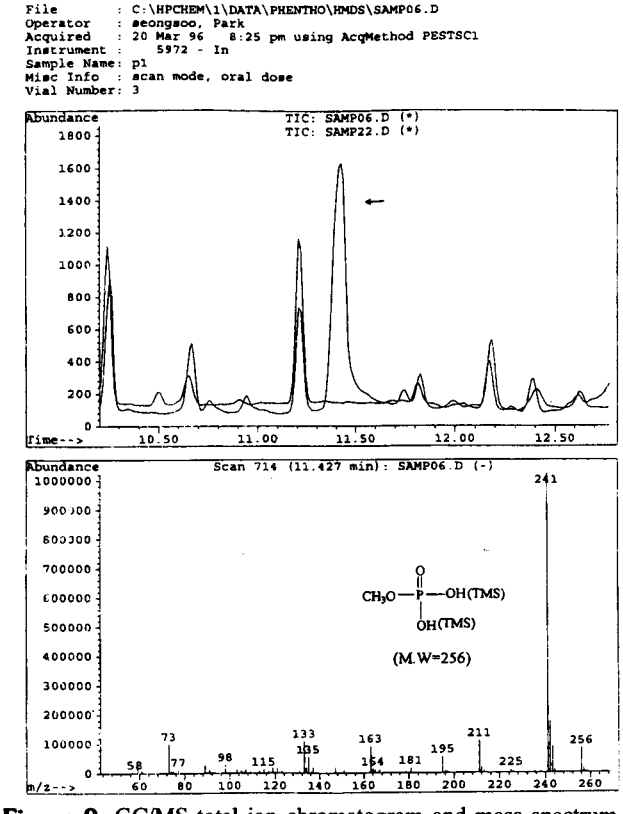


Figure 9. GC/MS total ion chromatogram and mass spectrum of urinary metabolite, monomethylphosphate. Molecular ion at m/z 256, and major fragment at m/z 241 (due to the loss of a methyl group).

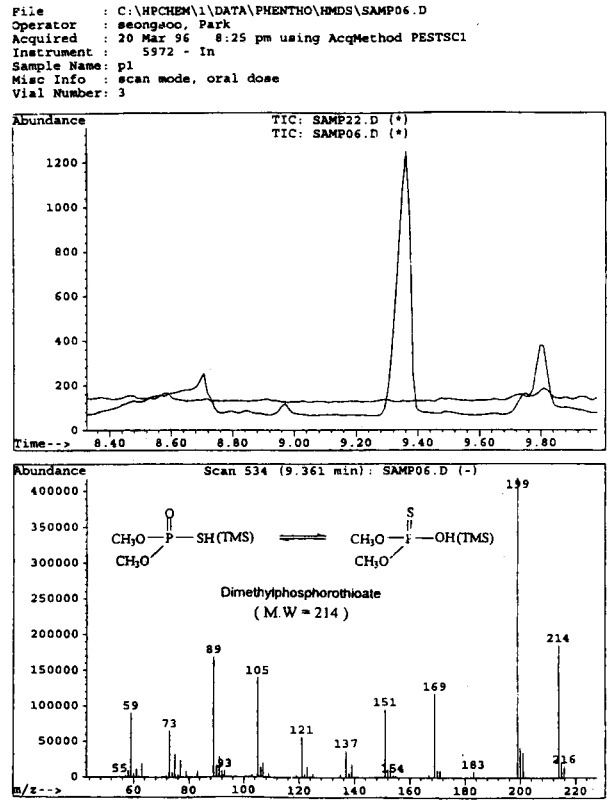


Figure 10. GC/MS total ion chromatogram and mass spectrum of urinary metabolite, dimethylphosphorothioate. Molecular ion at m/z 214, and major fragment at m/z 199 (due to the loss of a methyl group).

were expected to be metabolites were actually detected in rat urine. Their TIC and mass spectral data are shown in Figure 9 and Figure 10, respectively.

Conclusions

The conclusion of this study on analysis for the metabolites of organophosphorus pesticide in biological samples is as follows:

Four common metabolites of organophosphorus pesticides were detected in urine samples, the simultaneous analysis method was studied using Ultra-2 capillary column (25 m) and GC/MS/SIM. The extraction of four metabolites from urine sample was made at pH 4.0 by the SPE method with the cyclohexyl silica cartridge. Recoveries were over 86% except for DMDTP and their relative standard deviations were within 10%. The standard calibration curve showed a good linearity in the range of 0.1-10 μg/ml and the detection limits of the compounds were in 0.05-0.1μg/ml. DMP, DMDTP, monomethyl phosphate and dimethylthiophosphate were detected in urine after oral administration of phenthoate to rats. The result of this study may be applied to development of exposure biomarker for monitoring of environmental pollutants.

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Organic Precipitate Flotation of Trace Metallic Elements with Ammonium Pyrrolidinedithiocarbamate (II). Application of Solvent Sublation for Determination of Trace Cd, Co, Cu and Ni in Water Samples

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A solvent sublation was studied for the determination of trace Cd, Co, Cu and Ni in water samples. Ammonium pyrrolidine dithiocarbamate (APDC) was used as a complexing agent. Experimental conditions such as pH of solution, amounts of APDC, the type and amount of surfactant, the type of solvent, etc. were optimized for the effective sublation of analytes. After metal-PDC complexes were formed in sample solutions of pH 2.5, the precipitate-type complexes were floated in a flotation cell with an aid of sodium lauryl sulfate as a surfactant and by bubbling with nitrogen gas. The precipitates were dissolved and separated into the surface layer of methyl iso-butyl ketone (MIBK). The analytes preconcentrated were determined by a graphite furnace atomic absorption spectrophotometry (GF-AAS). Extractability of each element was 88% for Cd(II), 86% for Co(II), 95% for Cu(II) and 76% for Ni(II), respectively. And this procedure was applied to the analysis of real samples. From the recoveries of more than 92%, it was concluded that this method could be simple and applicable for the determination of trace elements in various water samples of a large volume.

Introduction

Recently, the interests for health-related and environmental sciences are widely and rapidly increased and the information about trace heavy elements is also becoming more important. Therefore, developement of accurate determination mehtods for them is necessary to fit such requirements. As a result, various types of powerful and elegant instruments have been developed up to present for a qualitative and quantitative analysis of materials in view of composition as well as structure. Besides, various instrumental techniques have been extensively developed to improve the sensitivity, precision, selectivity and efficiency of the analytical methods, but the combination of an appropriate preconcentration technique with these methods remains unsettled in the field of trace inorganic analysis to achieve successful analyses in many kinds of samples.

The solvent extraction,[1-3] adsorption, ion-exchange,[5,6] and coprecipitation[4] are commonly used as preconcentration techniques for the atomic spectrometric determination of trace heavy elements. However, these methods need relatively long time and troublesome operation for a large volume of sample. On the other hand, a flotation technique[7-27] is rapid and conveniently compared to such preconcentration techniques and can be performed with an unskillful technique. And this mehtod is also able to concentrate many elements from a large sample volume of several liters to less than 10 mL, simultaneously.

The flotation technique is defined as a separation procedure by which various substances in a solution are selectively floated to the surface with the aid of tiny gas bubbles. The flotation technique can be classified as precipitate flotation[8-13,18-22] and ion flotation.[14-17] The precipitate technique is further divided into organic and inorganic precipitate flotations according to the type of precipitate.

In this work, a modified flotation technique so called "solvent sublation"[23,24] was investigated for separative preconcentration of heavy elements in water samples. The solvent sublation is a technique that analytes floated onto the surface layer by gas-bubbling are collected in a water-immiscible organic solvent just like in solvent extraction. Various surfactants are frequently used in the process of flotation to float analytes effectively by making the materials hydrophobic. Generally, the solvent sublation is more rapid

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