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Hollow-fiber-supported liquid phase microextraction with in situ derivatization and gas chromatography–mass spectrometry for determination of chlorophenols in human urine samples

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ABSTRACT

A simple and highly sensitive method that involves hollow-fiber-supported liquid phase microextraction (HF-LPME) with in situ derivatization and gas chromatography–mass spectrometry (GC–MS) was developed for the determination of chlorophenols (CPs) such as 2,4-dichlorophenol (DCP), 2,4,6-trichlorophenol (TrCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) in human urine samples. Human urine samples were enzymatically de-conjugated with β-glucuronidase and sulfatase. After de-conjugation, HF-LPME with in situ derivatization was performed. After extraction, 2 μl of extract was carefully withdrawn into a syringe and injected into the GC–MS system. The limits of detection (S/N = 3) and quantification (S/N > 10) of CPs in the human urine samples are 0.1–0.2 ng ml^{-1} and 0.5–1 ng ml^{-1}, respectively. The calibration curve for CPs is linear with a correlation coefficient of >0.99 in the range of 0.5–500 ng ml^{-1} for DCP and TrCP, and of 1–500 ng ml^{-1} for TeCP and PCP, respectively. The average recoveries of CPs (n = 6) in human urine samples are 81.0–104.0% (R.S.D.: 1.9–6.6%) with correction using added surrogate standards. When the proposed method was applied to human urine samples, CPs were detected at sub-ng ml^{-1} level.

1. Introduction

Chlorophenols (CPs) are used extensively as preservatives, fungicides, pesticides, disinfectants, and intermediates in many industries. CPs are generated from phenols during treatment of tap water with chlorine [1] and are considered to be one of the most obnoxious contaminants [2,3] because they deteriorate taste and produce an unfavorable smell. Moreover, they are thought to be serious health hazards because they accumulate in moderate amounts and show high toxicity [4,5]. CPs are usually detected in human urine because of the intake of food and water containing CPs and other chlorinated substances as metabolites present in the environment [6]. In order to assess human exposure to CPs, a reliable and sensitive analytical method is required. Many analytical methods, including capillary electrophoresis [7], high-performance liquid chromatography (HPLC) [8,9], and gas chromatography (GC) [10,11], are available for the determination of CPs in human urine samples. HPLC is a simple method as it does not require any derivatization steps; however, because of the relatively low concentrations of CPs in human urine samples, preconcentration is required. Solid phase extraction (SPE) is usually used for preconcentration. However, although SPE requires a small volume of organic solvent, the manual version is tedious and time-consuming. Moreover, a certain kind of SPE cartridge is expensive. Recently, solvent-free and solvent-minimized polymer sorption techniques, such as stir bar sorptive extraction (SBSE), were developed [12]. Kawaguchi et al. reported SBSE-thermal desorption (TD)-gas chromatography–mass spectrometry (GC–MS) for the determination of CPs in human urine sample [13]. In their method, the SBSE tool could be used repeatedly. In addition, the method had high sensitivity and selectivity. However, one shortcoming is that the method requires the use of an instrument for TD-GC–MS. Another method, liquid phase microextraction (LPME), requires a microsyringe and only single drop of organic solvent [14–16]. The extract (organic) solvent in the microsyringe is injected into the GC–MS system. The main advantages of LPME are simplicity of preparation and high-cost

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performance. LPME has been applied to environmental samples [17–21] and food samples [22,23]. Moreover, it has been applied successfully to the determination of alkylphenols (APs), chlorophenols (CPs), and bisphenol A (BPA) in water samples [24]. We considered applying LPME to the determination of CPs in human urine samples; however, because of interfacial activity of urine samples, it is too difficult to retain a single droplet on the microsyringe needle tip. In this study, hollow-fiber-supported (HF) LPME was developed to improve retention of a single droplet of extract on the needle tip, and used to determine CPs in human urine samples.

2. Experimental

2.1. Materials and reagents

2,4-Dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TrCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP), and pentachlorophenol (PCP) of environmental analytical grade and acetic acid anhydride for trace analysis were purchased from Kanto Chemical Inc. (Tokyo, Japan). 2,4-Dichlorophenol-d_4 (2,4-DCP-d_4), 2,4,6-trichlorophenol-$^{13}C_6$ (2,4,6-TrCP-$^{13}C_6$), 2,3,4,6-tetrachlorophenol-$^{13}C_6$ (2,3,4,6-TeCP-$^{13}C_6$), and pentachlorophenol-$^{13}C_6$ (PCP-$^{13}C_6$) were used as surrogate standards and were purchased from Hayashi Pure Chemical Inc. (Osaka, Japan). *E. coli* β-glucuronidase (25,000 U 0.4 ml^{-1}) and *H. pomatia* sulfatase (3650 U ml^{-1}) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Prior to use, β-glucuronidase was added to 0.1 M ammonium acetate to make a total concentration of 10,000 U ml^{-1}. Other reagents and solvents of pesticide or analytical grade were purchased from Wako Pure Chemical Inc. (Osaka, Japan). The water purification system was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). Concentrated solutions (1.0 mg ml^{-1} in methanol) of 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP were prepared independently. Then, mixture-standard solution (10 μg ml^{-1}) was obtained by mixing the four concentrated solutions. Urine samples were collected from five healthy volunteers and sample preparation was performed immediately. All the healthy volunteers did not eat anything after 10 p.m. the night before sampling the urine. In addition, the volunteers skipped breakfast, and the urine sample was collected before lunch. The volunteers were able to drink water freely.

2.2. Instrumentation

A 10-μl microsyringe for LPME was purchased from SGE Japan (Kanagawa, Japan). The microsyringe needle had a cone tip of 50 mm length and 0.63 mm o.d. Accurel Q 3/2 polypropylene hollow-fiber membrane of 600 μm i.d., 200 μm wall thickness, and 0.2 μm pore size was purchased from Membrana (Wuppertal, Germany). The hollow-fiber membrane was cut manually and carefully into 1.1 cm lengths. Then, the hollow-fiber segments were cleaned in acetone prior to use. For the extraction, 2 ml sample vials from Agilent Technologies (Palo Alto, CA, USA) were used.

2.3. GC–MS instrument and analytical conditions

GC–MS was performed with an Agilent 6890N gas chromatograph equipped with a 5973N mass-selective detector (Agilent Technologies). Injection was performed in the pulsed splitless mode, and injection volume was 2 μl. The splitless time was set to 1 min. The temperature of the inlet was 250 °C. Separation was conducted on a DB-5MS fused silica column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies). Oven temperature was programmed to increase from 100 °C (held for 1 min) to 220 °C at 5 °C min^{-1}, and then increased to 280 °C (held for 3 min) at 15 °C min^{-1}. Helium was used as carrier gas at the flow rate of 1.2 ml min^{-1}. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode with electron ionization (EI) (ionization voltage: 70 eV). The monitoring ions were as follows: *m/z* 162 and 164 for the acyl derivative of 2,4-DCP, *m/z* 196 and 198 for the acyl derivative of 2,4,6-TrCP, *m/z* 230 and 232 for the acyl derivative of 2,3,4,6-TeCP, and *m/z* 266 and 268 for the acyl derivative of PCP. The underlined numbers are the *m/z* of the ion used for quantification. The monitoring ions for the acyl derivatives of 2,4-DCP-d_4, 2,4,6-TrCP-$^{13}C_6$, 2,3,4,6-TeCP-$^{13}C_6$, and PCP-$^{13}C_6$ were *m/z* 169, 206, 240, and 276, respectively.

2.4. Human urine sample preparation by LPME

Human urine sample (1 ml) spiked with surrogate standards was buffered with 1 M ammonium acetate solution (100 μl). After adding β-glucuronidase (10 μl; 10,000 units ml^{-1}) and sulfatase (10 μl; 3650 units ml^{-1}), the sample was sealed in a glass tube and gently mixed. Enzymatic de-conjugation to release free CPs was performed by incubating at 37 °C for 3 h [13]. 1 M sodium hydroxide solution (NaOH; 20 μl) for pH adjustment and acetic acid anhydride (20 μl) as the derivatization reagent were added. Then, the sample was agitated. Finally, the sample was subjected to HF-LPME using a 10 μl microsyringe. Before extraction, the microsyringe was rinsed 10 times each with acetone and toluene to avoid carryover and air bubble formation. Three microliters of toluene was withdrawn into the microsyringe. Then, the microsyringe needle tip was inserted into the hollow fiber and the assembly was immersed in toluene for about 20 s to impregnate the pores of the hollow fiber. After impregnation, the fiber was removed from toluene, and air in the syringe was injected to flush the hollow fiber, removing excess organic solvent from inside the fiber. The prepared fiber assembly was set below the surface of the sample solution to prevent desiccation of the hollow fiber. LPME was performed at room temperature for 0–24 min while stirring at 500 rpm. After extraction, 2 μl of the extract was carefully withdrawn into the microsyringe and injected into the GC–MS system.

3. Results and discussion

3.1. Optimization of in situ derivatization and GC–MS conditions

The volumes of NaOH (0–200 μl) and acetic acid anhydride (0–50 μl) in the in situ derivatization step were optimized. As shown in Figs. 1 and 2, when 20 μl of NaOH and 20 μl of acetic acid anhydride were used for the in situ derivatization of CPs, relatively high responses were obtained.

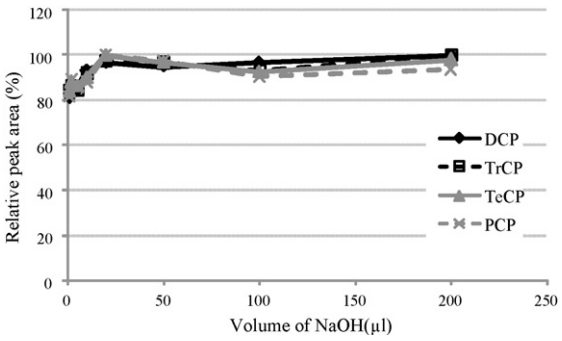

Fig. 1. Optimum volume of NaOH for in situ derivatization. In situ derivatization step was optimized. Profile of the optimum volume of NaOH for in situ derivatization of CPs in 1 ml standard solutions (5 ng ml^{-1}).

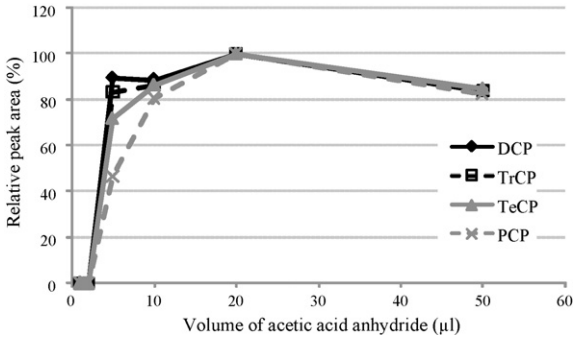

Fig. 2. Optimum volume of acetic acid anhydride for in situ derivatization. Optimum volume of acetic acid anhydride for in situ derivatization of CPs in 1 ml standard solutions (5 ng ml^{-1}) using LPME with in situ derivatization and GC–MS.

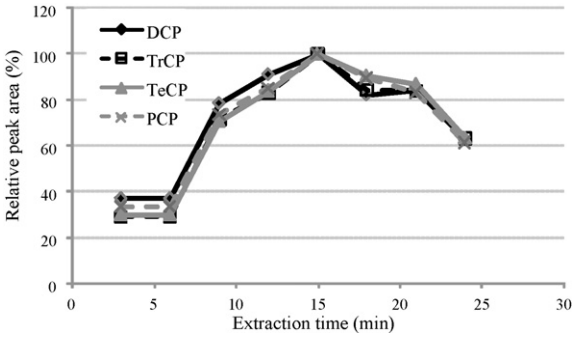

Fig. 4. Extraction time profiles of CPs by LPME. Optimum extraction time of acyl derivatives of CPs in 1 ml standard solutions (5 ng ml^{-1}) using LPME with in situ derivatization and GC–MS.

In EI-MS analysis of the standard solutions of 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP in the scan mode, the analytes were observed as acyl derivatives, and *m/z* 162, 196, 230, and 266 were obtained as the major signals, respectively, while *m/z* 164, 198, 232, and 268 were observed as the minor signals, respectively. Mass spectra of the acyl derivatives of CPs are shown in Fig. 3.

3.2. Extraction time

One of the most important parameters affecting LPME was the extraction time. To optimize the extraction time, 5 ng ml^{-1} standard solutions of CPs were used. The extraction time profiles of 1 ml standard solutions of the acyl derivatives of CPs using LPME with in situ derivatization and GC–MS are shown in Fig. 4. The highest responses were obtained when the extraction time was 15 min. One possible reason for the decrease in relative peak area was the reduced volume of toluene used as extraction solvent after extraction for 15 min. This condition was therefore used for the determination of CPs in human urine samples.

3.3. Analytical figures of merit

The limits of detection (LODs) (signal–noise ratio: S/N = 3) and the limits of quantification (LOQs) (S/N > 10) of CPs in human urine samples subjected to in situ derivatization were 0.1–0.2 ng ml^{-1} and 0.5–1 ng ml^{-1}, respectively. For CPs determination, calibration curves were obtained by plotting the peak area ratio (CPs/corresponding surrogate standards) versus CPs concentrations. For example, a calibration curve of PCP was obtained by plotting the peak area ratio (PCP/PCP-$^{13}C_6$) versus PCP concentration. The calibration curve for CPs was linear with a correlation coefficient of >0.99 in the range of 0.5–500 ng ml^{-1} for 2,4-DCP and 2,4,6-TrCP, and 1–500 ng ml^{-1} for 2,3,4,6-TeCP and PCP (Table 1). The relative recovery and precision of the method were assessed by replicate analyses (n = 6) of human urine samples spiked with 50 and 200 ng ml^{-1} surrogate standards. Non-spiked and spiked samples were subjected to LPME with in situ derivatization and GC–MS. The relative recoveries were calculated by subtracting the results for the non-spiked samples from those for the spiked samples. The results were obtained by using calibration curves of the standard solutions with surrogate standards. The average recoveries of CPs (n = 6) in human urine samples spiked with 50 and

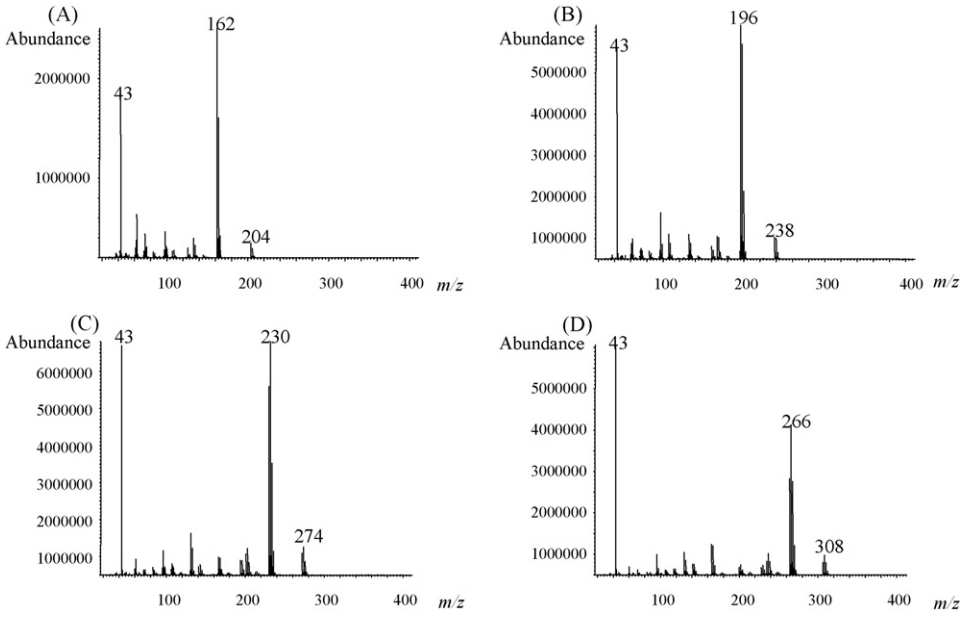

Fig. 3. Mass spectra of acyl derivatives of CPs. Mass spectra of acyl derivatives of 2,4-DCP (A), 2,4,6-TrCP (B), 2,3,4,6-TeCP (C), and PCP (D).

Table 1
Figures of merit of HF-LPME with in situ derivatizaion GC–MS

Analyte	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Linear range (ng ml^{-1})	Correlation coefficient (r)
2,4-DCP	0.1	0.5	0.5–500	0.99
2,4,6-TrCP	0.1	0.5	0.5–500	0.99
2,3,4,6-TeCP	0.2	1	1–500	0.99
PCP	0.2	1	1–500	0.99

LOD: limit of detection (S/N = 3); LOQ: limit of quantification (S/N > 10).

Table 2
Recoveries of CPs in human urine samples

Analyte	50 ng ml^{-1} spiked		200 ng ml^{-1} spiked	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
2,4-DCP	85.9	4.7	81.0	2.8
2,4,6-TrCP	91.4	1.9	104.0	6.6
2,3,4,6-TeCP	91.7	2.3	102.8	5.6
PCP	91.0	2.3	102.2	5.6

Recoveries and R.S.D. were also examined by replicate analyses (n = 6) of human urine samples. Recoveries of CPs were calculated as follows. Recovery (%) = (spiked sample − blank sample)/50 or 200 ng ml^{-1} CPs standard sample × 100.

200 ng ml^{-1} CPs were 85.9–91.7% (relative standard deviation, RSD; <4.7%) and 81.0–104.0 ng ml^{-1} (R.S.D. < 6.6%), respectively, with correction using the added surrogate standards (Table 2). Therefore, the method enables the precise determination of standards and can be applied to the determination of CPs in human urine samples.

3.4. Determination of CPs in human urine samples

Urine samples from five healthy volunteers were analyzed using the present method. As is obvious from the typical chromatograms shown in Fig. 5, CPs could be hardly detected in the samples before enzymatic de-conjugation. However, CPs of sub-ng ml^{-1} level were detected in the samples after enzymatic de-conjugation. When de-conjugation was performed, 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP were detected at 5.8–81.1 ng ml^{-1}, 1.09–5.16 ng ml^{-1}, ND to trace, and trace to 1.12 ng ml^{-1}, respectively (Table 3). Of note was that high levels of 2,4-DCP were detected in all the urine samples. It has been reported that the levels of 2,4-DCP, 2,4,6-TrCP, and PCP in human urine samples subjected to SPE and GC–MS were ND (<1.0) to 50 ng ml^{-1}, ND (<2.0 ng ml^{-1}), and ND (<2 ng ml^{-1}), respectively [25]. There is also another report stating that the levels of 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP in human urine samples subjected to SBSE-TD-GC–MS were 17.24–43.46 ng ml^{-1}, 0.38–2.42 ng ml^{-1}, 0.09–1.31 ng ml^{-1}, and 0.1–0.43 ng ml^{-1}, respectively [13]. The detected level of 2,4-DCP was almost the same as that determined by our method. The spot urine was used to confirm the potential of proposed method in this time, however, 24-h-urine or creatinine correction was worthy of consideration for exposure assessment.

Table 3
Concentrations of CPs in human urine samples

Analyte	A	B	C	D	E
2,4-DCP	10.3	81.1	17.9	5.8	44.2
2,4,6-TrCP	1.6	2.4	1.1	1.2	5.2
2,3,4,6-TeCP	Trace	ND	Trace	Trace	Trace
PCP	Trace	Trace	Trace	Trace	1.1

Urine samples were collected from healthy volunteers (A–E). Concentration is expressed in ng ml^{-1}. ND: below LOD level; Trace: between LOD level and LOQ level.

HF-LPME was compared with SPE and SBSE. SBSE had the highest sensitivity, followed by HF-LPME and SPE. HF-LPME was able to detect low concentrations of CPs sufficiently. In terms of cost performance, HF-LPME was the best. SBSE required a TD-GC–MS

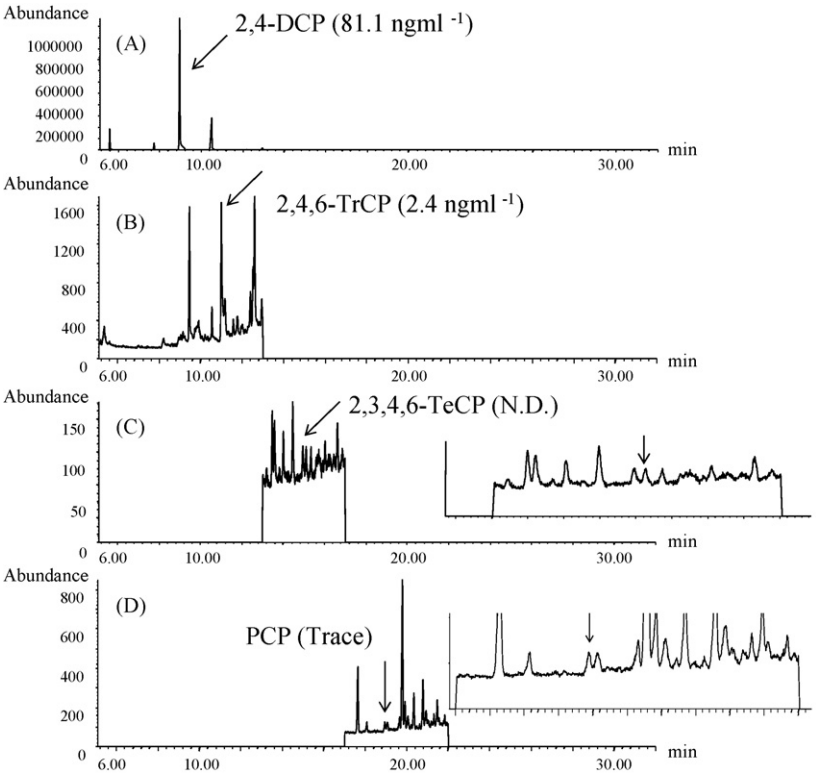

Fig. 5. SIM chromatograms of CPs in human urine sample. SIM chromatograms of CPs in urine sampled from a healthy human volunteer (Volunteer B). The chromatograms of 2,4-DCP (A), 2,4,6-TrCP (B), 2,3,4,6-TeCP (C), and PCP (D) were monitored at m/z 162, 196, 230, and 266 as acyl derivatives, respectively.

system, and the TD system had high running cost because liquid nitrogen was used. By contrast, HF-LPME did not require any special instrument for analysis; it required only a microsyringe and conventional GC–MS. Therefore, the present method may be useful for the assessment of human exposure to CPs.

4. Conclusions

This is a first paper to determine trace amounts of CPs in human urine samples using HF-LPME with in situ derivatization and GC–MS. The proposed method has many practical advantages, including simplicity of the extraction method, use of a small volume of organic solvent for extraction, and high sensitivity. The method was sufficiently applicable to the analyses of human urine samples. The limits of detection (S/N = 3) and quantification (S/N > 10) of CPs in human urine samples are 0.1–0.2 ng ml^{-1} and 0.5–1 ng ml^{-1}, respectively. The present method showed good linearity and high correlation coefficients using surrogate standards. In addition, the average recoveries of CPs ($n = 6$) in human urine samples were 81.0–104.0% with good precision (R.S.D.: 1.9–6.6%). This simple, accurate, and highly sensitive method is expected to have potential applications in human urine samples.

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