り，DNAメチル化とヒストン修飾が中心的な分子機構である．

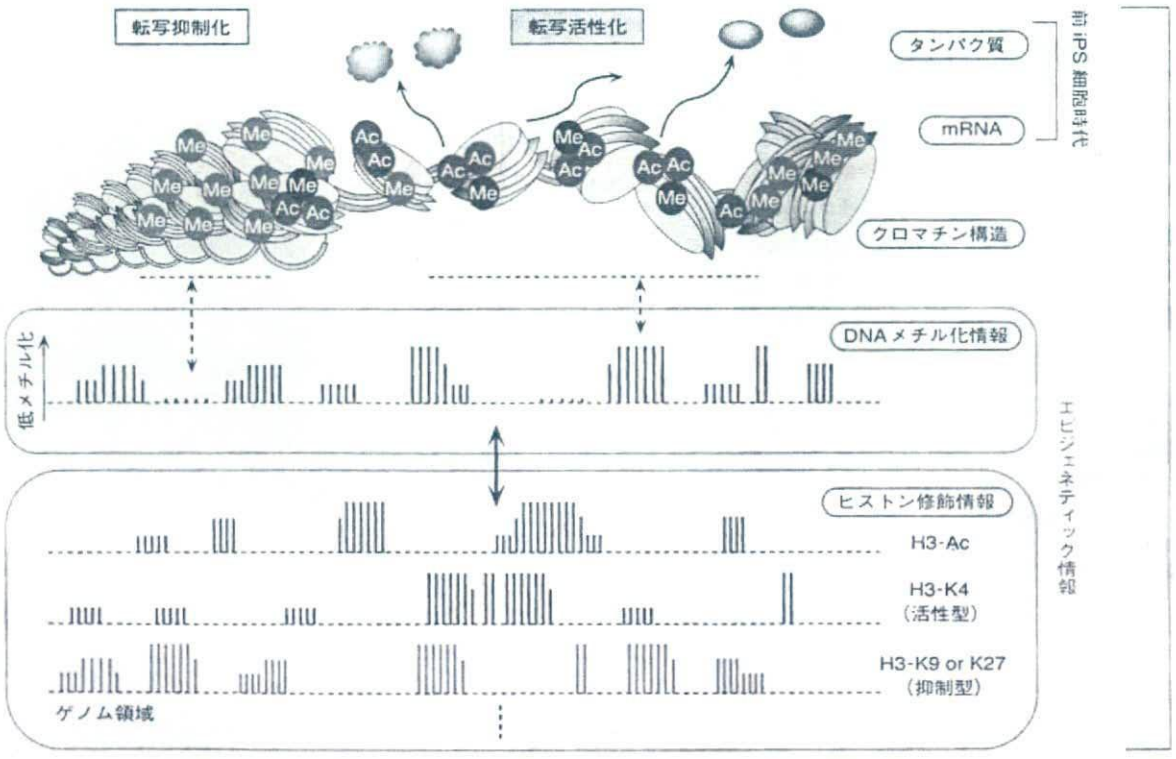
DNAのメチル化は哺乳類ゲノムにみられる唯一の化学修飾であり，おもに，シトシン（C），グアニン（G）の繰返し配列（CpG配列という）のシトシンがメチル化される．一方ヒストンにはさまざまな修飾があり，いくつかの修飾は，短時間に変化する．転写抑制型，転写活性型のヒストン修飾と，DNAのメチル化によりクロマチンの構造が変化し，転写が制御される（図2a）．

ES細胞など*Oct3/4*や*Nanog*が発現している細胞では，これら遺伝子の転写開始点や開始効率を決定するプロモーター領域で，DNAは低メチル化，ヒストンはアセチル化され，転写が活発な状況にある（文献4，5）．一方，*Oct3/4*や*Nanog*の発現が認められない体細胞では，DNAは高メチル化，ヒストンは脱アセチル化され，遺伝子発現が抑制されている．つまり，細胞ごとに異なる*Oct3/4*，*Nanog*の遺伝子発現パターンは，DNAメチル化，ヒストン修飾状況からなる，細胞に固有のエピジェネティックパターンに表されている（図2b）．

エピゲノム：ゲノム広域のエピジェネティクス情報

細胞や組織には，固有の細胞や組織に依存的メチル化領域（tissue dependent differentially methylated region，**T-DMR**という）があり，ゲノム上には膨大な数のT-DMRが存在する．細胞の種類ごとにT-DMRのメチル化状況は異なり，細胞の種類に特有のメチル化・非メチル化模様，すなわちDNAメチル化プロフィールが形成されている（文献7）．DNAメチル化プロフィールは，細胞のエピジェネティック状況の同一性，類似性，あるいは違いを知る上でよい指標となる．発生上で近縁あるいは同じ細胞系列の細胞（ES細胞と胚性生殖細胞など）の間では似たDNAメチル化プロフィールを示し，逆に発生上で異なった細胞（ES細胞と体細胞など）ではその違いは大きい（文献8）．

ヒトES細胞やマウスiPS細胞での一部のヒストン修飾についてはゲノム全域の解析が行われている（文献9）．ヒストン修飾情報とDNAメチル化情報は互いに密接な関連があり（図3），ヒストンの修飾状況を変化させると，DNAメチ



DNAメチル化情報とヒストン修飾情報は互いに密接な関係にあり，ヒストン修飾状況の変化と共にDNAメチル化も変化する．これらのエピジェネティック情報は，マーカー遺伝子発現を含めたこれらのES細胞，iPS細胞の新たな評価法として期待される．ヒストン修飾には60種以上が発見されており，ヒストン修飾の組合わせによるいわゆるヒストンコード説が成り立っている．

図3　エピジェネティック制御

ル化プロフィールが変化する．ゲノム全域のDNAメチル化状況を解析する方法は大きく二つ，

1）メチル化領域を検出する方法

2）非メチル化領域を検出する方法

に区分される．それぞれ一長一短であるが，1）に属するメチル化シトシン抗体を用いた方法（文献10）やメチル化シトシン結合タンパク質を利用した方法（文献11）は，正常細胞とがん細胞の違いなど，大幅にDNAメチル化状況が異なる場合に適している．一方，正常細胞や非がん細胞での比較には困難を伴う．なぜなら，繰返し配列を主体とする非遺伝子領域はほとんど（ゲノム全体の70～90%）がメチル化されており，ゲノム全体で遺伝子領域の占める割合が極少ないため，正常細胞間でのメチル化情報の違いが埋もれてしまうことが考えられるからだ．

その欠点を補う方法として，2）の制限酵素を用い，非メチル化領域を増幅して検出するHELP（Hap II tiny fragment enrichment by ligation-mediated PCR）法（文献12）やMIAMI（microarray-based integrated analysis of methylation by isoschizomers）法（文献13），および，D-REAM（T-DMR profiling with restriction-tag mediated amplification）法（文献14）が開発されている．HELP法やMIAMI法では，特定の制限酵素部位の解析が可能である．筆者らが開発したD-REAM法は，任意の制限酵素の組合わせが可能であるため，解析領域の選択肢が広がる利点をもっている．DNAメチル化プロフィールのデータを基に，T-DMRのメチル化状況と遺伝子発現がよく相関することが最近明らかにされている（文献14）．

このように，マイクロアレイを基盤に，ゲノム全域のエピジェネティック情報（エピゲノム）を調べることが可能となった．今後は，マイクロアレイに加え，メガ・シークエンシング技術を組合わせて，上記のヒストン修飾状況と重ね合わせ（図3），膨大なエピゲノムデータベースがつくられることになる．そのための国際エピゲノムプロジェクト構想も動き出している（文献15）．

ES細胞に限らず組織特異的な遺伝子発現は，マスター遺伝子産物とみなすことができるいくつかの転写因子の組合わせにより調節されている．ゲノム全域のDNAメチル化状況を解析したところ，成体の組織では，これらのマスター遺伝子，および下流の組織特異的な発現遺伝子は，組織特異的かつ協調的にメチル化制御されていることがわかった（文献14）．つまり，組織特異的な遺伝子発現プロフィールに重要な情報は，組織特異的な遺伝子発現を制御する転写因子と，それらによって制御される遺伝子のDNAメチル化情報の組合わせからなる．組織特有のDNAメチル化プロフィールに現れている．そして発生に重要な遺伝子の発現制御では，たとえば性決定に重要な因子である*Sry*遺伝子で示されているように，時期，部位特異的にT-DMRのメチル化が変化し，発現を制御している（文献16）．分化とは，ゲノムがこのような遺伝子発現の上流と下流が協調的に，時期，部位特異的DNAメチル化プロフィールを経て，最終的に組織特異的なプロフィールを形成していく過程と考えられる．

人工幹細胞評価法としてのエピゲノム解析

これまでES細胞やiPS細胞，およびそれらを分化誘導させてつくられる分化細胞の評価には，おもに限られた数の転写産物の解析，または転写産物を網羅的に解析するトランスクリプトーム解析が用いられてきた．しかしこれまで示したように，転写はヒストン修飾とDNAメチル化によって制御されており，さらに主要な組織特異的遺伝子発現プロフィールの根幹は，DNAメチル化プロフィールに現れている．

シトシンのメチル化は，正常DNAにみられる唯一の修飾であり，転写，ヒストンの修飾状況がDNAメチル化プロフィールに反映されていることを考え合わせると，DNAメチル化情報を中心として，ゲノム全域エピジェネティック情報の総体であるエピゲノム解析が，ES細胞やiPS細胞など人工幹細胞の重要な評価法となるであろう．

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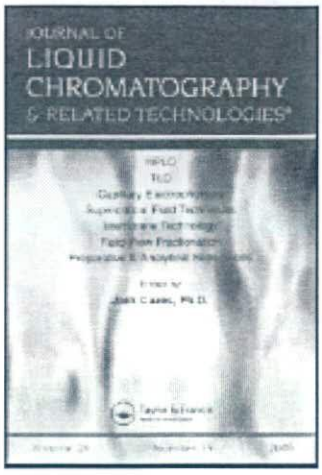

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Simultaneous Determination of Di(2-ethylhexyl)phthalate, Mono(2-ethylhexyl)phthalate, and Phthalic Acid Migrating from Gamma-Ray Irradiated Polyvinyl Chloride Sheet by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: The aim of the present study was to establish a liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the simultaneous determination of di(2-ethylhexyl) phthalate (DEHP), mono(2-ethylhexyl)phthalate (MEHP), and phthalic acid (PA). In the proposed method, the limits of detection of DEHP, MEHP, and PA were 5, 0.5, and 1 ng/mL, respectively, and the limits of quantification with standard solutions were 20, 2, and 5 ng/mL, respectively. Intra- and interday assays showed good accuracy and repeatability. The recoveries of DEHP, MEHP, and PA from respective extraction solvents ranged from 98.9 to 104.2% (relative standard deviation was below 10.3%). DEHP and its breakdown products migrating from gamma-ray irradiated polyvinyl chloride (PVC) sheets were determined simultaneously since DEHP is easily eluted from PVC medical devices. DEHP migration was noted from

both gamma-ray irradiated and control PVC sheets. Compared with the migration from the control PVC sheet, MEHP showed significant migration from the gamma-ray irradiated PVC sheet. In contrast, PA migration was noted only from the gamma-ray irradiated PVC sheet.

Keywords: LC-MS/MS, Di(2-ethylhexyl)phthalate, Mono(2-ethylhexyl)phthalate, Phthalic acid, Gamma-ray sterilization

INTRODUCTION

Phthalate esters, particularly di(2-ethylhexyl)phthalate (DEHP), are extensively used as plasticizers to increase the flexibility of polyvinyl chloride (PVC) products. PVC is one of the most widely used plastic polymers in such medical products as blood containers, blood tubing, and catheters. However, it has been reported that DEHP was easily eluted from PVC products into food, drugs, and body fluids.[1–4] DEHP is considered to exhibit reproductive and developmental toxicity,[5,6] carcinogenicity, and testicular toxicity.[7–9] It was also found to affect the reproductive organs and fertility.[10] It has been reported that DEHP is hydrolyzed enzymatically into mono(2-ethylhexyl)phthalate (MEHP),[11–13] and that MEHP may be even more toxic than the parent compound. In vitro studies have shown that MEHP inhibits FSH stimulated cAMP accumulation in cultured Sertoli cells,[14–18] in addition to reducing 17β-estradiol production and aromatase mRNA expression.[19,20] DEHP was determined to be the common plasticizer migrating from PVC medical devices into the blood.[2,3] MEHP was also determined to be the metabolite of DEHP. PA was not determined because it is not as toxic as DEHP or MEHP and the amount of PA in blood is negligible, although it is also produced by the enzymatic hydrolysis of DEHP.[21]

In our previous studies, we observed that not only DEHP but also MEHP migrated from PVC medical devices into simulated pharmaceuticals even without enzymatic hydrolysis.[4,22] In addition, we found that hydrolysis may occur during the sterilization process, particularly gamma-ray sterilization.[22] Therefore, a method for the simultaneous analysis of DEHP, MEHP, and PA was developed to confirm that MEHP and PA are produced from DEHP even without enzymatic hydrolysis. In addition, the method was used to determine DEHP, MEHP, and PA migrating from gamma-ray irradiated PVC sheets into purified water, 5% glucose solution,

EXPERIMENTAL

Chemicals and Materials

Environmental analytical grade DEHP and DEHP-d_4 were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). MEHP and MEHP-d_4 were purchased from Hayashi Pure Chemical Industries (Osaka, Japan). PA and PA-d_4 were purchased from CDN Isotope Central Chemicals Co., Inc. (Tokyo, Japan). Phthalic acid esters, analytical grade acetonitrile, and acetone were used in the experiments. Analytical grade formic acid was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The water purification system used was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA).

The test material was PVC sheets subjected to gamma-ray (^{60}Co; 24.2 kGy). The gamma-ray doses were set with reference to sterilization conditions used by commercial medical devices. The control sample was not irradiated gamma-ray. These PVC sheets were kindly supplied by the manufacturer.

The extraction solvents were 5% glucose solution for injection (Otsuka Pharmaceuticals Co., Tokyo, Japan), polyoxyethylated hydrogenated castor oil 60 (HCO-60) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and purified water.

Instrumentation and Chromatographic Conditions

A Series 1100 liquid chromatograph from Agilent Technologies (USA) was coupled to an API 4000™ (Applied Biosystems Japan, Tokyo, Japan) equipped with a Turbo Ionspray™ ionization source. Mass spectrometry data were processed with Analyst 1.3.2 software. An Inertsil-Ph3 column (50 mm × 2.1 mm, 5 μm particle size) from GL Sciences was used for the separation.

After 5 μL of the sample was injected with an autosampler, it was loaded onto the analytical column by flowing mobile phase at the flow rate of 0.2 mL/min. Acetonitrile (mobile phase A) and 0.05% formic acid in water (mobile phase B) were used. Separation was carried out with the following profile: mobile phase A/B was 15/85 (0–4 min) → 90/10 (4.01–15 min for elution) → 15/85 (15.01–25 min for equilibration) (v/v). The column oven was maintained at 40°C for LC.

MS/MS Conditions

The working parameters for turbo ion spray ionization MS/MS were as follows: curtain gas flow rates, 10 psi (DEHP and DEHP-d_4 for positive ion mode) and

20 psi (MEHP, PA, and their internal standards for negative ion mode); nebulizer gas (N_2) pressure, 20 psi for positive ion mode and 30 psi for negative ion mode; and turbo ion spray gas (N_2) pressure, 10 psi for positive ion mode and 80 psi for negative ion mode. The ion source temperature was maintained at 650°C and the turbo ion spray voltages for positive ion mode (DEHP, DEHP-d_4) and negative ion mode (MEHP, PA, and their internal standards) were 3500 and −4500 V, respectively. DEHP and DEHP-d_4 were detected in the positive ion mode, whereas MEHP, PA, and their internal standards were detected in the negative ion mode. The product ion mass spectra of DEHP, MEHP, and PA obtained by the LC-MS/MS system are shown in Figure 1. The combinations of precursor ion and product ions were as follows: DEHP (precursor ion → product ion, m/z 391 → 149), DEHP-d_4 (m/z 395 → 153), MEHP (m/z 277 → 134), MEHP-d_4 (m/z 281 → 138), PA (m/z 165 → 121), and PA-d_4 (m/z 169 → 125). The collision gas (N_2) pressures were set at 5 units (positive ion mode) and 4 units (negative ion mode).

Method Validation

After selection of the optimum conditions for sample preparation and LC-MS/MS, the method was thoroughly evaluated using DEHP, MEHP, and PA standard solutions. The linearity of the response of this system was examined with a calibration curve obtained at six different concentrations of the standard solution containing the certain amount of internal standard. Linear regression was performed using the ratio of DEHP peak area/DEHP-d_4 (internal standard) peak area plotted against the concentration. The calibration curves for MEHP and PA were also obtained in the same way. To assess the accuracy and precision of this method, low and high quality control samples were determined by replicate analysis. Intraday precision and accuracy were determined by replicate analysis of standard solutions in one day ($n = 3$), and interday precision and accuracy were determined over a span of three days.

The method was applied to 5% glucose solution and HCO-60 (0.02 mg/mL) samples that were spiked with 100 ng/mL DEHP, MEHP, and PA standards and certain amounts of internal standards. Each recovery was obtained from three replicates.

Migration Test

The migration of DEHP, MEHP, and PA from PVC sheets (1 × 3 cm) into 5 mL of each extraction solvent was examined. Five percent glucose solution, HCO-60, and purified water were used as extraction solvent, and served as simulated pharmaceuticals. HCO-60 is a surfactant that is

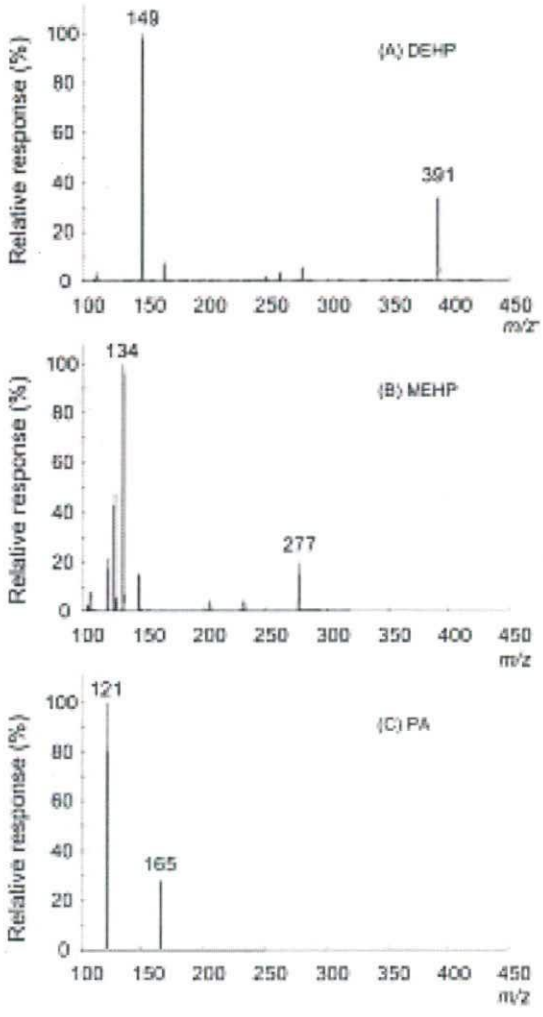

Figure 1. Product ion mass spectra of DEHP, MEHP and PA standard solutions. (A) 1 μg/mL DEHP standard solution, (B) 1 μg/mL MEHP standard solution, (C) 1 μg/mL PA standard solution. Each standard solution was infused directly into the MS system.

involved in the migration of DEHP into drugs such as Prograf®. The extent of DEHP migration was dependent on the concentration of HCO-60;[23] however, the injection of DEHP at high concentrations contaminated the MS system. Therefore, in this study, 0.02 mg/mL HCO-60 was prepared for the migration test. The samples were kept in test tubes and extraction was carried out by shaking at room temperature for 1 hr. A 1 mL aliquot of the extract was pipetted into another test tube, and DEHP-d_4, MEHP-d_4, and PA-d_4 were added. Then, the sample solution was appropriately diluted prior to LC-MS/MS analysis.

RESULTS AND DISCUSSION

Optimizing the LC-MS/MS Method

In the scan mode, DEHP, MEHP, and PA were monitored at m/z 391, 277, and 165 which were assigned to $[M + H]^+$, $[M\text{-}H]^-$, and $[M\text{-}H]^-$, respectively. Moreover, in the product ion MS/MS measurement, the selective reaction monitoring ions (SRM) of DEHP, DEHP-d_4, MEHP, MEHP-d_4, PA, and PA-d_4 were set depending upon their precursor ions. For the separation and the MS ionization, formic acid was added to purified water as the mobile phase. The optimum concentration of formic acid in purified water was 0.05% (Figure 2). In addition, the sample solution was acidified (1%) to improve separation. No interference from peaks of other compounds present in the extraction solvents was noted. The SRM chromatograms of DEHP, MEHP, and PA spiked into HCO-60 were shown in Figure 3.

Validation of the Method

In the proposed method, the limits of detection (LODs; signal-to-noise ratio = 3) of DEHP, MEHP, and PA were 5, 0.5, and 1 ng/mL, respectively. The limits of quantification (LOQs) (signal-to-noise ratio > 10) of DEHP, MEHP, and PA were 20, 2, and 5 ng/mL, respectively. For DEHP measurement, a calibration curve was obtained by plotting the peak area ratio (DEHP/DEHP-d_4) versus DEHP concentration, and was linear over the range of 20 to 1000 ng/mL ($r = 0.999$). For MEHP measurement, a calibration curve was obtained by plotting the peak area ratio (MEHP/MEHP-d_4) versus MEHP concentration, and was linear over the range of 2 to 1000 ng/mL ($r = 0.999$). For PA measurement, a calibration curve was obtained by plotting the peak area ratio (PA/PA-d_4) versus PA concentration, and was linear over the range of 2 to 1000 ng/mL ($r = 0.999$).

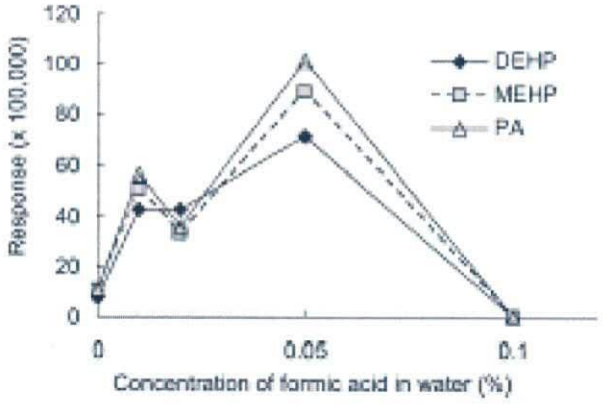

Figure 2. Effect of concentration of formic acid in purified water on response.

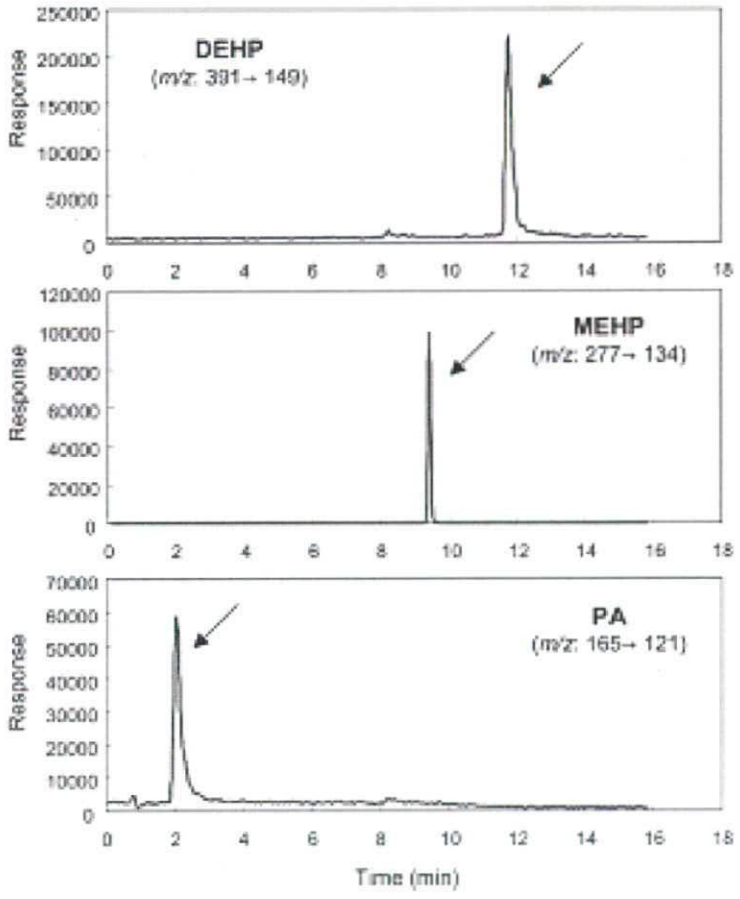

Figure 3. Chromatograms of DEHP, MEHP, PA spiked into HCO-60. 100 ng/mL of DEHP, MEHP, and PA were spiked into HCO-60 solution. SRM chromatograms were monitored as follows; DEHP (m/z 391 → 149), MEHP (m/z 277 → 134), and PA (m/z 165 → 121).

Intraday precision was expressed as relative standard deviation (RSD), which was calculated by measuring low (50 ng/mL) and high (500 ng/mL) concentrations of the standard solution three times (n = 3) in one day. Interday precision and accuracy were calculated using values measured at two concentrations (50 and 500 ng/mL) of the standard solutions over a span of three days. As Table 1 shows, all values of intra- and interday precision were less than 10%.

We also examined recovery using 5% glucose solution and HCO-60 as extraction solvents. For 5% glucose solution that was spiked with 100 ng/mL DEHP, MEHP, and PA, the average recoveries ranged from 100.4 to 104.2% (RSD <9.5%; Table 2). For HCO-60 that was spiked with 100 ng/mL DEHP, MEHP, and PA, the average recoveries ranged from 98.9 to 102.9% (RSD <10.3%; Table 2).

Table 1. Results of intra- and interday assays to validate proposed LC-MS/MS method

		Intraday			Interday		
Analyte	Concentration (ng/mL)	Detected average (ng/mL)	RSD (%)	Accuracy (%)	Detected average (ng/mL)	RSD (%)	Accuracy (%)
DEHP	50	54.4	4.0	106.8	50.3	8.9	100.6
	500	508.6	0.8	101.7	503.6	1.4	100.7
MEHP	50	48.9	3.5	97.8	48.6	1.1	97.1
	500	500.9	1.6	100.2	503.0	1.3	100.6
PA	50	49.9	4.8	99.9	49.4	1.6	98.9
	500	501.5	1.7	100.1	503.0	0.3	100.6

(n = 3).

Table 2. Recoveries of DEHP, MEHP and PA

Compound	Spiked amount (ng/mL)	Average recovery (%)		
		Water	5% Glucose solution	0.02 mg/mL HCO-60
DEHP	100	106.9 ± 6.9	104.2 ± 5.9	100.6 ± 10.3
MEHP	100	104.2 ± 2.4	103.4 ± 9.5	102.9 ± 5.8
PA	100	105.6 ± 7.6	100.4 ± 1.8	98.9 ± 8.2

(Mean ± SD, n = 3).

DEHP, MEHP, and PA Migration from Gamma-Ray Irradiated PVC Sheet

The proposed method was applied to the determination of DEHP, MEHP, and PA migration from the gamma-ray irradiated PVC sheet. DEHP migrated from both irradiated and unirradiated PVC sheets. The concentrations of DEHP that migrated from gamma-ray irradiated and unirradiated PVC sheets into purified water, or 5% glucose solution, were almost the same level (53.0–69.1 ng/mL). In contrast, the concentrations of DEHP that migrated from irradiated and unirradiated PVC sheets into HCO-60 were both high level (average concentration: 88.9 ng/mL) compared with the other solution. These concentrations were similar to those reported previously.[22] In our previous study, we noted that temperature and optical irradiation had an influence on DEHP release from the PVC sheet.[24] Therefore, DEHP release from the examined PVC sheet might have been influenced by temperature and/or optical irradiation, although the PVC sheet was stored in the dark at room temperature. The concentrations of MEHP that migrated from gamma-ray irradiated and unirradiated PVC sheets were also similar to those reported previously.[22] Gamma-ray irradiated PVC sheets released a high concentration of MEHP (Figure 4). In contrast, PA migration from unirradiated PVC sheets into any of the extraction solvents was not detected, whereas the gamma-ray irradiated PVC sheets released detectable levels of PA (Figure 4).

In our previous study, not only DEHP but also MEHP migrated from PVC medical devices into simulated pharmaceuticals. MEHP migration from PVC sheets was detected even though MEHP was not used as a plasticizer. In addition, MEHP was detected in gamma-ray irradiated PVC sheets but was not detected in PVC sheets sterilized by autoclaving or exposure to ethylene oxide gas.[22] The concentration of MEHP migrating from gamma-ray irradiated PVC sheets was significantly high compared with that from the unirradiated ones. Moreover, the concentration of PA migrating from gamma-ray irradiated PVC sheets was increased compared with unirradiated ones. MEHP and PA migrated from gamma-ray irradiated PVC sheets into

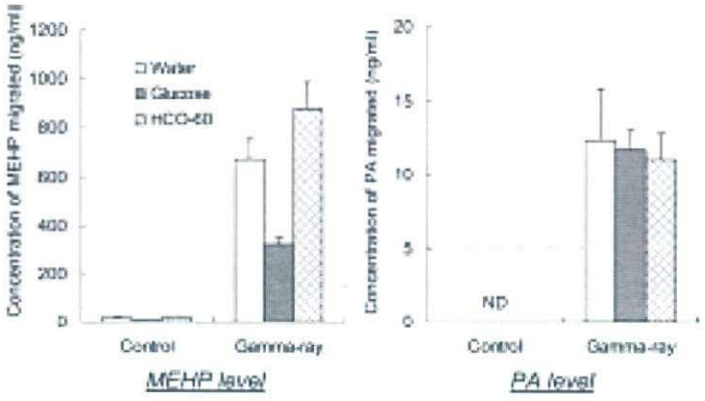

Figure 4. Concentrations of MEHP and PA migrating into various solutions from gamma-ray irradiated PVC sheets. Control is a PVC sample without gamma-ray irradiation. Each column is the mean of triplicate analysis (n = 3). Error bar represents standard deviation (S.D.). ND means "not detected." The dotted line at 5 ng/mL PA represents the limit of quantification.

both HCO-60 and purified water. We have already shown that MEHP was produced from DEHP as a breakdown product.[2,3] MEHP was produced by cleavage of one of two ester bonds in DEHP (Figure 5). We surmise that if MEHP was produced from DEHP by gamma-ray irradiation, PA would be produced by the same mechanism.

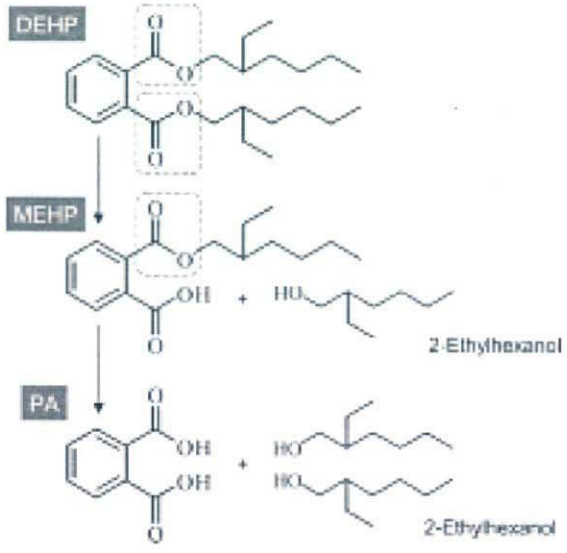

Figure 5. Chemical structures of DEHP, MEHP, and PA. The dotted circle represents the ester bond. MEHP was produced by cleavage of one of two ester bonds in DEHP. PA was produced by cleavage of two ester bonds in DEHP, and by cleavage of an ester bond in MEHP.

CONCLUSIONS

In this study, a method for the simultaneous determination of DEHP, MEHP, and PA was developed. The method had sufficient precision and accuracy to determine the concentrations of DEHP and its breakdown products migrating from PVC medical devices. Using the developed method, not only MEHP but also PA was found to be the breakdown product of DEHP. MEHP is thought to be more toxic than DEHP. The assessment of DEHP exposure in high risk patients is necessary to determine exposure to MEHP and PA, although PA is not as toxic as MEHP.

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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 160, 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.

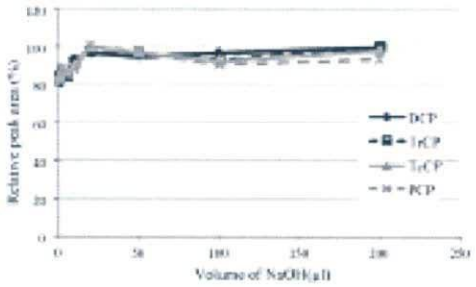


Fig. 1. Optimum volume of NaOH for in situ derivatization. In situ derivatization step was optimized. Profile of the optimum volume of NaOH for in situ derivatization of CPs in 1 ml standard solutions (5 ng ml^{-1}).

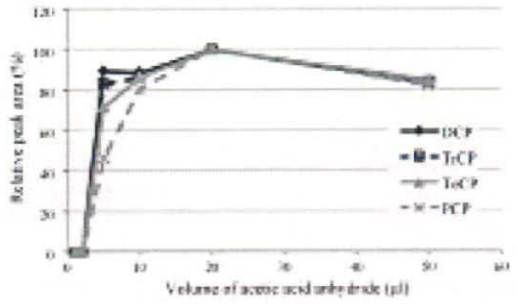


Fig. 2. Optimum volume of acetic acid anhydride for in situ derivatization. Optimum volume of acetic acid anhydride for in situ derivatization of CPs in 1 ml standard solutions (5 ng ml^{-1}) using LPME with in situ derivatization and GC–MS.

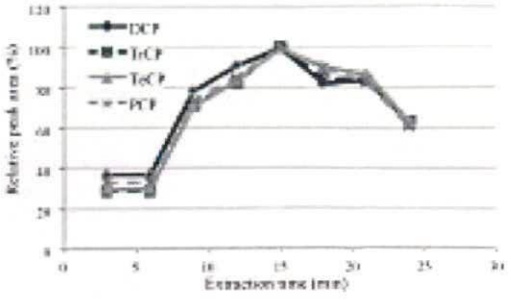


Fig. 4. Extraction time profiles of CPs by LPME. Optimum extraction time of acyl derivatives of CPs in 1 ml standard solutions (5 ng ml^{-1}) using LPME with in situ derivatization and GC–MS.

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

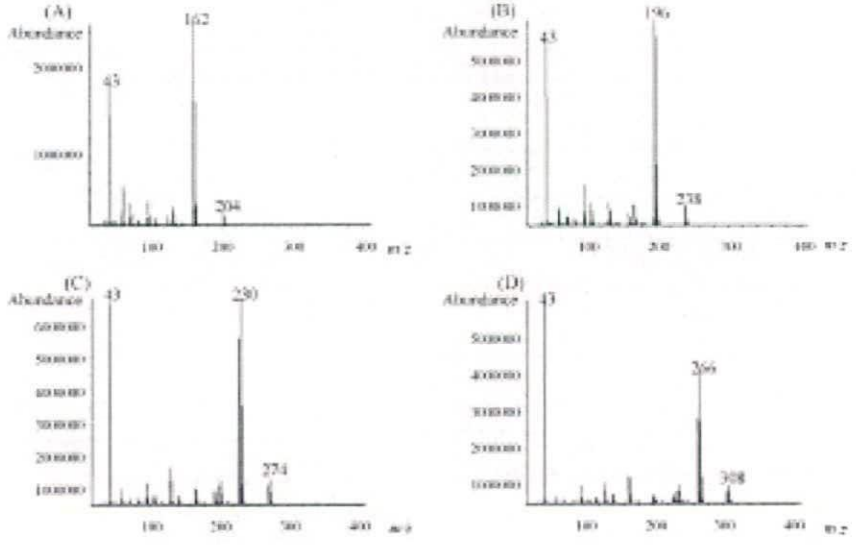


Fig. 3. Mass spectra of acyl derivatives of CPs. Mass spectra of acyl derivatives of 2,4-DCP (A), 2,4,6-TrCP (B), 2,3,4,6-TeCP (C), and PCP (D).

ble 1
gures of merit of HF-LPME with in situ derivatization GC–MS

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

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

ble 2
ecoveries of CPs in human urine samples

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

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

00 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 orrection using the added surrogate standards (Table 2). Therefore, the method enables the precise determination of standards nd can be applied to the determination of CPs in human urine amples.

A. Determination of CPs in human urine samples

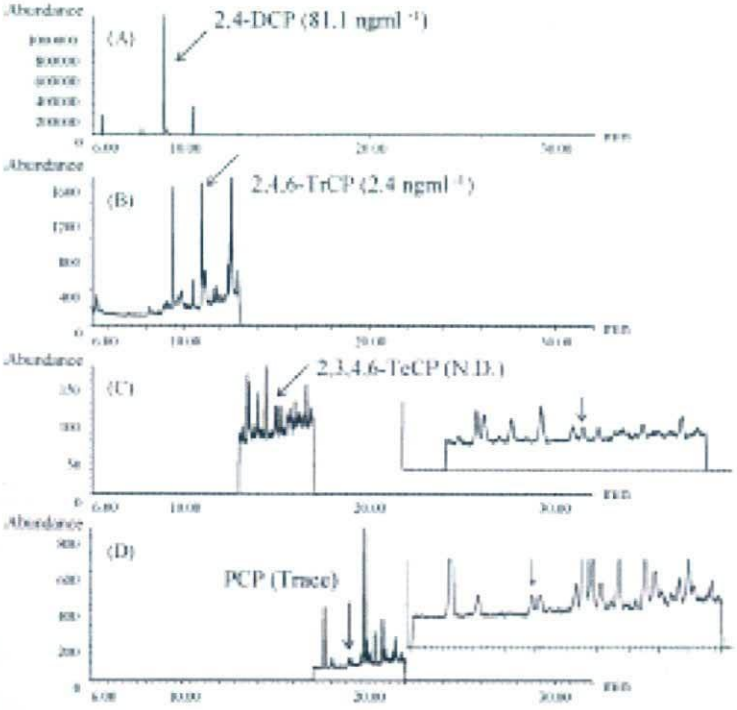
Urine samples from five healthy volunteers were analyzed using ne present method. As is obvious from the typical chromatograms shown in Fig. 5, CPs could be hardly detected in the samples before enzymatic de-conjugation. However, CPs of sub-ng ml^{-1} level were detected in the samples after enzymatic 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.

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

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.



g. 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 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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