

Original

**Determination of Benzene and Phenol in Body Fluids
by Headspace Solid-Phase Microextraction (SPME)
and Capillary Gas Chromatography**

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Abstract : Benzene and its metabolite phenol are extractable from human whole blood and urine using headspace solid-phase microextraction (SPME) with a Carboxen/polydimethylsiloxane fiber. Both compounds were assayed using ethylbenzene and 2,4-dimethylphenol as internal standards (IS) and capillary gas chromatography (GC) with flame ionization detection. The headspace SPME-GC gave sharp peaks for benzene, phenol, and IS-1, 2; with the whole blood and urine samples showing low background noise. The extraction efficiencies of benzene and phenol were 51.8–99.4% and 10.6–14.3%, respectively, for both whole blood and urine. Regression equations also showed excellent linearity in the range of 5–400 ng/0.5 ml for benzene and 10–500 ng/0.5 ml for phenol extracted from whole blood and urine. The detection limits (signal-to-noise ratio = 3) for the benzene and phenol were 5–10 ng/0.5 ml for whole blood and 2–5 ng/0.5 ml for urine. The coefficients of within-day variation in terms of extraction efficiency for both compounds in whole blood and urine samples were not greater than 13.8%. These data indicate that benzene and phenol can be successfully separated and determined from human samples using the established headspace SPME method.

Key words : solid-phase microextraction, capillary gas chromatography, headspace method, benzene, phenol

Introduction

Benzene is an aromatic hydrocarbon of commercial importance as a component of crude petroleum and gasoline; it has also become widespread in the environment¹⁾. Benzene is absorbed rapidly by inhalation and ingestion, and slowly through skin contact. A fraction of this internalized benzene is excreted unchanged in the exhaled air and the remaining fraction is metabolized in the liver and excreted in urine. The association between benzene exposure and

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leukemogenic effects is well established by peer review²⁾.

Phenol is a major metabolite of benzene that is excreted in the urine mainly as conjugates, principally of glucuronic acid, but also of sulphuric acid³⁾. The most commonly used methods for determining urinary phenol are gas chromatography (GC)^{4, 5)}, high performance liquid chromatography (HPLC)^{6, 7)}, and GC/mass spectrometry (MS)^{8, 9)}. Most of these methods use acid hydrolysis to release phenol from its conjugated derivatives and suffer from the possibility of incomplete hydrolysis. In many such analyses, no internal standard is used and thus accuracy and precision depend upon accurate manipulations throughout the sample preparation and chromatography, sometimes involving multiple solvent extraction steps. Therefore, no single assay is currently capable of quantitating urinary metabolites of benzene using these conventional methods.

Solid-phase microextraction (SPME), introduced by Arthur and Pawliszyn in 1990¹⁰⁾, is an excellent alternative to the above-listed classical methods. It is a solvent-free extraction technique that incorporates sample extraction, concentration, and introduction into a single procedure¹¹⁾. Several recent studies have confirmed the usefulness of SPME for analyzing drugs and xenobiotic substances in various biological samples¹²⁻²⁰⁾. In this paper, we have established a recommendable procedure for analyzing benzene and its metabolite phenol in human whole blood and urine samples using headspace SPME and capillary GC.

Materials and methods

Materials

Benzene and ethylbenzene for use as one of the internal standards (IS-1), and phenol and 2,4-dimethylphenol for IS-2 were obtained from Wako Pure Chemical Industries (Osaka, Japan). The SPME devices and 75- μ m Carboxen/PDMS fiber assemblies were purchased from Supelco Inc. (Bellefonte, PA, USA). Other common chemicals used were of analytical grade. Whole blood and urine were obtained from healthy subjects.

Conditioning of the SPME fiber

New Carboxen/PDMS fibers were conditioned in an injection port of a GC machine at 280°C for 30 min to remove fiber contaminants. Fibers for re-use were cleaned by heating at the conditioning temperature for 15 min before extraction.

Headspace SPME procedure

Stock solutions of benzene and phenol were prepared separately by dissolving an appropriate amount of each compound in methanol to 20 μ g/ml. The internal standard solution was prepared with 20 μ g/ml of ethylbenzene for benzene and 2,4-dimethylphenol for phenol. Working standard solutions of the compounds were prepared by serial dilution of the stock standard solutions with methanol. To 7.5-ml vials containing 0.5 ml of human whole blood or urine spiked with benzene, phenol, and their respective internal standards, we added 1.5 ml of distilled water, 0.6 g of NaCl, and a small Teflon-coated stirring bar. The vials were rapidly

sealed with silicon-septum caps and put on an aluminum block heater (Reacti-Therm™ Heating / Stirring Model, Pierce, Rockford, IL, USA) for heating and stirring. After heating at 100°C for 10 min, the septum-piercing needle of the SPME device was passed through the septum. The pretreated fiber was pushed out from the needle and exposed to the headspace of the vial at 100°C for 30 min to allow adsorption of the compounds. The fiber was then withdrawn into the needle, removed from the vial, and immediately injected into the GC port. The fiber was exposed in the injection port for 1.5 min for complete desorption of the compounds.

GC Conditions

GC analyses were carried out on a Shimadzu GC-14B gas chromatograph equipped with flame ionization detection (FID) (Shimadzu Co., Kyoto, Japan). GC separation was achieved with a DB-624 fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 1.80 μm; J & W Scientific, Folsom, CA, USA) under the following conditions: column temperature 30–240°C (2 min hold at 30°C, 30°C / min from 30 to 70°C and 20°C / min from 70 to 240°C); injection and detector temperature at 250°C; and helium flow rate of 3.0 ml / min. In the case of direct injection of the authentic compounds dissolved in methanol, a 0.2-μl aliquot (100 ng each on-column) was injected into the GC port. The samples were injected in the splitless mode at a column temperature of 30°C and the splitter was opened after 1.5 min.

Mass spectrometry (MS) conditions

GC / MS analysis was performed on a Shimadzu GC / MS-QP2010 instrument (Shimadzu Corp., Ltd., Kyoto, Japan) with a computer-controlled data analysis system. The mass spectrometer was operated in the positive-ion electron-impact mode at an ionization energy of 70 eV; the temperature of the interface was set at 250°C. GC separation was made with the above DB-624 capillary column. The GC conditions for the GC / MS were the same as those described for the GC-FID.

Actual determination of urinary phenols from petrol station workers

Urine samples collected from two petrol station workers were also analyzed to test the described method, after obtaining informed consent. The samples were stored in a refrigerator at –80°C until analysis. This study was approved by the Ethics Committees of Showa University School of Medicine (No. 2085).

Results

We first tested the effects of various salts (NaCl, Na₂SO₄, (NH₄)₂SO₄, and CaCl₂) on the extraction efficiencies of benzene, phenol, and two ISs from human whole blood analyzed by the present headspace SPME and a Carboxen / PDMS fiber (Table 1). Adding NaCl to the whole blood samples showed the highest efficiency of extraction for benzene; Na₂SO₄ showed the highest efficiency of extraction for phenol, and no salt gave the highest efficiency for the

Table 1. Effects of various salts on the extraction efficiencies of headspace solid-phase microextraction (SPME) of the present components from human whole blood using a Carboxen / PDMS fiber

Salt	Benzene	Ethylbenzene	Phenol	2,4-Dimethylphenol
NaCl	100.0	100.0	100.0	100.0
Na ₂ SO ₄	55.3	86.2	118.0	113.6
(NH ₄) ₂ SO ₄	27.0	63.2	59.3	78.0
CaCl ₂	32.8	61.0	50.4	69.9
No Salt	61.8	126.6	104.1	144.9

The amounts of analytes spiked to 0.5 ml of human whole blood in the presence of 1.5 ml of distilled water plus 0.6 g of each salt (400 ng of benzene, 1,000 ng of phenol,) using 200 ng of ethylbenzene and 1,000 ng of 2,4-dimethylphenol as the two internal standards (IS). The Carboxen / PDMS fiber was exposed in the headspace of the vial for 30 min at 100°C after pre-heating for 10 min at the same temperature. The amount of each compound extracted by the addition of NaCl was set at 100%. Each value represents the mean of duplicate determinations.

two ISs.

Fig. 1 shows gas chromatograms obtained from non-extracted authentic compounds (100 ng each on-column) dissolved in methanol and for headspace SPME extracts from human whole blood or urine samples spiked with benzene, phenol, and ISs. All compounds were well separated from each other and peaked sharply under the present GC conditions. The blank chromatograms gave a few impurity peaks over a wide range of temperatures, but no interfering peaks appeared around the test peaks (Fig. 1, lower panels).

To confirm that the detected peaks from human whole blood and urine in the headspace SPME analyses represent benzene, phenol, and the ISs, we measured the positive-ion electron-impact mass spectra for each peak. Molecular peaks at m/z 78, 94, 106, and 122 were observed in the spectra for benzene, ethylbenzene, phenol, and 2,4-dimethylphenol, respectively. There were also fragment ions detected at m/z 78 (base peak), 63, 52, and 39 for benzene, m/z 106, 91 (base peak), 77, 65, and 51 for ethylbenzene, m/z 94 (base peak), 66, and 39 for phenol, and m/z 122, 107 (base peak), 91, 77, and 65 for 2,4-dimethylphenol. The spectra for whole blood and urine extracts were almost identical to those of the authentic compounds, confirming the identities of the compounds in the body fluids.

The extraction efficiencies and their within-day variation (CV) measured by the method for benzene and phenol in human body fluid samples are presented in Table 2. The extraction efficiencies for benzene and phenol were 51.8–57.1 and 10.6–11.2%, respectively, for whole blood, and 93.8–99.4 and 11.9–14.3%, respectively, for urine (Table 2). The CV values were satisfactory and not greater than 13.8% for both body fluids.

Table 3 shows regression equations for the benzene and phenol in human whole blood and urine samples analyzed by the present method. The equations were constructed by plotting six different concentrations according to the peak-area ratios with ethylbenzene as IS for benzene, and with 2,4-dimethylphenol as IS for phenol. Benzene and phenol showed linearity

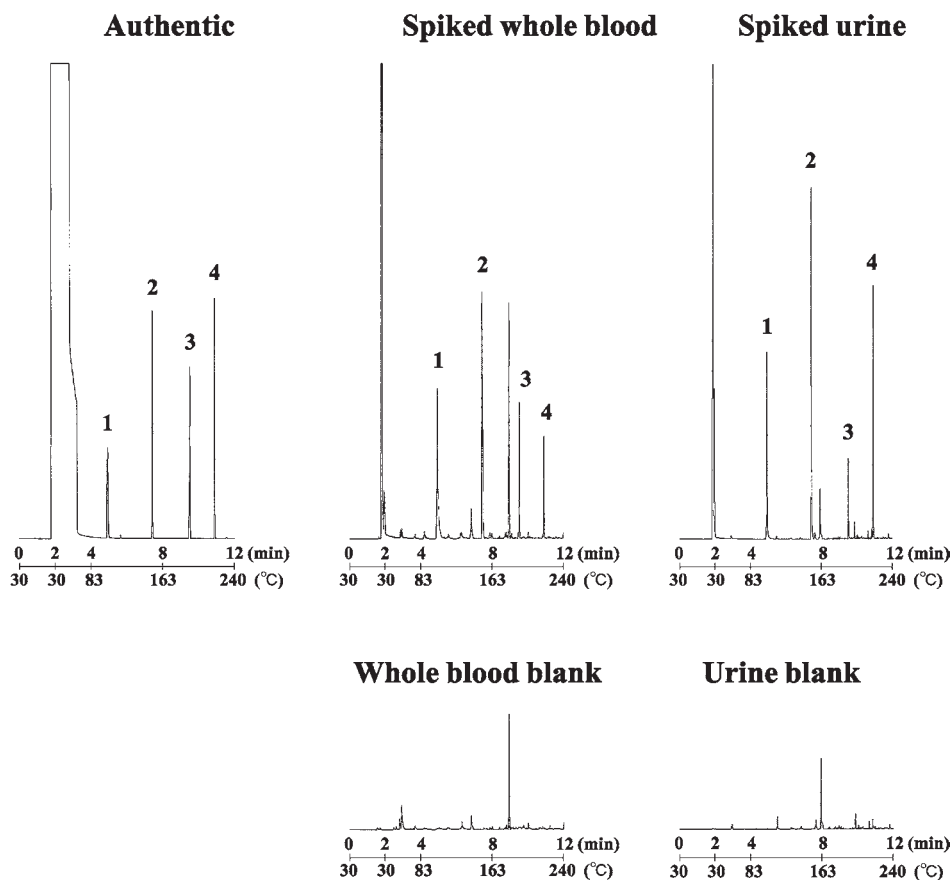


Fig. 1. Capillary GC-FID chromatograms for benzene, phenol, and two internal standards (IS) extracted from human whole blood and urine samples using SPME and a Carboxen/PDMS fiber. Both samples (0.5 ml) were spiked with either benzene and IS-1 (100 ng each) or phenol and IS-2 (500 ng) containing 1.5 ml of distilled water and 0.6 g of NaCl. After pre-heating for 10 min at 100°C, the fiber was exposed to the headspace of the vial for 30 min at the same temperature. The vertical scales of chromatograms for the authentic compounds were expanded (2 x) relative to those for the whole blood and urine samples. Peaks: 1) benzene; 2) ethylbenzene (IS); 3) phenol; 4) 2,4-dimethylphenol (IS). The large front peak appearing in the authentic chromatogram is the result of methanol used as a vehicle.

in the range of 5–400 ng/0.5 ml and 10–500 ng/0.5 ml, respectively, for whole blood, and 5–160 ng/0.5 ml and 10–320 ng/0.5 ml, respectively, for urine. The correlation coefficients (r) of each calibration curve were > 0.998 . The detection limits (signal-to-noise ratio = 3) for the benzene and phenol were 5–10 ng/0.5 ml for whole blood and 2–5 ng/0.5 ml for urine.

Headspace SPME/GC chromatograms of phenol extracted from the urine samples from petrol station workers showed peaks for phenol, but not benzene (Fig. 2). The positive-ion electron-impact mass spectra for these urine samples were almost identical to those for the authentic compounds (data not shown). As for the petrol station worker samples, after confirmation by GC-MS, GC-FID analysis showed phenol concentrations of 52.4–87.1 ng/0.5 ml urine (Table 4).

Table 2. Extraction efficiencies and their coefficients of within-day variation (CV) for benzene, phenol, and two ISs from human whole blood and urine samples by the present method

Compound	Amount added (ng / 0.5 ml)	Extraction efficiency (%)	CV (%)
Whole blood			
Benzene	100	57.1±5.07	8.9
	400	51.8±3.99	7.7
Phenol	500	10.6±1.46	13.8
	1,000	11.2±0.86	7.7
Urine			
Benzene	100	99.4±7.63	7.7
	400	93.8±1.37	1.5
Phenol	500	11.9±0.99	8.3
	1,000	14.3±0.51	3.6

The values are mean±SD of four experiments. The efficiencies were calculated by comparing the peak areas obtained from the extracts of the spiked human body fluid samples with those obtained by direct GC injection of non-extracted authentic compounds dissolved in methanol.

Table 3. Regression equations and detection limits for benzene and phenol from human whole blood and urine samples by the present method

Compound	Equation*	Correlation coefficient (<i>r</i>)	Correlation range (ng / 0.5 ml)	Detection limit (ng / 0.5 ml)
Whole blood				
Benzene	$y = 0.0055x + 0.0069$	0.9985	5–400	5
Phenol	$y = 0.0015x - 0.0153$	0.9978	10–500	10
Urine				
Benzene	$y = 0.0029x + 0.0233$	0.9995	5–160	2
Phenol	$y = 0.0009x + 0.0021$	0.9975	10–320	5

*The data were subjected to linear regression analysis of peak area ratios (*y*) of the compound to an IS against the spiking concentrations (*x*). Over six plots (each point represents the mean of duplicate determinations) with different concentrations for each compound were used.

Discussion

The phenomenon whereby solubility of organic compounds in an aqueous phase is reduced by salts is commonly known as “salting-out”. In biological samples, adding salting-out agents improves the extraction efficiency for some drugs and poisons^{14, 17, 18)}. In this study, although some impurity peaks appeared and overlapped the test peaks after the addition of Na₂SO₄, all compounds were effectively extracted by headspace SPME in the presence of NaCl. We also found that heating at 100°C for 25–30 min produced the best reproducibility when analyzing extracted benzene, phenol, and the ISs from whole blood and urine samples using a Carboxen / PDMS fiber.

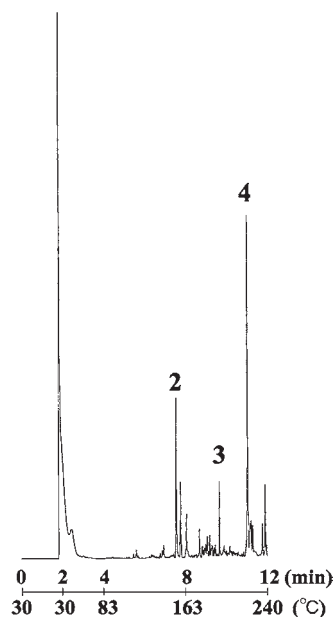


Fig. 2. Capillary GC-FID chromatograms for benzene and phenol extracted from urine samples of two petrol station workers. In the vial, 100 ng of IS-1 and 500 ng of IS-2 were spiked for extraction of benzene and phenol, respectively. Peaks: 2) ethylbenzene (IS); 3) phenol; 4) 2,4-dimethylphenol (IS).

Table 4. Concentrations of benzene and phenol in urine samples of control subjects and petrol station workers

Subject	Age (years)	Gender	Benzene (ng / 0.5 ml)	Phenol (ng / 0.5 ml)
Controls				
1	27	Male	0.33	18.5
2	30	Male	0.18	14.9
3	46	Male	0.25	16.1
Petrol station workers				
1	20	Male	1.62	52.4
2	30	Male	2.05	871

The low extraction efficiencies we achieved for phenol in this study are not surprising and merely reflect partitioning of the compounds among the stationary phase of the SPME fiber, headspace vapor, and the sample solution¹⁰⁾. In spite of these efficiencies, we still achieved low variation and excellent quantitation accuracy using the described headspace SPME.

The method also showed good linearity for the known components tested, with detection limits for benzene and phenol of 5–10 ng / 0.5 ml for whole blood and 2–5 ng / 0.5 ml for urine. In addition to the above spiked human whole blood and urine samples, we measured the levels of phenol in the urine of two petrol station workers. Concentrations of 5.0–78 ng / ml benzene are generally detected in human blood and urine following workplace exposure to benzene within acceptable limits (less than 2 ppm)^{21, 22)}. In contrast, reported post-mortem concentrations in blood and urine after benzene poisoning are 0.9–31.2 mg / ml for benzene^{23–25)} and 0.78–35.2 µg / ml for phenol^{4, 26)}. Thus, the detection limits by our method are sufficiently

low to adequately measure toxic levels of benzene and phenol.

In conclusion, we successfully extracted and assayed benzene and its metabolite phenol from human body fluids using a headspace SPME-GC method. To our knowledge, this is the first study dealing with headspace SPME using a Carboxen/PDMS fiber and capillary GC/FID for determining benzene and phenol in human whole blood and urine. With respect to simplicity, low background, sensitivity, excellent quantitative accuracy, and no requirement for organic solvents, the present method is recommended for extraction of compounds from biological specimens in the fields of forensic and clinical toxicology.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) KAKENHI grant (C) 26460886.

Conflict of interest disclosure

The authors have no conflicts of interest to declare.

References

- 1) Kalf GF, Post GB, Snyder R. Solvent toxicology: recent advances in the toxicology of benzene, the glycol ethers, and carbon tetrachloride. *Annu Rev Pharmacol Toxicol*. 1987;**27**:399-427.
- 2) Yardley-Jones A, Anderson D, Parke DV. The toxicity of benzene and its metabolism and molecular pathology in human risk assessment. *Br J Ind Med*. 1991;**48**:437-444.
- 3) Capel ID, French MR, Millburn P, *et al*. The fate of (14C) phenol in various species. *Xenobiotica*. 1972;**2**:25-34.
- 4) Van Roosmalen PB, Purdham J, Drummond I. An improved method for the determination of phenol in the urine of workers exposed to benzene or phenol. *Int Arch Occup Environ Health*. 1981;**48**:159-163.
- 5) Ahmed N, Hale K. A microassay for urinary phenol using capillary gas chromatography and optimised enzymic hydrolysis. *Clin Chim Acta*. 1994;**230**:201-208.
- 6) Ducos P, Gaudin R, Robert A, *et al*. Improvement in HPLC analysis of urinary trans, trans-muconic acid, a promising substitute for phenol in the assessment of benzene exposure. *Int Arch Occup Environ Health*. 1990;**62**:529-534.
- 7) Lee BL, New AL, Kok PW, *et al*. Urinary trans, trans-muconic acid determined by liquid chromatography: application in biological monitoring of benzene exposure. *Clin Chem*. 1993;**39**:1788-1792.
- 8) Dasgupta A, Blackwell W, Burns E. Gas chromatographic-mass spectrometric identification and quantitation of urinary phenols after derivatization with 4-carbethoxyhexafluorobutyl chloride, a novel derivative. *J Chromatogr B*. 1997;**689**:415-421.
- 9) Waidyanatha S, Rothman N, Li G, *et al*. Rapid determination of six urinary benzene metabolites in occupationally exposed and unexposed subjects. *Anal Biochem*. 2004;**327**:184-199.
- 10) Arthur CL, Pawliszyn J. Solid phase microextraction with thermal desorption using fused silica optical fibers. *Anal Chem*. 1990;**62**:2145-2148.
- 11) Hawthorne SB, Miller DJ, Pawliszyn J, *et al*. Solventless determination of caffeine in beverages using solid-phase microextraction with fused-silica fibers. *J Chromatogr*. 1992;**603**:185-191.
- 12) Lee X-P, Kumazawa T, Sato K. A simple analysis of 5 thinner components in human body fluids by headspace solid-phase microextraction (SPME). *Int J Legal Med*. 1995;**107**:310-313.

- 13) Kumazawa T, Watanabe K, Sato K, *et al.* Detection of cocaine in human urine by solid-phase microextraction and capillary gas chromatography with nitrogen-phosphorus detection. *Jpn J Forensic Toxicol.* 1995;**13**:207–210.
- 14) Lee X-P, Kumazawa T, Sato K, *et al.* Detection of organophosphate pesticides in human body fluids by headspace solid-phase microextraction (SPME) and capillary gas chromatography with nitrogen-phosphorus detection. *Chromatographia.* 1996;**42**:135–140.
- 15) Kumazawa T, Sato K, Seno H, *et al.* Extraction of local anaesthetics from human blood by direct immersion-solid phase micro extraction (SPME). *Chromatographia.* 1996;**43**:59–62.
- 16) Lee X-P, Kumazawa T, Sato K, *et al.* Detection of tricyclic antidepressants in whole blood by headspace solid-phase microextraction and capillary gas chromatography. *J Chromatogr Sci.* 1997;**35**:302–308.
- 17) Kumazawa T, Seno H, Lee X-P, *et al.* Detection of ethanol in human body fluids by headspace solid-phase micro extraction (SPME) / capillary gas chromatography. *Chromatographia.* 1996;**43**:393–397.
- 18) Hall BJ, Brodbelt JS. Determination of barbiturates by solid-phase microextraction (SPME) and ion trap gas chromatography-mass spectrometry. *J Chromatogr A.* 1997;**777**:275–282.
- 19) Lee XP, Kumazawa T, Kondo K, *et al.* Analysis of methanol or formic acid in body fluids by headspace solid-phase microextraction and capillary gas chromatography. *J Chromatogr B.* 1999;**734**:155–162.
- 20) Kumazawa T, Lee XP, Sato K, *et al.* Solid-phase microextraction and liquid chromatography / mass spectrometry in drug analysis. *Anal Chim Acta.* 2003;**492**:49–67.
- 21) Ghittori S, Fiorentino ML, Maestri L, *et al.* Urinary excretion of unmetabolized benzene as an indicator of benzene exposure. *J Toxicol Environ Health.* 1993;**38**:233–243.
- 22) Perbellini L, Faccini GB, Pasini F, *et al.* Environmental and occupational exposure to benzene by analysis of breath and blood. *Br J Ind Med.* 1988;**45**:345–352.
- 23) Winek CL, Wahba WW, Winek CL Jr, *et al.* Drug and chemical blood-level data 2001. *Forensic Sci Int.* 2001;**122**:107–123.
- 24) Barbera N, Bulla G, Romano G. A fatal case of benzene poisoning. *J Forensic Sci.* 1998;**43**:1250–1251.
- 25) Harada K, Ichiyama T, Ikeda H, *et al.* A fatal case of oral ingestion of benzene. *Am J Forensic Med Pathol.* 1999;**20**:84–89.
- 26) Khoshsorur G, Petek W. Rapid Determination of benzene metabolites phenol and *p*-cresol in the urine of petrol station workers by gas chromatography. *Anal Sci.* 2000;**16**:589–591.

[Received September 7, 2016 : Accepted October 6, 2016]