Introduction

The biomonitoring of workers occupationally exposed to aromatic vapours such as benzene is essential for health and safety purposes. Benzene is a known human carcinogen. An acute exposure to aromatic vapours such as benzene can produce haematopoietic toxicity while continuous and chronic exposure can lead to effects of bone marrow damages.

In the body, benzene is converted to benzene oxide by the liver microsomal mixed function oxidases as part of the detoxication process [1]. This oxide would then rearrange non-enzymatically to form the major metabolite, phenol. In the urine, benzene metabolites are found in the form of ethereal sulphates and glucuronides of the phenol-type metabolites, muconic acid and mercapturic acids from glutathione conjugation.

The use of urinary phenol as biomarker has been shown useful at benzene inhalation exposure that exceeds 5 ppm or 3 mg/m^3, and has been found to be linearly correlated to exposures as high as 620 mg/m^3 [2]. The importance of phenol and cresols as biomarkers from aromatic hydrocarbons exposure in workers has been established, where the Biological Exposure Index for the end of workshift sample is 50 mg/g of total phenol in creatinine or 50 mg/L urine.

References

[1] R Snyder and LS Andrews. In:Casarett and Doull's Toxicology. The Basic Science of Poisons.(CD Klaassen, editor). 5th ed. McGraw-Hill, New York; 1996; 741.

[2] O Inoue, K Seiji, M kasahara, H Nakatsuka, T Watanabe, S-G Yin, G-L Li, X-Z Wang, M Ikeda. Quantitative relation of urinary phenol levels to breath benzene concentrations: a factory survey. *Br. J. Ind. Med.* **43**: 692-697 (1986).

The Objective

The purpose of this study is to develop and validate a simple, reliable and accurate analytical method for the separation and quantitative determination of phenol and *p*-cresol concentrations in urine as the biomarker from benzene exposure in the workplace.

Experimental

Reagents

All chemicals were of reagent grade quality and were used as received without further purification. Phenol (99+%), 4-methylphenol (99+%) and 2,6-dimethylphenol (99+%), were all purchased from Sigma-Aldrich. Concentrated hydrochloric acid and both. gradient grade acetonitrile and methanol were all obtained from Fisher Chemicals, Fisher Scientific, UK. Bakerbond SPE octadecyl C_{18} cartridges (7020) were from JT. Baker (Phillipsburg, NJ, USA). Deionized, doubly-distilled water was used in all analyses.

Preparation of standard solutions

Stock solutions of phenol, *p*-cresol and 2,6-dimethylphenol (2,6-DMP) were prepared in methanol at a concentration of 200 μg/ml.

Stock solutions were diluted to appropriate working solutions for the preparation of the calibration standards. The 2,6-DMP is selected as internal standard in quantitative analysis. The appropriate final concentration of compounds prepared were as follows: 0,3,5,10,30,50,70,90,100 μg/ml.

Sample preparation

A 0.4 mL of concentrated hydrochloric acid was added to an aliquot of 1 ml of urine and heated in a water bath at 95 °C for 90 mins. After cooling to room temperature, the samples were loaded onto SPE columns. Spiked samples were prepared by adding the mix solution containing phenol, *p*-cresol and 2,6-DMP to the acid hydrolyzed urine.

Procedure for SPE

SPE column (silica C_{18}) was conditioned by pumping 2 mL of methanol and followed by 3 mL of distilled water. The aqueous sample was administered at a flow rate of 1 mL/min by applying a pressure of 1.3-1.5 KPa. The polar impurities were washed away with 2 mL of distilled water. The eluate was finally eluted with 1 ml of acetonitrile:methanol (1:10v/v) and collected in a 1.8-mL GC capped autosampler vial. The vials were kept cooled to 0°C until they were being analysed on the GC-FID detector. A volume of 1-μL eluate was injected onto the GC.

Chromatographic conditions

The chromatographic system includes a Hewlett-packard HP Model 6890 series GC with flame-ionization detector (FID), a HP 6890 series autosampler/autoinjector and a chemstation software for data analysis. Metabolite separation was done on the HP 5 (5% phenyl methyl siloxane) ID length (m)=30, diameter (μm)=250, film thickness (μm)=0.25 capillary column; injector temp, 220 °C; detector temp, 230 °C; oven temp, 60 °C for 1 min, then increased by 6 °C/min to 105; final temperature 220 °C for 1 min; carrier gas, hydrogen at a flowrate of 2.5 mL/min; injection volume 1μL; splitless mode.

Result and Discussion

A representative chromatogram analysis of purified extract of the hydrolysed spiked urine sample (1 ml) is as shown in Fig.1. The phenols were isolated in an acetonitrile concentrate following the extraction procedure to free the conjugated phenols. Separation of the phenol components was achieved using the phenyl containing-HP-5 column, which is suitable for adsorption of polar aromatic hydrocarbons structures. The peaks of analytes of interest were shown to be well separated from any interferences. The results obtained in this study showed good recoveries of both analytes, as shown in Table 1.

The detection limit (LOD) was defined as the lowest concentration of each of the phenolic compounds that can produce a signal-to-noise ratio of 3. The LOD for phenol and *p*-cresol is 0.1 and 0.3 μg/ml respectively. The limit of quantification (LOQ) was

defined as the lowest concentration of each of the phenolic compounds that can produce a signal-to-noise ratio of 10. The LOQ of both compounds were found to be at 3 μg/ml. This LOQ value was used as lowest point range in subsequent quantitative analysis.

The parameters of the calibration curves are as shown in Table 2. A linear relationship was found between the ratio of peak area of analyte to peak area of internal standard and the concentration of both phenols at 3, 5, 10, 20, 30, 50 and 100 μg/ml in studies that were performed in 3 consecutive days. The equations of the linear calibration graphs showed good correlation values (r^2 >0.9).

The precision and accuracy of the method is as shown in Table 3. Data for precision and accuracy were evaluated using urine samples spiked at the low, midpoint and high level concentrations (3, 50 and 100 μg/ml) respectively. Each point measurement on the curve was derived from a mean values of 6 spiked samples. The accuracy was expressed as the percentage deviation between the mean concentration values of 6 samples and the theoretical concentration. Each concentration was calculated on the basis of the ratio of analyte peak area to internal standard peak area, with respect to the calibration curves. In both analytes, the accuracy of the method is satisfactory especially in the middle and high point range. The precision is also satisfactory as reflected by the percentage in coefficient of variation that is mostly less than 30 %. Based on detection limits, the sensitivity of the GC method that is in the low to subppm range is adequate for purpose of measuring workplace exposure in workers whose BEI value is at least 50-fold higher.

Conclusion

An assay method using solid phase extraction with non-polar bonded octadecyl (C_{18}) sorbents for extraction method and simultaneous determination of phenols using simple GC-FID detection was established. The described method is useful for measurements of phenol compounds in aqueous solutions or air samples in areas of environmental and toxicological studies.

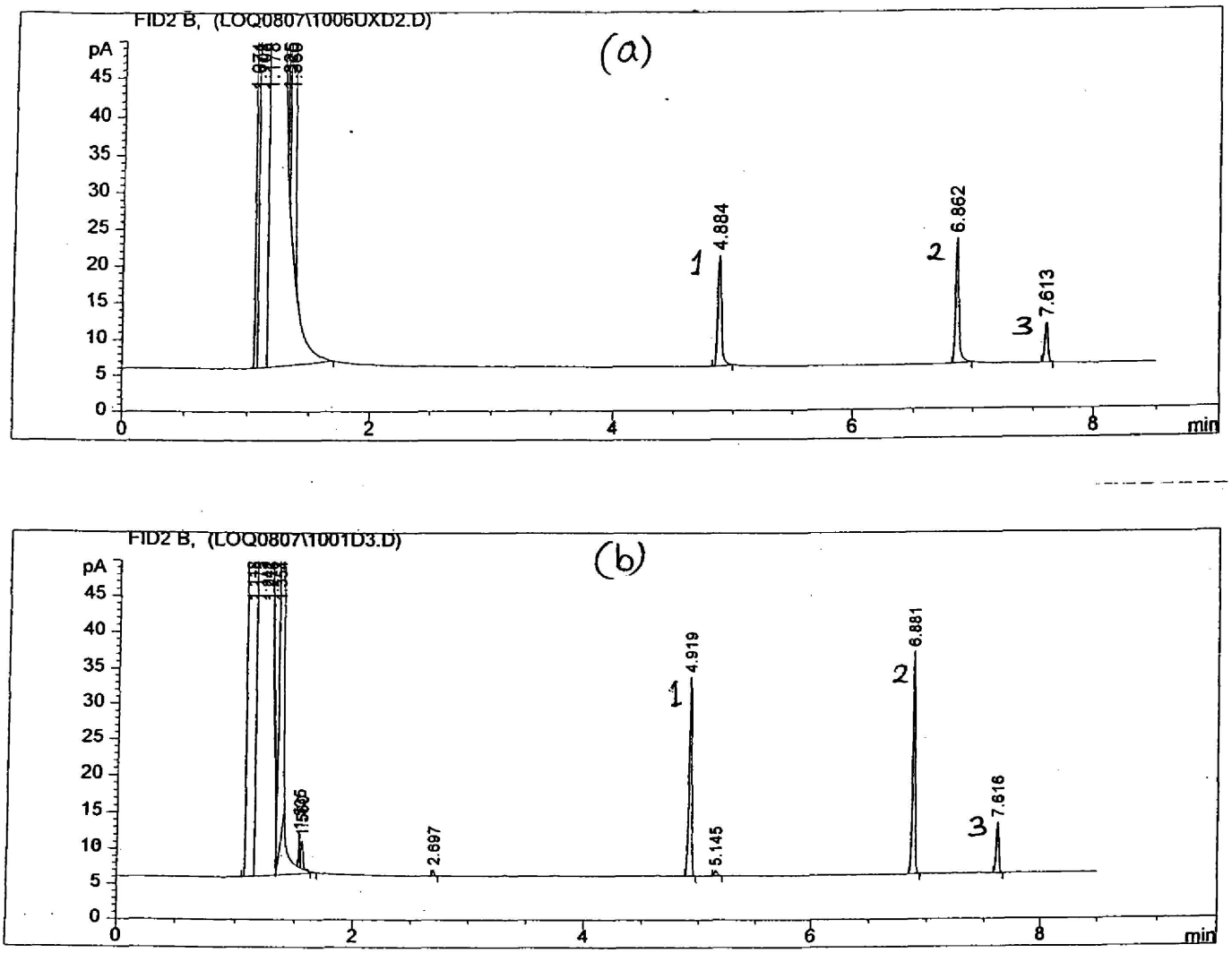

Fig 1 (a) Chromatogram of a standard solution containing 100µg/mL of phenol, 100µg/mL of *p*-cresol and 25µg/mL of 2,6-dimethylphenol in acetonitrile. Peaks: 1 = phenol, 2= *p*-cresol, 3= 2, 6-DMP.
(b) Chromatogram of a purified extract of hydrolysed spiked urine sample (100µg/mL of phenol, 100µg/mL of p-cresol and 25µg/mL of 2,6-dimethylphenol). Peaks: 1 = phenol, 2= p-cresol, 3= 2, 6-DMP.

Table 1: Solid-phase extraction of phenols using octadecyl (C_{18}) columns. Each result is the average of 6 repeated measurements. The concentration of each compound was at LOQ (3 µg/ml), middle point of the range (50 µg/ml), highest point of the calibration standard range (100 µg/ml).

Analyte	% Extraction Recovery [a]		
	LOQ	Mid point	Highest point
Phenol	165.96	105.76	101.71
p-cresol	160.09	115.80	112.20

[a] % Extraction Recovery = (mean extracted target analyte / mean unextracted target analyte) x 100
Both mean extracted target analyte and mean unextracted target analyte values were average concentration values obtained from a series of 6 measurements.

Table 2: Linearity – Calibration curve of standards

Analyte	Concentration added (µg/ml)	Concentration calculated (µg/ml)		
		Day 1	Day 2	Day 3
Phenol	0	0.00	0.00	0.00
	3	0.222		0.298
	5		0.154	0.347
	10		0.193	0.445
	20	0.811		
	30	1.457		
	50	1.883	0.947	2.009
	70			3.231
	90		1.923	
	100	4.147	2.368	4.398
r^2		**0.993**	**0.989**	**0.995**
***p*-cresol**	0	0.00	0.00	0.00
	3	0.338		0.251
	5		0.183	0.306
	10		0.228	0.424
	20	0.709		
	30	1.248		
	50	1.657	1.101	
	70			2.729
	90		2.244	
	100	3.725	2.933	4.196
r^2		**0.990**	**0.980**	**0.997**

r^2 is regression coefficient of the linear calibration curve of the peak area ratio (y-axis) vs added concentration in µg/ml (x-axis).

Table 3: Accuracy of the phenolic assays

Between Assay Accuracy for phenol

Validation Sample	Accuracy Day 1 (%)	Accuracy Day 2 (%)	Accuracy Day 3 (%)	Average Accuracy
Lowest point of the range	11.2	70.6	11.1	31.0
Mid point of the range	16.7	33.0	-9.31	19.7
Upper point of the range	16.2	28.9	-5.10	16.7

Between Assay Accuracy for *p*-cresol

Validation Sample	Accuracy Day 1 (%)	Accuracy Day 2 (%)	Accuracy Day 3 (%)	Average Accuracy
Lowest point of the range	64.7	70.3	-2.77	45.9
Mid point of the range	-19.8	26.6	-6.56	17.6
Upper point of the range	-3.48	22.2	3.22	9.63

Table 4a: Precision of the phenolic assays in terms of repeatability

Repeatability of phenol assay

Validation Sample	c.v. (%) Day 1	c.v. (%) Day 2	c.v. (%) Day 3
Lowest point of the range	34.11	29.10	10.70
Mid point of the range	27.75	18.23	11.38
Upper point of the range	35.53	14.89	34.94

% c.v. = (standard deviation/mean measured concentration) x 100

Repeatability of *p*-cresol assay

Validation Sample	c.v. (%) Day 1	c.v. (%) Day 2	c.v. (%) Day 3
Lowest point of the range	45.38	26.94	11.99
Mid point of the range	29.99	16.39	9.67
Upper point of the range	34.16	18.83	31.08

Table 4b: Precision of the phenolic assays in terms of reproducibility

Reproducibility of phenol assay

a) Within Assay precision

Validation Sample	c.v. (%) Day 1	c.v. (%) Day 2	c.v. (%) Day 3	Average c.v. (%)
Lowest point of the range	34.11	29.10	10.70	24.64
Mid point of the range	27.75	18.23	11.38	19.12
Upper point of the range	35.53	14.89	34.94	28.45

b) Between Assay Precision

Validation Sample	Day 1	Day 2	Day 3	Average	Precision (%)
Lowest point of the range	SD = 2.663 Mean =7.807	SD = 2.975 Mean =10.223	SD = 0.361 Mean = 3.373	SD = 1.999 Mean =7.13	28.04
Mid point of the range	SD = 11.560 Mean =41.662	SD = 13.614 Mean =74.673	SD = 5.206 Mean =45.740	SD = 10.127 Mean =54.03	18.75
Upper point of the range	SD = 29.781 Mean =83.817	SD = 20.911 Mean =140.657	SD = 33.242 Mean =95.152	SD = 27.978 Mean =106.54	26.26

SD = standard deviation

% precision = (SD/mean) x 100

Reproducibility of *p*-cresol assay

a) Within Assay precision

Validation Sample	c.v. (%) Day 1	c.v. (%) Day 2	c.v. (%) Day 3	Average c.v. (%)
Lowest point of the range	45.38	26.94	11.99	28.10
Mid point of the range	29.99	16.39	9.67	18.68
Upper point of the range	34.16	18.83	31.08	28.02

b) Between Assay Precision

Validation Sample	Day 1	Day 2	Day 3	Average	Precision (%)
Lowest point of the range	SD = 3.854 Mean =8.493	SD = 2.725 Mean =10.116	SD = 0.350 Mean = 2.919	SD = 2.31 Mean =7.176	32.2
Mid point of the range	SD = 12.509 Mean =41.716	SD = 11.163 Mean =68.101	SD =4.536 Mean =46.923	SD = 9.40 Mean =52.25	18.0
Upper point of the range	SD = 33.017 Mean =96.641	SD = 24.209 Mean =128.533	SD = 32.114 Mean =103.328	SD = 29.78 Mean =109.50	27.2