BIOMARKERS, 2001, VOL. 6, NO. 3, 190–203

A simple HPLC method to determine urinary phenylmercapturic acid and its application to gasoline station attendants to biomonitor occupational exposure to benzene at less than 1 ppm†

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Received 30 June 2000, revised form accepted 24 September 2000

The objective of this study was to establish a hand-saving method to measure phenylmercapturic acid (PMA) and to examine urinary PMA as a marker of occupational exposure to benzene at levels less than 1 ppm. A simple HPLC method was developed to analyse PMA by monitoring absorption at 195 nm of the effluent from an ODS-3 column with acetonitrile–methanol–perchloric acid–water as a mobile phase. The detection limit of the method was 0.2 μg l^{-1} with sufficient reproducibility. The method was applied to end-of-shift urine samples from 70 gasoline station attendants exposed to up to 107 ppb benzene, and 20 non-exposed controls of both sexes. Time-weighted average (TWA) exposure to benzene was measured by diffusive sampling. A regression analysis was applied to examine the quantitative relationship between the intensity of exposure to benzene and PMA in the end-of-shift urine samples. Multiple regression analysis showed no effects of age, sex, smoking and co-exposure to toluene and xylenes on urinary PMA. There was a linear relationship between TWA benzene exposure and urinary PMA ($r = 0.60$–0.67, $P < 0.01$). Background PMA in urine of the non-exposed controls was low and scattering of PMA around the regression line was narrow so that those with 20 ppb benzene exposure can be separated from the non-exposed by urinalysis for PMA. Thus, urinary PMA is sensitive enough for biological exposure monitoring of those exposed to less than 1 ppm benzene.

Keywords: benzene, biological monitoring, gasoline station, occupational exposure, phenylmercapturic acid, urinalysis.

Introduction

A number of epidemiological studies on benzene-exposed workers made it clear that leukaemia incidence was increased among the exposed populations (Aksoy 1980, 1985, Yin *et al.* 1996), which indicates that benzene is carcinogenic to humans (International Agency for Research on Cancer 1982, 1987). Nevertheless, benzene is in use in various organic synthesis processes in modern industries, and exposure to benzene may occur in petroleum refineries (Kawai *et al.* 1991, Verna *et al.* 2000) as well as cokeries in the iron and steel industries (Kivisto *et al.* 1997). In addition, benzene is present in commercial automobile gasoline (Ikeda *et al.* 1984, Kawai *et al.*

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† A part of this work was presented at the 73rd annual meeting of Japan Society for Occupational Health, 24–26th April 2000, Kitakyushu, Japan.

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500010009582

1991, Javelaud *et al.* 1998), and gasoline station attendants may also be exposed to benzene, even though at low levels, e.g. 1 ppm or less (Ong and Lee 1994, Hotz *et al.* 1997).

Accordingly, biological monitoring to detect benzene exposure has been the research subject of this study group, and the target analytes included catechol (Inoue *et al.* 1988a), quinol (Inoue *et al.* 1988a), 1,2,4-benzenetriol (Inoue *et al.* 1989a,b) as well as *t*,*t*-muconic acid (Inoue *et al.* 1989c) in addition to a traditional exposure marker of phenol (Inoue *et al.* 1986). Subsequently, the usefulness and limitation of each analyte as an exposure marker were also investigated; in short, none of them appears to be sensitive enough to bio-monitor benzene exposure at less than 1 ppm. Attention has been focused recently on urinary phenylmercapturic acid (PMA; N-acetyl-*S*-phenyl-L-cysteine), a glutathione conjugate of benzene, as a candidate marker to detect low-level benzene exposure (van Sittert *et al.* 1993, Popp *et al.* 1994, Boogaard and van Sittert 1995, 1996, Ghittori *et al.* 1995, Hotz *et al.* 1997, Kivisto *et al.* 1997, Dor *et al.* 1999).

The advantages of PMA from toxicological as well as practical viewpoints have been reviewed recently (De Rooij *et al.* 1998). Among the disadvantages of this marker is, however, the complexity of the analytical procedures such as derivatization followed by gas chromatography–mass spectrometry (GC–MS) (van Sittert *et al.* 1993), or clean-up through columns before instrumental analysis (Stommel *et al.* 1989, Maestri *et al.* 1993, Einig and Dehnen 1995, Einig *et al.* 1996, Angerer *et al.* 1998), which may hinder application of the method to routine exposure monitoring in occupational health.

Efforts have been made in this group to develop a practical HPLC method with minimum treatment of urine prior to chromatography. The earlier trials were successful in developing a method sensitive enough to detect benzene exposure at several dozen ppm (Inoue *et al.* 2000). The sensitivity of the method has been further improved in the present study to allow biological exposure monitoring of gasoline station attendants, who were exposed to levels of benzene below 1 ppm, by means of HPLC analysis of end-of-shift urine for PMA.

Materials and methods

Population studied, and collection of urine and air samples

The survey was conducted in gasoline stations in Hokkaido island in northern Japan on a workday in the latter half of a workweek. In total, 70 station attendants (48 men and 22 women) participated in this study; the subjects included smokers (22 men and 7 women) with daily consumption of 10–40 cigarettes. In addition, 20 non-smoking control subjects (10 men and 10 women) with no occupational exposure to organic solvents, including gasoline, also joined the study.

The time-weighted average (TWA) intensities of exposure to benzene and two other aromatics of toluene and xylenes (three isomers in combination) were measured by diffusive air sampling with 3M samplers (3M Health Care, Tokyo, Japan) followed by gas chromatographic analysis as previously described (Inoue *et al.* 2000). In short, each worker was equipped with a sampler on the cloth at chest pocket level from about 09:00 till about 17:00 (time duration recorded). After exposure, the exposed carbon cloth was extracted with carbon disulphide, following the operation manual, and the extract was analysed for TWA exposure concentrations of benzene, toluene and xylenes (three isomers in combination) by means of a gas chromatograph equipped with a fiame-ionization detector. The detection limit for each of the three solvents was 2 ppb when a signal to noise ratio of 3:1 was taken.

Analysis of urine for phenylmercapturic acid

Urine samples analysed were collected from both exposed workers and non-exposed controls near the end of an 8-h day-shift. Under standard assay conditions, each urine sample (2.0 ml; diluted as necessary with water to a final volume of 2.0 ml) was taken in a 12 ml glass-stoppered glass test tube, and

mixed step-wise with 2.0 ml water, 0.1 ml internal standard (IS) solution (20 mg 3,5-xylenol dissolved in 30 ml ethanol and diluted to a final volume of 1 l with water), and 0.8 ml 48.5 % sulphuric acid. Within 10 min after the sulphuric acid addition, the acidity of the mixture was weakened by addition of 0.8 ml 7.8 N potassium hydroxide. It was previously determined (Inoue *et al.* 2000) that 10 min or less is appropriate for conversion of pre-PMA [*N*-acetyl-*S*(1,2-dihydro-2-hydroxyphenyl)-L-cysteine] (Sabourin *et al.* 1988) to PMA prior to instrumental analysis (Sabourin *et al.* 1988, International Programme for Chemical Safety 1993).It was also known that about 80 % of total PMA is excreted in urine as a PMA precursor (probably as pre-PMA) in urine (Inoue *et al.* 2000). A preliminary experiment with authentic PMA dissolved at 20 μg l^{-1} showed that PMA is stable under the conditions of the acid–alkali pretreatment (Inoue *et al.* 2000).

The mixture (about 5.7 ml in volume) was extracted with 5 ml of an ether:methanol mixture (9:1 by volume) by 5 min vigorous mechanical shaking, and the two phases were separated by 10 min centrifugation at 1600*g*. The organic phase (about 4 ml) was transferred to another test tube, mixed with 0.2 ml of 200 g l^{-1} ascorbic acid solution in water, and evaporated to about 0.2 ml under a nitrogen stream with warming at 25 ¡C, and up to 70 μl of the remaining solution was injected into an HPLC.

The system employed consisted of an HPLC (Model LC-SPD-10AD, Shimadzu, Kyoto, Japan), connected with an automated liquid sampler (Model SIL-10AXL, Shimazdu), a degasser (Model DGU-14A, Shimadzu), a spectrophotometer (UV–VIS Detector Model SPD-10A; Shimadzu), a temperature controller for the detector (Shimadzu) and a personal computer (Dell Computer Corporation, Austin, Texas, USA) installed with Borwin chromatography software [obtained from Japanese Spectroscopic (JASCO), Tokyo, Japan]. A stainless steel column (400 mm in length and 4.6 mm in inner diameter) packed with ODS-3 (diameter, 5 mm; GL Science, Tokyo, Japan) was employed for separation at 60 ¡C.

A mixture of acetonitrile (100 ml) methanol (24 ml), 60 % perchloric acid (0.5 ml) and deionized water (to a total volume of 1 l) was introduced to the system as a mobile phase after thorough degassing by ultrasonication. The mobile phase was allowed to flow at 2.5 ml min^{-1}, and the absorption of the effluent was monitored at a wavelength of 195 nm; the wavelength was selected as a result of a balance between a more stable baseline and higher sensitivity. Care was taken for complete removal of foams in the flow system. Each determination was terminated in 92 min. A typical chromatogram of urinalysis is shown in figure 1.

In some instances, PMA concentration in urine was adjusted for creatinine concentration (hereafter

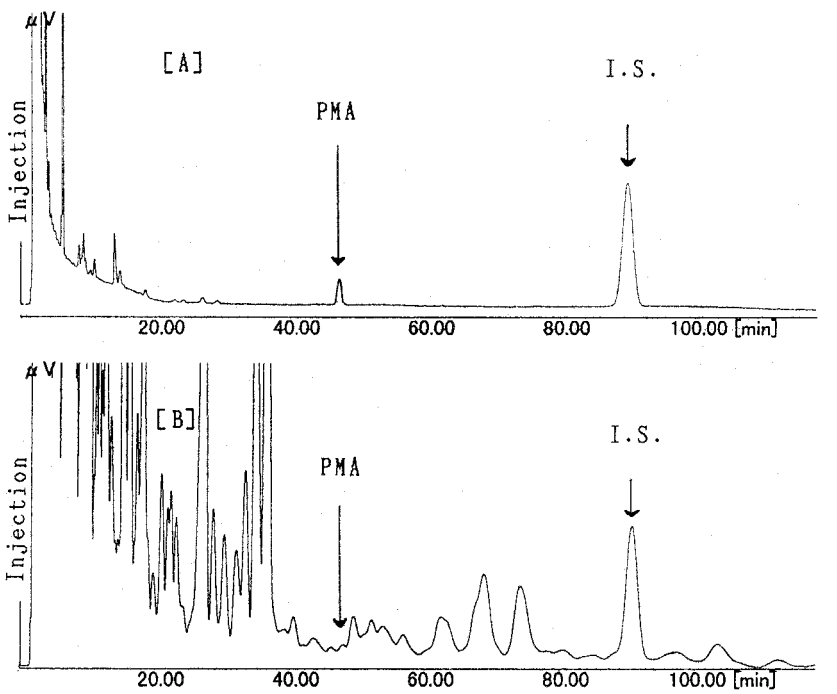

Figure 1. Typical HPLC charts of [A] authentic PMA dissolved at 200 μg l^{-1} in water which was spiked with 3,5-xylenol at 20 mg l^{-1} as an international standard (IS) and [B] 28.4 μg l^{-1} of PMA detected in the urine of a worker exposed to 72.9 ppb benzene.

abbreviated as cr; Jackson 1996) or a specific gravity of urine of 1.016 (sg; Rainsford and Lloyd Davies 1965). Creatinine concentration and specific gravity were measured by colorimetry and refractometry, respectively.

Reagents

D-L-Phenylmercapturic acid was purchased from Tokyo Kasei Chemicals (Tokyo, Japan). Acetonitrile and methanol (both of HPLC grade) were from Junsei Chemicals (Tokyo, Japan), and perchloric acid (fine analysis grade) was from Wako Pure Chemicals (Osaka, Japan).

Statistical analysis

Simple and multiple linear regression analyses were applied to detect possible correlation of urinary PMA with TWA intensity of exposure to benzene. AM, ASD, GM and GSD represent arithmetic mean, arithmetic standard deviation, geometric mean, and geometric standard deviation, respectively.

Results

Performance of the method developed

Under the HPLC conditions developed, the peak for PMA showed a retention time of about 48 min (figure 1). A baseline of the peak was set as a tangent line of the two local minima on both sides of the PMA and IS peaks in the chromatogram curve, and the areas under the curves (AUC) were measured. When a urine sample from a control subject with no endogenous PMA was spiked with authentic PMA at the final concentrations of 0, 20, 40, 60, 80, and 100 μl^{-1}, respectively, and subjected to the analysis (including the acid–alkali pretreatment), the AUC was proportional to the added concentration of PMA; the dose-dependent enlargement of the PMA peak is shown in figure 2, and the quantitative relationship of the ratio in AUC for PMA over that of IS with the added PMA is depicted in figure 3.

In order to examine the reproducibility of the measurement, five samples each of PMA dissolved in water at four concentrations of 5, 10, 20 and 40 $\mu g\, l^{-1}$ were analysed similarly, the coefficient of variation was 8.8, 6.9, 3.1 and 4.1 %, respectively. In the next experiment, PMA was dissolved either in five PMA-free urine samples or in water at four concentrations of 0, 5, 10 and 20 $\mu l\, l^{-1}$. The recovery (defined as the increment in the AUC for PMA in urine over the increment in water) was 92.0, 94.8 and 100.2 %, respectively. The detection limit was 0.3 $\mu l\ ;l^{-1}$, when a signal to noise ratio of 3:1 was taken, following Einig *et al.* (1996) and Angerer *et al.*, (1998).

Exposure of workers to benzene, toluene and xylenes

TWA intensities of the workers to benzene, toluene and xylenes were generally low but varied in wide ranges, being up to 107, 341 and 122 ppb for the three solvents respectively. An assumption of log-normal distribution for the concentrations gave 34 ppb for benzene, 38 ppb for toluene and 22 ppb for xylenes as GM, with GSD of about 2 for benzene and about 3 for other two aromatics (table 1). There was no significant ($P > 0.10$) difference in exposure intensity between men and women (data not shown).

GC analysis of liquid gasoline supplied at the stations showed that the contents of benzene, toluene and xylenes were 0.8, 1.3 and 1.7 % (weight by weight), respectively. Simple estimation by weight proportion between benzene and gasoline suggests that a subject with 100 ppb (= 320 $\mu g\ m^{-3}$) benzene exposure would be exposed to 40 mg m^{-3}

gasoline. Similar calculation with 340 ppb (1278 μg m^{-3}) toluene and 120 ppb (521 μg m^{-3}) xylenes gave 93 and 31 mg m^{-3} gasoline, respectively.

Possible effects of age, smoking habits and co-exposures to aromatics other than benzene

In order to identify possible effects of factors other than benzene exposure on urinary PMA, multiple regression analyses (MRA) were carried out with 70 exposed cases taking six factors of sex, age, daily cigarette consumption, and TWA intensities of exposure to benzene, toluene and xylenes as independent variables, in which sex was taken on a nominal scale and others on numerical scales. The dependent variable was either PMA as observed (PMAob), PMA divided by creatinine concentration (PMAcr) or PMA as corrected for a specific gravity of urine of 1.016 (PMAsg).

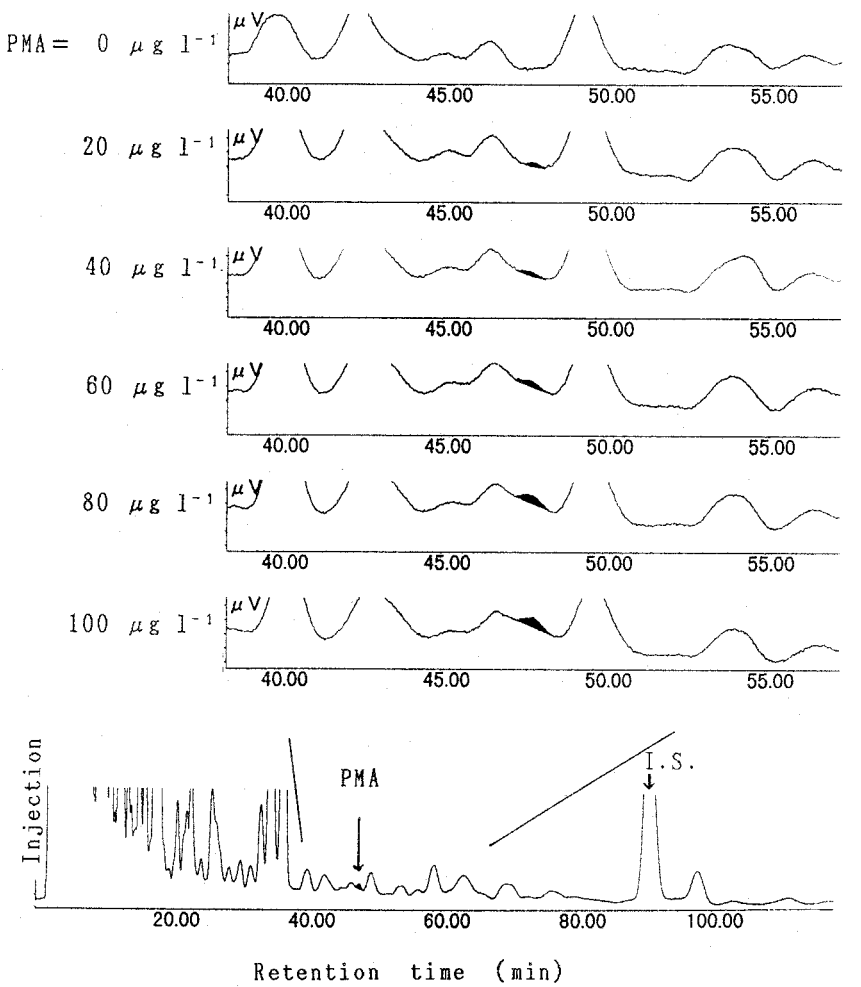

Figure 2. Enlargement of PMA peak as a function of added amount of PMA. PMA was added to a urine sample from a non-exposed subject (with no endogenous PMA) at the concentrations of 0, 20, 40, 60, 80, and 100 μg l^{-1}, respectively.

Table 1. Exposure to benzene, toluene and xylenes.

Solvent	Number of cases	TWA solvent concentration (ppb)		
		GM (GSD)	AM	Maximum
Benzene	70	33.8 (1.98)	40.7	107.1
Toluene	70	38.4 (2.98)	53.3	341.0
Xylenes[a]	70	22.0 (3.12)[b]	31.8	121.5

[a] Three xylene isomers in combination.

[b] Among the 70 exposed workers studied, four cases had no measurable (detection limit: 1 ppb) xylene exposures. The exposures were assumed as if they were at half the limit (i.e. 0.5 ppb) in calculating the GM and GSD.

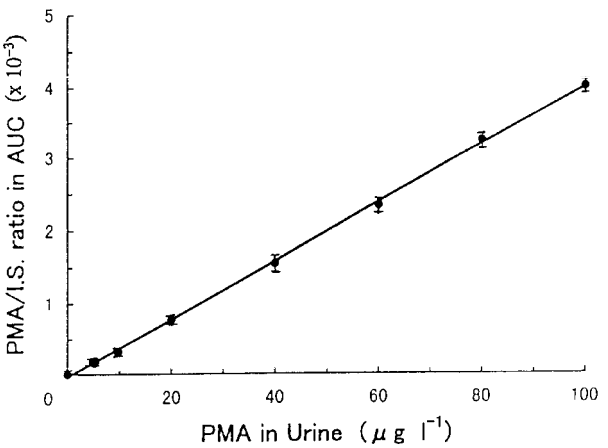

Figure 3. The area under curve proportional to the added concentrations of PMA. The results shown in figure 2 are quantitatively presented. Arrows show ASDs.

In all of the three cases studied, only one or two independent variables showed significant ($P < 0.10$) influence on urinary PMA, i.e. benzene as expected, and either sex, smoking habit or none. Benzene in air alone could explain 28–38% of total variation in urinary PMA. In contrast, the influence of either sex or smoking habit was quite limited: the cumulative R^2 indicated that only a 2–6% increase was achieved in the explanation of PMA variation when these second variables were introduced. Six independent variables in combination could explain 36–43% of the PMA variation (the right-most column in table 2). In other words, a single variable of benzene could contribute 79–94% of the total explanation for the variation (i.e., 0.282–0.383 out of 0.357–0.488).

The observation that partial correlation coefficients for benzene in the multiple regression analysis were identical with corresponding correlation coefficients in simple regression analysis (in the centre of table 2) also indicates that benzene had an almost exclusive effect on PMA, with very limited contribution of sex, smoking and co-exposures to other two aromatics. The results were essentially reproduced when a combination of the 70 exposed and 20 non-exposed cases were subjected to the MRA. Accordingly, no further consideration was made for effects of sex, smoking habits and co-exposures on benzene metabolism in further statistical analysis.

Table 2. Influential independent variable in multiple regression analysis.

Dependent variable[a]	Independent variable		
	1st variable	2nd variable	Total[b]
PMAob	Benzene (0.282; 0.531[c], 0.531[d])	Sex (0.343)	0.357
	Benzene (0.355; 0.596[c], 0.596[d])	*Sex (0.401)*	*0.420*
PMAcr	Benzene (0.376; 0.613[c], 0.613[d])	Smoking (0.395)	0.428
	Benzene (0.453; 0.673[c], 0.673[d])	*Smoking (0.476)*	*0.500*
PMAsg	Benzene (0.383; 0.618[c], 0.618[d])	None	0.407
	Benzene (0.453; 0.673[c], 0.673[d])	*Sex (0.467)*	*0.482*

Multiple regression analysis was conducted with 70 exposed cases. The results with a combination of the 70 exposed and 20 non-exposed cases are shown in italics. Only independent variables with significant ($P < 0.10$) influence are shown among the independent variables of age, cigarette consumption and TWA exposure concentrations of benzene, toluene and xylenes on numerical scales and sex on a nominal scale. Unless otherwise specified, the values in parentheses are cumulative R^2.

[a] Dependent variable: PMAob, PMAcr and PMAsg are phenylmercapturic acid as observed ($\mu g\ l^{-1}$)), corrected for creatinine [μg (g creatinine)$^{-1}$] or corrected for a specific gravity of 1.016 ($\mu g\ l^{-1}$), respectively.

[b] R^2 for all six variables in combination.

[c] Partial correlation coefficient obtained by the multiple regression analysis.

[d] Correlation coefficient obtained by simple regression analysis.

PMA in urine of non-exposed subjects

The possible presence of PMA was examined with 20 urine samples (from 10 men and 10 women). The analysis showed that PMA was not always present in the urine from the non-exposed control subjects; it was present only in urine samples from two men and three women out of 20 controls (table 3). When PMA levels below the detection limit of 0.2 $\mu g\ l^{-1}$ were assumed to be half the limit (i.e. 0.1 $\mu g\ l^{-1}$), the GM (in $\mu g\ l^{-1}$ without urine density correction) followed by GSD (dimensionless) in parenthesis was 0.13 (1.75) for 10 men and 0.18 (2.43) for 10 women; there was no significant ($P > 0.10$) difference between the two sexes irrespective of correction for urine density. The 95% upper limits of PMA in the urine from the non-exposed subjects, when calculated after the equation of $GM \times GSD^2$, were 0.7 $\mu g\ l^{-1}$, 0.7 μg (g cr)$^{-1}$, and 0.8 $\mu g\ l^{-1}$, after no correction for urine density (i.e. as observed), or when corrected for creatinine concentration or a specific gravity of urine of 1.016, respectively.

Exposure–excretion relationship

Pairs (90 in total, including 70 exposed and 20 non-exposed of both sexes) of TWA intensities of exposure to benzene and PMA levels in the end-of-shift urine samples were subjected to simple regression analysis to examine if PMA in urine can be taken as a marker of exposure to benzene. The calculations was made for PMA without correction (i.e. as observed), or after correction for urine density in terms of creatinine concentration or a specific gravity of 1.016; the results are shown graphically in figure 4, and the parameters of regression lines are summarized in table 4 together with correlation coefficients and their statistical significance. Correction for urine density did not result in an improvement in the correlation. Calculation with the 70 exposed cases only gave essentially the same results. It is clear from figure

Table 3. Phenylmercapturic acid levels in the urine from non-exposed and non-smoking control subjects.

Correction for urine density	Unit	Phenylmercapturic acid levels		
		GM	(GSD)	95% UL[a]
No correction	μg l^{-1}	0.15	(2.15)	0.69
Corrected for creatinine conc.	μg (g cr)$^{-1}$	0.15	(2.27)	0.79
sp. gr. (1.016)	μg l^{-1}	0.16	(2.28)	0.81

Values are calculated with 10 men and 10 women (all non-smokers), of whom urinary PMA levels were below the detection limit of 0.2 μg l^{-1} in eight men and seven women. The urinary PMA levels of such cases were assumed as if they were half the limit (*i.e.* 0.1 μg l^{-1}) in calculating GM and GSD.
[a] 95% upper limit of distribution, calculated as $GM \times GSD^2$.

Table 4. Correlation of PMA in end-of-shift urine samples with TWA benzene exposure.

Corrected for	Regression parameter[a]		
	α	β	*r*
None (i.e. as observed)	–1.39	0.116	0.596**
	–1.05	*0.129*	*0.531***
Creatinine concentration	–0.15	0.067	0.673**
	–0.54	*0.074*	*0.613***
Specific gravity (1.016)	–0.34	0.072	0.673**
	–0.95	*0.103*	*0.618***

Regression analysis is based on 90 cases in total, i.e. 20 non-exposed controls (10 men and women each) and 70 exposed workers (48 and 22 women) in combination. The results with the 70 exposed cases only are shown in italics.
[a] α and β are parameters of a regression line, $y = \alpha + \beta x$, where x is TWA benzene (ppb) in breathing zone air and y is PMA [μg l^{-1} or μg (g creatinine)$^{-1}$] in end-of-shift urine samples. r are correlation coefficients; all coefficients are statistically significant ($P < 0.01$).

4 and table 4 that PMA either with or without correction correlates significantly ($P < 0.01$) with benzene exposure with correlation coefficients of about 0.66–0.67.

Discussion

The intensity of the exposure of the gasoline station attendants to benzene was at 34 ppb as GM and 107 ppb as the maximum. With regard to the occupational exposure limit for benzene, all three of the occupational health-related organizations, i.e. of American Conference of Governmental Industrial Hygienists (ACGIH; 2000), Deutsche Forschungsgemeinischaft (DFG; 1999) and Japan Society for Occupational Health (JSOH; 1999), identify benzene as an established human carcinogen in agreement with International Agency for Research on Cancer (1987).

Thus, ACGIH (2000) set a TLV of 0.5 ppm for benzene and a BEI of 25 μg l^{-1} PMA in end-of-shift urine after benzene exposure. DFG (1999) established a technical exposure limit of 1 ppm for exposure to benzene in workplaces in general

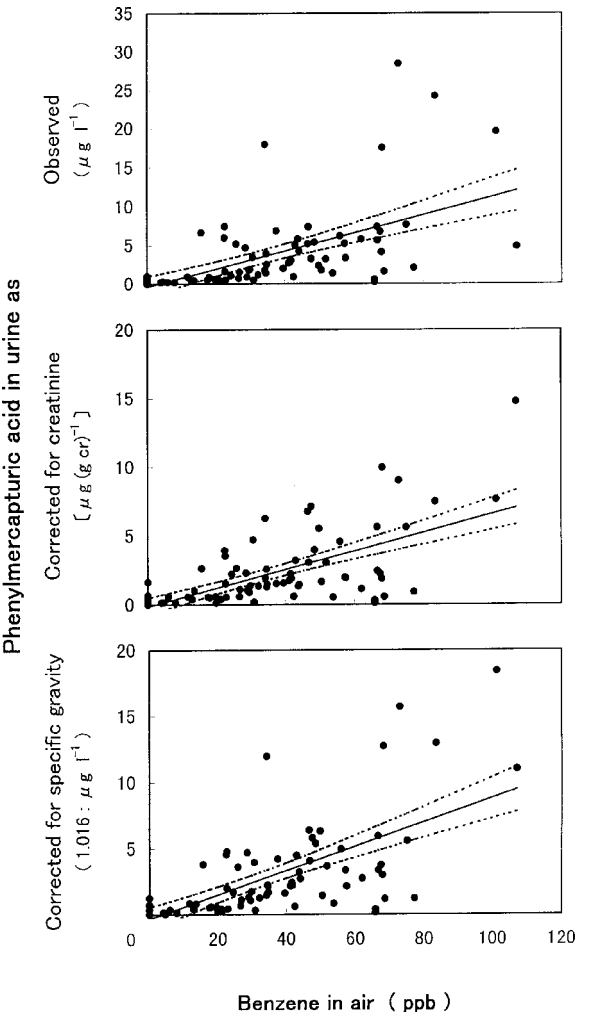

Figure 4. Scatter diagrams between TWA intensities of exposure to benzene and PMA in end-of-shift urine samples. In total, 90 subjects (70 exposed and 20 non-exposed of both sexes) were subjected to the analyses. Each dot in the figure represents one subject. The lines in the middle are calculated regression lines and the curves on both sides of the lines show the 95% confidence ranges of the group means. The detection limit is 0.3 μg l^{-1} when a signal to noise ratio of 3:1 is taken.

(i.e., other than coking, etc.). JSOH (1999) proposed two reference values for benzene of 1 ppm and 0.1 ppm which correspond to benzene-induced excess cancer risk for a lifetime of 10^{-3} and 10^{-4}, respectively. Although the meanings of the proposed criteria are different, as addressed above, it is plausible to conclude that the occupational benzene exposure of the subjects studied did not exceed any of the criteria given. Estimation of urinary PMA for a hypothetical exposure to benzene at 0.5 ppm (or 500 ppb) gives 55 μg l^{-1} as observed (table 4), which is about two times greater than the ACGIH's BEI of 25 μg l^{-1}.

The HPLC method developed in the present study enables urinary PMA to be determined using simple procedures. The only pretreatment necessary for the

Table 5. Quantitative correlation of PMA in end-of-shift urine samples with TWA benzene exposure: a literature survey.

References[a] Type of workers surveyed	Benzene exposure (ppm)	Regression parameter[b] α	β	*r*	PMA at 1 ppm benzene
A: Workers in chemical plants, natural gas refinery, etc.	< 2				46 μg (g cr)$^{-1}$[c]
B: Workers in car repair shop	< 2.5	4.1	35.1	0.81	39 μg l^{-1}
C: Workers in gas production chemicals production, oil refinery, etc.	< 3.2				47 μg (g cr)$^{-1}$
D: Workers in chemical plants	< 20	1.59	0.66[d]	0.63	39 μg (g cr)$^{-1}$
E: Workers in gas production, chemicals productions, oil refinery, etc.	< 6				44 μg (g cr)$^{-1}$
F: Workers in cookery, garage, etc.	< 7.3				12 μg (g cr)$^{-1}$
G: Workers in cokery	< 15	–8.2	66.3	0.99	58 μg (g cr)$^{-1}$
H: Shoe-makers					
(PMA as observed)	< 210	–41	64.5	0.78	24 μg l^{-1}
(PMA as corrected for creatinine concentration)		–19	77.7	0.82	59 μg (g cr)$^{-1}$
The present study:					
Gasoline station attendants	< 0.107				
(PMA as observed)		–0.4	116	0.60	116 μg l^{-1}
(PMA as corrected for creatinine)		–0.2	67	0.67	67 μg (g cr)$^{-1}$

[a] References: A; van Sittert *et al*. (1993). B; Popp *et al*. (1994), C; Boogaard and van Sittert (1995). D; Ghittori *et al*. (1995). E; Boogaard and van Sittert (1996). F; Hotz *et al*. (1997). G; Kivisto *et al*. (1997). H; Inoue *et al*. (2000).

[b] α and β are parameters of a regression line, $y = \alpha + \beta x$, where x is TWA benzene (ppm) in breathing zone air and y is PMA (μg l^{-1}) in end-of-shift urine samples; when benzene or PMA concentration was expressed in mg m^{-3} or mmol, it was converted to ppm or μg l^{-1}. r values are correlation coefficients; all coefficients are statistically significant ($P < 0.01$).

[c] Corrected for creatinine concentration.

[d] Double logarithmic regression.

[e] Originally 6 μg (g cr)$^{-1}$ for 0.5 ppm benzene.

determination is a short time acidification of urine samples for *in vitro* conversion of pre-PMA to PMA. One of major technical disadvantages is a long retention time in HPLC analysis (i.e., 92 min per determination); one analytical chemist may complete only 15 determinations a day, even though the HPLC can be run man-free overnight. It should be added that the PC software, Borwin, was effective in identifying the small peak for PMA and quantifying the AUC in the chromatogram.

The detection limit of the HPLC method developed was 0.2 μg l^{-1}, which is as low as or even lower than the limits described by other authors (i.e. 0.5 to 1 μg l^{-1}; van Sittert et al. 1993; Maestri *et al*. 1993, Popp *et al*. 1994, Boogaard and van Sittert 1995, 1996, Einig and Dehnen 1995, Angerer *et al*. 1998) for more complex methods. The limit is however substantially higher than the limit (0.06 μg l^{-1}) established by Einig *et al*. (1996) by means of a sensitive gas chromatographic method.

In order to make an overall quantitative evaluation of the sensitivity as an exposure marker, two parameters of the lowest separation concentration 1 (or

LSC-1) and 2 (LSC-2) have previously been proposed (Kawai *et al.* 1992). The LSC-1 is defined as the solvent vapour concentration at which the 95% lower limit of the distribution of the urinary analyte concentration is equal to the 95% upper limit at no exposure (i.e. 0 ppb or 0 ppm). In some cases, the urinary background level may be substantial, and the 95% upper limit of the background distribution can be estimated. Accordingly, the LSC-2 is defined as the vapour concentration at which the 95% lower limit of the distribution of the urinary analyte to concentrations is equal to the 95% upper limit of the background level. Both LSC-1 and LSC-2 are graphically determined by taking advantage of scatter diagrams such as figure 4. In practice, determination utilizing figure 4 and the 95% upper limits of urinary PMA among the non-exposed (the right-most column in table 3) gave both LSC-1 and LSC-2 in a range of 16 to 20 ppb regardless of correction for urine density (table 6). In other words, PMA in urine can separate those exposed to benzene at 20 ppb from those with no occupational exposure to benzene. No effects of co-exposures to toluene and xylenes were detected in the present study (table 2). Whereas the absence of metabolic interaction is quite conceivable considering co-exposure to solvents at very low concentrations, the lack of the effect is certainly an advantage of PMA as a biological marker of benzene exposure, because exposure to benzene in particular takes place generally in the presence of other solvents.

Table 5 summarizes quantitative correlation of urinary PMA with benzene exposure by inhalation, as reported in previous publications (van Sittert *et al.* 1993, Popp *et al.* 1994, Boogaard and van Sittert 1995, 1996, Ghittori *et al.* 1995, Hotz *et al.* 1997, Kivisto *et al.* 1997, Inoue *et al.* 2000). As the intensities of exposure to benzene were various depending on the studies, urinary PMA concentration after a hypothetical benzene exposure at 1 ppm was calculated from the regression parameters reported. Most papers (van Sittert *et al.* 1993, Boogaard and van Sittert 1995, 1996, Ghittori *et al.* 1995, Hotz *et al.* 1997, Kivisto *et al.* 1997, Inoue *et al.* 2000) gave the PMA as corrected for creatinine concentration, and the PMA levels ranged from 12 μg (g cr)$^{-1}$ (Hotz *et al.* 1997) to 59 μg (g cr)$^{-1}$ (Inoue *et al.* 2000). Comparison shows that the present value of 67 μg (g cr)$^{-1}$ is quite close to the highest value reported. Although the present results of 116 μg l^{-1} for uncorrected PMA appear to be higher than the levels previously reported, it is still difficult to make precise comparison because only two reports including one from this group (Popp *et al.* 1994, Inoue *et al.* 2000) gave uncorrected PMA levels.

Only limited effects of smoking on urinary PMA levels were observed in the present study (table 2), whereas many authors (Boogaard and van Sittert 1995,

Table 6. Lowest separation concentration by PMA.

Correction for urine density	Lowest separation concentration (LSC)	
	LSC-1[a]	LSC-2[b]
As observed (μg l^{-1})	20 ppb	16 ppb
For creatinine [μg (g cr)$^{-1}$]	17 ppb	19 ppb
For specific gravity (μg l^{-1})	17 ppb	18 ppb

[a] The lowest separation concentration set as 1-bromopropane (1-BP) concentration at which the 95% lower limit value is equal to the 95% upper limit value at 0 ppm 1-BP (Kawai *et al.* 1992).

[b] The lowest separation concentration set as 1-BP concentration at which the 95% lower limit value is equal to the 95% upper limit of the background level (Kawai *et al.* 1992).

Ghittori *et al*. 1995, Einig *et al*. 1996) reported a smoking-associated substantial increase in PMA-U, especially among heavy smokers (Ghittori *et al*. 1999). Although no quantitative data are available, observation during the survey indicates that the gasoline station attendants consumed only the top quarter (or one-third) of a cigarette when they smoke. Thus, the actual amount of cigarette-originated benzene inhaled should be much less than the number of cigarettes would suggest. Such a smoking pattern might explain the weak infiuence of cigarettes observed in the present study.

It is also worthy to note that the benzene exposure in the present survey (110 ppb or less) was substantially lower than the exposure examined in previous studies, and that all but two (Inoue *et al*. 2000 on Chinese and the present one on Japanese) dealt with Caucasian working forces. Taking possible dose-dependent changes in benzene toxicokinetics and ethnic differences into consideration, the extension of the survey to other Asian populations exposed to benzene at 1–100 ppm is currently in progress.

Acknowledgements

Thanks are due to Professor K. Yoshinaga, the Director of Tohoku Rosai Hospital, Sendai, Japan, for his support to this study.

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