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Original Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR DETERMINATION OF *S*-PHENYLMERCAPTURIC ACID (*S*-PMA) IN URINE

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ABSTRACT

Objective: The aim of this study is to obtain the optimum condition of HPLC method for the determination of benzene metabolite, *S*-phenyl mercapturic acid (*S*-PMA), in urine samples.

Methods: Liquid-liquid extraction (LLE) was used as sample preparation method for urine sample. HPLC analysis was performed using Hypersil ODS column 125 x 4 mm ID, 5 µm, the column temperature 25°C and the DAD detector was set at wavelength of 205 nm.

Results: Optimum separation was accomplished using gradient system with eluent consists of methanol and phosphate buffer pH 3. The result showed linear correlation with a regression coefficient (r) = 0.994. The detection and quantification limit of *S*-PMA were 0.7832 ± 0.0329 µg/mL and 2.6108 ± 0.0940 µg/mL, respectively. The recovery of the method was 88.34 - 117.16% with a mean of 99.96 % and coefficient variation (CV) = 8.98 %.

Conclusion: The proposed method has met the acceptance criteria for validation. The method is simple, fast, and has reproducible recoveries. Therefore, this method can be used for routine analysis of *S*-PMA.

Keywords: *S*-Phenylmercapturic acid, HPLC, Urine.

INTRODUCTION

Benzene is an important chemical compound used in the manufacturing of polymers, plastics, rubber, dyes, detergents and other products [1]. However, the use of benzene in industry results in environment pollution and consider as potential carcinogenic. Environmental exposure to benzene can cause accumulation of benzene in the body. People who could potentially have high levels of benzene in the body are the highway patrol, gas station employees and petroleum refinery employees in direct contact with oil. Continuous exposure to benzene may increase the likelihood of developing cancer. Several studies show correlation between benzene with leukemia [2, 3]. Depression of bone marrow and leukemogenesis caused by damage to several classes of hematopoietic cells and hematopoietic cell functions also has been reported [1]. Therefore, analysis of benzene metabolites in the urine of humans exposed to benzene can be useful in the cancer prevention and early detection of cancer risk. The analysis result can be used as a safety reference in industry, for example by organizing safety training on the hazards of benzene exposures and the importance of masks and gloves for those who directly contact with benzene.

The main metabolite of benzene (Figure 1) is phenol, hydroquinone (HQ), catechol (CAT) and 1,2,4-benzenatriol, *t,t*-muconaldehyde, and *t,t*-muconic acid (*t,t*-MA). Benzene oxide can react further with glutathione which is excreted in the urine as *S*-PMA [4]. *t,t*-MA and *S*-PMA are two metabolites that can be used as a biomarker for determine the occupational level of exposure to benzene. However, *S*-PMA is more common used as a biomarker than *t,t*-MA [5, 6]. American Conference of Governmental Industrial Hygienists (ACGIH) also requires industrial company to evaluate the *S*-PMA level in workers who work related with benzene [6]. Some methods have been developed for *S*-PMA determination, such as LC-MS/MS [7], LC-tandem MS [8,9], and ELISA [10]. However, all those methods are expensive and need a long sample preparation method. The aim of this study is to perform the optimization and validation of HPLC analytical method for determining benzene metabolite, *S*-PMA in urine samples. The HPLC method must be accurate, reliable and can be done in a short time.

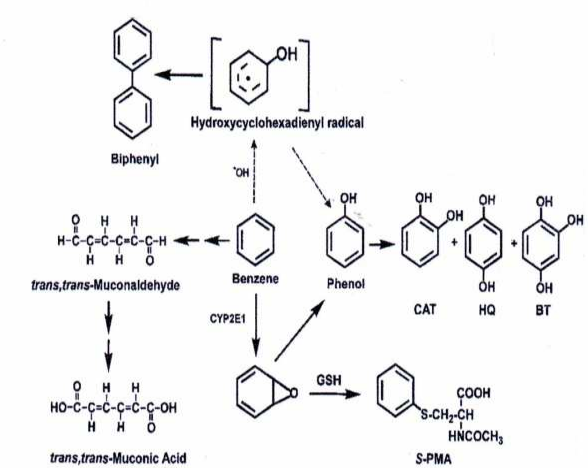

Fig. 1: Metabolism pathway of benzene [4]

MATERIALS AND METHODS

Sample: Urine samples were taken from healthy young men and recruited from the student and staff population of our University. The samples were collected early morning before activity. Informed consent was obtained before urine collection. The study protocol was approved by the institutional review board of Institute for Research and Community Services (IRCS), Airlangga University.

Reagents: *S*-PMA pro analysis was purchased from Tokyo Chemical Industry (Tokyo). Pro HPLC grade methanol, potassium dihydrgen phosphate (KH_2PO_4), phosphoric acid (H_3PO_4), ethyl acetate and benzoic acid (pro analysis grade) were obtained from E. Merck. High purity aquabidestilated water was obtained from Widatra.

Instruments: HPLC system (Hawlett Packard Agilent 1100) equipped with DAD detector, Hypersil ODS column (125 mm × 4.0 mm i.d., particle size 5µm). The injection volume 20 μl was used.

Mobile phase was prepared by mixing (A) methanol and (B) phosphate buffer pH 3, in a gradient system (specified as in Table 1). The UV detector was monitored at 205 nm. Sample preparation: Three mililiter of urine was acidified using 18N H_2SO_4 to pH 1.0. After 10 minutes, 10N KOH was added to make pH 1 – 2. Samples were extracted with ethyl acetate 3.0 ml (2 times) and continued with vortex for 30 sec. The solution then centrifuged at 5000 rpm for 30 seconds. Ethyl acetate phases were taken and dried with nitrogen gas to dryness, dissolved with 0.5 mL of water. Finally, the solution was ultrasonicated for 1 min and filtered through Whatman filter paper 0.2 μm prior to injection.

Selectivity

The selectivity was analyzed by injecting *S*-PMA standard into chromatographic system. The resolution (Rs) value was calculated and used as selectivity parameter.

Linearity, the limit of detection (LOD) and quantification (LOQ)

To obtain the linearity, working solutions containing *S*-PMA (1, 2, 3, 4, and 5 µg/mL) and internal standard (benzoic acid) were analysed in triplicate. The linearity were established by plotting ratio peak area of *S*-PMA with internal standard versus the concentration of each analyte. The limit of detection (LOD) and limit of quantification (LOQ) of *S*-PMA were determined by injecting a series of standard solutions until the signal-to-noise (S/N) ratio was 3 for LOD and 10 for LOQ, respectively.

Accuracy

Accuracy was calculated from the equation:

$$\text{Accuracy (\%)} = 100 \times (C_{real} - C_{determined})/C_{real}$$

where C_{real} denotes added amount of *S*-PMA while $C_{determined}$ is *S*-PMA calculated from the equation of calibration curve. Accuracy was performed by standard addition method. The urine samples were added with *S*-PMA, and then prepared as sample solutions procedure.

Precision

Intra- and inter- day precision were used to determine the precision. For intra-day test, the samples were analyzed for three times within the same day, whereas for inter-day test the samples were analyzed for three consecutive day.

Matrix effect

To see the matrix effect from different donors. *S*-PMA was diluted with two different urine sample. The matrix effect was observed by making calibration curve.

RESULTS

Selectivity

Blank drug free urine was analyzed by HPLC. No interfering peaks were observed at the retention time of *S*-PMA and benzoic acid as internal standard (IS). The Rs value of *S*-PMA was 1.93 and this value met the acceptance criteria of Rs (> 1.5)

Accuracy

The accuracy results are shown in Table 2. The % recovery of *S*-PMA was between 88.34 and 108.41% with an average of 99.96 ± 8.97 %.

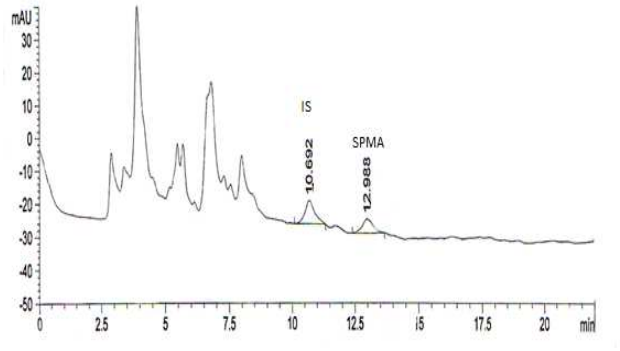

Fig. 2: Chromatogram of matrix urine spiked with standard *S*-PMA solution and benzoic acid solution as internal standard (IS)

Linearity, the limit of detection (LOD) and quantification (LOQ): Linearity of *S*-PMA with concentrations of 1, 2, 3, 4, and 5 µg/mL was evaluated. The calibration curve was y = 2.308 x + 0.004 with R^2 value = 0.981 (Figure 3). The detection limit of *S*-PMA was 0.7832 ± 0.0329 µg/mL whereas the limit of quantitation was 2.6108 ± 0.0940 µg/mL.

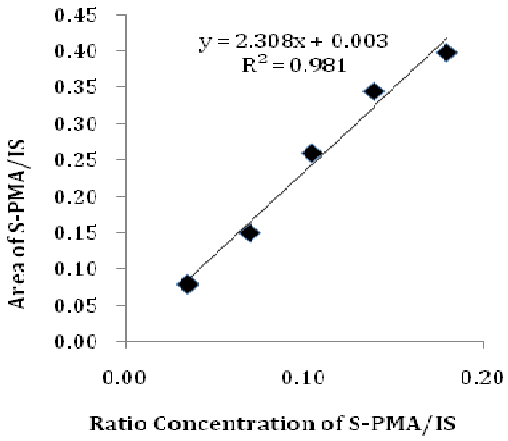

Fig. 3: Calibration curve of *S*-PMA with concentrations of 1, 2, 3, 4, and 5 µg/mL.

Table 1: Eluent composition for analysis of *S*-PMA in Urine

T (min)	Methanol (%)	Phosphate buffer pH 3 (%)	Flow (ml/min)
0-5	14.5	85.5	1
5-5.30	22.7	77.3	2
5.30-20	22.7	77.3	2
20-20.30	14.5	85.5	1
20.30-25	14.5	85.5	1

Precision

The intra-day and inter-day data precision are shown in Table 3. In the intra-day studies, 3 repeated injections of *S*-PMA standard and benzoic acid as internal standard in urine were made in a day and percentage RSD was calculated. Similarly with intra-day, inter-day precision performed on 3 consecutive days and percentage of RSD was calculated. Both inter- and intra- day result met the requirements according to the Horwitz equation.

Matrix Effect: The matrix effect data were analyzed using *paired t-test* mode. The result showed that urine matrix did not interfere the determination of *S*-PMA in samples (p > 0.05). The linearity of *S*-PMA in urine sample can be seen on Figure 4.

Table 2: Recovery data of *S*-PMA in urine sample

Replication	Peak Area of standard *S*-PMA	Peak Area of Sample	Concentration of *S*-PMA added (ug/ml)	Concentration of *S*-PMA obtained (ug/ml)	% Recovery
1	106.3410	93.9365	5.20	4.5935	88.34
2	106.3410	99.0423	5.20	4.8431	93.14
3	106.3410	114.8960	5.20	5.6184	108.05
4	106.3410	115.2790	5.20	5.6371	108.41
5	106.3410	108.3050	5.20	5.2961	101.85
				means	99.96
				SD	8.97
				CV (%)	8.98

Table 3: Intra-day and inter-day precision studies determination of *S*-PMA by HPLC

Concentration (µg/ml)	*S*-PMA % RSD (*n* = 3)	
	Intra-day	Inter-day
10.1	5.96	6.22
15.2	4.33	4.65
25.3	4.02	4.11

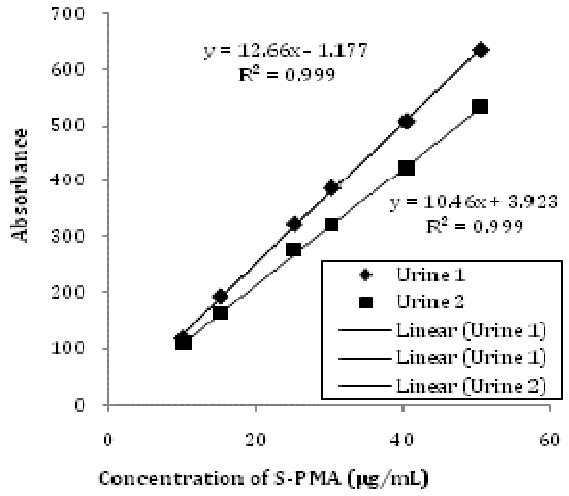

Fig. 4: Two different urine samples were used to dilute *S*-PMA. Both samples showed a good linearity (R > 0.999).

DISCUSSION

In this study, environmental benzene exposure is determined by detecting the levels of *S*-PMA in urine because it is not affected by sorbic acid levels in foods while *t,t*-MA levels are highly dependent [11]. Tuakila (2013) revealed the high benzene exposure in ambient air of the Kinshasa population can be determined by measuring the level of *S*-PMA [5]. Therefore, the determination of the level of *S*-PMA in workers associated with exposure to benzene needs to be done to protect their health. The proposed method was applied to two different urine. This step needs to be done to see the effect of the urine matrix on the determination of levels of *S*-PMA. Two healthy male volunteers aged 30-35 years who were selected should not smoke, drink alcohol or take medication and relatively unexposed benzene. The results showed that there was no significant difference in the determination of *S*-PMA in both urine matrix. In addition, the correlation coefficient of both urine samples were greater than 0.999. This condition proves that the method can be used in a various of matrices urine. Human genotype also affects the metabolism and excretion of *S*-PMA such as the influence of glutathione S-transferases polymorphisms on benzene metabolite excretion [12]. Beside that, smoking or non-smoking also affects the levels of *S*-PMA in urine [13]. These factors must be considered to determine *S*-PMA level.

Based on the result, HPLC method in this study has given satisfactory result and met the validation requirements that can be applied to monitor of benzene exposure in humans, especially the workers in petrochemical industry. We suggest to use this method for control the inhaled benzene levels in order to avoid air hazard.

CONCLUSION

The HPLC proposed method has met the acceptance criteria for validation. Furthermore, the proposed method are simple, fast, and has reproducible recoveries. Therefore, this method can be used for routine analysis of *S*-PMA.

ACKNOWLEDGEMENT

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