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A sensitive liquid chromatographic method for the spectrophotometric determination of urinary trans,trans-muconic acid

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Abstract

Benzene is a human carcinogen and its metabolite, urinary trans,trans-muconic acid (ttMA), is a biomarker for risk assessment. However, most of the existing methods were not sensitive enough for monitoring of low level exposure. This paper describes a HPLC-UV method for ttMA determination with enhanced selectivity and sensitivity. A 30 mg Oasis®MAX cartridge was used to clean-up 50 μl of urine sample and gradient elution was performed on a Zorbax SB-C_{18} column (30 °C). ttMA was detected at wavelength 263 nm using a UV diode array detector (DAD). The two mobile phases used were (A) 150 mM ortho-phosphoric acid containing of 9% (v/v) methanol; and (B) 125 mM ortho-phosphoric acid containing 30% (v/v) acetonitrile. The method was validated with 61 urine samples collected from non-occupationally benzene exposed individuals and 14 quality control specimens from an international quality assessment scheme. The urinary ttMA concentrations (mean ± S.D. μg/g creatinine) were 90 ± 34 for smokers (n =26), 49 ± 39 for non-smokers (n = 21) and 23 ± 18 for non-smoking hospital staff (n = 14). A correlation coefficient, r = 0.99 was found with 14 external quality specimens for ttMA ranged from 0.4 to 6.8 mg/l. The recovery and reproducibility were generally over 90% and the detection limit was 5 μg/l.

Keywords: Biomarker; Interference of sorbic acid; Low level benzene exposure; Cigarette smoking

1. Introduction

Benzene is known to be a human carcinogen [1] and its metabolite trans,trans-muconic (ttMA) in urine is one of the recognized biomarkers for risk assessment of exposure [2–4]. Existing reversed-phase (RP) HPLC-UV methods using solid phase exchange for sample clean-up are well suited for determining ttMA [5,6]. Although the sample clean-up can be done online with coupled columns technique [7,8], the column efficiency was found easily deteriorated due to contamination. In addition, the selectivity and sensitivity of existing UV detection methods are incomparable to LCMS/MS which offers a better detection sensitivity of 3 μg/l of ttMA [9–11]. However, the background levels of non-benzene exposure reported using LCMS/MS were similar or even higher than those reported for environmental studies [12,13]. Further, urinary ttMA results were occasionally found not correlated with low benzene exposures at ppb levels [14,15]. The high ttMA background level has been suggested to be owing to the potential interference contributed by sorbic acid (SA), a common food preservative [16,17].

As cost-effectiveness, ease of use and conveniences are important factors for routine laboratory analysis, it is generally agreed that UV detection is still preferable to mass spectrometry detection. Thus, we decided to develop a new method based on our earlier approach [5] but using more advance chromatographic technologies to enhance the sensitivity through improved selectivity and resolution. The urine sample volume was reduced from 1 ml to 50 μl and the strong anion exchanger was replaced with mixed-mode anion exchange reversed-phase sorbents for sample clean-up. To prevent dietary interference, vanillic acid which was previously used as an internal standard (IS) was replaced with isovanil-

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lic acid. The resolution was enhanced by using extremely high ionic strength (pH = 1.8) mobile phase and chemical stable reversed-phase C_{18} stationary phase. The accuracy and reliability of the assay were ensued by our participation in the Finnish Institute of Occupational Health (FIOH) Quality Assurance Programme for organic solvent metabolites. The method was also validated by determining the background levels of urinary ttMA among 61 non-occupational benzene exposed individuals. The effect of sorbic acid interference was intensively investigated and discussed.

2. Material and methods

2.1. Reagents and chemical

ttMA, sorbic acid and isovanillic acid used as internal standard (IS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ortho-phosphoric acid, formic acid, ammonia solution and hydrochloric (HCl) acid were analytical grade and were obtained from Merck (Darmstadt, Germany). Methanol, acetonitrile and ethyl acetate (HPLC/Spectro grade) were purchased from Tedia (Fairfield, OH, USA).

2.2. Samples collection

For method validation, spot urine samples were collected in the morning before meal from 21 non-smokers and 26 smokers working in a factory that does not use solvent and 14 non-smoking medical graduate students and hospital staff, with informed consent. For time-course study of SA interference, four medical staff volunteered to ingest specific amount of cheese sandwiches with SA content estimated to be 20, 40, 80 and 160 mg. The other 10 subjects included as the control group took egg and rice for breakfast. Home cooked lunch that avoided all types of preservative was prepared for both groups and urine samples excreted from 8.30 a.m. to 6 p.m. were collected on the same day. An aliquot of 2 ml urine was preserved with 20 μl 6 M of HCl and stored at −20 °C until analysis.

2.3. Standards preparations

Stock solutions of ttMA (500 mg/l) and IS (IVA, 2.5 mg/ml) were prepared individually with methanol–water (8:2, v/v). Working IS of 5 mg/l was prepared by diluting the stock solution 500-fold with 60 mM of HCl solution. Working standards with various concentrations (5–5000 μg/l) were prepared in a solution same as mobile phase A (9%, v/v of methanol in 150 mM of ortho-phosphoric acid). The calibrators were prepared by mixing 50 μl ttMA each of these working standards with equal volume of IS (5 mg/l).

2.4. Extraction procedure

The Supelco Visprep™ vacuum manifold (24-ports) was used for SPE extraction. Flow rate of <10 ml/min were applied for the conditioning and rinsing steps and <1 ml/min for the loading and elution steps. In a micro-centrifuge tube, a 50 μl aliquot of acidified urine was mixed with 50 μl of IS solution and 900 μl of 60 mM of HCl solution. After centrifugation (15,000 × *g*/2 min), 900 μl of this sample mixture was percolated through the 30 mg Oasis®MAX sorbents (Waters, MA, USA) preconditioned with 1 ml each of methanol and water. After rinsing with 1 ml each of 25 mM ammonia solution–methanol (95:5, v/v) and pure ethyl acetate, the cartridge was vacuum dried for 5 min. The analytes were than collected with 800 μl of eluent made up of formic acid–ethyl acetate (2:98, v/v). With the use of Supelco Visidry™ and Visprep™ device, samples were dried under a stream of nitrogen for 25 min. We reconstituted the dried residue in 90 μl of mobile phase A. The extract was transferred it to a micro-vial and 20 μl was injected on to the column.

Table 1
Reproducibility and recovery (*n* = 5)

Added (μg/l)	Mean (μg/l)	CV %		Recovery (%)
		Within-day	Between-day	
–	23	7.0	16.5	–
20	40	2.0	3.9	84
100	112	7.1	9.7	89
500	472	6.0	9.3	90
5000	4926	6.0	6.1	99

For reproducibility and recovery studies, five sets of spiked samples (Table 1) were prepared and analysed within 24 h for within day precision, and between-day variation was determined by repeating the same assay once a week for 5 consecutive weeks. To ensure the accuracy and reliability of determination, 14 external QC specimens from FIOH with ttMA concentrations ranging from 0.4 to 6.8 mg/l were analysed together with every batch of samples analysis. The results were presented as observed (μg/l) or after correction for urinary creatinine (μg/g creatinine).

2.5. HPLC condition

The Waters Alliance 2695 Separations Module (MA, USA) was connected with a guard cartridge (Jour Guard C_{18}, USA), an Agilent Zorbax SB-C_{18} stationary phase (5 μm, 150 mm × 4.6 mm (i.d.), NJ, USA) maintained at 30 °C and a Waters Model 996 DAD detector. The mobile phase A was made-up of 9% (v/v) methanol prepared in 150 mM ortho-phosphoric acid and B was 30% (v/v) acetonitrile in 125 mM ortho-phosphoric acid. The gradient elution was performed at 1 ml/min with an initial condition of 90% of mobile phase A for 4 min, it was increased to 100% at 5 min (hold 3 min), and linearly decreased to 10% at 25 min (hold, 7 min). The system was then returned to the initial condition from 32 to 35 min before the next injection. Peaks were identified using photodiode array detection at 263 nm for ttMA and IS, and at 220 and 254 nm to access the gravity of matrix interferences.

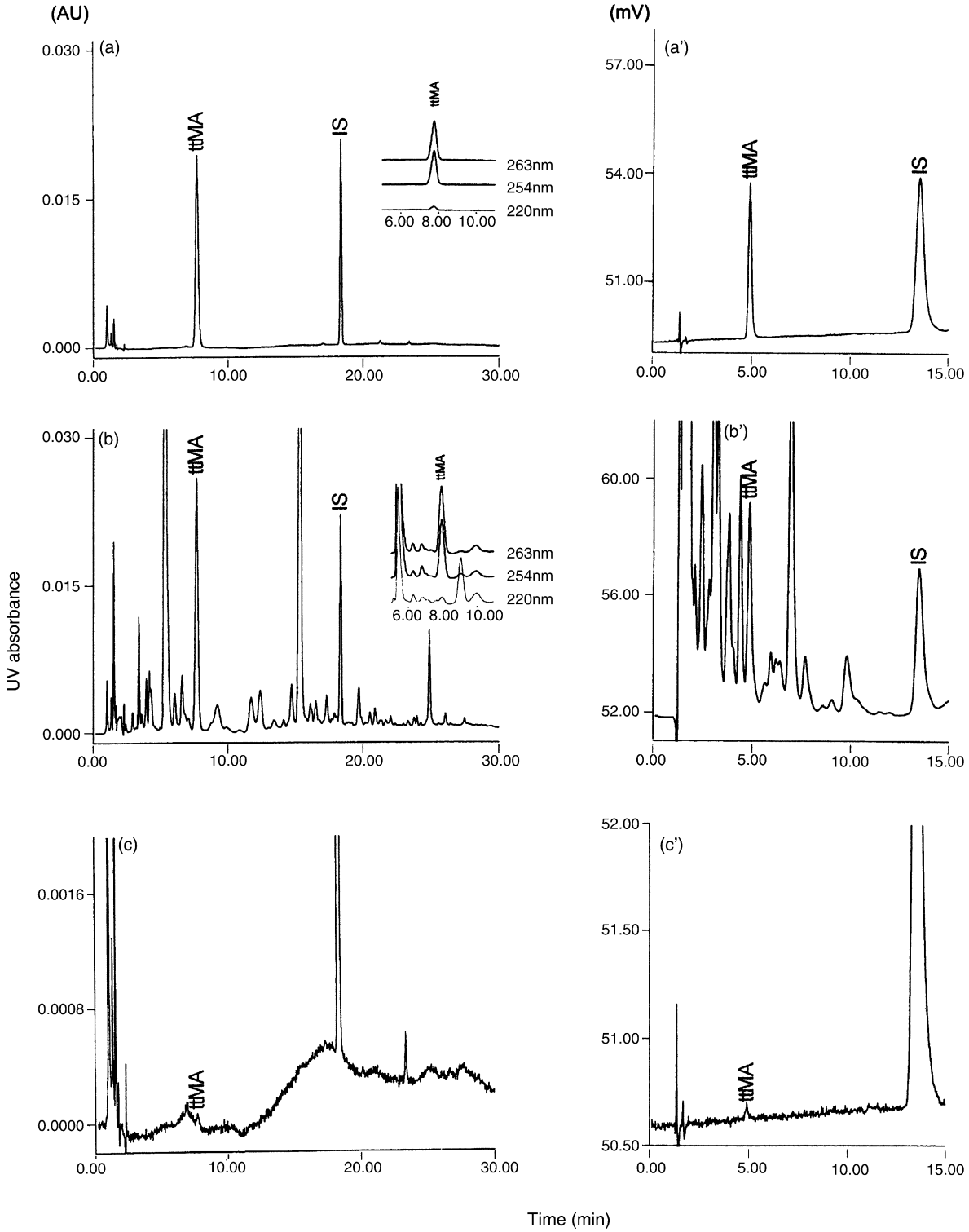

Fig. 1. Typical chromatograms of ttMA analysis performed by present method and extracted at 263 nm: (a) a standard solution containing 2 mg/l of ttMA and 5 mg/l of IS, (b) a quality control sample from FIOH and (c) lowest detection limit of 5 μg/l of ttMA. Illustrations are chromatograms extracted at 220, 254 and 263 nm for monitoring of matrix interferences. Chromatograms (a) and (b) are sample assayed using the proposed method and (a′) and (b′) are their corresponding samples assayed using the old method (5). Chromatograms (c) and (c′) are the lowest detection limits using the new and old method, respectively.

Data acquisitions were performed with the Waters Empower™ software.

3. Results and discussion

3.1. Chromatographic separation and efficiency

Owing to the recent concern on health effect of low level benzene exposure, existing HPLC-UV urinary ttMA methods were found to be insensitive and not reliable for exposure risk assessment. Although the use of LCMS/MS technique could be a better detection mode, the expensive equipment and complicated techniques limit its application for routine analysis. Thus, the development of a precise HPLC-UV technique with higher sensitive and reliability was necessary.

In this study we noted that there was peak coelution when the mobile phase pH ≥ 2 and the best resolution could only be achieved with a strong ionic mobile phase of pH as low as 1.8. It was also observed that ortho-phosphoric acid with pK_a value of 2.1 offered the best separation, compared to formic (pK_a = 3.7), acetic (pK_a = 4.7) or trifluro-acetic (pK_a = 0.3) acid reported earlier [5–11]. In order to avoid damage that might cause by the use of low pH on conventional C_{18} column, a Zorbax SB-C_{18} column was used instead. The chromatographic performance of this column was found to be stable and no degradation was observed with over 600 samples tested during the 4 months study. In addition, methanol was found to be the best modifier of mobile phase A for the separation of ttMA from the adjacent peaks, whilst the acetonitrile was a preferable modifier of mobile phase B as it offered stronger elution strength for the IS. Using the optimized condition, the maximum absorbance for ttMA of a calibrator (2 mg/l) eluted at 7.9 min was at 263.2 nm (Fig. 1a). The chromatogram of a FIOH QC sample known to contain 2.6 mg/l of ttMA having identical retention time as the calibrator is shown in Fig. 1b. It was noted that many urinary acids with maximal UV absorbance at 220 and 254 were also detectable at 260–268 nm. Hence, the selectivity and specificity of ttMA measurement was concurrently monitored at 220, 254 and 263 nm; as shown in the respective illustrations of Fig. 1a and b. The retention times of ttMA and IS (18.5 min) were highly reproducible with coefficient of variations (CV) <0.5%. Non-polar residues were effectively removed from column within 35 min. For comparison purpose, the same samples were also analysed using our earlier method [5] and the chromatograms are shown in Fig. 1a′ and b′. Although the run time of the new method was slightly longer, the resolution and selectivity were much better. Furthermore, the sample size required was 20-fold smaller and yet the sensitivity was four times higher (detection limit = 5 μg/l, Fig. 1c) than the earlier method of 20 μg/l (Fig. 1c′).

3.2. Efficiency of sample preparation

It is well known that the reliability of trace analysis in urine sample is always hampered by matrix interferences. Sample clean-up methods using anion-exchange sorbents for solid phase extraction were well established since last decade [5,6]. Laboratory packed cartridges were recently replaced with commercially available cartridges [10,11]. In this investigation, we used the recently developed mixed-mode polymeric cartridge with anion-exchange and reversed-phase functionalities for the sample clean up. The advantages of using this polymeric cartridge are; (1) the anion-exchanger imparted high selectivity for acidic compounds, (2) the reverse-phase function of the cartridge permits neutral and basic compounds to be removed earlier when pure solvent was used, (3) the required pH of below 2 could be auto-adjusted by diluting the sample with HCl solution and (4) the non-requirement of wet cartridges prior to sample loading, offered the convenience of continuous vacuuming that saved time in SPE extraction.

However, results of our initial investigation showed that the recovery rates varied markedly due to differences in urine concentration and matrices among individual samples. Higher recovery was usually achieved with less concentrated samples. In its place of a bigger capacity of sorbents to be used, the urine volume was reduced from 1 ml to 50 μl. Using conditions described above, the recovery rate was consistent and highly reproducible, as shown in Table 1. Using the proposed method, 24 urine samples can be prepared in less than 1 h and >160 samples can be continuously analysed within 5 consecutive days.

3.3. Calibration, reproducibility and recovery

In the present study we used internal standard additions method for calibration. Working solutions stored at 25 °C were found to be stable for at least a week, with CVs of <5%. The typical calibration curves for concentration ranged from 5 to 500 μg/l was $y = 2314x - 1$ with $r = 0.99$ and 50–5000 μg/l was $y = 2409x - 6$ with $r = 0.99$ (y = ttMA concentration, x = peak height ratio and r is the coefficient of correlation). The day-to-day variations ($n = 6$) of slope and linearity were <5 and <0.1%, respectively. The within-day precision, between-day variations and recovery rates were

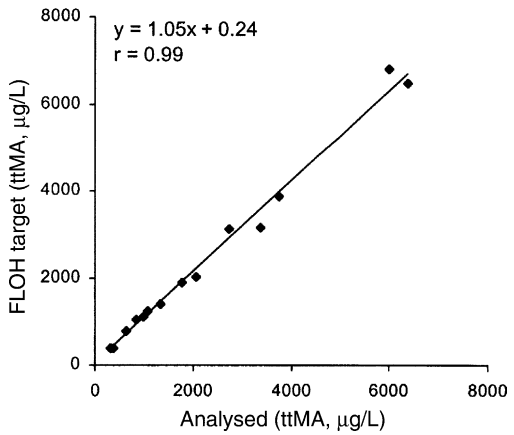

Fig. 2. Comparison between results obtained using the present method and FIOH target values.

examined using urine samples spiked with various concentrations of ttMA. The recovery rate based on direct peak height comparison of IS and spiked ttMA was around 95(±5)%. The corrected recovery rates with the use of internal standards were 84–99%, the coefficients of variations ($n = 5$) of within-day assays and between-day assays were generally <10% (Table 1).

3.4. Method validation

The specificity and reliability were validated using external QC specimens. As shown in Fig. 2, the ttMA results obtained were closely correlated with the target values for ttMA concentration ranged from 0.4 to 6.8 mg/l, with a correlation coefficient $r = 0.99$. In order to further validate the sensitivity of the present method, morning urine samples were collected from 61 non-occupationally exposed individuals, and the results are summarized in Table 2. Although the mean value obtained from the non-smoking group (49 ± 39 μg/g

Table 2
Urinary ttMA concentrations of non-occupational benzene exposure individuals with and without smoking habit

Groups	*n*	ttMA (μg/l)			ttMA (μg/g creatinine)		
		Mean	S.D.	Range	Mean	S.D.	Range
Hospital staff and students							
Non-smokers	14	26	20	<5–64	23	18	4–57
Factory workers							
Non-smokers	21	75	58	13–222	49	39	6–119
Smokers	26	150	81	41–432	90	34	24–150

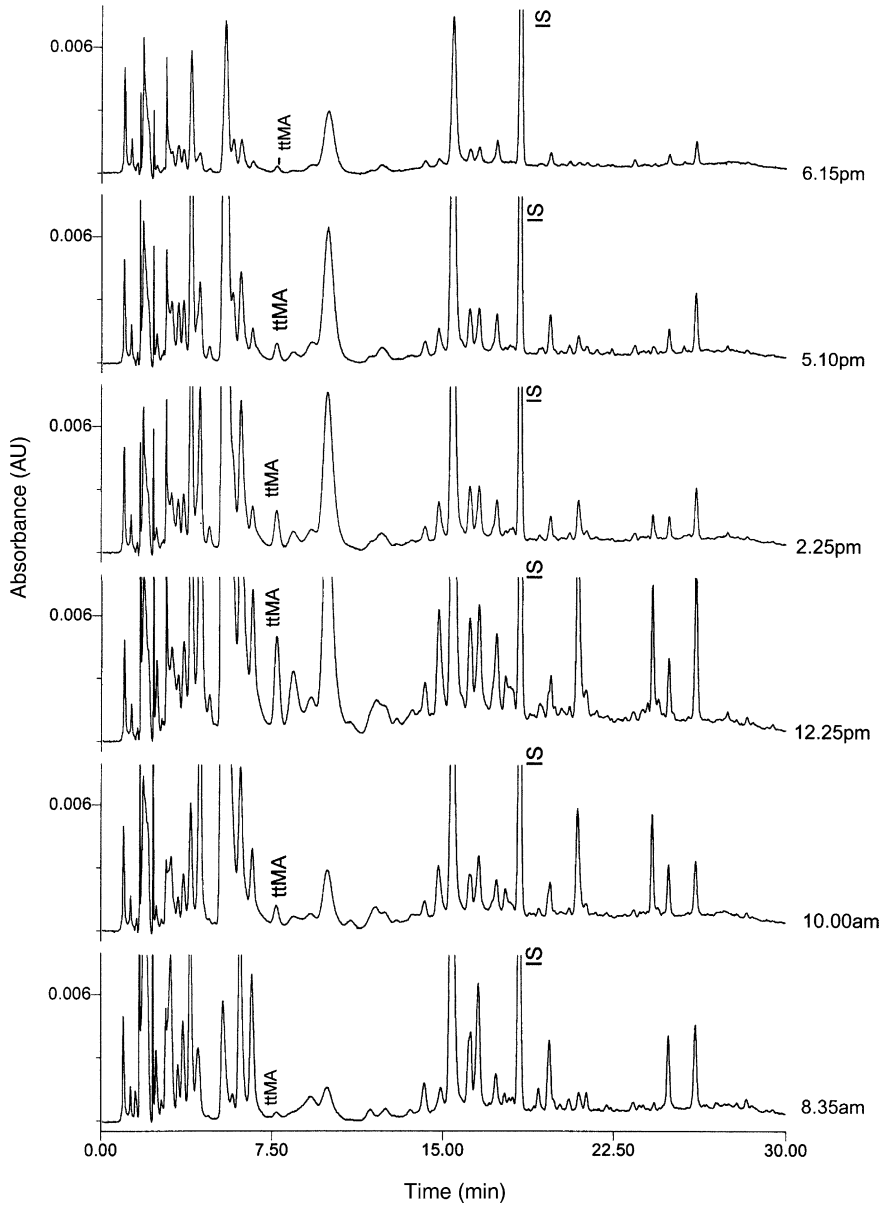

Fig. 3. Chromatograms of urine samples collected over 10 h by a volunteer taken breakfast containing approximately 160 mg of SA on the same day.

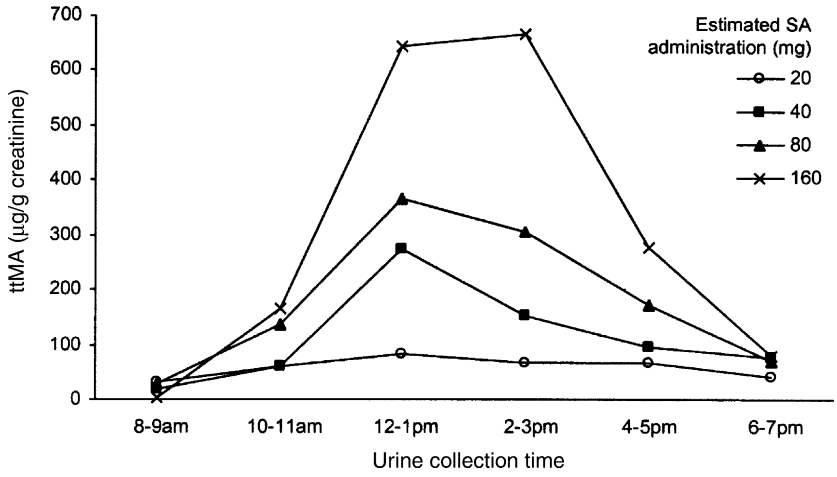

Fig. 4. Time course of urinary excretion of ttMA four volunteers ingested 20–160 mg of SA.

creatinine) was significantly lower than the smoking group (90 ± 34 μg/g creatinine) from the same factory, the value was two-folds higher than those obtained from hospital staff (23 ± 18 μg/g creatinine). The higher ttMA values of non-smoking factory workers could be due to exposure to passive smoking, as most of their colleagues are smokers and cigarette smoke is known to contain benzene [18].

3.5. SA interferences and time course study

The possible interference of sorbic acid (SA) intake on urinary ttMA measurement has been an issue of recent concern. In this study we collected urine samples of over 8 h from four volunteers consumed breakfast containing SA and 10 volunteers who took breakfast with no SA. It was noted that there were no significant differences for urine samples collected through out the day for control subjects, with an average value of 27 μg/g creatinine. The typical chromatograms of urine samples collected from a volunteer taken breakfast containing 160 mg of SA are shown in Fig. 3. It was noted that chromatograms of afternoon samples are more complex compared to the morning and evening samples. The urinary ttMA was lowest (7 μg/l) at 8.35 a.m., highest (663 μg/l) at 12.35 p.m. and then back to low level at 6.15 pm. The results of the time-course study of four volunteers consumed different concentrations of SA were plotted and shown in Fig. 4. It was found that regardless of the amount of SA taken, the highest concentration of ttMA detected was 3–5 h after SA ingestion. Thus, our results confirm the earlier findings that dietary intake of SA does not cause significant interference to the measurement of urinary ttMA as a biomarker for benzene exposure [19–21]. This finding is in line with the kinetic studies that SA has a half-life of less than 8 h [19] and benzene was around 13 h [21] and confirmed the finding of Pezzagno et al. that there was no correlation between SA intake and ttMA excretion when samples were collected 10–12 h after the last meal [20].

In summary, this paper shows that enhancing the sensitivity and specificity through chromatographic selectivity and resolution enable us to accurately determine low concentrations of ttMA in urine. This proposed HPLC-UV method has been proven to be robust, sensitive, and reliable.

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