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Determination of mercapturic acids in urine by solid-phase extraction followed by liquid chromatography–electrospray ionization mass spectrometry

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A novel method for the determination of five kinds of mercapturic acids, found in urine as metabolites of alkylbenzenes, based on liquid chromatography–electrospray ionization mass spectrometry is described. A solid-phase extraction procedure was used for the extraction of the mercapturic acids from urine and the separation was performed on a reversed-phase C_{30} column. The detection limits were in the range 2.4–3.2 ng ml^{-1}.

Volatile organic compounds (VOCs) have caused serious environmental problems in recent decades.[1] The main constituents of indoor VOC air pollutants are alkylbenzenes, such as toluene, xylenes and trimethylbenzenes. Alkylbenzenes are widely used as industrial solvents, and it is well known that these VOCs exist in the indoor environment at parts per billion levels.[2] Therefore, it is important to explore the influence of alkylbenzenes on vital functions and the behavior of these compounds *in vivo*.

van Doorn *et al*. have demonstrated the excretion of not only phenolic compounds and hippuric acids but also mercapturic acids in the urine of rats treated with toluene and isomeric xylenes.[3] The mercapturic acids were formed by reactions of toluene or xylenes with glutathione. Therefore, there is the possibility that the formation of mercapturic acids after exposure to alkylbenzenes leads to the depletion of glutathione. In fact, van Doorn *et al*. reported that the administration of toluene and xylenes to rats caused a decrease in the liver glutathione concentration. The depletion of glutathione may cause adverse effects, such as an increase in lipid peroxidation, elevated levels of unscheduled DNA repair synthesis and degeneration of the gastrointestinal epithelial cells.[4,5]

We have previously reported the synthesis of *o*-, *m*- and *p*-methylbenzylmercapturic acids (*o*-, *m*- and *p*-MBM) for use as standards for metabolites of xylene and we determined these metabolites in the urine of rats treated with xylenes by HPLC.[6] The results showed that the mercapturic acid pathway operates marginally as a metabolic route of *m*- or *p*-xylene when compared with *o*-xylene. Next, we synthesized 2,3- and 2,6-dimethylbenzylmercapturic acid (2,3- and 2,6-DMB) and determined them in the urine of rats treated with 1,2,3-trimethylbenzene. We found that the excreted mercapturic acid was only 2,3-DMB.[7]

In the above work, we determined the mercapturic acids in the urine of rats administered alkylbenzenes by HPLC with UV absorption detection (LC-UV). However, the sensitivity and selectivity of LC-UV are very low, and it is difficult to detect and separate minute amounts of these compounds from urinary contaminants. The development of a sensitive and selective method for the determination of mercapturic acids in urine is required for environmental, toxicological and clinical studies in order to clarify the metabolism of alkylbenzenes.

Liquid chromatography–electrospray ionization mass spectrometry (LC-ESIMS) has been shown to be a powerful technique for the determination of non-volatile compounds, which are difficult to detect by GC-MS.[8] Therefore, the technique has often been used for the determination of metabolic compounds in urine, because such compounds are mostly non-volatile. Furthermore, by using LC-ESIMS, there is the possibility of simplifying the clean-up procedure for the analyses of samples containing various contaminants, such as urine or serum samples, because mass spectrometry generally shows high selectivity in comparison with other detection methods coupled with LC.[9]

In this paper, a sensitive and selective method for the determination of mercapturic acid in urine based on LC-ESIMS following a solid-phase extraction is described. The targets of this method were five kinds of mercapturic acids, namely-benzylmercapturic acid (BMA), *o*-MBM and 2,3-, 2,6- and 3,4-DMB, which are metabolites of toluene, *o*-xylene and trimethylbenzenes (Scheme 1). By applying LC-ESIMS to the detection of the mercapturic acids, the instrumental detection limits were improved about 100-fold over those of LC-UV. Furthermore, the selectivity of the analysis for the mercapturic acids in urine samples was sufficient to overcome interfering contaminants in urine.

Experimental

Materials

All solvents were of HPLC grade and other chemicals were of analytical-reagent grade. The mercapturic acids were synthesized from the corresponding bromoalkylbenzenes with *N*-acetyl-L-cysteine according to the method described previously.[7] Ammonium acetate was obtained from Wako (Osaka, Japan). Ultra-pure water was produced with a Milli-Q system (Millipore, Bedford, MA, USA). A urine sample was obtained from a healthy volunteer at our institute.

HPLC conditions

Liquid chromatography was carried out on a Hewlett-Packard HP 1100 Series HPLC apparatus (Yokogawa Analytical Systems, Tokyo, Japan). A Nomura Chemical (Aichi, Japan) Develosil C30-OG column (5 μm particle size, 150 × 4.6 mm id) was used for the LC separation of the mercapturic acids. HPLC separation was carried out at 40 °C using a gradient

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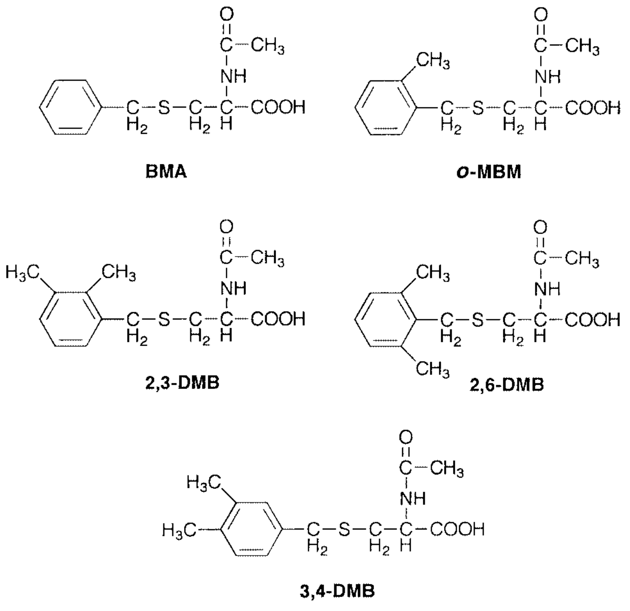
composed of mobile phase A (20 mM ammonium acetate solution adjusted to pH 4 by the addition of acetic acid) and mobile phase B (acetonitrile). The gradient was as follows: 0–20 min, a linear increase from 5 to 50% B; and 20–30 min, hold at 50% B. The mobile phase flow rate was 0.5 ml min^{-1}.

ESIMS

ESIMS was performed using a Hewlett-Packard HP-1100MSD system (Yokogawa Analytical Systems). The working conditions for ESI were as follows: the drying nitrogen gas temperature was set at 340 °C and the gas was introduced into the capillary region at a flow rate 12 l min^{-1}; the capillary was held at a potential of –3500 V relative to the counter electrode for the negative-ion mode; the fragmenter voltage was fixed at 60 V; and the injection volume of the sample solutions in the ESIMS system was 5 µl. When working in the selected ion monitoring (SIM) mode, the *m*/*z* 252, 266 and 280 ions, which were assigned as the [M − H]$^-$ ions of BMA, *o*-MBM and 2,3-, 2,6- and 3,4-DMB, respectively, were monitored.



Scheme 1

Clean-up procedure

Each sample was centrifuged at 1500 rpm for 10 min. The supernatant was subjected to a clean-up procedure by solid-phase extraction. A 1 ml volume of the supernatant was pre-mixed with 0.5 ml of 0 or 100 ng ml^{-1} mercapturic acid standards and 3.5 ml of distilled water. The urine samples were then extracted by solid-phase extraction using a Sep-Pak Plus tC$_{18}$ cartridge. The Sep-Pak Plus cartridge was preconditioned with 2.5 ml methanol and 5 ml mobile phase solution A. The sample solution (5 ml) was loaded on to the solid-phase extraction cartridge. The cartridge was rinsed with 10 ml of solution A and dried by centrifuging for 10 min. The cartridge was then eluted with 4 ml of acetonitrile into a glass tube. The eluate was dried by nitrogen purging and diluted to 1 ml. A 20 µl aliquot was injected into the LC-ESIMS system.

Results and discussion

Detection of mercapturic acids

In the case of the mass spectral investigation by ESIMS through flow injection of the five kinds of mercapturic acid solutions (2

Table 1 Instrumental detection limits and retention times for the mercapturic acids obtained using LC-ESIMS

Analyte	Monitored ion (*m*/*z*)	Retention time/min	Instrumental detection limit[a]/ ng ml
BMA	252	16.29	1.2
o-MBM	266	18.49	1.6
2,3-DMB	280	19.81	1.6
2,6-DMB	280	19.76	1.5
3,4-DMB	280	20.24	2.9

[a] Calculated as three times the baseline noise.

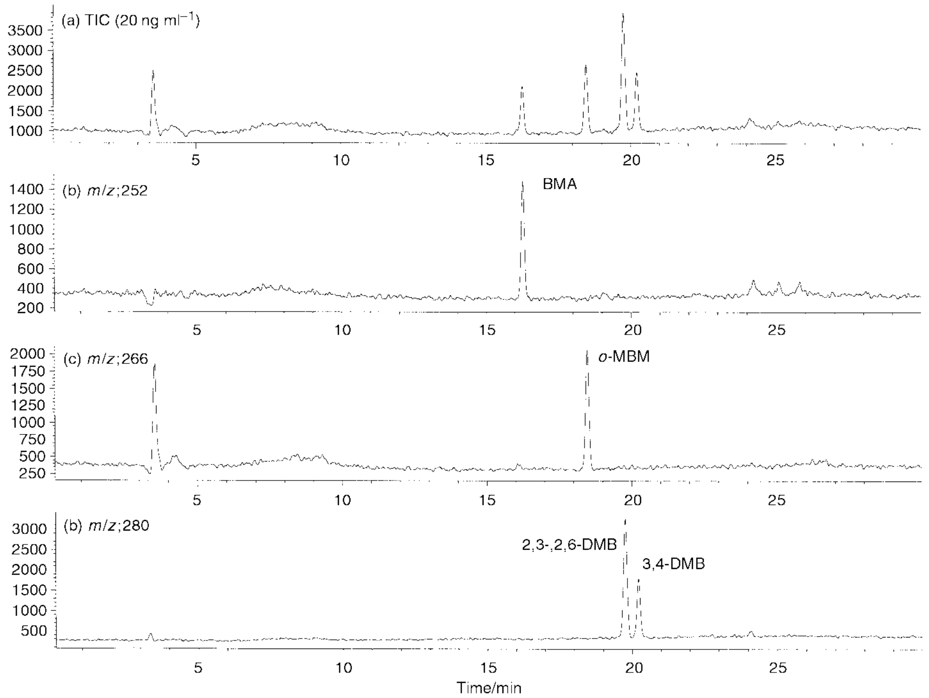


Fig. 1 LC-ESIMS of the mercapturic acids with injection of 20 µl of a standard solution (20 ng ml^{-1}) using SIM of the *m*/*z* 252, 266 and 280 ions.

μg ml^{-1}), the m/z 252, 266 and 280 ions, which were assigned as the [M − H]$^-$ ions, were observed as the main peaks.

In order to establish the optimum fragmenter voltage for the detection of the mercapturic acids, the signals of the m/z 280 ion of 2,3-DMB *vs.* fragmenter voltage were investigated. The [M − H]$^-$ peak of 2,3-DMB showed a maximum at 60 V. When the fragmenter voltage was > 70 V, the m/z 151 fragment ion peak was observed. This fragment ion would form through C–S bond cleavage by collision-induced dissociation. From this result, the optimum drift voltage was determined as 60 V for the present method.

LC-ESIMS

The mercapturic acid ions were detected by ESIMS after separation of the mercapturic acids through a reversed-phased C_{30} column. Fig. 1 shows the chromatograms of the 20 ng ml^{-1} mercapturic acids standard solution using SIM of m/z 252, 266 and 280 ions. The retention times of the mercapturic acids were very reproducible; 2,3-DMB and 2,6-DMB could not be separated under the LC conditions adopted. The retention times and the instrumental detection limits are summarized in Table 1. The instrumental detection limits calculated as three times the baseline noise were 1.2–2.9 ng ml^{-1}. The values for 2,3-DMB and 2,6-DMB were obtained by the injection of individual standard solutions.

The calibration equations were obtained for the mercapturic acids using a series of standard solutions over the concentration range 1–100 ng ml^{-1}. The peaks of 2,3- and 2,6-DMB were not separated. Therefore, the calibration equation was determined as the total of 2,3- and 2,6-DMB, and calculated using the sum of the peak areas of the two DMBs. The equations and correlation coefficients (r^2) are summarized in Table 2.

Determination of mercapturic acids

A solid-phase extraction procedure was developed for the clean-up treatment. For the recovery evaluation of the mercapturic acids by this method, the solid-phase extraction procedure was carried out after the human urine sample (1 ml) had been spiked with 60 ng of the mercapturic acids. The peaks of the mercapturic acids were not observed without spiking the standard solutions of the mercapturic acids. The sample ($n = 5$) was prepared for LC-ESIMS analysis as described in the Experimental section. The chromatograms of the sample spiked with the mercapturic acids standard are shown in Fig. 2 (obtained using SIM monitoring of the m/z 252, 266 and 280 ions). The peaks of the mercapturic acids were clearly detected without interference from the peaks of contaminants in the urine. The recoveries were evaluated by determining the peak areas based on the SIM chromatograms; 2,3- and 2,6-DMB were calculated as one species. The mean recoveries were 93.2–107.2% and the detection limits of the mercapturic acids by this method, defined as three times the standard deviation of the blank, were 2.4–3.2 ng ml^{-1}, as shown in Table 2.

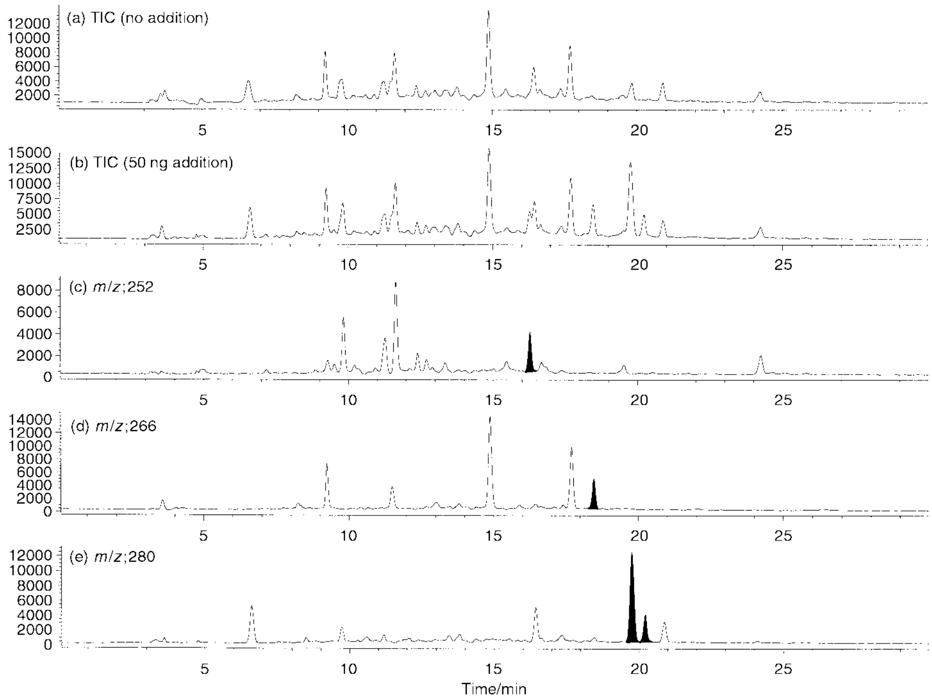


Fig. 2 LC-ESIMS of a urine sample and a spiked urine sample using SIM of the m/z 252, 266 and 280 ions.

Table 2 Calibration equations, recoveries of the spiked urine sample and detection limits of the method for the mercapturic acids obtained using LC-ESIMS

Analyte	Calibration equation[a]	Correlation coefficient (r^2)	Recovery (%)	Standard deviation (%)	Detection limit[b] of the method/ng ml^{-1}
BMA	$y = 543x - 1959$	0.998	107.2	2.1	3.2
o-MBM	$y = 772x - 2765$	0.998	105.9	2.0	3.0
2,3- and 2,6-DMB	$y = 2195x - 12861$	0.996	103.2	1.8	5.5
3,4-DMB	$y = 800x - 5325$	0.992	93.2	1.6	2.4

[a] Least-squares regression equation; y = peak area, x in ng ml^{-1}. [b] Calculated as three times the standard deviation.

Conclusions

The determination of mercapturic acid in urine samples by LC-ESIMS has, in the past, not been particularly well reported. In this study, LC-ESIMS was used for the determination of mercapturic acids in urine. The advantage of the proposed method using LC-ESIMS is that the selectivity and sensitivity are much better than those of previously published methods using LC-UV. The procedure could be useful in studies exploring the metabolism of alkylbenzenes.

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