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Identification and Quantification by LC–MS and LC–ICP MS of Arsenic Species in Urine of Rats Chronically Exposed to Dimethylarsinic Acid (DMAA)

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The potential of liquid chromatography–mass spectrometry (LC–MS) using electrospray ionization (ESI) was investigated for the identification and quantification of organoarsenic species excreted in rats urine chronically exposed to dimethylarsinic acid (DMAA). Quantification was performed by both LC–ESI-MS and LC–inductively coupled plasma mass spectrometry (ICP-MS). The detection limits of organoarsenic species in LC–ESI-MS with cation-exchange chromatography were 75–200 pg as arsenic. Although there are about ten times higher than that of LC–ICP-MS, LC–ESI-MS had a low enough detection limit to determine major metabolic arsenic species in the urine. LC–ESI-MS was applied to the identification of organoarsenic species in the urine. Major arsenic peaks in urine were identified as DMAA and trimethylarsine oxide using agreement of the spectra and retention times. Three unidentified arsenic peaks were found in the urine; one of these was determined to be tetramethylarsonium ion by agreement of both the spectrum and the retention time. LC–ESI-MS and LC-ICP-MS were also used to quantify organoarsenic in urine: good agreement between LC–ESI-MS and LC–ICP-MS was obtained. Copyright © 1999 John Wiley & Sons, Ltd.

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INTRODUCTION

The importance of identification and quantification of the chemical species present in biological and environmental samples is well recognized. One of the reasons is that different species of the same element may have very different chemical and toxicological properties, because their properties depend on their chemical form. A typical example is arsenic species. Inorganic arsenic species have long been known to be carcinogenic to humans, giving rise to both skin and lung cancer.[1] Most mammals, including humans, convert inorganic arsenic species to monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA) by methylation.[2–4] Furthermore, it has been reported that DMAA is further methylated to trimethylarsine oxide (TMAO),[5,6] which has been considered the ultimate methylated metabolite in mammals.

In general, the acute toxicity of organoarsenic compounds is much lower than that of inorganic arsenic. Methylation can be considered as a mechanism of detoxification of arsenic, since it renders arsenic less reactive to tissue and therefore facilitates its elimination from the body.[7] Recently, the potential of DMAA to the promotion of cancer in the urinary bladder, kidney, liver and thyroid gland was revealed by using a multiorgan carcinogenesis bioassay in rats.[8,9] In these studies, six-

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month exposure to 10 mg l^{-1} DMAA promoted the induction of tumors in carcinogen-treated rats. These results suggested that DMAA has the potential to promote rat carcinogenesis.

We reported previously on the speciation of arsenic species in urine using liquid chromatography (LC) with inductively coupled plasma mass spectrometry (ICP-MS).[10,11] LC–ICP-MS was applied to the determination of arsenic species in the urine of rats chronically exposed to DMAA in toxicological and carcinogenic studies:[8,9,12] a few unidentified arsenic species were found in the company of the major urinary metabolites MMAA, DMAA and TMAO.[11,12] However, LC–ICP-MS cannot give any information on chemical structure because of atomization at the ICP. Therefore, identification by LC–ICP-MS always depends only on agreement of the retention time with that of the reference compound. Consequently, the chemical structures of these unidentified arsenic species have not been determined.

In studies on the elucidation of arsenic metabolism, analytical methods which can characterize methylated arsenic species should be used. Mass spectrometry is a good analytical tool for the identification of organic and organometallic compounds. The identification of organoarsenic species has been performed by electron impact mass spectrometry (EI-MS)[13,14] and fast atom bombardment mass spectrometry (FAB-MS).[15] For the identification of chemical species in biological samples, however, combination of the identification method with some separation technique is indispensable. Arsenic species which can generate the corresponding hydride by reduction may be identified by hydride generation–gas chromatography–mass spectrometry (HG–GC–MS).[16,17] This technique has been applied to the analysis of arsenic species in marine organisms[16] and hamster urine.[17] On the other hand, liquid chromatography–mass spectrometry (LC–MS) is the best suited for the identification and quantification of nonvolatile and/or thermally unstable compounds. LC–MS with a source of electrospray ionization (ESI) has been applied to the measurement of several organometallic compounds, including arsenobetaine (AB).[18]

The purpose of the present study was to evaluate the availability of LC–ESI-MS for the determination of organoarsenic species in urine. It was also applied to the identification and quantification of organoarsenic species excreted in the urine of rats chronically exposed to DMAA; identification was by LC–ESI-MS, and quantification was performed by both LC–ESI MS and LC–ICP-MS.

EXPERIMENTAL

Reagents

The organoarsenic species MMAA, DMAA, TMAO, tetramethylarsonium (TeMA) iodide and AB, were purchased from Tri Chemical Laboratories Inc. (Yamanashi, Japan). Analytical-grade sodium arsenite [As(III)] was purchased from Wako Pure Chemical Industries (Osaka, Japan). Nitric acid, ammonium nitrate, formic acid, ammonium formate and ethanol (each of analytical grade) were also purchased from Wako Pure Chemical Industries. Deionized water was obtained from a Milli-Q system (Nihon Millipore, Tokyo, Japan). Solid-phase extraction cartridges of Toyopak ODS-M packed with octadecylsilanized silica gel was purchased from Tosoh Co. (Tokyo, Japan).

Instrumentation and analytical conditions

The LC–MS equipment was a Model HP1100 series HPLC with MS (Hewlett-Packard, USA). Electrospray ionization (ESI) was selected as the ionization system. An LC–ICP-MS, Model HP4500 ICP–MS (Hewlett-Packard) was used for arsenic-specific detection. According to the method of our previous paper,[11] the organoarsenic species in urine were mainly separated by cation-exchange chromatography. A Shodex RSpak NN-414 (Showa Denko, Tokyo, Japan) was chosen as the separation column. The NN-414 was 150 mm × 4.6 mm i.d. and was packed with a hydrophilic-resin-based cation-exchange resin (cation-exchange capacity, 0.1 meq g^{-1}). Although the cation-exchange chromatography was performed under conditions comparable with those of the previous paper,[11] NH_4NO_3 was added to the mobile phase in order to shorten the analytical time. HNO_3 (8 mM)/NH_4NO_3 (5 mM) was used as the mobile phase. For chromatographic identification by different separation modes, Gelpak GL-IC-A15S (Hitachi Chemical, Tokyo, Japan) was also used. The IC-A15S column was 175 mm × 3.0 mm i.d. and was packed with a resin-based anion-exchange resin (anion-exchange capacity, 0.05 meq g^{-1}). Ammonium formate (6 mM) buffer adjusted to pH 5.5 was used as the mobile phase. The liquid chromatograph was operated under the following conditions: mobile phase flow rate, 0.4 ml min^{-1}; column temperature, 40 °C; injection volume, 50 and 20 μl for LC–ESI-MS and LC–ICP-MS, respectively. The operating conditions for ICP–MS were set in accordance with

those reported by our previous papers.[10,11] The detection mass of ICP–MS was set to $m/z = 75$ (for $^{75}As^{+}$) and $m/z = 35$ (for $^{35}Cl^{+}$), respectively, and dwell time was 0.5 and 0.05 s, respectively.

Animals and pretreatment of urine

Adult male F344/DuCrj rats were obtained from Charles River Japan (Hino, Japan). Five rats were housed in each box cage and provided with a standard diet (CE2, Clea Japan, Tokyo, Japan) and water *ad libitum.* Rats (10 animals) were given 100 mg l^{-1} DMAA in drinking water. To control rats (10 rats), untreated water was given. Urine was collected by forced urination after 12 weeks of DMAA administration. The urine samples were centrifuged to remove particulate materials and stored at −20 °C until analysis. The urine sample for analysis was diluted 10- or 50-fold with deionized water, and was then passed through an ODS cartridge to eliminate hydrophobic compounds. The eluate (50 and 20 µl respectively) was injected into the LC–ESI-MS and LC–ICP-MS systems for analysis.

RESULTS AND DISCUSSION

Optimization of ionization conditions for organoarsenic species during LC–ES MS

Ionization efficiency during LC–ESI MS depends on the following conditions: the activation voltage (voltage of fragmentor); the flow rate of the drying gas; and the pressure of the nebulizer. Particularly, the fragmentor voltage controls the production efficiency of molecular ions. The effect of the fragmentor voltage was examined by a flow injection method. An acidic solution (8 mM HNO_3/5 mM NH_4NO_3) was used as the carrier solution for detection in the positive mode. Other conditions were as follows: flow rate, 0.4 ml min^{-1}; polarity, positive; drying gas, N_2 (13 l min^{-1}, 350 °C); nebulizer, N_2 (60 psi ≃ 414 kPa); and detection mode, scan. Pseudo-molecular ions of $[M+1]^+$ and $[M]^+$ were monitored for anionic and cationic species, respectively. Figure 1 shows the relationship of the fragmentor voltage and the abundance of the pseudomolecular ions. The abundance of all organoarsenic species increased as the fragmentor voltage increased, and maximum abundance was obtained in the range of about 80–100 V. The abundance of the anionic species, particularly MMAA, was lower than that of the cationic species. This result suggests that the relatively higher anionicity of MMAA might hinder the production of positive ions by ESI. On the other hand, the drying gas and nebulizer conditions were not so effective on the production efficiency of the molecular ions.

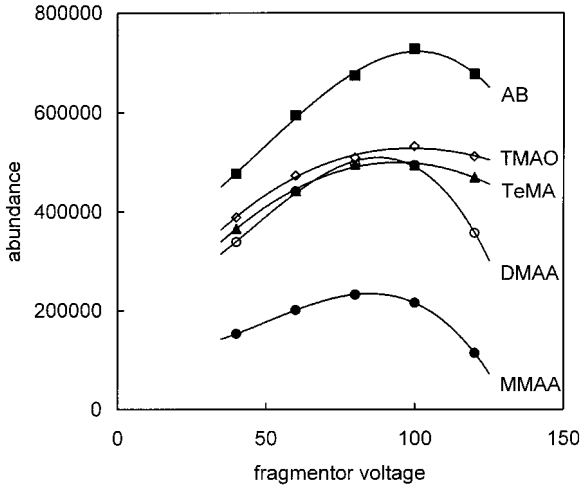

Figure 1 Effect of the fragmentor voltage on the abundance of organoarsenic species. Conditions: Carrier, 8 mM HNO_3 / 5 mM NH_4NO_3, 0.4 ml min^{-1} drying gas, N_2 (13 l min^{-1}, 350°C); nebulizer, N_2 (60 psi ≃ 414 kPa); polarity, positive, mode, scan; samples, 10 mg As l^{-1} each, 10 µl injections.

According to these results, the spectra of the organoarsenic species which were separated by cation-exchange chromatography were measured. Figure 2 shows the spectra of the organoarsenic species. Pseudo-molecular ions $[M+1]^+$ in relatively high abundance were obtained for the anionic and non-ionic species MMAA, DMAA and TMAO. For MMAA and DMAA, pseudo-molecular ions based on the dimer and trimer were also observed. On the other hand, $[M]^+$ ions were obtained for the cationic species TeMA and AB. Similar spectra were also obtained when anion-exchange chromatography was used.

Fragment ions were not observed, except for molecular ions based on the dehydration of MMAA and DMAA. In the EI-MS spectra, molecular ions due to fragmentation have been observed in relatively high abundance.[16] This suggests that ESI is not so radical ionization as compared with EI. Furthermore, the fragmentation of organoarsenic species might also depend on the degradation stability based on the strong As–C bonds. Consequently, the pseudo-molecular ions should be

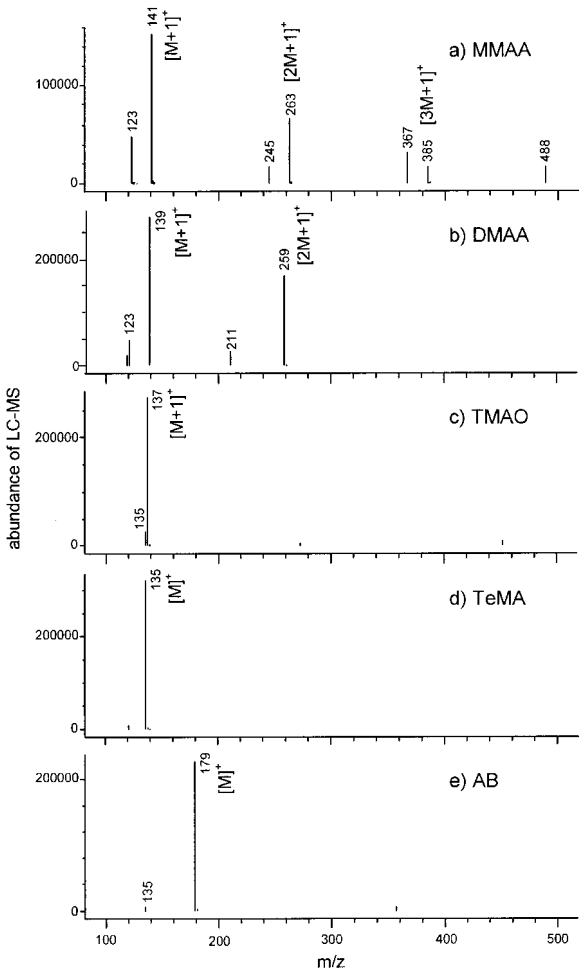

Figure 2 Spectra of organoarsenic species from LC–ESI-MS with cation-exchange chromatography. Conditions: column, Shodex RSpak NN-414; mobile phase, 8 mM HNO_3 / 5 mM NH_4NO_3, 0.4 ml min^{-1}; temperature, 40 °C; samples, 1.0 mg As l^{-1} each, 50 µl injections. Other conditions are those given in Fig. 1.

chosen as the detection mass for methylated arsenic species. On the other hand, the molecular ion with $m/z = 135$ $[(CH_3)_4As]^+$ was observed in the spectrum of AB. The fragment ion might be produced by decarboxylation or degradation of a C–C bond.

Detection limits of organoarsenic species

The chromatograms of arsenic species using LC–ESI-MS and LC–ICP-MS are shown in Fig. 3. The concentrations of arsenic species were 0.1 mg As l^{-1} each, and the injection volume was 20 and 50 µl for LC–ICP-MS and LC–MS, respectively. The selective ion monitoring (SIM) mode was used. According to knowledge on fragmentation, $[M+1]^+$ and/or $[M]^+$ were chosen as the detection masses. Organoarsenic species were completely separated by LC–ESI-MS without any interferences. In particular, TeMA was completely separated by LC–ESI-MS without interference from TMAO, in contrast to LC–ICP-MS.

The system detection limits ($S/N = 3$) of the organoarsenic species were calculated from 0.01 mg As l^{-1} standard solution. Table 1 shows a comparison of the system detection limits for LC–ESI-MS and LC–ICP-MS: for LC–ESI-MS with cation-exchange chromatography they were 75–113pg as As, except for MMAA. Even though these are 7–14 times higher than that of LC–ICP-MS, LC–ESI-MS had a sufficiently low detection limit to determine the major metabolic organoarsenic species in urine of rats exposed to large amounts of DMAA.

Identification and quantification of organoarsenic species in urine of rats chronically exposed to DMAA

LC–ESI-MS was applied to the identification and quantification of metabolic organoarsenic species in the urine of rats chronically exposed to DMAA. The analytical sample was 50-fold diluted urine. The same urine sample was also analyzed by LC–ICP-MS. Further, the same urine, diluted 10-fold, was also analyzed by LC–ESI-MS in scan mode to produce the spectra.

In our previous papers,[11] seven arsenic species were detected by LC–ICP-MS using cation-exchange chromatography in the urine of rats exposed to DMAA. Five peaks were identified as As(III), MMAA, DMAA, TMAO and AB, and there were two arsenic peaks that were unidentified on the bases of their retention times, and that were eluted immediately before DMAA and after TMAO, respectively. They were characterized as anionic and cationic species, respectively, due to their retention behavior.

The chromatograms of the urine obtained by LC–ICP-MS with cation-and anion-exchange chromatography are shown in Fig. 4. The elution order of arsenic species during cation-exchange chromatography was identical to that in the previous paper, except for the retention of an unidentified arsenic peak characterized as a cationic species in the previous paper. The unidentified peak in this experiment was eluted immediately before TMAO.

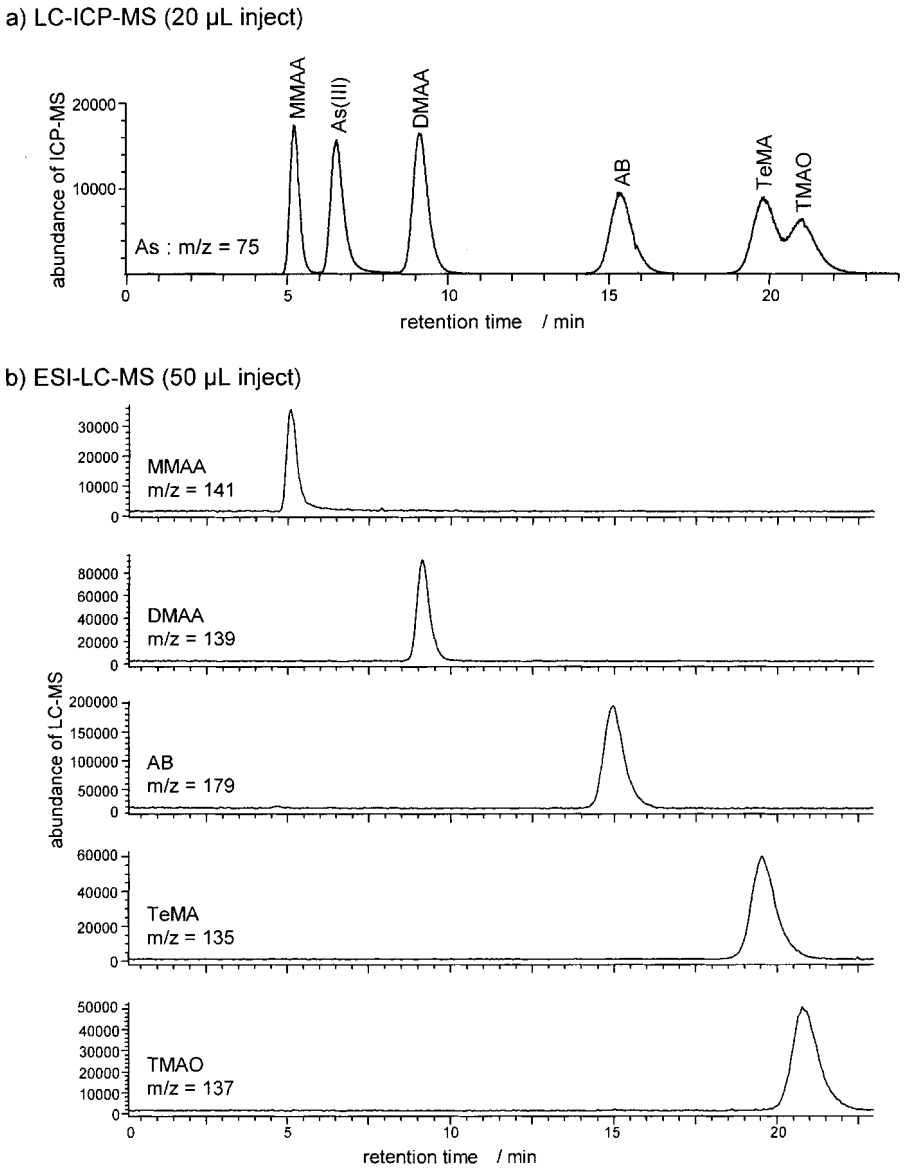

Figure 3 SIM chromatograms of organoarsenic standards on (a) LC–ICP-MS and (b) LC–ESI-MS. Conditions were those given in Fig. 2, except for mode and sample: mode, SIM; sample, 0.1 mg As l^{-1} each; 20 µl (LC–ICP-MS) and 50 µl (LC–ESI-MS) injections.

In the chromatogram, DMAA was the most abundant arsenic species in the urine. TMAO was present in a relatively higher proportion in arsenic species detected in the urine. Although a peak was detected at the retention time of As(III) on cation-exchange chromatography, no peaks was observed at the retention time of As(III) on anion-exchange chromatography. On the basis of the comparison of chromatograms using two separation modes, it was established that the peak was distinct from As(III), and also that the peak contained two unidentified arsenic peaks (peaks A and B). On the other hand, the retention time of the unidentified peak which was eluted immediately before TMAO agreed with that of TeMA.

The SIM chromatograms of the urine obtained by LC–ESI-MS are shown in Fig. 5. Major arsenic peaks, determined as DMAA and TMAO by LC–

Table 1 Comparison of the system detection limits of organoarsenic species for LC–ESI MS and LC–ICP MS

Method	Separation mode	Detection limits (pg As)				
		MMAA	DMAA	TMAO	TeMA	AB
LC–ESI MS	Cation	200	78	105	75	113
	Anion	153	140	68	33	70
LC–ICP MS	Cation	5.2	5.4	14.2	9.8	9.8
	Anion	52.8	10.8	10.4	9.2	9.6

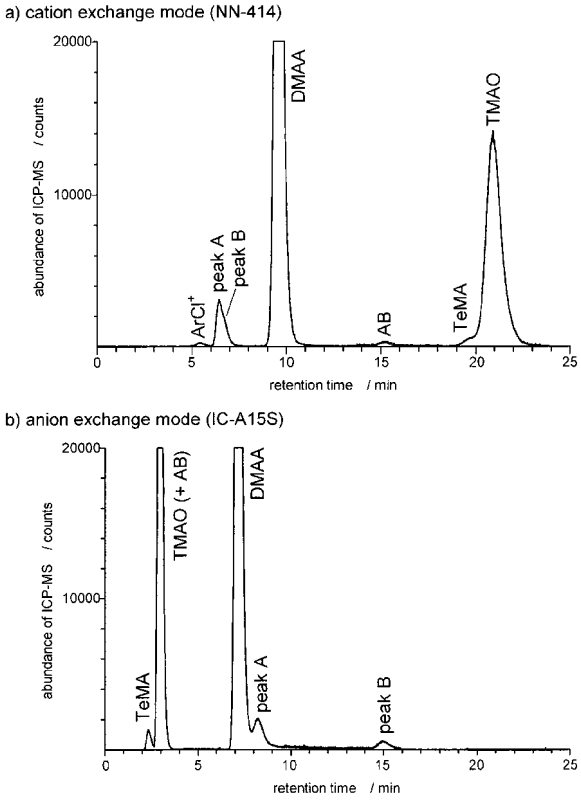

Figure 4 Chromatograms of rats urine exposed to DMAA on LC–ICP-MS from (a) cation-exchange chromatography and (b) anion-exchange chromatography. Conditions: column, Shodex RSpak NN-414 and Gelpak GL-IC-A15S; mobile phase, 8 mM HNO_3 / 5 mM NH_4NO_3 and 6 mM ammonium formate, pH 5.5, 0.4 ml min^{-1} each; temperature, 40 °C; sample, 50-fold diluted urine, 20 μl injections.

ICP-MS, were eluted at the retention times of DMAA and TMAO, respectively. Their spectra, which were obtained from the measurement of ten-fold diluted urine in scan mode, also agreed with the spectra of standards. According to the agreement of the spectra and their retention times, they were identified as DMAA and TMAO, respectively. In a similar manner to that described above, AB was also identified. It is generally thought that AB is not produced in mammals.[19] In the urine of control rats, AB was also detected at the same retention time. Its concentration determined by LC–ICP-MS agreed approximately with that in the urine of rats exposed to DMAA. Therefore, it is concluded that AB came from the feed. On the other hand, the retention time of the unidentified peak which was eluted immediately before TMAO on the mass chromatogram at $m/z = 135$ also agreed with that of TeMA. The spectra of TeMA and the unidentified peak at 19.4 min are shown in Fig. 6. The spectrum of the unidentified peak was not so clear, but $[M]^+$ ($m/z = 135$) of TeMA was included in the spectrum. These results suggested that this unidentified arsenic peak was TeMA. The retention times of the other peaks in each SIM chromatograms (Fig. 5) did not agree with that of the standard arsenic compounds. These seemed to arise from organic compounds in the rat urine. It was confirmed by the LC–ICP-MS analysis that the other peaks were free of arsenic atoms.

It is well known that TeMA is not produced in mammals. However, it was established that TeMA was not derived from the feed, because no peaks in the urine of the control rats were detected at the retention time of TeMA. Shiomi *et al.*[15] reported the presence of TeMA in small amounts in some marine animals. They also found that TeMA exhibits strong acute toxicity in mice. The finding of a further-methylated metabolite (TeMA) is very intriguing, because methylation has been considered to be a detoxification mechanism in mammals.

In this experiment using LC–ESI-MS, information on the other unidentified arsenic peaks (A and B) was not obtained because of the interference of organic matrices. However, it was assumed that they were anionic species, due to their retention behaviour on both anion- and cation-exchange

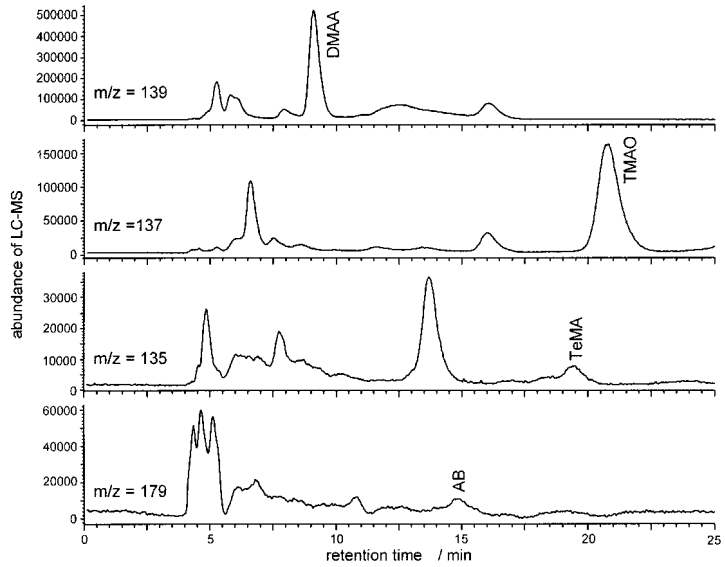

Figure 5 SIM chromatograms of urine of rats exposed to DMAA from LC–ESI-MS with cation-exchange chromatography. Conditions were those given in Fig. 2, except for mode and sample: mode, SIM; sample, 50-fold diluted urine, 50 μl injection.

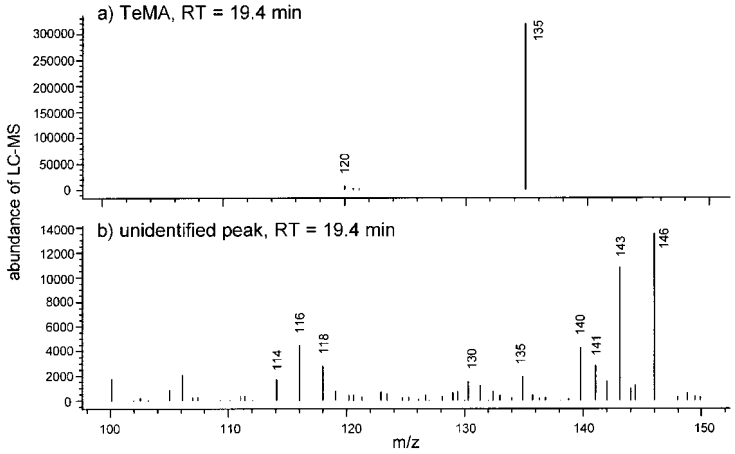

Figure 6 Spectra of TeMA and unidentified peak (retention time = 19.4 min) in urine. Analytical conditions were those given in Fig. 2. Sample, 10-fold diluted urine, 50 μl injections.

Table 2 Concentrations of organoarsenic species in the urine of rats exposed to DMAA determined by LC–ESI-MS and LC–ICP-MS

Method	Separation mode	Concentration of arsenic species in urine (mg As l^{-1})						
		MMAA	Peak A	Peak B	DMAA	AB	TeMA	TMAO
LC–ICP-MS	Cation	0.13	1.83[a]		32.8	0.20	0.37	15.7
	Anion	ND[b]	1.75	0.43	31.6	ND	0.47	16.2
LC–ESI-MS	Cation	ND	—	—	32.3	0.46	2.08	15.6
	Anion	ND	—	—	32.0	ND	1.81	14.4

[a] Shown as the sum of two peaks because of incomplete separation.
[b] ND, not detected.

chromatography on LC–ICP-MS. Furthermore, their retention times in anion-exchange chromatography were decreased by adding ethanol (10%) to the mobile phase, and they were eluted just before DMAA. This result suggested that these unidentified arsenic species might have hydrophobic groups. To elucidate the structures of the unidentified species, further experiments, such as purification, will be needed.

Finally, the quantification of organoarsenic species in urine of rats exposed to DMAA was performed by LC–ESI-MS and LC–ICP-MS. A 50-fold diluted portion of urine was used as the analytical sample. Both cation- and anion-exchange chromatography were used as separation modes. $[M+1]^+$ and/or $[M]^+$ were used as the detection masses with the external calibration method for LC–ESI-MS. The detection mass for LC–ICP-MS was set at $m/z = 75$. The results are shown in Table 2. MMAA on anion-exchange chromatography and LC–ESI-MS could not be detected because of its lower concentration, and AB on anion-exchange chromatography could not be determined because of the overlap of TMAO. Good agreement was obtained between the concentrations determined by LC–ESI-MS and LC–ICP-MS, except for TeMA. It seems that the disagreement over TeMA was caused by incomplete separation from TMAO during LC–ICP-MS. Therefore, the concentration of TeMA determined by LC–ESI-MS might be more reliable than that determined by LC–ICP-MS.

CONCLUSIONS

LC–ESI-MS is an excellent analytical tool for the identification and quantification of organoarsenic species in urine. The detection limits are about 10 times higher than those of LC–ICP-MS. However, simultaneous use of LC–ICP-MS and LC–ESI-MS also has sufficient capability to quantify arsenic species. For there identification, however, higher purification and preparation were needed in order to overcome the interferences of organic matrices.

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