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Speciation of arsenic compounds by using ion-pair chromatography with atomic spectrometry and mass spectrometry detection

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Abstract

Chromatography separation of arsenic compounds is important for chemical speciation studies; yet a complete separation of several biologically and environmentally important arsenic compounds has been difficult to achieve on a single column. We report here baseline resolution for arsenate, arsenite, monomethylarsonic acid, dimethylarsinic acid, arsenobetaine, arsenocholine, and tetramethylarsonium ion, by using mixed ion-pair reagents containing 10 m*M* hexanesulfonate and 1 m*M* tetraethylammonium hydroxide, as mobile phase. The complete separation of these anionic, cationic and neutral arsenic species on a reversed-phase C_{18} column took only 12 min. Inductively coupled plasma mass spectrometry, hydride generation atomic absorption spectrometry and atomic fluorescence spectrometry systems were used for element-specific detection. The speciation technique was successfully applied to studies of urinary excretion of arsenic compounds following one-time ingestion of shrimp. Arsenobetaine ingested from the consumption of shrimp was excreted into urine in its original form; and approximately 70% of the total arsenobetaine ingested was excreted into urine within 37 h after ingestion.

Keywords: Ion-pairing reagents; Detection, LC; Arsenic compounds

1. Introduction

Arsenic has been known as a poison for centuries, because of the toxic properties of some of its compounds. The use of arseneous oxide, a poisonous and tasteless powder as a favored homicidal agent during the Middle Ages provided early evidence of its acute toxicity. Lewisite, a highly toxic organoarsenic compound was used as a weapon (war gas) during the First World War. The International Agency for Research on Cancer, the US Environmental Protection Agency and the National Toxicology Program have classified arsenic as a human carcinogen [1,2]. The largest epidemiological study on arsenic, where a Taiwanese population of 40 421 was divided into three groups based on the arsenic content (presumably arsenate) of their drinking water, has shown a dose-response relationship between exposure to arsenic and the frequency of dermal lesions, blackfoot disease (a peripheral vascular disorder) and skin cancers [3].

However, the toxicity of arsenic varies dramatically with different chemical forms of arsenic [4–6]; the median lethal dose values (LD_{50}) in rats for some arsenic compounds being (in mg/kg): potassium arsenite 14, calcium arsenate 20, monomethylarsonic acid 700–1800, dimethylarsinic acid 700–2600, arsenobetaine >10 000, and arsenocholine, 6500. There-

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fore, total arsenic concentration is not an appropriate measure for assessing toxicity, environmental impact, and effect of occupational exposure [7–11]. Chemical speciation of arsenic compounds (i.e., identification and quantification of individual chemical forms of arsenic) is essential to a reliable assessment of health risk involving arsenic exposure.

Arsenite [As(III)], arsenate [As(V)], monomethylarsonic acid (MMAA), and dimethylarsinic acid (DMAA) are widely present in the natural waters [8,9,12–15]. Arsenobetaine [$(CH_3)_3As^+CH_2COO^-$], arsenocholine [$(CH_3)_3As^+CH_2CH_2OH$], the tetramethylarsonium ion [$(CH_3)_4As^+$], and arsenosugars are present in marine organisms [8,9,15]. A complete speciation of these arsenic compounds is challenging particularly when trace amounts of arsenic compounds are present in complex environmental and biological matrices.

The most commonly used speciation techniques often involve a combination of chromatographic separation with spectrometric detection. Both gas and liquid chromatography have been popular as separation techniques [16–21]. Some recent studies also involve the use of supercritical fluid chromatography [21] and capillary electrophoresis [22]. The coupling of chromatography with various sensitive detectors that are used in atomic spectrometry has lead to significant improvements in performing chemical speciation studies. Extensive discussions on these chromatography–spectrometry techniques can be found in a number of recent reviews [16–21].

Three major HPLC systems, ion-exchange, ion-pair, and gel permeation chromatography, have been used for the separation of arsenic compounds. At neutral pH, arsenate ($pK_{a_1}=2.3$), MMAA ($pK_{a_1}=3.6$), DMAA ($pK_a=6.2$), and arsenosugars are present as anions; arsenocholine [$(CH_3)_3As^+CH_2CH_2OH$] and the tetramethylarsonium ion [$(CH_3)_4As^+$] as cations; arsenobetaine as a zwitterion [$(CH_3)_3As^+CH_2COO^-$]; and arsenious acid ($pK_{a_1}=9.3$) as an uncharged species. Thus both cation-exchange [23–26] and anion-exchange [23–34] chromatography have been commonly used for the separation of ionic arsenic species. Reversed-phase ion-pair HPLC has also been used [23,29,30,35–38] with appropriate counter ions, e.g., tetramethylammonium cation or heptanesulfonate anion, in the mobile phase. The counter ion forms an ion-pair with oppositely charged analyte ions and therefore additional interactions are introduced resulting in better separation. Gel permeation HPLC has been used for the separation of arsenosugars [33,34]. Although much study has dealt with HPLC separation of some arsenic species, few systems have been able to resolve all commonly encountered arsenic species. A column switching approach involving a combination of anion-exchange and reversed-phase HPLC was required to separate both anionic and cationic arsenic species in a single run [39]. This approach required a careful selection of mobile phase to be suitable for both columns.

We report here convenient approaches to rapid speciation of seven biologically and environmentally important arsenicals, including arsenite, arsenate, MMAA, DMAA, arsenobetaine, arsenocholine, and the tetramethylarsonium ion. We discuss important parameters to achieve a complete separation of these arsenicals on a single column, using ion-pair chromatography. We also demonstrate the application of the techniques to the speciation of arsenic in human urine samples.

2. Experimental

2.1. Standards and reagents

Arsenite, arsenate, MMAA, and DMAA were obtained from commercial sources (Aldrich, Milwaukee, WI, USA). Arsenobetaine, arsenocholine, and tetramethylarsonium iodide were synthesized and characterized by literature methods [40–42]. Standard solutions of these arsenicals were prepared as described previously [11,30,43]. Creatinine standard solutions were obtained from Fisher (Pittsburgh, PA, USA).

A Standard Reference Material, Toxic Metals in Freeze-Dried Urine SRM 2670, was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The freeze-dried urine was reconstituted by the addition of 20.0 ml of deionized water as recommended by NIST. The certified value for total arsenic concentration is 0.48 ± 0.10 μg/ml in two bottles containing elevated levels of toxic metals.

All HPLC eluents, including various sulfonic acids

ranging from methanesulfonic acid to octanesulfonate (Aldrich), tetraethylammonium hydroxide (Aldrich) and tetramethylammonium hydroxide (Aldrich) were prepared in distilled deionized water and filtered through a 0.45 μm membrane. The pH of these eluents was adjusted by using sodium hydroxide and nitric acid. An appropriate amount of methanol (HPLC grade, Fisher) was added prior to the pH adjustment and the filtration.

Sodium borohydride (Aldrich) solutions in 0.1 *M* sodium hydroxide (Fisher) and microwave digestion reagents containing 0.1 *M* potassium persulfate (Fisher) and 0.3 *M* sodium hydroxide were prepared fresh daily. All reagents used were of analytical grade or better.

2.2. Instrumentation

Two HPLC systems have been used in the present study. One system consists of a Waters (Milford, MA, USA) Model 510 solvent delivery pump, a Waters U6K injector and a Phenomenex (Torrance, CA, USA) reversed-phase C_{18} column (10 μBondclone C_{18}, 300 mm×3.9 mm) or a Waters reversed-phase C_{18} column (Bondapak C_{18}, 300 mm×3.9 mm). Hydride generation atomic absorption spectrometry (HGAAS) and inductively coupled plasma mass spectrometry (ICP-MS) were used for detection. The other system consists of a Gilson (Middletone, WI, USA) Model 370 pump, a Rheodyne 6-port sample injector (a sample loop volume of 20 μl), and a Phenomenex C_{18} column (10 μBondclone C_{18}, 300 mm×3.9 mm) or ODS(3) column (Phenomenex Prodigy 250 mm×4.6 mm). Hydride generation atomic fluorescence spectrometry (HGAFS) was used for detection. A guard column packed with the same material was always used preceding the analytical column.

A VG PlasmaQuad 2 Turbo Plus ICP-MS system (VG Elemental, Fisons Instrument) equipped with a SX300 quadrupole mass analyzer, a standard ICP torch (Fassel configuration) and a Meinhard concentric nebulizer was used. A mini-chiller (Coolflow CFT-25, Neslab) was used to cool the spray chamber to approximately 4°C. The sampling position and ion lens voltages were optimized with respect to signal-to-noise ratio at m/z 75 by introducing a solution containing 30 ng/ml of arsenite in 1% nitric acid. The quadrupole mass analyzer was operated in the single ion monitoring mode. The instrumental operating conditions are the same as described previously [11,30]. A PTFE tubing (20 cm×0.4 mm I.D.) with appropriate fittings was used to connect the outlet of the HPLC analytical column directly to the inlet of the ICP nebulizer. Signals at m/z 75 were monitored by using a multichannel analyzer and data were automatically transferred to and stored in the VG data system. Once a chromatographic run was complete, a chromatogram was plotted on an Epson FX-850 printer.

Atomic absorption spectrometric (AAS) measurements were performed by using a Varian (Victoria, Australia) Model AA 1275 atomic absorption spectrophotometer equipped with a standard Varian air–acetylene flame atomizer, as described previously [30,43,44]. A conventional open-ended T-shaped quartz absorption tube (11.5 cm×0.8 cm I.D.) was mounted in the air–acetylene flame of the burner. The quartz tube was aligned to allow light from both an arsenic hollow cathode lamp and a deuterium lamp to pass through it. Hydrides were introduced to the quartz tube by way of its side arm for atomization and AAS measurements. Atomic absorption at 193.7 nm with deuterium background correction was measured. A Hewlett-Packard (Boise, ID, USA) 3390A integrator with both peak area and peak height measurement capability was used to record signals from the spectrometer.

The hydride generation system coupled with atomic absorption spectrometry (HGAAS) for HPLC detection was the same as previously described [30]. Briefly, effluent from the HPLC directly met at two PTFE T-joints with continuous flows of hydrochloric acid (3 *M*, 3.4 ml/min) and sodium borohydride (0.64 *M*, 3.4 ml/min), introduced by using a peristaltic pump (Gilson Minipuls 2). Upon mixing the HPLC effluent, acid and sodium borohydride solutions, hydride generation took place. Hydride generated from the reaction was carried by a continuous flow of nitrogen carrier gas (160 ml/min) to the flame heated quartz tube for atomic absorption measurement. Alternatively, the HPLC effluent underwent microwave assisted decomposition before hydride generation took place. As demonstrated previously [30,43], a solution containing 0.1 *M* potassium persulfate and 0.3 *M* sodium hydroxide

efficiently decomposed organoarsenicals to arsenate with the aid of microwave heating, and this mixture reagent was used in the present study. The HPLC effluent (1 ml/min) and the decomposition reagent (4 ml/min) met at a T-joint. This solution mixture flowed through a PTFE decomposition coil (5 m× 0.8 mm I.D.) located in a continuously operating microwave oven (500 W, 2450 MHz, Sharp Electronics, Japan), where the decomposition took place. The solution from the microwave oven then met the continuous flows of acid and borohydride at two T-joints. Arsines produced upon the hydride generation were introduced to the flame heated quartz tube for atomic absorption measurement. The time that an analyte spent in the microwave decomposition hydride generation system was approximately 30 s. This was determined by by-passing the HPLC column and measuring the time between the injection of an arsenate standard and the appearance of the signal maximum.

A similar microwave assisted decomposition system was also used to derivatize organoarsenicals before detection by hydride generation atomic fluorescence spectrometry (HGAFS). An Excalibur 10.003 instrument (PS Analytical, Kent, UK), consisting of an atomic fluorescence detector and a hydride generator [45], was used as a HPLC detector. A Hewlett-Packard 3390A integrator was connected to the fluorescence detector to record chromatogram traces.

2.3. Arsenic speciation in urine by using HPLC–ICP-MS

The columns (10 μBondclone C_{18}, 300 mm×3.9 mm; and Bondpak C_{18}, 300 mm×3.9 mm) were equilibrated with the appropriate eluent flowing at 0.8–1 ml/min for at least 2 h before any sample injection was made. Urine samples were filtered through 0.45 μm nylon filters (SPE, Ontario, Canada) to remove any suspended particulate before they were subjected to HPLC analysis. A 20 μl volume of a sample was injected for analysis. Three replicate determinations of arsenic species in each sample were carried out. Arsenic species in the urine samples were identified by matching the retention times of the chromatographic peaks of the urine samples with those of standards spiked into the sample. Concentration of arsenic species in urine samples was obtained by calibrating against known concentration of arsenic standards.

2.4. Arsenic speciation in urine by using hydride generation–gas chromatography–atomic absorption spectrometry (HG–GC–AAS)

Urine samples were also analyzed for inorganic arsenic, MMAA, and DMAA concentrations by using HG–GC–AAS, a similar set up as reported by Braman et al. [13].

The sample, hydrochloric acid, and sodium borohydride, each 3 ml, were used for hydride generation. Arsines generated from the reaction were purged from the reaction vessel by using helium carrier gas, passed through an acetone–dry ice bath to remove moisture, and trapped in a PTFE U-tube (30 cm×0.3 cm I.D.) cooled in liquid nitrogen. After 6 min of trapping, the liquid nitrogen was removed and the U-tube was warmed by using a hot water bath (70°C). The trapped arsines were released and swept with the aid of the helium carrier gas into a PTFE GC column (50 cm×0.3 cm I.D.) packed with Porapak-PS (80–100 mesh, Waters/Millipore). The GC oven temperature was kept at 50°C for the initial 0.5 min and then linearly increased to 150°C at a rate of 30°C/min. Individual arsine species were separated on the GC column and were subsequently introduced into a quartz absorption tube heated internally with a hydrogen–air flame for AA measurement [46]. Chromatograms were recorded on a Hewlett-Packard 3390A integrator capable of both peak height and peak area measurements.

2.5. Determination of creatinine in urine samples

Creatinine in urine samples was determined by using HPLC with UV–Vis absorption spectrophotometric detection, essentially as described by Achari et al. [47]. Urine samples were diluted by 50 times with deionized water and a 10 μl volume was injected onto a C_{18} column (Bondclone C_{18}, 300× 3.9 mm, Phenomenex). The eluent was 50 m*M* sodium acetate (pH 6.5) in water–acetonitrile (98:2, v/v). The flow-rate was 1.0 ml/min. A system consisting of a Dionex (Sunnyvale, CA, USA) Gradient Pump DX300, Waters 712 WISP Auto-

sampler, and Waters 484 Tunable Absorbance Detector was used. The detector wavelength was set at 254 nm. Creatinine peak area was measured for quantitation.

3. Results and discussion

We have previously reported on the speciation of arsenite, arsenate, MMAA, DMAA, and arsenobetaine using both ion-exchange and ion-pair chromatography with (HGAAS and ICP-MS) detection [30]. The anion-exchange chromatography is simple and is convenient for studies involving arsenite, arsenate, MMAA and DMAA. However, arsenobetaine (AB), a zwitterion $[(CH_3)_3As^+ CH_2COO^-]$, co-eluted with H_3AsO_3 ($pK_{a_1}=9.3$) under commonly used conditions (e.g., phosphate buffer pH 7.5).

We have shown that an ion-pair chromatographic system using heptanesulfonate was useful for arsenic speciation [30]. In principle, the use of an ion-pairing reagent (counter ion) introduces additional solute–solvent interactions, resulting in a change in retention behavior compared to a similar system which does not contain the counter ions [48]. We achieved baseline resolution for the five arsenic compounds (arsenite, arsenate, MMAA, DMAA and arsenobetaine) [30]. Unfortunately, arsenocholine (AC) and tetramethylarsonium ion (Me_4As^+) did not elute from the column, even after 40 min. Little information is available on a comprehensive chromatography study of these seven arsenic compounds. Because arsenocholine and the tetramethylarsonium ion are important arsenic compounds present in biological systems [8,9,15], we decided to carry out further speciation studies including these compounds. Our initial emphasis was directed to the reduction of retention time for AC and Me_4As^+ using ion-pair chromatography while maintaining baseline separation of other arsenic species.

Our first approach to reducing the retention of AB, AC and Me_4As^+ was to decrease the hydrophobicity of the ion-pair reagent (counter ion) by using a sulfonate that has a shorter hydrocarbon chain. We used a number of sulfonates as the HPLC eluents and studied the retention of the arsenicals on the same column. These sulfonates have hydrocarbon chain length ranging from one carbon (methanesulfonic acid) to eight carbons (sodium octanesulfonate). We found that as the length of hydrocarbon chain attached to the sulfonate group decreases the retention times of AC and Me_4As^+ on the reversed-phase C_{18} column also decrease. Fig. 1 shows that the Me_4As^+ ion elutes at approximately 20 min when propanesulfonate was used as the ion-pair reagent in the mobile phase; it did not elute from the column in a 40 min run when heptanesulfonate was used.

It has been shown that the ion-pair reagent in an aqueous mobile phase interacts with the hydrophobic stationary phase [48–50]. Sulfonic acid is ionized under the present pH condition (3.5). It acts as an anion-exchanger to interact with the solutes at the polar end while it interacts with the reversed-phase C_{18} at its hydrophobic end. The decreased interaction between the sulfonate and the stationary phase of the C_{18} column, due to a reduction of hydrophobicity of the sulfonate from heptanesulfonate to propanesulfonate, results in the shorter retention of the solutes.

The use of methanesulfonic acid as an ion-pair reagent further reduces the retention time of the

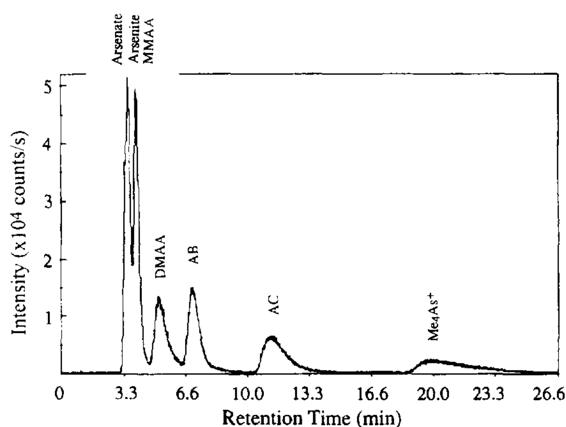

Fig. 1. Chromatogram of seven arsenicals (20 ng each) obtained on a reversed-phase C_{18} column with an aqueous eluent containing 10 m*M* propanesulfonate, 4 m*M* malonic acid, and 0.1% methanol (pH 3.5). Flow-rate was 1 ml/min, and column temperature was ambient (20–25°C). ICP-MS was used for detection. Peak labels are MMAA = monomethylarsonic acid; DMAA = dimethylarsinic acid; AB = arsenobetaine; AC = arsenocholine; Me_4As^+ = tetramethylarsonium ion.

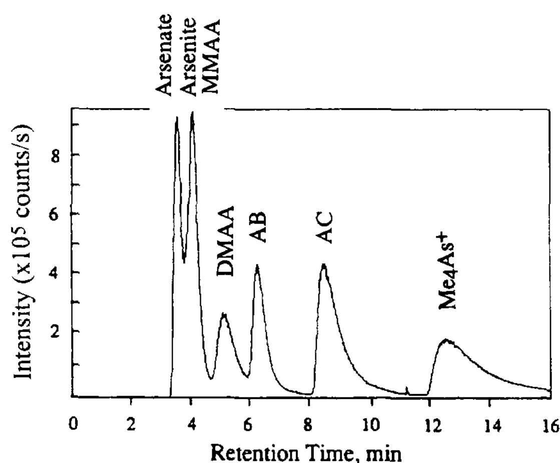

Fig. 2. Chromatogram of seven arsenicals (20 ng of arsenate, arsenite, MMAA, DMAA, and AB; 40 ng of AC and Me_4As^+) obtained on a reversed-phase C_{18} column with 10 mM methanesulfonic acid, 4 mM malonic acid, and 0.1% methanol as eluent (pH 3.5) and using ICP-MS detection. Other conditions were the same as in Fig. 1.

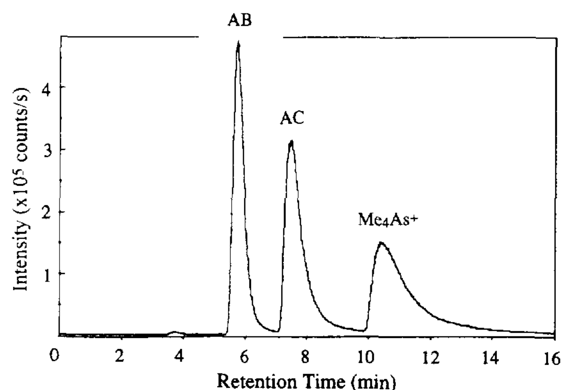

Fig. 3. Chromatogram of AB, AC, and Me_4As^+ (20 ng each) obtained on a reversed-phase C_{18} column with 10 mM methanesulfonic acid, 4 mM malonic acid, and 2% methanol as eluent (pH 3.5) and using ICP-MS detection. Other conditions were the same as in Fig. 1.

strongly retained analytes. As demonstrated in Fig. 2, the retention times of AC and Me_4As^+ in particular are further reduced to approximately 8.5 and 12.5 min, respectively, when methanesulfonic acid was used as the ion-pair reagent.

The addition of an organic modifier into the aqueous mobile phase also reduces the retention of analytes in an ion-pair chromatography system [48]. As demonstrated in Fig. 3, the retention times of AB, AC and Me_4As^+ are 5.7, 7.6, and 10.5 min, respectively, when 2% methanol was added to the mobile phase. This represents a reduction of the retention times of these same arsenicals by up to 2 min from 6.3, 8.5, and 12.5 min, respectively, when only 0.1% methanol was present in the mobile phase (Fig. 2). This is because the introduction of organic solvent into the mobile phase reduces the hydrophobic interaction between the C_{18} stationary phase and the ion-pair reagent to which analyte ions are paired. The use of a higher concentration of organic solvent is not advisable because the sample cone of the ICP-MS interface may become blocked due to deposition of carbon residue as a result of incomplete combustion of organic compounds [20,28,51]. The introduction of small amount of air into the ICP nebulizer gas can reduce this problem [28,51]. However, a shorter lifetime of the instrument sample cone has been observed [20,51] as a consequence of air introduction.

3.1.1. Step gradient

Although AB, AC, Me_4As^+ and DMAA are well separated from each other (Figs. 2 and 3) by using propanesulfonate or methansulfonic acid as eluents, arsenate, arsenite, and MMAA peaks partially overlap. In a routine analysis, two chromatographic runs of the same sample, one under the conditions stated previously [30] and the other under the conditions indicated in Fig. 3 could be performed to speciate all these seven arsenicals. Alternatively, a step gradient operation involving heptanesulfonate as an initial mobile phase followed by methanesulfonic acid as the mobile phase can be used. Fig. 4 shows an example obtained from a such step gradient chromatographic operation. A complete separation of the seven arsenicals is achieved on the single column in one run.

The elution time between AB and AC is about 13 min. Further improvement may be made by increasing the flow-rate after AB is eluted from the column. This should result in a better peak shape and a shorter retention time for AC and Me_4As^+. However, the step gradient approach is time-consuming because it took approximately 2–3 h to reequilibrate the column between chromatographic runs.

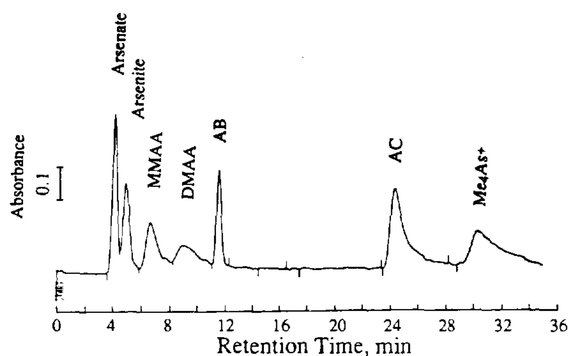

Fig. 4. Chromatogram of seven arsenicals (20 ng of arsenate, arsenite, MMAA, DMAA, and AB; 40 ng of AC and Me_4As^+) obtained by using a step gradient ion pair chromatography. Aqueous solution containing 10 m*M* heptanesulfonate (pH 3.5) was the eluent for initial 2 min. Next, another eluent containing 10 m*M* methanesulfonic acid, 4 m*M* malonic acid, and 2% methanol (pH 3.0) was used for the rest of the chromatographic run. Hydride generation with on-line microwave digestion and atomic absorption spectrometry was used for detection. Other conditions were the same as in Fig. 1.

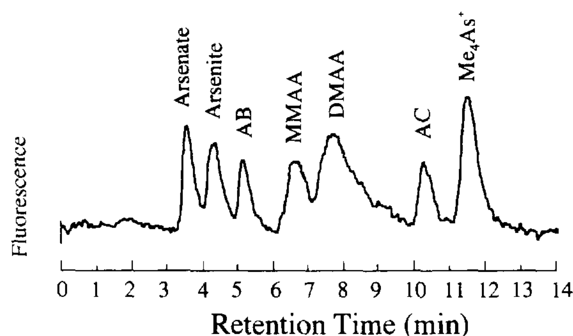

Fig. 5. Chromatogram of seven arsenicals (3 ng of arsenate and arsenite, 6 ng of MMAA, AB and AC, and 40 ng of DMAA and Me_4As^+) obtained on a reversed-phase C_{18} column with 10 m*M* hexanesulfonate, 1 m*M* $(C_2H_5)_4NOH$, and 0.5% methanol as eluent (pH 4.0) and using HGAFS detection. Flow-rate was 0.8 ml/min. Column temperature was 70°C.

3.1.2. Use of mixed ion-pair reagents

The addition of a small amount of second ion-pair reagent that has the same charge as the analytes of interest (positive charge in this case) into the mobile phase also reduces the retention of the analytes. Because of the repulsion between the ions of the same charge and also because of the competition between the introduced ions and the analyte ions for the same sites of the oppositely charged pairing ions, a shorter retention is expected.

We have carried out a series of studies using mixed ion-pair reagents containing both sulfonate and tetraethylammonium hydroxide [$(C_2H_5)_4NOH$], for the separation of arsenic species. We optimized the composition, pH and flow-rate of the mobile phase, to achieve optimum resolution of the seven target arsenic compounds. We found that an aqueous solution (pH 4.0) containing 10 m*M* hexanesulfonate, 1 m*M* tetraethylammonium hydroxide, and 0.5% methanol was an optimum mobile phase for the separation. The optimum flow-rate was 0.8 ml/min. We also observed an improved separation efficiency and a shorter analysis time using an elevated column temperature (70°C), and thus this column temperature was chosen. Fig. 5 shows a chromatogram of the seven arsenic species. Nearly baseline resolution is achieved for all the seven arsenic species within 12 min. To our knowledge, this is the first report demonstrating a complete separation of the seven arsenicals using isocratic run on a single HPLC column. Relative standard deviation (R.S.D.) of peak retention times from six replicate HPLC analyses is 0.27–1.3% for the seven arsenic species.

3.1.3. Comparison of separation efficiency under the above conditions

Table 1 summarizes the efficiency data for the separation of the seven arsenic species shown in Figs. 1–5. The efficiency ($N_{5\sigma}$) was obtained by using the following equation [52]:

$$N_{5\sigma} = 25(t_R / W_{4.4})^2$$

where $W_{4.4}$ is the width of a peak at 4.4% of the peak height. This asymmetry based equation was used because several peaks in the chromatograms were asymmetric. The asymmetric factors (F_{asym}) are also listed in Table 1. The results show that symmetric peaks have better efficiency than asymmetric ones have. However, the overall efficiency for all the peaks is poor. This is primarily because we used an old, poor column in the present study. Nonetheless, the study illustrates several approaches to resolving the seven arsenic species within a short time. It also demonstrates that the use of mixed ion-pair reagents

Table 1
A summary of efficiency ($N_{5\sigma}$) and asymmetry factors (F_{asym}) for the chromatographic peaks shown in Figs. 1–5

		Arsenate	Arsenite	MMAA	DMAA	AB	AC	Me_4As^+
Fig. 1	$N_{5\sigma}$	701	1538	1538	206	495	406	370
	F_{asym}	s	s	s	3.6	1.7	1.7	3.5
Fig. 2	$N_{5\sigma}$	1340	1462	1462	506	822	375	291
	F_{asym}	s	s	s	2.2	1.4	3.7	4.7
Fig. 3	$N_{5\sigma}$	–	–	–	–	899	494	217
	F_{asym}	–	–	–	–	3.0	2.9	4.5
Fig. 4	$N_{5\sigma}$	645	523	302	272	5877	997	1012
	F_{asym}	s	s	1.7	2.4	s	2.6	3.0
Fig. 5	$N_{5\sigma}$	573	759	697	298	1036	5256	2947
	F_{asym}	2.5	2.1	2.0	2.5	2.1	1.6	2.3

s = Symmetric peak; – = Not evaluated.

(Fig. 5) gives better or equivalent separation efficiency compared to the use of a single ion-pair reagent. This is particularly evident for the late eluting compounds, such as AC and Me_4As^-.

3.1.4. Application to arsenic speciation in human urine

A shrimp sample purchased from a local fish market was found to contain 2.3 μg/g (wet weight) of arsenic in the form of arsenobetaine. Urine samples collected from a volunteer who ingested 180.4 g of the shrimp were studied for arsenic speciation, using ion-pair liquid chromatography with ICP-MS detection. HG–GC–AAS was also used to determine concentration of inorganic arsenic, and DMAA. MMAA was not detected in the urine samples from either measurement. The detection limit for MMAA using HG–GC–AAS is 0.3 ng/ml (or 1 ng for 3 ml of sample). The concentrations of AB, DMAA, and inorganic arsenic (mainly arsenate) in the urine samples, normalized against their creatinine concentration, are shown in Fig. 6. Creatinine is known to be excreted constantly from an individual human body [53,54]. The concentration of arsenic relative to creatinine in the urine is a measure in which the variation in urine volume is taken into consideration. Therefore, the normalized results in Fig. 6 roughly illustrate the rate of excretion of the arsenic compounds. It is clear that arsenobetaine is rapidly excreted from the human body following the ingestion of shrimp. It is also clear that the ingestion of arsenobetaine due to the consumption of shrimp does not increase inorganic arsenic, MMAA and DMAA concentration in urine. This is understandable because arsenobetaine is simply excreted without metabolic change.

A comparison of the total amount of arsenobetaine ingested with that excreted indicates that a major portion of arsenobetaine is excreted rapidly into urine. The total amount of arsenobetaine found in urine samples collected for 37 h following the consumption of shrimp is 274 μg, which accounts for 66% of the total amount of arsenobetaine ingested (415 μg) from the shrimp (180.4 g). Another volunteer excreted 120 μg of arsenobetaine within 37 h following the ingestion of 163 μg of arsenobetaine from the shrimp. This accounts for 73% of the total arsenobetaine ingested. The results confirm that the urinary excretion is the major pathway for the elimination of arsenobetaine.

Speciation of arsenic in a standard reference material, SRM 2670 freeze-dried urine, was carried out by using HPLC separation with HGAFS de-

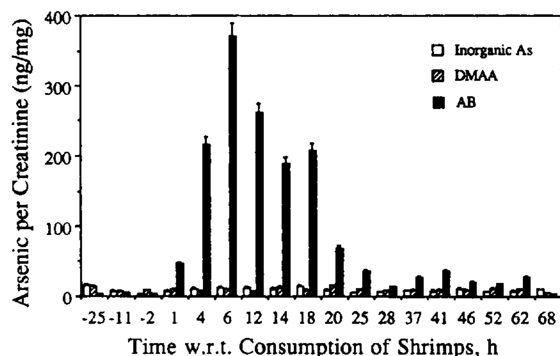

Fig. 6. Speciation of arsenicals in urine samples collected from a volunteer with respect to (w.r.t.) the consumption of shrimp.

tection. The freeze-dried urine was reconstituted with deionized water, and a sample of the solution was immediately subjected to the HPLC analysis. We found 413±20 ng/ml ($n=8$) of arsenic in the form of arsenate. No other arsenic species were above our quantitation limit (50 ng/ml). There is no certified value for arsenic speciation in any of the standard reference materials available. The total arsenic concentration (480±100 ng/ml) in SRM 2670 was certified. Our analysis of SRM 2670, by using direct HGAFS without HPLC separation, gives a total arsenic concentration of 484±10 ng/ml ($n=5$), which is in excellent agreement with the certified value.

We have successfully combined ion-pair chromatography with several detection systems, including ICP-MS, HGAAS and HGAFS. The urine analyses also demonstrated a successful application of these approaches to chemical speciation of trace element. Among these HPLC detection systems for speciation studies, ICP-MS appears to be the best choice. However, ICP-MS is also most expensive in both initial instrument purchasing and operating cost. Post-column microwave derivatization and hydride generation coupled with atomic absorption and atomic fluorescence detection provide inexpensive alternatives to ICP-MS for arsenic speciation using HPLC.

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