Determination of Arsenobetaine, Arsenocholine, and Tetramethylarsonium Cations in Seafoods and Human Urine by High-Performance Liquid Chromatography–Thermochemical Hydride Generation–Atomic Absorption Spectrometry

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A simple method was developed for the determination of arsonium compounds in edible marine tissues (lobster tail muscle, peeled and deveined shrimp, cod fillet, and cod liver oil) and human urine. The homogenized marine tissue [5–10 g (or an equivalent weight of freeze-dried powder)] was blended with methanol; the extracts were combined and flash evaporated. Alternatively, urine (5 mL) was diluted with 50 mL of ethanol and placed in a dry ice–acetone bath for 20 min. Supernatant was separated from the resulting precipitate by centrifugation and flash evaporated. Residues from either sample type were resuspended in water, filtered through an anion exchanger, and acidified. The arsonium analytes were partitioned into liquefied phenol, which was diluted with diethyl ether and back extracted with water. The combined water extracts were taken to dryness, redissolved in methanol, concentrated to 1 mL, and separated on a cyanopropyl bonded-phase column using a methanolic mobile phase containing 19% (v/v) diethyl ether, 1% (v/v) acetic acid, 0.12% (v/v) triethylamine, and picrylsulfonic acid (0.200 g/L). Analytes were detected *on-line* by thermochemical hydride generation–atomic absorption spectrometry. Recoveries from tissues or from urine which had been spiked at 0.1–3.4 μg of cation/g of fresh weight were 83% or greater from each of the five sample types.

INTRODUCTION

It has been recognized for many years that an appreciable portion of the total arsenic in marine animals is in the form of organosoluble compounds (Chapman, 1926; Coulson et al., 1935). Although continued progress was made to isolate and identify these compounds (Lunde, 1969, 1975), it was not until 1977 that arsenobetaine [$(CH_3)_3As^+CH_2COO^-$], isolated from the Western rock lobster, *Panulirus cygnus* (Edmonds and Francesconi, 1977), was identified conclusively. Since this discovery, arsenobetaine has been demonstrated to be the most abundant organoarsenical in many marine animals (Maher et al., 1988; Beauchemin et al., 1988; Cullen and Dodd, 1989). The presence of arsenocholine [$(CH_3)_3As^+CH_2CH_2OH$] has been reported in shrimp by Norin and Christakopulos (1982) and by Lawrence et al. (1986). Other researchers (Luten et al., 1983; Shiomi et al., 1984), however, were not able to confirm its presence in this organism. Tetramethylarsonium ion [$(CH_3)_4As^+$] has been detected recently in the clam *Meretrix lusoria* (Shiomi et al., 1987) (a plankton feeder) and is the major arsenic compound in gill tissues. This cation has also been detected in other marine bivalves (clams, mussels, and scallops; Shiomi et al., 1987; Cullen and Kenneth, 1989). Trimethylarsine oxide [$(CH_3)_3AsO$], trimethylarsine [$(CH_3)_3As$], and certain arsenosugars (Edmonds and Francesconi, 1982; Maher and Butler, 1988; Cullen and Dodd, 1989) have been identified as minor components of the total arsenic fraction in a limited number of marine tissues.

Arsonium compounds have been determined by ion-exchange or ion-pair HPLC with *on-line* detection by inductively coupled plasma (ICP) mass spectrometry (Beauchemin et al., 1988, 1989; Shibata and Morita, 1989a,b) and ICP optical emission spectrometry (Irgolic et al., 1983; Shiomi et al., 1987) or *off-line* detection by graphite furnace AAS (Lawrence et al., 1986; Cullen and Dodd, 1989). Whereas *off-line* detection provides a histogram which may compromise quantitation, the higher purchase and operating costs of ICP MS or ICP OES may limit the availability of these techniques to certain researchers. Recently, an HPLC–AAS quartz interface has been reported which thermochemically converts arsonium ions (Blais et al., 1990) or selenonium ions (Blais et al., 1991) in HPLC eluate to products which are efficiently atomized and detected by AAS. Subsequently, surface response methodologies were used to optimize the separation/detection of arsenobetaine, arsenocholine, and tetramethylarsonium cations by HPLC–AAS (Huyghues-Despointes et al., 1991).

Arsonium compounds are generally considered to be poorly absorbed by mammals and are rapidly eliminated via the urine (Cannon et al., 1981; Shiomi et al., 1988). In the current study, a method for selenonium [trimethylselenonium and (2-hydroxyethyl)dimethylselenonium ions] analytes in human urine is extended to arsonium compounds (arsenobetaine, arsenocholine, and tetramethylarsonium ions) in human urine and four marine tissues.

MATERIALS AND METHODS

Reagents and Standards. Solvents used were distilled in glass or HPLC grade (Caledon Inc., Georgetown, ON). Certified ACS reagent grade hydrochloric and acetic acids were used. Triethylamine was purified gold label grade, and picrylsulfonic acid was 99.9% pure (Aldrich Chemical Co., Milwaukee, WI). Water was double distilled and deionized. Arsonium standards were prepared from cacodylic acid (Aldrich) as described below. **Caution**: Synthetic procedures with arsenic compounds should be carried out in an efficient fume hood. Special precautions are necessary when dimethylarsenic acid (cacodylic acid), dimethyliodoarsine, and trimethylarsine are handled. Protective gloves and laboratory coat should be worn to prevent skin contact with these compounds. Trimethylarsine, dimethyliodoarsine, and cacodylic acid are very toxic. Trimethylarsine is highly volatile and can burst into flame on contact with air.

Dimethyliodoarsine was prepared by the reaction of dimethylarsenic acid with potassium iodide (Burrows et al., 1920). A solution of cacodylic acid (25 g) and potassium iodide (80 g)

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in water (100 mL) was saturated with sulfur dioxide, and dilute HCl (1 M) was added in small portions over the course of the reaction. The reduction proceeded rapidly with the separation of dimethyliodoarsine as a brown oil. The termination of the process was signaled by the separation of sulfur. The crude layer of oil was purified by distillation, which resulted in a yellow liquid (40 g, 95% yield) boiling at 154–157 °C.

Trimethylarsine was prepared by the reaction of methylmagnesium iodide with dimethyliodoarsine (Burrows et al., 1920). A solution of dimethyliodoarsine (40 g) in benzene was added dropwise to an ice-cold stirred solution of 1.1 equiv of methylmagnesium iodide in dibutyl ether (55 mL). The crude reaction mixture was permitted to warm to room temperature. After 2 h, a solution of 1 M HCl (100 mL) was added dropwise to the mixture. The mixture was heated to 80 °C under a nitrogen atmosphere and trimethylarsine (bp 47–55 °C) was recovered, in 80% yield, by distillation.

Arsenobetaine Bromide (AsBet). Bromoacetic acid (7.0 g), dissolved in 20 mL of benzene, was added dropwise to a stirred solution of trimethylarsine (3.8 g) in benzene (20 mL). The exothermic reaction yielded a white precipitate immediately. The mixture was stirred for 6 h, and the product was recovered by filtration, washed with benzene, and recrystallized from hot ethanol. White nonhygroscopic crystals were obtained upon cooling (mp 229–231 °C, lit. mp 229 °C). The yield, 10.2 g, was virtually quantitative.

Tetramethylarsonium Iodide (TMAs). Methyl iodide (7 g) was added to a solution of trimethylarsine (2.0 g) in benzene (20 mL). A white solid precipitate formed immediately. The mixture was stirred overnight, filtered, and washed with benzene. The filtered product was dissolved in hot methanol and cooled at room temperature to produce nonhygroscopic white crystals (95% yield) (mp 350–352 °C).

Arsenocholine bromide (AsChol) was prepared by the reaction of trimethylarsine with 2-bromoethanol (Irgolic et al., 1987). Trimethylarsine (3.4 g) in benzene (25 mL) was transferred to a 100-mL round-bottom flask, equipped with a condenser. A slight excess of bromoethanol (3.6 g) was added to the arsine. The mixture was refluxed and stirred with a magnetic bar for 48 h. Upon cooling, the liquid solidified. The solid was recrystallized from hot CH_3CN. The crude recrystallization mixture was chilled and filtered under nitrogen, and the crystals were dried in a desiccator over P_2O_5. The white hygroscopic crystals (88% yield) were stored under nitrogen (mp 224–227 °C, lit. mp 226.7 °C).

The purity of the arsonium salts was assessed spectroscopically (MS, ^1H and ^{13}C NMR) and chromatographically. Stock solutions (1.00×10^{-4} g/mL as the salt) of these standards were prepared in methanol and stored at −40 °C.

High-Performance Liquid Chromatography. The chromatograph consisted of a Beckman Model 100A pump, an autosampler (LKB Model 2157), a cyanopropyl bonded-phase (5-μm silica support, 0.46 cm i.d. × 15 cm, LC-CN, Supelco Inc., Bellefonte, PA) HPLC column, and an atomic absorption spectrometer (set at 193.7 nm, Philips, PU9,100), which was equipped with a high-energy hollow cathode lamp (Photron super lamp system, Australia) and a deuterium arc background system. The mobile phase, delivered at 0.65 mL/min, consisted of 80% (v/v) methanol, 19% (v/v) diethyl ether, and 1% (v/v) glacial acetic acid, containing 0.12% (v/v) triethylamine and 20 mg/100 mL picrylsulfonic acid. The HPLC–AAS interface was as previously described (Blais et al., 1990). Briefly, the all-quartz assembly consisted of a T-tube mounted within the optical beam of the AAS. The lower portion of the T-tube served as an inlet for O_2 to support a microdiffusion flame which was maintained just below the optical beam of the AAS. A separate sidearm, which met the lower T-tube at a 45° angle, contained a pyrolysis chamber fitted with inlets for O_2 and H_2. In operation, HPLC column eluate was thermosprayed into the pyrolysis chamber and combusted in an O_2 atmosphere. Downstream, the combustion products were mixed with H_2 to convert them to their hydrides, and the product hydrides were atomized in the cool microdiffusion flame. Calibration of the system was performed according to the method of external standards, and recoveries of standards from samples containing detectable quantities of analytes were determined according to the method of standard additions.

Samples. Samples of shrimp (whole), lobster (whole), cod (fillet), and cod liver oil were purchased locally. Shrimps were peeled and deveined, and the tail muscle of lobsters was removed on arrival. Excised tissues were homogenized in a Waring blender and then stored at −40 °C or, after homogenization, freeze-dried and then blended to a fine homogeneous powder (lobster) and stored at −40 °C to await analysis.

Extraction Procedure. A weighed sample (5 g of cod or shrimp, 10 g of cod liver oil, or 0.5 g of lobster powder) was placed in a 50-mL Pyrex centrifuge tube and vortexed (30 s) three times with 20 mL of methanol. The methanolic extracts were combined and rotary flash evaporated at 35 °C to near dryness, and the residue was taken up in 10 mL of water. Urine samples (5 mL) were diluted with 50 mL of ethanol and chilled in a dry ice–acetone bath for 20 min according to the method of Kraus et al. (1985). The resulting precipitate was separated from the liquid by refrigerated (−15 °C) centrifugation at 7000 rpm. The supernatant was taken to dryness, and the residue was redissolved in 10 mL of water. The aqueous solution (from either tissue or urine) was applied to the head of an anion-exchanger column (0.5 × 10 cm of Dowex 2X8). The column was washed with additional distilled deionized water to result in a total volume of 30 mL of eluate which was acidified to pH 3 with HCl. The acidified aqueous solution was extracted four times with liquefied phenol (2 × 10 mL and 2 × 5 mL). The phenol extracts were combined and back-washed with water (1 × 10 mL and 2 × 5 mL) to remove excess chloride and salts. The phenol layer was diluted with 75 mL of diethyl ether and reextracted three times with 5 mL of water. The aqueous extracts were combined, washed three times with 5 mL of diethyl ether, and evaporated to dryness. The residue was resolubilized in methanol and concentrated to 1 mL under a gentle stream of N_2. Aliquots (50 μL) were analyzed by HPLC–AAS.

Recovery Trials. A methanolic standard solution containing 0.1 mg/mL of arsenobetaine bromide, arsenocholine bromide, and tetramethylarsonium iodide was added to three replicate samples of each type (5 g wet weight homogenized tissue, 0.5 g of freeze-dried powder, 5 mL of urine, or 10 mL of cod liver oil). A further three samples of each type served as controls. All samples were extracted and analyzed as above.

RESULTS AND DISCUSSION

The prototype HPLC–AAS interface was developed for the detection of arsonium and selenonium analytes which are not amenable to *on-line* chemical hydride generation techniques. The interface AAS detector has been demonstrated (Huyghues-Despointes et al., 1991) to provide equivalent limits of detection (4–5 ng as As) for arsonium standards [arsenobetaine (AsBet), arsenocholine (AsChol), and tetramethylarsonium (TMAs)] in HPLC column eluate. The principal limitation of this prototype design remains the requirement for an organic rich mobile phase which acts as a fuel to support the combustion process. Because the AAS response was slightly dependent on the mobile-phase composition, an isocratic separation of AsBet, AsChol, and TMAs cations was developed by using a cyanopropyl bonded-phase column and a methanol–diethyl ether mobile phase containing triethylamine (TEA), picrylsulfonic acid, and acetic acid. The TEA was considered to mask active silanol groups on the stationary phase. In the previous study, it had been concluded that the principal interaction of selenonium analytes [trimethylselenonium and (2-hydroxyethyl)dimethylselenonium ions] with the cyanopropyl bonded phase was with underivatized surface silanol groups. Optimum chromatography was achieved with a mixture of trimethylsulfonium (TMS) and triethylammonium (TEA) ions in the methanolic mobile phase. Relative to TEA alone, the combination of TMS and TEA did not improve the peak shape of arsonium analytes perceptibly. By contrast, the addition of 2,4,6-trinitrobenzenesulfonic acid (picrylsulfonic acid) resulted in appreciably improved chromatography of these analytes. Limits of detection were improved

Table I. Recoveries of Arsenobetaine, Arsenocholine, and Tetramethylarsonium Cations from Lobster Tail Muscle, Whole Shrimp, Cod Fillet, Cod Liver Oil, and Human Urine

sample matrix	analyte	spiked sample,[a] μg/g	unspiked sample, μg/g	% recovery
lobster	Asbet	30.4 (9.3)[b]	27.0 (8.3)	85 ± 2.0
	Aschol	3.4 (1.1)	0.00	85 ± 1.3
	TMAs	3.3 (1.0)	0.00	83 ± 2.5
shrimp	AsBet	2.1 (0.65)	1.8 (0.54)	95 ± 1.2
	AsChol	0.37 (0.12)	0.00	93 ± 0.7
	TMAs	0.37 (0.11)	0.00	83 ± 2.3
cod fillet	Asbet	1.6 (0.48)	1.2 (0.38)	85 ± 1.1
	AsChol	0.33 (0.10)	0.00	83 ± 1.1
	TMAs	0.33 (0.10)	0.00	84 ± 2.4
cod liver oil	AsBet	0.18 (0.06)	0.00	91 ± 2.0
	AsChol	0.18 (0.06)	0.00	90 ± 0.6
	TMAs	0.17 (0.05)	0.00	87 ± 2.6
human urine	AsBet	0.28 (0.11)	0.10 (0.031)	97 ± 1.4
	AsChol	0.32 (0.13)	0.00	85 ± 0.5
	TMAs	0.21 (0.11)	0.00	94 ± 2.1

[a] Micrograms of cation per gram of fresh weight of sample. [b] Values in parentheses represent micrograms per gram as As.

approximately 3-fold relative to a methanolic mobile phase which contained only triethylammonium ion. The current studies were undertaken to demonstrate the applicability of this interface to the routine determination of arsonium analytes in biological matrices.

Extraction procedures were based principally on the earlier work of Cannon et al. (1981). Tissue, [5–10 g of homogenized muscle (or an equivalent weight of freeze-dried powder)] was blended twice with 20 mL of methanol in a 50-mL centrifuge tube. The supernatants from the extractions were combined and concentrated nearly to dryness. The residue was resolubilized in water and applied to the head of an anion-exchange column. The filtration through the ion-exchange medium was necessary to remove anions that interacted strongly with the arsonium analytes and appreciably modified their chromatographic behavior. The aqueous column eluate was acidified to pH 3, and the analytes were partitioned into liquefied phenol. The phenol extracts were diluted with diethyl ether, and the arsonium compounds were repartitioned back into distilled water. The aqueous extracts were concentrated virtually to dryness, and the residue was resolubilized in methanol and analyzed by HPLC–THG–AAS. For human urine, a desalting procedure which was developed for selenonium ions by Kraus et al. (1985) proved to be effective for the arsonium analytes as well. A convenient volume of urine was diluted with 10 volumes of ethanol, and the mixture was chilled in a dry ice–acetone bath for 15 min. Centrifugation facilitated separation of the liquid from the resulting precipitate. The supernatant was concentrated to dryness; the residue was redissolved in methanol and treated as above.

Recoveries from lobster tail muscle, whole deveined shrimp, and cod fillet were assessed by the method of standard additions. Fresh tissue homogenate (or an equivalent of freeze-dried powder) was spiked with a methanolic solution containing approximately equal amounts of the three arsonium standards. Spiking levels were chosen to be at least 10% of the anticipated levels of arsenobetaine in each tissue. Recoveries, at these levels, from lobster, shrimp, cod fillet, and cod liver oil were 83% or greater for each of the analytes (Table I). Despite the relatively simple extraction procedure, no problems were encountered with the chromatography of tissue extracts containing these analytes (Figure 1). Additionally, the

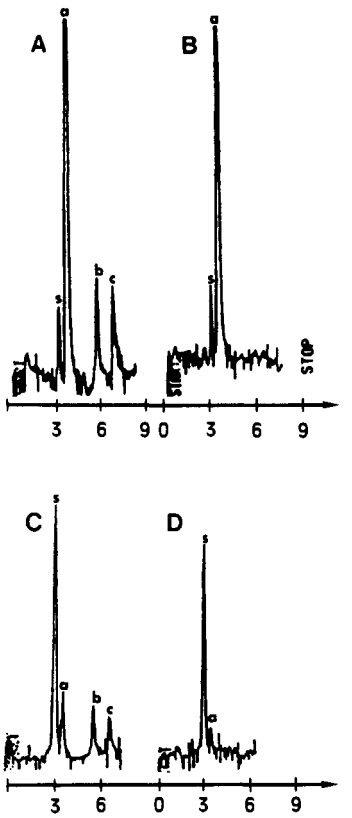

Figure 1. HPLC–THG–AAS chromatograms of (a) arsenobetaine, (b) arsenocholine, (c) tetramethylarsonium cation, and (s) solvent front recovered from spiked (A, C), and control (B, D) samples of shrimp (A, B) or human urine (C, D) collected 12 h after baked cod was consumed.

co-injection of tissue extract with the synthetic standards did not alter either the chromatography of, or the detector response to, these analytes. Thus, other "onium" coextractives, which must have been present in the extracts, did not interfere with either the chromatographic separation or the detection process. By contrast, if a mixture of triethylamine and trimethylsulfonium iodide was added to the methanol–1% acetic mobile phase, coextractives from the tissues, when co-injected with arsonium standards, appreciably modified the retention times of these standards.

No arsonium analytes were detected in cod liver oil, but arsenobetaine was present in each of the tissue samples (at levels ranging between 27.0 and 1.2 μg of cation/g of fresh weight). Neither arsenocholine (AsChol) nor tetramethylarsonium ion was detected in the samples, although AsChol has been reported in certain samples of shrimp. It has been suggested (Norin et al., 1983) that AsChol is a precursor to arsenobetaine and that the relative proportions of these analytes can vary with the metabolic rates of the species. The arsenocholine in a mixture of shrimp extract and AsChol standard was observed to be converted to arsenobetaine even when the mixture was stored at 4 °C. Although not investigated in detail, the half-life was estimated to be 28 days at this temperature.

Arsonium analytes were recovered from human urine somewhat more efficiently (Table I) than were the selenonium isologues (Huyghues-Despointes et al., 1991). In both cases the choline isologues were recovered less efficiently than the permethylated species. That the proposed method is applicable to metabolic trials isdemonstrated by the chromatograms in Figure 1 which represent either shrimp (A, B) or a human urine sample (C, D) taken 12 h after a serving of 150 g of baked cod was consumed. A control urine sample provided just prior to the meal did

not contain any detectable residues of arsonium ions. Levels of arsenobetaine in unspiked urine (Table I) ranged from nondetectable up to 0.10 μg/mL.

CONCLUSION

Arsonium analytes were recovered efficiently from seafoods, cod liver oil, and human urine by using a series of partitioning steps in combination with a filtration of the partially purified extract through an anion exchanger. The analytes were partitioned from the acidified column eluate into liquefied phenol and then repartitioned back into an aqueous phase after the polarity of the phenol phase was decreased with diethyl ether. These cleanup steps were sufficient to eliminate the influence of coextractives on the subsequent chromatography provided that picrylsulfonic acid was added, as a pairing ion, to the methanolic–1% acetic acid mobile phase. For spiking levels of 0.1–2.8 μg of cation/g of sample, recoveries from each of the five matrices were 83% or better. Thus, HPLC–thermochemical hydride generation–AAS provides an inexpensive alternative to conventional techniques for the determination of arsenobetaine, arsenocholine, and tetramethylarsonium cations in biological matrices.

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Registry No. Arsenobetaine, 64436-13-1; arsenocholine, 39895-81-3; tetramethylarsonium, 27742-38-7; dimethyliodoarsine, 676-75-5; dimethylarsenic acid, 75-60-5; potassium iodide, 7681-11-0; trimethylarsine, 593-88-4; methylmagnesium iodide, 917-64-6; arsenobetaine bromide, 71642-15-4; bromoacetic acid, 79-08-3; tetramethylarsonium iodide, 5814-20-0; methyl iodide, 74-88-4; 2-bromoethanol, 540-51-2; arsenocholine bromide, 71802-31-8.