APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. **13**, 765–770 (1999)

Arsenocholine- and Dimethylated Arsenic-containing Lipids in Starspotted Shark *Mustelus Manazo*

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We have investigated lipid-soluble arsenic compounds present in the tissues of a demersal shark, the starspotted shark *Mustelus manazo*. Arsenic compounds were extracted with chloroform–methanol from several tissues taken from each of five individuals, subjected to mild alkaline hydrolysis and fractionated into alkali-labile and alkali-stable fractions. Ordinary muscle, kidney and brain contained alkali-labile arsenic compounds; liver, stomach, heart and gall bladder contained alkali-stable compounds; and intestine, skin, dark muscle, spleen and bone contained both types of arsenic compounds. After further hydrolysis, the hydrolysates from ordinary muscle and liver were chromatographed with HPLC–ICP–MS. Arsenocholine was detected in the hydrolysates from the muscle, suggesting that arsenolecithins were present in the tissues. However, dimethylarsinic acid was detected in the hydrolysates from the liver, suggesting the presence of dimethylated arsenolipid in it. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: arsenolipid; arsenolecithin; dimethylated arsenic; arsenocholine; HPLC–ICP–MS; starspotted shark

Received 6 October 1998; accepted 13 May 1999

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INTRODUCTION

Since the isolation and identification of arsenobetaine in the tail muscle of the western rock lobster,[1] the study of water-soluble arsenic compounds in marine organisms has developed rapidly.[2–6] However, few data are available on lipid-soluble arsenic compounds. This may be due to the limited amounts of these compounds and to the difficulty of isolating them. The investigation of the structure of the lipid-soluble arsenic compounds is important for the elucidation of arsenic circulation in marine ecosystems. The starspotted shark *Mustelus manazo* is a demersal shark whose water-soluble arsenic compounds occur almost entirely in the form of arsenobetaine.[7,8] This species was chosen for investigation because of the high content of lipid-soluble arsenic in the polar lipid fraction in the liver.[7] In this study, in order to elucidate the structure of arsenolipids accumulated in shark tissues, lipid-soluble arsenic compounds extracted from liver and 11 tissue and organ samples were subjected to mild or severe hydrolysis to identify the derived water-soluble residues by HPLC–ICP–MS.

MATERIALS AND METHODS

Starspotted shark

Five fresh starspotted sharks (average weight 1300 g) were purchased from a market. Twelve tissue and organ samples, i.e. ordinary muscle (130 g), dark muscle (191.5 g), stomach (104.5 g),

 CCC 0268–2605/99/100765–06 $17.50

heart (6.9 g), gall bladder (2.6 g), intestine (60.0 g), skin (211.5 g), spleen (15.0 g), brain (8.5 g), liver (290.0 g), kidney (26.0 g) and bone (114.5 g), were taken and stored at −30 °C until they were subjected to extraction of lipid-soluble arsenic compounds. Care was taken to remove muscle and marrow from the bone sample.

Extraction of lipid-soluble arsenic compounds

After thawing and cutting into pieces, each sample was extracted twice with ten times its volume of chloroform–methanol (2:1). Water was then added to reach a water:chloroform–methanol ratio of 1:4. After being shaken for 2 min, the mixture was allowed to stand overnight.[9] The arsenic compounds that separated into the chloroform (lower) layer are referred to as lipid-soluble arsenic compounds.

Preparation of polar and neutral lipid fractions

The lipid-soluble arsenic compound fraction was fractionated into polar and neutral lipid frations as follows. After drying with a vacuum evaporator, the dried lipid-soluble arsenic compound fraction was dissolved in ten times its weight of chloroform and mixed with five times its weight of silicic acid (Mallinckrodt, 100-mesh) in a beaker. After filtration with No. 2 filter paper on a No. 3 glass filter, the silicic acid on the paper was washed six times with five times its weight of chloroform. The chloroform filtrates were gathered and concentrated as the neutral lipid-soluble arsenic compound fraction. The silicic acid on the paper was then washed four times with methanol ten times the weight of the dried lipid-soluble arsenic compound fraction.[10] The methanol filtrates were gathered and concentrated as the polar lipid-soluble arsenic compound fraction.

Partial hydrolysis of lipid-soluble arsenic compounds

According to Dawson's method, the polar lipid-soluble arsenic compound (methanol) fraction extracted from each tissue or organ was subjected to mild alkaline hydrolysis: the lipids in the fraction of each tissue or organ were incubated in 0.027 mol dm^{-3} sodium hydroxide for 20 min to prepare alkali-labile and alkali-stable fractions. The alkali-stable fraction of the ordinary muscle and liver were further hydrolysed also by Dawson's method (6 mol dm^{-3} CCl_3COOH) to prepare an acid-labile fraction.[11] As a severe hydrolysis, alkali-labile compounds from ordinary muscle were further hydrolysed with 6 mol dm^{-3} HCl in boiling water (alkali-labile/HCl fraction) and alkali-stable compounds from liver were further hydrolysed with saturated barium hydroxide under reflux for 5 h [alkali-stable/$Ba(OH)_2$ fraction].[12]

Arsenic determination

Arsenic was determined by arsine evolution–electrothermal atomic absorption spectrometry after the tissue or organ samples were digested with a mixture of nitric, sulphuric and perchloric acids as described previously.[13] For the dried chloroform fraction, an aliquot of each fraction was saponified (100 °C, 10 min) with 12.5 times its volume of 2.4 mol dm^{-3} ethanolic potassium hydroxide before the digestion.

High-performance liquid chromatography–inductively coupled plasma spectrometry

A Hewlett-Packard 1050 solvent delivery unit and a 100 μl injection loop of a Rheodyne six-port injection valve were used. Arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion (TETRA) were separated at a flow rate of 1.0 or 1.5 cm^3 min^{-1} on a Supelcosil LC-SCX cation-exchange column (250 mm × 4.6 mm i.d.) with a 0.01 mol dm^{-3} pyridine–formic acid buffer (pH 3.0 or 5.0). Arsenite {As(III)}, arsenate {As(V)}, methanearsonic acid (MMA) and dimethylarsinic acid (DMA) were separated at a flow rate of 1.5 cm^3 min^{-1} on a Hamilton PRP-X100 anion-exchange column (250 mm × 4.6 mm i.d.) with a 0.02 mol dm^{-3} phosphate buffer (pH 5.6). The exit of the column was connected to a Bobinton-type nebulizer via 50 cm of PEEK capillary tubing (0.13 mm i.d). An HP 4500 (Hewlett-Packard, Waldbronn, Germany) inductively coupled plasma mass spectrometer (ICP–MS) served as an arsenic-specific detector. Each sample was directly injected onto the column.

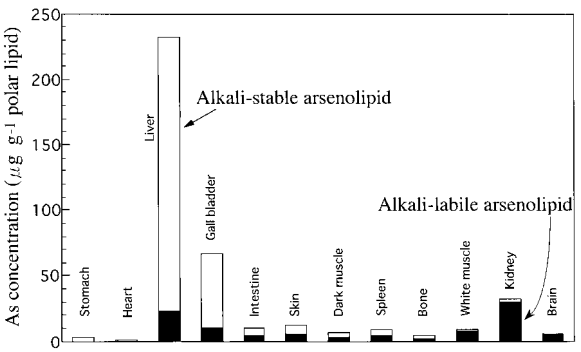


Figure 1 Distribution of alkali-labile and alkali-stable arsenolipids in the tissues and organs of starspotted shark.

RESULTS

Total arsenic in each tissue

The arsenic content of each tissue was 33.6 (ordinary muscle), 22.8 (dark muscle), 14.2 (stomach), 8.0 (heart), 9.4 (intestine), 1.5 (skin), 4.3 (spleen), 20.1 (liver), 4.6 (kidney) and 8.8 μg g^{-1} (bone). Those of brain and gall bladder were not determined because of insufficient amounts.

Arsenic contents in alkali-labile and alkali-stable fractions

Almost all of the lipid-soluble arsenic was detected in the polar lipid fraction in each tissue or organ: little or no arsenic existed in the neutral lipid fraction. Arsenic was then determined in both prepared fractions, obtained by mild hydrolysis.

There was considerable variation in the arsenic content of the polar lipid fractions from the 12 tissues. Furthermore, the arsenic in these tissues did not show a definite distribution into alkali-labile and alkali-stable fractions (Fig. 1).

Ordinary muscle, kidney and brain mainly contained alkali-labile arsenic compounds; liver, stomach, heart and gall bladder contained alkali-stable compounds, and intestine, skin, dark muscle, spleen and bone contained both types of arsenic compounds. Especially in the stomach and heart muscle, arsenic was detected only in the alkali-stable fraction. On the other hand, arsenic was detected only in the alkali-labile fraction in brain.

Arsenic contents in alkali-labile, acid-labile and alkali/acid-stable fractions from ordinary muscle and liver

Polar lipid fractions from ordinary muscle and liver from another specimen of the starspotted shark were prepared and fractionated into alkali-labile, acid-labile and alkali/acid-stable fractions. The arsenic content in each fraction is shown in Table 1. Most of the arsenolipids from ordinary muscle (20.0 μg g^{-1}, 96.6%) were fractionated into alkali-labile fraction. On the other hand, most of the arsenolipids (220 μg g^{-1}, 93.3%) from liver were resistant to mild alkaline or acid hydrolysis and fractionated into the alkali- and acid-stable fractions.

In order to identify the structures of the water-soluble residues bonded to the major arsenolipids, these lipids from the ordinary muscle or the liver were then subjected to severe hydrolysis.

HPLC–ICP–MS analysis of alkali-labile/HCl fraction from ordinary muscle

The alkali-labile fraction from ordinary muscle was further hydrolysed with 6 mol dm^{-3} HCl and analysed by HPLC–ICP–MS. In the prepared alkali-labile/HCl fraction, the major arsenic peak was identified as arsenocholine (Fig. 2).

HPLC–ICP–MS analysis of alkali-stable/Ba(OH)$_2$ fractions from liver

The alkali-stable fractions from liver were further

Table 1 Arsenic content in polar lipid, neutral lipid, alkali-labile, acid-labile and alkali/acid-stable fractions

	Mean (n = 2) arsenic concentration (μg g^{-1} polar lipid)				
Sample	Polar lipid	Neutral lipid	Alkali-labile	Acid-labile	Alkali- and acid-stable
Ordinary muscle	21.8	N.D.[a]	20.0	N.D.	0.71
Liver	276	N.D.	15.7	0.13	220

[a] N.D., not detected

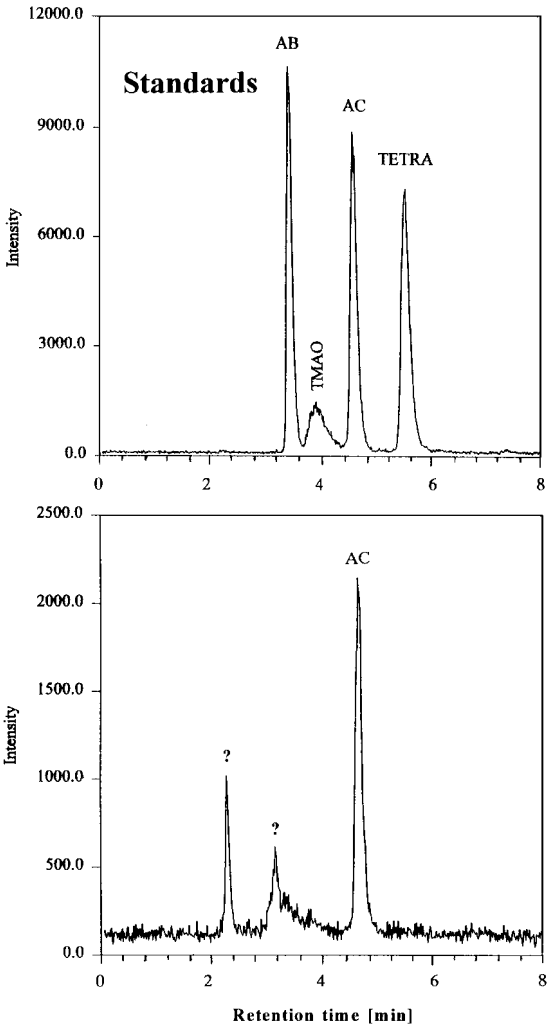


Figure 2 HPLC–ICP–MS chromatograms (Supelcosil LC-SCX) of authentic arsenicals (above) and the water-soluble arsenic residues obtained with severe acid hydrolysis of alkali-labile arsenic fraction prepared from ordinary muscle (below). Experimental conditions were as described in the text.

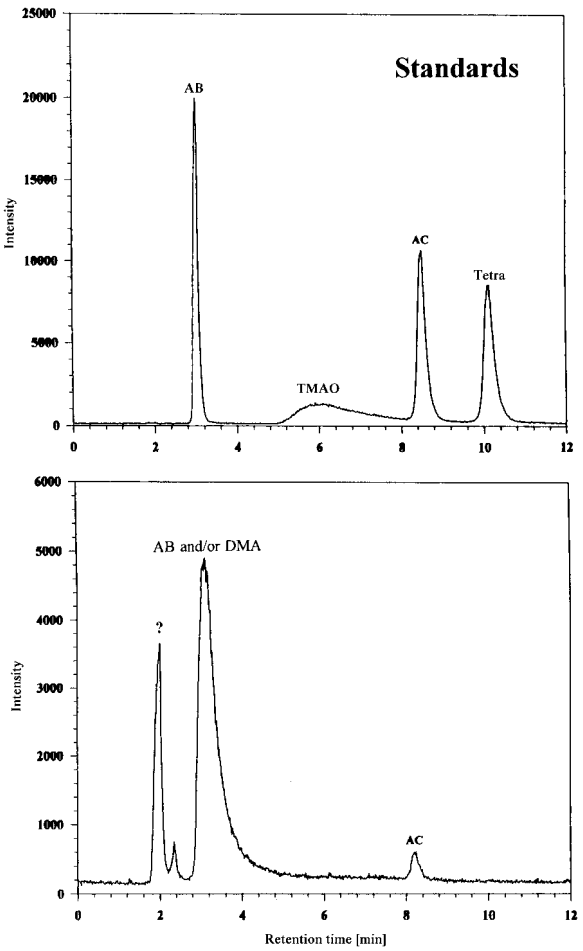


Figure 3 HPLC–ICP–MS chromatograms (Supelcosil LC-SCX) of authentic arsenicals (above) and the water-soluble arsenic residues obtained with severe alkaline hydrolysis of alkali-stable arsenic fraction prepared from liver (below). Experimental conditions were as described in the text.

hydrolysed with saturated $Ba(OH)_2$. Figure 3 and 4 show the HPLC–ICP–MS chromatograms of the derived water-soluble arsenicals in the alkali-stable/$Ba(OH)_2$ fraction. The HPLC was performed on either a Hamilton PRP column or a Supelcosil SCX column. Arsenocholine was detected in the liver with a Supelcosil SCX column (Fig. 3), as it was in ordinary muscle (Fig. 2). However, unlike the arsenocholine in the muscle, the arsenocholine in the liver was not the major peak. The retention time of the major peak was the same as that of arsenobetaine and dimethylarsinic acid. Figure 4 shows the chromatogram obtained with the Hamilton PRP column. The major arsenic peak was confirmed to be dimethylarsinic acid because, with this column, arsenobetaine elutes in the void volume.

Behavior of arsenocholine towards severe hydrolysis

A portion of the synthetic arsenocholine solution (0.5 μg g^{-1}) was heated under the same conditions used for the alkali-labile or alkali-stable fraction and analysed with HPLC–ICP–MS using a Nucleosil 10 SA column: 90.4% of arsenocholine was

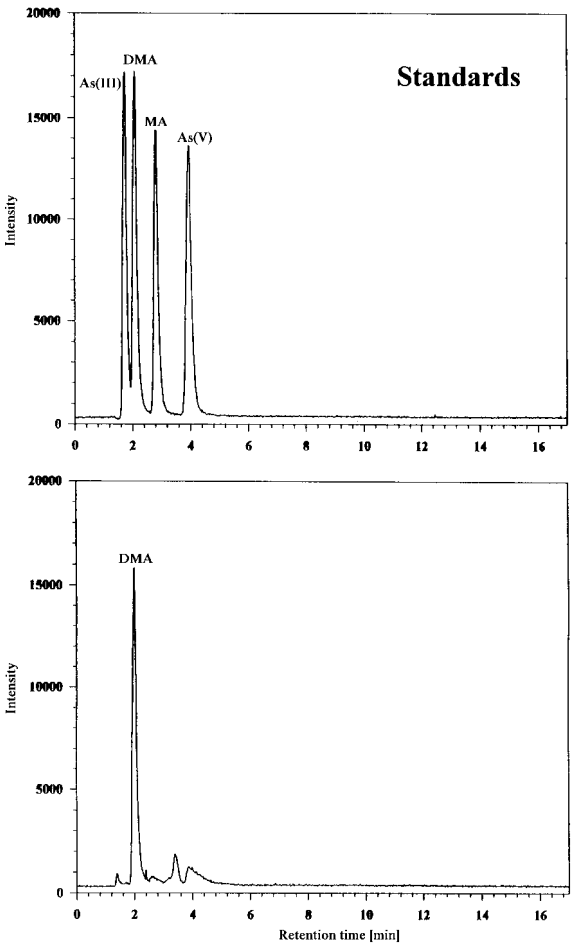
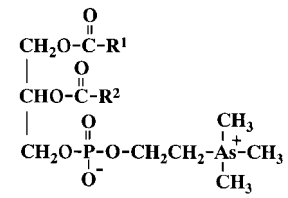


Figure 4 HPLC–ICP–MS chromatograms (Hamilton PRP-X100)) of authentic arsenicals (above) and the water-soluble arsenic residues obtained with severe alkaline hydrolysis of alkali-stable arsenic fraction prepared from liver (below). Experimental conditions were as described in the text.

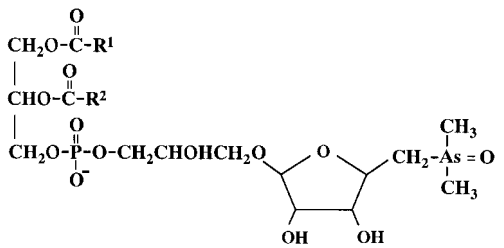
recovered after the HCl hydrolysis and 85.9% after barium hydroxide hydrolysis. Besides arsenocholine, no organoarsenicals including dimethylarsinic acid were detected.

DISCUSSION

Arsenic was localized in the polar lipid fraction in each tissue, suggesting that arsenic-containing groups are bonded to the lipid-soluble compound in such a way that choline is bonded to phosphatidic acid in phosphatydylcholine (lecithin). However,



Phosphatidylarsenocholine



Phosphatidylarsenosugar

Figure 5 The molecular structures of phosphatidylarsenocholine and phosphatidyl arsenosugar. —OCR[1], —OCR[2]: fatty acyl group.

the results were not so simple because at least two arsenolipids were clearly demonstrated in the tissues (Fig. 1, Table 1). One is an alkali-labile type that accumulates in ordinary muscle, kidney and brain and the other an alkali-stable type in liver, stomach, heart and gall bladder. This diversity in the structure of arsenolipids in a single species is interesting from the point of view of comparative biochemistry. Interspecies diversity should be investigated in future as well as intraspecies diversity.

Arsenocholine was derived as the major arsenical in the alkali-labile/HCl fraction from the polar lipids from ordinary muscle (Fig. 2). As the typical alkali-labile polar lipid or phospholipid, phosphatydylcholine is suggested. Furthermore, phosphatidylarsenocholine (Fig. 5) was reported to be present in the muscle of yelloweye mullet following oral administration of arsenocholine.[14] Together, these findings strongly suggested that phosphatidylarsenocholine (arsenolecithin) was present in ordinary muscle tissues. To confirm this, we are now trying to synthesize glycerylphosphorylarsenocholine, which is considered to be derived from phosphatidylarsenocholine with the liberation of two fatty acids following mild alkaline hydrolysis.

On the other hand, based on the fact that the major arsenolipid from liver remained lipid-soluble after hydrolysis with sodium hydroxide or hydro-

chloride, it could not have been analogous to a glycerophospholipid such as phosphatidylcholine. The arsenolipid was expected to be arsenosphingomyelin, because sphingomyelin is resistant to both alkali and acid hydrolysis.[11] However, dimethylarsinic acid was derived in the alkali-stable/$Ba(OH)_2$ fraction from the polar lipid fraction of liver (Figs 3 and 4), indicating that it is not an arsenocholine-containing lipid. As to the dimethylated arsenic compounds, arsenosugars have been confirmed in various algae as water-soluble arsenicals.[2–6] An arsenosugar-containing lipid (a phosphatidylarsenosugar, Fig. 5) was reported in the brown algae *Undaria pinnatifida*.[15] However, this compound cannot be the arsenolipid from liver because it is not an alkali- and acid-stable polar lipid but an alkali-labile one. Further experiments will be needed to identify the structure of the major polar arsenolipid in the liver. Thus, at this time, we say only that the major arsenolipid in the liver of starspotted shark is a dimethylated arsenic-containing lipid.

The chromatograms show the occurrence of minor arsenic compounds in both the ordinary muscle (Fig. 2) and the liver (Figs 3 and 4). The arsenocholine-containing lipid that was found to be the major arsenolipid in the muscle and the dimethylated arsenolipid in the liver may co-exist in both tissues, as a small arsenocholine peak was also detected in the liver. We are at present conducting studies to determine whether these arsenolipids are present in tissues other than ordinary muscle and liver.

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