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Simultaneous determination of glycine betaine and arsenobetaine in biological samples by HPLC/ICPMS/ESMS and the application to some marine and freshwater fish samples

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ABSTRACT

We describe a HPLC/mass spectrometry method for the simultaneous determination of glycine betaine and arsenobetaine in biological samples. The sample preparation included a water/methanol extraction followed by clean-up of extracts on a strong cation-exchange resin; the HPLC system consisted of a cation-exchange column with an ammonium formate buffer solution as mobile phase. Glycine betaine and arsenobetaine were quantified in a single chromatographic run by splitting the HPLC flow with an adjustable flow splitter and detecting glycine betaine selectively by electrospray MS in the positive single ion monitoring mode at m/z 118, and arsenobetaine with the arsenic-selective detector ICPMS at m/z 75. The proposed method was validated for arsenobetaine by analysis of CRM Dorm-2, and for glycine betaine by spiking the CRM Dorm-2 with a defined amount of glycine betaine. Finally, the developed method was applied to determine glycine betaine/arsenobetaine ratios in single specimens of four species of marine fish and one species of freshwater fish.

1. Introduction

Arsenobetaine (AB, $Me_3As^+CH_2COO^-$), the arsenic analogue of the quaternary ammonium compound glycine betaine (GB), was first discovered in the tail muscle of the western rock lobster in 1977 [1]. Since ABs discovery, further work over several years demonstrated that it is the major arsenical in a wide range of marine animals including fish, crustaceans and molluscs [2,3]. Although AB is strongly linked to marine ecosystems, it is also found, albeit at much lower concentrations, in freshwater and terrestrial organisms [4,5].

GB is widely distributed in the environment and serves as an important organic osmolyte in several organisms [6,7]. Such organisms have developed highly efficient uptake processes for GB [8]. The origin and possible function of AB remains unclear, although its similarity to GB in terms of chemical structure might suggest that AB is taken up (mistakenly) by the GB uptake process. Aquarium tests with the blue mussel *Mytilus edulis* demonstrated that AB, in contrast to other arsenic compounds, was strongly accumulated from seawater [9]; this accumulation was inversely proportional to the concentration of GB in the water suggesting that a competitive uptake mechanism was in place.

If AB is used by marine organisms in a way similar to GB, then its concentrations might be expected to be also osmotically controlled and hence vary with salinity. This hypothesis is supported by findings showing that the uptake and elimination of AB in blue mussels was related to the salinity of the water [10]. A similar relationship between the total arsenic concentration and salinity was observed in teleost fish from the Baltic and North Sea, where fish from high salinity waters showed higher total arsenic concentrations than the same species collected from waters with lower salinity [11]. In that case, the fish arsenic concentrations were thought to reflect the arsenic content of their food from the various waters. Investigations to obtain definitive evidence for a link between AB and GB would greatly benefit from a single analytical method capable of quantifying both GB and AB.

There has been one report of a method for determining GB and AB simultaneously. A HPLC tandem- mass spectrometry method was reported for the determination of both AB and GB together with other osmolytes in serum and plasma [12]. The method, consisting of a sample clean-up procedure and isotope dilution with deuterated internal standards, showed good recoveries for GB and AB in bovine serum and seal plasma. However, because of matrix effects and low concentrations, AB is rarely quantitatively determined by molecular mass spectrometry — the preferred method is based on the use of HPLC coupled to an elemental mass spectrometer such as an inductively coupled plasma mass spectrometer (ICPMS) as arsenic-selective detector.

GB and AB occur at very different concentrations in biological samples, with GB being by far the dominant compound. For example, in marine organisms, AB levels are commonly in the range of 1–10 μg g^{-1} (wet mass) [4], whereas GB is often present at levels up to 2000 μg g^{-1} (wet mass) [13]. This concentration difference can complicate the simultaneous

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quantification of both compounds when using molecular MS alone. A practical solution to covering the broad range of concentrations of GB and AB found in natural samples is to couple a HPLC system to both elemental MS, for As-selective detection of AB at trace levels, and molecular MS for detection of GB. We describe a robust and sensitive HPLC/ICPMS/ESMS split method for the simultaneous determination of GB and AB in biological samples, and report preliminary results from its application to some samples of marine and freshwater fish.

2. Experimental

2.1. Chemicals, standards and samples

Water (18.2 MΩ cm) provided from a Milli-Q Academic water purification system (Millipore GmbH, Vienna, Austria), was used throughout this work. Cation-exchange resin DOWEX™ 50WX8-200 in the hydrogen form and hydrochloric acid (≥37%, puriss p.a.) were purchased from Sigma Aldrich (Steinheim, Germany). Ammonium formate (≥95%, purum), formic acid (≥98%, p.a., ACS) and ammonia solution (≥25%, p.a.) were purchased from Carl Roth (Karlsruhe, Germany). Methanol (HPLC gradient grade) was purchased from J.T. Baker (Deventer, Netherlands). Glycine betaine anhydrous (BioUltra, ≥99.0%) was purchased from Sigma (Steinheim, Germany) and arsenobetaine (>99% by NMR) was previously synthesized in house; standard solutions of GB and AB were prepared in water.

Beetroot (*Beta vulgaris*; obtained as chips from Olewo GmbH, Lehrte-Arpke, Germany), which is naturally high in glycine betaine, was used as a model sample for the method development. It was purchased from a shop, ground to a fine powder with a commercially available household coffee grinder, and stored at room temperature prior to use. Five fish samples were obtained as follows: one brown trout (*Salmo trutta fario*) caught with rod and line from the Granitzbach (Granitztal—St. Paul, Austria); and one mackerel (*Scomber scombrus*) from the North Atlantic, one mullet (*Mugilidae* sp.) from the Mediterranean Sea, one red gurnard (*Chelidonichthys cuculus*) from the Southern Pacific Ocean, and one salmon (*Salmo salar*) steak (aquaculture, Norway) bought from a local central market (Metro GmbH, Graz). A fillet was taken from each of the whole fish and put through a commercially available kitchen extruder. The resulting paste was freeze-dried to constant mass with a CHRIST Gamma 1–16 LSC freeze-dryer (Martin Christ GmbH, Osterode am Harz, Germany), milled to a particle size <1 mm with a Retsch ZM 200 (Retsch GmbH, Haan, Germany), and stored at 4 °C prior to use.

The certified reference material DORM–2 (dog fish muscle) with the certified concentration of 16.4 ± 1.1 mg As kg^{-1} arsenobetaine (dry mass) was purchased from the National Research Council of Canada (Ottawa, Canada).

2.2. HPLC/ESMS and HPLC/ICPMS/ESMS analyses

HPLC/ESMS measurements were performed with an Agilent Series 1100 LC/MSD system consisting of a G1946 MSD single quadrupole mass analyser (Agilent, Waldbronn, Germany) equipped with an atmospheric pressure ionization (API) LC-MS interface and electrospray ionization (ESI) source coupled to an Agilent 1100 Series high performance liquid chromatography system with binary pump and solvent degasser. Masses m/z 118 (GB) and m/z 179 (AB) were monitored simultaneously in the positive single ion monitoring mode at a fragmentor voltage of 100 V. The optimization of fragmentor voltages for GB and AB was performed by flow injection experiments with a mixed aqueous standard solution containing 10 mg L^{-1} GB and 10 mg As L^{-1} AB at varying fragmentor voltages (20 V steps; range 20–200 V). The spray chamber settings of the electrospray ionization source were as follows: 10.0 L min^{-1} drying gas (N_2) at 350 °C, 50 psig nebulizer pressure, and 4000 V (positive) and 3500 V (negative) capillary voltages. HPLC/ICPMS/ESMS measurements were performed by coupling simultaneously an Agilent Series 1100 LC/MSD system with a 7500ce inductively coupled plasma mass spectrometer (Agilent, Waldbronn, Germany), equipped with a Scott type spray chamber and a Burgener Ari Mist HP nebulizer (Burgener, Research International, Berkshire, UK). The monitored mass in ICPMS measurements was m/z 75. The HPLC system for HPLC/ESMS and HPLC/ICPMS/ESMS analyses consisted of a cation-exchange column IonoSpher C (100 × 3 mm, particle size 5 μm; Varian, Darmstadt, Germany); for optimizing chromatographic conditions, ammonium formate (5–50 mM) and pyridine (1–10 mM) buffer solutions all at pH 2.50 (pH adjusted with formic acid); and the addition of methanol (1–10%, v/v) were tested. The final conditions consisted of a 20 mM ammonium formate buffer pH 2.50 with the addition of 10% MeOH (v/v). The flow rate of mobile phase was 1.00 mL min^{-1} and the injection volume was set to 10 μl. In the HPLC/ICPMS/ESMS setup, the HPLC flow (1.0 mL min^{-1}) was split 1:10 with a QuickSplit™ adjustable flow splitter (Analytical Scientific Instruments, Richmond, CA, USA). The low flow (10%) to the ESMS was supported with a make-up flow (0.8 mL min^{-1}) of the same composition of mobile phase as used for the HPLC main flow.

2.3. Extraction

Portions (*ca* 0.5 g weighed to 0.1 mg) of sample (beetroot spiked with 100 μg As AB by adding 100 μl of a 1000 mg As L^{-1} AB solution to the dry powder before adding the extraction solvent; freeze-dried fish samples; CRM Dorm-2; or CRM Dorm-2 spiked with 2.5 mg GB by adding 2.5 mL of a 1000 mg L^{-1} GB solution) were placed in 50 mL polypropylene tubes and extracted in 10 mL of water/methanol (1 + 1, v/v) overnight on a mechanical rotation device. Afterwards, the crude extracts were centrifuged for 25 min at 4500 rpm with a Hettich Rotina 420R centrifuge (Hettich, Tuttlingen, Germany) and supernatants were filtered through 25 mm Nylon™ syringe filters with a pore size of 0.2 μm (Markus Bruckner Analysentechnik, Linz, Austria). The crude extracts were stored in the dark at 4 °C prior to use.

2.4. Clean-up on DOWEX™ 50W and mass balance

Beetroot spiked with AB was used for the development of the clean-up procedure and the determination of the mass balance of the clean-up. A 150 mm glass Pasteur pipette was plugged with cotton wool and filled with wet conditioned DOWEX™ 50WX8-200 resin (suspended in 1 M hydrochloric acid) to a height of *ca* 5 cm, and washed with water until the effluent was neutral. The clean-up procedure for samples was as follows: A portion (1 mL) of crude extract was loaded on to the column. The "load" volume (1 mL; fraction 1) was collected in a 1.5 mL Eppendorf tube. Then, the column was washed with 10 mL of water/methanol (1 + 1, v/v; fraction 2) followed by 10 mL of an aqueous 1 M ammonia solution (fraction 3), each collected in 15 mL polypropylene tubes. Then, the column was washed with 3 mL of water/methanol (1 + 1, v/v), and regenerated with 5 mL of 6 M hydrochloric acid and 3 mL of 1 M hydrochloric acid. Before loading a new crude extract on to the column, 3 mL of water/methanol (1 + 1, v/v) were applied to remove excess H^+ from the column. The aqueous ammonia fractions from fish, CRM Dorm-2 or CRM Dorm-2 spiked with GB were evaporated under vacuum to dryness and the residues were weighed then dissolved in 500 μl (fish) or 5 mL (Dorm-2 and Dorm-2 spike) of water. The fish and Dorm-2 extracts were analysed by HPLC/ICPMS/ESMS with external calibration (calibration range: 1–300 μg As L^{-1} AB and 1–200 mg L^{-1} GB).

3. Results and discussion

To develop a HPLC method for the determination of GB and AB in biological samples capable of covering a broad range of concentration levels of both analytes, we investigated the simultaneous use of ESMS and ICPMS as detectors. The steps involved were (1) optimizing the HPLC conditions suitable for both GB and AB, and mass spectrometer