Abstract  The purpose of the study was to optimise analytical methods for determination of the chemical speciation of mercury in studies of protective mechanisms of selenium. Optimisation of the methods was performed using CRM DOLT-2 (Dogfish liver), both in its original form and after separation of various fractions. The sample was homogenised with 10 mM Tris-HCl buffer (pH 7.6) and ultracentrifuged. The soluble phase obtained was applied to a size exclusion chromatography column (Sephadex G-75 column) for separation of various protein fractions. Total mercury (total Hg), monomethyl mercury (MeHg) and selenium (Se) were determined in whole dogfish liver tissue and its soluble and insoluble phases (pellet). Different approaches for determination of total Hg and MeHg were compared. Simultaneous determination of MeHg and inorganic mercury (Hg\(^{2+}\)) was based on alkaline dissolution and/or acid leaching, followed by ethylation, room temperature precollection, isothermal gas chromatography (GC), pyrolysis and detection with cold vapour atomic fluorescence spectrometry (CV AFS). The sum of MeHg and Hg\(^{2+}\) was compared to total Hg results obtained by acid digestion and CV AAS detection. The accuracy of MeHg determination was checked by its determination using acid leaching at room temperature, solvent extraction, back extraction into Milli-Q water, ethylation, GC and CV AFS detection. For the insoluble phase it is recommended to use solvent extraction for MeHg and acid digestion CVAAS for total Hg. For determination of MeHg and Hg\(^{2+}\) in the lyophilised sample and water soluble fractions containing low concentrations of mercury species, the simultaneous measurement of MeHg and Hg\(^{2+}\) after alkaline dissolution is the most appropriate method.

1 Introduction

The capability of organisms to cope with harmful reactive metal ions in the environment is invariably linked to the existence of specific metal-binding proteins. Among them the low molecular weight proteins called metallothioneins (MTs) with SH-based clusters are believed to be important in short-term protection against toxic levels of some metal ions (Cd, Hg, Cu, Zn) [1], and in the case of mercury recent investigations have been focused particularly on selenoproteins [2].

For studies of metal metabolism the speciation of the element is very important. The total concentration of the element is of little value without knowledge of its chemical forms.

Mercury exists in a large number of different chemical and physical forms with a wide range of properties. The most toxic mercury compound is MeHg, the organic form of mercury. MeHg is mainly formed in the aquatic environment by biotic and/or abiotic processes. The accumulation of MeHg in biota, and its biomagnification in aquatic food chains are of particular concern due to its extreme toxicity and its ability to bioaccumulate in fish tissues.

During recent years many analytical techniques for determination of total Hg and MeHg in biological materials have been available. They involve, in most cases, a succession of analytical steps (extraction, derivatisation, separation, detection) which may all be prone to systematic errors [3]. Over ten years ago, these methods were poorly validated due to a lack of evaluation programmes and of certified reference materials. The situation improved recently thanks to the development of more sensitive and specific analytical techniques, the organisation of intralaboratory studies and the availability of certified reference materials (CRMs) [4].

The purpose of this work was to compare and optimise different analytical techniques in order to find the most suitable, simple, accurate and sensitive way of determining total Hg and MeHg in very diluted biological samples, where only small amounts of sample with a low concentration of MeHg are available. Experiments were performed on DOLT-2, Dogfish liver, a lyophilised certified reference material (CRM) produced by the National Research Council of Canada (NRCC). This reference material was selected in order to make our results comparable...
with those obtained in other laboratories engaged in similar studies. DOLT-2 is certified for total Hg, MeHg and Se, and contains relatively high concentrations of these metals.

2 Experimental

Reagents

1% (w/v) sodium tetraethylborate solution: prepared from NaBEt₄ (Strem Chemicals, Newburyport MA, USA) in Milli-Q water containing 1% KOH (analytical grade, Merck, Darmstadt, Germany); 2 M potassium acetate buffer: prepared from potassium acetate (extra pure, Merck) and acetic acid (Suprapur, Merck) in Milli-Q water; 25% (w/v) KOH in methanol solution: prepared from KOH (analytical grade, Merck) and methanol (SupraSolv, Merck); 6 M, 8 M HCl solutions: prepared from HCl (30%, Suprapur, Merck) in Milli-Q water; 5% SnCl₂ solution: prepared from SnCl₂ · 2H₂O (analytical grade, Merck) in Milli-Q water containing 3 M H₂SO₄ (Suprapur, Merck); 5% (v/v) H₂SO₄ solution: prepared from H₂SO₄ (Suprapur, Merck) in Milli-Q water; 18% (w/v) KBr solution: prepared from KBr (analytical grade, Carlo Erba, Milano, Italy) in Milli-Q water; 1 M CuSO₄ solution: prepared from CuSO₄ · 5H₂O (analytical grade, Merck) in Milli-Q water; CH₂Cl₂ (SupraSolv, Merck); HNO₃ (65%, Suprapur, Merck); Tenax (20/35 mesh, Alltech, Deerfield IL, USA); OV-3 15% (Sigma-Aldrich, Deisenhofen, Germany); argon; nitrogen; Milli-Q deionised water (> 18 MΩ cm, Millipore, Bedford MA, USA).

Standard solutions

1 ng/mL working standard solutions were used for MeHg (as Hg) and Hg²⁺ for calibration of the CVAFS system, and a 10 ng/mL Hg²⁺ working standard solution for CVAAS measurements. The standards were prepared in Milli-Q water by dilution of stock solutions. They were prepared daily and stored in the dark at 4 °C.

Cleaning procedure

Special precautions must be taken to avoid contamination. All glass and Teflon materials need to be cleaned carefully. All vessels were left to soak in a soap (Micro-90, Bioblock, Illkirch Cedex, France) solution overnight. They were thoroughly rinsed first with tap water then with Milli-Q water. The vessels were placed in 50% (v/v) concentrated HNO₃ solution and heated at 60 °C for 2 days. After being thoroughly rinsed with Milli-Q water, vials were transferred in 10% (v/v) concentrated HCl solution and left to soak for a further day at room temperature. They were thoroughly rinsed again with Milli-Q water, filled with 1% HCl and stored in polyethylene plastic bags. Vials were emptied just before use for sample processing.

Biochemical separation procedure

A schematic presentation of the biochemical separation is shown in Fig. 1. CRM DOLT-2 lyophilised liver tissue was used for separation of pellet and supernatant (cytosol) and for isolation of water soluble proteins with accompanying bound metals (Se, Zn, MeHg, Hg). Among the protein fractions, metallothionein protein was partly characterised by its molecular weight (MW), Zn content and the absence of a UV absorption peak at 280 nm.

For sample preparation, 2 g and 4 g of sample was homogenised (1:9 or 1:5.7 w/v) with a glass homogeniser and a motor-driven Teflon™ pestle (1000 rpm) in nitrogen-saturated Tris-HCl buffer (pH 7.6, at 1–4 °C), 10 mM diithiothreitol (DTT), 25% saccharose and 0.1 mM PMSF (phenylmethylsulfonyl fluoride). The 15 or 10% homogenate was centrifuged at 100 000 g for 90 min at 4 °C in a Centrikon T-2070 ultracentrifuge (Kontron instruments; rotor TFT 70.38). Supernatant was decanted from pellet and both parts were frozen until further analysis (metal determination, gel filtration).

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5 mL of the corresponding supernatant was applied to a calibrated 2.4 × 65 cm Sephadex G-75 column and eluted at 13.8 mL/h in a nitrogen atmosphere at 4 °C with 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM DTT. The UV absorption at 280 and 254 nm and the concentrations of metals (Se, Zn, Hg and MeHg) were determined in the column fractions (5 mL).

The column was standardised with marker proteins of known molecular weight (Pharmacia AB, gel filtration kit): blue dextran (MW 2000000), bovine serum albumine (MW 67000), chymotryptsinogen A (MW 25000), myoglobin (MW 17000) and cytochrome c (MW 12400). The exclusion range was from 70 kDa to 5 kDa.