Characterization of Metal Bound to Thiol Endogenous Ligands in the Liver of Rats Exposed to Methylmercury and Co-Exposed to Selenomethionine

C.M.L. Carvalho¹, Z. Pedrero², A.P.M. Santos¹, M.L. Mateus¹, Y. Madrid², C. Cámara² and M.C.C. Batoréu¹

¹Centro de Estudos de Ciências Farmacêuticas, Faculdade de Farmácia, Universidade de Lisboa, Portugal (Cristina.Carvalho@ff.ul.pt)
²Facultad Ciencias Químicas, Universidad Complutense de Madrid, España

Keywords: Methylmercury, Selenomethionine; Liver; Glutathione, Thiol groups, Proteins, Hepatotoxicity and Oxidative Stress
1. Introduction

The two major elements under study are very distinct as mercury (Hg) is a toxic heavy metal and selenium (Se) is an essential trace element that becomes toxic at higher doses. It has been established that the toxicity of mercury is mainly due to its ability to form stable complexes with the sulfhydryl cysteine groups of proteins and non-protein thiols, causing subsequent alterations in enzyme functions. The consequences are the increase in reactive oxygen species (ROS) and oxidative stress induction leading to cellular and organ injury.

Many studies related to Hg toxicity have been performed in the brain or in the kidney, however, the liver is the main organ involved in detoxification and the hepatic function should be implicated in the incorporation and transportation of this element to the target organs.

The liver is also considered the central organ of selenium metabolism. Most ingested selenium enters specific metabolism pathways after the uptake from the portal vein blood or removal from selenomethionine (SeMet) via the transsulfuration pathway, being this Se used for the synthesis of selenoproteins or excreted in urine in the form of metabolites. The selenoproteins that are synthetized by the liver include selenoenzymes that support liver function and Selenoprotein P that is released to the blood.

The selenoenzymes include glutathione peroxidase (GSHPx), glutathione reductase (GSH reductase) and thioredoxin reductases (TRs). Thioredoxin reductases such as TR1 (cytosol TR), thioredoxin glutathione reductase (TGR) and TR3 (mitochondrial TR) have been proven to exist in humans and animal models such as the rat and are responsible for the reduction of the active site disulfide in oxidized thioredoxin (protein thiol redox regulation); thioredoxin is approximately 500-fold more effective as a reducing substrate than GSH (Burk and Hill, 2005).

TGR is a selenoprotein oxidoreductase with specificity for thioredoxin and GSH systems (Sun et al., 2005) and exhibits also broad substrate specificity. Expression of TGR has been proven to be regulated by both selenium and tRNA status in liver. TR1 and TR3 were shown to have high priority for selenium supply, in comparison to stress-related selenoproteins such as GSHPx (Sun et al., 2005).

Selenium protective effects against mercury toxicity have been subject of discussion being the available results still controversial. In this process selenoproteins and glutathione anti-oxidant systems might play a key role in the cellular protection against pro-oxidants and this work aims to contribute for its understanding in relation to methylmercury (MeHg) toxicity.

2. Materials and Methods

In Vivo Study and Experimental Design

Twenty-four male Wistar rats (150-175g) supplied by Charles River Laboratories were kept for 1 week acclimatization period with food and water ad libid. After that they were divided in groups and were i.p. exposed during 2 weeks to 5 doses of 1.5 mg/Kg methylmercury hydroxide (MeHg) (G1), to 2 doses 1.5 mg/Kg MeHg (G2), to 2 doses of 1 mg/Kg selenomethionine from Sigma (SeMet) (G3) and co-exposed to 2 doses 1.5 mg/Kg MeHg and 2 doses of 1 mg/Kg SeMet (G4). Non-exposed rats were used as control (G5). On day 32 of the experiment (12 days after the last exposure) animals were sacrificed and livers were removed and kept at -20°C.

Methylmercury hydroxide was supplied by Alfa Aesar (Johnson Matthey, Germany) and seleno – DL-methionine by Sigma (Portugal).

Determination of Glutathione

To determine the GSH content, 0.5g of rat liver were homogenized with 4.5 ml 0.1M phosphate buffer at pH 7.4. To each homogenate were added 4.5 ml of 4% sulphosalicylic acid followed by
vortex mixing and centrifugation for 10 min at 2500 rpm. To 0.5 ml of the supernatant was added 0.05 ml DTNB (5,5-dithiobis-2-nitrobenzoic acid) and 4.5 ml 0.1M phosphate buffer pH 7.4 with vortex mixing; the absorbance at 412 nm was read after 2 min against blank solution of reactants. A standard calibration curve was prepared with 98% GSH solution from Sigma.

**Mercury Quantification**

Dried liver samples (~100 mg) were digested with 3mL of HNO$_3$ (65%) and 1mL of H$_2$O$_2$ (35%) from Panreac in an analytical microwave oven (CEM, 1000W MSP, Mattheus, NC, USA) with double-walled advanced composite vessels. The total Hg concentration was determined by external calibration of the signal obtained by the continuous Hg cold vapor system connected to atomic fluorescence spectrometer, AFS (Merlin 10.023, P.S. Analytical Ltd., UK) for 5 standard solutions. To extract the inorganic mercury, approximately 100mg of samples were treated with 5ml HCl 5M and then, sonicated for 15 min before being centrifuged for 10 min at 3500 rpm. The liquid phase was separated and diluted to 10ml before analysis by CV-AFS. The difference between the total amount of mercury and the inorganic form is assumed to correspond to the MeHg stored in liver.

**Total Selenium Quantification**

Dried digested samples as described in (3) were filtered and analyzed by ICP-MS for total selenium quantification. An inductively coupled plasma mass spectrometer (ICP) (HP-4500 Plus, Tokyo, Japan) was used as detector after the separation was performed by an HPLC pump (Milton Roy CM4000). Samples and standards for calibration were all analyzed in triplicate.

**Selenium speciation**

Samples for Se speciation were weighed in duplicate (100mg) with 20 mg protease type XIV from *Streptomyces griseus* (Sigma) and were added 3 ml 25mM Tris/HCl and 0.1M KCl pH 7.5. Digestion of samples was performed using a focused sonicator (Bandelin Sonoplus) for 120s with 20% of potency. The solutions were then centrifuged for 20 min with 7500 rpm at 20ºC. An inductively coupled plasma mass spectrometer (ICP) (HP-4500 Plus, Tokyo, Japan) was used as detector after the separation was performed by an HPLC pump (Milton Roy CM4000). The analytical peaks obtained were evaluated in terms of peak area by the standard additions method at m/z 82.

**Determination of elemental/protein fractions using SEC**

To attain the elemental/protein profile 0.5g of liver samples were added 3ml of 25mM Tris+0.1M KCl at pH 7.5 and homogenized in an ice bath. Homogenates were centrifuged for 30min at 4ºC using 14,000 rev/min. The supernatant was decanted and filtrated through 0.22 µm nylon filters. The samples were then kept in ice till chromatographic analysis by ICP-MS with an HP 4500 Plus and HPLC-UV (at 280 and 250 nm using spectrometer 5000 DAD LDC Analytical) for protein profile determination. Mobile phase with 25mM Tris and 0.05 mM KCl was set at pH 6.8 with HCl. Both analytical processes were carried out using a molecular size exclusion column Biosep-Sec-S 2000 from Phenomenex with 300×7.80 mm allowing identification of species in the range 1-300 kD. Calibration of this column with standards led to the following relation between retention time and molecular weight: \[ \log (MW) = (-0.4773) t_R + 5.5112. \]

The distribution of Hg, Se and other relevant elements such as Cr, Mn, Fe, Co, Cu, Zn, As, Cd, Pb, S, Mo and Ni in association to proteins was investigated.
3. Results and Discussion

The hepatic glutathione (GSH) content was evaluated at the sacrifice of animals (Table 1). The detrimental effects of MeHg in the liver are evidenced by the depletion of GSH with G1 presenting 78% and G2 88% of the GSH control content two weeks after the last MeHg administration. Total Hg accumulated in a dose-dependent manner in the liver and was not detected either by CV-AFS or by ICP-MS analysis in the non-exposed MeHg groups.

Table 1: Hepatic GSH content in the different groups of Wistar rats.

<table>
<thead>
<tr>
<th></th>
<th>GSH content (µmol/g liver)</th>
<th>STD</th>
<th>N</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.51</td>
<td>0.441</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>5 MeHg</td>
<td>6.62</td>
<td>0.951</td>
<td>4</td>
<td>77.8</td>
</tr>
<tr>
<td>2 MeHg</td>
<td>7.47</td>
<td>0.875</td>
<td>4</td>
<td>87.7</td>
</tr>
<tr>
<td>2 SeMet</td>
<td>7.67</td>
<td>1.09</td>
<td>3</td>
<td>90.1</td>
</tr>
<tr>
<td>2 MeHg+2SeMet</td>
<td>7.69</td>
<td>0.667</td>
<td>5</td>
<td>90.4</td>
</tr>
</tbody>
</table>

Fig. 1 shows that concentrations of Hg in liver 12 days after the last exposure are still high; G1 presented 15.8mg/kg and G2 6mg/kg (slightly above G4). This indicates that MeHg accumulates in liver. The quantification of inorganic Hg was carried out being the values between 0.9-1.8% in G1 and below the detection limit for the other groups. Thus, 98% of the mercury in liver is MeHg.

Total selenium quantification was carried out by ICP-MS and no significant differences were noticed (Fig.2) among the different groups, although the groups administered with SeMet displayed higher average values.

Speciation of selenium was also carried out and the only identified species were selenocysteine (SeCys) and SeMet, with SeCys representing 80-87% of total. Besides the area of peaks there were no significant changes in chromatograms and Fig. 3 shows the result obtained for the control group. Quantification results of the two species are displayed in Fig. 4 and 5.
The main variations occur in SeMet concentration (Fig.4) with the groups exposed (G1 and G2) or co-exposed (G4) to MeHg presenting a decrease in its content when compared with G5 and G3, respectively. Possible interpretations of these results include: 1) the presence of Se in the form of SeMet promotes the synthesis of selenoenzymes to hinder the detrimental effects of MeHg and increases SeMet consumption; 2) MeHg interacts with the selenol derived from the SeMet and accelerates the rate of conversion of SeMet decreasing its concentration. Previous works support these hypotheses.

The increase of hepatic Se concentration leads to Se induction of detoxificant enzymes such as GSHPx increase in aquatic birds (Hoffman, 2002). On the other hand there are reports that SeMet reacts with MeHg removing it partly (Seppanen et al., 1998). The proteins profiles attained with SEC and UV detection were similar with protein peaks overlapping. The typical profile is the first chromatogram displayed in Fig.s 6-9.

The distribution of S with the MW of proteins (Fig. 6) indicates a complex whole of three adjacent peaks that possibly includes the cellular proteins (200-300kD), selenoproteins and selenoenzymes (~50-60kD), and a group of smaller MW proteins 25-13kD that comprises thioredoxin (12kD) and enzymes such as GSHPx (22.2kD), GSH transferase (25.6kD), methionine sulfoxide reductase (19kD) and superoxide dismutase (15.8kD) among others. Small peaks were noticed at 3.5-5.5 kD, which might be metallothioneins.
that was exposed to the same doses of MeHg.

The glutathione conjugates such as \( \text{CH}_3\text{Hg-SG} \) complex (MW 0.49kD), catalysed by GSH-S-transferase, might have been formed in the cytosol of hepatocytes but once formed they are promptly excreted from cells, therefore 2 weeks after exposure they were not detected in liver (see Fig. 8 with Hg chromatograms). The association of the peak to GSSG also explains the increase of the peak in G4 as the reduction of hidroperoxides needs GSHPx (Se-containing enzyme) to form GSSG and the co-administration of SeMet may activate liver GSHPx as mentioned before.

However, the main difference in S distribution among the different animal groups was for a peak correspondent to a very small MW between 0.6-1.5kD. This peak is in the limit of SEC column applicability and the MW can not be expressed with more precision. It is displayed by all the groups but increases when there is exposure to MeHg especially in G1 (5 doses). Interestingly, the co-exposed G4 contains more of this low MW specie(s) than G2 that was exposed to the same doses of MeHg.

This <1kD peak can be attributed to GSH (0.3 kD) and to oxiglutathione (GSSG) (MW 0.61 kD), the dominance being to GSSG in G1-G4 since a depletion of GSH was noticed (Table 1). The glutathione conjugates such as \( \text{CH}_3\text{Hg-SG} \) complex (MW 0.49kD), catalysed by GSH-S-transferase, might have been formed in the cytosol of hepatocytes but once formed they are promptly excreted from cells, therefore 2 weeks after exposure they were not detected in liver (see Fig. 8 with Hg chromatograms). The association of the peak to GSSG also explains the increase of the peak in G4 as the reduction of hidroperoxides needs GSHPx (Se-containing enzyme) to form GSSG and the co-administration of SeMet may activate liver GSHPx as mentioned before.

Fig. 6: Chromatograms obtained by ICP-MS for sulphur fractions.

Fig. 7: Chromatograms obtained by ICP-MS for selenium fractions.
Moreover, it has been reported by other authors the pro-oxidative effects of selenium compounds and SeMet in particular, in rat liver (Farina et al., 2004; Hoffman, 2002) this justifying the synergic effect verified in group 4 towards the increase of GSSG.

Selenium is an essential element and intracellular concentrations of essential elements are normally maintained within a narrow range due to homeostatic control. As discussed above (Fig. 2) total selenium slightly varies in the different groups but the results are statistically similar. Also the chromatograms obtained with SEC (Fig. 7) maintain the main characteristics with a single poorly defined peak(s) in the range of 50-60kD.

Besides Selenoprotein P (50.5kD) and their isoforms, other selenoenzymes that support liver function such as GSH reductase (45.8kD) and thioredoxin reductase, TrxR (58kD) and thioredoxin/glutathione reductase, TGR, (65kD) are also included in this broad range. Some of these selenium-containing enzymes have been reported only in the last few years and their structure as well as their function is not fully known, but they might be a key for some of the detoxification mechanisms involved.

Mercury was not detected in G3 and in G5. In the remaining groups Hg interacts with high to medium MW cellular proteins of 300-40kD (Fig. 8).

Again the selenoproteins referred above on Se chromatograms, Selenoprotein P and selenoenzymes (GSH reductase, TrxR and TGR) have compatible MW and may be key proteins for Hg interaction due its high affinity for reduced sulfhydryl groups, including those of cysteine and GSH this being related to its transport by molecular mimicry (Clarkson, 1993; Ballatori, 2002).

The primary structure of selenoprotein P contains many potential redox centers in the form of cysteine (17) and selenocysteine (10) residues and this protein is predominantly produced by the liver with a rapid turn-over in rat plasma. Most authors associated Selenoprotein P with selenium transport but others suggest some anti-oxidant functions (Burk et al., 2003; Burk and Hill, 2005) and explain its rapid turnover to the fact that it could serve as an anti-oxidant molecule that cannot be reduced back to an active form. Due to the high histidine and cysteine content, selenoprotein P seems to be suitable to bind heavy metals such as mercury. According to this it was reported that the co-administration of selenite and mercuric chloride lead to the formation of a complex that binds to Se-P10 but works on that are scarce and still lack confirmation (Burk and Hill, 2005).

Fig. 8: Chromatograms obtained by ICP-MS for mercury fractions.

The most notorious association of mercury to liver proteins is showed by a sharp peak at 8.4 min for G1 and G2 and 8.8-9.0 for the G4 co-exposed group (Fig. 8), hence, the target proteins to
MeHg in liver seem to have a MW 16-32kD; in this range emphasis should be given to GSHPx (22.2kD) (hydroperoxide catabolism) and GSH transferase (25.6kD). Also superoxide dismutase (SDO) with 15.8kD and methionine sulfoxide reductase (MSrA) with 18.9kD can be considered. Until now there is evidence that MeHg inhibits GSHPx and a proposed mechanism is the direct chemical interaction of MeHg with selenolate at the active center of the enzyme, as the selenols(ates) are more reactive toward Hg than thiol(ates) (Farina et al., 2004).

Bando et al., 2005 also noticed that mercury (HgCl₂) induced liver responses including significant changes in endogenous antioxidant enzymes such as GSHPx, GR, G6PDH and SODs. TRs might also be target selenoenzymes to MeHg due to their structure. TR1 (TrxR) in rats is homologous of GSH reductase with a selenocysteine-containing carboxy-terminal elongation (Zhong et al., 1998) and that SeCys residue forms a redox active bridge with the neighboring Cys residue and such a Cys-SeCys bridge is present in the oxidized form of TrxR. The reduced form presents wide substrate specificity unlike GSH reductase. Inactivation of TrxR has been noted with low MW electrophilic compounds.

Since some of these enzymes are Se-containing proteins and the fact that the administration of SeMet has a synergic effect on oxidative stress justifies the magnitude of the sharp peak in different groups: G1-320; G2-125 and G4-800, although the experiment was not performed with quantifiable purposes but just to identify the species involved.

Concerning the Zn distribution (Fig. 9) G1 and G2 have a similar profile although different from G3-G5 groups, which chromatograms overlap. The difference is in the peak eluted at 9.6 min that corresponds to a MW around 8.5kD and the profiles show that MeHg administration removes Zn from this group of proteins except when SeMet is supplied. The MW is compatible with metallothioneins MT1-MT4 with 6 to 10kD. Furthermore, it was established by Leiva-Presa et al., 2004 that MeHg⁺ cation replaces Zn(II) in recombinant mammalian MTs with the concomitant unfolding of MTs. This fact corroborates our observation of Zn displacement from low MW proteins as well as explains the reason why Hg was not found associated to MTs.

![Fig. 9: Chromatograms obtained by ICP-MS for zinc fractions.](image)

### 4. Conclusions

Methylmercury accumulates in liver and cause oxidative stress associated to thiol depletion. Cellular responses of hepatocytes involve the activation of antioxidant defensive mechanisms, such as enzymes, scavengers of radicals or reductants. MeHg seems to interact strategically with some enzymes like GSHPx and GSH reductase, thioredoxin reductases and affect their activity, the result being the overwhelming of antioxidant defense mechanisms. Nevertheless, the role of some
selenoproteins and/or seleno-dependent enzymes is not well established nor on the detoxification process or on the protection against oxidative stress. This work reinforces the pro-oxidant effect of MeHg in rat liver and based on the multi-elemental association to proteins proposes some explanations to improve the understanding of molecular mechanisms underlying the toxic effects of MeHg as well as the interference of selenium on this process. This study is also a start-point for the search of metallomic biomarkers that could be useful to clarify the mechanisms of action and to detect early effects of toxicity.

Acknowledgements


REFERENCES


