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Title: Biological interactions between mercury and selenium in distribution and detoxification processes in mice under controlled exposure. Effects on selenoprotein.

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Abstract: Antagonistic interactions between mercury (Hg) and selenium (Se), were evaluated in mouse (Mus musculus), as a mammalian model, in a series of controlled exposure experiments. The beneficial effect of Se against Hg toxicity involves a variety of biochemical and toxicological processes that have not been clarified yet. For this purpose, a metallomic workflow based on the use of size-exclusion chromatography (SEC) with inductively coupled plasma mass spectrometry (ICP-MS) detection was complemented with the speciation of selenoproteins and low molecular mass selenium species in serum and liver cytosolic extracts using a multidimensional approach based on SEC-AF-HPLC-ICPMS, using species-unspecific isotope dilution (SUID)-ICP-MS for selenium quantification. The results showed potential interactions between Hg/Se in organs and serum related to accumulation and detoxification processes, in addition to the effects of mercury on selenoproteins in hepatic cytosolic extracts and bloodstream when both elements are administrated at the same time. These results provide information about elements distribution, interactions and homeostasis and reveal the potential of metallomic approaches in exposure experiments.

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Dear Editor,

Please find enclosed the amended version of the manuscript "BIOLOGICAL INTERACTIONS BETWEEN MERCURY AND SELENIUM IN DISTRIBUTION AND DETOXIFICATION PROCESSES IN MOUSE MUS MUSCULUS UNDER CONTROLLED EXPOSURE. EFFECTS ON SELENOPROTEINS" by M.A. García-Sevillano, G. Rodríguez-Moro, T. García-Barrera*, F. Navarro and J.L. Gómez-Ariza* to be published in Chemico-Biological Interactions. All the corrections have been marked in red in the new version of the manuscript and a detailed point-by-point response to each comment raised in the review is also provided. All the comments have been taken into account and the new version of the manuscript has been considerably improved.

Sincerely yours,

José Luis Gómez Ariza

Response to the comments

Editor Comments:

Your manuscript entitled "Biological interactions between mercury and selenium in distribution and detoxification processes in mice under controlled exposure. Effects on selenoprotein." has been reviewed by two well-qualified referees knowledgeable in this area of research. The Section Editor handling the review of your paper, has recommended to me that the manuscript should be acceptable, pending suitable revision, for publication in Chemico-Biological Interactions. I have read the manuscript and concur with their recommendation.

In general, the reviewers felt that your paper presented some interesting data, but they did raise some concerns that somewhat dampened their enthusiasm. The comments made by the reviewers are relatively straightforward and should be easily addressed. Some of the comments revolve around methodological questions whereas other comments pose questions based on your data.

Reviewer Comments:

Reviewer #1: I thank the authors for revising their paper to a considerably higher standard. The Tables and Figures are much clearer, and the text has few remaining errors.

Most important is to correct the Figure 1 legend, in which metal concentrations are alleged to be significantly different from controls ($p<0.05$), which can only refer to Se, since the control levels for Hg were beneath detection limits, and cannot be used for statistical analysis. (Assuming that the levels are the same as LOD is invalid.).

Legend of figure 1 has been corrected

Additional errors, requiring correction:

p.11, Introduction, 2nd sentence, "Mercury exists in three main chemical forms: elemental, inorganic, and organic;..." cannot be correct: mercury is not organic. A correct listing could be "elemental, as an inorganic salt, or bound to an organic ligand;..."

Under your suggestion, this sentence was replaced in the revised version of the manuscript.

p.11, next sentence, "thiol-binding elements" (not "element").

This mistake was replaced in the revised version of the manuscript.

p.11, same paragraph, "the essential character of Se has distinct limitations, toxic effects have been found in areas with either low or high levels of this element," is misleading: all essential elements have the potential for either deficiency (too low) or toxic (too high) health effects. An appropriate wording might be,"Se is an essential element in mammalian health, with both deficiency and toxicity effects apparent, depending on soil content, due to a fairly narrow range of optimal Se intakes."

Under your recommendation, this sentence was replaced in the revised version of the manuscript.

p.14, last line, please provide a reference for the LD50 cited [which does not agree with J.D. Park et al. (2001), Toxicology 163: 93-100].

Under your comment, we have checked the LD50 and this mistake has been corrected in the new version of this manuscript.

p.17, 2nd para., "Hg administered alone or together with Se significantly altered the level of mercury in the examined organs and serum of mice ($P<0.05$)," is misleading, since it implies that levels of Hg were significantly different from controls, but this cannot be the case, since control levels of Hg were <LOD, and can therefore not be used to determine significance. The best that can be concluded here, is that "Hg administered alone or together with Se appeared to lower the level of mercury in kidneys and increased the levels in liver and sera of Se-treated mice, compared to those that received Hg alone."

Under your recommendation, this sentence was replaced in the revised version of the manuscript.

p. 23, last line before Conclusions, "causes" is too much of a stretch of the imagination; please use 'was accompanied by," instead, as this accurately reflects your observations.

Under your suggestion, this word was replaced in the revised version of the manuscript.

p.31, Legend for Figure 1 must be corrected! Significant differences may have been detected for Se, for treated groups vs. control groups; however, they could not have been determined for

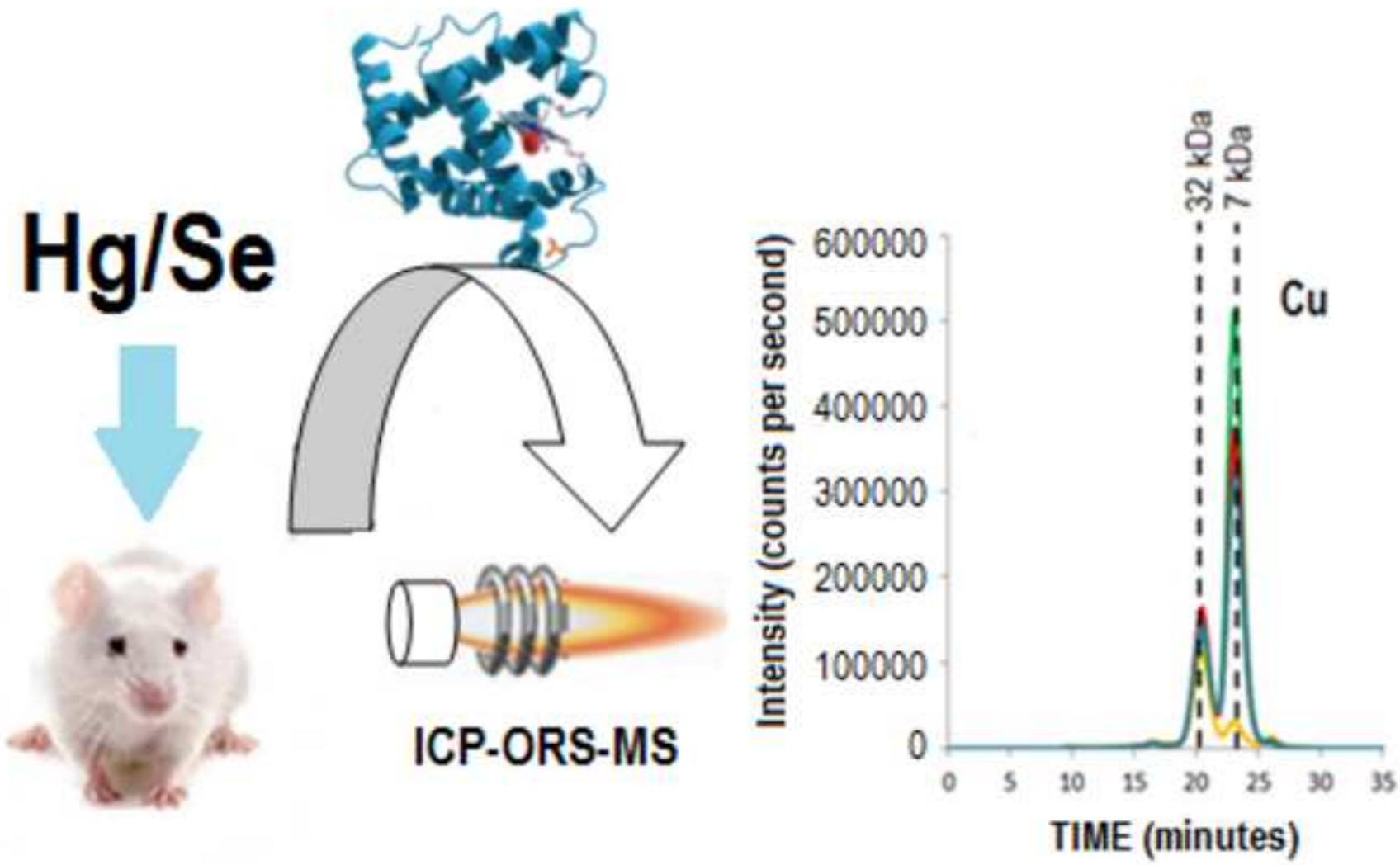
Hg (see above). A better solution would be to re-evaluate the statistical significance, comparing Hg+High-Se and Hg+Low-Se treatment, vs. Hg only. Based on the data in Table 2 [which can now be read clearly - thank you!], you may have some interesting results to report. Also, "subcutaneous administration" is the correct phrase (or 'subcutaneously administered dose', if you prefer).

The value used for Hg in control group was LOD for statistical analysis, but under your affirmation, this sentence has been depleted in the new version of this manuscript

Reviewer #2: This revised manuscript has been greatly improved by improving figures, adding the amount of Hg and Se used as supplements, and by correcting English errors. The authors have successfully and carefully addressed all the questions from the Editor and three reviewers. This reviewer does not have additional comments.

Thank you very much.

Graphical Abstract (for review)



***Highlights (for review)**

The most remarkable contributions of the present paper are summarized in the following points:

- Antagonistic interactions between Hg/Se related to accumulation and detoxification.
- Selenoproteins may have two important roles in protecting against Hg toxicity.
- Selenium affects the redistribution of Hg and induces binding to inert complexes.

Biological interactions between mercury and selenium in distribution and detoxification processes in mice under controlled exposure. Effects on selenoprotein.

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ABSTRACT:

Antagonistic interactions between mercury (Hg) and selenium (Se), were evaluated in mouse (*Mus musculus*), as a mammalian model, in a series of controlled exposure experiments. The beneficial effect of Se against Hg toxicity involves a variety of biochemical and toxicological processes that have not been clarified yet. For this purpose, a metallomic workflow based on the use of size-exclusion chromatography (SEC) with inductively coupled plasma mass spectrometry (ICP-MS) detection was complemented with the speciation of selenoproteins and low molecular mass selenium species in serum and liver cytosolic extracts using a multidimensional approach based on SEC-AF-HPLC-ICPMS, using species-unspecific isotope dilution (SUID)-ICP-MS for selenium quantification. The results showed potential interactions between Hg/Se in organs and serum related to accumulation and detoxification processes, in addition to the effects of mercury on selenoproteins in hepatic cytosolic extracts and bloodstream when both elements are administrated at the same time. These results provide information about elements distribution, interactions and homeostasis and reveal the potential of metallomic approaches in exposure experiments.

Keywords: *Mus musculus, mercury, selenium, metal interactions, metallomics, isotopic dilution analysis, selenoproteins.*

ABBREVIATIONS: AF - affinity chromatography; BSA - bovine serum albumin; CRMs - Certified reference materials; Cys - cysteine; DNA - Deoxyribonucleic acid; eGPx - extracellular glutathione peroxidase; GPx - glutathione peroxidase; GSH - reduced glutathione; HMM - high molecular mass; HPLC - High performance liquid chromatography; ICP-MS - Inductively coupling plasma-mass spectrometry; IDA - isotopic dilution analysis; LC - liquid chromatography; LMM - low molecular mass; MS - mass spectrometry; MT - metallothionein; ORS - octopole reaction systems; PMSF - Phenylmethanesulfonyl fluoride; RBCs - red blood cells; SeAlb - selenoalbumin; SEC - size exclusion chromatography; SelP - selenoprotein P; SeCys - selenocysteine; SUID - species-unspecific isotopic dilution; SOD - superoxide dismutase; ThxR - thioredoxin reductase; TCEP - tris(2-carboxyethyl)phosphine hydrochloride; 2D - two dimensional.

1. INTRODUCTION

Mercury (Hg) is a widespread environmental and industrial contaminant that induces rigorous toxic effects in body tissues of both human and animals [1], depending on its different chemical forms [2]. Mercury exists in three main chemical forms: elemental, as an inorganic salt, or bound to an organic ligand; although all the chemical species are potentially toxic. Divalent inorganic mercury (Hg^{2+}) is one of the strongest thiol-binding elements, which explains its toxicity by the ability to form stable complexes with the sulfhydryl-cysteine groups of proteins (-SH), such as numerous thiol-related enzymes [3]. Hg^{2+} can also give rise to free radicals that induce lipid, protein and DNA oxidation related to oxidative stress [4-6]. On the other hand, organic mercury compounds are mostly metabolized in the liver where they may suffer demethylation [7] or undergo conjugation reactions with glutathione (GSH) [2,8]. It is well known that Se presents a protective effect against Hg toxicity and inhibits oxidative damage caused by Hg in mammals [9-11]. The antagonistic interaction between Hg and Se was first reported in 1967 in rats treated with mercury chloride and selenite [11]. All the same, Se is an essential element in mammalian health, with both deficiency and toxicity effects apparent, depending on soil content, due to a fairly narrow range of optimal Se intakes [12-14]. However, it has been reported that simultaneous exposure to both Se and Hg compounds increased whole-body retention of Hg, possibly due to the formation of inert Se-Hg complexes, which has been considered as a preventive mechanism against Hg toxicity [11,15]. Nevertheless, although the Se and Hg co-accumulation in humans and other mammals is well known, the mechanism of interaction between these elements is still unsolved.

Hg^{2+} can also react with selenol groups (-SeH) that constitute a part of selenocysteine (SeCys), and as a consequence they can be incorporated to selenoproteins, prosthetic groups of selenoenzymes and peptides [16-17], since the -SeH in SeCys shows even stronger affinity for Hg that -SH groups, possibly due to the lower pKa of SeCys (~5.4) that provides it a higher reactivity than Cys (pKa~8.0). Moreover, selenoproteins play an important role in the maintenance of cellular homeostasis [18]. In the same way, Hg^{2+} can also react with selenides

(Se^{2-}) and hydrogen selenide or selenols to form Hg-Se-S complexes together with glutathione that can finally bond to selenoprotein P (SelP) forming a ternary complex in the bloodstream [19-21]. Se is an essential component of several selenoenzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (ThxR), which also contains Se as SeCys. In that respect, the perturbation of selenoproteins functions has been related to the development of several diseases such as carcinogenesis [22-23].

Although at the moment there are very powerful analytical techniques and sample preparation procedures for element speciation, biological systems require multi-elemental analytical approaches that make possible to characterize processes involving metals interactions, trafficking and homeostasis [24]. For this purpose, metallomics is a relatively new field to decipher changes in metal-biomolecules expression and identification in biological matrices. In this sense, it is necessary to consider that approximately one third of proteins need the presence of metals as cofactors to develop their function [24-27]. On the other hand, ICP-MS is a valuable technique in this field since it allows multi-isotopic analysis, detection capability, high sensitivity, tolerance to matrix and large linearity range of quantification [24], generally coupled to liquid chromatographic arrangements, and parallel molecular mass spectrometry for biomolecules identification in an integrated workflow [25-26]. As a consequence, metallomics provides a good option to deep insight into the fate of elements in exposed organisms to elements, and provides information about element distributions, retentions and interactions [28].

The aim of the present study was to characterize the biochemical response of mice *Mus musculus* in metal distribution and detoxification processes caused by simultaneous mercury exposure and selenium exposure based on suitable metallomic workflows. The metallomic approach included the use of size exclusion chromatography (SEC) coupled to ICP-MS as detector. In addition, the study was complemented by the speciation of selenoproteins and low molecular mass selenium species in serum and liver cytosolic extracts of mice based on species-unspecific isotope dilution (SUID)-ICP-ORS-MS on-line coupled to a SEC in tandem with an AF that integrate the analytical speciation platform.

2. MATERIALS AND METHODS

2.1. Instrumentation

The mineralization of samples to determine the total metal content in biological matrices was carried out by using a microwave accelerated reaction system model MARS (CEM Corporation, Matthews, Carolina del Norte, USA) and MARSXpress vessels.

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770) was used to prepare tissues homogenates. Metal-containing biomolecules were separated and detected with an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan) which was equipped with an octopole reaction system (ORS). Liquid chromatography separations were performed using a Model 1100 HPLC pump with detector UV (Agilent, Wilmington, DE, USA). ICP-MS conditions (Table 1) to measure in He mode were optimized using a HNO_3 5% (v/v) aqueous solution of ^{59}Co, ^{89}Y, ^{205}Tl (1 µg L^{-1}). A microflow nebulizer (Teflon; model ESI, Ohama, USA) was used to establish the hyphenation of the LC-ICP-ORS-MS system.

2.2. Standard solutions and reagents

All reagents that were used for sample preparation in the metallomic approach were of the highest available purity. Phenylmethanesulfonyl fluoride (PMSF) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (BioUltra grade, >98%) were obtained from Sigma Aldrich (Steinheim, Germany). Helium and hydrogen, which were used as collision and reaction gas (in the ICP-ORS-MS system), were of high-purity grade (>99.999%).

The standards which were used for mass calibration of analytical SEC columns (mass range 600-10 kDa) included horse ferritin (440 kDa) (purity 95%), bovine serum albumin (67 kDa) (purity 96%) and superoxide dismutase containing Cu and Zn (32 kDa) (purity > 70%). All reagents were purchased from Sigma-Aldrich (Steinheim, Germany). On the other hand, metallothionein (MT) containing Cu, Zn and Cd (isolated from rabbit liver) (purity 95 %) was purchased from Enzo Life Sciences (Madrid, Spain). The mobile phase buffer used for SEC was

50 mM of ammonium acetate (Suprapure grade) purchased from Merck (Darmstadt, Germany), which was prepared daily with ultrapure water (18 MΩcm) from a Milli-Q system (Millipore, Watford, UK). The pH was adjusted to pH 7.4 with ammonia solution, which was prepared by dilution of 20% (w/v) ammonia solution (Suprapur, Merck) with ultrapure water. The void volume was determined by injecting ferritin (440 kDa).

Human serum certified reference material (CRM) BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). A standard solution of 1000 mg L^{-1} of Se stabilized in 5% (v/v) nitric acid Suprapur and of 1000 mg L^{-1} of bromide stabilized in 5% (v/v) nitric acid Suprapur was purchased from Merck (Darmstadt, Germany). Enriched ^{74}Se powder was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) and dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to volume with ultrapure water.

2.3. Animals and experiment exposure

Mus musculus (inbred BALB/c strain) mice were obtained from Charles River Laboratory (Spain). Mice of 7 weeks of age were fed *ad libitum* with conventional pellets (rodent global diet from Harlan Laboratories Inc. Indianapolis, IN 46250, USA). The animals were allowed to acclimate for 5 days with free access to food and water under controlled condition (temperature (25-30ºC) and a 12 h light-dark cycle) prior to exposure.

Mice were randomly divided into four groups: one control and three treated groups including Hg group, Hg+Low-Se group and Hg+High-Se group. Hg, as mercuric chloride ($HgCl_2$) and Se, as selenite were subcutaneously and orally exposed, respectively, every day at different doses. Hg group was given a subcutaneously administration of 0.2 mg/kg bw/day $HgCl_2$. Hg+Low-Se and Hg+High-Se groups were given a subcutaneously administration of 0.2 mg/kg bw/day of $HgCl_2$ and supplemented orally with 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite, respectively. The control mice were subjected to subcutaneously administration of 100 µL of ultrapure water with 0.9% NaCl per day for 10 days. The acute subcutaneous mercury chloride dose of 0.2 mg kg^{-1} bw/day was selected taking into account the

LD_{50} for mice (3 mg kg^{-1}) which was not exceed the LD_{50} for the overall exposure experiment (10 days). On the other hand, selenium supplements of 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite were selected taking into account the recommended level of dietary selenium and about 3 times more, respectively.

Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, dissected using a ceramic scalpel and finally livers and kidneys transferred rapidly to dry ice. Liver and kidney are main targeted tissues for Hg as they are involved in its metabolism. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% NaCl solution, frozen in liquid nitrogen and stored at -80 ºC until they were used for extract preparation. Serum was obtained by centrifugation (4000g, 30 min, 4ºC) of whole blood after incubation during 30 min at 37 ºC in the dark. All animals received humane care in compliance with the guidelines of the animal care and use of the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

2.4. Total metal determination in organs and serum of mice by ICP-ORS-MS

For the determination of total metals in the organs and serum, individual samples were weighed (0.100 g) in 5-mL microwave vessels and 0.5 mg of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v) was added. After 10 min, the PTFE vessels were closed and introduced into the microwave oven. Mineralization was carried out at 400 W from room temperature ramped to 160 ºC for 15 min and held for 20 min at this temperature. Then the solutions were made up to 2.0 g with ultrapure water and the metals were analyzed by ICP-ORS-MS. Rhodium was added as internal standard (1µg L^{-1}). All analyses were performed in triplicate. The ICP-ORS-MS conditions are depicted in Table 1.

2.5. Analysis of mouse tissue cytosolic extracts and serum using SEC-ICP-ORS-MS

Serum and organs from 6 individual mice (male) per group were pooled and treated following a procedure described elsewhere [28]. Briefly, the metal-containing biomolecules were extracted with a solution (3 mL g^{-1}) containing 20 mM ammonium acetate buffer solution at pH 7.4, 1 mM TCEP (tris-2-carboxyethylphosphine) and 1 mM PMSF (phenylmethylsulfonyl

fluoride) using a glass/teflon homogenizer in a cold chamber at a constant temperature of 4 ºC. Then the extracts were centrifuged at 120,000 g for 1 h at 4 ºC. The obtained extracts were stored under nitrogen atmosphere to avoid oxidation at -80 ºC until analysis and filtered through Iso-disc Poly-vinylidene fluoride filters (PVDF, 25-mm diameter, 0.45-µm pore size) (low protein adsorption) to avoid column overloading or clogging. The SEC-ICP-MS on-line coupling was performed by connecting the outlet of the chromatographic column to the Microflow nebulizer inlet ESI (Ohama, USA) of the ICP-ORS-MS by means of PEEK tubing (0.6 i.d. mm). The quality control of the SEC-ICP-ORS-MS system to overcome problems related to contamination, loss and stability of species has been described elsewhere [28]. Separation of the analytes was performed with an analytical size exclusion column Superdex^{TM}-200 (10 mm x 300 mm x 13 µm) (GE Healthcare, Uppsala, Sweden) with an exclusion limit of 600 kDa (effective separation range from 10 to 600 kDa). Details pertaining to the chromatographic separation and the ICP- MS conditions are depicted in Table 1.

2.6. Quantification of selenium containing proteins and selenium metabolites in mouse serum and cytosolic extracts of tissues by 2D/SEC-AF-HPLC-(SUID)-ICP-ORS-qMS using unspecific-species isotopic dilution analysis.

For this purpose, 10 µL of 100 mM of PMSF and 100 mM of TCEP mixture were added to 100 mg of serum as proteases inhibitor and a reducing agent, respectively. To avoid changes of the Se species, serum samples were directly injected on to the column, without prior dilution. Liver cytosolic extracts were prepared as previously explained for SEC-ICP-ORS-MS. The fractionation of Se-containing proteins and selenometabolites was performed by two dimensional chromatographic separations based on SEC prior to the double AF column using ICP-ORS-MS as detection system (2D/SEC-AF-SUID-ICP-ORS-MS) following a procedure described elsewhere [29]. In brief, this involved two 5 mL HiTrap® Desalting Columns (GE Healthcare, Uppsala, Sweden) that were connected in series with a dual affinity column arrangement comprising a 1.0 mL heparin-sepharose column (HEP-HP) (GE Healthcare, Uppsala, Sweden) and a 1.0 mL blue-Sepharose column (BLU-HP) (GE Healthcare, Uppsala, Sweden), interconnected with a six-way switching column valve. The HiTrap column is a size

exclusion chromatography column that removed low molecular mass components (MW<1000Da) from high molecular mass molecules, such as DNA, proteins or peptides. The combination of these two columns increased the chromatographic resolution. The chromatographic separation parameters and the ICP-MS conditions are displayed in Table 1.

3. RESULTS AND DISCUSSION

3.1. Multielemental determination in serum and organs from mice exposed to mercury/selenium by ICP-ORS-MS.

Copper, zinc, selenium and mercury concentrations in important metabolic organs (liver and kidney) and serum of mice exposed to Hg/ Se were quantified and the results are presented in Table 2 and for a better visualization in Fig. 1. Recovery experiments were performed by spiking the extracts with 1, 5, 10 or 50 ng g^{-1} of metal, depending on the relative concentration of each metal in the extracts. Confirmation of quantitative recovery was obtained in all cases, with values ranging between 86-114 %. Instrumental limits of detention (LOD) are also provided in table 2.

Hg administered alone or together with Se appeared to lower the level of mercury in kidneys and increased the levels in liver and sera of Se-treated mice, compared to those that received Hg alone. Some authors have reported the reduction of Hg accumulation in kidney in the presence of Se, and correlatively the increases of Hg presence in the remaining body, especially in liver and serum [9,30]. These results are in good agreement with our findings (Table 2). In previous a work, Hg showed a non-uniform distribution after absorption, being accumulated mainly in mouse kidney after similar experimental conditions [31]. Therefore, our results show that accumulation of Hg in liver and serum of mice exposed to this element together with Se remains high, although other studies claims for the decrease of Hg in the presence of Se. Possibly, Se minimizes the toxic action of mercury enhancing the antioxidant ability of organisms and increasing contents of the antioxidants, as has been checked in rats [9,32]. On the other hand, beneficial action of Se can be attributed to the formation of non-toxic Se-Hg complexes that has been identified as a process involved in the prevention of Hg toxicity

by Se [11,15,31,33]. However, although Se and Hg co-accumulation in humans and other mammals is well known, the mechanism of interaction between Se and Hg is still unclear.

Se concentration in liver, kidneys and serum of mice only exposed to Hg was increased about 75 % and 11 %, respectively, however decreased about 12 % in serum in comparison with the control group (Table 2). This decrease of Se levels in serum was also observed in a preliminary study with *Mus musculus* subcutaneously exposed to a low dose of mercury (0.1 mg/kg bw/day $HgCl_2$) [31]. In this study similar trends were obtained for an $HgCl_2$ dose of 0.2 mg/kg bw/day (Table 3). The distribution of Se was significantly different between Hg+Low-Se and Hg+High-Se groups. Table 2 shows increasing of Se concentration in liver, kidney and serum, when the intake of this element increases.

Additionally, it is well known that Hg administration provokes perturbations in the distribution of Cu and Zn [31,34]. In this experiment, Cu accumulation in the kidneys and serum of Hg-exposed mice was perturbed about +101 % and -7 %, respectively compared with the control group. Changes in metals distribution is also observed in mice simultaneously exposed to Hg and Se, this later at different doses (Table 2). It is remarkable the decreased concentration of Cu in liver of Hg-exposed mice (about -12%) when high concentration of Se was administered (0.50 mg/kg bw/day of selenite) (Table 2). Alternatively, when Zn presence was evaluated in the different organs (liver and kidney) and serum, zinc concentrations change about +6 %, +32 % and +32 %, respectively, compared with the control group. The distribution of Cu, the Zn did not change significantly although Se was administered together with Hg at different doses (Table 2). These results agree with previous studies in mice subcutaneously exposed to lower dose of Hg [31].

3.2. Characterization of metal-biomolecules distribution in liver, kidney and serum extracts from mice under inorganic mercury and selenium exposure using SEC-ICP-ORS-MS.

For a better understanding of the antagonistic interaction between Hg and Se, the protein binding pattern distribution of Hg in hepatic and renal cytosol from different groups were analyzed to evaluate the biological response of mice exposed to Hg and Se for 10 days

based on the use of SEC-ICP-ORS-MS. Organs with high metabolic activity such as liver, kidneys, and fluids as serum were used for this purpose. The most interesting results were observed in relation to Cu, Zn and Hg. The figure 2 shows the Hg-traced chromatogram obtained by SEC-ICP-ORS-MS using a column with mass resolution ranged between 10-600 kDa. The relative presence of mercury reflected by the peaks intensity is in accordance with the total mercury content in the different biological matrices under consideration (Table 2).

In liver (Fig. 2A), exposure to Hg and Se for 10 days increased the intensity of three Hg-traced peaks, one with retention time at 11 minutes matching with the dead volume of the effective range of separation of the column (Superdex^{TM}-200), which reflects the binding of Hg to high molecular mass proteins (HMM), due to the high affinity of mercury to thiol groups at the moiety of proteins and endogenous metabolites including glutathione (GSH) or cysteine (Cys) [2,8]. In that respect, the proteins with higher molecular mass usually contain more Cys residues than proteins with lower molecular mass (LMM). The higher concentration of mercury in liver of Hg/Se-treated mouse (table 2), may explain the presence of this peak. A second Hg-containing peak matches well with that of Cu,Zn-BSA standard (17.5 min) (Fig. 2A). The intensity of Hg in this fraction decreases when the dose of Se increases, exhibiting a maximum when Hg is administrated alone. In this sense, it is well known that Hg forms stable complexes with albumin in human serum [35]. Furthermore, the high electrophilic character of Hg^{2+} enhances the bound to Cys or SeCys in different proteins/metalloproteins, such as Se containing proteins with similar molecular mass. Furthermore, selenoproteins play an important role in Hg detoxification, mainly selenoprotein P (SelP), since it contains 6-10 SeCys groups and the high reactivity of selenol groups increases the possibility of Se-Hg bound [31]. Finally, it can be observed in Fig 2A a third peak matching with Cu,Zn-SOD standard (32 kDa). The intensity of this peak is similar in the three groups exposed to Hg (Fig. 2A). However, the intensity of peak corresponding to putative Hg-SOD significantly increase with respect to control. This fact could be related with decreased enzymatic activity of SOD in hepatic cytosolic extracts observed in rats under Hg administration [36], even when Se is ingested simultaneously [37].

Two remarkable peaks are obtained by SEC-ICP-ORS-MS in mouse serum exposed to Hg/Se (Fig. 2B). In this case the SEC-chromatogram shows similar distribution to liver cytosolic extracts (Fig. 2A). A first Hg-peak related to Hg-attachment to HMM proteins rich in Cys residues (Fig. 2B), and a second peak, at about 67 kDa, related to transport proteins and selenoproteins, as discussed above for liver.

Alternatively, metallobiomolecules associated with Cu, Zn and Hg in kidney cytosolic extracts are shown in Fig. 3. The peaks traced by Hg (Fig. 3C) show higher intensity of peaks in kidney than in liver and serum which agree with total levels of this element in Fig 1. It is remarkable an Hg traced predominant peak at about 7 kDa matching with metallothionein standard (Cu,Zn,Cd-MT) (Fig. 3). Analogous peaks traced by Cu and Zn can be observed in Fig. 3A and 3B. These molecules are associated to metal detoxification processes, because MTs are cysteine-rich proteins [38]. In this sense, MTs are known as storage depots for metals such as Cu and Zn; indeed, these proteins scavenge sulfhydryl reactive metals that enter the cells, although they have a higher affinity for Hg than Zn or Cu, in this case metal-MTs are excreted by urine that constitutes a detoxification mechanism for Hg [39]. Plus, it has been reported that co-administration of selenite and Hg reduces urinary excretion of mercury in rats [40-42], which agrees with results in Fig. 3A, 3B and 3C that show a lower intensity of peaks traced by Cu, Zn and Hg, respectively, in the presence of Se, which seems to inhibit MTs production [30]. The inhibiting effect of Se in MTs triggering increases with the dose of Se (Fig. 3).

3.3. Characterization of selenoproteins distribution in liver cytosolic extract and serum from mice under inorganic mercury and selenium exposure using 2D/SEC-AF-SUID-ICP-ORS-MS.

In mammalian bloodstream, Se is mainly present in form of four selenium containing molecular entities, namely SelP [29], eGPx [43], SeAlb and free selenite [44]. In liver other selenoproteins can also be found, such as thioredoxin reductase (TrxR), a redox selenoenzymes with a molecular mass of 54 kDa that reduce thioredoxin (Trx) [45]. In that respect, some of these selenoproteins contains several SeCys and Cys residues, such as SelP [46], and could play

an important role as a vehicle for Hg detoxification. Therefore, a decrease in plasma SelP after Hg exposure has been reported by other authors [10]. In addition, GPx represents a family of antioxidant enzymes whose main function is to counteract the adverse effect of hydrogen peroxide and lipid hydroperoxides [17,47]. Related to this, a decreased activity of GPx in liver has been reported in mice after Hg exposure when a single dose of 20 μmole/kg bw was intraperitoneal injected [37]. On the other hand, it is well known that low molecular mass (LMM) selenium-species (such as selenite) are required for the synthesis of selenoproteins in liver, which are then transported to bloodstream [48]. The selenite in the bloodstream is readily taken up by red blood cells (RBCs) [49] and reduced to selenide (SeH^-), for latter effluxion into the bloodstream in the presence of albumin and transferred to the liver in the form of SeAlb for the synthesis of selenoproteins [48]. In contrast, selenate is not taken up by RBCs, but it interacts with hepatocytes and is used for the synthesis of SelP and eGPx, which are released into the bloodstream for final partial excretion into urine [21]. In this way, significantly decreased levels of LMM selenium species and correlatively increased SelP levels in plasma of Hg-treated mice were observed when 0.1 mg/kg bw/day of $HgCl_2$ was supplied alone [31]. These results suggest that LMM selenium species were initially consumed in the Hg detoxification process, to supply the requirements in SelP for Hg redistribution in the form of Hg-SelP, to be finally excreted [44]. In order to obtain more complete information about this Hg detoxification mechanism, a supplementation of selenite at different doses have been administered in this work to evaluate the effects in the levels of selenoproteins in serum and liver cytosolic extract using a multidimensional approach based on 2D/SEC-AF-SUID-ICP-ORS-MS. The speciation method has been validated using a CRM of human serum (BCR-637) certified for total Se content. The concentration of different selenium species obtained in the BCR-637 is in concordance with previous results published by the authors [44].

The results of our present study indicate that SelP levels increase in serum and hepatic cytosolic extracts after Hg exposure period, and this effect is more pronounced with increasing selenite supplementation (Table 3 and Fig. 4A-B). This fact highlights the synthesis of SelP from selenite and the antagonistic interaction between both elements in mice serum and liver,

since Hg-SelP complex plays an important role as a vehicle for Hg detoxification [10]. In that respect, decreased levels of LMM selenium species in serum and a correlatively increased in liver cytosolic extracts are concordant with the requirement of selenite for SelP synthesis in the liver, which are then transferred to the bloodstream [48]. Additionally, SelP levels have been upregulated in the liver, which can explain the elevated concentration of this selenoprotein in this organ after Hg exposure period, and this fact is also more marked with increasing selenite supplementation, as in serum (Table 3 and Fig. 4A-B). Related to this, decreased levels of SeAlb in mouse serum were obtained when Hg is supplied alone and was accompanied by increased liver SeAlb concentration (Table 3), since this transport selenoprotein is transferred to the liver for the synthesis of SelP [48]. More pronounced effects were observed when Se was administered together with Hg at different doses, 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite (Table 3).

Serum HMM selenium species (Fig. 4B) is attributed to eGPx selenoenzyme that is located in the mammalian bloodstream, representing about 5 % of selenium in mouse plasma [29**]**. Our results show up-regulation of eGPx induction in Hg-treated mouse serum in comparison with control group, practically independent of the Se supplementation dose (Table 3). In contrast, HMM selenoproteins fraction in liver cytosolic extract (Fig. 4A) may be containing other selenoproteins beside to eGPx, such as TrxR as previously discussed. Independently of the low resolution in this fraction, increased levels in both redox selenoenzymes are related to oxidative damage caused by Hg toxicity were obtained after Hg exposure, more pronounced when Se is administered together with Hg (table 3), since their antioxidative properties help to eliminate reactive oxygen species induced by Hg *in vivo* [10,45].

Finally, in Fig. 4C and 4D can be observed, with the use of affinity column (2D-SEC-AF-ICP-MS), that Hg is present in plasma in three remarkable peaks in liver cytosolic extracts and serum, respectively. One close to void volume, other more intense at retention time matching with SeP peak, and a third peak coincident with the retention time of SeAlb. However,

peaks traced by Hg are not observed in control samples or their intensities are significantly lower (Fig. 4C and 4D). Therefore, these results reflects the binding Hg to high molecular mass proteins (HMM), SelP and SeAlb fractions, since Hg traced peak at retention time of LMM selenium species is not present in both biological matrices (Fig. 4C and 4D). On the other hand, it seems that Se supplementation promote Hg binding in favor of binding to higher molecular mass proteins, especially when higher Se dose is administered together with Hg (Fig. 4C and 4D). We did not find any significantly differences in the accumulation of Hg associated to SelP in serum, with the exception of a notable increase in Hg content in SelP fractions after 0.5 mg/kg bw/day of selenite supplementation in mouse liver and serum (Fig. 4C and 4D). In this way, increased Hg intensity in SelP fraction was reported by other authors in human serum with the increasing Hg content in the bloodstream [10]. These results are in good agreement with our findings. As we can see, in Hg-SeAlb fraction similar tendencies were obtained (Fig. 4C and 4D). These findings seem to support the idea that Hg-SelP and Hg-SeAlb complex formation was accompanied by redistribution of Hg in the organism.

4. CONCLUSIONS

We have demonstrated in this work the high reliability of elemental mass spectrometry based on metallomic approaches to study the biochemical effects induced by Hg/Se in exposed mice on liver, kidney and serum metals distribution, interactions and homeostasis. The application of size exclusion chromatography coupled to ICP-MS to cytosolic extracts of metabolic active organs and biological fluids from the laboratory mice exposed to Hg/Se, allows deciphering the changes of metal-binding biomolecules induced by both elements. Likewise, selenium speciation in serum (2D/SEC-AF-SUID-ICP-ORS-MS) confirms the role of selenoproteins in Hg detoxification processes and the need for selenite intake after Hg exposure. The use of enriched stable isotopes is crucial to study the fate of trace elements in biological systems, employing isotopic dilution analysis measurements, which shows unequivocally the importance of selenium species for Hg protection. The protective mechanism includes the capability of Se to perturb the redistribution of Hg in organs and biological fluids and induces

binding of the inert Hg-Se-S complexes. Related to this, we propose that selenoproteins may have two important roles in protecting against Hg toxicity. First, they may bind more Hg through their highly reactive selenol group, and second, their antioxidative properties help compromise the reactive oxygen species induced by Hg in vivo. These results provide a better understanding of the interaction between Hg and Se in whole organisms during a 10 days exposure period. Finally, we can conclude that selenite could be a potential treatment of Hg toxicity in areas affected by this toxic metal, such as mining and industrial areas.

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FIGURE LEGENDS

Figure 1. Metal concentrations in the organs and serum of *Mus musculus* mice after exposure to Hg/Se. Values are mean ± SD for six mice per group. Hg group was given a subcutaneous administration of 0.2 mg/kg bw/day $HgCl_2$. Hg+Low-Se and Hg+High-Se groups were given a subcutaneous administration of 0.2 mg/kg bw/day of $HgCl_2$ and supplemented orally with 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite, respectively.

Figure 2. SEC-ICP-MS derived Hg-specific chromatograms obtained for the analysis of mouse liver cytosolic extracts (A), and serum (B). Chromatographic conditions: Superdex^{TM}-200 column; mobile phase: ammonium acetate 20 mM (pH 7.4); flow rate: 0.8 ml min^{-1}; injection volume: 20 µL.

Figure 3. SEC-ICP-MS derived Cu (A), Zn (B) and Hg (C) specific chromatograms obtained for the analysis of mouse kidney cytosolic extracts. Chromatographic conditions: Superdex^{TM}-200 column (10x300x13 µm); mobile phase: ammonium acetate 20 mM (pH 7.4); flow rate: 0.8 ml min^{-1}; injection volume: 20 µL.

Figure 4. A) Mass flow chromatogram with $^{78}Se/^{74}Se$ isotope ratios of selenoproteins and low molecular weight selenium species in mouse liver cytosolic extracts after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. (B) Mass flow chromatogram with 78Se/74Se isotope ratios of selenoproteins and low molecular weight selenium species in mouse serum after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. C) Hg specific chromatograms obtained for the analysis of mouse liver cytosolic extracts after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. D) Hg specific chromatograms obtained for the analysis of mouse serum after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. Hg group was given a subcutaneous administration of 0.2 mg/kg bw/day $HgCl_2$. Hg+Low-Se and Hg+High-Se groups were given a subcutaneous administration of 0.2 mg/kg bw/day of $HgCl_2$ and supplemented orally with 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite, respectively.

Table 1. Operating conditions of chromatographic separations and ICP-ORS-MS detection

Forward power	1500 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1 L min^{-1}
Carrier gas flow rate	0.15 L min^{-1}
Sampling and skimmer cones	Ni
Nebuliser	Microflow (ESI)
Torch	Shield (with long life platinum shield plate)
Q_{oct}	-18 V
Q_p	-16 V
Points per peak	1
Integration time	0.3 per isotope
Replicates	1
Isotopes monitored for total metals determination and SEC	^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{103}Rh, ^{80}Se, ^{78}Se, ^{201}Hg and ^{202}Hg.
$H_{e\ flow}$ for total metals determination and SEC	4.0 mL min^{-1}
Isotopes monitored for SUID	^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se, ^{79}Br, ^{81}Br and ^{83}Kr
$H_{2\ flow}$ for SUID	3.7 mL min^{-1}
Dead time detector	47 ns
Column	Superdex^{TM}-200 (10x300x13µm)
Resolution range	600-10 kDa
Mobile phase	Ammonium acetate 50 mM(pH 7.4)
Flow rate	0.7 mL min^{-1}
Injection volume	20 µL
UV detection	254 nm
Sample loop	100 µL
Flow rate	1.3 mL min^{-1}
Mobile phase A	0.05 M ammonium acetate pH 7.4
Mobile phase B	1.5 M ammonium acetate pH 7.4
Gradient	0-7 min 100% A, 6-18 min 100% B, 18-20 min 100% A
6-port valve position	1-10 min Inject 10-17 min Load 17-20 min Inject

Table 2. Concentration of elements (ng g^{-1}) in the different organs and serum of mice following inorganic mercury and selenium exposure for 10 days.

SAMPLES	GROUP	Cu (ng g^{-1})	Zn (ng g^{-1})	Se (ng g^{-1})	Hg (ng g^{-1})
Liver	Control Group	6742.3±31	34126±2045	314.21±18.7	<LOD
	Hg-exposed Group	6689.6±45	36224±3154	550.12±36.3	314.31±42.4
	Hg/Low-Se-exposed Group	6831.4±54	35721±2892	703.54±51.8	581.16±38.6
	Hg/High-Se-exposed Group	5983.7±26	35201±3546	711.26±62.4	603.81±48.8
Kidneys	Control Group	4293.6±42	18631±904.3	821.19±58.4	<LOD
	Hg-exposed Group	8641.8±68	24621±1625	913.54±46.3	9831.4±613
	Hg/Low-Se-exposed Group	8012.3±73	20036±1820	2304.6±184	7342.6±702
	Hg/High-Se-exposed Group	7814.6±54	21093±2014	3821.4±257	6103.4±524
Serum	Control Group	432.12±16	921.35±88.61	330.14±21.2	<LOD
	Hg-exposed Group	402.21±38	1214.5±114.4	289.62±14.6	240.21±21.8
	Hg/Low-Se-exposed Group	394.52±25	1024.2±122.5	489.22±31.5	368.74±33.5
	Hg/High-Se-exposed Group	398.21±17	1105.6±98.44	531.24±42.8	421.33±27.4
Limit of detention (LOD)		0.054	0.105	0.098	0.204

Table 3. Selenoproteins concentration (ng g^{-1}) in hepatic cytosolic extract and serum of mice following inorganic mercury and selenium exposure for 10 days.

SAMPLES	GROUP	HMM Se species	LMM Se species	SelP	SeAlb	Sum Se species
		Mean ± SD *(ng g^{-1})* (n=3)				
Liver cytosolic extracts	Control Group	46.5±5.21	47.7±6.21	195±15.4	20.2±3.16	309±9.14
	Hg-exposed Group	102±8.14	111±10.2	297±18.3	34.5±4.22	544±16.54
	Hg/Low-Se-exposed Group	117+11.2	90.3±12.1	422±31.4	48.6±7.16	678±24.7
	Hg/High-Se-exposed Group	129±9.87	118±8.42	434±18.8	59.4±10.1	740±17.3
Serum	Control Group	8.72±2.11	52.1±2.57	251±16.2	14.3±2.42	326±12.4
	Hg-exposed Group	14.4±3.04	5.41±1.30	266±12.7	9.66±1.68	295±10.8
	Hg/Low-Se-exposed Group	13.0±2.26	12.4±2.01	463±24.8	10.7±2.01	499±18.4
	Hg/High-Se-exposed Group	14.9±2.34	26.3±4.11	514±31.4	13.1±2.88	568±16.7
Certified Material	Mean ± SD (n=3)	11.2±1.51	<LOD	53.4±2.18	18.2±2.34	82±2
BCR-637	Certified value	Not certified				81±7
Limit of detention (LOD)		0.3	1.2	0.6	0.9	

Figure 1-

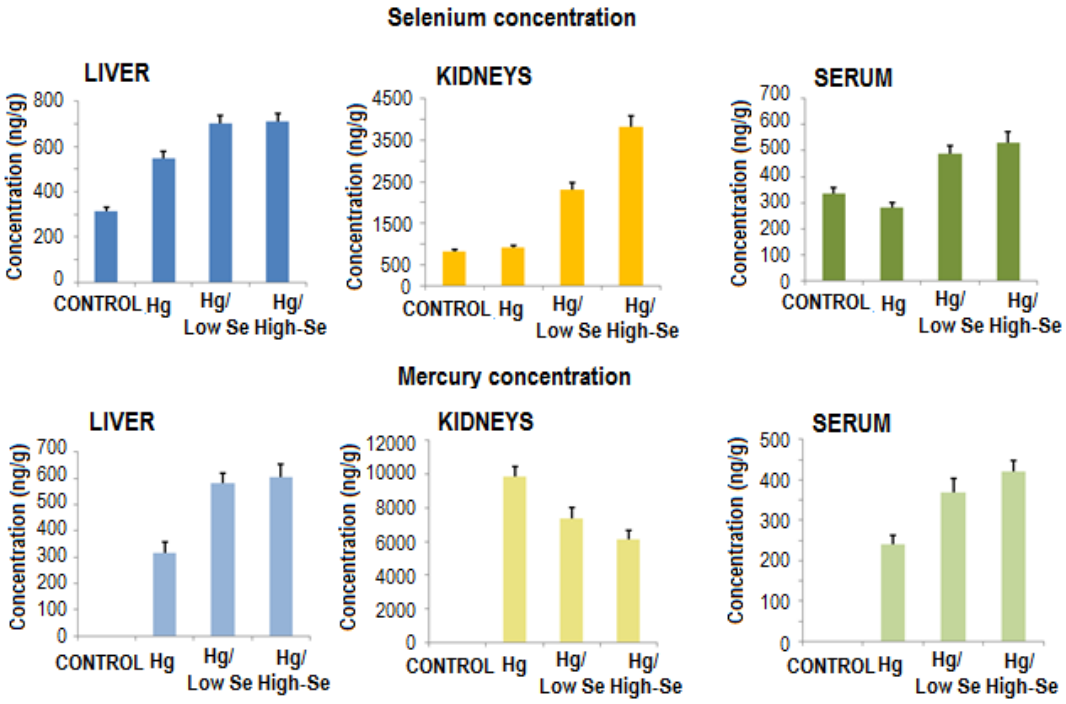

Selenium concentration
LIVER
KIDNEYS
SERUM
Concentration (ng/g)
CONTROL
Hg
Hg/ Low Se
Hg/ High-Se
Mercury concentration
LIVER
KIDNEYS
SERUM
Concentration (ng/g)
CONTROL
Hg
Hg/ Low Se
Hg/ High-Se

Figure 2-

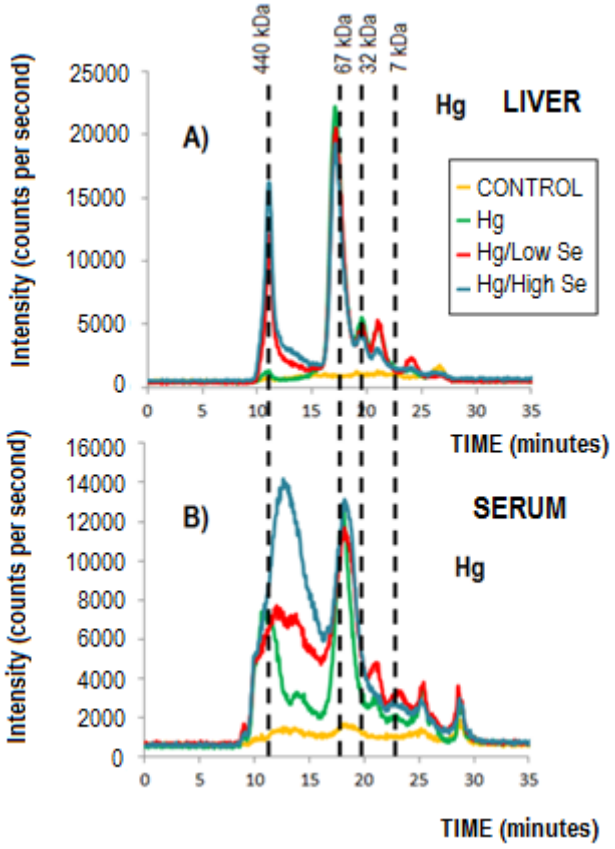

440 kDa
67 kDa
32 kDa
7 kDa
A)
Hg
LIVER
CONTROL
Hg
Hg/Low Se
Hg/High Se
Intensity (counts per second)
TIME (minutes)
B)
SERUM
Hg
Intensity (counts per second)
TIME (minutes)

Figure 3-

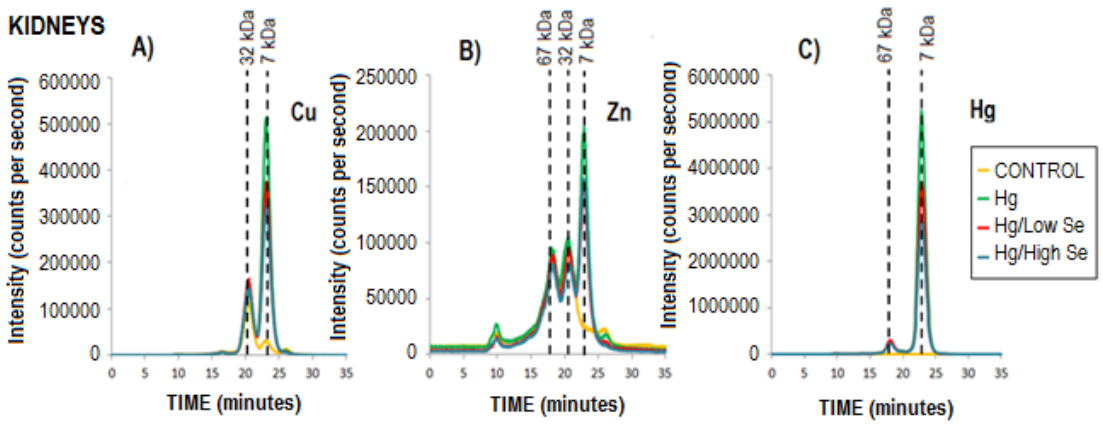
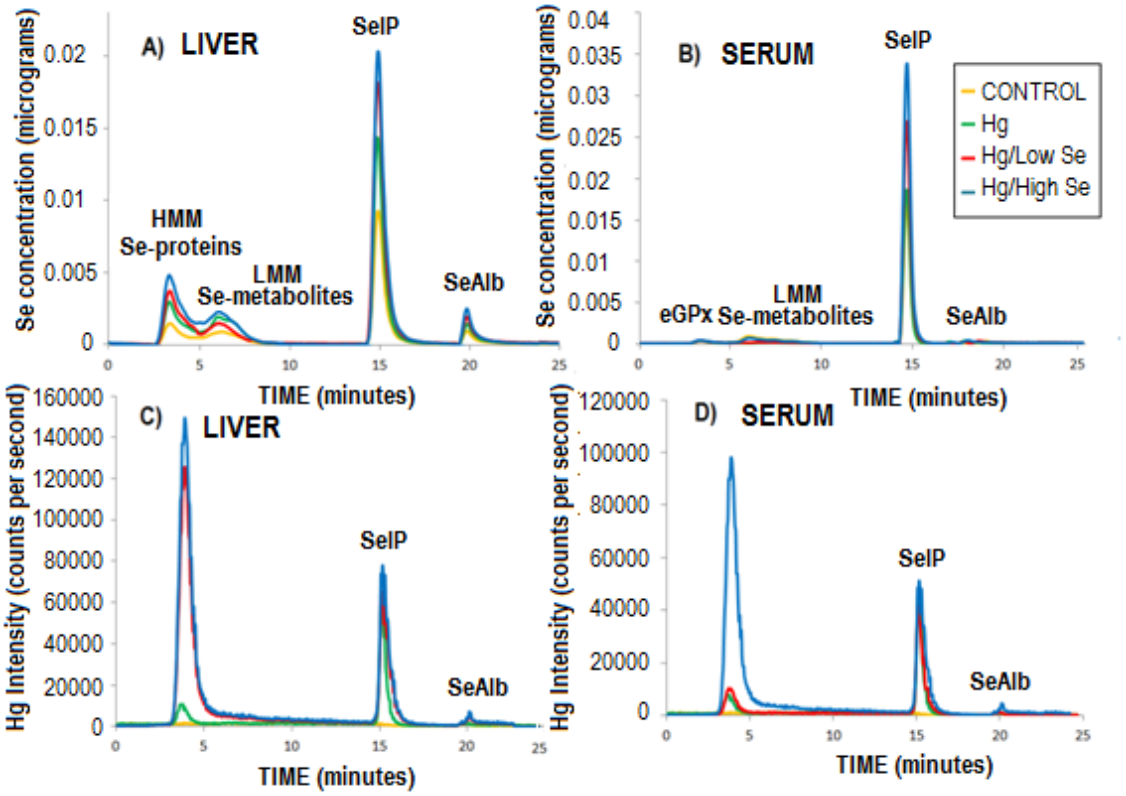

KIDNEYS
A)
32 kDa
7 kDa
Cu
Intensity (counts per second)
TIME (minutes)
B)
67 kDa
32 kDa
7 kDa
Zn
Intensity (counts per second)
TIME (minutes)
C)
67 kDa
7 kDa
Hg
Intensity (counts per second)
TIME (minutes)
CONTROL
Hg
Hg/Low Se
Hg/High Se

Figure 4-


A) LIVER
SelP
HMM
Se-proteins
LMM
Se-metabolites
SeAlb
Se concentration (micrograms)
TIME (minutes)
B) SERUM
SelP
LMM
eGPx Se-metabolites
SeAlb
CONTROL
Hg
Hg/Low Se
Hg/High Se
C) LIVER
SelP
SeAlb
Hg Intensity (counts per second)
TIME (minutes)
D) SERUM
SelP
SeAlb
TIME (minutes)

Figure(s)

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Figure 1.-

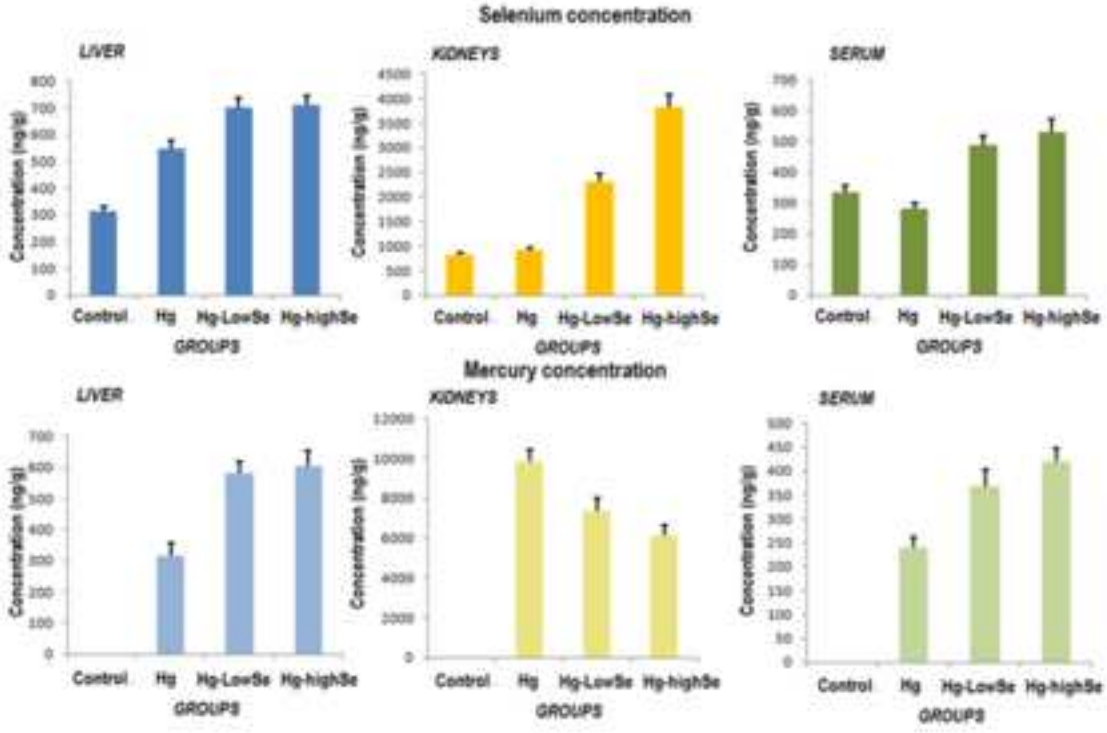

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Figure 2.-

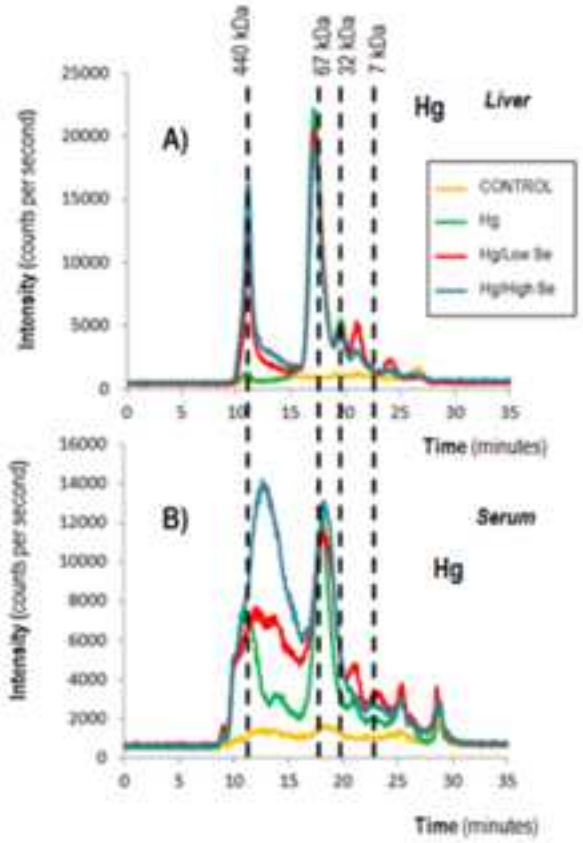

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Figure 3.-

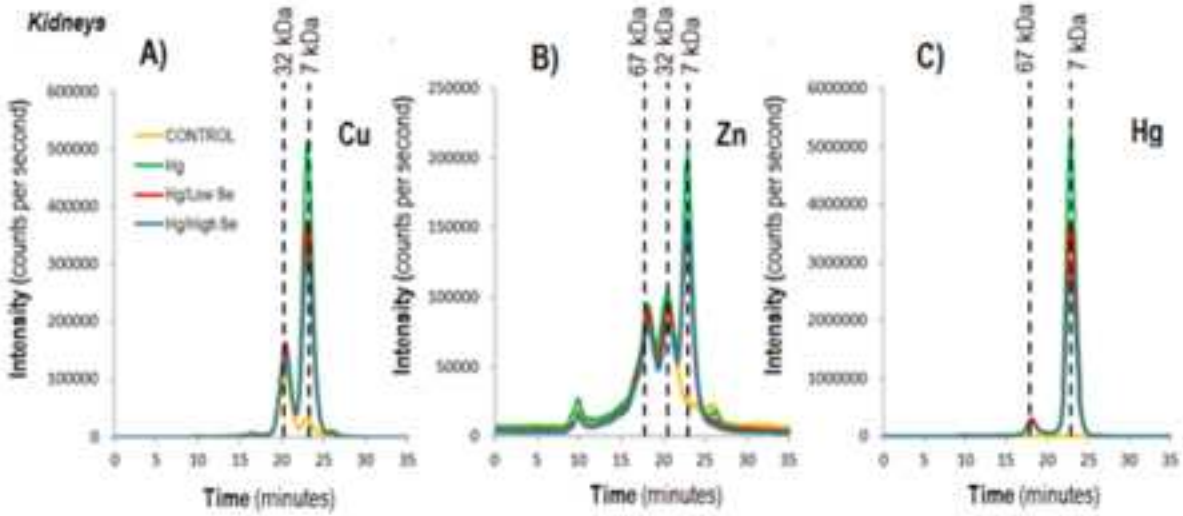

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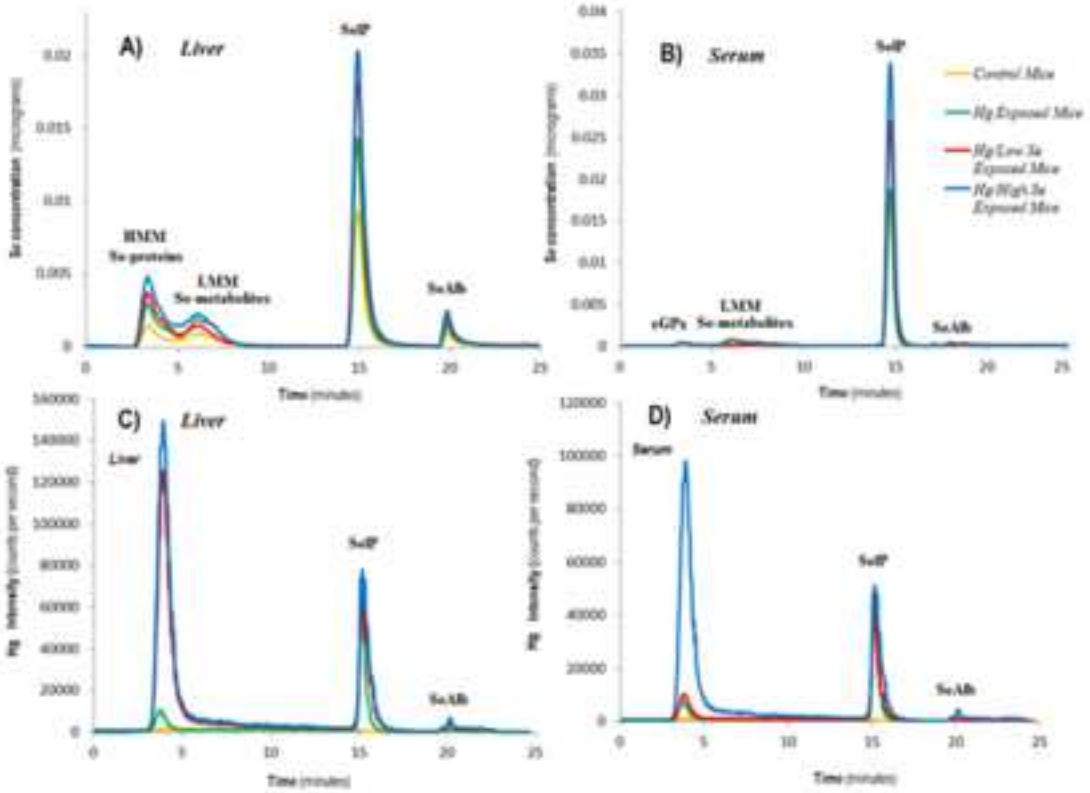
Figure 4.-



Biological interactions between mercury and selenium in distribution and detoxification processes in mice under controlled exposure. Effects on selenoprotein.

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ABSTRACT:

Antagonistic interactions between mercury (Hg) and selenium (Se), were evaluated in mouse (*Mus musculus*), as a mammalian model, in a series of controlled exposure experiments. The beneficial effect of Se against Hg toxicity involves a variety of biochemical and toxicological processes that have not been clarified yet. For this purpose, a metallomic workflow based on the use of size-exclusion chromatography (SEC) with inductively coupled plasma mass spectrometry (ICP-MS) detection was complemented with the speciation of selenoproteins and low molecular mass selenium species in serum and liver cytosolic extracts using a multidimensional approach based on SEC-AF-HPLC-ICPMS, using species-unspecific isotope dilution (SUID)-ICP-MS for selenium quantification. The results showed potential interactions between Hg/Se in organs and serum related to accumulation and detoxification processes, in addition to the effects of mercury on selenoproteins in hepatic cytosolic extracts and bloodstream when both elements are administrated at the same time. These results provide information about elements distribution, interactions and homeostasis and reveal the potential of metallomic approaches in exposure experiments.

Keywords: *Mus musculus, mercury, selenium, metal interactions, metallomics, isotopic dilution analysis, selenoproteins.*

ABBREVIATIONS: AF - affinity chromatography; BSA - bovine serum albumin; CRMs - Certified reference materials; Cys - cysteine; DNA - Deoxyribonucleic acid; eGPx - extracellular glutathione peroxidase; GPx - glutathione peroxidase; GSH - reduced glutathione; HMM - high molecular mass; HPLC - High performance liquid chromatography; ICP-MS - Inductively coupling plasma-mass spectrometry; IDA - isotopic dilution analysis; LC - liquid chromatography; LMM - low molecular mass; MS - mass spectrometry; MT - metallothionein; ORS - octopole reaction systems; PMSF - Phenylmethanesulfonyl fluoride; RBCs - red blood cells; SeAlb - selenoalbumin; SEC - size exclusion chromatography; SelP - selenoprotein P; SeCys - selenocysteine; SUID - species-unspecific isotopic dilution; SOD - superoxide dismutase; ThxR - thioredoxin reductase; TCEP - tris(2-carboxyethyl)phosphine hydrochloride; 2D - two dimensional.

1. INTRODUCTION

Mercury (Hg) is a widespread environmental and industrial contaminant that induces rigorous toxic effects in body tissues of both human and animals [1], depending on its different chemical forms [2]. Mercury exists in three main chemical forms: elemental, as an inorganic salt, or bound to an organic ligand; although all the chemical species are potentially toxic. Divalent inorganic mercury (Hg^{2+}) is one of the strongest thiol-binding elements, which explains its toxicity by the ability to form stable complexes with the sulfhydryl-cysteine groups of proteins (-SH), such as numerous thiol-related enzymes [3]. Hg^{2+} can also give rise to free radicals that induce lipid, protein and DNA oxidation related to oxidative stress [4-6]. On the other hand, organic mercury compounds are mostly metabolized in the liver where they may suffer demethylation [7] or undergo conjugation reactions with glutathione (GSH) [2,8]. It is well known that Se presents a protective effect against Hg toxicity and inhibits oxidative damage caused by Hg in mammals [9-11]. The antagonistic interaction between Hg and Se was first reported in 1967 in rats treated with mercury chloride and selenite [11]. All the same, Se is an essential element in mammalian health, with both deficiency and toxicity effects apparent, depending on soil content, due to a fairly narrow range of optimal Se intakes [12-14]. However, it has been reported that simultaneous exposure to both Se and Hg compounds increased whole-body retention of Hg, possibly due to the formation of inert Se-Hg complexes, which has been considered as a preventive mechanism against Hg toxicity [11,15]. Nevertheless, although the Se and Hg co-accumulation in humans and other mammals is well known, the mechanism of interaction between these elements is still unsolved.

Hg^{2+} can also react with selenol groups (-SeH) that constitute a part of selenocysteine (SeCys), and as a consequence they can be incorporated to selenoproteins, prosthetic groups of selenoenzymes and peptides [16-17], since the -SeH in SeCys shows even stronger affinity for Hg that -SH groups, possibly due to the lower pKa of SeCys (~5.4) that provides it a higher reactivity than Cys (pKa~8.0). Moreover, selenoproteins play an important role in the maintenance of cellular homeostasis [18]. In the same way, Hg^{2+} can also react with selenides

(Se^{2-}) and hydrogen selenide or selenols to form Hg-Se-S complexes together with glutathione that can finally bond to selenoprotein P (SelP) forming a ternary complex in the bloodstream [19-21]. Se is an essential component of several selenoenzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (ThxR), which also contains Se as SeCys. In that respect, the perturbation of selenoproteins functions has been related to the development of several diseases such as carcinogenesis [22-23].

Although at the moment there are very powerful analytical techniques and sample preparation procedures for element speciation, biological systems require multi-elemental analytical approaches that make possible to characterize processes involving metals interactions, trafficking and homeostasis [24]. For this purpose, metallomics is a relatively new field to decipher changes in metal-biomolecules expression and identification in biological matrices. In this sense, it is necessary to consider that approximately one third of proteins need the presence of metals as cofactors to develop their function [24-27]. On the other hand, ICP-MS is a valuable technique in this field since it allows multi-isotopic analysis, detection capability, high sensitivity, tolerance to matrix and large linearity range of quantification [24], generally coupled to liquid chromatographic arrangements, and parallel molecular mass spectrometry for biomolecules identification in an integrated workflow [25-26]. As a consequence, metallomics provides a good option to deep insight into the fate of elements in exposed organisms to elements, and provides information about element distributions, retentions and interactions [28].

The aim of the present study was to characterize the biochemical response of mice *Mus musculus* in metal distribution and detoxification processes caused by simultaneous mercury exposure and selenium exposure based on suitable metallomic workflows. The metallomic approach included the use of size exclusion chromatography (SEC) coupled to ICP-MS as detector. In addition, the study was complemented by the speciation of selenoproteins and low molecular mass selenium species in serum and liver cytosolic extracts of mice based on species-unspecific isotope dilution (SUID)-ICP-ORS-MS on-line coupled to a SEC in tandem with an AF that integrate the analytical speciation platform.

2. MATERIALS AND METHODS

2.1. Instrumentation

The mineralization of samples to determine the total metal content in biological matrices was carried out by using a microwave accelerated reaction system model MARS (CEM Corporation, Matthews, Carolina del Norte, USA) and MARSXpress vessels.

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770) was used to prepare tissues homogenates. Metal-containing biomolecules were separated and detected with an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan) which was equipped with an octopole reaction system (ORS). Liquid chromatography separations were performed using a Model 1100 HPLC pump with detector UV (Agilent, Wilmington, DE, USA). ICP-MS conditions (Table 1) to measure in He mode were optimized using a HNO_3 5% (v/v) aqueous solution of ^{59}Co, ^{89}Y, ^{205}Tl (1 µg L^{-1}). A microflow nebulizer (Teflon; model ESI, Ohama, USA) was used to establish the hyphenation of the LC-ICP-ORS-MS system.

2.2. Standard solutions and reagents

All reagents that were used for sample preparation in the metallomic approach were of the highest available purity. Phenylmethanesulfonyl fluoride (PMSF) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (BioUltra grade, >98%) were obtained from Sigma Aldrich (Steinheim, Germany). Helium and hydrogen, which were used as collision and reaction gas (in the ICP-ORS-MS system), were of high-purity grade (>99.999%).

The standards which were used for mass calibration of analytical SEC columns (mass range 600-10 kDa) included horse ferritin (440 kDa) (purity 95%), bovine serum albumin (67 kDa) (purity 96%) and superoxide dismutase containing Cu and Zn (32 kDa) (purity > 70%). All reagents were purchased from Sigma-Aldrich (Steinheim, Germany). On the other hand, metallothionein (MT) containing Cu, Zn and Cd (isolated from rabbit liver) (purity 95 %) was purchased from Enzo Life Sciences (Madrid, Spain). The mobile phase buffer used for SEC was

50 mM of ammonium acetate (Suprapure grade) purchased from Merck (Darmstadt, Germany), which was prepared daily with ultrapure water (18 MΩcm) from a Milli-Q system (Millipore, Watford, UK). The pH was adjusted to pH 7.4 with ammonia solution, which was prepared by dilution of 20% (w/v) ammonia solution (Suprapur, Merck) with ultrapure water. The void volume was determined by injecting ferritin (440 kDa).

Human serum certified reference material (CRM) BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). A standard solution of 1000 mg L^{-1} of Se stabilized in 5% (v/v) nitric acid Suprapur and of 1000 mg L^{-1} of bromide stabilized in 5% (v/v) nitric acid Suprapur was purchased from Merck (Darmstadt, Germany). Enriched ^{74}Se powder was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) and dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to volume with ultrapure water.

2.3. Animals and experiment exposure

Mus musculus (inbred BALB/c strain) mice were obtained from Charles River Laboratory (Spain). Mice of 7 weeks of age were fed *ad libitum* with conventional pellets (rodent global diet from Harlan Laboratories Inc. Indianapolis, IN 46250, USA). The animals were allowed to acclimate for 5 days with free access to food and water under controlled condition (temperature (25-30ºC) and a 12 h light-dark cycle) prior to exposure.

Mice were randomly divided into four groups: one control and three treated groups including Hg group, Hg+Low-Se group and Hg+High-Se group. Hg, as mercuric chloride ($HgCl_2$) and Se, as selenite were subcutaneously and orally exposed, respectively, every day at different doses. Hg group was given a subcutaneously administration of 0.2 mg/kg bw/day $HgCl_2$. Hg+Low-Se and Hg+High-Se groups were given a subcutaneously administration of 0.2 mg/kg bw/day of $HgCl_2$ and supplemented orally with 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite, respectively. The control mice were subjected to subcutaneously administration of 100 µL of ultrapure water with 0.9% NaCl per day for 10 days. The acute subcutaneous mercury chloride dose of 0.2 mg kg^{-1} bw/day was selected taking into account the

LD_{50} for mice (3 mg kg^{-1}) which was not exceed the LD_{50} for the overall exposure experiment (10 days). On the other hand, selenium supplements of 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite were selected taking into account the recommended level of dietary selenium and about 3 times more, respectively.

Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, dissected using a ceramic scalpel and finally livers and kidneys transferred rapidly to dry ice. Liver and kidney are main targeted tissues for Hg as they are involved in its metabolism. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% NaCl solution, frozen in liquid nitrogen and stored at -80 ºC until they were used for extract preparation. Serum was obtained by centrifugation (4000g, 30 min, 4ºC) of whole blood after incubation during 30 min at 37 ºC in the dark. All animals received humane care in compliance with the guidelines of the animal care and use of the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

2.4. Total metal determination in organs and serum of mice by ICP-ORS-MS

For the determination of total metals in the organs and serum, individual samples were weighed (0.100 g) in 5-mL microwave vessels and 0.5 mg of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v) was added. After 10 min, the PTFE vessels were closed and introduced into the microwave oven. Mineralization was carried out at 400 W from room temperature ramped to 160 ºC for 15 min and held for 20 min at this temperature. Then the solutions were made up to 2.0 g with ultrapure water and the metals were analyzed by ICP-ORS-MS. Rhodium was added as internal standard (1µg L^{-1}). All analyses were performed in triplicate. The ICP-ORS-MS conditions are depicted in Table 1.

2.5. Analysis of mouse tissue cytosolic extracts and serum using SEC-ICP-ORS-MS

Serum and organs from 6 individual mice (male) per group were pooled and treated following a procedure described elsewhere [28]. Briefly, the metal-containing biomolecules were extracted with a solution (3 mL g^{-1}) containing 20 mM ammonium acetate buffer solution at pH 7.4, 1 mM TCEP (tris-2-carboxyethylphosphine) and 1 mM PMSF (phenylmethylsulfonyl

fluoride) using a glass/teflon homogenizer in a cold chamber at a constant temperature of 4 ºC. Then the extracts were centrifuged at 120,000 g for 1 h at 4 ºC. The obtained extracts were stored under nitrogen atmosphere to avoid oxidation at -80 ºC until analysis and filtered through Iso-disc Poly-vinylidene fluoride filters (PVDF, 25-mm diameter, 0.45-µm pore size) (low protein adsorption) to avoid column overloading or clogging. The SEC-ICP-MS on-line coupling was performed by connecting the outlet of the chromatographic column to the Microflow nebulizer inlet ESI (Ohama, USA) of the ICP-ORS-MS by means of PEEK tubing (0.6 i.d. mm). The quality control of the SEC-ICP-ORS-MS system to overcome problems related to contamination, loss and stability of species has been described elsewhere [28]. Separation of the analytes was performed with an analytical size exclusion column Superdex^{TM}-200 (10 mm x 300 mm x 13 µm) (GE Healthcare, Uppsala, Sweden) with an exclusion limit of 600 kDa (effective separation range from 10 to 600 kDa). Details pertaining to the chromatographic separation and the ICP- MS conditions are depicted in Table 1.

2.6. Quantification of selenium containing proteins and selenium metabolites in mouse serum and cytosolic extracts of tissues by 2D/SEC-AF-HPLC-(SUID)-ICP-ORS-qMS using unspecific-species isotopic dilution analysis.

For this purpose, 10 µL of 100 mM of PMSF and 100 mM of TCEP mixture were added to 100 mg of serum as proteases inhibitor and a reducing agent, respectively. To avoid changes of the Se species, serum samples were directly injected on to the column, without prior dilution. Liver cytosolic extracts were prepared as previously explained for SEC-ICP-ORS-MS. The fractionation of Se-containing proteins and selenometabolites was performed by two dimensional chromatographic separations based on SEC prior to the double AF column using ICP-ORS-MS as detection system (2D/SEC-AF-SUID-ICP-ORS-MS) following a procedure described elsewhere [29]. In brief, this involved two 5 mL HiTrap® Desalting Columns (GE Healthcare, Uppsala, Sweden) that were connected in series with a dual affinity column arrangement comprising a 1.0 mL heparin-sepharose column (HEP-HP) (GE Healthcare, Uppsala, Sweden) and a 1.0 mL blue-Sepharose column (BLU-HP) (GE Healthcare, Uppsala, Sweden), interconnected with a six-way switching column valve. The HiTrap column is a size

exclusion chromatography column that removed low molecular mass components (MW<1000Da) from high molecular mass molecules, such as DNA, proteins or peptides. The combination of these two columns increased the chromatographic resolution. The chromatographic separation parameters and the ICP-MS conditions are displayed in Table 1.

3. RESULTS AND DISCUSSION

3.1. Multielemental determination in serum and organs from mice exposed to mercury/selenium by ICP-ORS-MS.

Copper, zinc, selenium and mercury concentrations in important metabolic organs (liver and kidney) and serum of mice exposed to Hg/ Se were quantified and the results are presented in Table 2 and for a better visualization in Fig. 1. Recovery experiments were performed by spiking the extracts with 1, 5, 10 or 50 ng g^{-1} of metal, depending on the relative concentration of each metal in the extracts. Confirmation of quantitative recovery was obtained in all cases, with values ranging between 86-114 %. Instrumental limits of detention (LOD) are also provided in table 2.

Hg administered alone or together with Se appeared to lower the level of mercury in kidneys and increased the levels in liver and sera of Se-treated mice, compared to those that received Hg alone. Some authors have reported the reduction of Hg accumulation in kidney in the presence of Se, and correlatively the increases of Hg presence in the remaining body, especially in liver and serum [9,30]. These results are in good agreement with our findings (Table 2). In previous a work, Hg showed a non-uniform distribution after absorption, being accumulated mainly in mouse kidney after similar experimental conditions [31]. Therefore, our results show that accumulation of Hg in liver and serum of mice exposed to this element together with Se remains high, although other studies claims for the decrease of Hg in the presence of Se. Possibly, Se minimizes the toxic action of mercury enhancing the antioxidant ability of organisms and increasing contents of the antioxidants, as has been checked in rats [9,32]. On the other hand, beneficial action of Se can be attributed to the formation of non-toxic Se-Hg complexes that has been identified as a process involved in the prevention of Hg toxicity

by Se [11,15,31,33]. However, although Se and Hg co-accumulation in humans and other mammals is well known, the mechanism of interaction between Se and Hg is still unclear.

Se concentration in liver, kidneys and serum of mice only exposed to Hg was increased about 75 % and 11 %, respectively, however decreased about 12 % in serum in comparison with the control group (Table 2). This decrease of Se levels in serum was also observed in a preliminary study with *Mus musculus* subcutaneously exposed to a low dose of mercury (0.1 mg/kg bw/day $HgCl_2$) [31]. In this study similar trends were obtained for an $HgCl_2$ dose of 0.2 mg/kg bw/day (Table 3). The distribution of Se was significantly different between Hg+Low-Se and Hg+High-Se groups. Table 2 shows increasing of Se concentration in liver, kidney and serum, when the intake of this element increases.

Additionally, it is well known that Hg administration provokes perturbations in the distribution of Cu and Zn [31,34]. In this experiment, Cu accumulation in the kidneys and serum of Hg-exposed mice was perturbed about +101 % and -7 %, respectively compared with the control group. Changes in metals distribution is also observed in mice simultaneously exposed to Hg and Se, this later at different doses (Table 2). It is remarkable the decreased concentration of Cu in liver of Hg-exposed mice (about -12%) when high concentration of Se was administered (0.50 mg/kg bw/day of selenite) (Table 2). Alternatively, when Zn presence was evaluated in the different organs (liver and kidney) and serum, zinc concentrations change about +6 %, +32 % and +32 %, respectively, compared with the control group. The distribution of Cu, the Zn did not change significantly although Se was administered together with Hg at different doses (Table 2). These results agree with previous studies in mice subcutaneously exposed to lower dose of Hg [31].

3.2. Characterization of metal-biomolecules distribution in liver, kidney and serum extracts from mice under inorganic mercury and selenium exposure using SEC-ICP-ORS-MS.

For a better understanding of the antagonistic interaction between Hg and Se, the protein binding pattern distribution of Hg in hepatic and renal cytosol from different groups were analyzed to evaluate the biological response of mice exposed to Hg and Se for 10 days

based on the use of SEC-ICP-ORS-MS. Organs with high metabolic activity such as liver, kidneys, and fluids as serum were used for this purpose. The most interesting results were observed in relation to Cu, Zn and Hg. The figure 2 shows the Hg-traced chromatogram obtained by SEC-ICP-ORS-MS using a column with mass resolution ranged between 10-600 kDa. The relative presence of mercury reflected by the peaks intensity is in accordance with the total mercury content in the different biological matrices under consideration (Table 2).

In liver (Fig. 2A), exposure to Hg and Se for 10 days increased the intensity of three Hg-traced peaks, one with retention time at 11 minutes matching with the dead volume of the effective range of separation of the column (Superdex^{TM}-200), which reflects the binding of Hg to high molecular mass proteins (HMM), due to the high affinity of mercury to thiol groups at the moiety of proteins and endogenous metabolites including glutathione (GSH) or cysteine (Cys) [2,8]. In that respect, the proteins with higher molecular mass usually contain more Cys residues than proteins with lower molecular mass (LMM). The higher concentration of mercury in liver of Hg/Se-treated mouse (table 2), may explain the presence of this peak. A second Hg-containing peak matches well with that of Cu,Zn-BSA standard (17.5 min) (Fig. 2A). The intensity of Hg in this fraction decreases when the dose of Se increases, exhibiting a maximum when Hg is administrated alone. In this sense, it is well known that Hg forms stable complexes with albumin in human serum [35]. Furthermore, the high electrophilic character of Hg^{2+} enhances the bound to Cys or SeCys in different proteins/metalloproteins, such as Se containing proteins with similar molecular mass. Furthermore, selenoproteins play an important role in Hg detoxification, mainly selenoprotein P (SelP), since it contains 6-10 SeCys groups and the high reactivity of selenol groups increases the possibility of Se-Hg bound [31]. Finally, it can be observed in Fig 2A a third peak matching with Cu,Zn-SOD standard (32 kDa). The intensity of this peak is similar in the three groups exposed to Hg (Fig. 2A). However, the intensity of peak corresponding to putative Hg-SOD significantly increase with respect to control. This fact could be related with decreased enzymatic activity of SOD in hepatic cytosolic extracts observed in rats under Hg administration [36], even when Se is ingested simultaneously [37].

Two remarkable peaks are obtained by SEC-ICP-ORS-MS in mouse serum exposed to Hg/Se (Fig. 2B). In this case the SEC-chromatogram shows similar distribution to liver cytosolic extracts (Fig. 2A). A first Hg-peak related to Hg-attachment to HMM proteins rich in Cys residues (Fig. 2B), and a second peak, at about 67 kDa, related to transport proteins and selenoproteins, as discussed above for liver.

Alternatively, metallobiomolecules associated with Cu, Zn and Hg in kidney cytosolic extracts are shown in Fig. 3. The peaks traced by Hg (Fig. 3C) show higher intensity of peaks in kidney than in liver and serum which agree with total levels of this element in Fig 1. It is remarkable an Hg traced predominant peak at about 7 kDa matching with metallothionein standard (Cu,Zn,Cd-MT) (Fig. 3). Analogous peaks traced by Cu and Zn can be observed in Fig. 3A and 3B. These molecules are associated to metal detoxification processes, because MTs are cysteine-rich proteins [38]. In this sense, MTs are known as storage depots for metals such as Cu and Zn; indeed, these proteins scavenge sulfhydryl reactive metals that enter the cells, although they have a higher affinity for Hg than Zn or Cu, in this case metal-MTs are excreted by urine that constitutes a detoxification mechanism for Hg [39]. Plus, it has been reported that co-administration of selenite and Hg reduces urinary excretion of mercury in rats [40-42], which agrees with results in Fig. 3A, 3B and 3C that show a lower intensity of peaks traced by Cu, Zn and Hg, respectively, in the presence of Se, which seems to inhibit MTs production [30]. The inhibiting effect of Se in MTs triggering increases with the dose of Se (Fig. 3).

3.3. Characterization of selenoproteins distribution in liver cytosolic extract and serum from mice under inorganic mercury and selenium exposure using 2D/SEC-AF-SUID-ICP-ORS-MS.

In mammalian bloodstream, Se is mainly present in form of four selenium containing molecular entities, namely SelP [29], eGPx [43], SeAlb and free selenite [44]. In liver other selenoproteins can also be found, such as thioredoxin reductase (TrxR), a redox selenoenzymes with a molecular mass of 54 kDa that reduce thioredoxin (Trx) [45]. In that respect, some of these selenoproteins contains several SeCys and Cys residues, such as SelP [46], and could play

an important role as a vehicle for Hg detoxification. Therefore, a decrease in plasma SelP after Hg exposure has been reported by other authors [10]. In addition, GPx represents a family of antioxidant enzymes whose main function is to counteract the adverse effect of hydrogen peroxide and lipid hydroperoxides [17,47]. Related to this, a decreased activity of GPx in liver has been reported in mice after Hg exposure when a single dose of 20 μmole/kg bw was intraperitoneal injected [37]. On the other hand, it is well known that low molecular mass (LMM) selenium-species (such as selenite) are required for the synthesis of selenoproteins in liver, which are then transported to bloodstream [48]. The selenite in the bloodstream is readily taken up by red blood cells (RBCs) [49] and reduced to selenide (SeH^-), for latter effluxion into the bloodstream in the presence of albumin and transferred to the liver in the form of SeAlb for the synthesis of selenoproteins [48]. In contrast, selenate is not taken up by RBCs, but it interacts with hepatocytes and is used for the synthesis of SelP and eGPx, which are released into the bloodstream for final partial excretion into urine [21]. In this way, significantly decreased levels of LMM selenium species and correlatively increased SelP levels in plasma of Hg-treated mice were observed when 0.1 mg/kg bw/day of $HgCl_2$ was supplied alone [31]. These results suggest that LMM selenium species were initially consumed in the Hg detoxification process, to supply the requirements in SelP for Hg redistribution in the form of Hg-SelP, to be finally excreted [44]. In order to obtain more complete information about this Hg detoxification mechanism, a supplementation of selenite at different doses have been administered in this work to evaluate the effects in the levels of selenoproteins in serum and liver cytosolic extract using a multidimensional approach based on 2D/SEC-AF-SUID-ICP-ORS-MS. The speciation method has been validated using a CRM of human serum (BCR-637) certified for total Se content. The concentration of different selenium species obtained in the BCR-637 is in concordance with previous results published by the authors [44].

The results of our present study indicate that SelP levels increase in serum and hepatic cytosolic extracts after Hg exposure period, and this effect is more pronounced with increasing selenite supplementation (Table 3 and Fig. 4A-B). This fact highlights the synthesis of SelP from selenite and the antagonistic interaction between both elements in mice serum and liver,

since Hg-SelP complex plays an important role as a vehicle for Hg detoxification [10]. In that respect, decreased levels of LMM selenium species in serum and a correlatively increased in liver cytosolic extracts are concordant with the requirement of selenite for SelP synthesis in the liver, which are then transferred to the bloodstream [48]. Additionally, SelP levels have been upregulated in the liver, which can explain the elevated concentration of this selenoprotein in this organ after Hg exposure period, and this fact is also more marked with increasing selenite supplementation, as in serum (Table 3 and Fig. 4A-B). Related to this, decreased levels of SeAlb in mouse serum were obtained when Hg is supplied alone and was accompanied by increased liver SeAlb concentration (Table 3), since this transport selenoprotein is transferred to the liver for the synthesis of SelP [48]. More pronounced effects were observed when Se was administered together with Hg at different doses, 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite (Table 3).

Serum HMM selenium species (Fig. 4B) is attributed to eGPx selenoenzyme that is located in the mammalian bloodstream, representing about 5 % of selenium in mouse plasma [29**]**. Our results show up-regulation of eGPx induction in Hg-treated mouse serum in comparison with control group, practically independent of the Se supplementation dose (Table 3). In contrast, HMM selenoproteins fraction in liver cytosolic extract (Fig. 4A) may be containing other selenoproteins beside to eGPx, such as TrxR as previously discussed. Independently of the low resolution in this fraction, increased levels in both redox selenoenzymes are related to oxidative damage caused by Hg toxicity were obtained after Hg exposure, more pronounced when Se is administered together with Hg (table 3), since their antioxidative properties help to eliminate reactive oxygen species induced by Hg *in vivo* [10,45].

Finally, in Fig. 4C and 4D can be observed, with the use of affinity column (2D-SEC-AF-ICP-MS), that Hg is present in plasma in three remarkable peaks in liver cytosolic extracts and serum, respectively. One close to void volume, other more intense at retention time matching with SeP peak, and a third peak coincident with the retention time of SeAlb. However,

peaks traced by Hg are not observed in control samples or their intensities are significantly lower (Fig. 4C and 4D). Therefore, these results reflects the binding Hg to high molecular mass proteins (HMM), SelP and SeAlb fractions, since Hg traced peak at retention time of LMM selenium species is not present in both biological matrices (Fig. 4C and 4D). On the other hand, it seems that Se supplementation promote Hg binding in favor of binding to higher molecular mass proteins, especially when higher Se dose is administered together with Hg (Fig. 4C and 4D). We did not find any significantly differences in the accumulation of Hg associated to SelP in serum, with the exception of a notable increase in Hg content in SelP fractions after 0.5 mg/kg bw/day of selenite supplementation in mouse liver and serum (Fig. 4C and 4D). In this way, increased Hg intensity in SelP fraction was reported by other authors in human serum with the increasing Hg content in the bloodstream [10]. These results are in good agreement with our findings. As we can see, in Hg-SeAlb fraction similar tendencies were obtained (Fig. 4C and 4D). These findings seem to support the idea that Hg-SelP and Hg-SeAlb complex formation was accompanied by redistribution of Hg in the organism.

4. CONCLUSIONS

We have demonstrated in this work the high reliability of elemental mass spectrometry based on metallomic approaches to study the biochemical effects induced by Hg/Se in exposed mice on liver, kidney and serum metals distribution, interactions and homeostasis. The application of size exclusion chromatography coupled to ICP-MS to cytosolic extracts of metabolic active organs and biological fluids from the laboratory mice exposed to Hg/Se, allows deciphering the changes of metal-binding biomolecules induced by both elements. Likewise, selenium speciation in serum (2D/SEC-AF-SUID-ICP-ORS-MS) confirms the role of selenoproteins in Hg detoxification processes and the need for selenite intake after Hg exposure. The use of enriched stable isotopes is crucial to study the fate of trace elements in biological systems, employing isotopic dilution analysis measurements, which shows unequivocally the importance of selenium species for Hg protection. The protective mechanism includes the capability of Se to perturb the redistribution of Hg in organs and biological fluids and induces

binding of the inert Hg-Se-S complexes. Related to this, we propose that selenoproteins may have two important roles in protecting against Hg toxicity. First, they may bind more Hg through their highly reactive selenol group, and second, their antioxidative properties help compromise the reactive oxygen species induced by Hg in vivo. These results provide a better understanding of the interaction between Hg and Se in whole organisms during a 10 days exposure period. Finally, we can conclude that selenite could be a potential treatment of Hg toxicity in areas affected by this toxic metal, such as mining and industrial areas.

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FIGURE LEGENDS

Figure 1. Metal concentrations in the organs and serum of *Mus musculus* mice after exposure to Hg/Se. Values are mean ± SD for six mice per group. Hg group was given a subcutaneous administration of 0.2 mg/kg bw/day $HgCl_2$. Hg+Low-Se and Hg+High-Se groups were given a subcutaneous administration of 0.2 mg/kg bw/day of $HgCl_2$ and supplemented orally with 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite, respectively.

Figure 2. SEC-ICP-MS derived Hg-specific chromatograms obtained for the analysis of mouse liver cytosolic extracts (A), and serum (B). Chromatographic conditions: Superdex^{TM}-200 column; mobile phase: ammonium acetate 20 mM (pH 7.4); flow rate: 0.8 ml min^{-1}; injection volume: 20 µL.

Figure 3. SEC-ICP-MS derived Cu (A), Zn (B) and Hg (C) specific chromatograms obtained for the analysis of mouse kidney cytosolic extracts. Chromatographic conditions: Superdex^{TM}-200 column (10x300x13 µm); mobile phase: ammonium acetate 20 mM (pH 7.4); flow rate: 0.8 ml min^{-1}; injection volume: 20 µL.

Figure 4. A) Mass flow chromatogram with $^{78}Se/^{74}Se$ isotope ratios of selenoproteins and low molecular weight selenium species in mouse liver cytosolic extracts after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. (B) Mass flow chromatogram with 78Se/74Se isotope ratios of selenoproteins and low molecular weight selenium species in mouse serum after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. C) Hg specific chromatograms obtained for the analysis of mouse liver cytosolic extracts after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. D) Hg specific chromatograms obtained for the analysis of mouse serum after exposure to Hg/Se for 10 days using 2D/SEC-AF-SUID-ICP-ORS-MS. Hg group was given a subcutaneous administration of 0.2 mg/kg bw/day $HgCl_2$. Hg+Low-Se and Hg+High-Se groups were given a subcutaneous administration of 0.2 mg/kg bw/day of $HgCl_2$ and supplemented orally with 0.15 mg/kg bw/day and 0.50 mg/kg bw/day of selenite, respectively.

Table 1. Operating conditions of chromatographic separations and ICP-ORS-MS detection

Forward power	1500 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1 L min^{-1}
Carrier gas flow rate	0.15 L min^{-1}
Sampling and skimmer cones	Ni
Nebuliser	Microflow (ESI)
Torch	Shield (with long life platinum shield plate)
Q_{oct}	-18 V
Q_p	-16 V
Points per peak	1
Integration time	0.3 per isotope
Replicates	1
Isotopes monitored for total metals determination and SEC	^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{103}Rh, ^{80}Se, ^{78}Se, ^{201}Hg and ^{202}Hg.
$H_{e\ flow}$ for total metals determination and SEC	4.0 mL min^{-1}
Isotopes monitored for SUID	^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se, ^{79}Br, ^{81}Br and ^{83}Kr
$H_{2\ flow}$ for SUID	3.7 mL min^{-1}
Dead time detector	47 ns
Column	Superdex^{TM}-200 (10x300x13µm)
Resolution range	600-10 kDa
Mobile phase	Ammonium acetate 50 mM(pH 7.4)
Flow rate	0.7 mL min^{-1}
Injection volume	20 µL
UV detection	254 nm
Sample loop	100 µL
Flow rate	1.3 mL min^{-1}
Mobile phase A	0.05 M ammonium acetate pH 7.4
Mobile phase B	1.5 M ammonium acetate pH 7.4
Gradient	0-7 min 100% A, 6-18 min 100% B, 18-20 min 100% A
6-port valve position	1-10 min Inject 10-17 min Load 17-20 min Inject

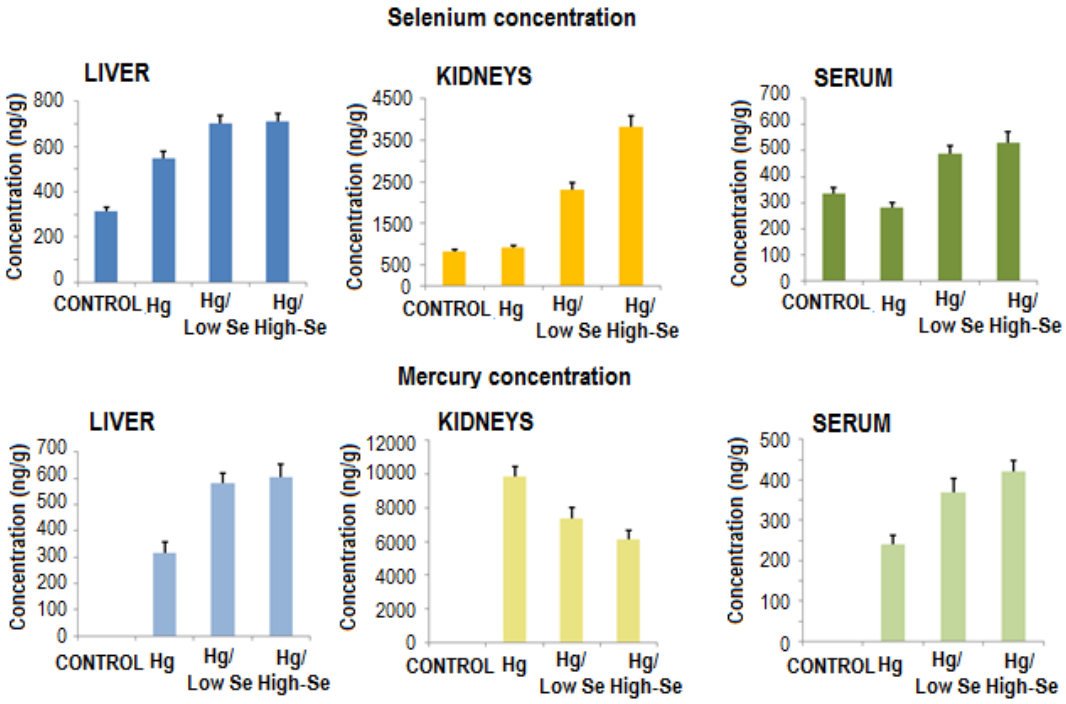
Table 2. Concentration of elements (ng g^{-1}) in the different organs and serum of mice following inorganic mercury and selenium exposure for 10 days.

Liver	Control Group	6742.3±31	34126±2045	314.21±18.7	<LOD
	Hg-exposed Group	6689.6±45	36224±3154	550.12±36.3	314.31±42.4
	Hg/Low-Se-exposed Group	6831.4±54	35721±2892	703.54±51.8	581.16±38.6
	Hg/High-Se-exposed Group	5983.7±26	35201±3546	711.26±62.4	603.81±48.8
Kidneys	Control Group	4293.6±42	18631±904.3	821.19±58.4	<LOD
	Hg-exposed Group	8641.8±68	24621±1625	913.54±46.3	9831.4±613
	Hg/Low-Se-exposed Group	8012.3±73	20036±1820	2304.6±184	7342.6±702
	Hg/High-Se-exposed Group	7814.6±54	21093±2014	3821.4±257	6103.4±524
Serum	Control Group	432.12±16	921.35±88.61	330.14±21.2	<LOD
	Hg-exposed Group	402.21±38	1214.5±114.4	289.62±14.6	240.21±21.8
	Hg/Low-Se-exposed Group	394.52±25	1024.2±122.5	489.22±31.5	368.74±33.5
	Hg/High-Se-exposed Group	398.21±17	1105.6±98.44	531.24±42.8	421.33±27.4
Limit of detention (LOD)		0.054	0.105	0.098	0.204

Table 3. Selenoproteins concentration (ng g^{-1}) in hepatic cytosolic extract and serum of mice following inorganic mercury and selenium exposure for 10 days.

Liver cytosolic extracts	Control Group	46.5±5.21	47.7±6.21	195±15.4	20.2±3.16	309±9.14
	Hg-exposed Group	102±8.14	111±10.2	297±18.3	34.5±4.22	544±16.54
	Hg/Low-Se-exposed Group	117+11.2	90.3±12.1	422±31.4	48.6±7.16	678±24.7
	Hg/High-Se-exposed Group	129±9.87	118±8.42	434±18.8	59.4±10.1	740±17.3
Serum	Control Group	8.72±2.11	52.1±2.57	251±16.2	14.3±2.42	326±12.4
	Hg-exposed Group	14.4±3.04	5.41±1.30	266±12.7	9.66±1.68	295±10.8
	Hg/Low-Se-exposed Group	13.0±2.26	12.4±2.01	463±24.8	10.7±2.01	499±18.4
	Hg/High-Se-exposed Group	14.9±2.34	26.3±4.11	514±31.4	13.1±2.88	568±16.7
Certified Material	Mean ± SD (n=3)	11.2±1.51	<LOD	53.4±2.18	18.2±2.34	82±2
BCR-637	Certified value	Not certified				81±7
Limit of detention (LOD)		0.3	1.2	0.6	0.9	

Figure 1-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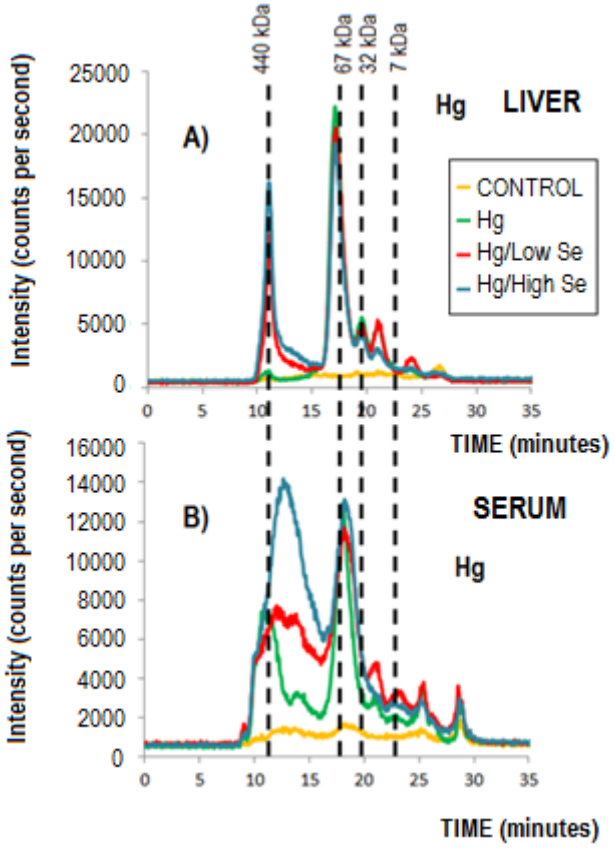
LIVER
KIDNEYS
SERUM
Concentration (ng/g)
CONTROL
Hg
Hg/ Low Se
Hg/ High-Se
Mercury concentration
LIVER
KIDNEYS
SERUM
Concentration (ng/g)
CONTROL
Hg
Hg/ Low Se
Hg/ High-Se

Figure 2-


440 kDa
67 kDa
32 kDa
7 kDa
A)
Hg
LIVER
CONTROL
Hg
Hg/Low Se
Hg/High Se
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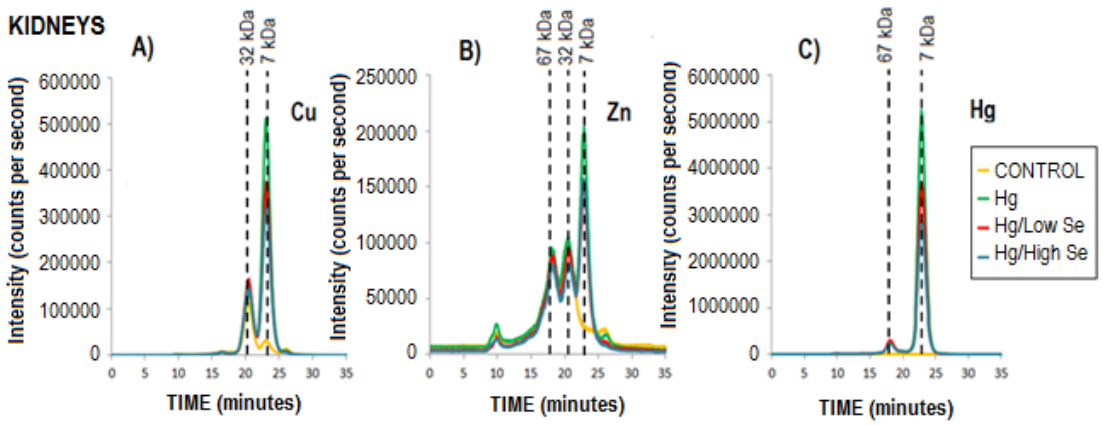
TIME (minutes)
B)
SERUM
Hg
Intensity (counts per second)
TIME (minutes)

Figure 3-


KIDNEYS
A)
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Intensity (counts per second)
TIME (minutes)
B)
67 kDa
32 kDa
7 kDa
Zn
Intensity (counts per second)
TIME (minutes)
C)
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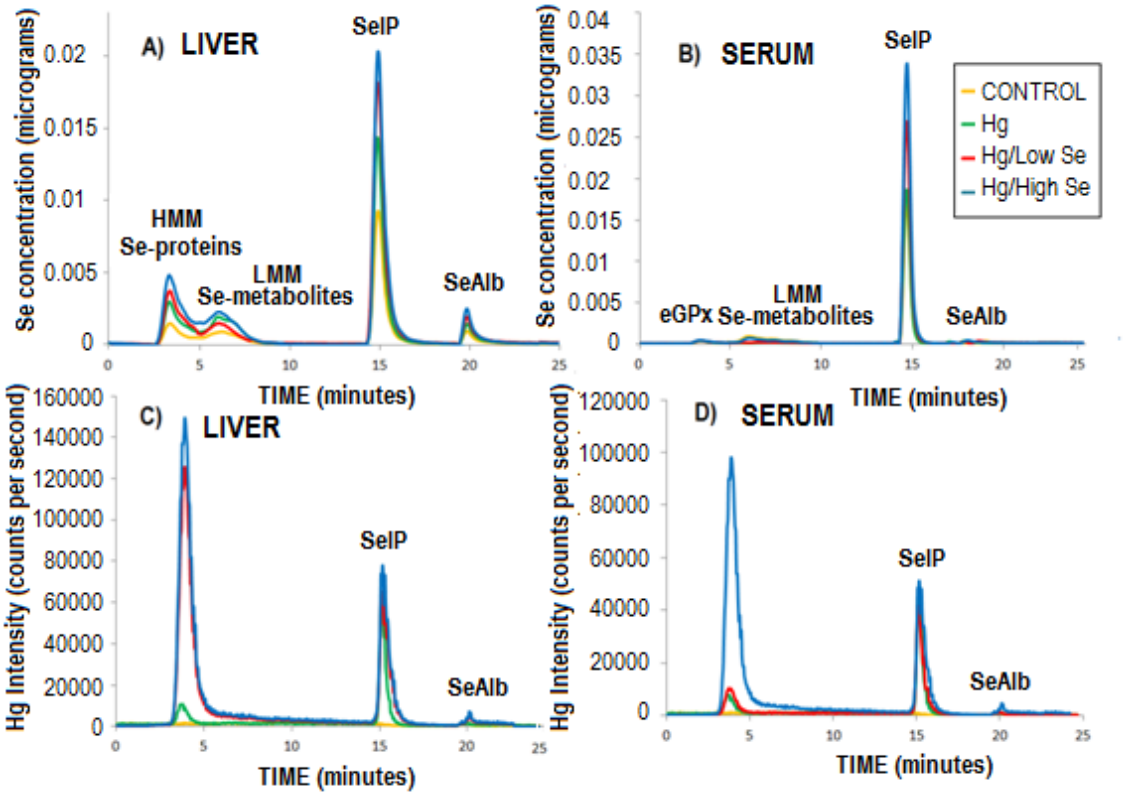
Hg
Intensity (counts per second)
TIME (minutes)
CONTROL
Hg
Hg/Low Se
Hg/High Se

Figure 4-


A) LIVER
SelP
HMM
Se-proteins
LMM
Se-metabolites
SeAlb
Se concentration (micrograms)
TIME (minutes)
B) SERUM
SelP
LMM
eGPx Se-metabolites
SeAlb
CONTROL
Hg
Hg/Low Se
Hg/High Se
C) LIVER
SelP
SeAlb
Hg Intensity (counts per second)
TIME (minutes)
D) SERUM
SelP
SeAlb
TIME (minutes)

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