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ARTICLE TYPE

Cadmium toxicity in *Mus musculus* mice based on metallomic and metabolomic approaches. Antagonistic interaction between Se and Cd in the bloodstream

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Cadmium (Cd) is an important inorganic toxicant in the environment and health, which is the origin of numerous environmental issues and health problems, such a cancer. In this work, a metallomic approach based on size-exclusion chromatography (SEC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS) to achieve a better understanding of the function, detoxification processes and regulation of metals in mice (*Mus musculus*) under controlled Cd and Cd/^{77}Se exposure. In addition, isotopic dilution analysis (IDA) was performed for quantifying the selenium containing proteins in plasma with a ICP-qMS as a multielemental detector. Additionally, isotope pattern deconvolution (IPD) was applied to study the fate of enriched ^{77}selenite in mice subjected to cadmium exposure and the effect in plasma selenoproteins. On the other hand, intended to get as much metabolic information as possible, liver and plasma taken from laboratory mouse *Mus musculus* have been studied in different period of time of Cd exposure. The analysis was carried out by mass spectrometry of high-resolution by direct infusion (DI-ESI(±)-QTOF-MS) and gas chromatography-mass spectrometry (GC-MS). Statistical analysis of results allowed us to compare the different metabolic profiles, establishing those metabolites that are altered in the presence of these contaminants. This work illustrates the high reliability of the integrated use of organic mass spectrometry for metabolomic study of biochemical effects in metabolic pathways induced by Cd, with inorganic mass spectrometry for metallomics approach provides a good alternative to deep insight into the fate of elements in exposed organisms, and gives information about metals trafficking, interactions and homeostasis.

Introduction

Cadmium (Cd) is a widespread, highly toxic environmental pollutant, naturally or because of industrial use, known to accumulate in human body. Several studies have indicated a carcinogenic potential for the heavy metal Cd in humans[1-3] and experimental animals, such as mice[4-5]. Another major source of Cd is tobacco smoke, resulting in higher blood Cd concentrations in smokers compared to non-smokers[6]. Cd has a long half-life in the body, and adverse effects of chronic Cd exposures on kidneys and bone are well documented[7-8]. Recently, Cd exposure has been associated with several endocrine effects[9-11]. In addition, it is well known that selenium (Se) present numerous antagonistic interactions with Cd, such as Se prevents the Cd-induced oxidative stress[12], Se protects against Cd-induced nephrotoxicity and hepatotoxicity[13] and Se antagonizes the Cd-induced inhibition of hepatic drug metabolism[14].

In contrast, the toxicological effects in metabolism, trafficking and alterations in metabolic cycles are still unclear. For this reason, the use of methods of massive information, such as metallomic and metabolomic approaches are mandatory to evaluate these effects in *Mus musculus* mice under controlled exposure of Cd and ^{77}Se/Cd[15]. Nowadays, using these metallomics and metabolomics methodologies there is a lot

information about the biological function of elements in the literature, although most of methods are focused on only one element or very well-defined species family linked to an element. However, most of these biological mechanisms and metabolic pathways are based on the interactions of several elements or their species that can counteract the action of others in cooperation or availability mechanisms[16].

The metabolism of the different metals is related with the source of exposure. Most metals from natural sources usually come from to anthropogenic processes, such as industrial, agricultural, mining and livestock, or the traffic itself, which should be considered as sources of Cd. Cd can not be degraded or destroyed and can enter the human body through drinking water, either by ingestion or skin absorption (Figure 1). May also be ingested, inhaled or absorbed dermally from resuspended dust particles coming from the soil. In air, air pollution particles containing levels of cadmium involves a wide variety of potential adverse health effects[17]. Independently regardless of the route of entry of this toxic metals in organisms, bloodstream plays a fundamental role in the transport of species among different organs[18](Figure 1). Additionally, when cadmium chloride is absorbed an important aspect in exploring the mechanisms of Cd toxicity is to identify the most likely binding sites for the toxic cation. Among them, the active sites of different metalloproteins are prominent candidates as they are designed to accommodate divalent Cd. For instance cadmium in metallothionein (MT) is exclusively bound to sulfur[19], but cadmium coordination is mixed in one zinc site of alcohol dehydrogenase[20].

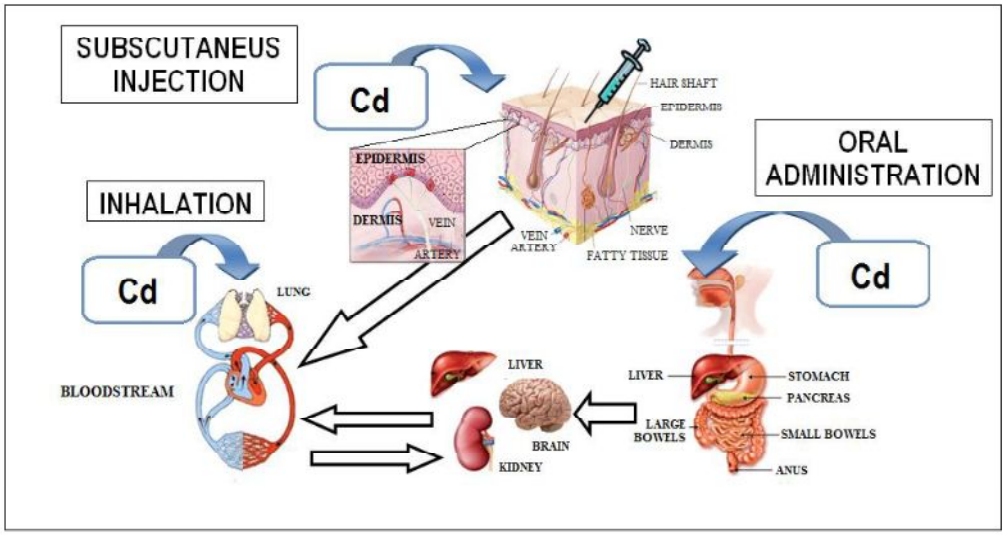

Fig.1. Schematic representation of different sources of exposure to cadmium in mammals.

Metallomics[21] uses metals or metalloids, present in one third of biomolecules in cells, as heteroatomic markers or tags to track these molecules in complex biological matrices[22]. In these approaches it is fundamental the use of high sensitivity atomic detectors mainly inductively coupled plasma mass spectrometer (ICP-MS)[23], generally coupled to a previous chromatographic step (in single or multidimensional arrangements), and mass spectrometry for parallel biomolecules identification in an integrated workflow[24] (Fig. 2a). The combination of metallomics with organic mass spectrometry is mandatory for unknown species in biochemical issues, especially electrospray ionization (ESI) or matrix assisted laser desorption (MALDI). The use of ESI-MS is more suitable than MALDI-MS for tandem mass spectrometry and on-line couplings with separation techniques (HPLC, CE) using offline two dimensional chromatography or online multidimensional chromatography (Fig. 2a). Several mass analyzers can be used to obtain structural information of species such as triple quadrupole (QqQ), triple quadrupole time of flight (QqQ-TOF), ion trap (IT), quadrupole trap (QTrap) or Fourier transform ion cyclotron resonance (FT-IRC).

On the other hand, metabolomics is based on the complete study of metabolites involved in different metabolic processes in cells of organisms, and the metabolome can be defined as the entire cellular set of endogenous low molecular weight biomolecules (typically <1000Da)[25]. Metabolomics plays a key role in understanding the results of complex biological processes, such as metal exposure experiments[15]. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are major analytical tools for metabolomics approaches[26-27]. MS is usually coupled to high performance liquid chromatography (HPLC)[28-29], gas chromatography (GC)[30], or capillary electrophoresis (CE)[31], in order to reduce the isobaric interferences in the mass spectra and making possible metabolite identification and quantification (Fig. 2b). Gas chromatography, especially when interfaced with mass spectrometry (GC-MS), is one of the approaches most widely used in metabolomics, since it offers very high chromatographic resolution, but requires chemical derivatization for many metabolites (Fig. 2b). Nevertheless, a number of studies propose the use of sample direct infusion to mass spectrometer without any previous separation step[32-35], this approach makes analysis simpler and faster and the traditional isobaric interferences affecting direct infusion are overcome with the use of high resolution mass analyzers, such as hybrid systems triple quadrupole-time-of-flight (QqQ-TOF)[15] (Fig. 2b) or Orbitrap[36].

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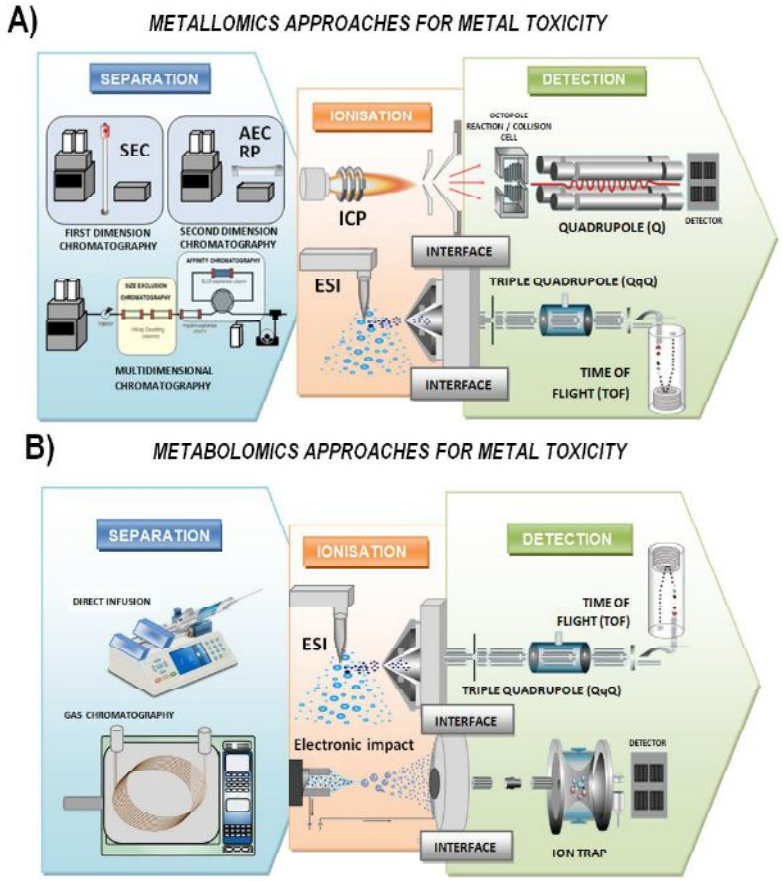

Fig. 2. Metallomics and metabolomics workflow for metal toxicity in mice *Mus musculus* under controlled exposure

In addition, isotope dilution analysis (IDA) is a well known analytical technique based on the measurement of isotope ratios in samples where its isotopic composition has been altered by the addition of a known amount of an isotopically enriched element[37]. Post-column isotope dilution allows the accurate determination of elemental species even if the structure of the compounds is unknown while multiple isotopically enriched species can be applied for the evaluation and correction of species interconversion reactions[37]. Species-unspecific isotope dilution mode (SUID) is especially useful either when the structure and composition of analysed species is not exactly known or the corresponding isotopically labelled compound is not commercially available[38]. In contrast, when the isotopically labelled compound is commercially available, species-specific isotopic dilution mode (SSID) is applied[39]. Moreover, enriched stable isotopes are crucial to study the fate of trace elements in biological systems. Dual isotope ratio measurements require selenium equilibration between natural existing selenium in the sample and added isotopically enriched selenium. In this work we have applied isotope pattern deconvolution (IPD) for insolating distinct isotope signatures from mixtures of natural (endogenous) abundance and enriched tracers (exogenous)[40].

The aim of the present study is to determine the toxicological effects in mice of Cd exposure over 12 days using MS. Divalent inorganic cadmium chloride is administered by subcutaneous injection (0.1mg/kg body weight and per day) in *Mus musculus* mice were investigated using metallomics and metabolomics approaches. A metallomic approach based on the use of SEC-ICP-MS is applied to characterize the biological response of Cd and Cd/^{77}Se exposed mice. In addition, selenoproteins were quantified using SEC coupled to multiaffinity chromatography(AF)-ICP-MS by IDA. In addition, ^{77}Se (in the form of selenite) was administered by oral ingestion to evaluate the antagonistic interaction between Cd/Se in the bloodstream. On the other hand, metabolomic approaches based on the use of by direct infusion to a mass spectrometer (DI-ESI-QTOF-MS) followed by Partial Least Squares-Discrimination Analysis (PLS-DA) and gas chromatography-mass spectrometry (GC-MS) allows characterize metabolic changes provoked by toxic xenobiotics as Cd in animal tissues or biological fluids.

Materials and methods

Instrumentation

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770) was used to prepare the homogenates. Homogenized tissues were subsequently disrupted with a glass/teflon homogenizer. The extraction was followed by ultracentrifugation with an ultracentrifuge Beckman model L9-90 K (rotor 70 Ti). Polycarbonate bottles of 10 ml with cap assembly (Beckman Coulter) were used for this purpose. A microwave oven (CEM Matthews, NC, USA, model MARS) was used for the mineralization of extracts.

Trace metals and metal-linked biomolecules were analyzed with an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan) equipped with an octopole collision/reaction cell. Chromatographic separations were performed using a Model 1100 HPLC pump with detector UV (Agilent, Wilmington, DE, USA) as the delivery system.

Metabonomic experiments were performed in a mass spectrometer QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using the electrospray (ESI) source. The parameters for QqQ-TOF system were optimized to obtain the higher sensitivity with minimal fragmentation of molecular ions, both in positive and negative ion mode. To acquire MS/MS spectra, nitrogen was used as collision gas.

Gas chromatographic analysis were performed in a Trace GC ULTRA gas chromatograph coupled to an ion trap mass spectrometer detector ITQ900, both from Thermo Fisher Scientific, using a Factor Four capillary column VF-5MS 30m×0.25mm ID, with 0.25 μm of film thickness (Varian).

Standard solutions and reagents

All reagents used for sample preparation for 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).

Standards used for mass calibration of analytical SEC columns (mass range 70-3 kDa) were: ferritin (440 kDa) (purity 95%), bovine serum albumin (67 kDa) (purity 96%), superoxide dismutase containing Cu and Zn (32 kDa) (purity > 70%), myoglobin (14 kDa) (purity > 98%), metallothionein I containing Cd, Cu and Zn (7 kDa) (purity > 95%) and arsenobetain (179 Da) (purity > 98%). All these reagents were purchased from Sigma-Aldrich (Steinheim, Germany). The mobile phase solution used in SEC was 20 mM of ammonium acetate (Suprapur grade) purchased from Merck (Darmstadt, Germany), which was prepared daily with ultrapure water (18 MΩcm) from a Milli-Q system (Millipore, Watford, UK) and the pH adjusted to pH 7.4 with ammonia solution, this later prepared by dilution of 20% (w/v) ammonia solution (Suprapur, Merck) with ultrapure water. The void volume was determined using blue ferritin (440kDa).

The 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 Br- stabilized in 5% (v/v) nitric acid Suprapur were purchased from Merck (Darmstadt, Germany). Enriched ^{74}Se and ^{77}Se were obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder and it was dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to volume with ultrapure water. The concentration of this solution was established by reverse isotope dilution analysis as described elsewhere[38].

All the solvents used in sample preparation for metabolomic study of liver tissue and plasma were of HPLC-grade. Methanol and chloroform were purchased from Aldrich (Steinheim, Germany), while dichloromethane and formic acid were supplied by Merck (Darmstadt, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK).

Derivatizing agents, methoxylamine hydrochloride and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS), were obtained in Sigma-Aldrich. Alanine, valine, isoleucine, proline, glycine, serine, threonine, glutamic acid, phenylalanine, fructose, galactose, glucose, tyrosine, tryptophan, urea, aspartic acid, glutamine, cholesterol, α-ketoglutarate, isocitric acid, citric acid, lactic acid and uric acid, purchased from Sigma-Aldrich, were used as standard substances in gas chromatography quantification.

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 feed selenium deficient pellets. 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 start exposure experiment.

Firstly, 32 *Mus musculus* mice were divided into two groups, one used as control and the other exposed to Cd(II) (in the form $CdCl_2$), using subcutaneous injection of 100 μL of a dose of 0.1 mg Cd per kg of body weight per day during a total period of 12 days. The control mice were subjected to subcutaneous injection of 100 μL of ultrapure water with 0.9% NaCl per day during 12 days. Mice were sacrificed after the 6^{th} and 12^{th} day from the beginning of the experience to assess the effect of exposure conditions and the diet.

Secondly, 60 *Mus musculus* mice were divided in four groups, one group (GROUP A) exposed to oral administration of 0.15 mg ^{77}Se per kg of body weight per day during a total period of 15 days. A second group (GROUP B) exposed to oral administration of 0.5 mg ^{77}Se per kg of body weight per day during a total period of 15 days. The other groups (GROUP C and D) were exposed to the same selenium concentration by oral administration and 0.1 mg Cd per kg of body weight per day by subcutaneous injection with the objective to evaluate the interactions between Se/Cd using different concentrations of Se.

Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, dissected using a ceramic scalpel and finally the organs transferred rapidly to dry ice. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% NaCl solution, frozen in liquid nitrogen and stored at -80 ºC until their use for extract preparation. Mice were handled according to the norms stipulated by the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

Metallomic approach based on analytical scale SEC-ICP-MS of plasma, liver and kidney of mice (*Mus musculus*) under under Cd or Cd/^{77}Se exposure

Pools of organs tissue from male mice of different groups of exposure were treated following a procedure described elsewhere[41]. Plasma collection was carried out by centrifugation (4000 g, 30 min, 4ºC) after addition of heparin (ANTICLOT) as a anticoagulant for separation into plasma and red blood cells (RBCs). In addition, 10 mg of 100 mM of PMSF and 100mM of TCEP mixture were added as proteases inhibitor and reductants agent, respectively.

The retention times corresponding to the peaks of standards used for Superdex^{TM}-75 column calibration were the following: ferritin 11.5 min, bovine serum albumin (BSA) 13.7 min, superoxide dismutase containing Cu and Zn (Cu,Zn-SOD) 16.3 min, myoglobin (an iron containing protein) 18.9 min, metallothionein I containing Cd, Cu and Zn (Cd,Cu,Zn-MT1) 21.0 min, and arsenobetain (AsB) 26.1 min.

Metallomic approach based on analytical scale SEC-AF-SUID-ICP-ORS-qMS of plasma of mice (*Mus musculus*) under Cd exposure.

To avoid changes in selenium species, the samples were directly injected into the column, without prior dilution.
The fractionation of selenium containing proteins by two dimensional chromatographic separations based on SE prior double affinity column was carried out following a procedure described elsewhere[42] in series stacking of two 5 ml HiTrap® Desalting Column (GE Healthcare, Uppsala, Sweden), which are in series connected with a dual affinity column arrangement comprising by a 1 ml heparin-sepharose column (HEP-HP) (GE Healthcare, Uppsala, Sweden) and a 1 ml blue-sepharose column (BLU-HP) (GE Healthcare, Uppsala, Sweden) interconnected by a six-way switching column valve. The HiTrap column is based on size exclusion chromatography, that removes low molecular mass components (MW<1000Da) from high molecular mass molecules, such as DNA, proteins or peptides (MW>5000Da), the combination of two columns increase the resolution of separation. On the other hand, HEP-HP column is able to retain selectively selenoprotein P (SeP) whereas BLUE-HP column retains both SeP and selenoalbumin (SeAlb) which has been previously described[43].

The quantification of selenium containing proteins and selenium-metabolites in the different chromatographic peaks was carried out by post-column specie-unspecific isotopic dilution (SUID) analysis as described by C. Sariego-Muñíz et al.[44]. Briefly, the intensity of different Se isotopes and polyatomic interferences were converted to mass flow chromatogram for the quantification of the selenium species in plasma and serum samples. Dead time correction was carried out using the procedure described by F. Vanhaecke et al[45], which result 47 ns in this study. Mathematical treatments were applied to correct BrH^+ and SeH^+ polyatomic interferences. Mass bias corrections were applied using the $^{78}Se/^{74}Se$ and $^{80}Se/^{74}Se$ isotope ratios calculated (exponential mode) as previously described by J. Ruiz-Encinar et al.[46]. Finally, an online dilution equation was applied to each point of the chromatogram and the amount of selenium in each chromatographic peak calculated using Origin 8.5.1. software (Microcal Software Inc., Northampton, MA, USA).

Total determination of endogenous and exogenous Se concentration by IPD-ICP-ORS-qMS of plasma of mice (*Mus musculus*) under Cd/^{77}Se exposure

For human serum reference materials, sample amount of 0.1 g were weighted directly in a glass vials of 10 mL. An appropiate amount of the ^{77}Se spike and 800 µL of a mixture 4:1 HNO_3:H_2O_2 were added. The samples were incubated during 8 hours at 80ºC and then diluted with ultrapure water to 5 g. Finally, the digested certified reference materials were filtered using a PTFE 0.45 µm syringe filters before the analysis by IPD-ICP-ORS-MS. Mice plasma were digested using the same procedure previously described but in this case the addition of ^{77}Se spike is not necessary.

For isotope ratio determination of endogenous and exogenous selenium the isotopes 77, 78, 80 to 100 ng g^{-1} were prepared to obtain the maximum signal for these masses. A solution of 5% (w/w) HNO_3 was used to correct the background level caused by polyatomic argon interferences. In addition, the correction for SeH^+ and BrH^+ formation was carried out using mathematical equations by monitoring also the signals at masses 76, 82 and 83 (for SeH^+) and 79 and 81 (for BrH^+). On the other hand, the optimum spike to sample ratio was calculated as a previously described by García-Alonso47. Finally, the concentration of the analyte was calculated using the isotope dilution equation described previously[47]. Selenium determination in mice plasma by IPD-ICP-ORS-MS was carried out using the operating conditions summarized in Table 1. All the analyses were performed using two replicates.

Table 1. Operating conditions of IPD-ICP-ORS-MS detection

ICP-MS conditions		
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 depth	7mm	
Sampling and skimmer cones	Ni	
H_2 flow	4 mL min^{-1}	
Nebulizer	Micromist (Glass Expansion)	
Torch	Shield (with long life platinum shield plate)	
Qoct	-18 V	
Qp	-16 V	
	IPD Analysis	***IPD Post column***
Points per peak	3	1
Integration time	4 s per isotope	0.3 s per isotope
Replicates	5	1
Isotopes monitored	^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se, ^{79}Br, ^{81}Br and ^{83}Kr	
Dead time detector	47 ns	

Metallomic approach based on analytical scale SEC-AF-IPD-ICP-ORS-qMS of plasma of mice (*Mus musculus*) under Cd/^{77}Se exposure

In to validate the SEC-AF-HPLC-IPD-ICP-ORS-MS methodology for quantitative selenium speciation in plasma samples, the human serum CRM was used. The CRM was

spiked with a optimum amounts of ^{77}Selenite. After the online separation, the quantification of selenium in the different chromatographic peaks was performed by post-column isotope dilution analysis using a solution of enriched ^{74}Se for quantification of the endogenous and exogenous selenium species in plasma of mice under enriched ^{77}Se and Cd. Finally, the IPD technique was applied of each point of the chromatogram to obtain the mass flow chromatogram for endogenous Se and exogenous Se[40].

Metabolomic approach of liver and plasma from mice (*Mus musculus*) under cadmium exposure by direct infusion organic mass spectrometry (DI-ESI(±)-QTOF-MS)

For metabolomic analysis, metabolites extraction from individual tissues was carried out in a two-step approach following a procedure described elsewhere[48]. The polar and lipophilic extracts were reconstituted to 200 µL of(1:1) chloroform/water mixture before the analysis by ESI-MS.

For DI-ESI(±)-QTOF-MS of plasma samples, proteins were removed from blood plasma by adding 400µL of 1:1 methanol/ethanol mixture to 100 µL of plasma in an Eppendorf tube followed by vigorous vortex shaking for 5 min at room temperature and centrifugation at 4000 g for 10 min at 4 °C. The supernatant was carefully collected avoiding contamination with the precipitated proteins, transferred to another Eppendorf tube. and the resulting supernatant was taken to dryness under nitrogen stream and stored to -80ºC until analysis. The pellet was homogenized again, with 200µL of a mixture of (2:1) chloroform/methanol, using a pellet mixer (2 min), to extract lipophilic metabolites and centrifuged (10000 g at 4 °C for 10 minutes). Finally, the resulting supernatant was taken to dryness under nitrogen stream and stored to -80ºC until analysis.

The polar and lipophilic extracts were reconstituted to 200µL of (1:1) chloroform/water mixture before the analysis by ESI-MS. For data acquisitions from positive ionization, 0.1% formic acid was added to polar extract and 30 mM of ammonium acetate to lipophilic extract. In the case of negative ionization intact extracts were directly infused to the mass spectrometer.

Metabonomic approach of plasma from mice (*Mus musculus*) under cadmium exposure by gas chromatography-mass spectrometry (GC-MS)

Plasma was thawed at 4ºC and vortex-mixed before use. For the extraction of metabolites from plasma, 100 µL of plasma samples are mixed with 400 µL of 1:1 methanol/ethanol mixture in an Eppendorf tube and vortexed for 5 min at room temperature, followed by centrifugation at 4000 g for 10 min at 4°C. The supernatant is transferred to another Eppendorf tube and dried under nitrogen stream. All the dried samples were derivatized with 50 µL methoxylamine hydrochloride (20 mg mL^{-1} in pyridine) at 70ºC for 40 min for protection of carbonyl groups by methoximation, followed by 50 µL MSTFA with 1% of TMCS at 50ºC for 40 min for derivatization of primary amines and primary and secondary hydroxy groups in the case of MTSFA. In addition, TMCS aids in the derivatization of amides, secondary amines and hindered hydroxy groups. Finally, the derivatized samples were vortex-mixed for 2 min before GC analysis, centrifuged at 4000 g for 5 min and supernatant is collected for analysis.

Chromatography was performed on a Factor Four capillary column VF-5MS 30m×0.25mm ID, with 0.25 µm of film thickness (Varian). The injector temperature was kept at 280ºC. Helium carrier gas was used at a constant flow rate of 1 mL/min. To acquire a good separation, the column temperature was initially maintained at 60ºC for 5 min, and then increased from 60 to 140 ºC at a rate of 7 ºC/min for 4 min. Then, the column temperature was increased to180 ºC at 5º C/min for another 6 min. After that, the temperature was increased to 280ºC at 5 ºC/min, and held for 2 min. For mass spectrometry detection, ionization was carried out by electronic impact (EI) with a voltage of 70 eV, by full scan mode in the m/z range 35–650, with an ion source temperature of 200ºC. Typical GC/MS total ion current (TIC) chromatograms are shown in Fig. 3 from mice plasma from control group. For analysis, 1 µl of sample is injected in splitless mode. The identification of endogenous metabolites was based on comparison with the corresponding standards according to their retention times and mass spectra characteristics or searching the NIST Mass Spectral Library (NIST 02).

Data analysis

Markerview™ software (Applied Biosystems) was employed to filter the mass spectrometric results. Statistical data analysis (Partial least squares discriminant analysis (PLS-DA) were performed by means of statistical software packages SIMCA-P™ (version 11.5, published by UMetrics AB, Umeå, Sweden). The data was processed in order to find differences between the groups of study in the different areas. For this purpose, the altered metabolites under arsenic exposure were identified considering their molecular mass and the fragments resulting from MS/MS experiments. The altered metabolites were characterized using different databases of metabonomics based on DI-ESI-QqQ-TOF/MS, such as Human Metabolome Database (http://www.hmdb.ca), METLIN (http://metlin.scripps.edu) and Mass Bank (http://www.massbank.jp). In the case of GC-MS, identification of metabolites responsible of the discrimination between groups was performed using the NIST Mass Spectral Library (NIST 02).

Results and discussion

SEC-ICP-ORS-MS in liver and plasma of mice (*Mus musculus*) under cadmium exposure

To check the presence and potential interactions of metal-biomolecules in organs of *Mus musculus* exposed to Cd the coupling SEC-ICP-MS was used to obtain the Cu, Zn and Cd-traced peaks in the cytosolic fractions of liver, kidney and plasma (Fig. 3). The distribution, accumulation and transference of zinc, copper and cadmium in living organisms have been considered in detail in the literature. Several facts such as the modulation of zinc concentration by homeostatic mechanisms[49] and the importance of transport mechanisms on copper distribution[50] are relevant issues to explain the relative presence of metal-binding molecules in the different organs of exposed organisms. In relation to this, the induction of Cd and Zn-metallothioneins in mice (*Mus musculus*) exposed to higher concentrations of Cd has been reported[51], and these experimental data confirm the antagonistic interactions among Cd, Zn, Cu, as well as the differential rate of excretion of these elements from kidney/liver under increasing exposure[52]. It can be observed that Cd-containing biomolecules in plasma present

two remarkable peaks at retention time of high molecular-weight (HMW) proteins (55 kDa) and a second peak that Cd elutes with the MT fraction at 19 min. However, peaks traced by Cd are not observed in this control samples (Fig. 3a). The accumulation of Cd in plasma along the exposure time in the fraction of 55kDa can be related with the high affinity of cadmium by selenoprotein P[53].

It is well known that Cd exposure causes change in the distribution of endogenous Zn and Cu in tissues and biological fluids, playing both metals a protective role against Cd toxicity due to their contribution in MTs induction[54-56]. It is possible that Cd intake causes an elevated content of Cu in plasma about 67 kDa (Fig 3b), molecular mass of two important copper transport proteins in the bloodstream, such as BSA and transferrin (Tf) of 67kDa and 79kDa of molecular mass, respectively.

Our results showed peaks related to Cd and Zn bound to MT fraction which increased consistently with Cd exposure in liver (Fig. 3c and 3d). The induction of MT is accompanied by a small increase of the intensity of Zn associated with MT fraction (Fig. 3d), which fact has been previously observed by Srivastava et al.[57]. The same effect was observed for Zn associated mostly with the HMW protein fractions.

On the other hand, higher concentration of Cd associated to MT is observed in comparison with liver and plasma (Fig. 3e). This fact is in concordance with the urine is a principal way of MTs excretion[58]. Finally, it is interesting to observe the presence of Cu-peaks (Fig. 3f) in kidney extract at about 7 kDa that contrast with the absence of the equivalent peak of Zn (data no shown). Decreased levels associated to this fraction are observed along the exposure time (Fig. 3f) Possibly the difference in binding affinity of MTs for metal ions can contribute to this result, since Cd, Hg or Cu can displace Zn from metal-MT complex[59]. In addition, it has been checked that when Cd-MT subcutaneously injected rats were exposed to Cu, the concentration of Cd-MT in kidney decreases, but no effect was observed when treatment was performed with Zn[60]. For this reason the lack of Zn-MT peak can be associated to the interactive relationship between Cd, Cu and Zn in terms of renal metabolism of these metals.

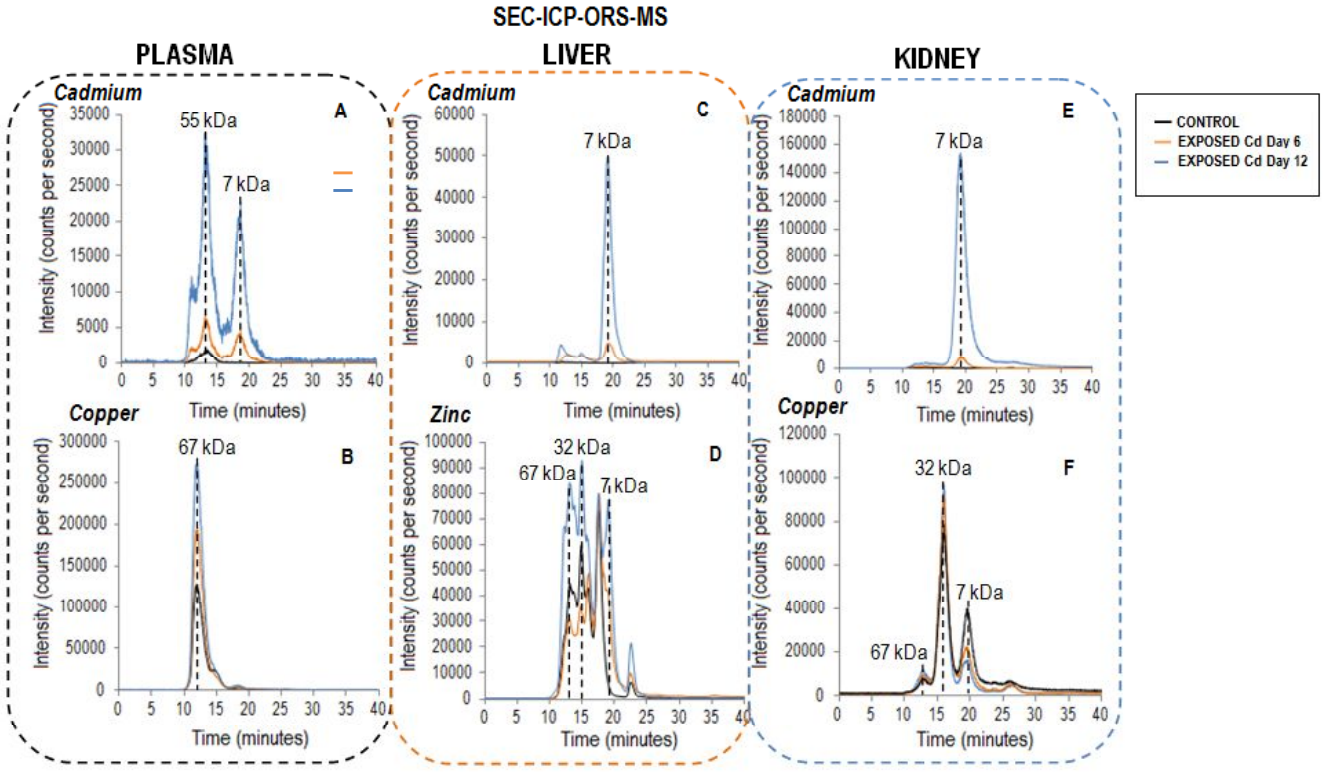

Fig.3. Up/downregulation of metal-biomolecule complexes in plasma (a, b), liver (c, d) and kidney (e, f) of *Mus musculus* exposed to cadmium assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex^{TM}-75 (10x300x13 µm); mobile phase, ammonium acetate 20 mM (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 20 µL

Selenoproteins in mice plasma (*Mus musculus*) under cadmium exposure by SEC-AF-HPLC-SUID-ICP-ORS-MS

Quantification of Se containing proteins and low molecular weight Se species has been performed in mice plasma using the proposed speciation method. Se concentration in selenoproteins is in good accordance with total Se concentrations determined by IDA-ICP-ORS-MS after acid digestion (table 2).

Table 2. Quantification of selenium species in mice plasma (*Mus musculus)* under cadmium exposure.

Selenium Species	*Plasma from mice Mus musculus*			
	CONTROL MICE (n=5)	*Cd EXPOSED MICE Day 6 (n=5)*	*Cd EXPOSED MICE Day 12 (n=5)*	**Detection limits** (LD, ng g^{-1})
GPx (ng g^{-1})	5.5±0.3	14±0.6	6.5±0.4	0.2
SeP (ng g^{-1})	132±9	146±8	154±10	0.7
SeAlb (ng g^{-1})	16.2±0.4	10.4±0.7	8.8±0.6	1.3
Se metabolites (ng g^{-1})	38.4±8	<LOD	<LOD	0.8
Sum of species (ng g^{-1})	193	170.4	169.3	-----
Total selenium[a] (ng g^{-1})	196±5	172±3	171±5	0.1

[a] Quantification of total selenium by IDA-ICP-ORS-MS.

For a better visualizing of the results obtained, the chromatogram of the mass flown of each group are shown in Fig. 4.

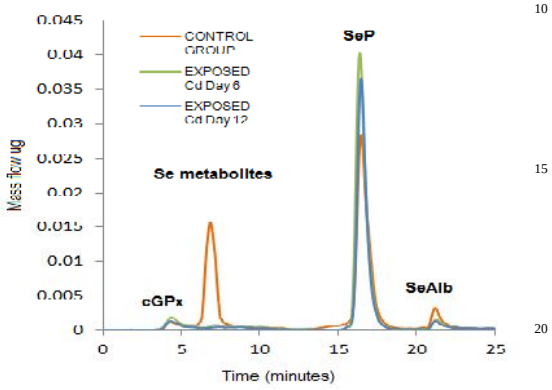

Fig. 4. Mass flow chromatogram with $^{78}Se/^{74}Se$ isotope ratios using 2D-SE-AF-HPLC-IDA-ICP-ORS-qMS of mice plasma from different groups of cadmium exposure.

It has been documented that Cd and Se interact in the body of mammals, and the co-administration of both reduces the toxicity of each other[61]. On the other hand, it is well known that Se levels in plasma is decreased under Cd exposure in rats subjected to oral administration[61].

In mammalian plasma, Se is incorporated mainly into three selenium containing proteins-SeP, eGPx (especially abundant in plasma[62]) and SeAlb. However, SeP is a unique selenoprotein that contains several selenocysteine (SeCys) and cystine (Cys) residues, indicating that it is capable of transporting Se and binding cadmium for excretion. For this reason, increased levels of SeP has been found along the exposure time. In addition, the peak of Cd about 55 kDa in Fig. 3a shows a significant increase with exposure, since SeP plays an important role in the traffic of cadmium between the organs.

Nevertheless, Se metabolites and SeAlb are required by the synthesis of selenoproteins in liver and then transported to plasma[63]. Decreased levels of selenium metabolites have been observed in mice after the administration of Cd supporting this idea (Fig. 4). Moreover, a little decrease of SeAlb levels has been observed along the exposure time (Table 2). Finally, eGPx are a family of antioxidant enzymes. Its role is antioxidant in the plasma and it can also reduce lipid hydroperoxides[64]. Increased levels of eGPx are observed in our study. This fact, could be related by the transport of this enzyme from liver to plasma to neutralize lipid hydroperoxide during lipid peroxidation.

Metabolomic approach in liver and plasma of mice (*Mus musculus*) under cadmium exposure by DI-ESI(±)-QTOF-MS and GC-MS

In order to discriminate between the groups, a partial least squares discriminant analysis (PLS-DA) was performed employing the intensities of the signals in the polar and lipophilic plasma and liver tissue extracts, using positive and negative mode of acquisition (Fig. 5). The models built with polar and lipophilic metabolites using positive and negative mode allow a good classification of sample in the different groups, which are showed by the respective scores plots. To identify which variables were responsible for this separation, the *Variable Influence on the Projection* (VIP) parameter was used. VIP is a weighted sum of squares of the PLS-DA weight which indicates the importance of the variable to the whole model. Thus is possible to select variables with the most significant contribution in discriminating between metabonomic profiles corresponding to groups of exposure against controls. Only metabolites with VIP > 2 have been considered good biomarkers of Cd exposure. The values of R^2Y (cum) and Q^2 (cum) of the combined model are 0.90-0.0.95 and 0.8-0.90, respectively, indicating that a combination of datasets between groups provides the best classification and prediction. In addition, it is remarkable the complementarily of using both ionization modes for polar and lipophilic metabolites (see Table 3).

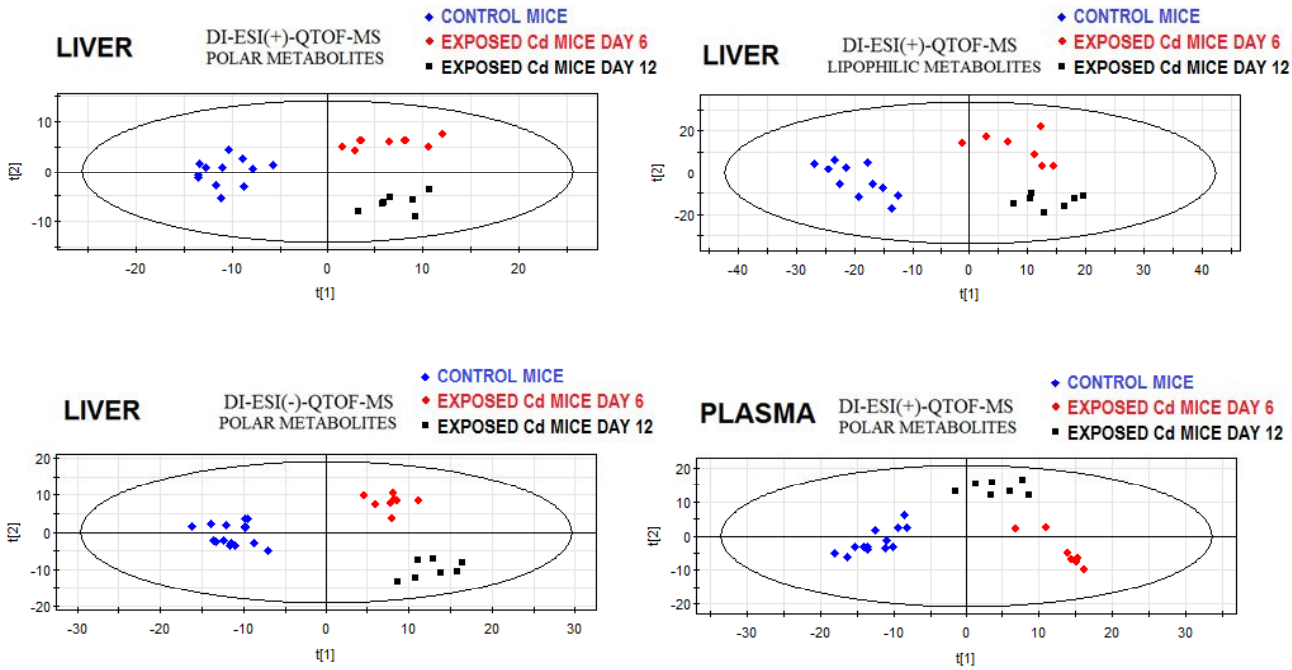

Fig. 5. Scores plots of PLS-DA for ESI+ and ESI– ionization modes of polar and lipophilic liver extracts and plasma. Blue spot: control mice; red spot: exposed to Cd mice day 6; black spot exposed to Cd mice day 12.

Table 3. Biomarkers from liver and plasma of mice (*Mus musculus*) exposed to cadmium during 12 days

Metabolites	*m/z*	*Mode of acquisition*	*Target Organ*	*Variation following Cd exposure*
Taurine	126.01 (H^+)	ESI(+)	Liver and plasma	↓
Choline	104.09 (H^+)	ESI(+)	Liver and plasma	↑
Glutamic acid	148.05 (H^+)	ESI(+)	Liver and plasma	↓
Citric acid	193.03 (H^+)	ESI(+)	Liver and plasma	↓
Glucose	203.05 (Na^+)	ESI(+)	Plasma	↓
	215.03 (Cl^-)	ESI (-)	Plasma	↓
Pipecolic acid	130.08 (H^+)	ESI(+)	Liver	↑
Arachidonic acid	303.24 (H^-)	ESI(-)	Liver	↑
Palmitic acid	255.42 (H^-)	ESI(-)	Liver	↑
Lyso-phosphatidylcholines (Lyso-PC)	450-600	ESI(+)/ESI(-)	Liver	↑
Phosphatidylcholines (PC)	700-850	ESI(+)	Liver	↓
Diglycerides	600-700	ESI(+)/ESI(-)	Liver	↑
Triglycerides	850-950	ESI(+)/ESI(-)	Liver	↑

Variations compared to control mice: ↑, increasing signal intensity, ↓, decreasing signal intensity

In addition, GC-MS was applied as complementary metabolomic approach to the confirmation and quantification of the altered metabolites obtained by DI-ESI(±)-QTOF-MS as complementary approach. For this purpose, three derivatizant agents were used for plasma samples from mice in order to get as much metabolic information as possible. Plasma metabolic profiles of five mice plasma of each group of exposure along the exposure time were obtained by GC-MS with the method as described above. The concentration of mice plasma metabolites are shown in table 4.

The metabolites responsible for the separation between control and Cd exposed groups were obtained from the PLS-DA analysis. The observed perturbations in triglycerides (TGs) and diglycerides (DGs) levels, perturbations in membrane phospholipids composition, elevation in choline, polyunsaturated fatty acids (PUFAs) and decrease in glucose, taurine, glutamic acid, citric acid levels have been obtained using DI-ESI(±)-QTOF-MS (table 3). On the other hand, decrease in glucose, isoleucine, glutamic acid, α-ketoglutarate, isocitric acid and citric acid concentrations and increase in lactic acid, glutamine and cholesterol concentrations have been obtained using GC-MS (table 4). Our results show the complementarity between the two techniques used in this work to evaluate the biological response to Cd exposure experiments in mice.

Cd is known to cause cellular acidosis in renal tissue by inhibiting carbonic anhydrase enzymatic activity. This decrease in pH can also lead to inhibition of the TCA cycle within mitochondria, resulting in a decrease in TCA cycle intermediates, and an elevation of glycolytic flux[65]. This mechanism also accounts for the observed lowering of plasma glucose concentration and increases in the concentration of lactic acid. In addition, laboratory rats given chronic intra-peritoneal Cd^{2+} doses of 9-24 µmol/kg of body weight[66] had demonstrated metabolic abnormalities including reduced urinary citrate, 2-oxoglutarate and 2-oxaloacetate concentrations. In this work, the alteration in intermediates from energy metabolism, such as citric acid, isocitric acid, α-ketoglutarate, glutamic acid, lactic acid and glucose supporting this idea. Reduced energy production within renal mitochondria produces aminoaciduria, by energetically limiting re-uptake of these compounds in the glomerulus and proximal tubule,

possibly induces increased expression of mitochondria, but ultimately culminates in cellular necrosis or apoptosis, as seen in high Cd^{2+}. exposure studies[67]. Moreover, glutamine is the main source of NH_4^+ ions appearing within the glomerular filtrate, with NH_4^+ production in turn being the main mechanism to reduce cellular acidosis in the kidney[69]. Increased levels of glutamine in plasma has been obtained along the exposure time in plasma by GC-MS (table 4).

Perturbations of amino acid concentrations seem to be a common response to many toxins in several species and are likely to be indicative of a general response to toxicant exposure rather than a specific response to a specific toxicant[68]. Alterations in plasma concentrations of taurine and isoleucine have not previously been reported following cadmium exposure, although it has been suggested that taurine may be an important osmoregulatory compound within the cell[70].

Perturbations in TGs levels in response to Cd has previously been demonstrated in both bank voles[71] and rats[72]. Although exposure to Cd has not previously been shown to affect lipid metabolism directly, studies in laboratory rats have shown that Cd can elevate concentrations of malondialdehyde (MDA), an indicator of lipid peroxidation[12,73]. In addition, it is also interesting to consider the increasing levels of choline and choline containing metabolites, such as phosphocholine detected in liver after Cd treatment which have been associated with drug induced breakdown of cell membrane[71]. The majority of phospholipids (PL) in cellular membranes consist of glycerophospholipids such as phosphatidylcholine (PC). PCs are involved in pathological mechanisms and crucial cellular functions such as signal transduction, apoptosis, necrosis and protein sorting[74-76]. Lysophosphatidylcholines (LPC) derive from phosphocholine by degradation produced by the enzymatic action of phospholipase A2 that removes one fatty acid from PC. LPC is rapidly hydrolyzed to glycerol-phosphocholine, and finally phosphorylcholine and choline. The intensity of MS peaks from different PCs in the m/z range 650-850 is decreased under cadmium exposure (Fig. 6). In addition, LPCs (m/z 450-650) increase in exposed mice in comparison with the controls. Results obtained demonstrate degradation of membrane phospholipids (cell apoptosis). In addition, this hypothesis is corroborated by the altered levels of free fatty acids found by the ESI(+) and ESI(-) analysis of lipophilic extract, increasing the presence of PUFAs (Fig. 6), as a consequence of PC degradation (Table 3).

Table 4. Quantification of mice plasma metabolites (*Mus musculus*) exposed to cadmium (Cd) by GC-MS.

	PLASMA			
Plasma Metabolites (mmol L^{-1})	***Retention Time (min)***	***CONTROL MICE (n=5)***	***Cd EXPOSED MICE Day 6 (n=5)***	***Cd EXPOSED MICE Day 12 (n=5)***
		Mean ± SD	Mean ± SD	Mean ± SD
Lactic acid	5.6	**1.47 ± 0.10**	**1.63 ± 0.14**	**1.94 ± 0.21**
Alanine	6.0	0.314 ± 0.018	0.303 ± 0.014	0.320 ± 0.019
Valine	11.6	0.196 ± 0.022	0.178 ± 0.011	0.212 ± 0.018
Urea	12.5	344 ± 16	331 ± 25	327 ± 18
Isoleucine	13.2	**0.0721 ± 0.008**	**0.0614 ± 0.009**	**0.0482 ± 0.010**
Proline	13.3	0.122 ± 0.008	0.116 ± 0.007	0.118 ± 0.014
Glycine	13.4	0.201 ± 0.011	0.205 ± 0.014	0.215 ± 0.016
Serine	14.5	0.102 ± 0.006	0.0982 ± 0.006	0.105 ± 0.008
Threonine	15.0	0.118 ± 0.007	0.120 ± 0.010	0.117 ± 0.011
Aspartic acid	17.2	0.092 ± 0.007	0.099 ± 0.004	0.101 ± 0.012
Glutamine	22.1	**0.568 ± 0.031**	**0.621 ± 0.026**	**0.672 ± 0.033**
Glutamic acid	23.1	**0.141 ± 0.09**	**0.123 ± 0.011**	**0.104 ± 0.012**
α-Ketoglutarate	23.5	**0.138 ± 0.011**	**0.121 ± 0.014**	**0.102 ± 0.011**
Phenylalanine	25.5	0.0632 ± 0.003	0.0618 ± 0.004	0.0593 ± 0.005
Isocitric acid	25.9	**0.521 ± 0.016**	**0.499 ± 0.034**	**0.478 ± 0.022**
Citric acid	27.2	**3.33 ± 0.41**	**3.14 ± 0.16**	**2.84 ± 0.18**
Fructose	27.9	0.876 ± 0.027	0.902 ± 0.031	0.886 ± 0.022
Galactose	29.0	0.116 ± 0.014	0.121 ± 0.009	0.111 ± 0.008
Glucose	29.2/29.9	**7.72 ± 0.51**	**7.41 ± 0.37**	**6.54 ± 0.69**
Tyrosine	31.0	0.113 ± 0.009	0.116 ± 0.007	0.108 ± 0.008
Uric acid	37.0	0.126 ± 0.008	0.122 ± 0.007	0.131 ± 0.006
Tryptophan	38.1	0.104 ± 0.006	0.109 ± 0.009	0.0102 ± 0.004
Cholesterol	54.6	**1.46 ± 0.09**	**1.55 ± 0.12**	**1.68 ± 0.096**

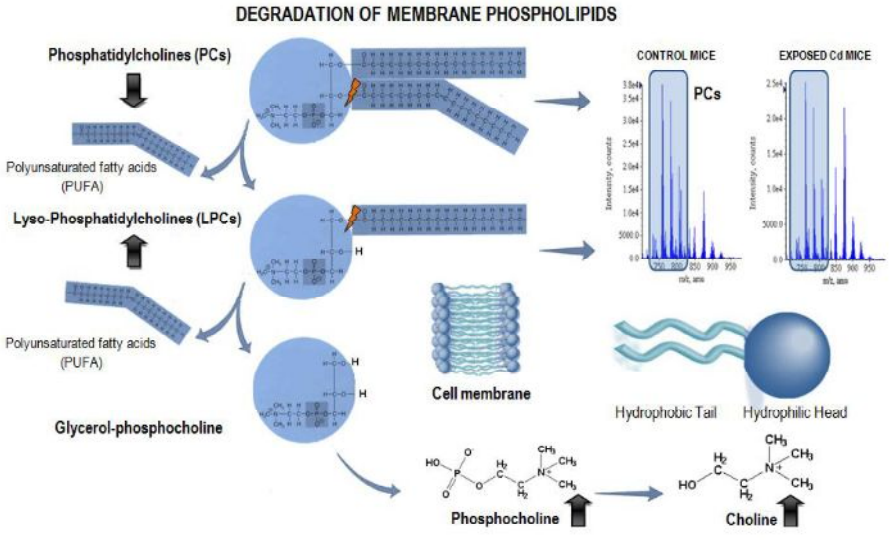

Fig 6. Schematic representation of the breakdown of membrane phospholipids

Antagonistic interaction between Cd/Se in plasma mice (*Mus musculus*) under ^{77}Se and cadmium controlled exposure

As a previously documented, Se levels in plasma is reduced when cadmium is administered by subcutaneous injection (Table 2). In addition, this observation has been affirmed by others author in rats under cadmium exposure subjected to oral administration[61]. This interactions has been studied by others authors in the bloodstream *in vitro* by means of the HPLC-ICP-MS method using the SeP fraction isolated from the serum of rats[53]. In our study, IPD was carried out to reveal the mechanism underlying the interactions between Cd and Se in the bloodstream *in vivo*. In this sense, ^{77}Se was orally administrated to evaluate the fate of enriched ^{77}selenite in mice subjected to cadmium exposure and the effect in plasma selenoproteins.

Our results show an increase in selenium requirement in plasma is necessary when cadmium is supplied simultaneously, more pronounced when higher doses of enriched selenium are administrated (Fig. 7a). In addition, the concentration of cadmium in the bloodstream is lower when high levels of enriched selenium are administrated (Table 5). The validation of the methodology has been carried out using a spiked CRM of human serum with enriched ^{77}selenite (Table 5). Moreover, when SEC-AF-IPD-ICP-ORS-qMS is applied an increase in the concentration of ^{77}SeP is obtained (Fig. 7b and 7c). Similar results are obtained when enriched ^{82}selenite is injected intravenously in rats[77]. In our study, this increment is more pronounced when Cd is administrated simultaneously. Based on the results, SeP play a fundamental role in Cd detoxification. The lower concentration of cadmium in plasma when high levels of enriched ^{77}Se is ingested, decreased levels of endogenous SeP (Fig. a) and the decreased levels of SeP in plasma of mice subjected to controlled exposure to Cd during 12 days (Table 2) supporting this idea.

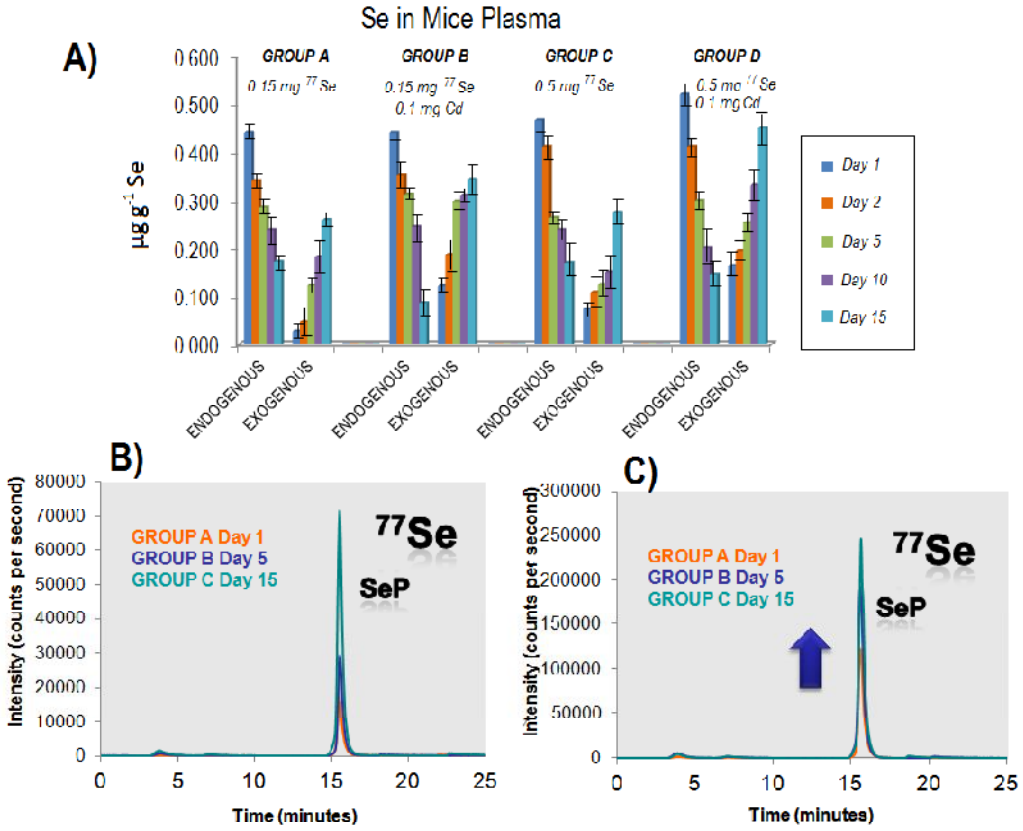

Fig. 7. A) Endogenous and exogenous selenium in plasma of mice under ^{77}selenite and cadmium administration by IPD-ICP-ORS-MS; B) Mass flow chromatogram with ^{78}Se/^{74}Se (natural) isotope ratios using SE-AF-HPLC-IPD-ICP-ORS-MS of plasma from different groups of Se/Cd exposure of *Mus musculus* mice. C) Mass flow chromatogram with ^{77}Se/^{74}Se (supplemented) isotope ratios using SE-AF-HPLC-IPD-ICP-ORS-MS of plasma from different groups of Se/Cd exposure of *Mus musculus* mice.

Table 5. Quantification of cadmium in mice plasma under ^{77}Se/Cd exposure by ICP-ORS-MS and selenium species in human serum (BCR-637) and BCR-637 spiked samples (50 ng of ^{77}Se g^{-1}; as sodium selenite) by IPD-ICP-ORS-MS

Selenium Species	Certified Human serum BCR-637 (ng g^{-1}± SD) Endogenous Se	SD (n=5)	Human serum BCR-637 (SEC-AF) (ng g^{-1}± SD) Endogenous Se	SD (n=5)	Human serum BCR-637 (SEC-AF) (ng g^{-1}± SD) Exogenous Se	SD (n=5)	Cadmium concentration	Plasma GROUP B 0.15 mg of ^{77}Se and 0.1 mg Cd (ng g^{-1}± SD)	SD (n=5)	Plasma GROUP D 0.50 mg of ^{77}Se and 0.1 mg Cd (ng g^{-1}± SD)	SD (n=5)
eGPx	15	± 4	12	± 2	< LOD		**Day 1**	12.36	± 1.1	11.72	± 0.9
SeP	60	± 7	53	± 2	< LOD		**Day 2**	38.24	± 5.4	24.66	± 4.9
SeAlb	13	± 4	14	± 3	<LOD		**Day 5**	52.22	± 3.7	38.51	± 3.2
^{77}Selenite spiked	-----	-----	<LOD		51	± 3	**Day 10**	132.2	± 7.8	60.54	± 7.1
Sum of species	79	± 3	79	± 2	51	± 3	**Day 15**	162.2	± 11	88.21	± 9.4
TOTAL Se	-----	-----	82	± 1	52	± 1					
Certified value (ng.mL-1)	81	± 7	81	± 7	-----	-----	**LOD (ng. g^{-1})**			0.012	

Conclusions

This work illustrated the high reliability of inorganic and organic mass spectrometry based on metallomic and metabolomic approach, respectively, on the study of the biochemical effects induced by cadmium chloride in exposed mice and their effects in liver, kidney and plasma. The application of size exclusion chromatography coupled to ICP-MS in cytosolic extracts of metabolic active organs and biological fluids from the laboratory mice *Mus musculus* exposed to Cd, allows decipher changes in metal-binding biomolecules induced by this metal, such as induction of MTs. Selenium containing proteins play important roles in protecting against toxicological effects caused by Cd and As. In addition, enriched stable isotopes are crucial to study the fate of trace elements in biological systems. IPD measurements require selenium equilibration between natural existing selenium in the sample and added isotopically enriched selenium. In this sense, enriched ^{77}selenite (orally) and cadmium (subcutaneously) are administrated simultaneously during 15 days. In this work we have applied IPD for insolating distinct isotope signatures from mixtures of natural abundance and enriched tracers. Our results shows that SeP plays a fundamental role in cadmium detoxification.

On the other hand, the time-dependent changes in endogenous metabolites by subcutaneously injection of cadmium were identified by PLS-DA discriminant analysis. Mass spectrometry analysis of polar and lipophilic metabolites of liver and plasma by DI-ESI(±)-QTOF-MS and GC-MS highlighted a number of perturbations in the endogenous metabolic profiles, which could be explained to cadmium induced biochemical pathways alterations, such as energy metabolism, aminoacids pathways and breakdown of membrane phospholipids.

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