ARSENIC SPECIES-BINDING PROTEINS IN HUMAN CARDIOVASCULAR AND MUSCLE TISSUES

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SUMMARY

The intracellular As-protein binding in cytosol and methanol–water extract of the auricle and saphene tissues of As impacted people was evaluated by bidimensional size exclusion FPLC-UV-ICP-MS. The fractionation of cytosol using Superdex, Phenomenex and MonoQ HR 5/5 columns, shows that As was distributed in a wide range of contiguous fractions of each column, being 8, 25, 50 % the percentages of As in the collected fractions, respectively.

Arsenic a sulphur coelute when FPLC–UV–ICP–MS was applied, which could implicate that As is bound to bio-compounds of different molecular mass through vicinal sulphur groups. The monitoring of S, Cu and P. In the methanol: water extracts a similar study than performed with the cytosol using preparative gel chromatography on Sephadex G-75 and Shephadex G-100 columns. A very low As and protein contain were found in the different fractions of both SEC fractionating series. A similar As–protein association to that found in the cytosol after fractionating with MonoQ HR 5/5 was observed for auricle and saphene.

Inorganic and methylated As speciation in the 20 - 26 cytosol fractions obtained within the Phenomenex column was performed by HPLC–ICP–MS using the Hamilton PRP-X100 column. Only As(III) and As(V) were present and the results obtained shows that the As(III)/As(V) ratio is constant in most cases.

Direct evidence of the existence of As–binding peptides in auricle and saphene vein from arsenic impacted human beings has have been obtained which was previously reported by means *of novo* peptide synthesis.

INTRODUCTION

The danger of As for human health is associated with cancer and non-cancer effects, apart from that its genotoxicity is broadly reported[1]. However also a certain essential character had to be assigned to As, in this case, experiments with mammalian model species[2], not associated to enzymes. The toxicity and the mitigation damaging effect may be related with promote of the folding or degradation of altered proteins or with limiting the synthesis of new proteins that may be altered by arsenite. Another aspect to take into account is detoxification mechanisms of As in the organisms[3]

The reactivity of As(III), as a soft metal ion which forms strong bonds with functional groups such as thiolates of cysteine and the imidazolium nitrogens of histidine residues, permits to deduce that the understanding of interaction of As ions with proteins is essential in order to understand the mechanisms governing the bioinorganic chemistry related with the biological activity of arsenic. As(III) is toxic because it is able to form organo-metal bondings with metal-thiol sidegroups in vicinal cysteines of enzymes such as pyruvate dehydrogenase at their activity centres[4]. As(V) disrupts oxidative phosphorilation by substituting phosphate in the formation of ATP. As binding to various cytosolic proteins and methylation through the methylating agent S–adenosylmethionine (SAM) governed by the ATP as well as arsenite and arsenate methyltransferase are considered competitive mechanisms for As detoxification[5]. However, compelling experimental evidence obtained by several laboratories is suggesting that biomethylation, particularly the production of methylated metabolites that contain As(III), is a process that activates As as a toxin and a carcinogen[6]. Thus there is evidence that the attachment of inorganic As to proteins and subsequent arsenic extrusion mechanisms by means of cytosol As binding proteins may be the prevalent detoxification mechanism.

The most important findings referring to As binding on proteins may be summarized as follows: i) Arsenic binds a variety of cytosolic proteins and macromolecular constituents of tissues from both, methylating and non methylating (Mamoset monkey) animals[7]; ii) As is bound to hemoglobin through vicinal –SH groups in spleen, bone marrow (high hemoglobin content) and in plasma and packed cells. iii) As is bound to 100 kDa, 450 kDa and > 2000 kDa size proteins in liver cytosol in the livers of mammalian animals[8] Relatively few studies about As binding to proteins in humans have been carried out[9]. The inorganic As content in serum from peritoneal dialysis patients was attached to both, high molecular mass protein (about 80 000 Da) and low molecular mass species (< 1000 Da), whereas arsenobetaine (AsB), although present in the former fraction, was not attached to the proteins[8].

There are four arsenic binding proteins of 50, 42, 38.5, and 19.5 kDa. Two of them were tentatively identified as turbulin (50 kDa) and actin (42 kDa), which are induced by As(III) in human lymphoblastoid cells[8]. As(III) binding to proteins is most likely to be an As(III) association to three thiol groups arranged in a specific spatial relationship, as proposed by Rosen[4]. Recently, it has been shown by *de novo* peptide synthesis that As(III)–cysteine interactions stabilise three–helix bundles in aqueous solutions[4].

The high concentration of As associated with copper mining activity and water supply in the Chilean II Region (about 800 μg L^{-1} in the period 1950-1970, decreasing in the actuality to values around 50 μg L^{-1}) is probably related with occurrence of cancer in this region, as well as with non-cancer symptomns such as abnormal pigmentation, acrocyanosis, hyperkeratosis, gangrene of fingers, ischemia of the tongue, diabetes, Raynand´s syndrome, thrombosis, cerebral vascular disease, coronary artery occlusions and other cardiovascular diseases (CVD)[10,11].

In a precedent study the distribution of As and As species in three heart tissues (auricle, mammary artery and fat) and in saphene vein (used as by-pass) was investigated. Samples were taken from individuals chronically exposed to arsenic in the Chilean II Region, suffering cardiovascular diseases (CVD) and subjected to hearth surgery in Antofagasta, Chile. It could be demonstrated that the main species in the auricle and saphene tissues was As(III), followed by As (V). DMA could only be detected in the saphene tissue[12].

In this work we have evaluated the intracellular As-protein binding by size exclusion (SE)-Fast Protein Liquid Chromatography (FPLC) with visible range (UV) and Inductively Coupled Plasma Mass Spectrometer (ICP-MS) detection in auricle and saphene tissues of the same group of persons in order to understand the effect of long term exposure to high concentrations of As on the cardiovascular system. Besides S, P and Cu were monitored together with As.

The As species present in SE relevant fractions have further been determined by Anion Exchange Liquid Chromatography (AELC-ICP-MS).

EXPERIMENTAL

Samples

The auricle tissue and saphene vein of six persons operated in the Antofagasta Hospital of coronary thrombosis and presenting high As content in both tissues were taken for this study. The samples were obtained from a population under study, made up of patients who have lived at least five years in the II Region of Chile[13]. Specific patient characteristics can be found in precedent work[10] and also in Table 1.

Instrumentation.

Before size exclusion and gel fractionating medium pressure liquid chromatography experiments (SE–GF–MPLC), the homogenisation and centrifugation of the tissues were made at 4 º C in a agate mortar and a Eppendorf centrifuge 5804 R (Germany). These gel chromatography procedures for protein fractionating were made employing tap water thermostated jacket and inert Merck Superformance Universal Glass Cartridge System (50 x 26 mm id) (Germany) packed with Sephadex G–75 and G–100 gels, coupled to a Shi-

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madzu LC– 10AS (Japan) chromatography pump via a Pharmacia Fine Chemicals SRV–3 valve and a SA–50 sample applicator (Uppsala, Sweden), and to a Gilson FC 203 fraction collector (France).

A Hydride Generation Atomic Fluorescence Spectrometer (HG-AFS) (Excalibur, PSA, UK) to determine the total As content was used.

An Inductively Coupled Plasma Mass Spectrometer (ICP–MS) HP 4500 (Yokogawa Analytical Systems, Tokyo, Japan) was used fitted with a Babington glass nebulizer and a Scott double pass spray chamber cooled by a Peltier system, which was employed as an "off line" detector after gel filtration chromatography (GFC) fractionation, and as an "on-line" detector for arsenic speciation by anion exchange liquid chromatography (AELC-ICP-MS) and for arsenic binding protein fractionation by anion protein exchange,namely Fast Protein Liquid Chromatography (FPLC-UV-ICP-MS). Single ion monitoring at m/z 75 was used to collect the data.

Anion exchange liquid chromatography (AELC) for inorganic and methylated As speciation was performed with an High-performance liquid chromatography (HPLC) system Division Riviera Beach, Florida, USA). 100 µL of sample were introduced into the injection valve Rheodyne 9125 (USA). A PRP-X100 analytical and guard anion–exchange columns (Hamilton, Reno, NV, USA) were used.

The analytical anion – exchange column MonoQ HR 5/5 (50 x 5 mm id.) (Pharmacia Biotech. Uppsala, Sweden) FPLC was used for fractionating of cytosol and methanol:water extract.

A gradient HPLC pump Jasco Pu 2089 plus (Tokyo, Japan) and LKB model 2151 UV detector provided with a 10 µL flow cell was used for absorbance monitoring during separation and measurements in the tandem FPLC – UV – ICP – MS.

Superdex (300 mm x 10 mm) and Phenomenex (300 mm x7.8 mm) (Pharmacia Biotech. Uppsala, Sweden) analytical gel filtration columns were used to perform protein fractionating in the cytosol, and the above mentioned Sephadex G – 75 and G – 100 to perform protein fractionating after methanol : water extract.

Solvent evaporation of methanol – water extracts and chromatographic fractions were performed in an Univapo100H – Unijet II system (Uniequip, USA).

Materials, reagents and standards

As(III) and As(V) standards were prepared from sodium arsenite and sodium arsenate (Sigma Aldrich, St Quintin, Fallavier, France), dimethylarsinic acid (DMA) and methylarsonic acid (MMA) were obtained from Merck, and arsenobetaine (AsB) and arsenocholine (AsC) from Tri Chemical Laboratory Inc. (Japan).

High-purity nitric and hydrochloric acids were obtained by distillation of the analytical-grade reagent (Merck) in an I.R. distiller (Berghof, BSB-939IR, Germany).

Proteins used for Size exclusion Chromatography (SEC) calibration: Blue dextran 2000 (>2000 kDa); alcohol dehidrogenase (150 kDa); bovine albumin (66 kDa); carbonic anhidrase (29 kDa), cytochrome C (12.4 kDa); aprotine (6.5 kDa), B – 12 vitamin (1576 Da), Se-cystine (334 Da), Se-methionine (198 Da) and Se-urea (123 Da) (Sigma Aldrich, St Quintin, Fallavier, France) and other chemicals were analytical reagent grade or the highest quality obtainable.

Total arsenic determination and extraction for As species.

Details regarding the mineralization of tissues for total As determination and extraction of inorganic and methylated species in water:methanol extracts are given in previous papers[10].

Cytosol preparation

The tissue was cut into little pieces, minced and homogenised in 2-3 mL of Tris solution [10 mM Tris, pH 7.4; 0.1 mM phenylmethylsulfoxyl fluoride (PMSF) and 25mM NaCl]. The mixture was centrifuged at 30 000 g. The supernatant was further heated at 55ºC for 15 min to precipitate thermally labile proteins and then centrifuged at 30 000 g for 90 min to obtain the supernatant cytosol. This procedure is based on a modified protocol of G.M. Bogdan et al.[14].

Procedures for As- protein fractionation in the cytosol by SEC and anion exchange protein chromatography

The fractionating for cytosol and reconstituted methanolic extracts was carried out following a procedure similar to that reported by Ferrarello et al.[15]. The general procedure performed is summarised in *FIGURE 1*. 200 µL of the cytosol were applied to the Superdex peptide analytical column (100 – 7000 Da). After the void volumes, 30 fractions of 1 mL each were collected using 10 mM Tris–HCl [pH=7.4]– 0.1mM PMSF and 25 mM NaCl as mobile phase at 0.5 mL min^{-1} [15].

The void volume, (about 30 mL), was evaporated up to 1 mL. The solution was filtrated through a 0.22 µm cellulose filter, then it was again size fractionated using the analytical Phenomenex (1000 – 80 000 Da) column under analogous conditions to those of the Superdex column. The new pooled void volume was evaporated up to 1 mL. This volume was fractionated in the MonoQ HR 5/5 anion protein exchange column under analogous chromatographic conditions. The chromatographically separated fractions of the different columns (5 mL each), were evaporated up to 3 mL and analysed for total protein content by the Bradford´s method[16] and for total As by Atomic Fluorescence Spectrometer (AFS). The fractions having high protein and high As content were further analysed by FPLC-UV-ICP-MS for As – protein binding (200 µL injection volume) and by AELC-ICP-MS for As speciation of As(III), As(V), MMA, DMA, AsB and AsC (100µL injection volume). The proteins were measured at 280 nm using an UV detector for the first coupling. ^{32}S, ^{65}Cu and ^{31}P were simultaneously monitored to ^{75}As by ICP-MS.

A fractionating procedure similar to that performed for the cytosol was used for the 1:1 methanol: water buffer reconstituted extracts using the Sephadex G-75 (3000 – 80 000 Da) and G–100 (4000 – 150 000 Da) preparative columns, and finally the MonoQ HR 5/5. For the Sephadex columns, 5 mL of sample were introduced on the column head and fractions of 3 mL were collected. The dead volume of the G-100 column (about 30 mL) was evaporated to 1 mL and introduced into the Mono Q HR 5/5. *TABLA 1* shows the chromatographic conditions used for the FPLC-UV-ICP-MS and AELC-ICP-MS.

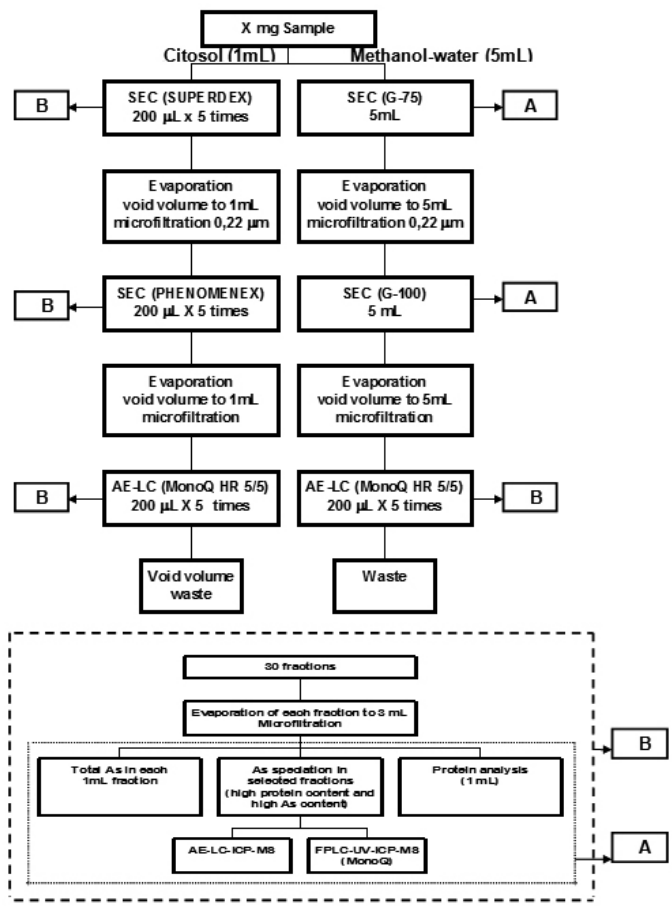

FIGURE 1:. Procedure: Size exclusion, anion protein exchange chromatography and anion exchange chromatography of As species in the cytosol and methanolic extract.

TABLE 1:. Chromatographic conditions for protein separation by FPLC-UV-ICP-MS and As speciation by AELC-ICP-MS

FPLC-UV-ICP-MS				AELC-ICP-MS
Anion Column:		MonoQ HR 5/5		Anion Column: Hamilton PRP-X100
Injection volumes :		200 µL		Injection volumes : 100 µL
Flow rate :		O.8 mL min^{-1}		Flow rate : 1.0 mL min^{-1}
Mobile Phase (gradient) : A: 10 mM TRIS-HCl (pH= 7.4); B: 250 mM ammonium acetate + 10 mM TRIS-HCl (pH= 7.4)				Mobile Phase (isocratic) : 10 mM Phosphate ammonium (pH = 6)
Time (min)	B%	Time (min)	B%	
0	0	10	17	
1.5	2	11	18	
2	3	12	99	
5.5	4	19	100	
6.0	10	23	0	

RESULTS AND DISCUSSION

TABLA 2 shows the total As concentration of the cardiovascular tissues and some important characteristics of the evaluated impacted persons. Samples S–1 and S-2 were used for the studies performed in the cytosol. Samples S–3, S–4, S–5 and S–6 for the studies performed in the water–methanol batch extracts. Similar results were achieved from additional analysed samples.

TABLA 2:. Individual information characteristics of the impacted people evaluated and total As concentration (µg g^{-1}), in some of their cardiovascular tissues.

Total As content in the tissue (µg/g^{-1})				Specific patient characteristics			
Sample		Auricle	Saphene	Age of the cardiac infarct	Working (living)	As stigmas	Others
S-1		3.9 ±0.2	2.5 ±0.2	37	Mine (Calama)	yes	Diabetes, dislipidemie
S-2		4.5 ±0.3	3.2 ±0.2	44	Mine	yes	Diabetes
S-3		5.4 ±0.4	2.6 ±0.3	45	Mine (Calama)	Yes	--
S-4		4.8 ±0.3	4.7 ±0.3	50	Mine (Antofagasta)	yes	--
S-5	4.8 ±0.4	5.2 ±0.4	53	Mine(Chuquicamata)	yes	Diabetes, anaemia	
S-6	6.1 ±0.5	5.1 ±0.4	65	Person from I Region	no	--	

Cytosol extracts.

FIGURE 2 (a – f), shows the total As (µg) and protein (µg) profiles of auricle and saphene tissues in the different fractions obtained for S–1 after Superdex, Phenomenex and MonoQ HR 5/5 fractionating. Analogous results were obtained for the auricle and saphene of S–2 sample (data not shown).

A–J dotted vertical lines, show the fraction at which the maximum peak of the proteins set used for calibration appeared. As was distributed within the three columns in a wide range of fractions (about 9 – 22 in the Superdex, 19 – 26 in the Phenomenex and 20 – 34 in the MonoQ HR columns). The similarity for the As and protein profile for both cytosol tissues (especially for saphene) for most of the fractions obtained by the three columns could indicate an As – protein association.

The percentages of As in the collected fractions from the Superdex column was 8%. The molecular weight of the peptides that would be associated with As from the Superdex column, considering the biomolecular mass calibration (lg MW= 3.92 - 0.44 K, r = 0.997), was within the ranges of 330-4600 Da and 630-3600 Da for the auricle and saphenous tissues, respectively (*FIGURE*. 2(a) and 2(b).

From the Phenomenex fractionation the percentage of As in fraction collected (*FIGURES*. 2(c) and 2(d).Was 25%. Considering the biomolecular mass calibration lg MW= 5.37 - 0.86 K, r = 0.980) we can infer the fraction of maximum As and protein content belong to species with a molecular weight within the 300 – 12 400 Da range. The presence of lower molecular weight[16] species than were expected could be due to either the fragmentation of high molecular weight proteins inside the column or to other non – controlled processes.

The percentage of As in the fractions collected from the anion exchange MonoQ HR 5/5 column was 50% (*FIGURE*. 2(e) and 2(f).

Calibration in the Superdex column: A = Aprotine (6500Da), B = B-12 Vitamin (1576 Da), C = Se Cystine (334 Da), D = Se Methionine (198 Da), E = Se urea (123 Da).

Calibration in the Phenomenex column: F = Bovine serum (69 000 Da), G = Carbonic anhidrase (29 000 Da), H = Citochrome C (12 400 Da), I = Se cystine (334 Da) J = Se methionine (198 Da).

In order to evaluate possible As association to the proteins, some representative Superdex, Phenomenex and MonoQ HR 5/5 fractions of both auricle and saphene cytosol tissues were applied to FPLC–UV– ICP–MS. Besides, S, P, and Cu were monitored together with As. Cu was evaluated because the persons were exposed to copper mining activity impact, it is an important component of some metallothioneins and also of some high molecular mass proteins (HMMP) and enzymes[17]. S is present in amino acids such as cysteine residues involved in binding, transport, and storage of metals in the cells[18]**.** P was considered because is also an important component of some proteins and other biomolecules, such as ATP.

In *FIGURE 3 (a and b*) the most representative FPLC–UV–ICP–MS chromatograms from auricle and saphene cytosol fractions with high As content after the Superdex fractionation show that As, Cu, and S elute together, and at the same retention time as the maximum of the UV chromatogram (maximum

content of proteins). Thus, it can be deduced that As and Cu are in most cases bound to the same type of protein species.

The monitoring of the arsenic was not interfered with by the formation of $ArCl^+$, since the chloride concentration in the mobile phase raised the chromatographic baseline only slightly, and chloride concentration in the cytosolic extract does not exceed the minimum interferent level.

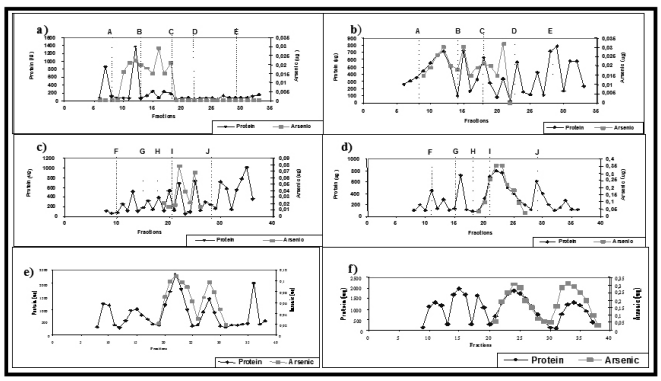

FIGURE 2.: As (μg) and protein (μg) content in the auricle and saphene cytosol of S-1 after fractionating. (a): auricle in Superdex column ; (b): saphene in Superdex column; (c) auricle in Phenomenex column; d) saphene in Phenomenex column; (e) auricle in MonoQ HR 5/5 column; (f) saphene in MonoQ HR 5/5 column.

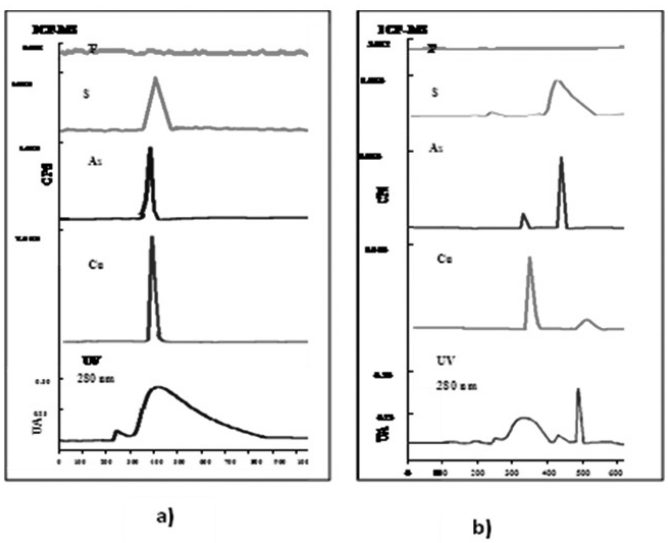

FIGURE 3:. FPLC-UV-ICP-MS chromatograms of selected fractions from the Superdex column. (a) auricle S-1 fraction 18. ; (b) saphene S-1 fraction 18.

It has been previously reported that As (III) is able to interact with metallothioneins (6–10 kDa), with glutathione, forming an $(AsSG)_3$ complex (1040 Da), and also with cysteine to produce an $(As\ Cys)_3$ complex (501 Da), which gives a 3- coordinate cysteine environment for As[18]. These species have molecular masses within the range of the Superdex fractions. However, it is also important to note that P is not present in these species and therefore, the ATP or similar mass protein peptides should not be present.

FIGURE 4, the profile obtained by the FPLC–UV–ICP–MS coupling of one of the most representative fractions of the Phenomenex column for the auricle (S1) shows to peaks with very different retention times for As, which coelutes with S and P. This fact clearly indicates their association with different types of proteins, both containing P. Cu also appears in the less retained protein. Arsenic may be bound to transferrin (80 kDa) and haemoglobin (64.5 kDa) at its binding sites and through vicinal –SH- groups respectively[18], and both have a molecular weight within the Phenomenex fractionating range. Similar results were obtained in other fractions and for the saphene tissue.

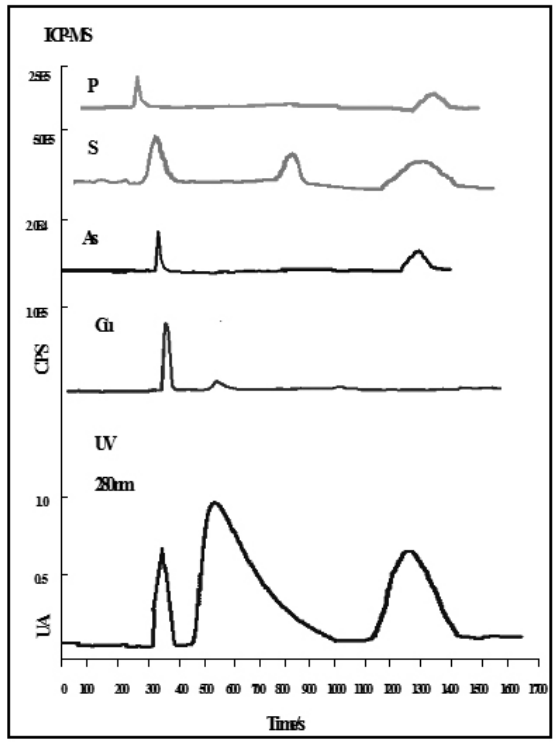

FIGURE 4. FPLC-UV-ICP-MS chromatogram of auricle S1; fraction 25 after Phenomenex fractionating.

FIGURE 5 (a - b) show two of the most representative chromatograms after MonoQ HR 5/5fractionation (obtained from fractions 22 and 24 for the auricle and saphene tissue, respectively in the Phenomenex column) containing one or two As peaks. The first one in *FIGURE* 5a is always associated with Cu and S and also with P. As expected, most of the As is associated with polar proteins (as they elute at a high retention time from the anion exchange MonoQ column) of a molecular mass higher than 300 kDa (void volume of Phenomenex). *FIGURE 2b,* it is important to highlight that these fractions represent about 50% of the As content and, therefore, that the higher molecular mass of the cytosol protein (after Phenomenex fractionation) supports the higher As content.

Methanol : water extracts.

1:1 methanol:water mixtures are widely used for As speciation in biological tissues. This extractant mixture was used previously in preparative gel chromatography for arsenosugar detection in algae. In order to evaluate whether this mixture extracts the As bound to the cytosol proteins in its associated protein form, a similar study than performed above with the cytosol was carried out. Preparative gel chromatography using Sephadex G–75 (3–80 kDa) and Shephadex G–100 (4–150 kDa) as reported and the anion protein exchange MonoQ HR 5/5, were used as it is shown in *FIGURE 1*. A very low content of As (<1% of total As) and protein were found in the different fractions of both SEC / GFC fractionating series. However, a similar association As – protein to that found in the cytosol after MonoQ HR 5/5 fractionating was observed for the auricle and saphene. *FIGURE 6* shows this association for the auricle of sample S-1. The As recovery found within the 25 – 33 fraction (corresponding to the higher MW cutting was 80% of the total arsenic. When FPLC–UV–ICP–MS was applied to the most representative As fractions of MonoQ HR 5/5, chromatograms (not shown) similar to those obtained for the cytosol were obtained. Therefore, we can assume that most of the As content in the methanol: water extract is bound to species that can probably be agglomerated and solubilized in this extract. Cu, As, S and P elute together in the same protein profile as in some of the preceding chromatograms. No differences in behaviour were detected between auricle and saphene.

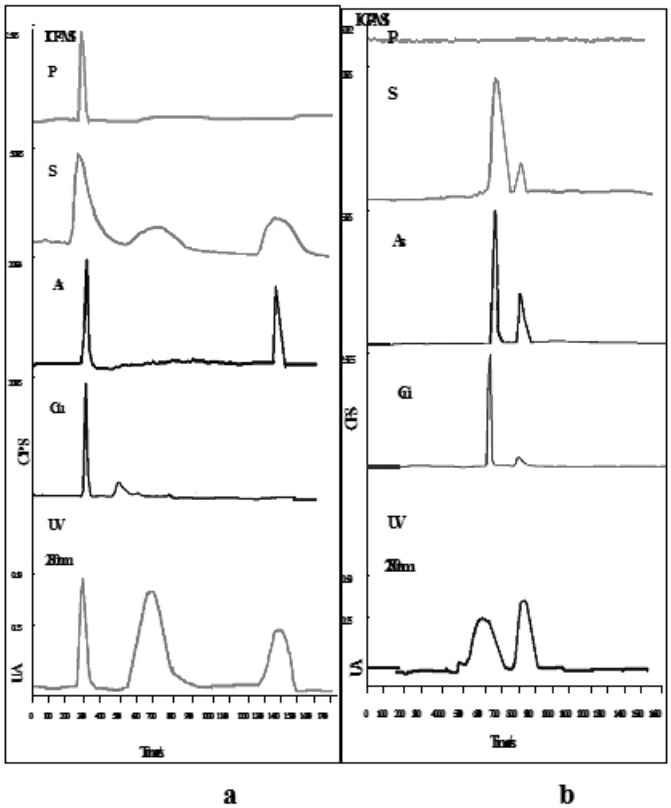

FIGURE 5. FPLC-UV-ICP-MS chromatograms of selected fractions from the MonoQ HR 5/5 column. (a) auricle S-2, fraction 22; (b) saphene S-2, fraction 24.

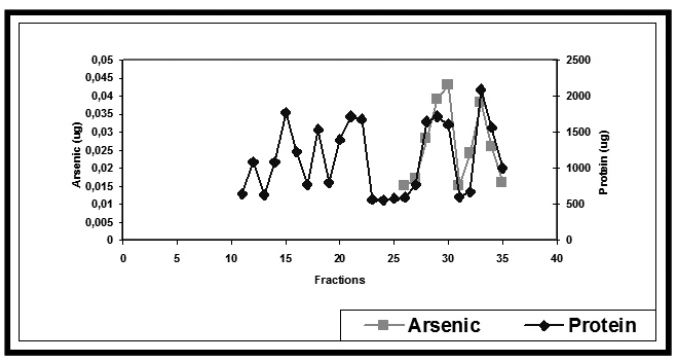

FIGURE 6.: As (µg) and protein (µg) content in 1:1 methanol:water extract after fractionating of auricle S-1 in the MonoQ HR 5/5 column.

As speciation in cytosol and methanol extracts.

As speciation within phenomenex 21–26 fractions of the cytosol from saphene S-1 sample in which an As–protein overlapping occurs was performed to ascertain which As species are attached to the proteins.

AELC–ICP–MS system with the Hamilton PRP–X100 column under conditions given in *TABLE 2*, shows the presence of only As(III) and As(V) in all the fractions *FIGURE 7* Similar chromatograms were obtained in the methanol:water fractions of both tissues. The As(III) / As(V) ratio is constant in most of the fraction analysed. A recovery of 90 – 95% [As(III) + As(V)] was found when total As was analysed in each fraction.

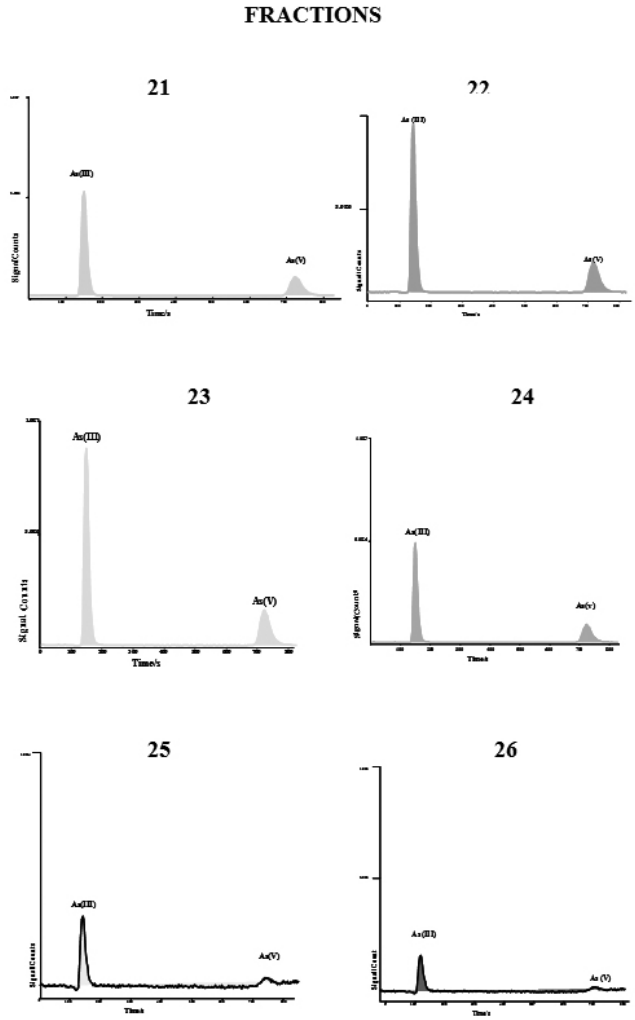

FIGURE 7: As(III) and As(V) chromatograms from consecutive fractions (20-26) of the phenomenex column from saphene S-1 cytosol.

CONCLUSIONS

This work can be considered as a first step in investigation on As bioaccumulation and the biochemical response of cardiovascular tissues proceeding from individuals chronically impacted by inorganic As.

After methanol – water extraction, accounting for more than 80% of the total As content in the tissue, exclusively non-methylated species were found in the auricle. A very small amount of DMA was present in saphene tissue. The absence of methylated species of As demonstrates the neglible capability of the vascular tissues for As methylation.

To summarise, in all the fractions analysed by FPLC-UV-ICP-MS from the different columns and tissues, the As and S are similarly associated, which could indicate that As is totally bound to biocompounds of different molecular mass through vicinal sulphur groups. The similarity of As and Cu behaviour in most cases indicates similar type of binding to the biomolecules. At the current stage no final conclusion could be drawn as to the specific protein with which As is associated. Although it is possible for the molecular weight of the native form of this As-binding protein to higher than 300 kDa, molecular fragmentation may occur experimental procedures.

For a deeper insight in As – protein association as part of the metabolism pathways additional studies are evidently necessary. Ongoing work is focussed on this aspect.

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