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Sb(V) and Sb(III) distribution in human erythrocytes: Speciation methodology and the influence of temperature, time and anticoagulants

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ABSTRACT

In this research a new method was developed and optimized for the determination of Sb(V) and Sb(III) in human erythrocytes fractions (plasma and cytoplasm) by high performance liquid chromatography with hydride generation atomic fluorescence spectrometry. The method considers the first step of samples cleaning by protein precipitation by salting out followed by C18 solid phase extraction, EDTA elution, and finally a chromatographic separation by using anion exchange PRPX-100 (100 mm × 4.1 mm) and EDTA 20 mmol L^{-1} as mobile phase. The method was optimized by experimental design with a recovery of 90% for Sb(V) and 55–75% for Sb(III) approximately. The analytical method was applied to study the distribution of Sb(V) and Sb(III) in human erythrocytes considering temperature and time of incubations and with special attention about the influence of the anticoagulant. Results showed that both Sb(V) and Sb(III) are capable to enter the red blood cell in a proportion of approximately 40–60%. On the other hand, both species are then excreted from the interior of the cell, where the percentage considerably decreased from approximately 60 to less than 30% within the cell. An increase in the culture temperature increases the capacity of Sb(V) and Sb(III) to penetrate the membrane barrier and reach the cytoplasm. In order to preserve the original distribution of Sb in blood, heparin seems to be the best anticoagulant for sample preservation.

1. Introduction

Furuta et al. in Japan [1], Gao et al. in the USA [2], Weckwerth in Germany [3] and Gómez et al. in Argentina [4] have reported an enrichment of Sb in airborne particulate matter (APM), particularly in fine particles in areas of high vehicular traffic. Brake discs contain a solid lubricant containing Sb_2S_3, which reduces wear on friction materials under high-load conditions [5]. Therefore, the abrasion powder generated by friction brakes could be a possible source of Sb enrichment in airborne particulate matter (APM) [1,3]. Furthermore, Uexküll et al. [6] have suggested that Sb_2S_3 could be transformed into Sb_2O_3, a possible carcinogenic compound because, during braking, friction generates heat.

In 2009, our group found a relationship between vehicular traffic and Sb levels in the blood of port workers that were exposed to this source. Sb accumulated primarily in the cytoplasm of their erythrocytes [7].

Little is known about the Sb uptake mechanism in cellular systems, and most studies are related to the Leishmania parasite and fungi [8]. In addition, no experiments have been conducted on blood cells due to the lack of analytical methods to study the unstable blood matrix. In addition, there has been little scientific interest in this subject because there are no major questions related to Sb and blood.

Because we found in 2009 that port workers that were exposed to heavy vehicular traffic had an accumulation of Sb in their erythrocyte cytoplasm [7], we believe it is important to determine which Sb species can enter the erythrocyte cytoplasm and elucidate the mechanism and uptake channels of these species. These questions are important because erythrocytes are responsible for

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Sb distribution to human organs [9] and excretion in hair, nails [10] and urine [11].

The purpose of this study is to determine the presence of Sb species in erythrocytes and elucidate the uptake mechanism of these species. The capacity of Sb species to interact and penetrate the erythrocyte membrane is of particular interest. Thus, the main goals of this study are as follows:

- develop an analytical method for the determination of Sb (V) and Sb(III) content in human erythrocyte plasma and cytoplasm.
- Evaluate the capacity of Sb(V) and Sb(III) to enter the cytoplasm in real human blood cultures.
- Study the Sb(V) and Sb(III) distribution kinetics in plasma and in cytoplasm of erythrocytes.

2. Experimental

2.1. Apparatus

The chromatographic separation of Sb species was performed using a Jasco (Easton, MD 21601, USA) HPLC system PU-2089S Plus model equipped with quaternary pumps, degassers, an auto sampler, an injector with a 100 μL loop and a short Hamilton PRP-X100 column (100 mm × 4.1 mm) with small particle size (5 μm).

To determine the Sb concentrations using hydride generation-atomic fluorescence spectrometry (HG-AFS), a PSA Analytical (Ornpington, Kent, UK) Millennium model (10055) atomic fluorescence spectrometer was used. This instrument has a continuous flow system for hydride generation coupled to a commercial dryer membrane (Perma Pure product, dryer model MD-110-12 FP) attached to the fluorescence spectrometer. Stibine was purged with an atomization flame under an argon flow. H_2, which was produced in the hydride generation reaction between $NaBH_4$ and HCl, sustained the flame. In addition, a supplementary H_2 flow was injected to maintain a stable argon/hydrogen diffusion flame.

The instrument was equipped with a Sb-boosted-discharge hollow cathode lamp (BDHCL) from Photron PTY Ltd. (Victoria, Australia) that operated at 18.3 mA. Data acquisition was performed using a microcomputer with Avalon software from PS Analytical.

2.2. Standard solutions and reagents

The purities of all chemicals and reagents used in this study were of analytical-grade or higher. De-ionized water (18.2 M Ω cm^{-1}) was obtained using a Nanopure system (Barnstead, Dubuque, IA, USA). Glass and polyethylene containers were cleaned by soaking for 1 day in 10% v/v nitric acid (analytical grade) and were then rinsed several times with de-ionized water before use. Nitric acid (65% w/v, super pure grade) and hydrogen peroxide (H_2O_2) (30% w/v) were used for digestion, and both were purchased from Merck in Darmstadt, Germany.

Individual Sb stock solutions were prepared from potassium hexahydroxoantimonate ($KSb(OH)_6$, 99.95%). Sb(V) stock solutions (100 mg L^{-1}) were prepared by dissolving an appropriate amount of the respective compounds in de-ionized water and storing the resulting solutions in the dark at 4 °C until further use. Lower concentrations of Sb standards were prepared daily by appropriately diluting the stock solutions with de-ionized water.

Sb(III) standard solutions were prepared immediately before use by dissolving potassium antimonyl tartrate in de-ionized water. Lower concentration antimony standards (individual and/or mixed species) were prepared daily by performing an appropriate dilution.

To determine the total Sb content by HG-AFS, the hydride generation system conditions were as follows: the 1.5 mol L^{-1} HCl carrier solution was prepared from hydrochloric acid (32%, analytical reagent grade, Merck, Darmstadt, Germany), the 0.75% w/v $NaBH_4$ solution was prepared daily by dissolving appropriate amounts of powdered $NaBH_4$ (analytical reagent grade Merck, Darmstadt, Germany) in 0.2% w/v NaOH (analytical reagent grade Merck, Darmstadt, Germany) and Sb(V) was reduced to Sb(III) using an aqueous solution of 1% w/v L-cysteine (Sigma, USA).

2.3. Sample collection

Blood samples were obtained from two different sources.

1 A private institution provided us with 300 mL of blood remnant from routine clinical work. This blood stock was used for the speciation analytical method development to determine the Sb (V) and Sb(III) distribution in human blood fractions and for kinetic studies.
2 Seven healthy and non-exposed university students provided us with venous blood (approximately 3 g from each donor). A trained nurse used syringes to draw the blood, which was drained into plastic tubes and carefully mixed with the three different anticoagulants examined in this study (EDTA, heparin and citrate). This blood was used to determine the Sb distribution in human erythrocyte plasma, cytoplasm and membrane experiments.

2.4. Spiking and recovery experiments

Human blood samples mixed with different anticoagulants were used for the spiking and recovery studies. All samples were spiked separately with 56 ng g^{-1} of Sb(V) and Sb(III). The total Sb content in the blood fractions was determined using HG-AFS after acid digestion, and the % recovery was calculated. Species stability was evaluated qualitatively by matching the retention times using the HPLC–HG-AFS hyphenated system.

2.5. Blood fractionation procedures

To separate the whole blood, plasma and red blood cells (RBCs), approximately 2.5-3.0 mL of the whole blood was separated in vacutainer tubes. To obtain the RBCs, the whole blood sample was centrifuged at 1000*g* for 10 min at 4 °C in graduated centrifuge tubes using a swing-out rotor centrifuge. The plasma and buffy coat (the layer of white blood cells over the RBCs) were removed, and the RBCs were washed 3 times by centrifugation at 600*g* for 10 min at 4 °C and were then resuspended in approximately 30–40 mL of 5 mmol L^{-1} isotonic phosphate buffer at pH 7.4.

To obtain the erythrocyte membranes and the cytoplasmic fraction, the washed RBCs were lysed by adding approximately 30–40 mL of hypotonic phosphate buffer at pH 7.4 (at 4 °C) under constant stirring. After the mixture was cooled on ice for 30 min, the lysed RBC membranes and the cytoplasmic fraction were separated by centrifugation at 20,000*g* for 10 min at 2 °C, and the supernatant was reserved for determining the Sb species concentration in the cytoplasmic fraction. The erythrocyte membranes were then washed three times by resuspension in approximately 30–40 mL fresh buffer and centrifuged under the same conditions. The blood fractionation procedures are summarized in Fig. 1.

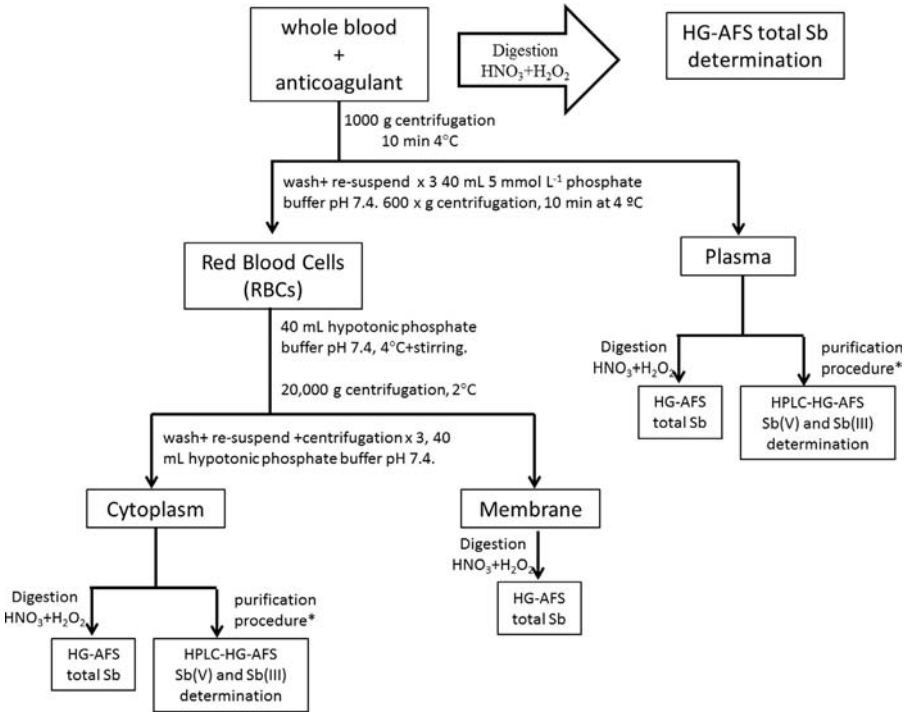

Fig. 1. Procedure scheme for blood analysis and fractionation procedure (*purification procedure is detailed in Fig. 2).

2.6. Sample digestion

All blood fractions were digested using the protocol previously described in 2009 [7]. The same procedure was applied to the plasma and cytoplasmic fractions. Analytical validation of the methodology was performed using certified reference materials, and these results were reported previously by our group. Briefly, the open vessel digestion method used for the total Sb concentration in blood was validated by the Seronorm™ Certified Reference Material L-2 and L-3. Complete recovery was observed using both materials.

The validated method includes using aliquots of whole blood with an initial mass of approximately 3 g, which were weighed to 0.1 mg precision and transferred to glass vessels. Nitric acid (6 mL) was added to the samples, and the solution was mixed by shaking. The mixture was kept closed for 24 h and then heated at 90 °C for 30 min. H_2O_2 (6 mL) was added, and the mixture was heated slowly to avoid sample loss due to violent bubble formation. The samples were diluted to 25 mL in a polypropylene volumetric flask and transferred to clean polyethylene bottles for storage prior to the HG-AFS analysis. Blank solutions were prepared by applying the entire digestion procedure to the samples.

2.7. HG-AFS determination

Aliquots of Sb(V) standard and sample solutions were placed in 50 mL volumetric flasks. The solutions were reduced to Sb(III) in an acidic solution by adding 1.5% KI in 0.2% ascorbic acid and HCl to obtain a final concentration of 1.5 mol L^{-1}. The solutions were analyzed using HG-AFS after 30 min of pre-reduction time. The measuring conditions for the HG-AFS system are provided in Table 1.

2.8. Sb(V) and Sb(III) determination in blood fractions

The chromatographic conditions for the separation of Sb species are summarized in Table 1. To prepare the blood fractions, 3 mL samples were used for both the cytoplasm and the plasma. First, 900 μL of 0.1 mol L^{-1} EDTA was added to the samples. Next, to precipitate the proteins, 3 mL of saturated $(NH_4)_2SO_4$ solution was added. The samples were mixed in 50 mL falcon tubes, and the resulting solution was centrifuged at 5000 rpm for 45 min until the supernatant was completely clear.

The supernatant was cleaned by elution through a C18 cartridge. To quantitatively recover the Sb retained in the cartridge, 3 mL of EDTA was added as the mobile phase. Finally, 1 mL of the resulting solution was collected, filtered using a 0.2 μm filter and then re-injected into the HPLC–HG-AFS system. The cytoplasm and plasma purification procedures for Sb(V) and Sb(III) determination are summarized in Fig. 2.

2.9. Data analysis

The Sb oxidation state in the plasma and cytoplasm was determined qualitatively using Sb(V) and Sb(III) chromatographic retention times according to the chromatographic standards previously reported by our group [12]. Sample aliquots of 100 μL were injected into the HPLC system, and quantification of Sb(V) and Sb(III) was performed using the standard addition method using 50 μg L^{-1} to 200 μg L^{-1} additions for the peak area measurements.

Spiked Sb(V) and Sb(III) recovery assays were determined by calculations based on the total Sb concentration. The total Sb concentration was determined in the plasma and cytoplasm using HG-AFS and external calibration according to previously developed procedures that were validated and published by our group. Briefly, the method consists of an external calibration using Sb(V) standard aliquots placed in 50 mL volumetric flasks. The calibration curve is prepared in the linear concentration range of 100-1000 ng L^{-1}. The Sb(V) solutions were reduced to Sb(III) by adding 0.25 g L^{-1} of cysteine and HCl to the flask to obtain a final concentration of 1.5 mol L^{-1}. The solutions were analyzed with HG-AFS 30 min after the reduction. The HG-AFS instrumental conditions, and additional details were previously reported by our group [7].

A screening study was performed using a two-level factorial experimental design. Two additional experiments were performed for each condition to calculate the standard deviation of the measured responses (σ). The factor effects, their interactions and their respective precision were evaluated using the least-squares method [13].

Table 1
Summary of conditions for HPLC coupled to HG-AFS detection system.

HPLC (Jasco HPLC system PU-2089S Plus)	
Column	Hamilton PRP-X-100 (100 × 4.1 mm id, particle size 5 μm)
EDTA mobile phase (mmol L^{-1})	20, pH = 4.5
Flow rate (mL min^{-1})	1.5
Injection volume (μL)	100
Detection HG-AFS (PS Analytical Ltd., Millennium Excalibur system)	
Sb BDHCL	
Primary current(mA)	15
Boosted (mA)	18
HCl (mol L^{-1})	1.5
HCl flow (mL min^{-1})	9
$NaBH_4$ (% w/v)	0.75 (in 0.4% NaOH)
Flow $NaBH_4$ (mL min^{-1})	0.35
Argon flow	
(Primary) (mL min^{-1})	300
(Secondary) (mLmin^{-1})	20
Hydrogen (auxiliary) (mL min^{-1})	60

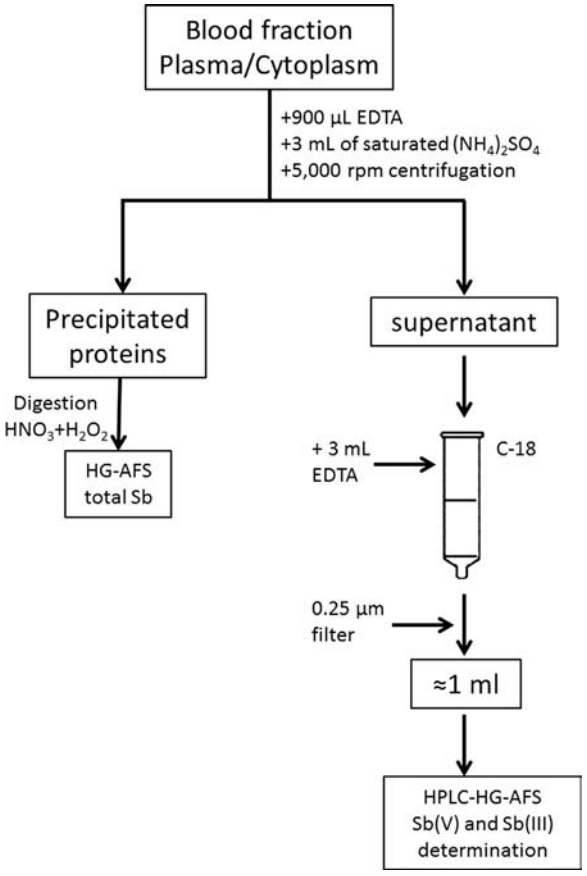

Fig. 2. Scheme for plasma and cytoplasm purification for the determination of Sb (V) and Sb(III) by HPLC–HG-AFS.

The factor or interaction was considered significant if the corresponding effect was higher than the precision. The statistical calculations were performed using the Statgraphics Plus 5.0 software package.

3. Results and discussion

3.1. Speciation methodology

A high organic content and protein presence in the blood cytoplasm and plasma are incompatible with the hydride generation system both for the generation of the foam and for the formation of precipitates. For these reasons, development of a method for the determination of Sb(V) and Sb(III) primarily focused on the sample treatment. The treatment of plasma and cytoplasm samples involved five steps:

1 Addition of 0.1 mol L^{-1} complexing agent (EDTA or oxalic acid).
2 Protein precipitation using 3 mL of a saturated solution of $(NH_4)_2SO_4$. This variable was kept constant throughout the study. To determine if any analyte loss was observed when precipitated proteins were used, the total Sb content was determined using a precipitate that was below the limit of quantification.
3 Centrifugation at 5000 rpm for 45 min. This variable was kept constant throughout the study.
4 Solid phase extraction cleaning (using a C8 or C18 column).
5 Elution from the solid phase using the same complexing agent as in step 1.

Table 2
Codified and natural variables, for cleaning optimization step.

Factors	**Codification**	
	−1	**1**
Stabilizing agent	EDTA	Oxalic acid
Concentration (mol L^{-1}) (for steps 1 and 5)	0	0.1
Stationary phase cleaning	C8	C18

Based on the above procedure, three variables were analyzed. First, two complexing agents were compared (1) EDTA, which is recognized for its capacity to extract or stabilize Sb in seawater [12], urine [11], sediments [14] and marine biota [15], and (2) oxalic acid, which has been used for soil [16] and vegetables [17]. Second, the concentration of the complexing agents (from 0.0 to 0.1 mol L^{-1}) was analyzed. Finally, the solid phase extraction (C8 and C18 columns) polarity was analyzed. Experiments were designed to optimize the recovery of each species. The variables and their concentrations are reported in Table 2. Ten experiments were performed, and three of them were repeats. The recovery study was conducted by adding 60 μg L^{-1} of Sb(V) and Sb(III) separately to each blood fraction. The Sb(V) and Sb(III) recoveries obtained in both fractions are provided in Table 3.

The statistical weight of each variable for the Sb(V) and Sb(III) recovery in blood plasma and cytoplasm are provided in the Pareto charts in Fig. 3. The black bar describes the statistical weight of the

Table 3
Experimental design for Sb(III) and Sb(V) in plasma and cytoplasm.

Experiment	Stabilizing reagent	Concentration	Stationary phase	Plasma % recovery		Cytoplasm % recovery	
				Sb(V)	Sb(III)	Sb(V)	Sb(III)
1	1	1	1	71	47	88	37
2	1	−1	1	70	42	64	32
3	1	−1	−1	58	43	64	22
4	−1	1	−1	99	76	87	55
5	−1	−1	−1	78	76	90	59
6	−1	1	1	85	88	85	59
7	1	1	−1	76	44	56	23
8	−1	−1	1	63	97	86	61
9	−1	−1	1	87	83	73	59
10	−1	−1	1	67	88	70	60

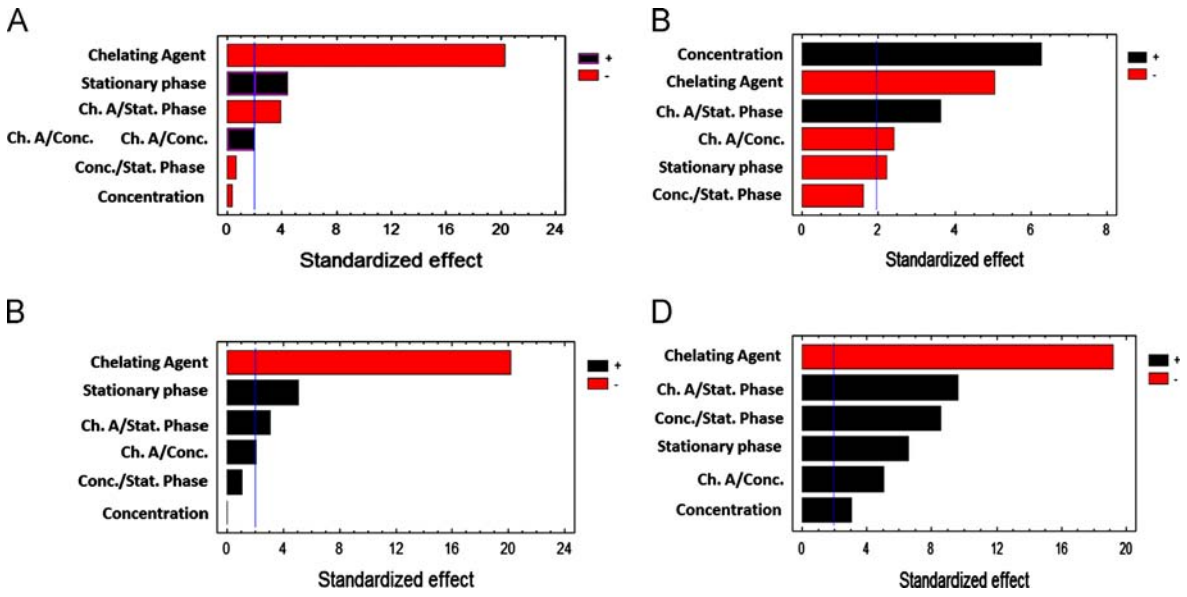

Fig. 3. Pareto charts for Sb recovery optimization for (A) Sb(V) in plasma; (B) Sb(III) in plasma; (C) Sb(V) in cytoplasm; (D) Sb(III) in cytoplasm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

variable when it generates a positive effect on the response, which means an increase in the variable (from −1 to +1) magnitude causes an extraction yield increase. In contrast, the red bar represents the statistical weight of the variable when the effect is negative, which means that the variable decreases the recovery of the species. The blue line perpendicular to the x-axis represents the limit at which the statistical weight of the variable is greater than the experimental design precision at 95% confidence.

Fig. 3A, C and D demonstrates that oxalic acid has a negative effect, which corresponds to the recovery of Sb(V) in the plasma and cytoplasm (A and C) and Sb(III) in the cytoplasm (D). A positive effect is detected only for Fig. 3B, which represents the recovery of Sb(III) in the plasma. These results confirm that EDTA is the best choice as the eluting agent because in 3 of the 4 possibilities EDTA produced an increase in antimony recovery. As shown in Figs. 3B and 1D, we observed that the complexing agent concentration is statistically significant for Sb(III) both in the plasma and cytoplasm. These results are different from Sb(V), in which the complexing agent concentration is not significant (Figs. 3A and 1C). These results can be interpreted according to the thermodynamics of both species. Sb (V) is anionic at a neutral pH ($Sb(OH)_6^-$) [18], and, thus, EDTA cannot form complexes with Sb(V). However, Sb(III) is neutral and readily complexes with EDTA. Indeed, complexes between Sb(III) and EDTA have been reported in the literature [19].

Finally, C18 was the stationary phase and exhibited improved performance for both species, except for the case of Sb(III) in the cytoplasm, which initially demonstrated better performance with the C8 cartridge. The stationary phase variable exhibited a lower statistical weight when compared with the nature and concentration of the complexing agent.

An interesting observation can be made regarding the recovery of Sb(III) in the cytoplasm, as shown in Table 3. Overall, the recoveries obtained are relatively low. We found no chromatographic signal for Sb(V), and, thus, the low recovery cannot be attributed to Sb(III) oxidation. One possible explanation may involve the transformation of Sb(III) into complexes with glutathione or trypanothione [20] or chemical reactions of inorganic Sb(V) and Sb(III) to form complexes with amino carboxylic acids or with organic ligands [18]. However, the reduction of Sb(V) to Sb (III) has been previously reported in macrophages [21]. Sb(V) could also undergo chemical transformations during sample treatment, such as precipitation with proteins or irreversible retention during the C-18 cleaning step, that could cause analyte loss. However, “analyte loss” during chromatographic separation could be another explanation for low recovery. For example, chemical transformation into undetectable or unidentifiable species during the chromatographic analysis may also explain the low recovery of Sb(V) and Sb(III) as shown in Table 3.

The chromatogram provided in Fig. 4 is empirical evidence for the presence of an unidentified chemical species. Thus, issues with analytical method development to identify new Sb species in the plasma and cytoplasm remain a challenge for the scientific community.

Finally, the Sb(V) and Sb(III) maximum recovery results in the plasma and cytoplasm (Table 3 and Fig. 3) indicate that the best conditions for plasma and cytoplasm treatment involve using C18 as the solid phase for cleaning and EDTA (0.1 mol L^{-1}) for stabilization and as the eluent.

The analytical characteristics for Sb speciation in blood plasma and cytoplasm obtained under optimum conditions are provided in Table 4.

Results from Table 4 indicate that the LOD of the calibration curve is at a $\mu g\,L^{-1}$ concentration level, which is a very low detection limit for Sb speciation. However, 3 g of blood sample and a high dilution was needed to obtain the cytoplasm fraction. For Sb (V) and Sb(III), 40 and 30 $\mu g\,g^{-1}$ LOD, respectively, in cytoplasm was not low enough for antimony speciation in this fraction considering the native Sb concentration. Therefore, improving method sensitivity in the cytoplasm in future studies is imperative. However, the current analysis is useful for spiking and recovery determinations.

In contrast, 4 ng g^{-1} and 3 ng g^{-1} LOD for Sb(V) and Sb(III), respectively, in plasma is enough to detect both species in expected concentrations (approximately 10 $\mu g\,g^{-1}$) in non-exposed people.

The optimized method was applied to Sb speciation in blood. Of the seven volunteers, Sb(V) and Sb(III) were detected only in one case. The chromatograms are provided in Fig. 4. Three unknown species were detected that can be attributed to Sb(V) or Sb(III) complexes with organic ligands such as glutathione or trypanothione, which has been described in previous in vivo and in vitro studies [22].

3.2. Native Sb distribution in human blood fractions

In our previous study, the Sb levels detected in the whole blood samples ranged from 1.5 to 35 ng g^{-1}, and we demonstrated that these data were highly variable and depended on the surrounding environment. With respect to the Sb distribution in human blood fractions, our previous study was extremely limited because only one anti-coagulant was considered (EDTA) [7]. For this reason, in the present study, the Sb distribution was evaluated in the presence of the three most commonly used blood preserving anti-coagulants. The Sb distribution in blood plasma, cytoplasm and membrane was determined in blood samples from seven donors. The mean results are reported in Fig. 5.

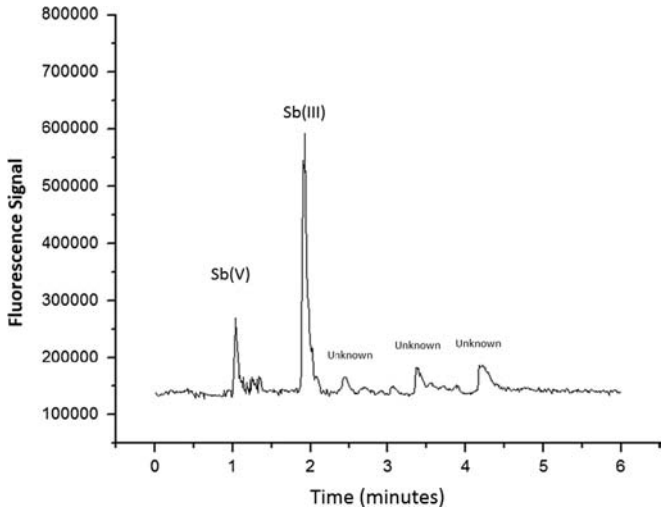

Fig. 4. Chromatogram for non-spiked human plasma sample by using optimized method.

Table 4
Analytical characteristics for antimony speciation in plasma and cytoplasm.

Characteristics	Sb(V) plasma/ cytoplasm	Sb(III) plasma/ cytoplasm
Working linear range ($\mu g\,Sb\,L^{-1}$)	2–100	1–100
Correlation coefficient	0.9971	0.9992
Detection limit in calibration curve (μgL^{-1})	0.5	0.3
Detection limit of methodology (ngg^{-1})	4/40	3/30
Quantification limit ($\mu g\,L^{-1}$)	2	1
Reproducibility (%CV for 56 ng g^{-1})	20/12	8/5

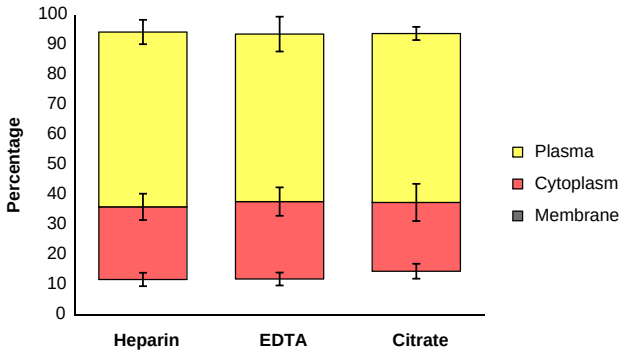

Fig. 5. Percentage of total Sb distribution in human erythrocyte plasma, cytoplasm and membrane ($n=7$ blood samples).

Results provided in Table 5 reveal that the native Sb concentration in the whole blood samples is 15 ng g^{-1}, which is approximately two to three times lower than was previously determined in exposed port workers [7]. The native Sb concentration also has a RSD of less than 10%. These results reveal the following Sb distribution in native blood: 60–70% in the plasma fraction and 30–40% in the cytoplasm fraction. Interestingly, the native Sb distribution is statistically independent of the anti-coagulant used.

In addition, these results show an inverse distribution compared with our previous results, in which 90% of Sb was found in the cytoplasmic fraction. This can be explained by the lower exposure to heavy vehicular traffic in the populations analyzed in this study. In contrast, the subjects in our previous study were exposed significantly to heavy vehicular traffic [7].

Finally, Table 5 shows that the levels of Sb obtained in the membrane fraction are kept constant at 1 ng g^{-1}, as was described previously [7], indicating that the lipid bilayer could be saturated with Sb. This finding explains why the Sb in the erythrocyte membrane fraction remains constant.

3.3. Anticoagulant selection: Sb(V) and Sb(III) distribution in plasma and cytoplasm fractions

Because most anticoagulants have the capacity to form complexes with metals and metalloids, the effect of anticoagulants on the Sb(V) and Sb(III) distributions in plasma and cytoplasm was analyzed by spiking the whole blood sample with 56 ng g^{-1} of Sb (V) and Sb(III), separately, and incubating for 30 min. This procedure used the total Sb concentration found in people exposed to pollution from heavy traffic, as reported previously [7]. The distribution of native Sb (white bar) and the recovery of spiked Sb

Table 5
Distribution of Sb in human blood fractions (plasma, cytoplasm and membrane) from seven volunteers in three anticoagulants. Concentrations are reported in ng of Sb g^{-1} of whole blood.

Volunteer	Heparin			EDTA			Citrate		
	Plasma	Cytoplasm	Membrane	Plasma	Cytoplasm	Membrane	Plasma	Cytoplasm	Membrane
1	8.3	6.4	1.1	9.6	6.2	1.2	8.5	5.6	1.2
2	8.6	5.3	1.3	8.6	6.8	1.3	8.0	4.9	1.1
3	8.2	5.8	0.9	7.9	5.8	1.1	7.6	6.2	1.3
4	8.5	6.3	0.8	7.4	6.3	1.2	8.1	5.6	1.1
5	7.8	6.4	0.9	8.8	7.0	0.9	7.9	5.9	1.0
6	8.6	6.3	0.8	9.8	6.4	0.8	8.3	6.9	1.3
7	9.7	5.4	1.1	8.3	8.4	0.9	8.4	7.2	0.9

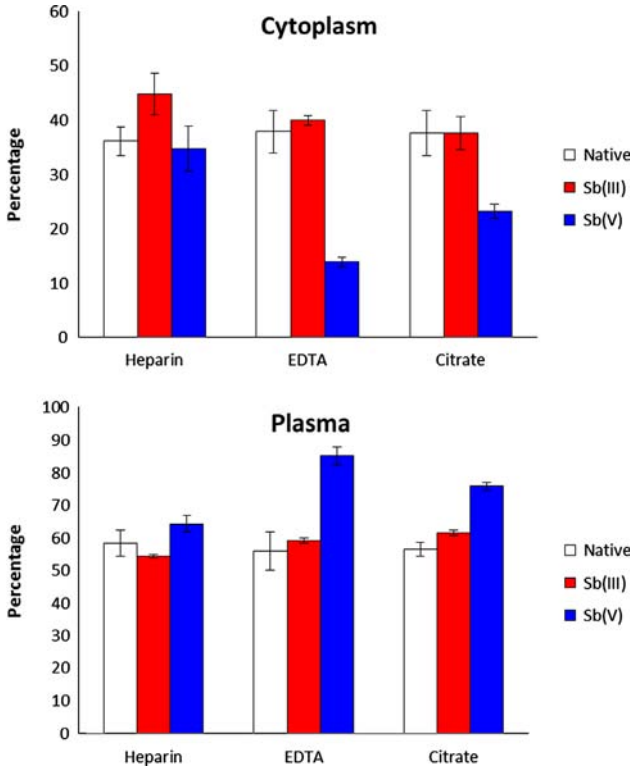

Fig. 6. Distribution of native Sb (white bar) and recovery of spiked Sb(III) (red bar) and spiked Sb(V) (blue bar) in plasma and cytoplasm. Sb(III) and Sb(V) experiments were performed in triplicate by spiking separately 56 ng g^{-1} of each species in whole blood. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(III) (red bar) and spiked Sb(V) (blue bar) in plasma and cytoplasm using the three anticoagulants is provided in Fig. 6.

Sb(III) shows a similar distribution to that seen for total Sb in native blood (comparisons were made assuming a redox equilibrium that is similar for both spiked and non-spiked samples) as shown in Fig. 6. However, the Sb(V) distribution is highly influenced by the anticoagulant used, and a significant decrease is observed when EDTA or citrate are used. Thus, we propose that heparin is the best anticoagulant for blood preservation.

Results presented in Fig. 6 are the first direct empirical evidence supporting the ability of Sb(V) to permeate red blood cells and reach the cytoplasm. We determined that Sb(III) can penetrate red blood cells more than Sb(V) for the three anti-coagulants used. These results are reasonable considering the thermodynamics of the species: Sb(III) is neutral at physiological pH.

In contrast to the Sb(III) EDTA complex [19], the citrate ligand is capable of complexing both species of Sb [23,24]. Our results contradict the theory that charged species are more energetically restricted from entering cell systems compared to neutral species [25]. For example, one would expect that when using EDTA or citrate as the anti-coagulant, Sb(III) uptake would decrease in comparison with heparin. However, we did not observe this. To further explain these results, future studies should determine the influence of protein channels on the Sb species distribution in human erythrocytes.

Considering the above results, we suggest heparin as the anticoagulant choice for future studies for the following reasons:

- heparin does not significantly affect the native Sb distribution.
- There are no reports of heparin and Sb(V) or Sb(III) complexes.
- Previous studies have been performed related to Sb(III) and erythrocytes by Poon et al. [26] that successfully used heparin as the anticoagulant.

Despite heparin's structural disadvantage (it is classically anionic) as defined in the “sample collection guidelines for trace elements in blood and urine” designed by Cornelis et al. [27], the anticoagulant choice for trace element analysis in blood must be evaluated for each element. In this case, both EDTA and citrate have more potential to affect measurements than heparin.

3.4. *Effect of time*

Considering the unstable nature of red blood cells, a study was performed to determine the incubation time effect on the native antimony distribution in blood fractions (whole and non-spiked blood). These experiments were performed in triplicate using three containers of a stock solution of whole blood with heparin as the anticoagulant. The total Sb content was determined in blood fractions after 0, 30, 60 and 90 min of incubation. The native Sb distribution in the plasma and cytoplasm is provided in Fig. 7.

The results presented in Fig. 7 reveal two interesting effects. First, the native Sb distribution in human blood appears to be extremely dynamic. After the blood sample is taken, the distribution begins to completely reverse from the initial cytoplasmic fraction to the blood plasma fraction after only 30 min. No hemoglobin in the blood plasma fraction was detected. Thus, these results are not due to erythrocyte membrane destruction. The implications of these results are bio-analytical. From an analytical chemistry standpoint, this dynamic behavior implies that any study trials that requires knowledge of the original metalloid distribution in the human blood fractions must be separated immediately after blood samples are taken to avoid bias in the original distribution. From a biochemical point of view, our results suggest that Sb is able to permeate the erythrocyte membrane from the cytoplasm to plasma or vice versa. This permeability raises additional questions regarding the nature of the channel and the physicochemical mechanism involved.

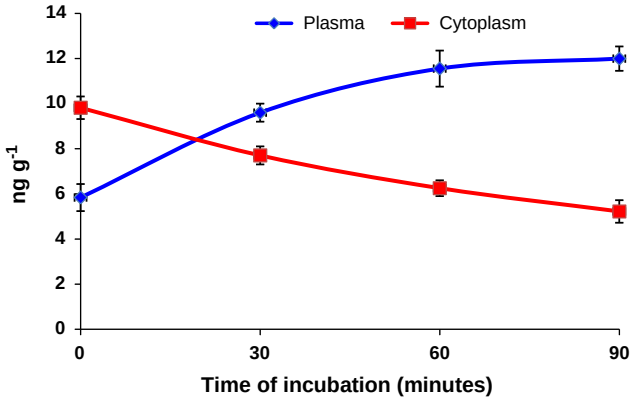

Fig. 7. Native Sb distribution in plasma and cytoplasm in function of time of incubation. Heparin was used as anticoagulant.

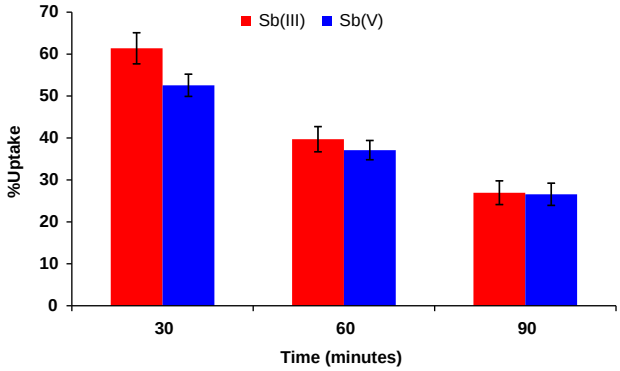

Fig. 8. Percentage spiked Sb(III) and Sb(V) uptake (which penetrate erythrocyte membrane until cytoplasm fraction) in function of time of incubation. Whole blood were spiked with 56 ng g^{-1} of each specie separately.

To evaluate the capacity of Sb(V) and Sb(III) to enter the erythrocyte cell, incubation of whole blood samples spiked with 56 ng g^{-1} of Sb(V) or Sb(III) was performed. The results of the permeation of the erythrocyte membrane of both species are provided in Fig. 8.

The results shown in Fig. 8 indicate that a high percentage of both species can enter the erythrocyte cell. However, both species are then excreted from the cell interior where there was a considerable decrease from approximately 60 to less than 30% within the cell.

We propose that some degradation of the cell potentially takes place; however, no evidence of hemolysis was detected in the 9 analyzed samples. In addition, we propose an internal detoxification mechanism to explain this excretion, which was previously described for Sb in the Leishmania parasite in which trypanothione amino-acid was used as a ligand for the excretion of Sb(V) from the inner cell [28].

3.5. Culture medium temperature

The influence of temperature on the native Sb, spiked Sb (V) and Sb(III) distributions in the blood fractions was examined by culturing at three different temperatures: 4 °C to simulate the storage temperature in a refrigerator, 20 °C to simulate room temperature and 37 °C to simulate the physiological temperature. The results are provided in Fig. 9.

The data in Fig. 9 confirm that increasing the culture temperature increases the capacity of spiked Sb(V) and Sb(III) to penetrate the membrane barrier and reach the cytoplasm. This fluidity would, therefore, be associated with either passive diffusion [29–31] or protein-channel mediation, which operates through a conformational change to facilitate the entry of one or both species [32].

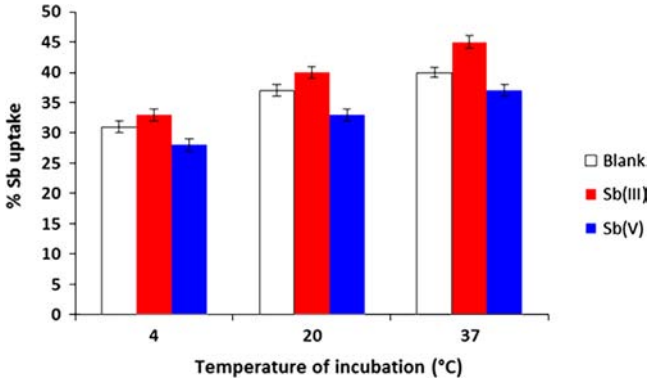

Fig. 9. Percentage of native Sb, spiked Sb(V) and spiked Sb(III) uptake until cytoplasm fraction in function of temperature of incubation.

4. Conclusions

A new method for Sb(V) and Sb(III) determination in human blood plasma and cytoplasm was developed in this study. A cleaning procedure is proposed that consists of precipitating proteins followed by a C18 solid phase column. In addition, an eluting solution of 0.1 mol L^{-1} EDTA allows the plasma and cytoplasm matrix to be more compatible with the chromatographic separation.

Results of the three blood cultures revealed that both species, Sb(III) and Sb(V), are able to enter the erythrocyte and reach the cytoplasm. Additionally, we demonstrated that this species distribution is highly dependent on the anticoagulant and the time and temperature of the cell culture.

Among the anticoagulants studied, heparin is the anticoagulant of choice because it generated the lowest change in native Sb distribution. Therefore, we suggest that all future studies regarding the presence of Sb in blood use heparin instead of EDTA or citrate for sample preservation. Heparin leads to the lowest degree of modification of the native Sb distribution in the matrix.

With respect to incubation time and from an analytical perspective, our results demonstrate that blood fractionation should be performed immediately after obtaining the sample to avoid bias in the final mass balances. A culture time of 30 min generated an output of greater than 20% intracellular Sb.

The incubation temperature is a key variable that generated an increase in the ability of Sb (V) and Sb (III) to enter the erythrocyte cytoplasm. This phenomenon is associated with an increase in fluidity of the plasma membrane.

Finally, this study reveals the need to elucidate the Sb(V) and Sb (III) uptake mechanisms in human erythrocytes. Hypotheses for passive diffusion and protein-mediated uptake need to be evaluated.

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