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Monitoring of total antimony and its species by ICP-MS and on-line ion chromatography in biological samples from patients treated for leishmaniasis

Abstract Results from a study are reported in which patients with leishmaniasis were monitored by whole blood, blood plasma, urine, and hair analysis, before, during, and after intramuscular administration of N-methyl meglumine antimoniate. Quadrupole ICP-MS was used for the detection of antimony and on-line ion chromatography for the separation of its species. After typically 30 consecutive daily injections of 5 mg antimony per kg of body weight, Sb concentrations of up to 250 μg L^{-1} in whole blood and plasma, and 60 mg of Sb per gram of creatinine in urine, were measured 24 h after drug administration. Antimony in hair samples of these patients showed concentrations of up to 24 μg g^{-1}. Speciation studies of Sb^{5+} and Sb^{3+} in drug, urine, and plasma samples were performed by ion chromatography using a Hamilton PRP-100X anion exchange column and EDTA (2 or 20 mM, pH 4.7) as the mobile phases. Repeatability of elution time and peak area measurements for a 0.125 ng spike were <1.2% and <3.5%, respectively. Method detection limits for both species, using a 1:10 diluted urine or plasma sample, were typically 1.6 μg L^{-1}. The procedure was capable of separating the very intense drug peak from its inorganic species, thus permitting the first studies on the bio-transformation of N-methyl meglumine antimoniate to Sb^{5+} and Sb^{3+} in the human body.

Keywords Antimony speciation · Ion chromatography · ICP-MS · Leishmaniasis

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Introduction

Contrary to its chemical analogue arsenic (As), little is known on the environmental and biological behavior of antimony (Sb) and nearly nothing of its speciation [1, 2]. The clinical application of pentavalent antimonial compounds in the treatment of leishmaniasis, a tropical disease that currently infects about 30 thousand people per year in Brazil and over 400,000 per year world-wide [3], is a rare opportunity to study the metabolism of antimony and its species in the human body. Among the many open questions concerning the therapeutic and toxic actions of antimonials, the following are of major importance and closely related to speciation: (1) to what extent is Sb^{5+} reduced to Sb^{3+} within the human body and how does this process contribute to the toxic side effects associated with long-term, high-dose therapies (this hypothesis was formulated by Goodwin and Page in 1943 [4], however not confirmed until now); (2) to what extent does the more toxic Sb^{3+} species participate in the eradication of leishmaniasis causing parasites; (3) what are the detoxification mechanisms for this element in humans and other mammals: methylation, binding to albumin, metallothionein or other biomolecules?

This study reports on antimony concentrations in whole blood, blood plasma, urine and hair samples obtained from patients with leishmaniasis before, during, and after intramuscular administration of N-methyl meglumine antimoniate at low doses. Results on the application of an ion chromatographic procedure for Sb speciation in biological fluids and in N-methyl meglumine antimoniate will be presented and the first data are shown which give experimental evidence for the metabolic alteration of the drug and the bio-reduction of Sb^{5+} to Sb^{3+}.

Experimental

Clinical samples

Urine and blood samples were taken from patients before, during, and after low-dose treatment (5 mg Sb per kg of body weight) with

N-methyl meglumine antimoniate (Glucantime, Rhodia-Farma). All patients were carefully instructed about their treatment and then asked for their adherence to the study as part of the routine protocol in use at the CPqHEC. During the administration period, typically 30 days with daily injections and in a few cases 60 days, samples were collected 3 times a week at about 24 h after injection of the drug. After discontinuing administration, samples were taken at short intervals (hours or daily) during the rapid excretion period and weekly or monthly during the slower one (see Fig. 3a,c). Blood specimens were obtained by venepuncture and sampled in vacutainers (Becton Dickinson, Franklin Lakes, NY, USA, ref. no. 366480) containing Na-heparin as anticoagulant. Urine samples were stored in acid-cleaned polyethylene bottles without acidifying. Blood plasma samples were obtained from whole blood samples by centrifugation (3500 rev min^{-1} over 15 min). As antimony concentrations in urine, whole blood, and plasma were highly elevated in most samples (see results), contamination problems were not considered to be relevant to this study. Nevertheless, blank values were measured and subtracted in all cases. All samples were kept in a refrigerator (<5 °C), or frozen when longer storage was intended. During transportation from the hospital to our lab at PUC-Rio, samples were kept in an ice-box. Normally, blood and urine samples were analyzed within 4 h of collection. Urine data were normalized by their creatinine content. Whenever applicable, the guidelines for collection of clinical samples suggested by Cornelis et al. [5] were followed.

Scalp hair samples from the occipital area were taken from patients before, during, and after treatment. For uptake studies, samples of 0.5–2.0 cm length (measured from the scalp) were collected as soon as possible, meaning that new hair growth resulted in this increase in hair length after about 15 days to one month, depending on the sex and physiology of the patient. For identification of the new hair portion, a hair strand at the neck was marked very close to the scalp with an appropriate water resistant color not containing Sb in interfering amounts. For retrospective studies of women treated up to 10 month before, hair strands were collected at full length (>15 cm) and cut into 1–2 cm segments.

Antileishmanial drug, reference materials, and reagents

All patients in this study were treated with *N*-methyl meglumine antimoniate sold by Rhodia-Farma (Brazil) under the commercial name Glucantime. For clinical applications and also for complementary speciation studies, freshly opened ampoules were always used. Total Sb, Sb^{5+}, and Sb^{3+} where measured in these drug samples. For the validation of total Sb determinations, different clinical reference materials were used throughout this study, however only results obtained on urine (lot: 403125) and whole blood (lot: 404108), both SERONORM (Nycomed AS, Pharma Diagnostics, Oslo, Norway), will be shown here. Reference materials with certified antimony species were not available for this work; validations were therefore performed with spiked samples. All reagents used were at least of analytical grade. Deionized water (18 MΩ cm) and HNO$_3$ (twofold sub-boiled) were of the highest quality. All pH adjustments were made with an ammonia solution (25%, p.a. Merck) or nitric acid. For analytical performance studies of Sb^{3+} and Sb^{5+} separation by IC or FI-HG using aqueous solutions or spiked urine and blood plasma samples, appropriate amounts of potassium antimonyl tartrate (C$_4$H$_4$KO$_7$Sb·0.5H$_2$O, p.a. Merck) and potassium hexahydroxyantimonate (KSb(OH)$_6$, p.a. Merck) were used for the preparation of stock solutions (1,000 mg L^{-1}).

Analytical methodologies

Inductively coupled plasma mass spectrometry

ICP-MS was the only method applied for antimony determination. The instrument used was an ELAN 5000A (PerkinElmer-Sciex, USA) equipped with a Meinhard nebulizer and a cycloclonic spray chamber (Glass Expansion, AU) in most applications. Antimony was measured at its *m/z* ratios of 121 and 123 and In (*m/z*=115)

was used as an internal standard. Spectral interferences on these masses were negligible for the sample matrices used in this study and therefore no corrections were required. Standard operational conditions for the ICP-MS measurements were used [6, 7]. For the determination of total Sb by solution nebulization ICP-MS, whole blood, plasma, and urine samples were diluted with MilliQ water (1:10 or 1:100). Most samples could be analyzed in the quantitative external calibration mode, however, for the less dilute samples (1:10), the analyte addition procedure was preferred. Hair samples were washed with acetone and water and after drying at low temperature (ambient air or 50 °C in a drying oven) wet-ashed with HNO$_3$-H$_2$O$_2$ in closed polyethylene tubes, using a procedure described previously [6, 7]. The diluted digest (1:100) was then analyzed by ICP-MS in the quantitative external calibration mode.

NaBH$_4$ hydride generation (HG)

As a complementary method for the determination of total Sb and Sb^{3+}, flow injection hydride generation (FI-HG) was applied using a procedure similar to that described by Petit de Penã et al. [8]. A flow injection system (FIAS-200) coupled to an autosampler AS-90 (both PerkinElmer, USA) was used for automation of the hydride generation procedure. Samples were introduced by a 500 μL loop and reduction was performed by freshly prepared NaBH$_4$ solutions (0.4% w/v, p.a. Merck in NaOH 0.05% w/v) using HNO$_3$ (3% v/v) as the carrier phase. For total Sb determination, pre-reduction was performed with a mixture of H$_2$SO$_4$ (0.5 M), KI (10% w/v), and ascorbic acid (1.0% w/v) at ambient temperature for 30 min.

Ion chromatography

IC was performed with the anion exchange column PRP-X100 (150 mm×4.1 mm i.d., Hamilton, USA) using EDTA as the mobile phase. This procedure is similar to that described by other authors [9, 10, 11]. EDTA concentrations of 2 mM or 20 mM at pH 4.7 were used depending on the peak resolutions required (see Results). The HPLC system consisted of a LC-200 pump (Perkin-Elmer, USA) equipped with a Rheodyne six-port injector valve (mod. 4595) and a 100 μL injection loop. A constant pressure of 1,000 psi (1 psi=6894.48 Pa) was used in all experiments, resulting in a constant eluent flow rate of 1.5 mL min^{-1}. All connections were made by PEEK tubings. Quantification of the chromatographic peak areas was performed using the software package Origin 6.0 (Microcal Software Inc., USA). Urine and blood plasma samples for IC-separations were membrane-filtered (<0.2 μm, no. FGLP02500, Millipore, USA) and then diluted (10- or 100-fold) with the mobile phase used in the HPLC method, generally EDTA (2 or 20 mM), immediately before the run.

Results and discussion

Analytical methodologies

Table 1 summarizes typical performance and validation data for the determination of total Sb by solution nebulization and hydride generation ICP-MS showing the suitability of the methodologies for the purpose of this study.

The separation of Sb^{3+} and Sb^{5+} by ion chromatography, using the PRP-X100 column available in our laboratory, was not so straightforward. Based on published data, several mobile phases were tested, including EDTA [9, 10, 11], HNO$_3$ [12], and phthalic acid [13, 14]. Until now, the best results in terms of separation power and detection limits were obtained with EDTA. The adequateness of ni-

Table 1 Typical analytical performance data of the ICP-MS procedures used for the determination of total antimony, including validation results obtained on two clinical reference materials. (All concentration data are in $\mu g\ L^{-1}$; n=3 for reference materials, n=10 for LOD and BEC determination; method detection limits are higher by the corresponding dilution factor; confidence intervals are given for p=0.05)

| Method	Seronorm™ Urine – 403125	Seronorm™ Whole Blood – 2
SN-ICP-MS (dilution 1:10)	104.1±2.9 (2.7%)	25.5±0.4 (1.7%)
FI-HG-ICP-MS (microwave digestion)	100.6±0.5 (0.5%)	25.9±0.7 (2.7%)
Reference (spike value)	101±2 (100)	(25)
Limits of detection (LOD, 3σ) and background equivalent concentrations (BEC)		
ICP-MS with Meinhard nebulizer and cyclonic spray chamber	Diluted sample (10×): LOD=0.008; BEC=0.025 Decomposed (dilution 10×): LOD=0.024; BEC=0.096	
FI-HG-ICP-MS	Diluted sample (40×): LOD=0.002; BEC=0.041	
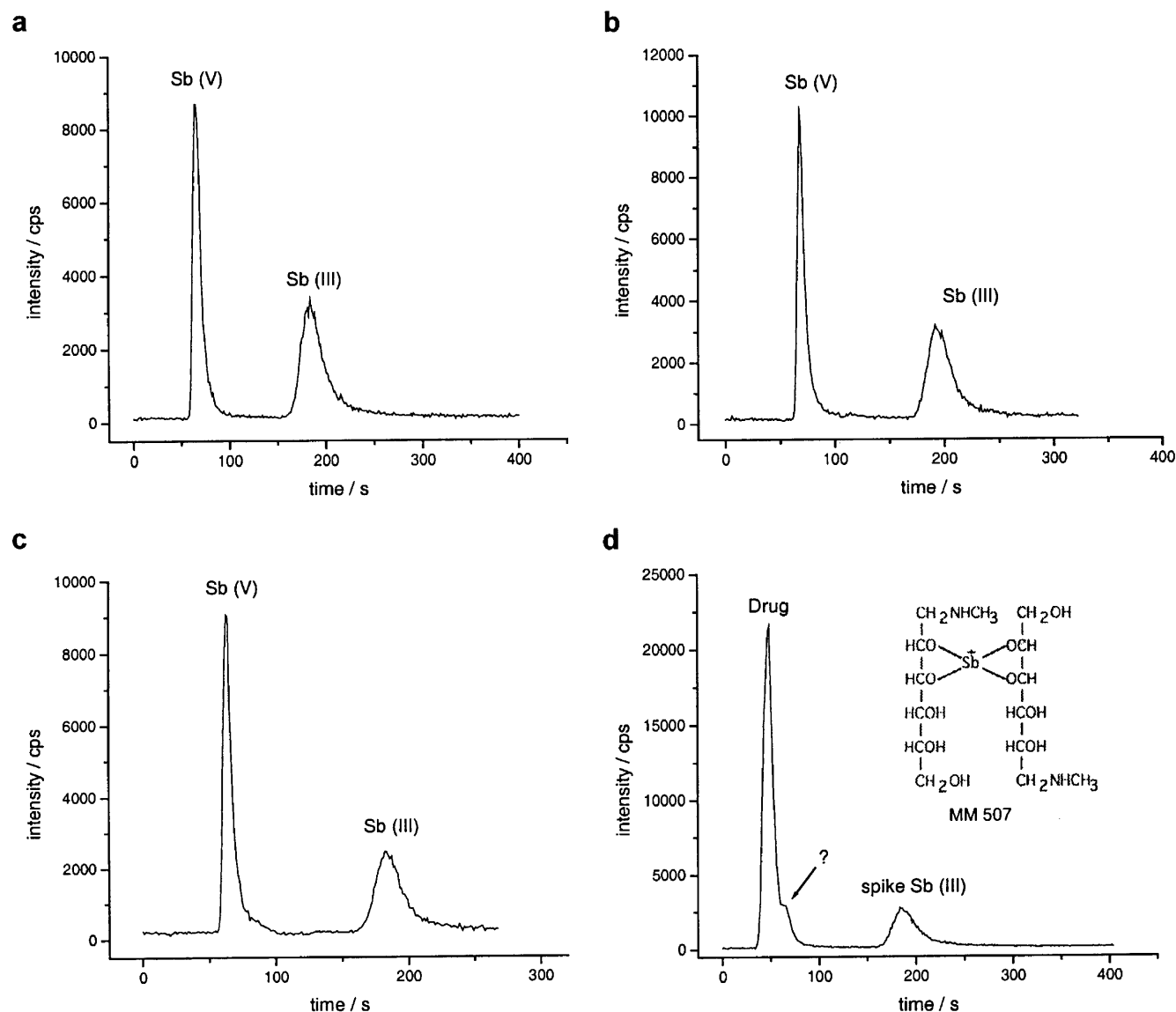
Fig. 1 Ion chromatograms of antimony species in: (**a**) 1 ng spike of Sb^{5+} and Sb^{3+} in EDTA (20 mM); (**b**) 1 ng spike of Sb^{5+} and Sb^{3+} in diluted urine (1+9) and (**c**) in diluted plasma (1+9); (**d**) *N*-methyl meglumine antimoniate solution (diluted about 1: 2×10^6) spiked with 1 ng of Sb^{3+}; the presence of Sb^{5+} close to the drug peak is indicated; one of the possible molecular structures of the drug is shown [15]. Other experimental conditions: PRP-X100 column; mobile phase: EDTA (20 mM, pH 4.7), flow rate: 1.5 mL min^{-1}; pressure: 1000 psi; injected sample volume: 100 µL
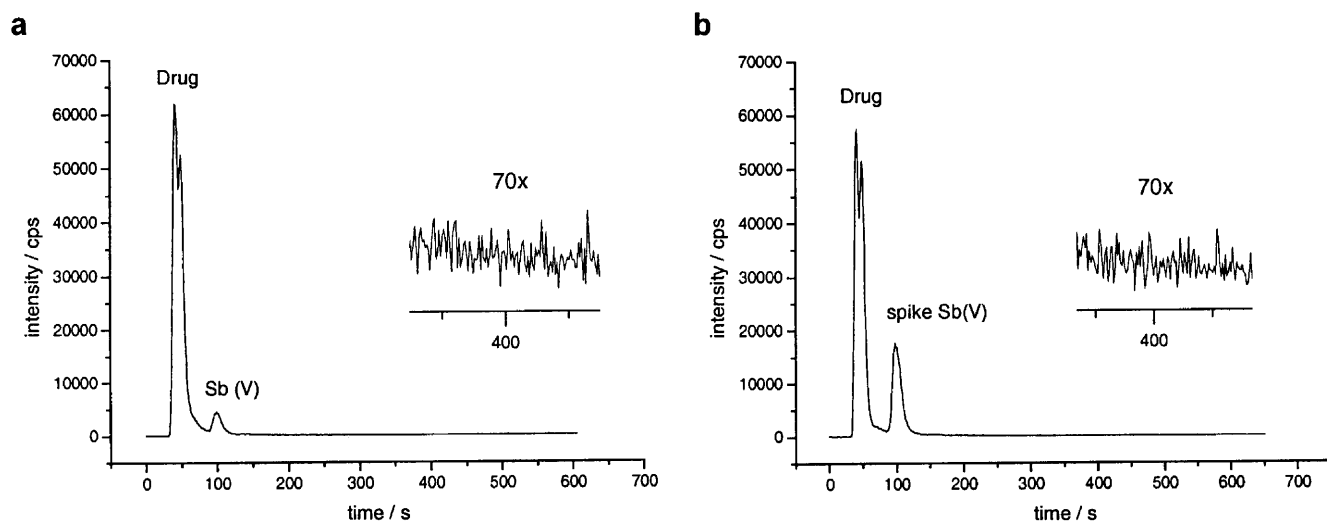
a

b

Fig. 2 Ion chromatograms of a diluted ($1:5\times10^5$) *N*-methyl meglumine antimoniate solution using EDTA (2 mM, pH 4.7) as mobile phase. Other experimental conditions as shown in Fig. 1. (**a**) sample without spike indicating the presence of anionic Sb^{5+} in the drug; (**b**) spiked sample (3 ng of Sb^{5+}) confirming the elution position of Sb^{5+}. The better resolution at the solvent front can be seen (compare to Fig. 1d)

tric acid (15 mM adjusted with KOH to pH 6) as the mobile phase for the separation of Sb^{3+} and Sb^{5+}, as proposed in [12], could not be confirmed in our study in which no separation of the species could be achieved. Phthalic acid (2 mM containing 2% acetone) resulted in good peak separation similar as shown in [13, 14], however, an elevated Sb background (>600 cps) was confirmed for this reagent and in addition, a shift of the Sb^{3+} peak to lower retention times was observed during repeated sample injections.

For the aforementioned reasons, EDTA was preferred in this study. Fig. 1a–c show representative chromatograms obtained with EDTA (20 mM, pH 4.7) for Sb^{3+} and Sb^{5+} in spiked water, 1:10 diluted urine, and plasma samples, respectively. An adequate separation of Sb^{3+} and Sb^{5+} can be observed and also very similar chromatographic features in all three matrices used. Fig. 1d displays a chromatogram of the *N*-methyl meglumine antimoniate drug in which overlapping of Sb^{5+} (naturally present in the drug, see Fig. 2a and further discussions) with the organic drug species, eluted at the solvent front, can be seen. Fig. 1d includes one of the proposed structure formulae of *N*-methyl meglumine antimoniate [15], which may suggest that the organic Sb-containing drug species is predominantly in a cationic form, thus explaining its elution position, which corresponds to the void volume of the column. This volume (1.04 mL) was determined by repeated injections of a Cs^+-spike (*m/z*=133) and corresponds to an elution time of 41.6 ± 0.6 s (n=10). This poor resolution between the Sb drug peak and Sb^{5+} would prevent the quantification of the latter species, but not that of Sb^{3+}.

Lowering the eluent concentration of EDTA to 2 mM (pH 4.7) resulted in a small shift to higher elution times and some peak broadening at the Sb^{3+} position, however

this improved greatly the resolution at the solvent front (Fig. 2a). In addition to the well resolved Sb^{5+} peak, whose identity was confirmed by spike addition (Fig. 2b), the drug peak also showed better resolution. A further benefit of this peak shift at lower EDTA concentration was the better separation of Sb^{5+} from the solvent front at which Na^+, present in higher concentrations in urine and plasma samples, will be eluted similar as mentioned for Cs^+ (see above). The pejorative effect of the co-elution of Na^+ on the Sb^{5+} signal has been observed and discussed by Krachler and Emmons [11].

Typical figures of merit for the determination of Sb^{3+} and Sb^{5+} in different matrices by IC-ICP-MS using EDTA (2 mM, pH 4.7) as the mobile phase are summarized in Table 2. The data show a good repeatability of the method with respect to peak position (elution time) and peak area. Spike recoveries for Sb^{3+} and Sb^{5+} in plasma samples were in the range 90–100%, but lower in urine samples (60–70%) at the minor spike addition tested (0.25 ng). This lower recovery in urine could be the result of a matrix effect, which is expected to be more pronounced in urine than in blood plasma samples. In any case, the urine samples analyzed in this study had considerably higher Sb concentrations so that this effect would not invalidate the results. Limits of detection (LOD), estimated from the standard deviation (3σ) of the lowest spike of the analytical curve (0.125 ng) and its sensitivity (slope), were about 0.016 ng for Sb^{5+} and Sb^{3+}, respectively, or 0.16 µg L^{-1} expressed as solution concentration, using a 100 µL loop for sample injection. Method detection limits for urine or plasma samples were typically 10 times higher due to the sample dilution involved.

For data interpretation it was important to verify that Sb^{5+} and Sb^{3+} concentrations in urine and plasma samples were the consequence of metabolic transformations and not artifacts of alterations occurring between collection and analysis. This possibility was tested and discarded by stability measurements on urine and plasma samples spiked with Sb^{3+} and Sb^{5+}. Within a time period of 240 min, no alteration in the Sb^{5+}-to-Sb^{3+} ratio could be observed, thus confirming the data of Garbos et al. [16]. Also, urine

Table 2 Performance data on the ion chromatography procedure coupled to ICP-MS. Experimental conditions: PRP-X100 column; mobile phase: EDTA (2 mM, pH 4.7), flow rate: 1.5 mL min^{-1}, pressure: 1000 psi; injected sample volume: 100 µL. Definition of detection limits see text

| Figures of merit	Sb^{5+}		Sb^{3+}	
Working range tested and variation coefficient (R^2)	0–100 µg L^{-1}		0–100 µg L^{-1}	
	0.9985		0.9989	
Repeatability of the elution time	0.8%		1.2%	
Repeatability of peak area for a 0.125 ng spike	2.3%		3.5%	
Detection limits (3σ), peak area measurements, Meinhard nebulizer and cyclonic spray chamber	0.16 µg L^{-1} or 0.016 ng (100 µL loop)		0.15 µg L^{-1} or 0.015 ng (100 µL loop)	
	Urine	Plasma	Urine	Plasma
Spike recovery (0.25, 0.5, and 2.0 ng, respectively) from 1:10 diluted urine and plasma samples (n=3)	72±12%	108±4%	64±4%	88±4%
	95± 6%	92±6%	111±6%	94±4%
	117± 2%	103±3%	113±2%	96±3%
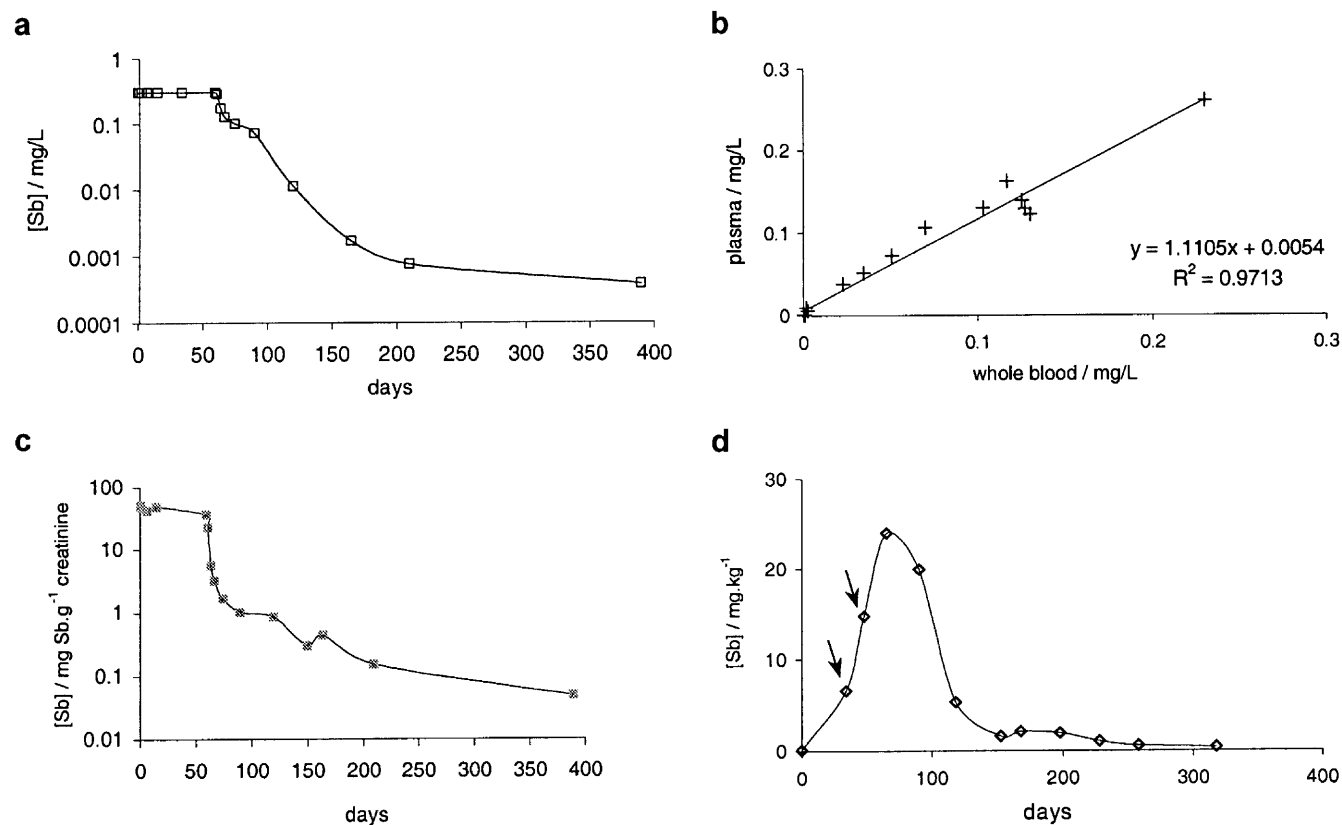
Fig. 3 Total antimony concentrations in clinical samples from a male patient (cutaneous and mucosal lesions, 64 years old, 70 kg body weight, treatment: 350 mg Sb per day over 60 days): (**a**) whole blood during and after administration of *N*-methyl meglumine antimoniate and (**c**) urine; (**b**) correlation graph: Sb-plasma versus Sb-whole-blood; (**d**) scalp hair; arrows indicate samples taken during the drug administration phase on day 30 and 60

and plasma samples spiked with the drug showed no alterations over this time interval, which was sufficient for the collection of samples, transportation from the hospital to our laboratory at PUC-Rio, and speciation analysis.

Total antimony concentrations in clinical samples

To date, 10 patients have been monitored for their total Sb concentrations in whole blood, plasma, urine, and scalp hair during the drug administration phase (typically 30 or 60 days) in which all patients received a daily intramuscular dose of 5 mg antimony per kg of body weight as *N*-methyl meglumine antimoniate, and during the elimination phase after discontinuing drug administration. Typical results are shown in Fig. 3a,c. Additional data on the correlation between Sb concentrations in whole blood and plasma are given in Fig. 3b. As the focus of this paper is not on the clinical and pharmacological aspects of antimony therapy, the data will be discussed only shortly to

indicate the range of total Sb concentrations in body fluids and hair from such patients. In addition, it will be shown later that speciation results are very dependent on the time period in which samples are collected (Fig. 3a,c).

Fig. 3a shows that daily incorporation of the drug, under the aforementioned conditions, leads to a stationary antimony concentration in whole blood, plasma (see good correlation between both specimens in Fig. 3b), and urine, with mean concentrations of 220 ± 7 μg L^{-1} for whole blood and 38 ± 8 mg of Sb g^{-1} creatine for urine, calculated from the data of 10 patients monitored so far. The mean concentrations of Sb during the stationary phase are therefore higher by a factor of about 320 for blood and about 19,000 for urine compared to the mean concentrations before administration of the drug (plasma: 0.69 μg L^{-1}; urine: 0.002 mg of Sb g^{-1} creatine). The slope of the curve in Fig. 3b, which is close to unity, also shows that only a very small fraction of Sb can be bound to erythrocytes. Plasma samples, which are easier to handle in chromatographic separations, were therefore used for the further studies. Subsequent to drug administration, rapid blood-clearing occurred in the first 3 days, in which over 50% of antimony was excreted by urine. This rapid excretion phase, which had a half live of about 24–72 h, was followed by a much slower one with a half life >50 days (Fig. 3a and 3c), which is different from previously reported results (e.g., mean half life of 76 h [17]). In the case presented, 150 days after the last administration of the drug (day 60), an increased Sb concentration could still be measured in whole blood and urine samples (Fig. 3a and 3c).

Fig. 3d shows that hair is not only an excellent bio-indicator for antimony body burden but also that concentrations in this tissue reflect well the administration history. A peak concentration of 24 mg kg^{-1} was measured 30 days after the last drug administration (day 60), which was 375 times higher than before treatment, or about 800 times higher when compared to the reference value for this element in human scalp hair (<0.03 mg kg^{-1} [6]). Normal levels were not reached within a period of 150 days after the end of drug administration, similar to what was observed for blood and urine samples. High antimony concentrations in the hair of leishmanial patients treated with Glucantime were also reported by Dórea et al. [18].

Speciation of antimony in drug, urine,
and blood plasma samples

Speciation studies on antimony in biological samples from our patients are just beginning. So far, the hypothesis of the metabolic alteration of *N*-methyl meglumine antimoniate and the bio-reduction of Sb^{5+} to Sb^{3+} have been the focus of our work. To achieve this, the drug had to be first be characterized with respect to its expected metabolic alteration products.

The total concentration of Sb in the original drug was 72.6 ± 1.5 g L^{-1} (n=3, different ampoules from the lot no. 9115 only were used in this work) and the percentage fraction of Sb^{5+} compared to organic antimony, determined by IC-ICP-MS, was $6.4 \pm 0.5\%$. The presence of Sb^{3+} could not be detected by IC-ICP-MS (see Fig. 2a), but HG-ICP-MS indicated a small fraction of Sb^{3+} (<1% of the total Sb). It cannot be excluded however, that this minute amount of Sb^{3+} may be an artifact due to the reduction of Sb^{5+} (as the free ion or bound to the drug) by $NaBH_4$.

Fig. 4a shows a typical ion chromatogram of an urine sample from one patient collected at day 28 of his treatment period (total of 30 days). The drug peak at the solvent front is still the predominant one, however, an increase of the Sb^{5+} peak can be observed. The ratio of Sb^{5+} to drug-Sb increased from 0.064 (6.4% of Sb^{5+}) in the pure drug to about 0.22 (22% of Sb^{5+}) in this urine sample. Also a small Sb^{3+} peak can already be seen in this chromatogram, which was not detected in the pure drug sample (Fig. 2a,b). Both observations indicate that *N*-methyl meglumine antimoniate is metabolized in the human body and in a small fraction during the administration phase. This finding may explain why low doses of the drug (5 mg per kg of body weight) result in similar therapeutic efficiency as high doses (20 mg per kg of body weight, or more [19]): the excess of the drug is excreted in its nearly unaltered form. In a blood plasma sample from the same patient collected at the same time, the presence of Sb^{3+} could not be detected by IC-ICP-MS. However, the Sb^{5+} peak is clearly seen and this accounts for a similar increased fraction of Sb^{5+} to drug-Sb (16% of Sb^{5+}) as was observed in the urine sample.

Fig. 4c and 4d show ion chromatograms of a urine and plasma sample from the same patient, collected 11 days after the last *N*-methyl meglumine antimoniate application, i.e., already during the elimination phase of the drug (see Fig. 3a,c). The increased proportions of Sb^{5+} and Sb^{3+} compared to the pure drug (Fig. 2a) and also to the urine sample collected during the treatment/absorption phase (Fig. 4a), can clearly be observed in this urine sample. The ratio of Sb^{5+} to drug-Sb increased from about 0.064 in the pure drug to 7.9 in this urine sample. In addition, a remarkable Sb^{3+} peak can be observed which accounts for an Sb^{3+} concentration of about 111 μg L^{-1} in the original urine sample.

Both observations constitute the first experimental evidence of the in vivo conversion of *N*-methyl meglumine antimoniate into the ionic species Sb^{5+} and Sb^{3+}. The bioreduction of Sb^{5+}, leading to considerable concentrations of Sb^{3+} in body fluids, could especially play a very important role in the therapeutic action and in the long term side effects of drug administration, due to the ca. 10-fold higher toxicity of the Sb^{3+} species.

In addition to the Sb^{5+} and Sb^{3+} peaks in Fig. 4c, a new peak, intermediate between both, can be seen which has not yet been identified. In the corresponding plasma sample (Fig. 4d), the concentrations of total Sb and its species were already close to the method detection limit of ICP-MS and only a very weak signal of Sb^{5+} can be suspected in this chromatogram. Comparing the results of plasma and urine samples, two conclusions can be drawn and which
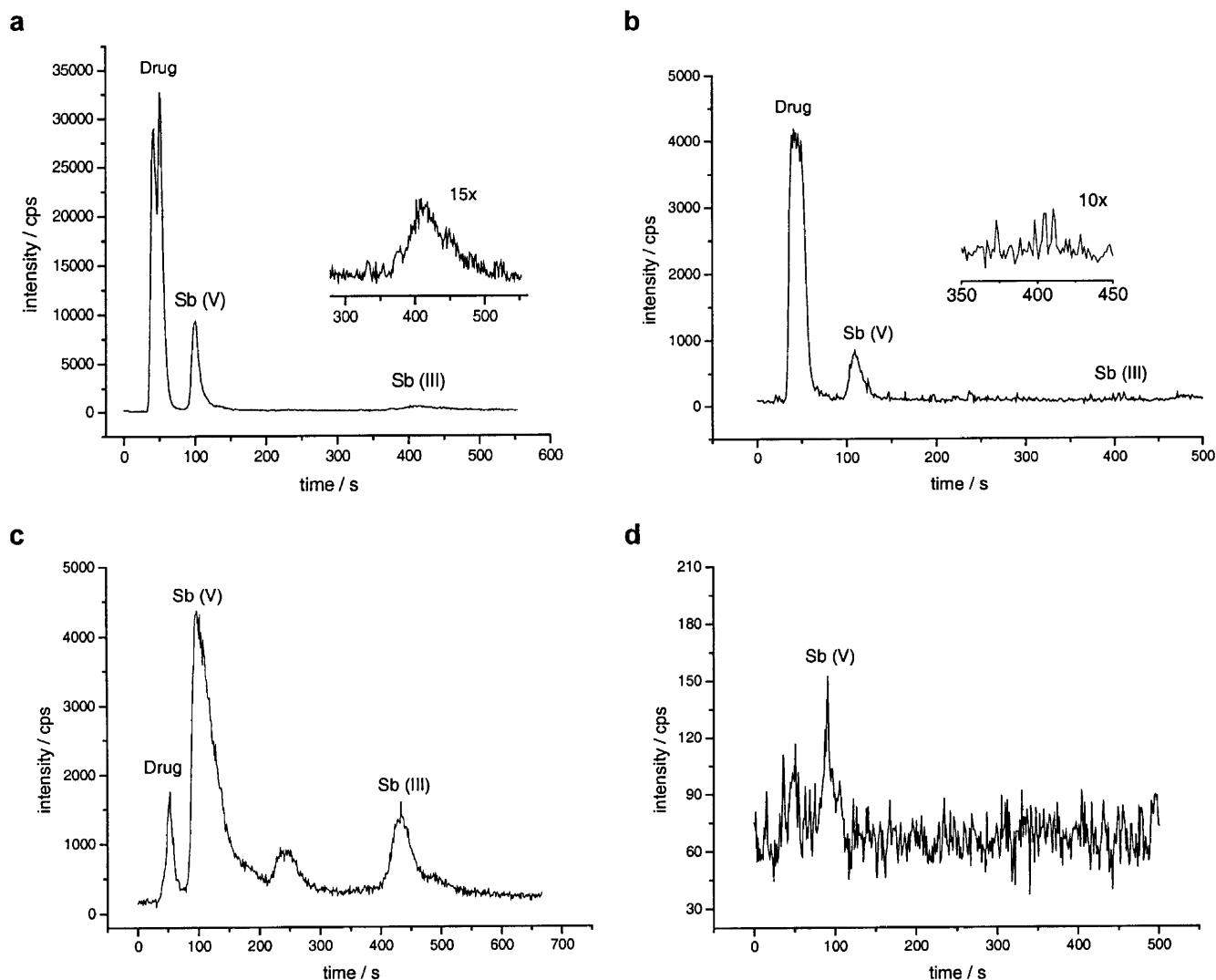
Fig. 4 Ion chromatograms of antimony species in urine (**a**, **c**) and plasma samples (**b**, **d**) from a male patient (mucosal lesions, 35 years old, 77 kg body weight, treatment: 385 mg Sb per day during 30 days). Figures (**a**) and (**c**) are for samples taken during the drug administration phase on day 28; Fig. (**c**) and (**d**) correspond to samples collected 11 days after discontinuing drug administration. Sample dilution factors: (**a**) urine: 200×; (**b–d**) urine, plasma: 10×

will be only briefly mentioned here: (1) Sb is rapidly eliminated from the central compartment (blood and plasma) after discontinuing drug administration, confirming earlier studies [4]. During this rapid phase, only a small conversion of the drug into other species seems to occur; (2) There are other peripheral body compartments for which the excretion kinetics of antimony is much slower and in which, apparently, active in vivo conversion of the organic antimonial drug occurs, including the formation of the more toxic Sb^{3+} species.

Other species than the already those mentioned, especially methylated forms of antimony [11, 20], have not be detected by the methodologies used in this work so far. The presence of the *N*-methyl meglumine antimoniate peak with very high intensity right at the solvent front would obscure a potential peak of trimethylstibonic species or other methylated forms that might appear close to it. The use of KOH (2 mM) as an alternative mobile phase to improve separation of these species, as proposed in [9], did not show a peak identifiable as a TMSb-species. Unfortunately, spikes of methylated Sb compounds were not available for this work, so that the analytical methodology could not be improved (until now). Other techniques, including CE-ICP-MS, will be used in the future to further address this issue.

Conclusions

Speciation studies of antimony in urine and plasma samples from patients treated for leishmaniasis gave the first experimental evidence of the in vivo conversion of *N*-methyl meglumine antimoniate. Ion chromatography on-line coupled to ICP-MS was used and the best results concerning peak separations (Sb-drug peak from the anionic Sb^{5+}) and detection limits were obtained with EDTA (2 mM, pH 4.7) as the mobile phase and using a Hamilton

PRP-X100 anion exchange column. Ion chromatograms of pure aqueous drug solutions showed that only a smaller fraction of the total antimony is present as anionic Sb^{5+} (<7%), the remaining is bound to the organic structure of the drug and eluted in the void volume of the column. The Sb^{5+} fraction increased significantly in urine and plasma samples from patients treated with the drug, indicating its metabolic conversion. Additionally, a peak of Sb^{3+} became apparent, especially in samples collected during the slow elimination phase of the drug, suggesting the bio-reduction of Sb^{5+} to its more toxic trivalent form. This finding supports the hypothesis that the formation of Sb^{3+} may be responsible for the observed long-term toxicity of the drug and, eventually, for its therapeutic action.

As for the total antimony concentrations in urine and blood samples from these patients, the data indicate that elimination of Sb from the human body seems to be slower than previously reported. This finding may be related to the in vivo conversion of the drug and the different biological behavior of its metabolites (Sb^{5+}, Sb^{3+}, and others not yet identified).

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