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Article

Kinetics of Antimony(V) Reduction by L-Cysteine. Pharmacological Implications and Application to the Determination of Antimony in Pentavalent Antimonial Drugs

Fernanda B. de Oliveira,[a] Dante A. Schettini,[a] Cláudio S. Ferreira,[b] Breno Rates,[a] Olguita G. F. Rocha,[c] Frédéric Frézard[a] and Cynthia Demicheli[*,b]*

[a]*Departamento de Fisiologia e Biofísica, ICB and* [b]*Departamento de Química, ICEx Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31270-901 Belo Horizonte - MG, Brazil*

[c]*Laboratório de Análise de Traces Metálicos, Fundação Centro Tecnológico de Minas Gerais, Belo Horizonte - MG, Brazil*

Como o antimônio é a espécies ativa no medicamento leishmanicida, antimoniato de meglumina (AM), o conhecimento da concentração exata do metal é crítico para o seu uso experimental e clínico. Por outro lado, o papel da cisteína no metabolismo dos antimoniais deve ser esclarecido. No presente trabalho, a reação de redução do Sb^{V} pela cisteína foi caracterizada cineticamente. As ordens de reação com relação ao Sb e à cisteína foram iguais a 1,0 e 3,3, respectivamente. A constante de velocidade da reação de redução foi dependente do pH. Essa reação foi usada para reduzir o Sb^{V} no AM, para subseqüente determinação fotométrica do Sb^{III} com o cromóforo vermelho de bromopirogalol. Eficiências de redução de 100% foram alcançadas em pH 3. Soluções apresentando concentrações de Sb baixas como 0,5 mmol L^{-1} foram dosadas com sucesso. O método foi também aplicado para a determinação de Sb em formulações lipossomais do AM.

Since antimony is the active species in the antileishmanial drug meglumine antimoniate (MA), the knowledge of the exact metal concentration is critical for its experimental and clinical use. On the other hand, the involvement of thiols, such as cysteine (Cys), in the metabolism of this drug remains to be clarified. In the present work, the reduction reaction of Sb^{V} by Cys was kinetically characterized. The reaction orders with respect to Sb and Cys were equal to 1.0 and 3.3, respectively. The rate constant for the reduction reaction was pH-dependent. This reaction was exploited to effectively reduce Sb^{V} in MA, for the subsequent photometric determination of Sb^{III} using the chromogen bromopyrogallol red. Reduction efficiencies of 100% were achieved at pH 3. Solutions with Sb concentration as low as 0.5 mmol L^{-1} were successfully assayed. The method was also applied to the determination of Sb in liposomal formulations of MA.

Keywords: cysteine, antimony, reduction, liposomes, meglumine antimoniate

Introduction

Pentavalent antimonials, including meglumine antimoniate, are the drugs of choice for the treatment of leishmaniases.[1] Since antimony is the active species, the knowledge of the exact metal concentration in commercial formulations is critical for its clinical use. Indeed, its concentration was found to vary from one lot to another in commercial formulations.[2] Moreover, the determination of Sb^{V} concentration in the liposomal formulations of antimonial drugs is essential for their pharmacological evaluations and therapeutic use.[3]

The most common analytical methods used to determine Sb are atomic absorption spectroscopy, plasma atomic emission spectrometry, ICP mass spectrometry, neutron activation analysis and voltammetry.[2] However, these methods usually require the pre-digestion of the organic material present in the sample, are time-consuming and are neither cost-effective nor appropriate for routine analysis.

A photometric assay has been also proposed for Sb^{V}.[2,4] As a first step, Sb^{V} was reduced to Sb^{III} by iodide in acidic medium. As a second step, iodine was inactivated by

*e-mail: demichel@netuno.lcc.ufmg.br

ascorbic acid and the resulting solution was neutralized with sodium hydroxide. Finally, Sb^{III} concentration was determined photometrically, exploiting the specific interaction of Sb^{III} with the chromogen bromopyrogallol red (BPR) and the fact that the absorbance of BPR at 560 nm decreases proportionally to the amount of Sb^{III} in the analyte solution, as a consequence of the formation of the 1:1 BPR-Sb^{III} complex.[4] However, the quantification of Sb^{V} using this procedure was complicated by the fact that its reduction by iodide was only 72% efficient.

In previous studies, L-cysteine (Cys) was proposed as a pre-reducing agent of Sb^{V} for its determination by flow-injection hydride generation atomic-absorption spectrometry and atomic-emission spectrometry.[5]

Since Cys is the predominant thiol in the lysosomes of mammalian cells,[6] in which *Leishmania* parasites reside,[7] and Sb^{III} is much more active than Sb^{V} against the parasite,[8] the pharmacological relevance of the reduction reaction of Sb^{V} by Cys has been investigated.[9] It was suggested that this reaction may contribute to the conversion of Sb^{V} to Sb^{III}, previously demonstrated in cell extracts, the vertebrate host and the parasite.[10] The reduction of Sb^{V} by Cys was found to occur at 37°C at both pH 5 and pH 7, but was favored at acidic pH.[9] Other physiologically-relevant thiols were also evaluated:[9] glutathione, which is the main thiol in the cytosol of mammalian cells;[11] and the glutathione-spermine conjugate, trypanothione ($T(SH)_2$), which is the predominant thiol within the parasite.[12] Strikingly, the initial rates of reduction of Sb^{V} were much greater in the presence of Cys and $T(SH)_2$ than in the presence of glutathione.[9] These data supported the hypothesis that Sb^{V} is reduced *in vivo* by $T(SH)_2$ within *Leishmania* parasites and by Cys within the acidic compartments of mammalian cells where the parasites reside. On the other hand, recent studies have also suggested the participation of enzymes in the process of reduction of Sb^{V} to Sb^{III} [13] and the possible role of Sb^{V}-ribonucleoside complexes in the mode of action of pentavalent antimonials.[14]

Further progress towards the elucidation of the pharmacological importance of Cys, as well as the use of this thiol in analytical procedures, depends on the full kinetic characterization of this reaction.

In the present work, the reaction of Sb^{V} with Cys was kinetically characterized. As a consequence, a new insight into the pharmacological role of Cys was achieved and the photometric assay for Sb^{V} was improved. Cys was also chosen for this assay because of its low cost and the relatively high rate of reduction promoted by this thiol. The resulting method was successfully applied to the determination of Sb in meglumine antimoniate and in liposomal formulations of the antimonial compound.

Experimental

Photometric determination of Sb^{III} and influence of Cys concentration

The procedure used to determine Sb^{III} was described in detail previously.[9] It is based on the specific interaction of Sb^{III} with BPR. The absorbance of BPR at 560 nm decreases proportionally to the amount of Sb^{III} in the analyte solution, as a consequence of the formation of the 1:1 BPR-Sb^{III} complex. Briefly, 0.5 mL of analyte solution was prepared from 0.1 mL of 0.1 mol L^{-1} phosphate, 0.01 mL of 5% (m/v) tartrate, 0.05 mL of 350 μmol L^{-1} BPR solution in 1:1 water/ethanol (v/v) and 0.34 mL of water. The pH was then adjusted to 6.8 using sodium hydroxide. The absorbance was registered, at 560 nm before (A_o) and after (A_m) adding 5 μL of the sample to be analyzed, so as to obtain a Sb^{III} concentration in the range of 1.2×10^{-6} to 25×10^{-6} mol L^{-1}. For each experiment, a calibration curve was established, in the presence of the same amount of Cys as that used in the reduction step. Potassium antimony(III) tartrate was used as the source of Sb^{III} and the change in BPR absorbance (A_o-A_m) was plotted as a function of Sb^{III} concentration.

Materials

N-methyl-D-glucamine (NMG, 99%), $SbCl_5$ (99%), bromopyrogallol red (BPR, 70%) and potassium antimony(III) tartrate (>99%) were obtained from Aldrich Chemical Co. (Milwaukee, Wis) and potassium hexahydroxo-antimoniate ($KSb(OH)_6$, >99%) from Fluka Chemie GmbH (Switzerland). Cholesterol (CHOL), dicetylphosphate (DCP), L-cysteine (Cys, 98%) and Triton X-100 (>99%) were purchased from Sigma Co. (St. Louis, MO, USA). Distearoylphosphatidylcholine (DSPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). An antimony standard ($SbCl_3$ in 24% HCl, Titrisol®) was purchased from MERCK (São Paulo, SP, Brazil). Double-distilled-deionized water was used throughout the experiments.

Preparation of meglumine antimoniate and of liposomal formulations

Meglumine antimoniate was synthesized, according to Demicheli *et al.*,[15] from an equimolar mixture in water of NMG and freshly precipitated and hydrated antimony pentoxide obtained from $SbCl_5$ previously hydrolyzed in

water. After precipitation with acetone, the resulting product was dried. Meglumine antimoniate product contained 29% of Sb by weight, as determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Perkin Elmer plasma emission spectrometer, model Optima 3000.

Liposomal formulations of meglumine antimoniate, DRVs and FDELs, were prepared from DSPC, CHOL and DCP (molar ratio of 5:4:1) in the absence or presence of sucrose, as described previously.[3] The concentration of Sb in the liposomal samples was determined by ICP-OES, after submission of the samples to digestion with concentrated nitric acid. Sb concentrations in the range of 8 to 16 g L^{-1} and Sb/lipid mass ratio in the range of 0.2 to 0.35 were found.

Determination of the linear range of the photometric assay and of the interference of Cys

The linear range and sensitivity of the method were evaluated through the linear regression of the (A_o-A_m) *vs.* [Sb^{III}] data, in the concentration range of 1.2×10^{-6} to 25×10^{-6} mol L^{-1}, and the determination of the linear correlation coefficient and the slope of the curve. To determine the interference of Cys on these parameters, the thiol was introduced at varying concentrations (0, 0.8, 2 or 4 mmol L^{-1}) in the analyte BPR solution.

Determination of the partial orders of the reduction reaction

Two different experiments were performed. In the first one, Sb concentration was kept constant at 1 mmol L^{-1} and Cys concentration was varied form 5 to 12.5 mmol L^{-1}. In the second one, Cys concentration was kept constant at 10 mmol L^{-1} and Sb concentration was varied from 0.5 to 2 mmol L^{-1}. All aqueous solutions contained 0.1 mol L^{-1} KCl, 0.02 mol L^{-1} phosphate and 0.02 mol L^{-1} acetate. The pH was adjusted to 5. Reaction was run at 37°C and under argon to prevent the oxidation of Cys by oxygen. The initial rate of Sb^{V} reduction (V_i) was calculated from the concentration of Sb^{III} determined photometrically, as described above, in the initial conditions of reaction. The reaction was considered in initial conditions when the amount of reduced Sb did not exceed 10% of the total Sb^{III} amount at equilibrium. The reaction partial orders, n and m, with respect to Cys and Sb respectively, were determined from the following equations:

$$V_i = k_{ap}[Cys]^n[Sb]^m \quad (1)$$

$$\ln(V_i) = n \ln([Cys]) + m \ln([Sb]) + \ln(k_{ap}) \quad (2)$$

where [Cys] = initial molar concentration of Cys, [Sb] = initial molar concentration of Sb and k_{ap} = apparent rate constant for reduction.

Determination of the activation energy of the reduction reaction

The reaction medium contained 5 mmol L^{-1} Cys and 1 mmol L^{-1} Sb, in the presence of 0.1 mol L^{-1} KCl, 0.02 mol L^{-1} phosphate and 0.02 mol L^{-1} acetate. The pH was adjusted to 5 and solutions were kept under argon. The reaction was run at 15°C, 25°C, 37°C or 47°C. The apparent rate constants for reduction (k_{ap}) were calculated using equation (1) and the concentration of Sb^{III}, determined photometrically, as described above, in the initial conditions of reaction. The activation enthalpy (ΔH^*) was obtained from the slope of the plot of arrhenius equation:

$$\ln(k_{ap}) = \ln[\omega \exp(\Delta S^*/R)] - \Delta H^*/RT \quad (3)$$

Influence of pH on the rate of Sb^{V} reduction

The reaction medium contained 5 mmol L^{-1} Cys, 1 mmol L^{-1} Sb, 0.1 mol L^{-1} KCl, 0.02 mol L^{-1} phosphate and 0.02 mol L^{-1} acetate. Different samples were prepared with a pH value varying from 3 to 6 (with intervals of 0.25 pH units) and the reaction was run under argon. The apparent rate constant for reduction (k_{ap}) was calculated using equation (1) and the concentration of Sb^{III} determined photometrically, as described above, in the initial conditions of reaction.

Efficiency of Sb^{V} reduction in meglumine antimoniate: influence of pH, time of reaction, Sb^{V} concentration and temperature

To evaluate the influence of pH, temperature, time of reaction and Sb^{V} concentration on the efficiency of Sb^{V} reduction in meglumine antimoniate, aqueous solutions containing meglumine antimoniate and 0.05 mol L^{-1} Cys were freshly prepared and reaction was run under argon.

In the study of the influence of pH and time of reaction, meglumine antimoniate was added at 0.5 mmol L^{-1} of Sb, pH was adjusted at 5, 6 or 7 and the reaction was run at 50°C for 1 or 3 h.

In the study of the influence of Sb^{V} concentration and temperature, meglumine antimoniate was added at a Sb concentration varying from 0.05 to 0.5 mmol L^{-1}, pH was adjusted to 3 and the reaction was run for 1 h at 25°C or 50°C.

Each sample was prepared in quadruplicates. The concentration of Sb^{III} was then determined using the BPR photometric assay, as described above.

Procedure for determination of Sb^{V} in samples of liposomal meglumine antimoniate

Samples of liposomal meglumine antimoniate, prepared as described above at a lipid concentration typically in the range of 30 to 65 g L^{-1}, were diluted five-fold in water and then mixed with an equal volume of 20% (m/v) Triton X-100 aqueous solution. The mixture was then incubated for 1 h at 60°C to induce the release of the encapsulated antimonial compound. 50 μL of the resulting suspension was then mixed with 250 μL of a freshly-prepared Cys solution at pH 3 and 200 μL of water. The resulting solution was then immediately incubated for 1 h at 60°C under argon to promote the reduction of Sb^{V} into Sb^{III}. The concentration of Sb^{III} was then determined photometrically, as described above.

To determine the possible interference of the liposome matrix (selectivity) and the precision of the method, samples with varying concentrations of Sb (from Titrisol® standard) (0.04, 0.08, 0.12, 0.16 and 0.2 mol L^{-1}) were prepared in water, in the absence or presence of empty liposomes made from DSPC, CHOL and DCP (molar ratio of 5:4:1) at a final lipid concentration of 65 g L^{-1}. For each Sb concentration, seven identical samples were prepared independently. These samples were treated as described above (treatment with Triton X-100 and Cys). After measurement of the sample-induced change in BPR absorbance, (A_o-A_m) was plotted as a function of the final Sb concentration in the cuvet. The Snedecor F Test was then applied to compare the variances, followed by the student t Test to compare the means. The mean (M) and standard deviation (D) of the measurements obtained for each Sb concentration were used to calculate the relative standard deviation (*%RSD*) for a confidence level of 95%, as follows:$\%RSD = (D/M)\times 100$.

To evaluate the accuracy of the method, samples with Sb concentrations (from Titrisol® standard) of 0.02 (sample 1), 0.08 (sample 2) and 0.16 mol L^{-1} (sample 3) were prepared in the presence of empty liposomes made from DSPC, CHOL and DCP (molar ratio of 5:4:1) at a final lipid concentration of 65 g L^{-1}. To samples 1, 2 and 3, known amounts of Sb were added at the final concentrations of 0.02, 0.04 and 0.04 mol L^{-1}, respectively. These samples were then treated as described above (treatment with Triton X-100 and Cys) and Sb^{III} concentration was determined photometrically using the BPR assay. The recovery was then calculated from$\%R = (C_1\text{-}C_2/C_3)$, where C_1 = Sb concentration determined in the sample after addition, C_2 = Sb concentration determined in the sample before addition; C_3 = concentration of added Sb. In addition to the study of Sb recovery, the values of Sb^{V} concentration determined for four different liposomal preparations of meglumine antimoniate were compared to those obtained by the classical ICP-OES method.

Results and Discussion

Kinetics of Sb^{V} reduction by Cys

Previous studies have established that Cys can promote the reduction of Sb^{V} into Sb^{III} in potassium antimoniate and meglumine antimoniate,[5,9] however, this reaction was not fully characterized kinetically.

As a first step, experiments were performed to determine the partial orders of the reaction with respect to Cys and antimoniate (Sb). Figures 1a and 1b show the plots of $\ln(V_i)$ as a function of ln([Cys]) and ln([Sb]), respectively. Linear relationships were obtained and the slopes gave the partial orders of reaction: m = 3.26 ± 0.16 and n = 1.05 ± 0.07. Therefore, the following relation could be established: $V_i = k_{ap}[\text{Cys}]^{3.3}[\text{Sb}]$

Using these data, the apparent rate constant for Sb^{V} reduction (k_{ap}) could also be estimated at pH 5 and 37 °C: $k_{ap} = 256 \pm 37$ (mol $L^{-1})^{-3.3}$ s^{-1}

It is noteworthy that it is the first time that this reaction is fully characterized kinetically. The demonstration of the strong dependence of the rate of reaction upon the concentration of Cys is an important contribution of the present study. The high order of the reaction in Cys, together with the fact that it is fractional, indicates that we are not dealing with a simple single-step mechanism. Although two Cys molecules would theoretically be sufficient to promote the reduction of Sb^{V} into Sb^{III}, the high order of reaction in Cys suggests that at least one additional Cys molecule is involved in the composition of the rate-limiting transition state.

Determination of k_{ap} at different temperatures and the plot of arrhenius equation (equation 3), as shown in Figure 2, allowed for the calculation of the activation enthalpy for Sb^{V} reduction: $\Delta H^{*} = 42.4 \pm 2.8$ kJ mol^{-1}.

As the rate of Sb^{V} reduction was found previously to depend on pH,[9] k_{ap} was determined at different pH values in the range of 3 to 6. Figure 3 displays the plot of k_{ap} as a function of pH. k_{ap} was found to be maximum at a pH value of about 4.7 and k_{ap} was significantly lower at both lower and higher values of pH.

When analyzing the pH-rate profile according to Loudon,[16] one can identify two upward bends at pH values

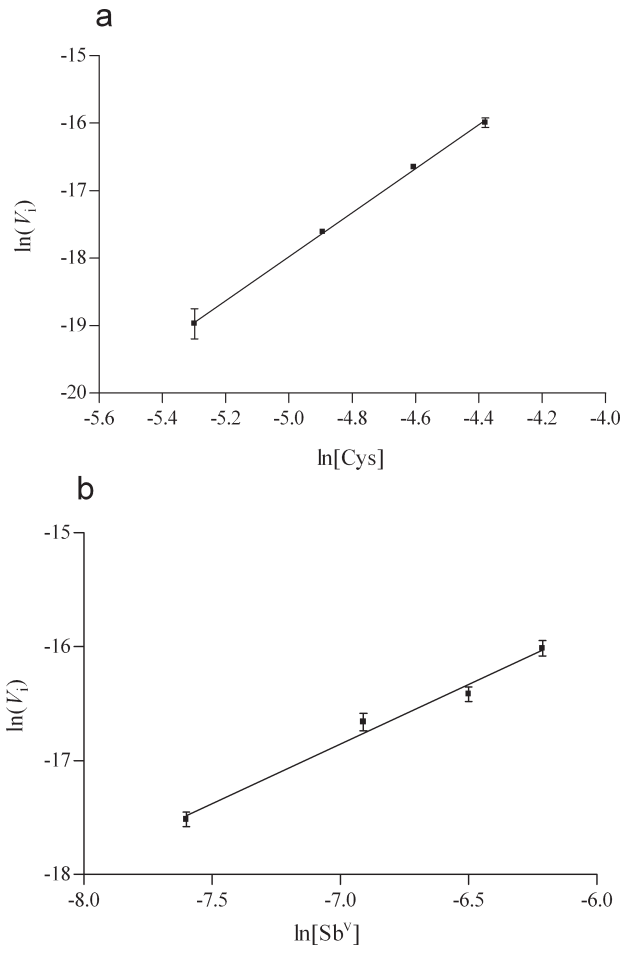

Figure 1. Plots of the logarithm of the initial rate of reduction of Sb^V by Cys (V_i/mol L^{-1} s^{-1}) determined at pH 5 and 37 °C as a function of the logarithm of Cys molar concentration (a) ([Sb] = 1 mmol L^{-1}) and Sb molar concentration (b) ([Cys] = 10 mmol L^{-1}). Sb^V was presented as potassium antimoniate. Data are shown as means ± SD (n = 3).

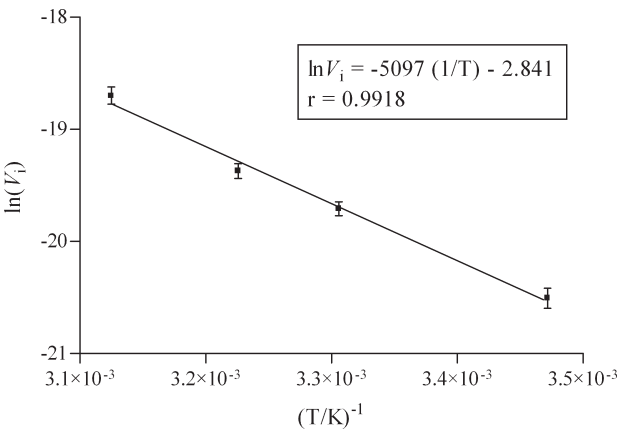

Figure 2. Arrhenius plot obtained with the initial rate of Sb^V reduction (V_i/mol L^{-1} s^{-1}). [Sb] = 1 mmol L^{-1}, [Cys] = 5 mmol L^{-1}, pH 5. Data are shown as means ± SD (n = 3).

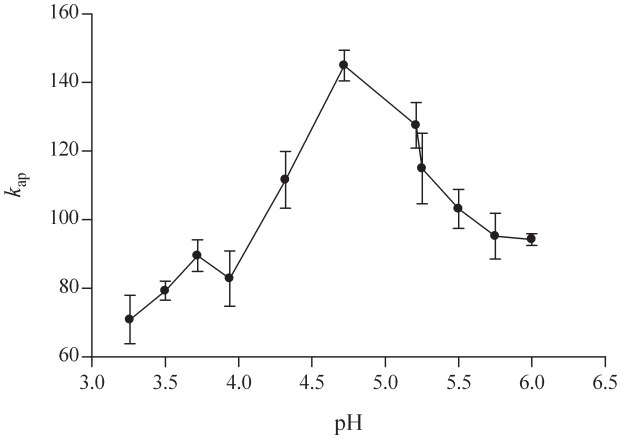

Figure 3. Influence of pH on the apparent rate constant (k_{ap}/(mol L^{-1})$^{-3.3}$ s^{-1}) for the reduction of Sb^V by Cys at 37 °C. Sb^V was presented as potassium antimoniate. Data are shown as means ± SD (n = 3).

of about 4.0 and 5.5, that can be attributed to changes in the mechanism of the reaction. Two downward bends were also observed at pH values of about 4.6 and 5.1, that may be explained, either by a titration of the reagents and/or by a change in rate-limiting step.[16] Titration of the reagents Cys and Sb^V is unlikely, especially if we consider their reported pK_a values. The carboxylic acid group of Cys has a pK_a in range of 1.7 to 2.1. Moreover, $Sb(OH)_5$ and $Sb(OH)_6^-$ are considered to be the predominant acid and base Sb^V species in aqueous solution,[17] which are in equilibrium according to the following reaction (pK_a{$[Sb(OH)_6^-]/[Sb(OH)_5]$}≈2.7):

$$Sb(OH)_5 + H_2O \leftrightarrow Sb(OH)_6^- + H^+ \quad (4)$$

Since Cys is considered as the predominant thiol in the lysosomes of mammalian cells[6] and *Leishmania* parasites reside inside the phagolysosomes of macrophages,[7] Cys may be involved in the *in vivo* reduction of Sb^V into more toxic Sb^{III} [10] and, ultimately, in the mechanism of action of pentavalent antimonial drugs againt leishmaniasis.

The pH value of the lysosomes, in the range of 4.5 to 5, is expected to favour the reduction reaction. However, whether or not such a reaction effectively takes place, depends on the local concentrations of Sb and Cys and on the state of complexation of Sb. As reported previously, the complexation of Sb^V with N-methyl-D-glucamine slows down the reduction reaction by a 7-fold factor.[9] On the other hand, a recent study aimed to the speciation of Sb in the serum and urine of humans submitted to chemotherapy with meglumine antimoniate indicated a partial dissociation of the drug into antimoniate.[18] The concentration of Sb in the lysosomes of infected cells during antimonial chemotherapy is not known precisely. However, considering that the Sb concentration achieved in the serum of humans during treatment is about 0.15 mmol L^{-1} and that mammalian cells exposed to meglumine

antimoniate exhibit comparable intra- and extra-cellular concentrations at equilibrium,[19,20] one can assume a Sb concentration of 0.15 mmol L^{-1} inside the lysosomes. Since the effective concentration of Sb^{III} against *Leishmania* parasites is about 0.04 mmol L^{-1} and macrophages retain Sb for at least three days after a short exposure to the antimonial drug,[8,21] one can infer that a minimum rate of reduction of 0.013 mmol L^{-1} day^{-1} would be necessary to achieve efficacy against the intracellular parasite. Using equation (1) and k_{ap} = 256 (mol $L^{-1})^{-3.3}$ s^{-1} (37 °C, pH 5) and assuming a Sb^{V} concentration of 0.15 mmol L^{-1}, one can estimate that the intralysosomal Cys concentration should be at least 3 mmol L^{-1} to reach such a rate of reduction.

Values for the concentration of Cys inside lysosomes have not been published so far, however, considering the millimolar range of glutathione concentration in the cytosol (2 to 10 mmol L^{-1}),[11] one would expect a similar range of Cys concentration.

Application of the reduction reaction of Sb^{V} by Cys to the determination of Sb in meglumine antimoniate

In the present study, a novel procedure has been evaluated for the determination of Sb^{V} in meglumine antimoniate. Two steps are proposed. As a first step, Sb^{V} is reduced to Sb^{III} by Cys. As a second step, Sb^{III} concentration is determined exploiting the specific interaction of Sb^{III} with BPR.[4]

Since Cys and Sb^{III} are expected to form a $Sb(Cys)_3$ complex,[22] competition should take place between Cys and BPR for the binding of Sb^{III}. This led us to evaluate the influence of different concentrations of Cys on the linearity and sensitivity of the photometric assay. Figure 4 shows the plot of the change in BPR absorbance as a function of Sb^{III} concentration, in the presence of varying concentrations of Cys. In the absence of Cys, the method was linear in the range of 1.2×10^{-6} to 23.5×10^{-6} mol L^{-1} with a correlation coefficient of 0.9989. When the Cys/BPR molar ratio was increased from 0 to 64, the slope of the curve suffered a slight diminution but its linearity remained almost unaffected (correlation coefficient > 0.9984). On the other hand, at the Cys/BPR molar ratio of 128, the slope decreased sharply and the linearity was maintained only in the range of 7.25×10^{-6} to 23.5×10^{-6} mol L^{-1}. According to these data, Cys/BPR molar ratio less or equal than 64 should be used in the photometric assay and the calibration curve should be established in the presence of the same amount of Cys as that used in the reduction step.

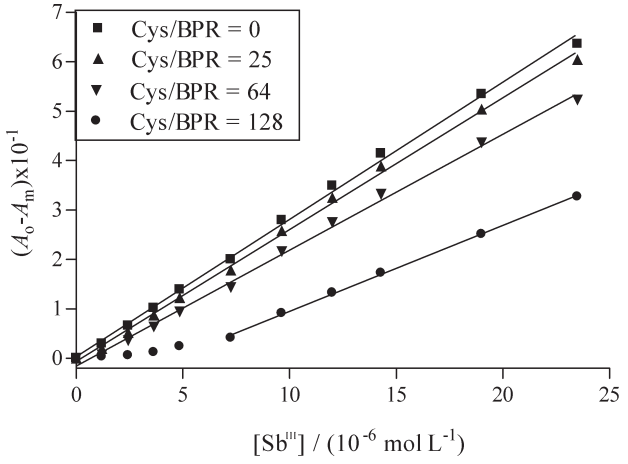

Figure 4. Influence of the Cys/BPR ratio on the calibration curve, representing the change in BPR absorbance at 560 nm (A_0-A_m) as a function of Sb^{III} concentration. Linear curve fits were performed using all the data in each condition, except for the Cys/BPR ratio of 128 that used [Sb] from 7.25×10^{-6} mol L^{-1}.

One of the objectives of the present study was to identify some experimental conditions leading to 100% of Sb^{V} reduction in meglumine antimoniate. A previous study has established that the initial rate of Sb^{V} reduction was about 7-fold lower with meglumine antimoniate than with potassium antimoniate,[9] most probably because Sb atom is less accessible in its complexed form.[15]

Figure 5 shows the efficiency of Sb^{V} reduction achieved, when meglumine antimoniate at 0.5 mmol L^{-1} was incubated with 50 mmol L^{-1} Cys at 50 °C. Strikingly, the reduction of Sb^{V} reached an efficiency of 100% only at pH 3. At pH 5 and 7, even after 3 h of incubation, the reduction of Sb^{V} was not complete. These results indicate a strong pH-dependence of the reaction and suggest a higher reactivity of $Sb(OH)_5$, the acid form of Sb^{V}.

The range of Sb concentration in the sample which leads to 100% of Sb^{V} reduction is also an important data to be determined. Figure 6 shows the efficiency of Sb^{V} reduction achieved, when meglumine antimoniate was incubated with 50 mmol L^{-1} of Cys at Sb concentration varying from 0.05 to 0.5 mmol L^{-1} (pH 3, 25 °C or 50 °C). It can be observed that the efficiency of Sb^{V} reduction was less than 100% at Sb concentration below 0.25 mmol L^{-1}. This concentration limit was identical at 25 °C and 50 °C. From this data, specific experimental conditions allowing for the determination of Sb^{V} in meglumine antimoniate could be defined. Those are summarised in Table 1.

It is noteworthy that these conditions may also apply to the determination of Sb in other Sb^{V} complexes, even though the method would still require validation.

When compared to the method proposed by Rath *et al.*,[2] our method presents the significant advantages of

Table 1. Recommended experimental conditions for the determination of Sb^V in meglumine antimoniate, either free or encapsulated in liposomes

	Meglumine antimoniate	Liposomal Meglumine antimoniate
Step 1. Lysis of liposomes	—	[Triton X-100] = 10% (m/v) [Phospholipid] < 15 g L^{-1} 1 h, 60 °C
Step 2. Reduction of Sb^V into Sb^{III} by Cys	[Cys] = 50 mmol L^{-1} 0.25 ≤ [Sb] ≤ 5 mmol L^{-1} pH 3, 1 h, 25-60°C	[Cys] = 50 mmol L^{-1} 0.4 ≤ [Sb] ≤ 5 mmol L^{-1} pH 3, 1 h, 60 °C
Step 3. Photometric determination of Sb^{III}	[Cys] ≤ 2 mmol L^{-1} or [Cys]/[BPR] ≤ 64 2.5 µmol L^{-1} ≤ [Sb] ≤ 20 µmol L^{-1}	

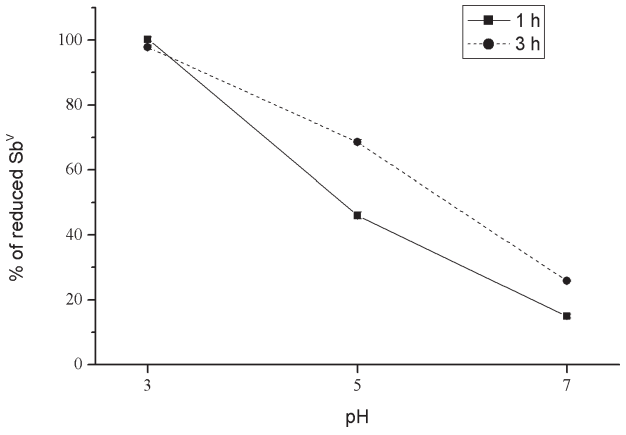

Figure 5. Influence of pH and reaction time on the reduction efficiency of Sb^V in the form of meglumine antimoniate by Cys. [Cys] = 50 mmol L^{-1}, [Sb] = 0.5 mmol L^{-1}, pH = 5. Data are shown as means ± SD (n = 4).

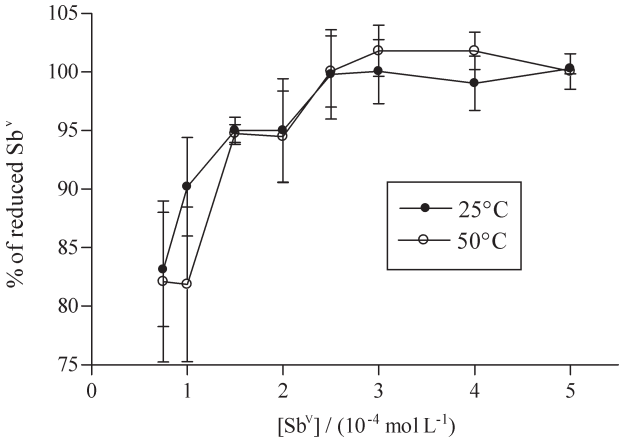

Figure 6. Influence of Sb^V concentration and temperature on the reduction efficiency of Sb^V in the form of meglumine antimoniate by Cys. [Cys] = 50 mmol L^{-1}, time of reaction = 1 h. Data are shown as means ± SD (n = 4).

being simpler (two-step *vs*. three-step method) and offering a 100% reduction efficiency of Sb^V.

Application of the reduction reaction of Sb^V by Cys to the determination of Sb in liposomal formulations of meglumine antimoniate

We described previously the preparation of liposomal formulations of meglumine antimoniate, DRVs and FDELs, from DSPC, CHOL and DCP (molar ratio of 5:4:1) in the absence or presence of sucrose, which have been evaluated in dogs for their pharmacokinetics.[3] Because of the high concentration of lipid in these liposomal preparations and the high phase transition temperature of the phospholipids used in their composition, the acid digestion of these samples is a time-consuming process. Therefore, there is a great need for simpler and more rapid procedures.

The experimental conditions proposed for the determination of Sb^V in liposomal meglumine antimoniate are also summarised in Table 1. When compared to the assay used for meglumine antimoniate, an additional step was included to permit the release of encapsulated meglumine antimoniate from liposomes. The temperature of 60 °C is important to allow for the action of Triton X-100, which is more effective above the phase transition temperature of DSPC (55 °C).

In order to investigate the possible interference of the liposome matrix (method selectivity), samples with varying concentration of Sb were prepared in water in the absence or presence of empty liposomes at a final lipid concentration of 65 g L^{-1}. These samples were treated with Triton X-100 and Cys and were then evaluated by the BPR photometric assay. Figure 7 compares the changes in absorbance as a function of Sb concentration, in the absence and in the presence of the liposome matrix. Application of the *F* Test indicated that the variances did not differ significantly. Application of the student *t* Test showed that the means were not significantly different, demonstrating the absence of interference of the liposome matrix in the photometric assay.

The precision of the method was also evaluated, by calculating the relative standard deviations for measurements performed with samples containing

Table 2. Precision of the Triton/Cys/BPR method for the determination of Sb^{V} concentration in liposomal formulations of meglumine antimoniate[a]

Sb concentration in initial preparation / (mol L^{-1})	Mean	Standard deviation	Relative standard deviation / (%)
0.04	0.1541	0.0033	2.17
0.08	0.3217	0.0022	0.69
0.12	0.4660	0.0115	2.47
0.16	0.6010	0.0106	1.76
0.2	0.7686	0.0135	1.76

[a]The mean and standard deviation refer to the measurement (A_o-A_m) of 7 preparations, prepared independently, containing empty liposomes at 65 g L^{-1} of lipid; Relative standard deviation (%) = (standard deviation/mean)×100.

Table 3. Sb concentration values determined in different liposomal formulations of meglumine antimoniate[a] by the Triton/Cys/BPR method and with the conventional ICP-OES method

Liposomal preparation	[Sb]/(mol L^{-1}) by Triton/Cys/BPR method	[Sb]/(mol L^{-1}) by ICP-OES method
DRV liposomes	0.097	0.095
DRV liposomes, prepared in the presence of sucrose	0.076	0.072
FDEL liposomes	0.067	0.069
FDEL liposomes, prepared in the presence of sucrose	0.069	0.071

[a]Liposomal formulations were made from DSPC, CHOL and DCP (molar ratio of 5:4:1) in the absence or presence of sucrose, as described previously.[3]

liposomes (65 g L^{-1}) and at different levels of Sb concentration. Table 2 shows the results of analysis of the method precision. Since the relative standard deviations were inferior to 5%, the method was considered precise.

The accuracy of the method was also satisfactory, since the recoveries of known amounts of Sb^{V} after addition to Sb-containing samples and empty liposomes varied between 96 and 104%. The Sb concentration values determined by the proposed method (Triton/Cys/BPR) and by conventional ICP-OES after nitric acid digestion of the samples are shown in Table 3 for comparison. Importantly, the concentrations of Sb determined by the two methods were found to be very similar for the four different liposomal formulations.

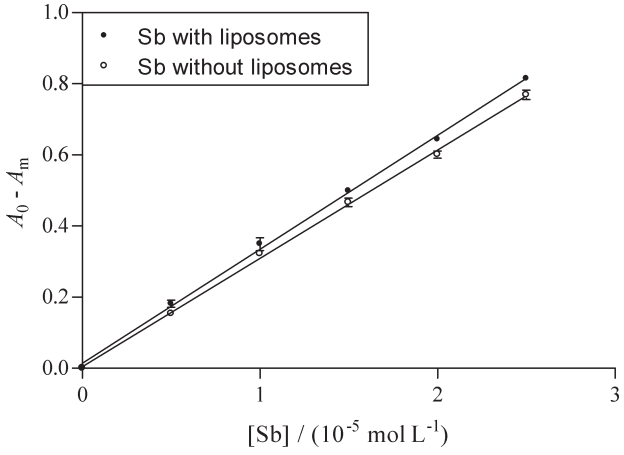

Figure 7. Change in BPR absorbance at 560 nm (A_0-A_m) as a function of the final Sb concentration. Samples with varying concentration of Sb (standard, Titrisol®) (0.04, 0.08, 0.12, 0.16 and 0.2 mol L^{-1}) were prepared in water, in the absence or presence of empty liposomes made from DSPC, CHOL and DCP (molar ratio of 5:4:1) at a final lipid concentration of 65 g L^{-1}. For each Sb concentration, seven identical samples were prepared independently. These samples were treated with Triton X-100 and Cys, as described in the Experimental Section. Data are shown as means ± SD (n = 7).

Conclusions

In the present work, the reduction reaction of Sb^{V} by Cys was kinetically characterized. The reaction orders with respect to Sb and Cys were equal to 1.0 and 3.3, respectively. The rate constant for the reaction of reduction was found to be pH-dependent. According to these data, the pharmacological relevance of this reaction depends on the Cys concentration in the macrophage lysosomes. It is suggested that Cys should have a concentration of at least 3 mmol L^{-1} in order to exert a significant pharmacological role. This reaction was exploited to effectively reduce Sb^{V} in meglumine antimoniate, for the subsequent photometric determination of Sb^{III} using BPR. In the case of meglumine antimoniate, reduction efficiencies of 100% were achieved at pH 3 in the presence of 50 mmol L^{-1} Cys and Sb concentrations higher than 0.25 mmol L^{-1}. The photometric assay was linear in the range of 1.2×10^{-6} to 23.5×10^{-6} mol L^{-1} of Sb^{III} with a correlation coefficient higher than 0.998, even in the presence of Cys at a Cys/BPR ratio of 64. Aqueous solutions with Sb concentration as low as 0.5 mmol L^{-1} were successfully assayed. The method was also applied to the determination of Sb in liposomal formulations of

meglumine antimoniate. In this case, an additional step was introduced before the reduction step, consisting of the Triton X-100-induced release of encapsulated Sb. The latter method showed good precision and accuracy.

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