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Determination of pentavalent antimony in antileishmaniotic drugs using an automated system for liquid–liquid extraction with on-line detection

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Abstract

An automated system for liquid–liquid extraction flow analysis (LLE-FA) for the determination of Sb(V) in antileishmanial drugs is presented. The method is based on extraction in a 5 mL glass extraction chamber of an ion pair formed between hexachloroantimoniate anion and rhodamine B cation into toluene. The detection system consists of a green light emitting diode (LED) and a photodiode. The system is controlled by a microcomputer using a program written in Visual Basic 3.0. The extraction process was optimized and the following experimental parameters were established: sample loop of 150 μL; reagent loop of 900 μL; stirring time of 100 s; phase separation time of 80 s; volumetric ratio of 1:1 (aqueous/organic). The method was *in-house* validated for the determination of Sb(V) in meglumine antimoniate. The following performance criteria were obtained: linearity of 0.9989, linear range of 7.0×10^{-5} to 7.2×10^{-4} mol Sb(V) L^{-1}, sensitivity of $1.61 \times 10^{6} \pm 2$ arbitrary units $L\,mol^{-1}$ ($P < 0.05$), intra-assay precision of 3.5% ($n = 5$; 4.1×10^{-4} mol L^{-1} Sb(V). Whereas the method is selective in the presence of Sb(III), As(III) and Pb(II) at concentrations up to one tenth of the concentration of Sb(V), As(V) interferes. The accuracy of the method was evaluated through comparison of results obtained from analyses of pharmaceutical formulations by the proposed LLE-FA method with those obtained by inductively coupled plasma optic emission spectrometry (ICP OES) and differential pulse polarography for total antimony and Sb(III), respectively. The proposed method presented an analytical frequency of eight analysis per hour and is suitable for Sb(V) determination in the quality control of drugs employed for the treatment of leishmaniasis.

Keywords: Automated system for liquid–liquid extraction; Pentavalent antimony; Meglumine antimoniate; Rhodamine B

1. Introduction

Antimony preparations have been the drugs of choice for the treatment of Leishmaniasis for over 90 years. This disease currently affects 12 million people worldwide and is endemic in 88 countries on five continents with a total of 350 million people at risk. Meglumine antimoniate and sodium stibogluconate are the pentavalent antimonial drugs of choice employed in French and English speaking countries, respectively [1].

The structure and mechanism of action of the antimonial drugs are not well known, contaminants such as trivalent antimony, arsenic and lead have been detected [2] and the quality control of these drugs has not been standardized. Thus, the development of simple, low cost, fast methodologies is important, especially for developing countries.

Several analytical methods have been described to determine antimony in matrices like sediments, soil and vegetables [3,4], steels and cast irons [5], biological material [6,7] and pharmaceutical products [8–10]. Due to the fact that the toxicity of antimony depends on the oxidation state, methods that allow speciation are recommended.

Flores et al. [11] described a method for the determination of trivalent and total antimony by hydride generation atomic absorption spectrometry in pharmaceuticals used for the Leishmaniasis treatment. Bloomfield et al. [9] developed a method using flow injection analysis for determination of pentavalent antimony in sodium stibogluconate. Rath et al. reported a spectrophotometric method for the determination of trivalent antimony in meglumine antimoniate, where the pentavalent antimony was determined after reduction with iodide [10]. Even though, these methods are selective to the oxidation state, the pentavalent antimony was usually determined indirectly. Traditional techniques, employed for the determination of antimony, such as atomic absorption spectrometry or inductively

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coupled plasma optical emission spectrometry, are not selective for the antimony speciation.

Pentavalent antimony, as hexachloroantimoniate, reacts with cationic dyes such rhodamine B [12,13], brilliant green [14] and crystal violet [15], forming an ion pair that can be extracted into an organic layer and determined by spectrophotometry. However, side reactions occur and a lack of stability of the ion pair was usually verified, which results in low precision and inadequate accuracy of the method. Furthermore, manual liquid–liquid extraction procedures require the employment of large volumes of solvents and involve laborious and tedious procedures that turn the method unattractive for quality control in routine analyses. These disadvantages can be overcome by performing the extraction in automated systems.

In the literature several flow systems have been described for the automation of liquid–liquid extraction processes. Kalberg and Thelander [16] and Bergamin et al. [17] proposed systems to carry out liquid–liquid extraction in flow injection systems. Based on these ideas different flow injection solvent extraction systems have been proposed [18–27]. Liquid–liquid extraction has been evaluated employing monosegment continuous-flow analysis (MCA) [28,29], sequential injection analysis (SIA) [30,31] and multicommutation [32]. Recently, Diniz et al. [33] described an automatic system for liquid–liquid extraction using a micro-batch extraction chamber for the preconcentration and determination of copper(II).

The present paper reports a selective method for pentavalent antimony determination in meglumine antimoniate, based on ion pair (IP) formation between a hexachloroantimoniate anion and a rhodamine B cation with extraction of the IP into toluene using an automated system for liquid–liquid extraction and a micro-batch extraction chamber.

2. Experimental

2.1. Reagents and solutions

All reagents used were of at least analytical grade. Distilled–deionised water was used throughout.

A 1.00×10^{-2} mol L^{-1} stock solution of Sb(V) was prepared by dissolving 0.6275 g of $KSb(OH)_6$ (99%, Sigma) in 250 mL water. Working standard solutions of Sb(V) were prepared daily by dilution of the standard stock solution.

A 1.00×10^{-2} mol L^{-1} rhodamine B stock solution was prepared by dissolving 0.47902 g rhodamine B chloride (Nuclear) in 100 mL of a 6 mol L^{-1} HCl solution. This solution was stable at least 1 month.

The Pb(II) solution were obtained from a reference lead standard solution 1000 mg L^{-1} (Aldrich). Standard stock solution, 1000 mg L^{-1} As(III), was prepared by dissolution of 0.132 g As_2O_3 (Merck) in 2 mL NaOH (Merck) 20% (m/v); the solution was acidified to pH 2 by adding HCl and diluted to 100 mL with water. Standard stock solution of 1000 mg L^{-1} As(V) was prepared by dissolution of 0.418 g $Na_2HAsO_4{\cdot}7H_2O$ (Aldrich) in 100 mL water.

2.2. Instrumentation

An Shimadzu UV–vis spectrophotometer (Japan) was used for obtaining the absorption spectrum.

A Fisatom Magnetic Stirrer (Brazil) was used to mix the solutions inside the extraction chamber.

A Unique USC 700 ultrasonic apparatus (Brazil) was used for sample pretreatment.

The polarographic measurements were carried out with a potentiostat–galvanostat, Autolab PGSTAT30 Eco Chemie B.V. (The Netherlands). Platinum wire and a mercury drop electrode were used as counter and working electrodes, respectively, and all potentials were recorded against a Ag/AgCl, KCl_{sat} reference electrode.

The inductively coupled plasma optical emission spectrometric (ICP OES) measurements were done with an Optima 3000 DV equipment from Perkin-Elmer (USA).

2.3. Flow analysis system

A diagram of the automatic system assembled to evaluate the liquid–liquid extraction procedure is shown in Fig. 1. The system is controlled by a microcomputer through a parallel interface (Advantech 711S) using a program written in Visual Basic 3.0. The extraction chamber was made in glass with total capacity of 5 mL. The propulsion of the Sb(V) standard solution or sample and the reagents were performed with a IPC—high precision multichannel peristaltic pump (Ismatec) with Tygon tubes. Samples were injected using a proportional injector [34]. The organic solvent (toluene, VETEC) was introduced into the system by pumping distilled water into the reservoir (100 mL) of the solvent. This procedure was employed in order to avoid contact of this solvent with the peristaltic pump tubes. The detection system used was a homemade detector consisted of a green (560 nm) light emitting diode (LED) and a photodiode (Centronic, OSI5K) [35]. The detector is attached to the upper exit of the extraction chamber, which consists of a glass tube with 2 mm i.d. (optical path). The extraction chamber and the sequence of the liquid–liquid extraction procedure is similar to that described by Diniz et al. [33].

2.4. Liquid–liquid extraction flow analysis procedure

The operational sequence of the liquid–liquid extraction procedure is summarized in Table 1.

Briefly, in step 1 the acid solutions of samples or standards and rhodamine B are pumped through loops L1 and L2. The solenoid valves V1–V4 (NResearch Model 161T031; 12 V; 80 mA) and the magnetic stirrer are off. The extraction process is started by commuting the proportional injector to the injection position and the segment composed of the rhodamine B acid solution and the standards or sample with Sb(V) are carried to extraction chamber E. When the segment reaches the optical sensor SO2 (opto-switches, PCTS-2103, RS Components) [36], the V3 cycle valve is automatically turned on and a defined volume of toluene is added to the extraction chamber, step 2 (Table 1). During the addition of toluene, the mixture of sample

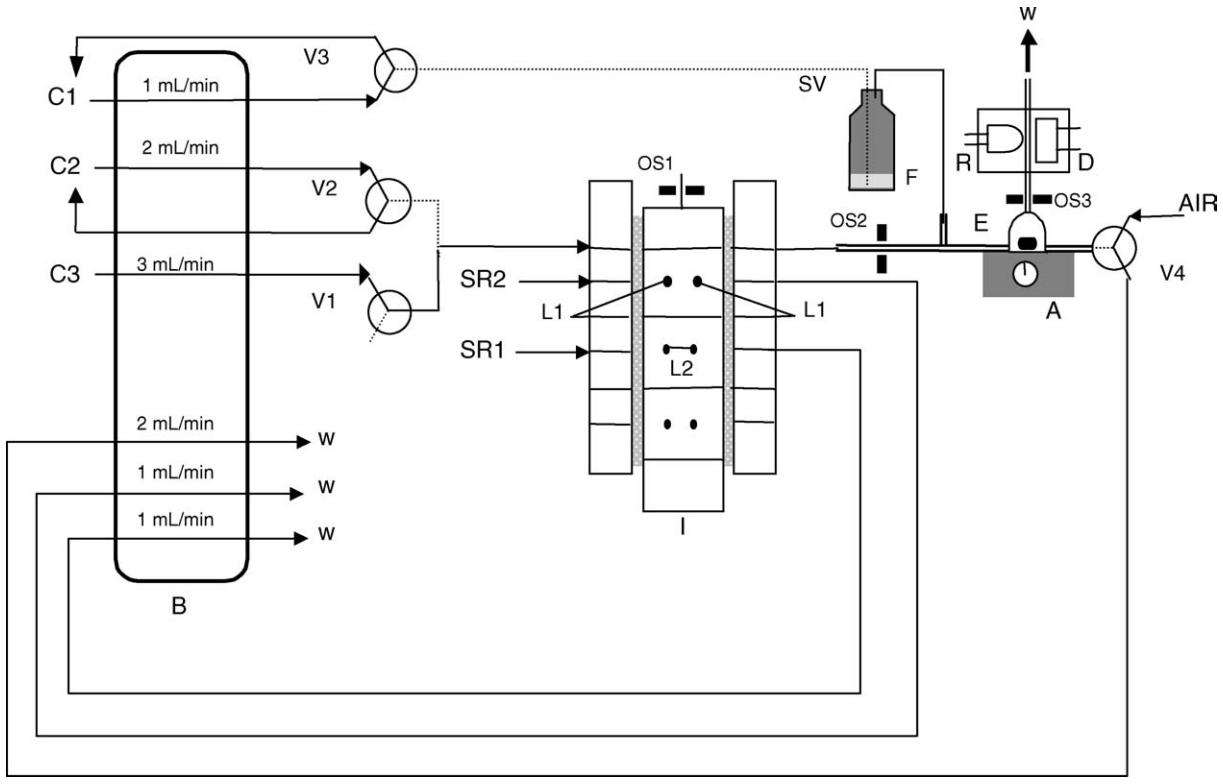

Fig. 1. Manifold used to evaluate the liquid–liquid extraction procedure. C1 and C2, carrier solution (water); C3, carrier (air); SR1, rhodamine B acid solution; SR2, sample or standard acid solutions; L1, reagent loops; L2, sample or standard loop; SV, organic solvent propulsion; F, organic solvent (toluene) reservoir; OS1–OS3, optical sensors; V1–V4, solenoid valves; E, extraction chamber; R, green (560 nm) light emitting diode (LED); D, photodiode; A, magnetic stirrer; B, peristaltic pump; I, proportional injector; W, waste.

(Sb(V)), reagent (rhodamine B) and solvent inside the extraction chamber is stirred for a period of 100 s for the formation and the extraction of the ion-pair (step 3). After the extraction of the ion-pair, the agitation is stopped and 80 s are allowed for the phase separation inside the extraction chamber (step 4). Valve V2 is then turned on and the carrier stream is pumped through the system to the extraction chamber, filling it and transporting the less dense organic phase containing the ion-pair towards the detector (step 5). When the organic phase is sensed by optical sensor SO3, analytical data acquisition is started (step 6). At the end of signal recording, the organic phase is discarded through the up-exit, carried by the water stream. After this, valves V1 and V4 and the magnetic stirrer is turned on and valve V2 is turned off. The residual solution inside the extraction chamber is aspirated (step 7). To wash the extraction chamber valve V2 is turned on and valves V1 and V4 are turned off. Water and air are pumped simultaneously to remove the residual solution present in the extraction chamber (step 8). Agitation is carried out throughout all the washing time. Then, to remove the residual washing solution present in the extraction chamber valves V1 and V2 is turned off and valve V4 are turned on (step 9).

2.5. *Efficiency of the extraction in the LLE-FA system compared with manual extraction*

The extraction efficiency of Sb(V) in the LLE-FA system was compared with that obtained using the manual

Table 1
Sequence for liquid–liquid extraction

Step	Function	V1	V2	V3	V4	Agitation	Detection	Air	Water	Organic solvent
1	Start	Off	Off	Off	Off	Off	Off	Pump	Recycle	Stop
2	Solvent injected	Off	Off	On	Off	On	Off	Pump	Recycle	Pump
3	Extraction	Off	Off	Off	Off	On	Off	Pump	Recycle	Stop
4	Phase separation	On	Off	Off	Off	Off	Off	Stop	Recycle	Stop
5	Carrier stream	On	On	Off	Off	Off	Off	Stop	Pump	Stop
6	Detection	On	On	Off	Off	Off	On	Stop	Pump	Stop
7	Chamber emptying	On	Off	Off	On	On	Off	Stop	Recycle	Stop
8	Chamber washing	Off	On	Off	Off	On	Off	Pump	Pump	Stop
9	Chamber emptying	Off	Off	Off	On	Off	Off	Pump	Recycle	Stop

extraction in a batch system, with a separation funnel, to which 9500 μL of 1.36×10^{-3} mol L^{-1} rhodamine B and 550 μL of 8.20×10^{-3} mol L^{-1} Sb(V) were added. The solution was homogenized and 10 mL of toluene were added. The mixture was vigorously agitated for 60 s and after a rest time of 30 s, in which phase separation occurs, the organic layer was separated and introduced through the detection device of the LLE-FA system. The analytical signals were compared with those obtained after extraction of Sb(V) in the LLE-FA system, using a similar volume ratio of aqueous/organic phase and Sb(V) concentration.

2.6. Influence of interferents

For this study, solutions containing Sb(III), As(III), As(V) or Pb(II), at two concentration levels (2.73×10^{-5} and 2.73×10^{-6} mol L^{-1}), were added to a 2.73×10^{-4} mol L^{-1} Sb(V) working standard solution. The analyses were performed as described in Section 2.4, using L2: 150 μL; L1: 900 μL; concentration of rhodamine B: 8.2×10^{-3} mol L^{-1} in 6 mol L^{-1} HCl; stirring time: 100 s; phase separation time: 80 s; volume of the extractor (toluene): 1000 μL.

2.7. Sample analysis by LLE-FA

Samples of meglumine antimoniate, commercialized as 5 mL ampoules, were purchased from local drug stores in the State of São Paulo, Brazil.

An aliquot of the meglumine antimoniate contained in the 5 mL ampoule is taken and diluted 1:100 (v/v) with 6 mol L^{-1} HCl. The acidified sample is sonicated at 55 kHz for 15 min. Subsequently, the solution is diluted 6:100 (v/v) with 6 mol L^{-1} HCl. The diluted sample is immediately introduced into the LLE-FA system, according procedure described in Section 2.4, using L2: 150 μL; L1: 900 μL; concentration of rhodamine B: 8.2×10^{-3} mol L^{-1} in 6 mol L^{-1} HCl; stirring time: 100 s; phase separation time: 80 s; volume of the extractor (toluene): 1000 μL.

2.8. Determination of Sb(III) by polarography

The polarographic determination of Sb(III) was carried out according to the procedure described by Franco et al. [37], using 2 mol L^{-1} HCl as supporting electrolyte.

2.9. Determination of total Sb by ICP OES

The total antimony content in the pharmaceutical formulations was obtained by ICP OES with an external calibration graph. The calibration graph was linear in the range of 10–120 mg L^{-1} Sb. For quantitation the following wavelengths were employed: 206.8, 217.5 and 231.1 nm. The meglumine antimoniate samples were diluted before analysis in 0.2% HCl (v/v).

3. Results and discussion

The formation of an ion pair between $SbCl_6^-$ and protonated rhodamine B was firstly described by Ramette and Sandel [38].

Rhodamine B shows, in aqueous solution, an absorption maximum at 560 nm. In a strongly acidic solution (6 mol L^{-1} HCl) three maxima at 465, 494 and 523 nm were observed. It was not possible to differentiate between free rhodamine B and the IP formed in aqueous acidic solution by spectrophotometry, due to the fact that both species presents the same absorption maxima. As a consequence, the determination of Sb(V) as an IP would be only possible if the IP formed is separated from the aqueous layer before measurement.

For this purpose, toluene was employed. In this solvent only the IP is transferred; the protonated rhodamine B remains in the aqueous layer. It was also verified that the IP is not stable in the organic layer. The IP decomposition reaction was monitored spectrophometrically and is a first-order reaction ($\ln A = \ln A_0 - 0.00327t - 1$, where A is the absorbance at 560 nm, t the time and A_0 is the absorbance at initial time).

It is also well known that $Sb(Cl)_6^-$ is not stable in aqueous solution, where insoluble products such as $Sb(OH)Cl_5^-$ are formed by hydrolysis. These products do not react with rhodamine B and in consequence the standard antimony working solutions were prepared in 6 mol L^{-1} HCl just before use.

Due to the fact that both species, $Sb(Cl)_6^-$ and the IP, are not stable in the medium, the analytical determinations needs to be carried out under rigorous time control to avoid lack of precision. Thus, automation of the method is especially indicated.

For the development of an automated method for the determination of Sb(V) by LLE-FA (Fig. 1) the chemical reaction, as well as the main variables that affect the extraction efficiency and detection of the IP were investigated. To optimize the conditions for extraction of the IP in the automated system, the following parameters were evaluated: HCl concentration (2–10 mol L^{-1}), phase separation time (0–120 s), stirring time (0–120 s), aqueous/organic volume ratio (1.5:0.3) and Sb(V)/rhodamine B ratio 0.5:6.

3.1. Analytical signal

Fig. 2 shows a typical signal obtained for the extracted IP in toluene in the LLE-FA system. The base line is identified as A and corresponds to the signal generated when the optical pathway is full of air. B1 and B1 correspond to the transmittance signal of the organic phase (toluene) without and with the analyte, respectively, and C is the signal of the acidic aqueous phase. The analytical signal is the difference (S) between B1 and B2 and corresponds to the absorption due to the IP in the organic phase.

3.2. Effect of HCl solution on extraction efficiency

The formation of the $Sb(Cl)_6^-$ ion, as well as the protonation of the rhodamine B and, consequently, IP-formation

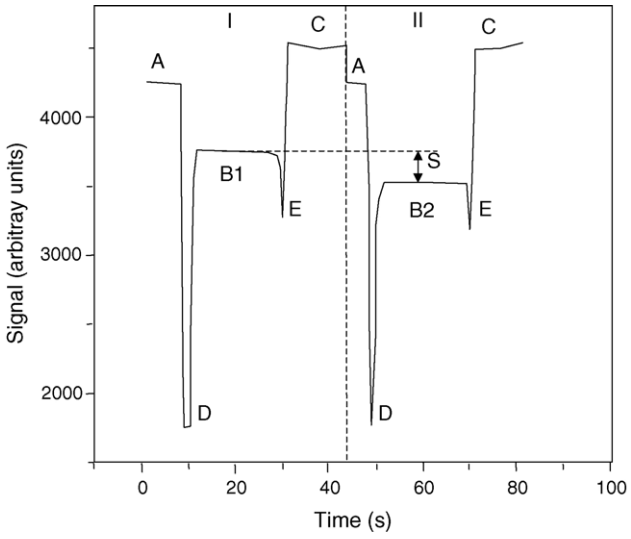

Fig. 2. Typical analytical signal obtained from LLE-FA Sb(V)determination. (I) Blank and (II) ion-pair in organic phase after liquid–liquid extraction of 4.1×10^{-3} mol L^{-1} Sb(V) standard solution. A, air signal; B1, blank organic phase signal; B2, ion-pair in the organic phase signal; C, aqueous phase signal; D, air-organic phase interface signal; E, organic–aqueous interface signal; S, difference signal between blank (B1) and analytical solution (B2).

depend on the HCl concentration. The dependence on the formation and extraction of the IP was evaluated over the range of 2–10 mol L^{-1} HCl. For this study, a 8.2×10^{-3} mol L^{-1} solution of Sb(V), which corresponds to 1 mg Sb(V) mL^{-1}, prepared in 2, 4, 5, 6, 8 and 10 mol L^{-1} HCl and a 8.2×10^{-3} mol L^{-1} solution of rhodamine B in 6 mol L^{-1} HCl were employed. The reagent (L1) and sample (L2) loop were 500 μL and 250 μL, respectively, and the volume of toluene was 1500 μL, resulting in an aqueous/organic volume ratio of 0.5.

The results (Fig. 3) indicate that the formation of the anionic ion $Sb(Cl)_6^-$ and the extraction efficiency of the IP from the aqueous solution are maximized at HCl concentrations higher than 6 mol L^{-1}.

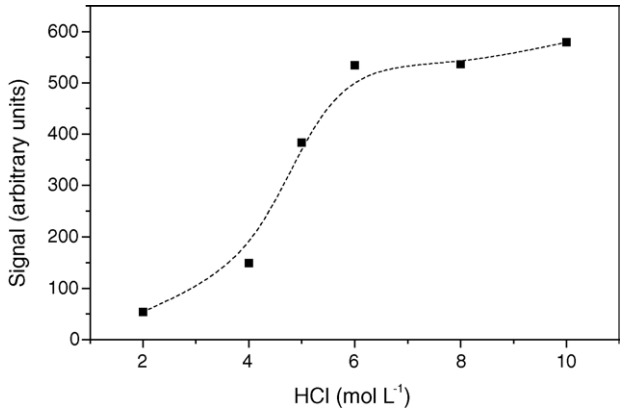

Fig. 3. Effect of HCl concentration on IP extraction efficiency. 8.20×10^{-3} mol L^{-1} solution of Sb(V) prepared with 2, 4, 5, 6, 8 or 10 mol L^{-1} HCl; 8.20×10^{-3} mol L^{-1} stock solution of rhodamine B in 6 mol L^{-1} HCl solution; L1: 500 μL; L2: 250 μL; stirring time: 60 s; aqueous/organic volume ratio: 0.5.

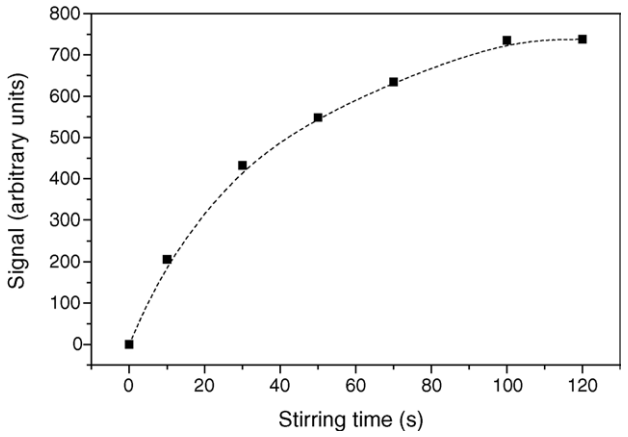

Fig. 4. Effect of stirring time on extraction efficiency. 8.20×10^{-3} mol L^{-1} solution of Sb(V) and 8.20×10^{-3} mol L^{-1} solution of rhodamine B, both in 6 mol L^{-1} HCl solution; L1: 500 μL; L2: 250 μL; phase separation time: 80 s; aqueous/organic volume ratio: 0.5.

3.3. Effect of stirring time on extraction efficiency

The extraction efficiency depends on the stirring time in the extraction chamber. The stirring promotes contact between the two phases, allowing transfer of the IP from the aqueous phase to the organic layer. The effect of stirring time was investigated over the range of 10–120 s. The stirring velocity was maintained constant at the maximum value of the magnetic stirrer used. The results (Fig. 4) indicate that the analytical signal becomes constant after a stirring time of 100 s, and this time was used in further studies.

3.4. Effect of phase separation time on extraction efficiency

The liquid–liquid extraction process involves the distribution of a solute between two immiscible liquid phases. The concentration of the species in the two phases after the extraction is represented by the distribution coefficient (K_D). After vigorously stirring of the aqueous–organic mixture for 100 s, the solution remained unstirred to promote the complete phase separation. In this stage, the uncharged organic molecules (IP) remain in the organic layer, while the charged, not complexed anion from the ionized molecules remains in the polar aqueous layer.

The extraction efficiency will be independent of the original concentration of the solute. The effect of time on the phase separation was investigated up to 120 s (Fig. 5). The results indicate that equilibrium was reached after 60 s. For subsequent studies a time of 80 s was established.

3.5. Effect of aqueous/organic volume ratio on extraction efficiency

The effect of the aqueous/organic volume ratio on the extraction efficiency, in an extraction chamber of 5 mL, was investigated. A volume of 750 μL aqueous phase (250 μL of 8.2×10^{-3} mol L^{-1} Sb(V) and 500 μL 8.2×10^{-3} mol L^{-1} rhodamine B) and volumes of 500, 1000, 1500, 2000 and 2500 μL toluene as organic phase were used, giving aqueous/organic

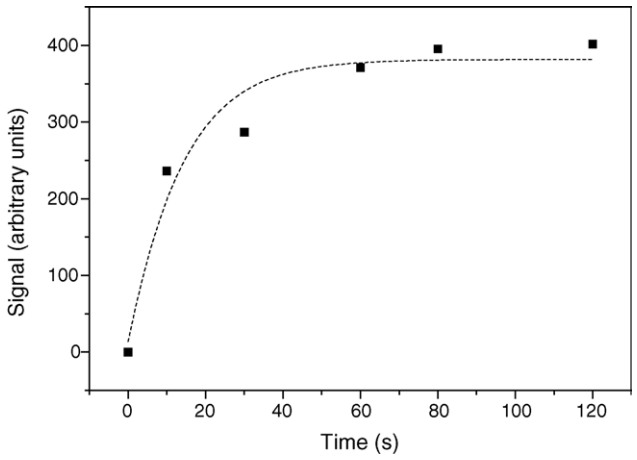

Fig. 5. Effect of time for phase separation on extraction efficiency. 8.20×10^{-3} mol L^{-1} solution of Sb(V) and 8.20×10^{-3} mol L^{-1} solution of rhodamine B, both in 6 mol L^{-1} HCl solution; L1: 500 μL; L2: 250 μL; stirring timing: 60 s; aqueous/organic volume ratio: 0.5.

volume ratios of 1.5, 0.75, 0.50, 0.38 and 0.30, respectively. The results are presented in Fig. 6. It can be observed that the analytical signal decreases exponentially with the increase in the toluene volume, i.e., with the decrease of the aqueous/organic volume ratio. The Sb(V) concentration in the pharmaceutical formulations is high (on the order of mg mL^{-1}) and no pre-concentration step is necessary. Thus, an optimal range of 1.5–0.75 for the aqueous/organic volume ratio was established and for further studies a value of 1.0 chosen. Higher volumes of toluene (>1000 μL) affect the sensitivity of the method and smaller volumes (<500 μL) influence the linear range of the analytical curve, where deviations from Beer's law at Sb(V) concentrations lower than 5×10^{-5} mol L^{-1} was observed.

3.6. Effect of rhodamine B concentration in the formation of the IP

The effect of the rhodamine B concentration on the IP-formation during the extraction was investigated. Molar ratios

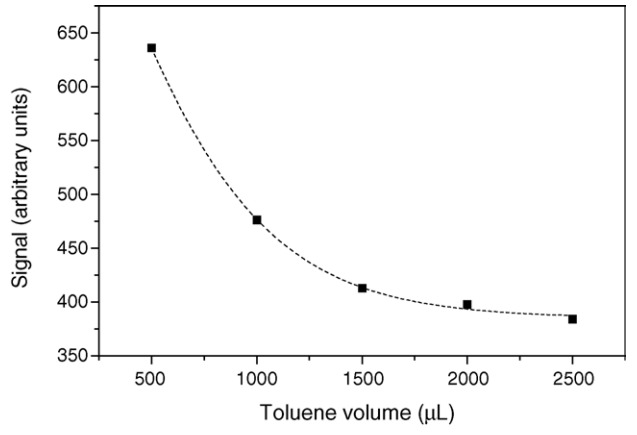

Fig. 6. Effect of the toluene volume on extraction efficiency. Two hundred and fifty microliters of 8.20×10^{-3} mol L^{-1} Sb(V) and 500 μL 8.20×10^{-3} mol L^{-1} rhodamine B, both in 6 mol L^{-1} HCl solution; L1: 500 μL; L2: 250 μL; phase separation time: 80 s; stirring time: 100 s.

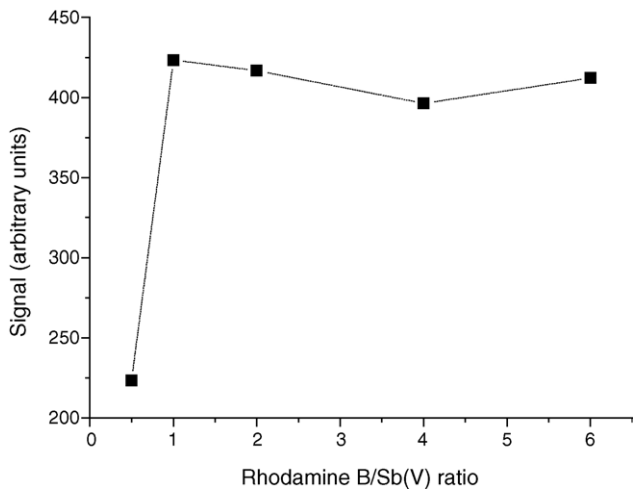

Fig. 7. Effect of the molar ratio of rhodamine B/Sb(V) on IP formation and extraction efficiency. 4.1×10^{-3} mol L^{-1} Sb(V) (L2: 450 μL) and 4.1×10^{-3} mol L^{-1} rhodamine B (L1: 225, 450, 900, 1800 and 3600 μL); phase separation time: 80 s; stirring time: 100 s; 1.0 mL toluene as organic phase.

of rhodamine B/Sb(V) of 0.5/1, 1/1, 2/1, 4/1 and 6/1 were evaluated. For this study the concentration of Sb(V) was set at 4.1×10^{-3} mol L^{-1} (L2: 450 μL). The quantity of rhodamine introduced in the extraction chamber was varied by changing the volume of the reagent loop (L1) from 450 to 3600 μL, using a 4.1×10^{-3} mol L^{-1} rhodamine B solution. According to the results (Fig. 7) the analytical signal increases up to a 1:1 rhodamine B/Sb ratio. When the concentration of rhodamine B is higher than the Sb(V) concentration in the extraction chamber the signal becomes independent of the rhodamine B concentration, even though the aqueous/organic volume ratio increases with the proportion of rhodamine B/Sb(V). The results indicate that the IP has a stoichiometric ratio of 1:1. An excess of rhodamine B does not interfere in the Sb(V) determination.

3.7. Efficiency of the extraction in the LLE-FA system compared with manual extraction

The extraction efficiency of Sb(V) in the LLE-FA system was compared with that obtained using manual extraction in a batch system according to the procedure described in Section 2.5. It was verified that the extraction efficiency of the IP in the manual batch system is lower (50.5%, R.S.D. = 7.7%, $n = 3$) than that obtained with the automated LLE-FA method. The lack in precision of the manual extraction procedure could be attributed to hydrolysis reactions.

3.8. Influence of interferents

Recently contamination of some commercial lots of *N*-meglumine antimoniate with trivalent antimony, lead and arsenic have been reported [2]. Due to this fact, an interference study was evaluated in order to verify if the presence of Sb(III), As(III), As(V) and Pb(II) influence the determination of Sb(V) by the proposed LLE-FA method. The possible interferents were added to a Sb(V) standard solution in a concentration ratio of 1/100 and

Table 2
Recovery of Sb(V) in the presence of potential interferents

Interferents	Sb(V) concentration (mol L^{-1})	Sb(V)[a] recovery (%), $n = 3$	R.S.D. ($n = 3$)
Sb(III)	2.73×10^{-6}	103	1.1
	2.73×10^{-5}	102	1.8
As(III)	2.73×10^{-6}	96	2.9
	2.73×10^{-5}	102	1.8
As(V)	2.73×10^{-6}	114	0.8
	2.73×10^{-5}	140	1.0
Pb(II)	2.73×10^{-6}	104	0.7
	2.73×10^{-5}	97	0.4

R.S.D., relative standard deviation.
[a] Sb(V) concentration: 2.73×10^{-4} mol L^{-1}.

10/100 as described in Section 2.6. The results are presented in Table 2. Whereas Sb(III), As(III) and Pb(II) do not cause significant interference in the Sb(V) determination at the two investigated concentration levels, As(V) enhances the analytical signal and leads to a positive error. Nevertheless, the concentrations of As(V) determined previously in meglumine antimoniate were always lower than 0.1%, and, as this ion should not be present in pharmaceutical formulations, it was not considered a potential interfering ion for Sb(V) quantitation.

3.9. Sample preparation

The antimony in the meglumine antimoniate formulation is complexed by the organic compound *N*-methylglucamine. Furthermore, the structure and composition of meglumine antimoniate is not completely elucidated, although results suggest [39] that a complex mixture of carbohydrate-antimony oligomers coexist in solution.

It was verified that the Sb(V) needs to be liberated from the organoantimonial compound to undergo reaction with rhodamine B. In previous work, it was found that ultrasound energy is suitable for this purpose. When the sample is only diluted with HCl and analyzed by the proposed LLE-FA method, recoveries lower than 72% were obtained. However, recovery rates higher than 92% were obtained if the sample is diluted 1:100 (v/v) with 6 mol L^{-1} HCl and exposed for 15 min on ultrasound energy (55 kHz) before analysis.

3.10. Validation

With the established experimental conditions for the LLE-FA system, the method was *in-house* validated for the Sb(V) determination in pharmaceutical formulations, using the following performance criteria: linearity, linear range, sensitivity, selectivity, intra-assay precision (repeatability), detectability and accuracy.

The following optimized experimental parameters for the LLE-FA method developed were used: volume of the extraction vessel: 5 mL; sample loop: 150 μL; reagent loop (rhodamine B): 900 μL; concentration of rhodamine B: 8.2×10^{-3} mol L^{-1}; stirring time: 100 s; phase separation time: 80 s; volume of the extractor (toluene): 1000 μL.

Table 3
Validation parameters

Linear range (mol L^{-1})	7.0×10^{-5} to 7.2×10^{-4}
Linearity	0.9989
Sensitivity (au L mol^{-1})	1.61×10^{6}
Intra-assay precision (% R.S.D., $n = 5$) Sb(V) 4.1×10^{-4} mol L^{-1})	3.5
Detectability (mol L^{-1})	2.9×10^{-5}

au, arbitrary units.

The validation parameters are presented in Table 3. The linearity, range and sensitivity were obtained from an analytical curve using the external standard method with nine concentration levels, with triplicate analyses. The linearity and the sensitivity were expressed as the linear correlation coefficient and the slope of the analytical curve, respectively.

The detectability of the method was calculated as 2.9×10^{-5} mol L^{-1}. This value represents the lowest concentration that could be detected in the extraction chamber of the LLE-FA system. The determination limit of the method is not presented, due to the fact that the Sb(V) is a major constituent of the formulation and this parameter is not required for method validation destined for the quality control of pharmaceutical products. Furthermore, the LOD would depend on the sample dilution before analysis.

The selectivity of the method was evaluated by the interference study previously described. No potential interferences were verified by the presence of other inorganic elements, as well as constituents of the matrix.

The accuracy of the method was evaluated through comparison of results obtained by the analyses of drugs by the proposed LLE-FA method with those obtained by inductively coupled plasma optic emission spectrometry and differential pulse polarography for total antimony and Sb(III), respectively (Table 3).

The proposed method presented an analytical frequency of eight analysis per hour. The automated LLE-FA method is suitable for Sb(V) determination in the quality control of antileishmaniotics drugs.

3.11. Sample analyses

Samples of meglumine antimoniate used in Brazil for the treatment of leishmaniasis were analyzed. The products are commercialized in 5 mL ampoules and according to the manufacturer, each ampoule contains 425 mg mL^{-1} of *N*-meglumine antimoniate, which corresponds to 85 mg of Sb(V) mL^{-1}. The Sb(V) content in the samples were determined by the proposed LLE-FA method and compared with values obtained by the difference of the total antimony determined by ICP OES and the Sb(III) content determined by polarography (Table 4).

For the different samples analyzed the Sb(V) content varied from 89 to 110 mg mL^{-1}. The mean values obtained by the proposed LLE-FA method and the difference between the total antimony determined by the ICP OES and the Sb(III) determined by polarography did not differ significantly ($P < 0.05$). In all

Table 4
Sb(V) content in samples determined on LLE-FA and ICP OES (Sb total) and polarography (Sb(III))

Sample	Average content of Sb(V) ± S.D. (mg mL^{-1})	
	ICP OES and polarography[a]	LLE-FA
1	109 ± 6	110 ± 2
2	114 ± 6	108 ± 2
3	94 ± 5	93 ± 1
4	85 ± 4	89 ± 1
5	112 ± 5	112 ± 1
6	105 ± 5	100 ± 2
7	104 ± 5	105 ± 5
8	102 ± 5	98 ± 3

S.D., estimate standard deviation ($n = 3$).
[a] Sb(V) were calculated between the difference of Sb total determined by ICP OES and Sb(III) determined by polarography.

samples Sb(III) was determined at concentrations lower than 10 mg mL^{-1} and corroborate other studies [10,34].

4. Conclusions

The automated liquid–liquid extraction system proposed was demonstrated to be an advantageous extraction technique for pentavalent antimony determination in pharmaceutical formulations employed for the treatment of leishmaniaisis. The proposed automated LLE-FA system is simpler, easier to handle and more precise than batch procedures. Furthermore, it allows control with good performance of the IP extraction and, the control of steps such as efficient mixture between the aqueous and organic phases, easy separation of the phases and detection of the content in the organic phase without its isolation. The automated system allows extractions on very small volumes of samples, and requires only small volumes of reagents and organic solvents. Moreover, the proposed method is faster than the conventional manual extraction method (eight injections per hour). Another characteristic of this system is that all the steps, except detection, occur in the extraction chamber contributing to decreases in the error produced by each step, when compared with manual extraction. Finally, the merits of the proposed automated system for liquid–liquid extraction with on-line detection can be expressed in terms of the increase in sampling frequency, the decrease in sample size, reduced solvent and chemical consumption, the possibility of measuring samples containing high concentrations of Sb(V), the decrease in interference effects from the matrix, the reduction of hazards associated with inflammable solvents, the reduction of solvent evaporation into the laboratory atmosphere and selectivity and sensitivity increases due to the separation process of this methodology.

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