*Quim. Nova,* Vol. 32, No. 2, 337-342, 2009

Artigo

# FLOW ANALYSIS-HYDRIDE GENERATION-GAS PHASE DERIVATIVE MOLECULAR ABSORPTION SPECTROPHOTOMETRIC DETERMINATION OF ANTIMONY IN ANTILEISHMANIAL DRUGS

**Máximo Gallignani y María del Rosario Brunetto**
Departamento de Química, Facultad de Ciencias, Universidad de Los Andes, Apartado postal 440, Mérida 5101-A, Mérida, Venezuela
**Fernando Ovalles***
Departamento de Análisis y Control, Facultad de Farmacia, Universidad de Los Andes, Mérida 5101-A, Mérida, Venezuela

Recebido em 21/2/08; aceito em 25/8/08; publicado na web em 26/1/09

In the present work, the development of a method based on the coupling of flow analysis (FA), hydride generation (HG), and derivative molecular absorption spectrophotometry (D-EAM) in gas phase (GP), is described in order to determine total antimony in antileishmanial products. Second derivative order ($D^2_{224nm}$) of the absorption spectrum (190 – 300 nm) is utilized as measurement criterion. Each one of the parameters involved in the development of the proposed method was examined and optimized. The utilization of the EAM in GP as detection system in a continuous mode instead of atomic absorption spectrometry represents the great potential of the analytic proposal.

Keywords: gaseous phase UV molecular absorption spectrophotometry; antimony; leishmaniasis.

## INTRODUCTION

The pharmaceutical literature on antimony mainly deals with the use of organic compounds as chemotherapeutic agents. It has lapsed almost a century from the introduction of organic antimonials into the treatment of leishmaniasis. This parasitic disease is widespread in the world. The World Health Organization (WHO) estimates a worldwide annual incidence of about 12 million and a population of 350 million people at risk of acquiring one of the forms of the disease.[1] Sodium stibogluconate (Pentostam ) and meglumine antimoniate (Glucantime®) are the pentavalent antimonial drugs of choice in the treatment of leishmaniasis. In Venezuela, another pentavalent antimonial drug for the treatment of leishmaniasis is also used. This drug called Ulamina is synthesized from antimony pentachloride and N-methyl glucamine (meglumine).[2]

Confidence knowledge of the antimony content in the pharmaceutical formulations is very important for prescribing the right dose. Stibiointoxication cases have been reported.[3] Although being old medicines, safety and quality control of these drugs have not been comprehensively evaluated so far.[4,5] Thus, the development of simple, low cost, fast methodologies is important, especially for developing countries.[6]

Several analytical methodologies have been developed for the determination of antimony in different aqueous and solid matrices, which have been reviewed by different authors.[7-10] Formation of volatile $SbH_3$ (hydride generation, HG) followed by its determination is a common practice in flow-injection atomic spectrometry using absorption (AAS) or emission with inductively coupled plasma (ICP-AES)[11] as the detection technique. A good deal of research on antimony has been, and continues to be, devoted to trace analysis of antimony.[8] However, the hyphenated instrumental techniques used for that purpose are usually sophisticated and expensive.

Works on antimony in formulated dosage forms are scanty. In this context, the analytical methods reported by the specialized literature dealing with the determination of antimony in pharmaceutical products are based -mainly- on redox reactions. In this fashion, Bloomfield *et al.*[12] developed a method using flow injection analysis (FIA) for determining Sb(V) in sodium stibogluconate. Franco *et al*.[13] established a chemical method for the determination of the levels of antimony to investigate proprietary formulas used to treat leishmaniasis by differential pulse polarography and iodine titration. Adsorptive stripping voltammetry using pyrogallol as a complexing agent has also been proposed for antimony speciation in commercial samples containing meglumine antimoniate.[14]

Meglumine antimoniate is mentioned in the Brazilian Pharmacopoeia[15] whereby a HG-AAS method is recommended for its determination, whether in its pure form or in injectable. Flores *et al*.[16] also reported the determination of Sb(III) and total Sb by HG-AAS in samples of injectable drugs used for leishmaniasis treatment. Later on, Flores *et al*.[17] described a procedure for the selective determination of Sb(III) in antileishmanial drugs by HG-AAS using a flow injection system. More recently, metal furnace heated by flame as a hydride atomizer for AAS was proposed for determining total antimony in pharmaceutical samples containing meglumine antimoniate.[18] Unfortunately, voltammetry, polarography and AAS are in a few special cases valuable tools in most of the official pharmacopoeias when they are compared to titrimetric, spectrophotometric and chromatographic methods.[19]

Spectrophotometry, due to its simplicity, is by far the most widespread method of analysis and is also used in antimony determination in pharmaceuticals.[20-24] Spectrophotometry with bromopyrogallol red has been proposed to determine Sb(V) in pharmaceuticals.[20,21] However, it involves several stages and lengthy pre-treatment of samples and some knowledge about the mathematical approaches which are not in common use in routine analysis.[21] Gallignani *et al*.[25] developed a flow analysis -hydride generation- Fourier transform infrared coupled system (FA-HG-FTIR) for the determination of antimony in pharmaceuticals being its major weakness the low sensitivity as compared to many available methods.

Some research has been made in the last two decades, in order to use UV/Vis molecular absorption spectrophotometry in gas phase as detection system. This technique is known as gas phase molecular

*e-mail: ovallesd@ula.ve

absorption spectrometry (GPMAS).[26] It was introduced for the first time in 1973 by Sity.[27] This technique involves the separation of the gaseous phase from the liquid phase using a gas-liquid separator and then is sweeping it into a flow through cell, which has been positioned in the cell compartment of an UV/Vis spectrophotometer.[28] Recently, Gallignani *et al*.[29] developed a method to determine Sb(III) in homeopathic products using FA-HG-GPMAS. The major weakness of that method is the low sensitivity (in mg $L^{-1}$ working range) as compared to many available methods based on AAS or ICP-AES, which have a working range in μg $L^{-1}$ or below. However, because pharmaceutical products containing organo-antimonials are formulated with relatively high content of antimony (80-100 mg $L^{-1}$) the proposed method could be very useful. On the other hand, conventional spectrophometer is common and less expensive equipment than AAS or ICP-AES.

Based on the above statements we have further developed the system proposed by Gallignani *et al*.[29] but utilizing a modification of the manifold and chemical parameters to allow the routine determination of total antimony in antileishmanial drugs. To the best knowledge of the authors, no work has been reported for the antimony determination in pharmaceutical samples by using a FA-HG-GPMAS coupled system. The effect of mineralization/oxidation of the organic pentavalent antimony of the samples, pre-reduction and hydride generation were optimized and adapted to the experimental manifold proposed in the present work.

## EXPERIMENTAL

### Apparatus

The FA-HG-GPMAS system used in this work is shown in Figure 1S (Supplementary material). It was mainly assembled from commercially available instruments and accessories. The continuous flow system consisted of an IPC peristaltic pump (*P1*) from Ismatec (Glattbrugg, Switzerland) of four channels in conjunction with a Varian HG system, Model VGA-77 (Springvale, Australia), which contains three channels peristaltic pump (*P2*); both pumps furnished with Tygon tubes for the transport of reagents and samples ($C_{Ac}$, $C_{PRed}$, $C_{Ox}$, $C_{Sam}$, $C_{Red}$, respectively), and three PTFE reaction coils (*R1-R3*). The Varian HG system incorporates a gas-liquid phase separator (*GLPS*), and provides a regulated supply of nitrogen divided into two branches (*Nb1 and Nb2*). The manifold includes a Rheodyne (Alltech, Waukegan, USA) manual selecting valve (*SV*), which permits the selective introduction in the system of either the carrier ($C_{Car}$) or the sample ($C_{Sam}$). A home made gas trap (*GT*) was used to eliminate the toxic stibine gas.[25] All measurements were made with a Perkin Elmer, Model Lambda 20, double beam spectrophotometer (Norwalk, CT, USA). The instrument was equipped with two Wilmad -10 x 2 cm- quartz flow cells (Buena, NJ. USA) placed in the optical path of the conventional UV/Vis spectrophotometer. A PC 586 equipped with a Perkin Elmer UV-WinLab Software was used for controlling the instrument and for data acquisition. Further details are given in Table 1S and Figure 1S.

### Reagents

All chemicals were of analytical-reagent grade from either Riedel-de Haën Sigma-Aldrich (GMBH Seelze, Germany) or J. T. Baker (NJ, USA), unless another source is indicated. Milli-Q water (18 MΩ $cm^{-1}$) was obtained from a Milli-Q purification system (Millipore Corporation, USA). It was used to prepare all solutions and to rinse the previously cleaned laboratory material. Stock standard solution (1000 μg $mL^{-1}$) of trivalent antimony was prepared by dissolving antimony potassium tartrate ($C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$ from Riedel-de Haën, Hannover, Germany) in 0.5 mol $L^{-1}$ tartaric acid. Working standard solutions were prepared by serial dilution of the stock solutions with water, immediately before use. Standard solution (500 μg $mL^{-1}$) of pentavalent antimony was prepared by dissolving potassium *pyro*-antimoniate ($K_4Sb_2O_7$ from General Chemical Division, AC&DC, NY, USA) in boiling water, cooled, and finally diluted with cold water. Working standard solutions of Sb(V) were prepared daily by serial dilution of a intermediate solution (20 μg $mL^{-1}$) with water. The $N_2$ used in this work was supplied by AGA (Maracaibo, Venezuela) with a certified purity of 99%.

### Drug samples

Three samples of the same lot of a commercial sample of meglumine antimoniate (Glucantime from Aventis Pharma Ltda, Brazil), here called GCT-1, GCT-2 and GCT-3 were used in this work. These samples are manufactured in ampoules of 5 mL containing 1.5 g of meglumine antimoniate equivalent to 0.405 g of Sb(V). Two samples of Ulamina from the same lot, called here as ULM-1 and ULM-2 were also used in this work. The dosage form of ULM is 10 mL unit dose vial of solution for injection with a nominal content under clinical study of about 0.100 g Sb(V). These last samples were kindly provided by the manufacturer (Centre of Parasitological Research José Witremumolo Torrealba, University of Los Andes, Mérida-Venezuela). The whole content of one ampoule (either 5 or 10 mL, respectively) was always diluted up to 1000 mL with water. Afterwards, for the determination of total antimony, intermediate solutions of samples were sonicated for 10 min and diluted up to 20 μg Sb $mL^{-1}$ with water. Samples within the dynamic linear calibration range were prepared at 5 μg Sb $mL^{-1}$ by appropriate dilution with water. The working sample solutions were prepared the same day of the analysis.

Standard addition studies were carried out by adding various aliquots (0, 5, 10, 15 and 20 mL, respectively) of a Sb(V) standard solution (20 μg $mL^{-1}$) to 50 mL of a sample solution containing a fixed amount of pentavalent antimony (about 2 μg $mL^{-1}$). This procedure was carried out using either GCT samples or ULM samples. Spiked parenteral pharmaceutical formulations were prepared following the above procedure.

### Procedure

The procedure runs by using simultaneously the two pumps (*P1* and *P2*), at room temperature (20-23 ºC), as indicated in Figure 1S, under the operating conditions indicated in Table 1S. In the set-up, *SV1* is switched to the acid agent position ($C_{Ac2}$) and *SV2* is switched to the sample position ($C_{Sam}$). Sample solution merges with the acid agent ($C_{Ac2}$) at the confluence point that goes toward the reaction coil *R1*. At the same time, the pre-reducing agent ($C_{PRed}$) merges with the acid solution ($C_{Ac1}$) at the confluence point *z* ($C_z$), where it merges with the acid sample stream toward the reaction coil *R2* in order to ensure the pre-reduction step. Subsequently, the obtained mixture (Sb-reduced) is then driven to the reaction coil *R3*. At the same time, the reducing agent ($NaBH_4$) flows through another channel ($C_{Red}$) towards the reaction coil *R3*. Then, in coil *R3*, the Sb(III) specie is quantitatively converted into its corresponding gaseous hydride form ($SbH_3$). The first branch of $N_2$ supplies inert gas to the inlet side of the reaction coil *R3* to improve the stripping of the stibine gas to the *GLPS*. Once the mixture reaches the *GLPS*, the liquid phase flows directly into the waste flask (*W*) and the gaseous phase ($SbH_3$, $H_2O$, $CO_2$, $H_2$, $N_2$, etc.) runs throughout the flow-gas cell (*SGC*) with the direct aid of the second branch of $N_2$ (carrier gas). At this time, the absorption spectrum is acquired in a continuous mode (1.5 min) by

triplicate. The baseline is established switching *SV2* to the position of the carrier solution ($C_{Car}$) keeping *SV1* in the acid medium position ($C_{Ac2}$). During this step, the reference gas cell (*RGC*) and the sample gas cell (*SGC*) in a continuous flow mode are cleaned and filled. After 3 minutes, the *RGC* is sealed and the *SGC* connected to the gas trap (*GT*). In this way, blank solution (all reagents) is introduced into the system.

## RESULTS AND DISCUSSION

### Preliminary tests

The previous test and the optimization -using the proposed FA-HG-GPMAS system- were carried out using standard solution of 5 µg Sb(III) $mL^{-1}$, standard solution of 5 µg Sb(V) $mL^{-1}$, sample solutions of GCT of 4.86 µg Sb(V) $mL^{-1}$ (nominal content) and ULM with a nominal content equivalent to 5 µg Sb(V) $mL^{-1}$, unless otherwise stated. The absorption spectra of $SbH_3$ were acquired in a continuous mode between 190 and 300 nm. A scan speed of 960 nm with 1 nm of nominal resolution was selected in this study as a compromise between analysis time and sample/reagent consumption by FA. Figure 1, shows representative gaseous $SbH_3$ UV-spectra from each species under study: (a) gaseous blank; (b)-(f) pentavalent antimony standard solutions at different concentrations; (g) standard solution of trivalent antimony; (h) sample solution of GCT; and (i) sample solution of ULM. As it can be seen, the gaseous blank (Figure 1a) has good transparency in the showed spectral region, while the UV spectra of the gaseous $SbH_3$ generated from Sb(V) and Sb(III) standard solutions at different concentrations (Figures 1b-g) show a broad band below about 240 nm, with a maximum absorption well defined at 195 nm. No maximum wavelength displacements were observed by modifying the antimony concentration. The analyzed samples showed the same pattern as the standards (Insert in Figure 1). Nevertheless, slight baseline displacements in the absorption spectra were observed.

The quantification of antimony in the described spectral system could be carried out -in principle- using the absorbance at 195 nm corrected by means of an horizontal baseline established at 250 nm (Insert in Figure 1). However, the absorption maximum of gaseous

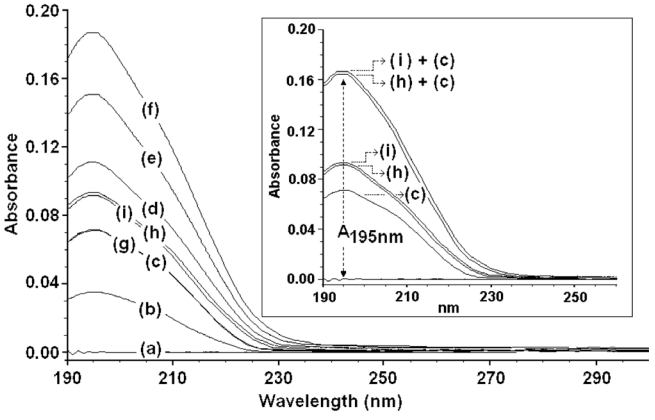


***Figure 1.*** *Representative UV absorption spectra of $SbH_{3(g)}$ evolved from Sb presents in both standard and sample solutions: (a)-(f) standard solutions containing, 0.0, 2.0, 4.0, 6.0, 8.0, and 10.0 µg Sb(V) $mL^{-1}$, respectively, (g) standard solution containing 4.0 µg Sb(III) $mL^{-1}$, (h) sample solution of GCT containing about 4.86 µg Sb $mL^{-1}$, (i) sample solution of ULM containing about 5.0 µg Sb $mL^{-1}$. Insert: representative spectra of sample solutions fortified with a standard solution of 4.0 µg Sb(V) $mL^{-1}$. Experimental conditions are described in Table 1S using the FA-HG-GPMAS system shown in Figure 1S*

$SbH_3$ is located close to the instrumental spectral limit, which is 190 nm. Additionally, it is evident that by using the described instrumentation, it is not possible to know the behaviour of the absorption band at wavelength lower than 190 nm. Keeping this in mind, the alternatives offered by the derivative spectroscopy were explored. As it is known, derivative spectroscopy is a powerful tool in UV-molecular absorption spectrophotometry to minimize the effects of the spectral interferences and baseline displacements. Initially, all the instrumental available derivative spectra ($D^1$ - $D^4$) were explored. From this last study, we concluded that second-order derivative spectroscopy ($D^2$) provides a more complete view about the behaviour of the stibine zero-order derivative spectrum ($D^0$). Insert in Figure 2 shows a representative spectra $D^2$ of $SbH_3$ generated from a Sb(V) standard -previously reduced-, recorded at different working times (3, 10 and 25 min, respectively). Certainly, the second order derivative spectra ($D^2$) showed an automatic correction of the displacement of the absorption spectra in regard to the baseline as the time of analysis lapses (Figure 2).

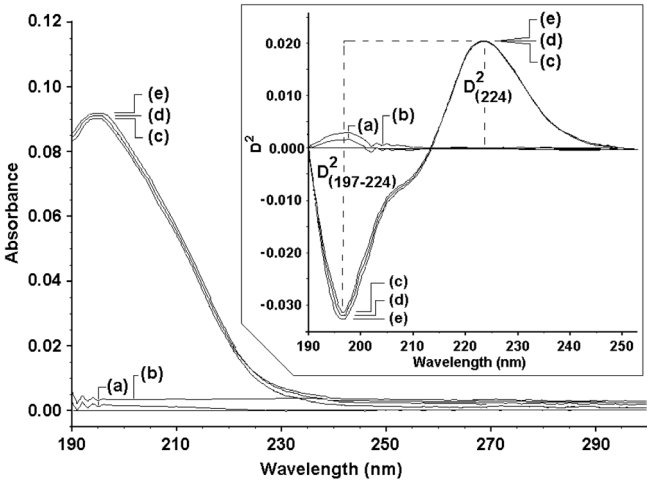


***Figure 2.*** *Representative spectra of $SbH_{3(g)}$ generated from a standard solution of 5.0 µg Sb(V) $mL^{-1}$: (a) baseline at start time; b) baseline at end time (c) standard measured at 3 min, (d) standard measured at 10 min, and (e) standard measured at 25 min. Insert: second-order spectra ($D^2$) show an automatic correction of the displacement of the absorption spectra in regard to the baseline as the time of analysis lapses; as it can be seen in the peak to zero baseline [$D^2_{(224nm)}$]. Experimental conditions are specified in Table 1S using the FA-HG-GPMAS system shown in Figure 1S*

In order to select a definitive measurement criterion, two $D^2$ potential analytical signals were evaluated: the absolute value of the peak (224 nm) to valley (197 nm) or [$D^2_{(224\text{-}197\ nm)}$]; and the value of the peak to zero baseline or [$D^2_{(224\ nm)}$]. The experimental determination was carried out evaluating all the analytical signals using a standard of 5 µg Sb $mL^{-1}$ (n= 20). The preliminary results clearly indicated the viability of using the peak to zero baseline of the second-order derivative [$D^2_{(224\ nm)}$] as measurement criterion for the determination of total antimony.

Complementary studies were carried out to select the appropriate smooth conditions by using different derivative windows -offered by the used UV-WinLab software- between 5 and 41 points. A value of 15 was selected as a commitment among the peak height (sensitivity), the band-shape, and the precision of the analytical signal.

As final preliminary tests, the nature and concentration of the dissolution medium of the samples were investigated because the yield of the stibine is highly dependent on these parameters. Relatively concentrated HCl has been the common acid medium used in HG.[30]

Based on the above fact, hydrochloric acid was selected as a medium able to generate an equivalent response signal for both species of antimony under study, Sb(III) and Sb(V).

On the whole, instrumental-spectroscopic parameters and reagent concentrations were optimized to achieve the best analytical performance of the FA-HG-GPMAS system for the quantification of total antimony in pharmaceutical samples. The optimization procedure was carried out using a single-variation method.

**Physical parameters**

*Flow rate*

The flow rate values were distributed according to the total volume arriving to the *GLPS* (Figure 1S), which used to be 10 mL for the vapour generation system Varian VGA-77. In all instants the mayor flow rate was kept for the sample as recommended by the operation manual; in this particular case, 4 mL $min^{-1}$, and the other 6 mL $min^{-1}$ were distributed as needed depending on the HG-sensitivity and the concentration of the reagents. The flow rates of the two branches of $N_2$ were kept under the specifications of the vapour generation system (Varian VGA-77). Well-defined peaks were obtained using the flow rates of the two branches of $N_2$. Table 1S shows the selected flow rate values for the proposed FA-HG-GPMAS system.

*Transfer line length*

The tubing length was kept as short as possible in regard to the designed manifold; mainly that one (25 cm) which separates the VGA-77 system from the sample compartment of the spectrophotometer.

*Reaction coil length*

Coil *R1*: mixing coils of different lengths, from 25 to 250 cm (0.5 mm, id), were inserted between the confluence point of $C_{Ox}/C_{Ac2}$ and $C_{Car}/C_{sam}$ (Figure 1S). According to the results, no apparent differences in the response signal were observed with variation of coil length longer than 50 cm (Figure not shown). Therefore, a length of 100 cm and an internal diameter of 0.5 mm was considered sufficient in subsequent studies to ensure the maximum $SbH_3$ response signal. Coil *R2*: mixing coils of different lengths, from 0 to 250 cm, were inserted between the confluence point of $C_{Ac1}/C_{PRed}$ and coil *R1* (Figure 1S). Results did not show apparent differences in the response signal using coil length longer than 25 cm, but higher response signal was obtained with an internal diameter of 0.8 mm instead of 0.5 mm (Figure not shown). Therefore, a length of 50 cm and an internal diameter of 0.8 mm were considered sufficient in subsequent studies to ensure the on-line pre-reduction procedure. Coil *R3*: mixing coils of different lengths were not explored because the appropriate length was supplied by the Varian VGA-77 vapour generation system. Selected values of all these experimental parameters are summarized in Table 1S.

**Chemical parameters**

*Oxidizing/mineralizing agent*

Today, it is well known that meglumine antimoniate and sodium stibogluconate are pentavalent organic antimonials (Sb-O-C) that exist in equilibrium as a complex mixture of carbohydrate-antimony polymers.[31] When no digestion of the organic matrix is applied to the samples, recovery of antimony from these matrices might not reach 100% because of non-hydride forming organic antimony species. Consequently, an efficient mineralization step to destroy organic antimony compounds in the samples is a pre-requisite for reliable determination of antimony based on HG.[32] The influence of potassium persulfate ($K_2S_2O_8$) employed for the mineralization of the organic antimonials (Sb-O-C) from pharmaceutical samples has been used successfully in a previous work.[25] In the present work, $K_2S_2O_8$ -prepared in HCl 10%, v/v- at different concentrations ($C_{Ox}$) was used for this purpose. The results showed that the presence of $K_2S_2O_8$ did not increase the response signal as it was expected (Figure 2S-A). On the contrary, the $SbH_3$ response signal was falling at concentrations of $K_2S_2O_8$ higher than 0.5%, m/v. This behaviour was attributable to the possibility of the prior consumption of KI by the strong oxidant; which would ultimately decreases the efficiency of the pre-reduction. Moreover, the attenuation of the $SbH_3$ response signal at higher concentrations than 0.5% (m/v) of $K_2S_2O_8$ could be attributable to the diluting effect caused by the gaseous species generated as subproducts by the oxidation process.

The removal of $K_2S_2O_8$ from the proposed FA-HG-GPMAS produced concentrations of total antimony in analyzed real samples as expected. This behaviour can be explained in two ways: the diluted samples are sonicated by ten minutes prior sample analysis, which could facilitate the sample hydrolysis; and when high dilutions of pentavalent-antimony carbohydrates take place (as it occurred in this assay), the polymerized species suffer hydrolysis into simpler ones, producing relatively labile adducts between Sb(V) and the matrix.[31] Therefore, this last behaviour will facilitate the pre-reduction and reduction steps with KI and $NaBH_4$, respectively, without interference of the oxidant agent.

As consequence of this study, $K_2S_2O_8$ was not used in further studies. Instead of this, *SV1* was switched to the acid position ($C_{Ac2}$) using 50% (v/v) of HCl. Under this procedure, standards and samples were prepared in just milli-Q water for further studies.

*Pre-reducer agent*

It is well known that HG is highly dependent on the oxidation state of antimony. Optimum stibine generation is found starting from Sb(III), and slowly stibine generation is obtained starting from Sb(V).[32] The approach most known to reduce Sb(V) to Sb(III) in HG systems is using KI in acidic medium. Therefore in this work, Sb(V) was reduced to Sb(III) with KI in acid medium, previous to the HG. In order to obtain the maximum $SbH_3$ response signal, the effect of KI concentration within the range 0-25% m/v ($C_{PRed}$) was tested at a constant HCl concentration of 20%, v/v ($C_{Ac1}$). The results (Figure 2S-B) showed that there was a steady increase of the analytical signal generated from Sb(V) reaching the optimum value near to 10% (m/v) of KI. At higher concentrations than 10% (m/v), a plateau was reached, which was a clear indication of the completeness of the pre-reduction of Sb(V) to Sb(III). As expected stibine generation efficiency from Sb(III) was not affected. With the purpose of guarantying a quantitative pre-reduction of Sb(V), a concentration of 15% (m/v) KI was chosen for further studies.

*Acid concentration*

The acid medium has effect on: sample hydrolysis in coil *R1*, pentavalent antimony pre-reduction in coil *R2*, and stibine generation in coil *R3*. As it was exposed in the preliminary studies, hydrochloric acid was selected as acid medium. The effect of the concentration of HCl on $SbH_3$ generation was firstly tested by changing it within 0 and 50% (v/v) on channel $C_{Ac1}$, and then on channel $C_{Ac2}$. After that a concentration of 20% HCl was kept constant on the channel $C_{Ac1}$, and the HCl concentration of the channel $C_{Ac2}$ was then rechecked varying it within 0 and 50% (v/v). As can be appreciated in Figure 2S-C, the effect of acid concentration on stibine generation efficiency was very marked: The maximum $SbH_3$ response signal was reached at 30% v/v HCl. Consequently, in order to guarantee an appropriate acidic medium for the quantitative stibine generation efficiency, a concentration of 20% (v/v) HCl was selected for the channel $C_{Ac1}$ and 50% (v/v) for channel $C_{Ac2}$. This last selection ensured low dispersion on reaction coil *R2*.

*Sodium borohydride concentration*

Having established that the efficiency of hydride generation is dependent simultaneously on the concentration of other related reagents, the influence of $NaBH_4$ concentration, within the range 0.01 up to 1.0% (m/v) -prepared in 0.1% (m/v) of sodium hydroxide- was explored at a constant and optimized KI and HCl concentrations (Figure 2S-D). The results showed that, in the FA-HG-GPMAS system, the analytical signal reaches a maximum at very low concentrations of $NaBH_4$ (0.1%, m/v), decreasing significantly at higher concentrations than 0.4% m/v, due certainly to the dilution of the hydride -in the flow gas cell- by the hydrogen. As a result, a concentration of 0.2% (m/v) of $NaBH_4$ was selected as the appropriate to ensure quantitative generation of stibine at the maximum concentration of antimony in the linear calibration.

By concluding the optimization and in view of the results, it could be inferred that the FA-HG-GPMAS system could be reconstructed and simplified by eliminating the acid channel $C_{Ac1}$, the valve *SV1* and the coil *R1*. In this case the standard solutions and sample solutions must be prepared in HCl at an appropriate concentration.

**Analytical performance of the system**

The analytical figures of merit achieved by the proposed method are listed in Tables 1-2. A linear dynamic range up to 10 μg $mL^{-1}$ for Sb(V) was achieved with a limit of quantification (10σ) of 0.39 μg $mL^{-1}$. The results showed a variation coefficient better than the unit (% RSD). The accuracy of the analytical method was established by determining total antimony in spiked samples of GCT and ULM. In both cases, the recoveries of antimony were not less than 95% and not more than 101% of total antimony, with a relative standard deviation better than 1%, which was an indication of the reliability of the proposed method (Table 3). The sample throughput was up to 30 samples $h^{-1}$, which could be considered appropriate for quality control. Regarding the analytical sensitivity, it was lower than those obtained by using either HG-AAS or ICP techniques, but comparable to that obtained by using HG-FTIR.[25]

**Interference study**

Interference studies were undertaken by means of two approaches: using derivative spectroscopy and by means of standard addition studies. The first approach eliminated at first instant those gaseous interferences very close to the UV cut limit (190 nm). The second approach allowed determining any physical or chemical interference from the matrix and also from the on-line chemical procedure. In this study, the slope obtained by the external calibration method did not differ significantly ($p$= 0.05) from the slope obtained by the standard addition method (Table 2).

Other possible concomitants usually present in the pharmaceutical formulation -as antioxidants-, such as anhydrous sodium sulphite (0.18 mg $mL^{-1}$) and potassium bisulphite (1.6 mg $mL^{-1}$) apparently not showed any remarkable effect on antimony determination by using the selected measurement criterion.

The interference in HG coming from inter-element effects, which suppress or enhance the true signal, is very important. Contamination of leishmanicidal pharmaceutical products with this type of species such as lead and arsenic is not expected unless poor quality control of manufacturing process allows the use of impure active chemicals.[4] Arsenic is the most probable impurity of antimonial drugs.[4] Arsenic can generate gaseous $AsH_3$ which could enhance or suppress the true response signal of $SbH_3$. Therefore, the presence of this inter-element as impurity of real samples was explored by FA-HG-FTIR at different concentration ratios.[25] As can be seen in Figure 3S, the presence of As in the tested drugs was not detected. Under these conditions, arsine interference was discarded as potential interfering specie in the developed method.

**Table 1.** Analytical figures of merit of the proposed FA-HG-GPMAS method for total antimony determination in commercial samples of antileishmanial drugs

| Feature | Sb(III) | Sb(V) |
|---|---|---|
| Dynamic range (μg $mL^{-1}$) | Up to 10 | Up to 10 |
| Linear equation [a] | $Y = -6.5x10^{-4} + 4.89x10^{-3}$ [X] | $Y = -5.3x10^{-5} + 4.91x10^{-3}$ [X] |
| Correlation coefficient | 0.9998 | 0.9999 |
| SD (y/x) | $3.9 \times 10^{-4}$ | $1.9 \times 10^{-4}$ |
| LOD (μg $mL^{-1}$) | 0.24 | 0.12 |
| LOQ (μg $mL^{-1}$) | 0.81 | 0.39 |
| Precision (% RSD; n = 10) [b] | 0.81 | 0.58 |
| Sample throughput ($h^{-1}$) | 30 | 30 |

[a] Y = a + b(X), where Y = second-order derivative signal ($d^2A/d\lambda^2$) of the peak to baseline at 224 nm; X = concentration of Sb (μg $mL^{-1}$). [b] Instrument precision estimated by measuring ten replicates of one sample solution expressed as relative standard deviation.

**Table 2.** Results obtained for the analysis of commercial samples of antileishmanial drugs

| | | FA-HG-GPMAS | | FA-HG-FTIR [d] |
|---|---|---|---|---|
| Sample [a] | Calibration mode | Equation [b] Y = a + b [X] | [Sb] [c] (g $ampoule^{-1}$) | [Sb] [c] (g $ampoule^{-1}$) |
| GCT-1 | Simple | | 0.405 (± 0.70) | 0.401 (± 0.25) |
| GCT-2 | Simple | | 0.402 (± 0.76) | |
| GCT-3 | Simple | $Y = -5.30 \times 10^{-5} + 4.91 \times 10^{-3}$ [X] | 0.403 (± 0.24) | |
| ULM-1 | Simple | | 0.102 (± 0,51) | 0.101 (± 1.0) |
| ULM-2 | Simple | | 0.101 (± 0,89) | |
| GCT | Standard addition | $Y = -1.03 \times 10^{-2} + 4.92 \times 10^{-3}$ [X] | 0.406 (± 0.40) | - |
| ULM | Standard addition | $Y = -1.08 \times 10^{-2} + 4.91 \times 10^{-3}$ [X] | 0.100 (± 0.92) | - |

[a] GCT = Glucantime: (1, 2 and 3) are from the same lot with a nominal content of 0.405 g $ampoule^{-1}$); and ULM = Ulamina: (1 and 2) are from the same lot with a nominal content of 0.100 g $ampoule^{-1}$). [b] Y = a + b(X), where Y = second-order derivative signal ($d^2A/d\lambda^2$) of the peak to baseline at 224 nm; X = concentration of Sb (μg $mL^{-1}$). [c] Mean ± relative standard deviation in per cent (n=3). [d] Reference method.[25]

**Table 3.** Recovery of Sb(V) added to commercial samples of antileishmanial drugs

| Sample | [Sb] endogenous (μg mL$^{-1}$) [a] | [Sb] added (μg mL$^{-1}$) | [Sb] found (μg mL$^{-1}$) [b] | Recovery (%) |
|---|---|---|---|---|
| GCT [c] | 2.03 (± 0.02) | 2.00 | 3.94 (± 0.96) | 95.5 |
| | | 4.00 | 5.92 (± 0.06) | 97.3 |
| | | 6.00 | 8.02 (± 0.13) | 99.8 |
| | | 8.00 | 9.95 (± 0.40) | 99.0 |
| ULM [d] | 2.00 (± 0.01) | 2.00 | 3.96 (± 0.64) | 98.0 |
| | | 4.00 | 5.96 (± 0.96) | 99.0 |
| | | 6.00 | 8.01 (± 0.42) | 100.2 |
| | | 8.00 | 9.97 (± 0.03) | 99.6 |

[a] Concentration (± standard deviation; n = 3). [b] Concentration (Relative standard deviation, n = 3). [c] Glucantime. [d] Ulamina.

### Analysis of real samples

The proposed FA-HG-GPMAS system was successfully applied to the determination of total antimony in two parenteral pharmaceutical formulations. Total antimony was determined in diluted commercial samples of GCT and ULM using the conditions described in Table 1S. The total antimony concentration in the analyzed samples of GCT and ULM (Table 2) was in good agreement with that of the prescribed amount (0.405 g Sb ampoule$^{-1}$ and 0.100 g Sb ampoule$^{-1}$, respectively). In addition, the real samples were also analyzed by an alternative method (FA-HG-FTIR),[25] and no significant differences were achieved at 95% confidence levels (*t*-test) by comparing the two sets of results (Table 2). These results again demonstrated the general reliability of the developed method.

## CONCLUSIONS

The determination of total antimony in antileishmanial drugs is very important for the quality control in the pharmaceutical field. In this context, the development of a new method for the determination of total antimony in samples of injectable drugs used for leishmaniasis treatment, based on the coupling of flow analysis, hydride generation, and derivative molecular absorption spectrophotometry in gas phase is proposed. As the leishmanicidal drugs content high level of antimony, molecular absorption spectrophotometry in gaseous phase seems ideal for analysing the total antimony content in these products.

This method provides some significant advantages such as accessibility of instrumentation and low cost, low reagent consumption, reduced sample handling, and adequate sample throughput, which could make the procedure feasible for pharmaceutical quality control either in the pharmaceutical industry or official governmental agencies in developing countries. To the best of our knowledge, this is the first study dealing with total antimony determination in meglumine antimoniate parenteral products using the proposed approach.

Finally, taking into account the promising results obtained using the developed method; further detailed studies to demonstrate its potential in speciation analysis of antimony in pharmaceuticals products are underway.

## SUPPLEMENTARY MATERIAL

Table 1S and Figures 1S, 2S and 3S. This material is available at http://www.quimicanova.sbq.org.br, in PDF file.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of the 'Council of Scientific, Humanistic and Technological Development' (CDCHT) of the University of Los Andes (ULA) from Venezuela, Project No FA-371-06-08-B, and SE-FA-03-07-08.

## REFERENCES

1. Ridley, R. G.; *Leishmaniasis. Special Program for Research and Training in Tropical Diseases (TDR)*; World Health Organization; Switzerland 1211, Geneva, 2005; http://www.who.int/tdr/diseases/leish/pubs.htm, accessed in January 2007.
2. Yepez, J. Y.; Scorza-Dager, J. V.; *Bol. Mal. Salud Amb*. **2003**, *43*, 9.
3. Masmoudi, A.; Maalej, N.; Mseddi, M.; Souissi, A.; Turki, H.; Boudaya, S.; Bouassida, S.; Zahaf, A.; *Med. Mal. Infect.* **2005**, *35*, 42.
4. Romero, G. A. S.; Flores, E. M. M.; Noronha, E. F.; Macedo, V. O.; *Mem. Inst. Oswaldo Cruz* **2003**, *98*, 145.
5. Miranda, E. S.; Miekeley, N.; Carvalho, R. R.; Paumgartten, F. J. R.; *Reprod. Toxicol.* **2006**, *21*, 292.
6. Trivelin, L. A.; Rohwedder, J. J.; Rath, S.; *Talanta* **2006**, *68*, 1536.
7. Quentel, F.; Filella, M.; *Anal. Chim. Acta* **2002**, *452*, 237.
8. Krachler, M.; Emons, H.; Zheng, J.; *Trends Anal. Chem.* **2001**, *20*, 79.
9. Smichowski, P.; Madrid, Y.; Cámara, C.; *Fresenius´ J. Anal. Chem.* **1998**, *360*, 623.
10. Nash, M. J.; Maskal, J. E.; Hill, S. J.; *Environ. Monit.* **2000**, *2*, 97.
11. Das, A. K.; Guardia, M.; Cervera, M. L.; *Talanta* **2001**, *55*, 1.
12. Bloomfield, M. S.; Dow, D. A.; Prebble, K. A.; *J. Pharm. Biomed. Anal.* **1992**, *10*, 779.
13. Franco, M. A.; Barbosa, A. C.; Rath, S.; Dorea, J. G.; *Am. J. Trop. Med. Hyg.* **1995**, *52*, 435.
14. González, M. J.; Renedo, O. D.; Martínez, M. J. A.; *Talanta* **2007**, *71*, 691.
15. *Brazilian Pharmacopoeia*; *Meglumine antimoniate*, 4th ed., Brazilian Pharmacopoeia Convention: Sao Paulo, 2002.
16. Flores, E. M. M.; Santos, E. P.; Barin, J. S.; Zanella, R.; Dressler, V. L.; Bittencourt C. F.; *J. Anal. At. Spectrom.* **2002**, *17*, 819.
17. Flores, E. M. M.; Paula, F. R.; Silva, F. E. B.; Moraes, D. P.; Paniz, J. N. G.; Santos, E. P.; Dressler, V. L.; Bittencourt, C. F; *At. Spectrosc.* **2003**, *24*, 15.
18. Figueiredo, E. C.; Dedina, J.; Arruda, M. A. Z.; *Talanta* **2007**, *73*, 621.
19. Görög, S.; *J. Pharm. Biomed. Anal.* **2005**, *36*, 931.
20. Rath, S.; Jardim, W. F.; Dórea J. G.; *Fresenius´ J. Anal. Chem.* **1997**, *358*, 548.
21. González, M. J. G.; Renedo, O. D.; Arcos, M. J.; *Talanta* **2005**, *68*, 67.
22. Figueiredo, E. C.; Luccas, P. O.; Arruda, M. A. Z.; *Anal. Lett.* **2006**, *39*, 543.
23. Trivelin, L. A.; Rohwedder, J. J.; Rath, S.; *Talanta* **2006**, *68*, 1536.
24. Almeida, V. G. K.; Lima, F. M.; Cassella, R. J.; *Talanta* **2007**, *71*, 1047.
25. Gallignani, M.; Ayala, C.; Brunetto, M. R.; Burguera, M.; Burguera, J. L.; *Talanta* **2003**, *59*, 923.
26. Cresser, M. S.; *Anal. Chim. Acta* **1976**, *85*, 253.
27. Sity, A.; *Anal. Chem.* **1973**, *45*, 1744.
28. Haghighi, B.; Kurd S. F.; *Talanta* **2004**, *64*, 688.
29. Gallignani, M.; Ovalles, F.; Brunetto, M. R.; Burguera M.; Burguera, J. L.; *Talanta* **2005**, *68*, 365.
30. Dedina, J.; Tsalev, D. L.; *Hydride generation atomic absorption spectrometry,* John Willey & Sons: Chichester, 1995.
31. Roberts, W. L.; McMurray, W. J.; Rainey, P. M.; *Antimicrob. Agents Chemother.* **1998**, *42*, 1076.
32. Krachler, M.; Burow, M.; Emons, H.; *Analyst* **1999**, *124*, 923.

**FLOW ANALYSIS-HYDRIDE GENERATION-GAS PHASE DERIVATIVE MOLECULAR ABSORPTION SPECTROPHOTOMETRIC DETERMINATION OF ANTIMONY IN ANTILEISHMANIAL DRUGS**

**Máximo Gallignani y María del Rosario Brunetto**
Departamento de Química, Facultad de Ciencias, Universidad de Los Andes, Apartado postal 440, Mérida 5101-A, Mérida, Venezuela
**Fernando Ovalles***
Departamento de Análisis y Control, Facultad de Farmacia, Universidad de Los Andes, Mérida 5101-A, Mérida, Venezuela

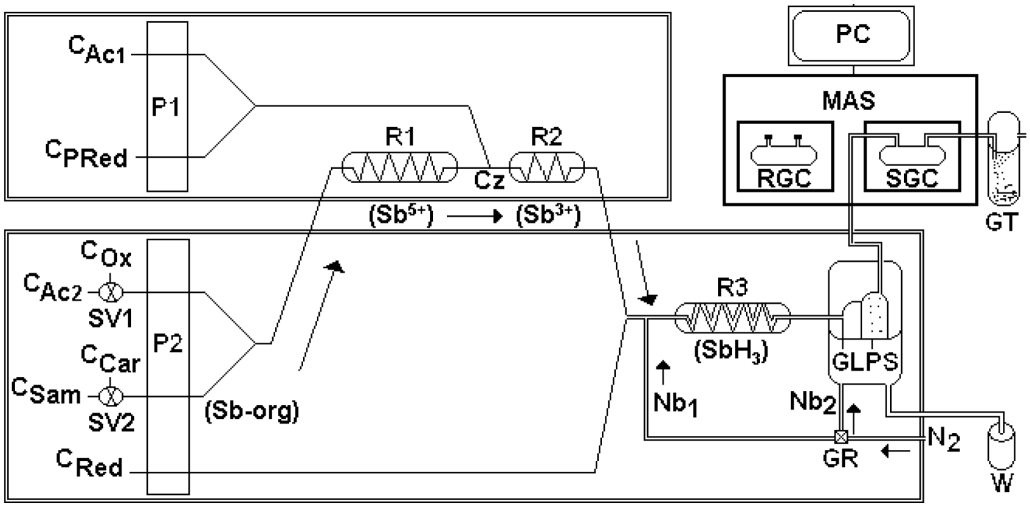


***Figure 1S.*** *Schematic diagram of the FA-HG-GPMAS coupled system proposed. $C_{Ac}$, acid agent; $C_{PRed}$, pre-reducing agent; $C_{Ox}$, oxidizing agent; $C_{Car}$, carrier; $C_{Sam}$, sample/standard; $C_{Red}$, final reducing agent; P1, independent peristaltic pump; P2, peristaltic pump from the Varian GLPS system; SV, manual selecting valve; R1, oxidation/mineralization reaction coil; R2, pre-reducing reaction coil; R3, hydride generation coil; Cz, confluence point; GLPS, gas-liquid phase separator VGA-77; $N_2$; nitrogen gas entry; Nb, nitrogen branch; GR, regulated supply of nitrogen; MAS, molecular absorption spectrophotometer; RGC, reference gas cell; SGC, sample gas cell; PC, Computer; GT, gas trapper; W, waste; Sb-org, organic antimony. Further details are given in Table 1S*

*e-mail: ovallesd@ula.ve

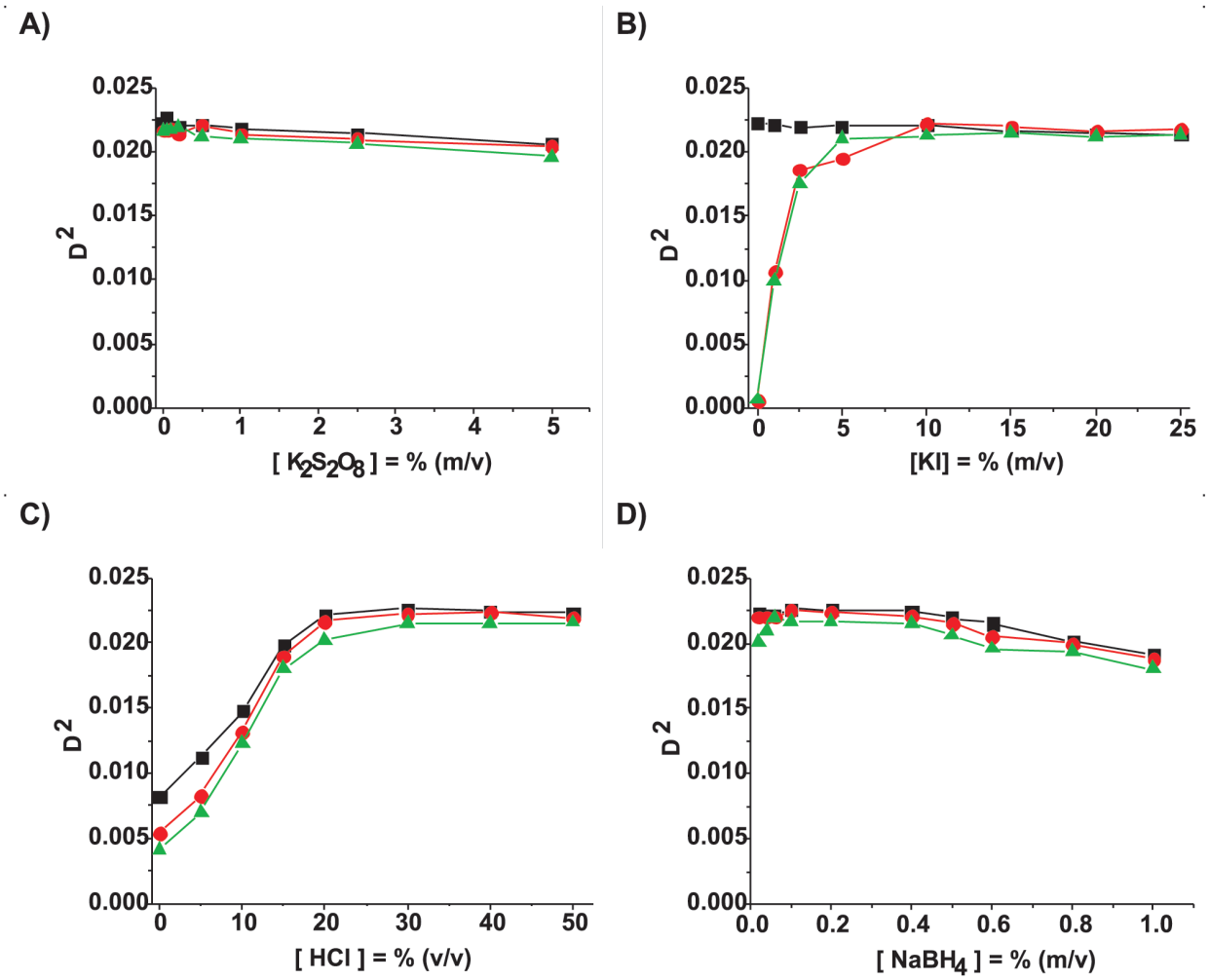


***Figure 2S.*** *Effect of chemical parameters on the analytical signal: A) oxidizing agent; B) pre-reducing agent; C) acidic medium; D) reducing agent. (■) 5.0 µg Sb(III) mL$^{-1}$; (●) 5.0 µg Sb(V) mL$^{-1}$; and (▲) sample solution containing an equivalent concentration to the standards (≅ 4.86 µg Sb mL$^{-1}$). D$^2$, signal of the second derivative spectrum [peak to zero baseline D$^2_{(224\ nm)}$]*

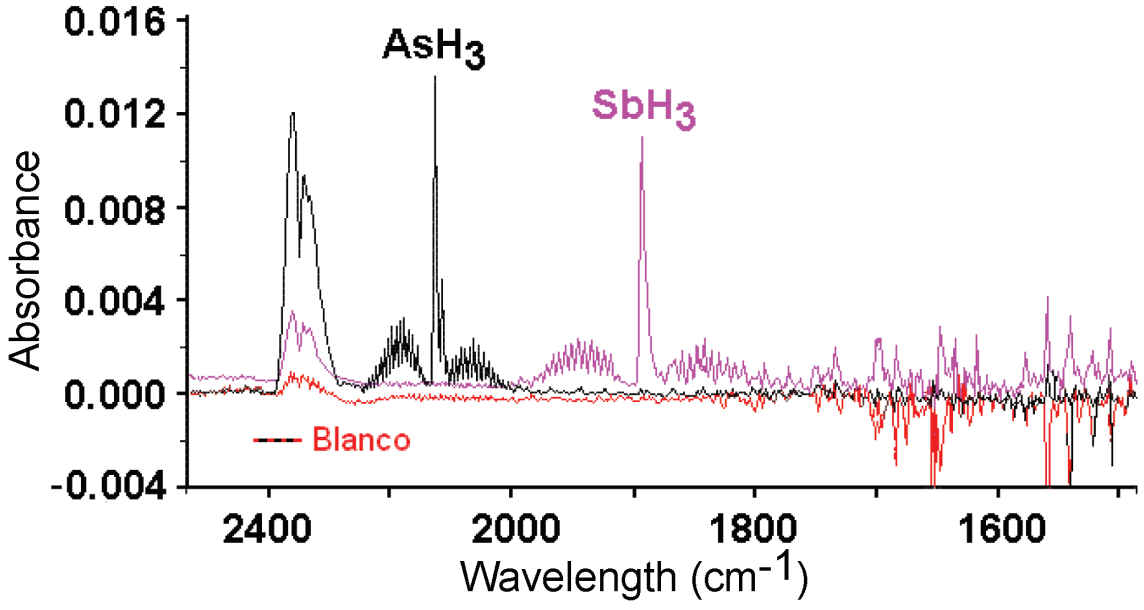


***Figure 3S.*** *Representative FTIR spectra of (a) blank, (b) sample of GCT 100 µg Sb(V) mL$^{-1}$, and (c) sample of GCT 100 µg Sb(V) mL$^{-1}$ enrichment with a standard of 10 µg As(III) mL$^{-1}$. Spectra were obtained following the FA-HG-FTIR method proposed by Gallignani et al..*[25]

**Table 1S.** Operating conditions of the FA-HG-GPMAS coupled system

| Instrumental and spectroscopic parameters | | | Description |
|---|---|---|---|
| UV-MAS | Spectral range | | 190-300 nm |
| | Nominal resolution | | 1 nm |
| | Scan speed | | 960 nm |
| | Measurement criteria | | Second order derivative of the peak to zero baseline at 224 nm |
| | Gas cell type: | Manufacturer | Wilmad |
| | | Path length | 100 mm |
| | | Internal diameter | 22 mm |
| | | Windows material | Quartz (circular shape) |
| | | Dead volume | 31.4 mL |
| | | Operating temperature | Room (20-23 ºC) |
| **Flow analysis-Hydride generation system parameters** | | | **Description** |
| FA-HG | ($C_{Ac1}$) acid channel composition | | HCl (20%, v/v) |
| | ($C_{Ac1}$) acid channel flow rate | | 2 mL $min^{-1}$ |
| | ($C_{PRed}$) pre-reducing agent composition | | Aqueous KI (15%, m/v) |
| | ($C_{PRed}$) pre-reducing agent flow rate | | 2 mL $min^{-1}$ |
| | ($C_{Ac2}$) acid channel composition | | HCl (50%, v/v) |
| | ($C_{Ac1}$) acid channel flow rate | | 1 mL $min^{-1}$ |
| | ($C_{Car}$) carrier composition | | $H_2O$ |
| | ($C_{Car}$) carrier flow rate | | 4 mL $min^{-1}$ |
| | ($C_{Sam}$) sample composition | | Sb(V) from Glucantime® and Ulamina; about 5 µg $mL^{-1}$ |
| | ($C_{Sam}$) standard composition | | Sb(V) from $K_4Sb_2O_7$; 0 - 10 µg $mL^{-1}$ |
| | ($C_{Sam}$) diluting agent of samples/standards | | $H_2O$ |
| | ($C_{Sam}$) sample/standard flow rate | | 4 mL $min^{-1}$ |
| | ($C_{Red}$) reducing agent composition | | $NaBH_4$ (0.2%, m/v) in NaOH (0.1%, m/v) |
| | ($C_{Red}$) reducing agent flow rate | | 1 mL $min^{-1}$ |
| | ($N_{b1}$) stripping gas flow rate | | 45 mL $min^{-1}$ |
| | ($N_{b2}$) carrier gas flow rate | | 45 mL $min^{-1}$ |
| | (GR-$N_2$) gas pressure | | 50 psi |
| | (GT) gas trapping composition | | $AgNO_3$ (0.5%, m/v) |
| | (R1) acidifying coil | | PTFE (1000 x 0.5 mm) |
| | (R2) pre-reducing reaction coil | | PTFE (500 x 0.8 mm) |
| | (R3) hydride generation coil | | PTFE (600 x 1.5 mm) |