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Quantitation of 2-chlorovinylarsonous acid in human urine by automated solid-phase microextraction–gas chromatography–mass spectrometry

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Abstract

Lewisite [dichloro(2-chlorovinyl)arsine] is a highly toxic chemical warfare agent with vesicant properties. The accidental exposure to lewisite or its intentional use as a chemical terrorism weapon are a public health threat and warrant investigations for the development of analytical methods to detect biomarkers of exposure to lewisite. Under aqueous conditions, lewisite rapidly hydrolyzes to the non-volatile 2-chlorovinylarsonous acid (CVAA). We have developed a sensitive, simple, and automated method for measuring CVAA in human urine. The assay is based on the use of solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) after derivatization of the CVAA with 1,3-propanedithiol (PDT). The volatile CVAA-PDT is adsorbed onto a SPME fiber and analyzed by GC–MS. The assay was validated on human urine samples spiked with CVAA to determine the accuracy, precision, and limit of detection (LOD). The LOD was 7.4 pg in 1 ml of urine. Published by Elsevier Science B.V.

Keywords: 2-Chlorovinylarsonous acid

1. Introduction

Lewisite [dichloro(2-chlorovinyl)arsine], a highly toxic chemical warfare agent with vesicant properties, was developed during World War I [1]. Lewisite, like the sulfur and nitrogen mustards, irritates the skin and eyes and is also poisonous when inhaled; but, unlike the mustards, its clinical effects appear within seconds of exposure [1]. Lewisite has been produced by several countries, including the USA, and large stockpiles are still present around the world [2]. The accidental exposure to chemical warfare agents by workers involved in the destruction of chemical weapons, and the confirmed use of chemical warfare agents in past conflicts (e.g., Iran–Iraq, Soviet Union–Afghanistan) [1] are a public health threat that warrants investigations for the development of analytical methods to detect biomarkers of exposure to chemical warfare agents, such as lewisite.

Lewisite is very reactive; in aqueous media, it rapidly hydrolyzes to a stable, non-volatile, water soluble derivative, 2-chlorovinylarsonous acid (CVAA), which is also toxic [3]. The occurrence of

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CVAA has never been shown except as a hydrolysis product of lewisite [4].

Several methods, based on the detection of CVAA by using gas chromatography (GC), have been reported for the identification of lewisite in environmental [4–6] and biological [7,8] samples. Since CVAA is non-volatile, all of these methods involve the derivatization of CVAA by a vicinal dithiol to form a volatile cyclic disulfide amenable to GC analysis. Fowler et al. described a GC method with sulfur-specific flame photometric detection for the quantitation of CVAA in water after derivatization with 1,2-ethanedithiol (EDT) and subsequent extraction with toluene [4]. The method limit of detection (LOD) was 5.5 ng/ml. CVAA was also detected in the urine of guinea pigs exposed to lewisite [8]. Urine samples were extracted using solid-phase extraction (SPE), the SPE extract was derivatized with EDT, and the CVAA-EDT cyclic disulfide was detected using both GC–mass spectrometry (MS) and GC coupled with atomic emission spectroscopy. The method was linear from 200 to 4000 ng/ml and was able to detect CVAA at levels $\leq$100 ng/ml in 1-ml urine samples [8]. Similarly, Fidder et al. extracted CVAA from urine and blood samples of guinea pigs exposed to lewisite using SPE after derivatization with 2,3-dimercapto-1-propanol (BAL, British Anti-Lewisite). The CVAA-BAL in the SPE extract was subsequently derivatized with heptafluorobutyryl imidazole and analyzed by GC–MS [7].

Solid-phase microextraction (SPME) coupled to GC–MS has been applied to the analysis of CVAA in environmental samples [5,6]. CVAA was determined in water samples and soil/sediment extracts after derivatization with EDT or 1,3-propanedithiol (PDT) followed by SPME–GC–MS; the LOD was 2 ng/ml in aqueous solution [5]. Also, CVAA was simultaneously extracted and derivatized from soil samples using an extraction solvent containing ascorbic acid and PDT prior to the analysis by SPME–GC–MS or SPME–GC with sulfur-specific flame photometric detection; the LOD ranged between 0.1 to 0.5 μg/g soil [6].

SPME is a rapid, sensitive, and solvent-free extraction method that involves exposing a fused-silica fiber coated with a non-volatile polymeric material to a sample or its headspace. During exposure, the volatile and semi-volatile components of the sample with a chemical affinity for the SPME fiber coating are absorbed and retained on the fiber. Subsequently, the absorbed analytes may be thermally desorbed in the injector of a gas chromatograph for separation and quantitation [9]. SPME has found many applications in pharmaceutical, environmental, food and flavor, toxicology, and forensic analysis [10]. SPME coupled to GC–MS and GC with nitrogen–phosphorous detection has been used to detect nerve agents in natural water samples at trace levels (e.g., ng/ml) [11].

In this paper, we describe the first SPME–GC–MS method for measuring CVAA in human urine. This sensitive, simple, and highly automated assay is suitable for the assessment of lewisite exposure in clinical and forensic specimens.

2. Materials and methods

2.1. Chemicals

We obtained CVAA from the US Army Medical Research Institute of Chemical Defense (USAMRICD, Aberdeen Proving Ground, MD, USA). Ammonium acetate buffer (10% ammonium acetate–20% glacial acetic acid) was obtained from Lab Depot (Atlanta, GA, USA). Phenyl arsonic acid (PhAs) and PDT (99%) were obtained from Pfaltz & Bauer (Waterbury, CT, USA) and Sigma–Aldrich (Milwaukee, WI, USA), respectively. HPLC-grade water (J.T. Baker, Phillipsburg, NJ, USA) was used for the preparation of all solutions. Helium gas (99.999%) was obtained from Air Products (Atlanta, GA, USA). All chemicals and solvents were used without further purification.

2.2. Preparation of standard solutions and quality control (QC) materials

A 4.5 μg/ml stock solution of CVAA was prepared by diluting 5 μl of a 4.5 mg/ml aqueous solution of CVAA in 5 ml of water. Next, 100 μl of the stock solution was diluted into 25 ml of water to give a final concentration of 18 ng/ml. This latter

CVAA solution, stored at 4 °C, was used to prepare seven standard solutions whose concentrations encompassed the entire linear range of the method; the concentration of each standard was adjusted such that a 25 μl aliquot in 1 ml of urine gave the desired CVAA amount (i.e., 41–1373 pg). A stock solution of PhAs was prepared by dissolving 50.0 mg of PhAs in 25 ml water. To prepare the 16 ng/ml working reference standard solution of PhAs, the stock solution was diluted with water in two steps, such that a 25 μl aliquot into 1 ml of urine provided an approximate standard amount of 400 pg. The CVAA and PhAs standard solutions were divided into 150–300 μl aliquots, flamed-sealed in ampules, and stored at 4 °C until used; the CVAA and PhAs standard solutions were stable for at least 3 months at 4 °C.

Note: The concentrations of the CVAA and PhAs solutions are expressed in amount of compound per volume.

The QC materials were prepared from a base urine pool obtained from several anonymous volunteers. The urine pool was filtered with a sterile apparatus and divided into three subpools that were diluted (2:3) with a solution of ammonium acetate buffer–water (1:4). Two of the subpools were enriched with CVAA as needed to afford low (~200 pg/ml) and high (~1200 pg/ml) level pools. The third subpool was not spiked with CVAA and was used as a blank QC. The subpools were uniformly mixed, dispensed into small aliquots (ca. 3–11 ml) in pre-rinsed glass vials, and stored at −20 °C until used; QC materials were stable for at least 3 months at −20 °C. The QC materials were characterized to define the mean and the 95th and 99th confidence intervals of CVAA concentration by the analysis of at least 20 repeat measurements.

2.3. Instrumentation and analysis

2.3.1. SPME–GC–MS analysis

The automated SPME–GC–MS analyses were performed on a HP 6890 gas chromatograph coupled to a HP5973 mass-selective detector (Agilent Technologies, Wilmington, DE, USA), and interfaced to a CTC Combi PAL LEAP GC sampler with SPME system (LEAP Technologies, Carrboro, NC, USA).

The twin-tier PAL autosampler, mounted on top of the GC system, included a vial tray holder, a sample heater block, and two movable heads housing syringe holders. These movable heads were programmed to perform motions in the *x*-, *y*-, and *z*-axes and to actuate the syringe plungers. One head (which ran along a track on the top tier) held a 10 μl liquid handling syringe (Hamilton, Reno, NV, USA) that was used to add PDT to the sample vials. The other head (which ran along a track on the bottom tier) housed the SPME fiber assembly and transferred the sample vials from the sample tray to the heater block for the SPME process. The SPME analyses were done using a 100 μm poly(dimethylsiloxane) (PDMS) fiber (Supelco, Bellefonte, PA, USA). Before initial use, the fibers were pre-conditioned in the GC inlet at 270 °C for 60 min. Fibers were replaced as needed, usually after about 200 injections. Following the addition of 3 μl of PDT to the sample vial, the twin-tier PAL autosampler moved the vial from the sample tray to the sample heater, where the vial was heated at 70 °C with continuous agitation (600 rpm) for 20 min. Next, the SPME fiber was exposed to the sample headspace for 10 min, retracted, and desorbed in the GC inlet, where it was kept for 14 min (purge valve was opened 10 min after the injection); we did not detect any carryover, even after analyzing the most concentrated standard samples. The GC inlet port, maintained at 270 °C in splitless mode, was fitted with a narrow-bore 0.75 mm I.D. SPME injection liner (Supelco). The gas chromatograph was equipped with a 30 m×0.25 mm, 0.25 μm film thickness, DB-5MS capillary column (J&W Scientific, Folsom, CA, USA). The helium flow-rate was maintained at 0.6 ml/min. The following oven temperature program was used: initial temperature 45 °C, held for 5 min, next ramped at 20 °C/min to 165 °C, followed by 8 °C/min to 210 °C, and finally ramped at 50 °C/min to 300 °C, and held at final temperature for 5 min (23.43 min total run time). The GC–MS transfer line was held at 200 °C and the MS quadrupole and source heaters were maintained at 150 and 230 °C, respectively. Total analysis time per sample was ~60 min (including sample preparation and GC run); when the derivatization reaction and fiber exposure were conducted during the GC run of the previous sample, the method throughput was 2 samples/h.

2.3.2. Selected ion monitoring (SIM) parameters

Mass spectra were acquired in SIM mode. The CVAA-PDT and PhAs-PDT ions used for quantitation and confirmation are given in Table 1. The presence of a double bond in CVAA results in the existence of *cis* and *trans* isomers, with the *trans* isomer being the predominant species [1].

Furthermore, the 3:1 isotopic ratio of $^{35}Cl/^{37}Cl$ yields two peaks at an interval of two mass units in the ratio 3:1 for ion species containing one chlorine atom, such as CVAA-PDT. The mass spectra of CVAA-PDT and PhAs-PDT have been published previously [5,6]. We used the molecular ion peaks at $[M]^+$ and $[M+2]^+$ as the quantitation and confirmation ions, respectively, for both CVAA-PDT isomer chromatographic peaks; the presence of the two SIM *cis*-CVAA-PDT ions at 15.2 min was used to further confirm the presence of *trans*-CVAA-PDT at 15.6 min. The peak area for the internal reference, PhAs, was determined from the molecular ion $[M]^+$; the confirmation ion was $[M+1]^+$. The ratio of the areas of the quantitation ion and the confirmation ion was used as an interference quality control check.

2.3.3. Sample preparation

A 1-ml volume of urine and 1.5 ml of an ammonium acetate buffer–water solution (1:4) were dispensed into a 10-ml vial. To each sample, 25 μl of 16 ng/ml PhAs was added to the urine to serve as an internal reference. For the standards, 25 μl of the appropriate CVAA analytical standard was also added to the urine. The vials were crimp-sealed and placed on the sample tray of the twin-tier PAL autosampler. The derivatization of CVAA was performed automatically using the PAL system by adding 3 μl of PDT to each vial immediately before starting the incubation process.

2.3.4. Data analysis

All of the samples, including blanks, standards, and QC materials were processed identically, and the data were evaluated for accuracy of integration. If necessary, data were manually reintegrated. We used the response factor (i.e, peak area ratio of *trans* CVAA-PDT to PhAs-PDT) for quantification. Seven standard CVAA amounts (41, 72, 180, 360, 720, 1017 and 1373 pg), which encompassed the entire linear range of the method, were used to construct a calibration curve of response factor versus standard amount. The calibration curve weighted by the reciprocal of the standard amount was used for quantification.

Data were automatically processed using the quantitation software of the GC–MS system. The

Table 1
Single ion monitoring analysis of 2-chlorovinylarsonous acid (CVAA) and phenyl arsine oxide (PhAs) after derivatization with 1,3-propanedithiol (PDT)

Analyte	Retention time (min)	Ion (m/z)	Use	Quality assurance parameter
trans-CVAA-PDT	15.6	242	Quantitation	N/A
		244	Confirmation	Presence of and ratio to m/z 242 confirmed
cis-CVAA-PDT	15.2	242	Confirmation	Presence of m/z 242 (15.6 min) confirmed
		244	Confirmation	Presence of m/z 242 (15.6 min) confirmed
PhAs-PDT	18.1	258	Quantitation	N/A
		259	Confirmation	Presence and ratio to m/z 258 confirmed

The 3:1 isotopic ratio of $^{35}Cl/^{37}Cl$ yields two peaks at interval of two mass units for the CVAA species.

integrated peak areas for each analyte and retention times were saved in a report file in ASCII format. The ASCII files were imported into an R:BASE (Microrim, Redmond, WA, USA) database specifically created for this analysis using an automated, custom-written routine. Statistical analysis of data was performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. Automated derivatization of CVAA with PDT

CVAA is non-volatile; therefore, its detection by GC is only possible after chemical derivatization to a volatile species. Previous methods have used the reaction of CVAA with a dithiol [4–8]. Our method is also based on the conversion of CVAA to a volatile cyclic dithiol; we selected PDT as the derivatizing agent on the basis of earlier studies by others [5]. A significant improvement of our method relative to previous methods is the use of a commercially available automated SPME–GC–MS system that allows, for the first time, a fully automated derivatization procedure of CVAA with PDT prior to SPME–GC–MS analysis.

3.2. SPME analysis of CVAA-PDT

The controlled addition of PDT, the derivatizing agent, was followed by a fully automated SPME and GC–MS analysis. We used a 100 μm PDMS SPME fiber, which has been used by other investigators to extract CVAA from soil and water samples [5,6], because of its ruggedness and reliability compared with other fibers. We selected a temperature of 70 °C and an extraction time of 10 min for the SPME procedure; Szostek and Aldstadt reported that 70 °C was the optimal temperature for the SPME analysis of CVAA-PDT and PhAs-PDT [5], and we found that optimal peak areas for both CVAA-PDT and PhAs-PDT were obtained after 10 min SPME extraction time.

A typical gas chromatogram of a spiked urine sample is shown in Fig. 1. The retention times of *trans*-CVAA-PDT, PhAs-PDT, and *cis*-CVAA-PDT were 15.6, 18.1, and 15.2 min, respectively. The

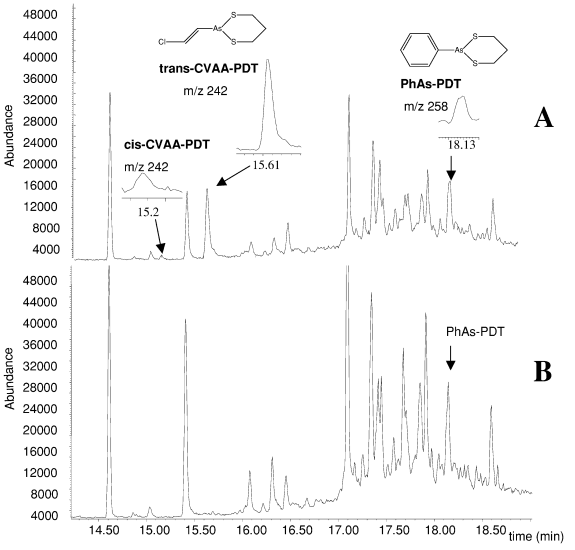

Fig. 1. Selected ion monitoring GC–MS chromatogram and quantitation ion profiles of the cyclic dithiols formed from the reaction of 2-chlorovinylarsonous acid (CVAA) and phenyl arsine oxide (PhAs) with 1,3-propanedithiol (PDT) in a spiked urine sample (CVAA concentration=1017 pg/ml) (A) and in a non-spiked urine blank (B). The chromatograms were acquired by monitoring both the quantitation and confirmation ions for the CVAA-PDT species and PhAs-PDT. The compound, and quantitation and confirmation ions monitored are as follows: *cis*-CVAA, 242, 244; *trans*-CVAA, 242, 244; PhAs, 258, 259.

chromatogram included many peaks other than those of interest that were also present in blank urine samples not spiked with CVAA (Fig. 1); we did not attempt to identify any of these peaks. The *trans*-CVAA-PDT and PhAs-PDT peaks were sharp and well resolved. As expected, the peak for *cis*-CVAA-PDT was smaller than that for the *trans* isomer. Therefore, we used the signal of *trans*-CVAA-PDT for quantitation and that of the minor *cis* isomer to further confirm the presence of CVAA (Table 1).

3.3. Effect of the buffer on the reaction of CVAA with PDT in urine

To determine the effect of the ammonium acetate buffer on the reaction of CVAA spiked in urine with PDT at 70 °C, we conducted a series of experiments using urine diluted both with water and with buffer. One urine sample was diluted (2:3) with water (final pH~7) and spiked with CVAA to a level of ~144

pg/ml. A second urine sample was diluted (2:3) with ammonium acetate buffer–water (1:4) (final pH~ 4.5) and spiked with CVAA to afford a CVAA concentration of ~144 pg/ml. A 2.5-ml volume of the diluted urine was spiked with the internal reference, PhAs, and the CVAA, and PhAs were derivatized with 3 μl of PDT, as described in Section 2.3.3. We observed that when the urine was diluted with buffer before spiking, the response factor (RF) was ~35% higher than for the urine diluted with water. Furthermore, the absolute area counts for the CVAA-PDT and PhAs-PDT chromatographic peaks were ~five times and ~four times higher, respectively, when the urine was diluted with buffer than with water. These results suggested that the presence of ammonium acetate buffer facilitated the reaction of both CVAA and PhAs in urine with PDT. We speculate that the effect of the buffer may be related to the reduced solubility of the cyclic dithiols in the buffered urine which results in their increased concentration in the headspace; alternatively, the low pH in the buffered urine playing a role in the increased reactivity of these arsenical compounds with PDT cannot be ruled out.

3.4. Calibration curve

The calibration curve of CVAA in urine analyzed over a 6-week period is shown in Fig. 2.

Each individual calibration curve showed excellent linearity with correlation coefficients generally ≥0.98. The calibration curve spanned one order of magnitude with a linear working range from the lowest to the highest standard. The standard deviations given in this figure show the excellent reproducibility of repeated measurements at each standard level.

3.5. Limit of detection

The LOD was calculated as $3s_0$, where s_0 is the standard deviation value as the concentration approaches zero [12]. We determined s_0 from the replicate analysis of low level standards (i.e., 41, 72, and 180 pg). The calculated method LOD was 7.4 pg in 1 ml of urine. This LOD value is between 1000 to 20 000 times smaller than that of previous methods [4,5,8]. To date, this is the most sensitive method for the determination of CVAA in a biological or environmental matrix.

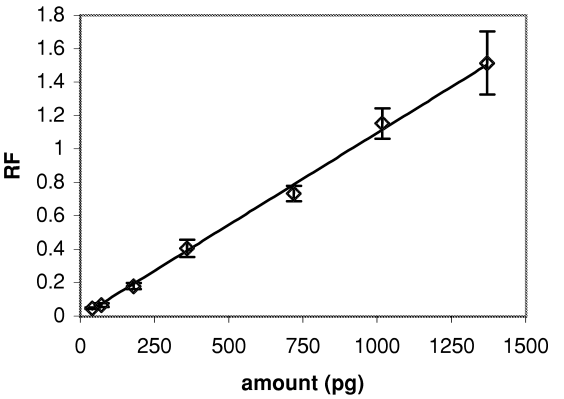

Fig. 2. Average calibration curve of 2-chlorovinylarsonous acid (CVAA) in urine based on replicate analysis of seven standard solutions across the linear range (±standard deviation) over a 6-week period (r^2=0.958). RF is the ratio of the peak areas of the cyclic dithiols *trans*-CVAA-PDT and PhAs-PDT [phenyl arsine oxide (PhAs), used as an internal reference]. The calibration curve, weighted by the reciprocal of the standard amount, was used for quantitation.

3.6. Accuracy and precision

The accuracy and intra-day precision were established by determining the relative recovery of spiked urine samples. In order to examine the consistency of the recovery over the linear range, the measurements were taken by quintuplicate at three different spiked amounts (i.e., 180, 720, and 1373 pg). The mean recoveries of CVAA in urine, expressed as a percentage of the spiked amount, were consistent across the concentration range and varied from 98 to 114%. The average mean recovery, a measure of the accuracy of the method, was 103%, and the slope of a linear regression analysis of the calculated versus the expected concentration was 0.97; these data confirmed the good accuracy of the method. The intra-day variability of the assay was excellent: the relative standard deviations (RSDs) at the low, medium, and high spike amounts (i.e., 180, 720, and 1373 pg) were 10, 6.5, and 1.5%, respectively (average RSD=6%). The inter-day precision, determined by calculating the RSD of 23 repeated measurements on the QC materials, was also very good (Fig. 3). Each point on the Shewhart QC plot

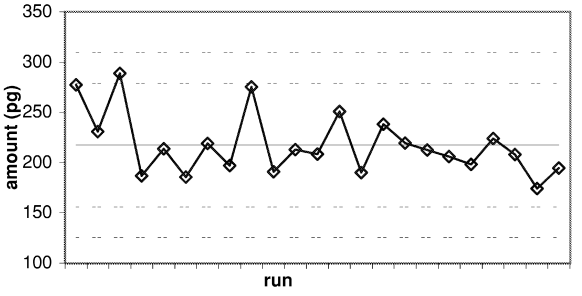

Fig. 3. Quality control (QC) plot for 2-chlorovinylarsonous acid (CVAA) levels in the low QC pool. QC urine was characterized by, at least, 20 separate analyses and subsequently used to monitor the long term precision and accuracy of the method. The mean concentration, and the 95th and 99th confidence intervals are indicated.

represents a determination of levels in the same QC material analyzed over a period of 6 weeks. Therefore, these measurements reflect both within- and between-run variations. The RSDs of the QC analyses for the low and high QC pools were 14.1 and 14.7%, respectively, which indicate the good reproducibility of the assay.

4. Conclusions

We have developed the first SPME–GC–MS method for the quantitative detection of CVAA, a specific hydrolysis product of the chemical warfare agent lewisite, in human urine. The method is simple and accurate, even at low pg levels. Furthermore, the method uses a small amount of urine (1 ml), is not labor intensive, involves minimal manual sample preparation (<2 min/sample), and uses an automated SPME–GC–MS system commercially available. To date, this is the most sensitive method for the detection of CVAA (LOD=7.4 pg); the low LOD of this method is suitable to assess lewisite exposure in clinical and forensic specimens.

5. Disclaimer

The use of trade names and commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or the US Department of Health and Human Services.

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