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Lewisite exposure biomarkers in urine by liquid chromatography – inductively coupled plasma tandem mass spectrometry: with an accelerated matrix-matched stability study

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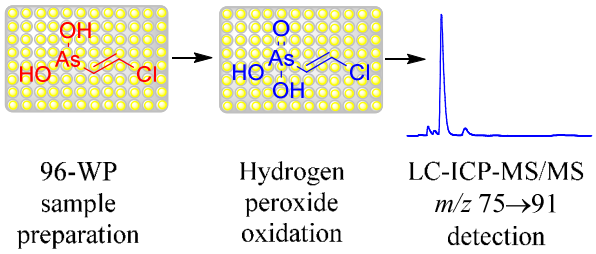

Abstract

A simple and robust LC-ICP-MS/MS method is described for quantitative analysis of human urine for (2-chlorovinyl)arsonic acid (CVAOA), a metabolite of Lewisite. This method oxidizes (2-chlorovinyl)arsenous acid (CVAA) with the addition of hydrogen peroxide to measure total Lewisite-1 metabolites as CVAOA, with *m/z* 75→91 detection specific for arsenic. The percentage of CVAA to CVAOA is clinically insignificant, because the amount of CVAA conversion to CVAOA is dependent upon residence time in the body. Once excreted into the urine, conversion of CVAA to CVAOA is dependent upon temperature and oxidative potential of the urine. The method also allowed for qualitative analysis for *bis*(2-chlorovinyl)arsinic acid (BCVAOA) and (1-chlorovinyl)arsonic acid (*gem*-CVAOA), minor Lewisite metabolites. Traditional methods have ignored these minor metabolites; the *bis*-metabolites can comprise ~30% of total Lewisite metabolites from chemical munitions and must be accounted for in the exposure measurement. The ion-pairing chromatography method results in a 5.73 min injection-to-injection cycle time with adequate retention (k' = 2.9) of CVAOA. The weighted ($1/x^2$) linear least squares regression results have correlation coefficients ($r^2 > 0.998$) for the clinically relevant calibration range of 50-3500 µg/L. The selectivity of the method is measured by chromatographic resolution from other common arsenic compounds that may interfere with the analysis. The 96-well plate preparation of 0.1 mL sample of human urine results in a method detection limit of 2.2 µg/L. Quantitative results from proficiency testing specimens demonstrate the accuracy (−7.1 to +4.3%) of the method. Quality control data demonstrate inter-analyst precise (3.1 to 3.3%) quantitative results of the method. An accelerated Arrhenius matrix-matched stability study demonstrates Lewisite metabolites are stabile in urine far greater than a year. The trivalent arsenic, CVAA oxidation half-life is estimated at normal body temperature *in*

vitro at 6.2 days. The combined sample preparation and analysis portions of this emergency response method have a throughput of 250 samples per day.

Introduction

Lewisite is a highly toxic vesicant, lung, and skin irritant causing immediate pain upon contact and during World War I was mass produced as a chemical weapon.[1-3] Lethal human exposures to Lewisite were estimated at 1500 mg-min/m^3, via inhalation route. The clinical signs and symptoms of Lewisite exposure include; dyspnea, coughing, vision impairment, eye and skin lesions, and in severe cases necrosis.[4] Lewisite lethality may be attributed to an increased capillary permeability causing loss of blood plasma, referred to as "Lewisite shock".[5] An *in vitro* study determined that all cell proliferation was inhibited by Lewisite at concentrations as low as 0.3 μg/mL.[6] Industrially-produced Lewisite has a strong penetrating geranium odor; however, pure Lewisite is odorless. Determination of Lewisite exposure biomarkers is essential for short- and long-term patient care.

Potential hazards include rare accidental exposures caused by catching disposed chemical weapon munitions from the sea. [7] There are also public health concerns of exposure near former Lewisite production facilities, such as the one in Willoughby, OH which was once thought to have produced up to 150 tons of the blistering agent.[8] Lewisite ordnance rounds were unearthed in the Spring Valley neighborhood in Washington, D.C., former site of the American University Experimental Station.[9] Occupational exposures associated with the destruction of stockpiled chemical weapons are another concern. High toxicity, availability of large amounts of stockpiled agent throughout the world, and relative ease of synthesis have made Lewisite a significant chemical threat.

Julius Arthur Nieuwland first synthesized Lewisite by the catalytic reaction of arsenic trichloride and acetylene.[10] The product was described as a black tarry substance that had a strong penetrating odor causing nausea and nervous depression. Winford Lee Lewis developed the highly toxic substance into a chemical weapon by purifying the material with hydrochloric acid, hence the namesake "Lewisite".[11] Lewisite (L) forms a mixture of products; (2-chlorovinyl)dichloroarsine (L1), *bis*(2-chlorovinyl)chloroarsine (L2), and *tris*(2-chlorovinyl)arsine (L3). The ratio of each depends upon reaction conditions and varies greatly between munitions. Weapons-grade Lewisite contains, on average, 64.0% (wt %) L1, 28.5% L2, and < 0.2% L3 with the remainder as impurities such as unreacted arsenic trichloride.[12] The *geminal* isomer (*i.e.*, 1-chlorovinyl) has been identified as a minor product.[13] Molecular orbital *ab initio* calculations have predicted that the *trans* geometric isomer was dominate.[14] The multiple products, isomers, and byproducts make Lewisite metabolite detection complex.

The primary metabolism pathway for Lewisite is hydrolysis[15], which occurs rapidly and the native agent is rarely found in nature.[16] Trivalent arsenic compounds are toxic[17] and methyl analogues have been shown to be carcinogenic.[18] Careful attention to the handling of these metabolites must be taken, and they should be considered as toxic as the native agent. The trivalent arsenic metabolites, (2-chlorovinyl)arsenous acid (CVAA) and *bis*(2-chlorovinyl)arsinous acid (BCVAA), are formed by the hydrolysis of L1 and L2, respectively (Figure 1), which can be oxidized to the pentavalent arsenic metabolites, (2-chlorovinyl)arsonic acid (CVAOA) and *bis*(2-chlorovinyl)arsinic acid (BCVAOA). Pentavalent CVAOA was the major metabolite found when trivalent CVAA was administrated to a single mouse[19] and

multiple rats[20]. Pentavalent arsenic metabolites can also be directly formed by reaction of the native agent with hydrogen peroxide.[21]

Emergency response methods must be capable of measuring exposure of several hundreds to thousands of patient specimens. Several analytical separation methodologies have been used to biologically monitor Lewisite exposure. Reductive-elimination oxidative-addition reaction of dithiols with Lewisite metabolites was used to create thermally labile derivatization products amendable to gas chromatographic analysis.[22-26] GC methods have their advantages; however, lengthy sample preparation with an incubation period and long run-times reduce their effectiveness for high-throughput emergency response. Metabolite methods can only measure from several hours to a few days after an exposure. For example, guinea pigs were subcutaneously exposed to Lewisite, the mean CVAA urinary concentrations were 3.5 μg/mL, ≤ 100 ng/mL, and trace levels at 0-8, 8-16, and 24-40 hour sample collection intervals.[22] This exposure study established the clinically relevant calibration range for Lewisite metabolites used in the method described. Liquid chromatographic separation has been used with electrospray ionization tandem mass spectroscopic detection (ESI-MS/MS).[20, 27] These molecular methods have potential with additional specificity using confirmation ions. However, CVAOA was not well retained by reverse-phase liquid chromatography, therefore subject to other non-retained urinary interferences. Also, lengthy run-times were necessary to remove endogenous hydrophobic compounds. Results become unreliable without the use of an isotopically labeled internal standard to compensate for differences in solid phase extraction recoveries. Arsenic-specific LC-ICP-MS methods were used to detect L1 metabolites in urine.[19, 28] Ion-pairing

chromatography was used to retain CVAA and CVAOA, and resolve the L1 metabolites from other common arsenic species found in urine.[28] However, the L2 metabolites were unaccounted.

In this paper, we describe modifications to the LC-ICP-MS method[28] that include: an oxidation step added to simplify the chromatography. A $1/x^2$ weighted least squares (WLS) linear regression implemented to improve precision across the linear dynamic range to minimize heteroscedasticity. Column dimensions changed to elute BCVAOA in a timely fashion to maintain demands of high-throughput emergency response. Additionally, a stability study of L1 metabolites in urine matrix is presented.

Methods

Liquid Chromatography

An Agilent 1200 Series LC was used consisting of a degasser, a quaternary solvent pump, a temperature-controlled autosampler, temperature-controlled column compartment with an integrated 2-position 6-port switching valve, and a manual controller (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved with a reverse-phase polar embedded amide column (Supelco Ascentis RP-amide, 100 mm × 2.1 mm i.d.; 5 μm particle size; Sigma Aldrich, Milwaukee, WI, USA), equipped with a pre-column filter (KrudKatcher™; Phenomenex, Torrance, CA, USA).

The mobile phase contained an ion-pairing reagent, 11.5 mM tetrabutylammonium hydroxide (TBAH) solution (1.5 M, Acros Organics, Thermo Fisher Scientific, Fair Lawn, NJ, USA), 5.0 mM succinic acid (99%, Acros Organics), and 2% (v/v) isopropanol (IPA) (99.8%, electronic

use, Acros Organics). The pH of the mobile phase was adjusted to 5.5 with nitric acid (Optima™ grade, Thermo Fisher Scientific, Fair Lawn, NJ, USA) and used unfiltered.

At the end of each working day, the LC column was washed with 4 column volumes of water (LC-MS chromasolv®, Fluka, Sigma Aldrich), followed by 54 column volumes of a linear binary gradient to acetonitrile (ACN) (Optima™ grade, Thermo Fisher Scientific) then held at 100% ACN for another 4 column volumes before the LC pump was automatically turned off. At the beginning of each working day, the LC column was conditioned in the reversed washing conditions and then equilibrated for a minimum of 60 column volumes of mobile phase prior to starting the analytical run.

Interface

The LC effluent could be directed to either waste or to the Telfon® construction PFA-ST nebulizer (Elemental Scientific Inc., Omaha, NE, USA) by the post-column 2-position 6-port switching valve. While the LC effluent was directed to waste, the ICP-MS/MS peristaltic pump could manually infuse optimization solutions to the nebulizer. A T-joint (Agilent Technologies) was used between the column and the nebulizer. The T-Joint was plugged when performing ICP-MS/MS optimizations, and for LC analysis was directed to the waste via the ICP-MS/MS peristaltic pump (at 0.1 rps). This alleviated the 1.0 mL/min LC effluent from the double-pass, quartz spray chamber (Agilent Technologies) to remove excessive waste. Additionally, a 65% increase in response resulted when the LC effluent was split between the nebulizer and waste, compared to when the T-joint was plugged. The spray chamber was peltier-cooled to 2 °C.

ICP-MS/MS

The 8800 ICP-MS/MS (Agilent Technologies) was operated as an arsenic-specific detector. Oxygen (research grade, >99.999%, Airgas, Radnor, PA, USA) was introduced into the third generation octopole reaction system (ORS^3) cell at 0.7 mL/min. The cell entrance, cell exit, and plate bias were optimized to –70 V, with the octopole bias and kinetic energy discrimination (KED) optimized at –10 V. Under these optimized settings there was > 97% conversion of $^{75}As^+$ (mass filtered by the first quadrupole) to $^{91}AsO^+$ (mass filtered by the second quadrupole) in the ORS^3 cell (located between the two quadrupoles), and background counts were an order of magnitude less compared to when the ORS^3 cell was evacuated measured at *m/z* 75→75 in MS/MS mode. The signal at *m/z* 75→91 was monitored and time-resolved analysis data acquired using the MassHunter (ver. C.01.01) software. The LC and ICP-MS/MS operated with the parameters summarized in Table 1. Detection filtering at every 5 points was used to smooth the chromatograms prior to integration.

Quantitative analysis of CVAOA in urine using single quadrupole ICP-MS instruments, without a reaction/collision gas, can be performed with method validation analytical figures of merit provided in the electronic supplementary information. The LC ruggedness, LC selectivity, and stability studies were collected using the 7500ce ICP-MS (Agilent Technologies) with the octopole reaction cell evacuated with instrument parameters in Table S1. The signal at *m/z* 75 was monitored and data acquired using the ICP-MS ChemStation (ver. B.04.00) software. The software required collection of data for another mass, *m/z* 76 as $^{38}Ar_2^+$, but was not used as a chromatographic internal standard to correct for ICP-MS drift. The ICP-MS ChemStation software was not able to control the functions of the LC, thus the ICP-MS and LC sequences

were required to be identical. The ICP-MS sequence was started using the LC manual controller, and data acquisition waited until the signal from the LC sequence was triggered by the first injection. PlasmaChrom (ver. C.01.00) chromatographic data software was used to integrate chromatograms, without smoothing, acquired from the time-resolved analysis data.

Reagents

Hydrolyzed Lewisite (L), semi-purified, was provided by U.S. Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD, USA) to the Centers for Disease Control and Prevention (CDC) (Chamblee, GA, USA). The 2.778 µg/mL aqueous stock solution contained predominately L1 metabolites, CVAA and CVAOA. Aqueous calibration materials were prepared by the Wisconsin State Laboratory of Hygiene (WSLH) (Madison, WI, USA) by dilution of stock solution and measured for total arsenic by ICP-MS traceable to a National Institute of Standards and Technology (NIST) arsenic standard. The aqueous calibration solution concentrations were corrected for arsenic impurities determined by CDC using arsenic speciation (94%, CVAA + CVAOA), and corrected for the CVAA molecular/elemental arsenic molecular weight ratio (170.4/74.9). The seven aqueous calibration materials were assigned concentrations of 500, 1200, 2300, 4700, 8400, 22000, and 35000 µg/L (lot CVAA4). These aqueous calibration materials were diluted 1:10 in water at our laboratory. DI water 18 MΩ•cm (Super-Q Plus, EMD Millipore, Billerica, MA, USA) was used for all aqueous solutions in our laboratory. Matrix-matched quality control materials were spiked into urine using the same aqueous stock solution. Three levels were prepared by WSLH at nominal concentration ranges of 50-125, 500-1000, and 2500-3500 µg/L for QL, QM, and QH, respectively. Sodium arsenoacetic acid (AsAc) (CDC purchased from TCI America, Portland, OR, USA) was dissolved in water (30 µg/mL) by

WSLH, and used as a surrogate internal standard. The surrogate internal standard, matrix-matched quality control, and aqueous calibration materials were provided to state public health laboratories for emergency response determination of human Lewisite exposures as partners in the Laboratory Response Network – Chemical (LRN–C). As a member of the LRN-C, our laboratory participated in proficiency testing (PT) of Lewisite metabolites in urine, which were provided by New York State Department of Health (NYS DOH) (Wadsworth Center, Albany, NY, USA).

Individual aqueous arsenic species solutions were used to identify retention under the described chromatographic conditions. Inorganic arsenic standard solutions, Arsenite (As^{III}) and arsenate (As^{V}), 1000 mg/L (Inorganic Ventures, Christiansburg, VA, USA) were used. Disodium methyl arsonate ($MMAA^{V}$), dimethylarsinic acid ($DMAA^{V}$) (both from Chem Service Inc., West Chester, PA, USA), and trimethylarsineoxide ($TMAO^{V}$) (purchased from WAKO USA, Richmond, VA, USA) were used to identify retention of the methylated arsenic species, human metabolism products of inorganic arsenic.[29] Arsenocholine bromide (AC) and arsenobetaine (AB) (WAKO USA) standards were used to identify retention of organic arsenic species associated with human consumption of seafood. A poultry-feed additive, 4-hydroxy-3-nitrobenzene arsonic acid, commonly known as roxarsone (ROX) (Acros Organics) was also evaluated. Phenylarsonic acid (PAA) (Alfa Aesar, Ward Hill, MA, USA) and diphenylarsinic acid (DPAA) (WAKO USA) were used to identify metabolites of phenyldichloroarsine (PFIFFIKUS, PD), diphenylchloroarsine (Clark I, DA), and diphenylcyanoarsine (Clark II, DC) incapacitating agents.[30] Crude BCVAOA was provided as a gift from Dr. Frederic Berg (U.S.

Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA) for confirmation of the L2 metabolite retention time.

Matrix-matched urine specimens were also spiked with hydrolyzed Lewisite by CDC for an accelerated Arrhenius stability study performed at our laboratory. Two different sets of matrix-matched urine specimens were prepared, buffered with succinic acid and unaltered "normal" urine. Low, medium, and high levels were prepared, which differed in concentration from the WSLH quality control materials described above. The stability data was collected using the CDC method[28] using a 150 mm × 4.6 mm i.d., 5 μm particle size Ascentis RP-amide column (Sigma Aldrich) for better resolution of the L1 metabolites. The aqueous calibration materials used for the stability study were from a previous lot (CVAA3), which were not adjusted for the arsenic impurities and differed in concentration. Similar LC-ICP-MS operating parameters were used, except: RF power was 1500 W, nebulizer gas flow rate was 0.85 mL/min (0.15 mL/min makeup gas), octopole cell was evacuated, injection volume was 20 μL, integration time was 1.2 s, and acquisition time was 15 min. Stability study samples were shipped frozen to our laboratory and stored for 1 day at –80 °C before collection of week 0 data. Each of the four sets of buffered and four sets normal urine specimens were then stored at 40 °C (incubator), 4 °C (refrigerator), 21 °C (room temperature), and –35 °C (freezer). Each level was analyzed in triplicate at the following weekly intervals; 1, 2, 3, 4, 6, 8, 13, 26, and 52 weeks.

Base Urine Collection

Collection of urine from laboratory volunteers was needed for matrix-matched calibration curves. Laboratory volunteers were not persuaded nor rewarded for participation, and there was

no repercussion for non-participation. Urine specimens were collected with recommended 24-hour seafood abstinence as the only dietary restriction to reduce arsenic exposure. Urine specimens were kept strictly anonymous, with absolutely no identifiers collected with the specimens. Unlabeled specimen containers were placed in laboratory restrooms for privacy, and gathered at the end of each day to be refrigerated. Urine specimens collected from laboratory volunteers constituted their informed consent. Participant signatures were not collected for enhanced anonymity. Individual anonymous urine specimens were analyzed for total arsenic by ICP-MS (Perkin-Elmer DRCII, Shelton, CT, USA). Briefly, urine specimens were diluted 1:10 with 2% (v/v) HNO_3 containing 10 µg/L Iridium (SPEX CertiPrep, Metuchen, NJ, USA) as an internal standard. The ICP-MS was calibrated against a NIST traceable arsenic standard (High Purity Standards, Charleston, SC, USA) between 5-4000 µg/L, with arsenic measured as *m/z* $^{91}AsO^+$ using 1.2 mL/min oxygen in the dynamic reaction cell ($RP_a = 0$, $RP_q = 0.75$). Those individual urine specimens with total arsenic concentration < 20 µg/L were pooled together creating the base urine, and then filtered (0.20 µm; EMD Millipore) to prevent/retard biological growth. Those individual urine specimens with total arsenic concentration > 20 µg/L were properly disposed. The collection protocol was approved by the Florida Department of Health Institutional Review Board.

Sample Preparation

All urine samples were thawed to room temperature prior to analysis. A 100 µL aliquot of urine was gently mixed with 100 µL of water and 300 µL of diluent into a 1 mL 96-well plate. Diluent consisted of 150 µg/L AsAc, and 6% hydrogen peroxide (30%, Acros Organics) prepared in mobile phase. AsAc was used as a surrogate internal standard, with hydrogen peroxide in molar

excess to oxidize As^{III} to As^{V} species. Matrix-matched calibration standards were prepared similarly; 100 µL aliquot of base urine, 100 µL of 1:10 diluted aqueous calibration standard, 300 µL of diluent. The calibration concentrations were assigned by the 1:10 dilution of the WSLH aqueous calibration material in base urine: 50, 120, 230, 470, 840, 2200, and 3500 µg/L. The 1 mL 96-well plate, with prepared samples, was mixed on an orbital micro-titer shaker (IKA® Works, Inc., Wilmington, NC, USA) for 4 min. The prepared samples were centrifuged for 16 minutes at 1300 × g and 4 °C (Model 5430R; Eppendorf AG, Hamburg, Germany). An aliquot of the supernatant fluid was transferred to a 200 µL 96-well plate and heat foil sealed (ALPS™ 25 manual heat sealer; Thermo Fisher Scientific). The 200 µL 96-well plate was placed in the autosampler maintained at 4 °C, ready for analysis.

Results and Discussion*Method Selectivity*

Isocratic elution of CVAA and CVAOA using ion-pairing chromatography was described elsewhere.[28] This modified method oxidizes CVAA to CVAOA with hydrogen peroxide to simplify the chromatography, with the RP-amide column dimensions changed to 100 mm × 2.1 mm i.d.; 5 µm particle size. These dimensions maintained adequate resolution of CVAOA from the internal standard (AsAc) and also allowed more rapid elution of BCVAOA metabolite. The CVAOA metabolite was eluted at 0.93 min (retention factor, k' = 2.9) as shown in the QH urine sample chromatogram (Figure 2). The Lewisite material that was used for calibration and quality control contained approximately 1:100 BCVAOA: CVAOA ratio. Confirmation of the BCVAOA retention time at 4.28 min (k' = 16.6) was achieved with the Edgewood Chemical Biological Center (ECBC) aqueous sample (data not shown). Three weapons-grade Lewisite munitions contained on average 1:10 L2:L1, but with one as high as 1:4 ratio.[12] The analysis

would be significantly impacted unless run-time (4.5 min) was extended to account for carryover of BCVAOA, which eluted > 9 min using the CDC method.[28] Modifying the column dimensions allowed BCVAOA elution for high-throughput emergency response and qualitative assessment of the L2 metabolite.

Polyatomic interferences $^{40}Ar^{35}Cl^{+}$ and $^{40}Ca^{35}Cl^{+}$ can compromise arsenic detection at *m/z* 75 by single quadrupole ICP-MS. Chlorine present in the urine can be abundant, which was measured *m/z* 35 and eluted at 0.71 min under the chromatographic conditions described (Figure S1). The *gem*-CVAOA peak was tentatively assigned based upon relative intensity compared to CVAOA and oxidative data. Weapons-grade Lewisite contained on average ~1% wt. *gem*-L1; however, in some munitions the *geminal* isomer was not detectable.[12] The *gem*-CVAOA was not detectable in the ECBC aqueous sample (data not shown). In the method described the tentatively assigned *gem*-CVAOA eluted at 0.74 min (k' = 2.1) which closely elutes with chlorine. Even though the polyatomic interferences were not detectable in urine blanks, we recommend that the *gem*-CVAOA not be integrated due to co-elution of the polyatomic interferences with single quadrupole ICP/MS. However, with *m/z* 75→91 ICP-MS/MS detection with oxygen introduced into the ORS^3 cell allowed for removal of these polyatomic interferences. The chlorine interference was effectively removed from arsenic by using oxygen mass-shift *m/z* 75→91 of aqueous samples containing up to 0.48 M hydrochloric acid.[31] Four L1 metabolites can be resolved using different column dimensions without the oxidation step during sample preparation (Figure 3). Three L1 metabolites were closely eluted: CVAA, CVAOA, and the tentatively assigned *gem*-CVAA and resolved on a 150 mm × 4.6 mm i.d., 5 μm particle size RP-amide column (Figure 3a). The 50 mm × 4.6 mm i.d., 3 μm particle size RP-amide column used in the

CDC method[28] did not resolve the *gem*-CVAA, which co-eluted with CVAOA, and thus not previously identified. Spiked urine samples stored for one week were oxidized by temperature. While the *gem*-CVAA decreased intensity upon oxidation by temperature, a fourth peak increased in similar intensity and was tentatively assigned to *gem*-CVAOA (Figure 3b). There was an unidentified peak from rat urine (high dose of CVAA exposure), albeit larger as relative to *gem*-CVAOA: CVAOA ratio, that had similar retention time as the tentatively assigned *gem*-CVAOA using similar column dimensions.[28] An analogous decrease and increase in intensity was observed as trivalent-arsenic CVAA was oxidized to pentavalent-arsenic CVAOA with respect to temperature increase.

Other more common arsenic species were individually spiked in urine to evaluate selectivity of the chromatographic method. Arsenocholine, arsenobetaine, trimethylarsine oxide, and dimethylarsinic acid eluted shortly after the void volume of the column, retention factors (k') were < 0.44 indicating little interaction of these arsenic species with the stationary phase. When hydrogen peroxide was not added to the diluent, arsenite (As^{III}) was not well-retained and eluted at 0.32 min (k' = 0.32). The trivalent arsenic metabolites CVAA and *gem*-CVAA were retained under chromatographic conditions at 0.99 and 0.86 min (k' = 3.1 and 2.6), in the absence of hydrogen peroxide. In the presence of hydrogen peroxide the trivalent arsenic metabolites were not detectable, simplifying the chromatography. Hydrogen peroxide oxidized As^{III} to arsenate (As^{V}) which was unresolved from monomethylarsonic acid with retention times at 0.56 and 0.52 min (k' = 1.3 and 1.1), respectively. Less common phenyl arsenic species were also evaluated. The riot control agent phenyldichloroarsine (PFIFFIKUS, PD) metabolizes to phenylarsonic acid (PAA) with a 1.16 min (k' = 3.8) retention time. The chromatographic run-time of the method

described would need to be extended if exposure to incapacitating agents, diphenylchloroarsine (Clark I, DA) or diphenylcyanoarsine (Clark II, DC) was expected. The diphenylarsinic acid (DPAA) metabolite was strongly retained at 17.3 min (k' = 70.5). Roxarsone (ROX), 4-hydroxy-3-nitrobenzene arsonic acid, an obsolete poultry-feed additive eluted at 2.72 min (k' = 10.2) which was more retained than the arsenoacetic acid (AsAc) internal standard at 1.57 min (k' = 5.5). The CVAOA peak tended to tail at higher concentrations such as the QH urine sample (Figure 2). Therefore, adequate resolution of CVAOA from AsAc (R_s = 5.8, new column) must be maintained throughout the column lifetime, typically 400-500 injections prior to peak degradation. This chromatographic method successfully resolved CVAOA and AsAc from other common arsenic species demonstrated the method selectivity.

Method Calibration & Sensitivity

Three linear regression calibration models were evaluated; ordinary least squares (OLS), inverse of concentration weighted least squares (WLS, 1/x), and inverse of the square of concentration weighted least squares (WLS, $1/x^2$). The linear equation ($y = mx + b$) was obtained from twenty daily calibration curves for each of the three linear regression calibration models. The area response ratios (y = CVAOA/AsAc) from the seven calibrators were solved for concentration (x). The precision of each linear regression calibration model was evaluated by plotting the coefficient of variation (CV) at each nominal concentration level on the logarithmic scale to emphasize the differences (Figure 4, and S2). Weighting should be evaluated for improved curve performance with hetereoscedastic data, where the standard deviation increases with concentration.[32] The precision using OLS for the three lowest calibrators were 13.3, 4.7, and 1.7%, showed the heteroscedasticity of the linear dynamic range which covered nearly three

orders of magnitude. The precision of the S2–S6 calibrators using WLS 1/x and $1/x^2$ were nearly identical, CVs ranged from 0.8 to 1.5%. The CV of the highest S7 calibrator was slightly lower at 0.8% using 1/x, compared to $1/x^2$ at 1.4%. However, there was improvement at the lowest calibrator when using $1/x^2$, CV of 0.6% compared to 2.2% using 1/x. The WLS $1/x^2$ calibration model was used for method validation, where correlation coefficients were (r^2) > 0.998. Matrix-matched quality control (QC) materials provided by WSLH were analyzed with twenty independent runs over a 32 day period by three different analysts. Analytical runs were rejected if QC values did not meet any of the four Westgard criteria; 3σ, 2×2σ, 4σ, and $10\bar{x}$.[33] The average low, medium, and high QC results (104, 779, and 3220 µg/L, respectively) were in good agreement with the provided nominal concentration ranges 50-125, 500-1000, and 2500-3500 µg/L, respectively. In addition, the QL, QM, and QH characterization CVs were 3.2%, 3.1%, and 3.3%, respectively.

The method detection limit was determined from the twenty QC characterization runs using the Taylor method.[34] A linear regression plot of standard deviation of the three lowest calibrators against the nominal concentration resulted in a negative y-intercept; there was not sufficient variability in the lowest calibrator using WLS $1/x^2$ data. The three lowest calibrators were reprocessed using WLS 1/x, and the plot of the standard deviation against the nominal concentration resulted in a positive y-intercept (S_0, the standard deviation at 0 µg/L concentration). The method detection limit defined as 3 times S_0 was 2.2 µg/L. The method detection limit of CVAA in urine by LC-ICP-MS was reported as 1.3 µg/L.[28] However, the detection limit was obtained using a larger 20 µL injection volume. A 10 µL injection was used in the LC-ICP-MS/MS method described to frequently allow pulse detection of all calibrators.

Occasionally, the apex of the highest calibrator peak was collected with analog detection. The method quantitation limit defined as 10 times S_0 was 7.4 µg/L. The method quantitation limit was well below the lowest calibrator with more than adequate signal-to-noise ratio (S/N). Instrument software calculated the S/N of the 50 µg/L calibrator; the average was 47 with the minimum and maximum of 29 and 66 for twenty independent runs. The analytical method described demonstrated excellent sensitivity, with no detectable carryover observed in urine blanks run immediately after the highest calibrator.

Accuracy & Precision

The accuracy of the method was evaluated by comparison of proficiency testing (PT) results (Table S2, and S3). Eighteen of the twenty urine specimens were spiked at concentrations ranging across the linear dynamic range by NYS DOH; two urine specimens were not spiked and correctly reported as not detected (ND). Eighteen laboratories participated in the PT, including our laboratory using the method described. A survey of the participating LRN-C laboratories indicated that some have implemented an oxidation step in sample preparation. While some laboratories were still using OLS, others had implemented WLS 1/x linear regression with ICP-MS detection of Lewisite metabolites as earlier recommended by these authors (personal communication). The Z-scores, derived from difference between the reported value and the consensus mean and then divided by the consensus standard deviation, were $\leq \pm 0.46$ (satisfactory PT results have $\leq \pm 2.0$ Z-score; and Z-scores between ±2.0 and ±3.0 were considered as a questionable result, but receive a passing grade). The consensus mean was used as the true value to calculate the bias of the reported PT results. Two spiked urine specimens were less than 50 µg/L, and reported as present below the lowest calibrator (PLC); one of which

had the highest bias at −7.1%. The number of laboratories reporting quantitative results for these specimens was not provided, with fewer laboratories expected to report a quantitative result may explain the higher bias. The average bias was +1.2% for the other sixteen spiked urine specimens, and ranged from –1.6% to +4.3% demonstrated excellent accuracy of the method. The average bias was +2.3% for nine spiked urine specimens, and ranged from −0.7% to +5.4% using the described chromatographic conditions with single quadrupole ICP-MS detection.

The precision of the method was further evaluated by a seven-day/run, seven-replicate precision study (Table 2, and S4). The day/run sample preparation included a blank, calibrators, and the three matrix-matched QC materials. The QC materials were analyzed seven times against the daily calibration curve. The within day/run, between day/run, and intermediate CVs were calculated with the aid of Excel® (Microsoft®, Redmond, WA) analysis of variation (ANOVA) statistical add-on package. The between day/run and grand CV were nearly identical, CVs < 2.2%. The within-day/run was extremely precise, CVs < 1.5% for the three QC levels. Only one skilled analyst preformed the precision study to remove inter-analyst variability. A similar precision study was performed on the single quadrupole ICP-MS instrument, with slightly higher CVs < 5.2%. The QC characterization, which used three different analysts, demonstrated slightly more imprecision as expected. The QC characterization CVs were between 3.1% and 3.3%, these and the precision study results demonstrated superb reproducibility of the described method.

Method Ruggedness

The ruggedness of the LC-ICP-MS/MS method was evaluated by the significance of mobile phase composition and octopole reaction system (ORS^3) cell parameters on sample stability.

Blanks, calibrators, and quality control samples were obtained under normal and stressed conditions. The mobile phase composition ruggedness data was collected in the evacuated cell mode on the single quadrupole LC-ICP-MS system. The stationary phase was allowed to equilibrate for 30 min prior to analysis when the mobile phase composition changed. Flow rate did not impact quality control results for 1.0 ± 0.5 mL/min; however, the run-time was extended for the 0.5 mL/min flow rate. There was no significant difference in quality control results when the column temperature was varied 30 ± 5°C, succinic acid concentration was modified 5.0 ± 2.5 mM, pH was adjusted 5.5 ± 0.8, and the alcohol content was switched to methanol. The tetrabutylammonium hydroxide (TBAH) was modified 0.30 ± 0.15%. The arsenoacetic acid (AsAc) surrogate internal standard retention time shifted from 1.59 to 1.30 min when TBAH content was 0.45%. The loss of resolution between CVAOA and AsAc led to an elevated response for the QH sample because of CVAOA tailing at higher concentrations. The ORS^3 cell parameters were stressed using the tandem quadrupole LC-ICP-MS/MS system. Three injections of diluent to equilibrate (> 15 min) the ORS^3 cell were collected prior to injection of the calibration curve. The quality controls were acceptable, within ±3σ of the mean, when the oxygen cell gas flow was changed 0.7 ± 0.2 mL/min. When the cell entrance, cell exit, and plate bias were stressed −70 ± 20 V, quality controls were within acceptable range. Acceptable results were also obtained when the kinetic energy discrimination (KED) was modified −10 ± 10 V. When the octopole bias was 0 V, the quality controls were acceptable. However, when the octopole bias was −20 V, there was significant loss in sensitivity. The 50 µg/L calibrator S/N was 7.1, with less than ideal correlation coefficient (r^2 = 0.9876), resulting in an elevated QL response; however, the QM and QH were within ±3σ range. The various mobile phase

compositions and ORS^3 cell parameters demonstrated the ruggedness of the LC-ICP-MS/MS method.

Material Stability & Oxidation

An accelerated Arrhenius matrix-matched stability study was performed as described elsewhere[35] to determine the shelf-life of L1 metabolites in urine. Normal urine and succinic acid buffered urine specimens were spiked at three different levels. The frozen urine specimens were analyzed on a 150 mm × 4.6 mm i.d., 5 µm particle size Ascentis RP-amide column at the start of the study (week 0). Subsequently, the urine specimens were stored at four different temperatures; –35 °C, +4 °C, +21 °C, and +40 °C and analyzed at 1-4, 6, 8, 13, 26, and 52 weeks. The samples were prepared similarly, but without the addition of hydrogen peroxide in the diluent. The combined integration of both the CVAOA and CVAA determined the total L1 metabolites concentration. There was no significant decrease in L1 metabolite concentration with respect to time or temperature over the course of one-year (Figure 5). There was no significant difference between normal urine (Figure 5a) and succinic acid buffered urine (Figure 5b), WSLH quality controls were subsequently prepared in normal urine. The rate constants of L1 metabolite degradation at each temperature were indistinguishable. Therefore, the slope of the Arrhenius plot was approaching zero indicating infinite stability. Based on this stability data the CDC has assigned a conservative 5-year shelf-life of the WSLH materials, with retest thereafter.

The first-order oxidation half-life of CVAA was estimated from the stability study data, the CVAA remaining nearly completely oxidized to CVAOA with increased temperature after one week (Figure 3). The relative recovery of CVAA was plotted against time of the medium level

normal urine, from the initial eight weeks of stability data. The slopes were equal to the oxidation rate constants at each temperature (Figure 6a). The natural logarithm of the oxidation rate constant at each of the four temperatures was plotted against the reciprocal temperature (Figure 6b). The oxidation rate (k_{oxd}) was $1.12e^{-1}$ (CVAA relative recovery day^{-1}) as determined by extrapolation of the Arrhenius plot linear equation at body temperature (37 °C). The *in vitro* CVAA oxidation half-life ($t_{50} = 0.693/k_{oxd}$) was 6.2 days, assuming that loss of CVAA was completely due to oxidation, and that the degradation was first order in CVAA. Trivalent arsenic methyl analogues monomethylarsonous acid ($MMAA^{III}$) and dimethylarsinous acid ($DMAA^{III}$) have been shown to completely oxidize in urine at room temperature in 3 days and 90 min, respectively.[36] There was 91% CVAOA, with respect to CVAA, when stored at 40 °C for 7 days (Figure 3); however, there was already 18% CVAOA present at the beginning of the study. The 73% difference of CVAA experimentally oxidized after 7 days at 40 °C, the Arrhenius plot theoretically calculated the time to 27% of CVAA remaining ($t_{27} = 1.31/k_{oxd}$) was 9.8 days. Therefore, the theoretical calculation of oxidation half-life was conservative compared to experimental data. The shelf-life ($t_{90} = 0.105/k_{oxd}$) of the trivalent oxidation state CVAA at −70 °C was 51 years, the time for 10% of the original amount of CVAA to oxidize to CVAOA. If stored at −20 °C, 10% of the CVAA metabolite would oxidize in 64 days. Therefore, CVAA materials were recommended to be stored as cold as possible, preferably ≤ −70 °C, to preserve the oxidation state.

Naseri *et al* recently detected CVAA (trivalent arsenic, soft acid) forming complexes with free cysteine (sulfhydryl, soft base) by GC-MS according to hard-soft acid-base (HSAB) theory.[37] Increasing the oxidation state to pentavalent arsenic, CVAOA becomes a borderline to hard acid,

decreasing the ability to bind to free cysteine or cysteine residues in proteins. Therefore, CVAA can form protein adducts and would be less likely to be detected in urine as a metabolite. CVAOA would unlikely form protein adducts, supporting CVAOA as the major metabolite being found in the urine of Lewisite exposed rodents.[19, 20] The LC-ICP-MS/MS method described used hydrogen peroxide to instantaneously oxidize any CVAA present in urine. The clinically relevant calibration range of the described method was 50-3500 µg/L (0.269-18.8 µmol/L, as CVAA). Free cysteine excreted in urine would be expected to be in molar excess, with reference range mean urinary concentrations of cysteine ranging between 28-49 µmol/mmol of creatinine.[38] Quality controls in urine were spiked (1:1) with 100 µmol/L of cysteine (Nacalai Tesque, Inc., Kyoto, Japan) and incubated at 40 °C for 1 hr. Diluent with hydrogen peroxide was added after the quality controls equilibrated to room temperature, and analyzed per the method described. The addition of cysteine to quality controls did not impact quantitative results, all were within ±1σ of the mean, and retention time shift was not noticeable. Either (a) hydrogen peroxide oxidized CVAA-cysteine complexes to CVAOA; (b) theoretical CVAOA-cysteine complex co-eluted with CVAOA under the chromatographic conditions described; or (c) CVAOA has already formed a complex with the free cysteine present in the urine. Regardless, the method was not compromised by the additional cysteine with elemental ICP-MS/MS detection (*m/z* 75→91). Whereas, direct molecular ESI-MS/MS detection of the CVAA precursor ion $[M+H]^+$ at *m/z* 171 would be impacted upon forming CVAA-cysteine complexes $[M+H+Cys–H_2O]^+$ measured at *m/z* 256 (with ^{35}Cl isotope). Further ESI-MS/MS investigation is required into the oxidation of the free and protein-bound CVAA-cysteine complexes.

Conclusions

An LC-ICP-MS/MS method was optimized for the separation and quantification of the pentavalent arsenic CVAOA Lewisite metabolite in urine. The trivalent arsenic metabolites were easily oxidized by the use of hydrogen peroxide for additional qualitative assessment of BCVAOA and tentatively assigned *gem*-CVAOA. The method has a potential forensic application, being able to find the ratio of L1,L2, and geminal metabolites matching the source chemical warfare agent material. The method was shown to be accurate, precise, selective, robust, and sensitive with a 2.2 μg/L detection limit. To our knowledge, this is the first stability study performed of L1 metabolites (CVAA + CVAOA), which were extremely stable in urine. The *in vitro* oxidation half-life of CVAA at body temperature was estimated at 6.2 days, indicating the stability of the trivalent arsenic Lewisite metabolite. The addition of cysteine did not impact CVAOA results, therefore oxidation with hydrogen peroxide or oxidative-addition reductive-elimination dithiol derivatization are appropriate sample preparation methods for Lewisite metabolite identification. The method is suitable to biomonitor Lewisite exposure in human urine by LC-ICP-MS/MS analysis, with CVAOA measured as the primary metabolite.

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Table 1 LC-ICP-MS/MS instrumentation parameters.

HPLC	
System	Agilent 1200 Series
Column	Ascentis 5 µm RP-amide, 100 mm × 2.1 mm i.d.
Column temp. (°C)	30 ± 0.8
Autosampler temp. (°C)	4 ± 1
Mobile phase (isocratic)	11.5 mM TBAH, 5 mM succinic acid, 2% IPA, pH 5.5
Mobile phase flow rate (mL/min)	1.0
Injection volume (µL)	10
ICP-MS/MS	
System	Agilent 8800
RF power (W)	1550
Plasma gas flow rate (L/min)	15.0
Auxiliary gas flow rate (L/min)	0.90
Nebulizer gas flow rate (L/min)	1.20
Nebulizer	Concentric Teflon® PFA-ST
Spray Chamber temp. (°C)	2
Ion lens voltages	Optimized daily
Oxygen cell gas flow rate (mL/min)	0.7
Cell exit (V)	–70
Cell entrance (V)	–70
Plate bias (V)	–70
Octopole bias (V)	–10
Kinetic energy discrimination (V)	–10
Integration time (s)	0.3
Isotopes monitored	Q1 *m/z* $^{75}As^{+}$ Q2 *m/z* $^{91}AsO^{+}$
Scan Type	time resolved analysis
Acquisition time (min)	5
Repetition	1

Table 2 Analysis of variation (ANOVA) calculation of coefficients of variation (CV) from seven day/run, seven replicate CVAOA precision data (n = 49)

Level	Grand Mean (µg/L)	Grand SD (µg/L)	Grand CV (%)	Within day/run CV (%)	Between day/run CV (%)	Intermediate CV (%)
QL	102	1.87	1.8	1.5	1.1	1.9
QM	776	10.5	1.3	0.8	1.1	1.4
QH	3200	69.5	2.2	0.8	2.1	2.3

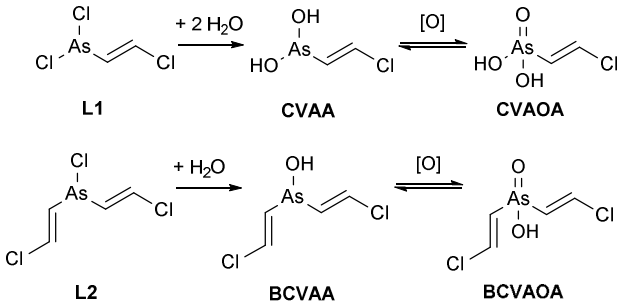

Figure 1 Hydrolysis and oxidation metabolites of Lewisite (L1, L2), structures are shown in the most abundant *trans*-2-chlorovinyl configuration.

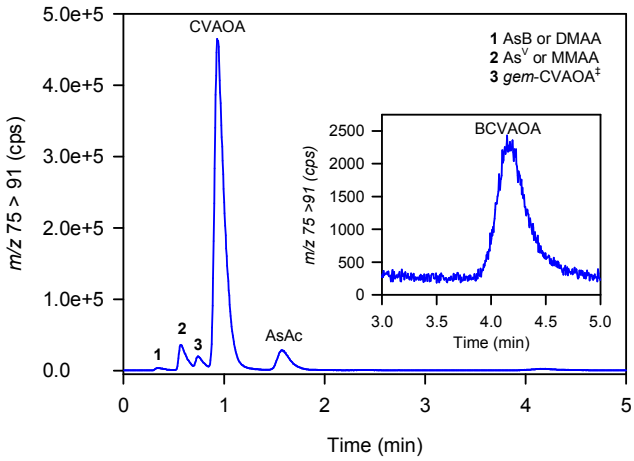

Figure 2 Chromatogram of a WSLH QH urine sample detected at *m/z* 75→91 using an Agilent 8800 ICP-MS/MS. The BCVAOA metabolite was more highly retained (inset). An aqueous sample of crudely purified L2 hydrolysis products was provided as a gift from Dr. Frederic Berg (U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA). The crude aqueous sample was oxidized with hydrogen peroxide and buffered with mobile phase, which confirmed the BCVAOA metabolite retention time (data not shown).

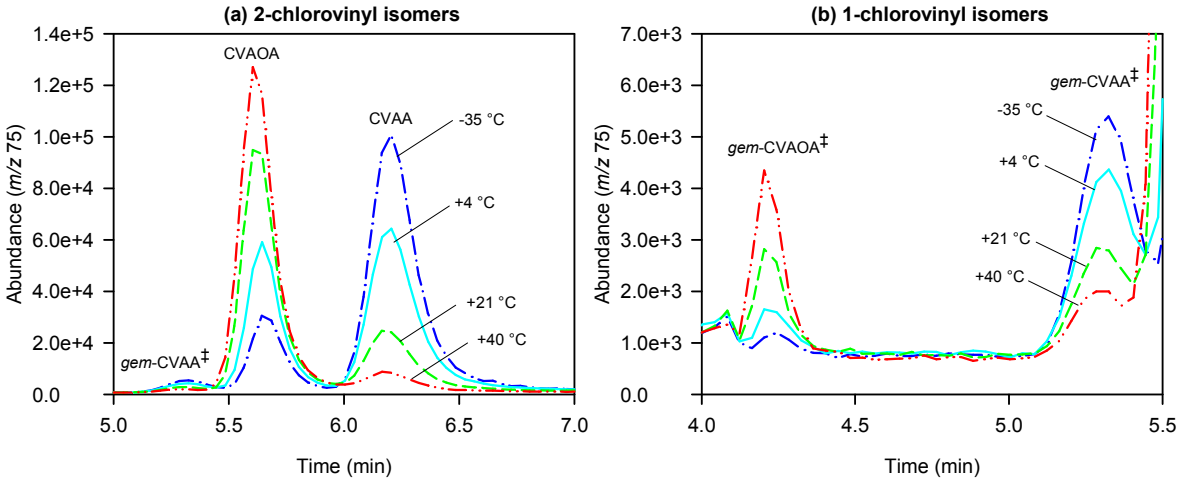

Figure 3 Separation of L1 metabolites was achieved with the 150 mm × 4.6 mm i.d., 5 µm particle size Ascentis RP-amide column. The overlaid chromatograms were measured with the octopole reaction cell evacuated with data collected at *m/z* 75 using the Agilent 7500ce ICP-MS. Sample preparation was performed without the addition of hydrogen peroxide to the diluent. The trivalent arsenic metabolites decreased, while the pentavalent arsenic metabolites increased in abundance as temperature was increased (stored at temperature for one week). The chromatograms are shown (a) between 5.0 and 7.0 min for clarity of the 2-chlorovinyl isomers, (b) between 4.0 and 5.5 min for the tentatively assigned (‡) *geminal* isomers.

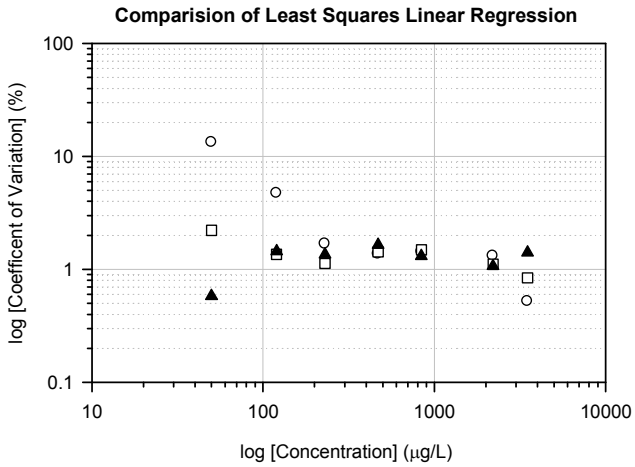

Figure 4 The coefficient of variation was plotted (logarithmic scale) for each standard reprocessed using three least squares linear regression models; ordinary least squares (○, OLS), inverse of concentration weighted least squares (□, WLS 1/x), and inverse of the square of concentration weighted least squares (▲, WLS $1/x^2$). The concentrations were calculated from the standards area response ratio (CVAOA/AsAc), slope, and intercept obtained from the daily calibration curve (n = 20). The plot showed WLS $1/x^2$ minimized the effect of heteroscedasticity across the linear dynamic calibration range.

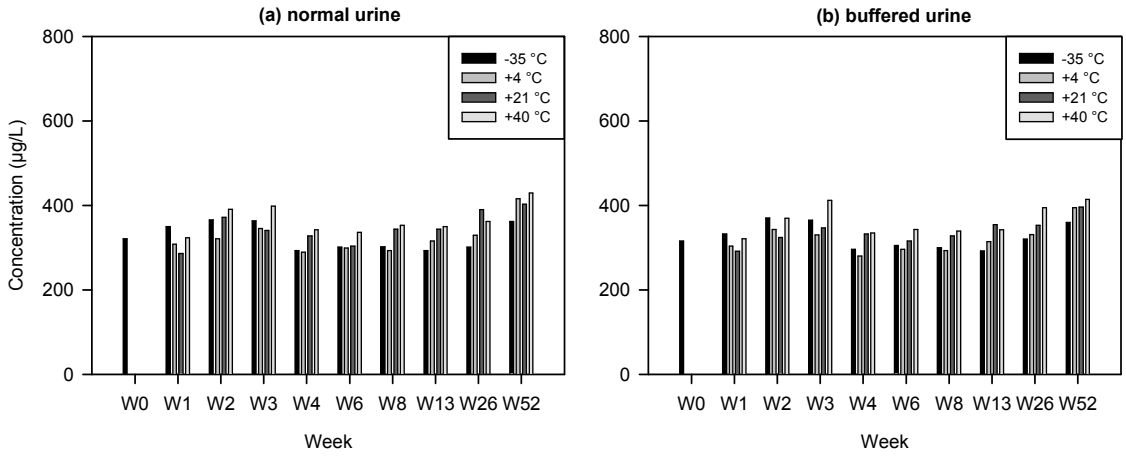

Figure 5 Accelerated matrix-matched stability study of L1 metabolites in (a) normal and (b) succinic acid buffered urine. The medium level concentration (shown) demonstrated the CVAA and CVAOA metabolites were stable for one year in both normal and buffered urine (low and high levels, not shown). There was no observed decrease in concentration with increased temperature. The stability study results were assigned a conservative 5-year shelf-life to the WSLH quality controls, with material stability retest thereafter.

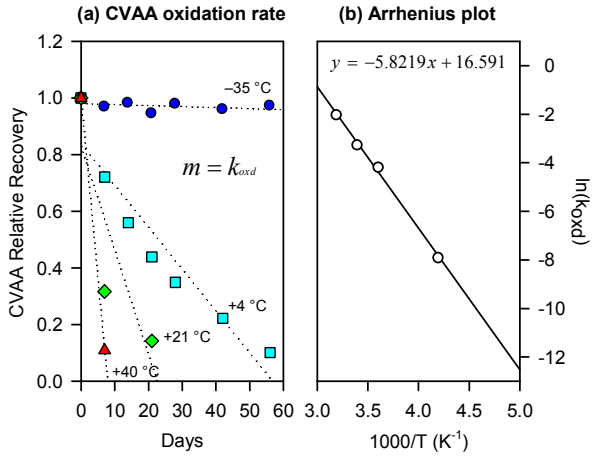

Figure 6 (a) The oxidation rates (k_{oxd}) were determined from the slopes of the relative recovery of CVAA from the first eight weeks of the accelerated stability study data. The relative recoveries were referenced at week 0, from the medium level in normal urine at −35, +4, +21, and +40 °C. (b) The Arrhenius plot was used to determine the rate constant at body temperature extrapolated from the equation of the line $[\ln(k) = \frac{-E_a}{R}\frac{1}{T} + \ln(a)]$. The oxidation half-life of CVAA was 6.2 days estimated from first-order rate constant at +37 °C, assuming that all loss was due to oxidation.

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