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Extraction tool and matrix effects on arsenic speciation analysis in cell lines

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Abstract

Arsenic glutathione (As-GSH) complexes have been suggested as possible metabolites in arsenic (As) metabolism. Extensive research has been performed on the toxicological and apoptotic effects of As, while few reports exist on its metabolism at the cellular level due to the analytical challenges. In this study, an efficient extraction method for arsenicals from cell lines was developed. Evaluation of extraction tools; vortex, ultrasonic bath and ultrasonic probe and solvents; water, chemicals (methanol and trifluoroacetic acid), and enzymes (pepsin, trypsin and protease) was performed. GSH effect on the stability of As-GSH complexes was studied. Arsenic metabolites in dimethylarsino glutathione (DMA(GS)) incubated multiple myeloma cell lines were identified following extraction. Intracellular GSH concentrations of myeloma cell lines were imitated in the extraction media and its corresponding effect on the stability and distribution of As metabolites was studied. An enhancement in both extraction recoveries and time efficiency with the use of the ultrasonic probe was observed. Higher stabilities for the As species in water, pepsin and trypsin were obtained. The presence of 0.5 mM GSH in the extraction media (PBS, pH = 7.4) could not stabilize the As-GSH complexes compared to the 5 mM GSH, where high stabilization of the complexes was observed over a 5 day storage study. Finally, the speciation analysis of the DMA(GS) culture incubated cell lines in the presence or absence of GSH revealed the important role GSH plays in the preservation of DMA(GS) identity. Hence, caution is required during the extraction of arsenicals especially the As-GSH complexes, since their identification is highly dependent on GSH concentration.

Keywords

Arsenic speciation; extraction; glutathione; liquid chromatography inductively coupled mass spectrometry

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1. Introduction

Arsenic trioxide (As_2O_3) is a double-edged sword. It is a well known environmental contaminant and human carcinogen.[1, 2] Reports exist for arsenite (As^{III}) inhibiting DNA repair by interfering with its methylation and signal transduction pathways or through causing changes in the cellular redox levels by generating free radicals and reactive oxygen species. Through binding to sulfhydryl groups of proteins that have catalytic functions, arsenicals can also damage protein molecules and hence cause cellular malfunction.[3] The toxicity of trivalent arsenicals on hepatocytes, lymphocytes, keratinocytes, melanocytes, dermal fibroblasts, dendritic cells, monocytes, T-cells, microvascular endothelial cells has been stated.[3-5] The cytotoxic effects of dimethylarsino glutathione ($\text{DMA}(\text{GS})$) on rat liver cells[6] and multiple myeloma (MM) cell lines have also been studied.[7] Paradoxically, As species; As_2S_2 , As_2S_3 , As_2O_3 have been used for medicinal purposes since ancient Greece and Rome.[8] As_2O_3 is a successful therapeutic agent for the treatment of acute promyelocytic leukemia (APL) and has shown some success in multiple myeloma (MM) clinical trials.[9]

Extensive research has been performed on the toxicological and apoptotic effects of arsenicals, their pharmacokinetics, mechanisms of action and cellular uptake pathways,[5, 9-11] however, very few reports exist for its metabolism, transformation and excretion at cellular level.[12-14] Since As toxicity is species dependent, it is essential to obtain speciation information at the cellular level to identify active As metabolites responsible for its toxicity and in some cases therapeutic efficacy.

Arsenic speciation analysis in biological samples remains a great challenge due to variations in As species identity and stability.[15-17] In addition to the need for a selective separation method, proper sample handling, preparation, extraction, and storage are prerequisites for correct species identification. Lack of suitable reference materials containing the various As species is an obstacle in developing proper extraction methods. The use of proper solvents is mandated for efficient and selective extractions. Common solvents and solutions reported in the literature for extracting As species from various samples are methanol/water (50/50), trifluoroacetic acid (TFA), dilute phosphoric acid, hydroxylammonium hydrochloride and ammonium oxalate.[18] However, these solvents either yield low extraction recoveries or cause oxidation of trivalent arsenicals. On the cellular level, few reports exist for speciation analysis of As.[12-14, 19, 20] Methanol/water (50/50) mixture is a common solvent used for As extraction from red blood cells (RBC).[12, 19] The extraction procedure implemented with this solvent involves continuous shaking 20 minutes to overnight with procedure replicates to confirm complete extraction.[12, 19] The use of ultrasonic bath for a period of 20 minutes followed by centrifugation for 30 minutes is another method reported by Kroening et al with phosphate buffer saline (PBS) as a solvent.[14] These aforementioned procedures are time-consuming,[14] yield low recoveries,[19] and could potentially alter As species identity.[12] Various reports exist for the instability of some As species present in biological samples. Arsenic glutathione ($\text{As}(\text{GS})_3$), monomethyl arsenic glutathione ($\text{MMA}(\text{GS})_2$) and $\text{DMA}(\text{GS})$ have half-lives in the minute to hour range in the absence of GSH depending on solution pH.[21-23] Other arsenicals such as monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) are highly susceptible to oxidation.[23, 24] Species conversion and oxidation is highly probable with the use of some of these reported extraction methods. The only reported rapid extraction method that could possibly preserve As species identity during extraction is to lyse the cells using Triton-X on ice for 5 minutes.[13] However, the stability of the various arsenicals using this extraction method has not been tested. Furthermore, the employed separation technique based on hydride generation is only specific for trivalent (As^{III} , MMA^{III} and DMA^{III}) and pentavalent (arsenate (As^{V}), methylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V})) As species. It is impossible

to discriminate arsenic glutathione complexes (As-GSH) from their trivalent counterparts using this separation method. All extraction methods discussed above have been more specific towards extraction of trivalent and pentavalent As species. To the best of our knowledge, no work has been reported for the extraction of As-GSH complexes from mammalian cell lines and no stability studies have been performed in the extraction media. The stability of pentavalent arsenicals is well known. However, proper extraction and storage conditions for trivalent and GSH-complexed forms are lacking although these species have been identified in some biological samples, such as urine, bile and cultured carcinoma cells.[12, 13, 25-28] Therefore, developing an efficient extraction method for biologically relevant As metabolites such as As^{III}, MMA^{III}, DMA^{III}, As^V, MMA^V, DMA^V, As(GS)₃, MMA(GS)₂ and DMA(GS) is crucial for proper species identification.

Enzymatic extraction along with sonication is a technique implemented for extraction of As from plants, soil, rice, hair, baby food, chicken, fish, straw and nails.[18, 29-32] Enzymes typically reported for As extraction are protease, amylase, trypsin, viscozyme, lipase, pancreatin.[18, 29, 30, 32, 33] Sonication has various applications. For analytical purposes, two different geometric designs for different applications are of interest, the ultrasonic bath, where ultrasonic waves are applied over a large surface area from the bottom of a vesicle, and ultrasonic probe, a small piece of rod through which sound waves pass over a small surface area and hence provide more focused and localized sample zone. The use of these two techniques, sonication and enzymatic treatment, in concert could produce more efficient extractions with higher recoveries, reduce extraction times and possibly conserve As species.

The aim of the present study was to develop an in vitro extraction method allowing quantitative, fast and efficient extraction of As species from cell lines while conserving species integrity. Due to the interest in the comparative toxicity and activity of As^{III} and darinaparsin ((DMA(GS), DAR) as therapeutic agents for MM [7, 34] and the availability of MM cell lines in our laboratory, these cells were employed for method development purposes. For As extraction recovery experiments, three different extraction devices (vortex, ultrasonic bath and ultrasonic probe) were compared. Stability of As species prepared in various extraction media (water, chemicals and enzymes) and the chromatographic separation of these species were evaluated. The effect of reduced glutathione (GSH) concentration on the species stabilities in PBS was assessed after spiking trivalent and glutathione complexed forms of trivalent arsenicals in cellular matrix. Finally, the developed method was employed for the identification of As metabolites in DMA(GS) incubated MM cell lines and the effect of GSH concentration on the speciation of the arsenicals in the cell extracts was evaluated.

2. Materials and methods

2.1. Chemicals

All reagents used were of analytical grade or better. Argon (Ar) purged deionized (DI) water (18 MΩ Barnstead Nanopure Diamond, NY, USA) was used throughout the experiments. Sodium metaarsenite (98%, As^{III}), sodium arsenate dibasic (99%, As^V), cacodylic acid sodium salt (98%, DMA^V), L-glutathione reduced (98-100%, GSH), trifluoroacetic acid (98%, TFA), and protease, Type XIV Bacterial (*Streptomyces griseus*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monosodium acid methane arsonate (99.5%, MMA^V) was purchased from Chem Service (West Chester, PA, USA). Acetonitrile, methanol (MeOH), formic acid (99%), pepsin and trypsin (immobilized) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Sodium chloride (NaCl), sodium phosphate, dibasic (Na₂HPO₄·7H₂O), potassium chloride (KCl), potassium dihydrogen phosphate (KH₂PO₄) used to prepare phosphate buffer saline (PBS) and ammonium hydroxide (NH₄OH) and

nitric acid (HNO_3) used for pH adjustments were also purchased from Thermo Fisher Scientific. MMA^{III} , DMA^{III} , $\text{As}(\text{GS})_3$, $\text{MMA}(\text{GS})_2$ and $\text{DMA}(\text{GS})$ were synthesized in our lab following the procedures previously reported.[23]

2.2. Cell lines

8226/S multiple myeloma cell line was purchased from the American type Culture Collection (ATCC, Manassas, VA), while KMS11 cell ed by Dr. P. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ, USA). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 on RPMI-1640 media, supplemented with 100 U. mL^{-1} of penicillin, 100 $\mu\text{g. mL}^{-1}$ of streptomycin, 10% heat inactivated fetal bovine serum and 2 mM L-glutamine (all culture reagents from Cellgro, MediaTech, Herndon, VA, USA).

2.3. Instrumentation

2.3.1. HPLC-ICP-MS—A Perkin Elmer Series 200 HPLC system equipped with a peltier controlled column compartment operated at 10 °C was coupled with a Perkin Elmer Elan DRC-e ICP-MS. The ICP-MS was equipped with a cyclonic spray chamber and a Meinhard nebulizer and was used in the standard mode. ICP-MS signals at m/z 75 for As and 77 for ArCl interference were monitored. As^{V} was injected post column as an internal standard to correct for instrumental drifts. Instrument performance was monitored daily against a tuning solution recommended by the manufacturer and if necessary nebulizer gas adjustments were made for maximum sensitivity (Table 1). Data was collected and processed using Elan v. 3.4 and Chromera v. 1.2 softwares (Perkin Elmer, San Jose, CA, USA) for total As and speciation analysis, respectively.

2.3.2. HPLC-ESI-MS—A Thermo Finnigan Surveyor HPLC system equipped with a peltier-controlled autosampler and column compartment was coupled with an LCQ Deca XP MAX (Thermo Finnigan, Waltham, MA, USA) MS. The ESI-MS was used in the positive ionization mode for GSH quantitation in the cell lines. Total ion chromatograms (TIC) were acquired from $m/z = 100$ to $m/z = 1050$ and data was collected and treated using Xcalibar software (Thermo, Waltham, MA, USA). The instrumental parameters were optimized using the flow injection mode with a 5 $\mu\text{g.mL}^{-1}$ caffeine standard (Table 1).

2.4. Procedures

2.4.1. Cellular essays—Cells were cultured at a concentration of 2.5×10^5 cells. mL^{-1} in 10 mL (T-25 Flasks) in supplemented RPMI-1640 media and incubated with or without 2 μM DAR (Ziopharm Oncology, Inc., Boston, MA, USA) for 30 min. Following incubation, control and treated cells were harvested by centrifugation, washed once with PBS and the pellets were stored at -80°C until analysis.

2.4.2. Extraction and digestion for total As analysis and recovery—DI water (1.5 mL) was added to control and treated cell pellets. Sample extractions were conducted using a vortex (Fisher Scientific Touch Mixer Model 232, USA), an ultrasonic bath (Bransonic Model 1510, Danbury, CT, USA) and a probe (Fisher Scientific Sonic Dismembrator Model 100, Waltham, MA, USA). Vortexing was performed for 15 min. at power level 10,[12] the ultrasonic bath was tested at two time periods; 30 min. and 1 hr.,[14] while 3 pulses for the ultrasonic probe were used at power level 2 with each pulse being performed for around 3 sec. For ultrasonic probe extractions the centrifuge tube was dipped in ice to prevent overheating. Digestion of the As-treated and control cell pellets was performed with concentrated HNO_3 and H_2O_2 following a previously reported procedure.[7] For extraction and digestion of the cell pellets, experiments were conducted in triplicate and 5 point external calibration was used for quantification with correlation coefficient of $r^2 > 0.99$.

2.4.3. Speciation analysis—Separation of As was achieved on a Waters Spherisorb C₈ column (150 × 4.6 mm in dimension and 5 micron particle size) following a previously published method.[23] The analytical figures of merits for As species were reported previously.[23] Briefly, the mobile phase consisted of 0.1% formic acid and acetonitrile in gradient mode at a flow rate of 1 mL.min⁻¹. The column effluent was separated into two (one part going into the detector and the other going into waste). All connections were made of inert PEEK material. The relative intensity of the HPLC-ICP-MS chromatographic peak for each As species in solution was calculated as the ratio of the peak area of the individual As species in that solution divided by the sum of the peak areas of all the species. GSH in DMA(GS) culture incubated cells was quantitated against a five point calibration curve of GSH (0.25 – 10 mM) with HPLC-ESI-MS.

2.4.4. Comparison of chemicals vs. enzymes for As species stability—The final concentration of the As species in the test solutions was 1.33 μM achieved by proper dilutions from their concentrated stocks in Ar-purged solvents of interest. Solvents tested were DI water (pH = 5.20), MeOH/water (50/50), 0.1% TFA (pH = 2.00), 0.5 mg.mL⁻¹ pepsin (pH = 4.01), 5 μL.mL⁻¹ trypsin (pH = 6.50), and 0.5 mg.mL⁻¹ protease (pH = 6.32). The solutions of trivalent arsenicals (As^{III}, MMA^{III} and DMA^{III}) and As-GSH complexes in the presence of 2.5 mM GSH were prepared in triplicate, and filtered through a 0.45 micron nylon syringe filter (Fisherbrand, Waltham, MA, USA) prior to speciation analysis. The As species stability was monitored over a period of five days and the samples were stored at -20°C after each analysis.

2.4.5. Effect of GSH concentration on stability of As-GSH complexes—For determination of the effect of GSH on the stability of the As-GSH complexes in cell extracts, a 1.33 μM standard of each trivalent As species (As^{III}, MMA^{III} and DMA^{III}) mixed with varying GSH concentrations (0.5 and 5 mM) was prepared in PBS buffer at pH = 7.4 in triplicate. The mixture was left at room temperature under normal room light for 20 hours for the complexes to be formed. Then, 600 μL of the prepared standards was added to the cell pellets, following probe sonication and filtration through 0.2 micron sterile nylon syringe filters (Whatman, Piscataway, NJ, USA), analysis was performed over a period of 5 days. Between analyses, the samples were stored at -20°C. Prepared standards of each As-GSH species (in 0.5 or 5 mM GSH) in the absence of cellular matrix were also analyzed to evaluate the effect of mobile phase on the distribution of As species in cell extracts.

For determination of GSH effect on the speciation of As metabolites in the DMA(GS) culture incubated cell lines, extraction was performed with or without 2.5 mM GSH in (pH = 7.4). Following speciation analysis using HPLC-ICP-MS, the relative intensities of As metabolites was calculated.

3. Results and discussion

3.1. Comparison of extraction recovery using different tools

To determine the efficiency of the different extraction tools used, total As analysis was performed for the extracted cell pellets. The results were compared to the total As values obtained from HNO₃/H₂O₂-digested cell lines. For an extraction tool to be effective, it has to i) break down the cell membrane, and ii) provide high recoveries in a short period of time to prevent As species conversion. As shown in Fig. 1, vortexing for 15 min. provided the lowest extraction efficiency (< 30%). Using the ultrasonic bath for 1 hr. provided higher recoveries (~ 70%). Longer times for vortexing or ultrasonic bathing were not appropriate because of the possibility of changes in arsenic speciation. On the other hand, the ultrasonic probe yielded around 92% extraction recovery within a short period of time (total 9

seconds). The strong wave pulses generated by sonication could be the reason why higher recoveries were obtained through the use of ultrasonic probe and bath. Since the probe has smaller surface area compared to the bath, higher energies were distributed along the solvent surface area, hence providing faster and more efficient extraction. Because some As species are not stable over a long period of time,[23] it is crucial to have fast sample preparation methods. Ultrasonic probe was employed for further optimization experiments.

3.2. Comparison of chemicals vs enzymes as extraction media

Cells vary in structure, some are easily broken, while others require the use of mechanical tools along with appropriate solvents for extraction purposes.[35] Sonication probe was able to break up the MM cells and provided high recoveries for total As. To determine the optimum solvent that can provide best stabilization for the As species and prevent them from decomposition during storage and analysis, different solvents for extraction were tested. A comparison among water, chemicals, and enzymes was performed. MeOH was considered as an extraction media due to its polarity, and both its hydrophilicity and hydrophobicity. TFA was considered for its high polarity and ionic form. Enzymes were also employed due to their ability to break down cell membranes. Pepsin, trypsin and protease were chosen for this study. Arsenic species stability and their chromatographic separation were evaluated with these solvents. With the use of MeOH/water (50/50), good recoveries were observed for As^{III} and its corresponding GSH complex, As(GS)₃ (Fig. 2). However, MeOH/water was unable to preserve MMA^{III}, DMA^{III} and their corresponding GSH complexes even in the presence of around 2.5 mM GSH (a biologically relevant concentration[36]). Pentavalent forms of these species were observed in the chromatograms (data not shown), indicating rapid oxidation of MMA^{III} and DMA^{III}. In addition, the use of MeOH/water interfered with the chromatographic separation for some As species. Broad chromatographic peaks were obtained for As^{III} and As(GS)₃ and peak tailing was observed for MMA^{III} and MMA(GS)₂ (data not shown). Use of 0.1% TFA was acceptable for As(GS)₃ and excellent for MMA(GS)₂ stabilization, while it yielded fast oxidation of MMA^{III} and DMA^{III} (Fig. 2). Decomposition and oxidation of DMA(GS) to DMA^V during storage was also observed. With respect to the enzymes, protease provided stability for MMA(GS)₂, while large decomposition and oxidation was observed in case of MMA^{III}, DMA^{III} and DMA(GS) over a period of 5 days. As^{III} oxidation and As(GS)₃ decomposition was also observed with this enzyme. Protease is an enzyme derived from a bacterial source, and As oxidation due to bacterial activity is well documented.[37] Bacterial activity leading to As species oxidation with the use of protease was suspected. Experiments performed to remove bacterial activity using 0.4 % formaldehyde decreased the trivalent As species oxidation to below 30% over 5 days (data not shown). Utilization of pepsin and trypsin yielded similar stability patterns for the As species. Although the stabilities of the complexes in these enzymes were similar to that of protease, the stabilities for As^{III}, MMA^{III} and DMA^{III} were higher in the presence of pepsin and trypsin. It is interesting to note that water alone provided similar stabilities as pepsin and trypsin. Since As species can be absorbed by cellular compartments or chemically bound to proteins or peptides as complexes, the use of enzymes along with mechanical tools could enhance break down of the cellular material, and hence provide better opportunity for the analysis of all As species. Therefore, pepsin and trypsin are alternative candidates as extraction solvents for cells that cannot be effectively broken by the use of water along with sonication.

3.3. Effect of GSH concentration on the formation and stability of As-GSH complexes in cell extracts

The dependence of the stability of As-GSH complexes on GSH concentration has been reported previously.[23] The effect of GSH concentration on formation and stability of the complexes in the cellular extracts was studied. As shown in Fig. 3A, As(GS)₃ was very

unstable both in the cell samples and in the standards containing 0.5 mM GSH. Speciation analysis revealed only the presence of As^{III} , indicating $\text{As}(\text{GS})_3$ was either not formed or decomposed immediately at the experimental conditions employed. Although GSH was present in excess of the amount needed stoichiometrically (3 GSH molecules for 1 As^{III}), it could have been oxidized rapidly. A similar pattern was observed for $\text{MMA}(\text{GS})_2$ in the standards and cell samples containing 0.5 mM GSH with MMA^{III} being the major species identified (Fig. 3B). Only a small amount of $\text{MMA}(\text{GS})_2$ was detected with $\sim 5\%$ relative intensity at the beginning of the test period. Upon storage, $\text{MMA}(\text{GS})_2$ completely decomposed into MMA^{III} . On the other hand, $\text{DMA}(\text{GS})$ was detected with $\sim 10\%$ relative intensity initially and over time, higher amounts of this species was formed both in the standards and in the cell samples containing 0.5 mM GSH (Fig. 3C). Compared to 0.5 mM GSH, the ability of 5 mM GSH to form and stabilize As-GSH complexes at pH = 7.4 is clearly depicted in Fig. 3. $\text{As}(\text{GS})_3$ and $\text{MMA}(\text{GS})_2$ were present at high relative intensities throughout the trial period. Initially, $\text{DMA}(\text{GS})$ had low relative intensity. Over time an increase in its intensity was observed reaching 65 and 86% on day 5 in standards and cell lysates, respectively. This increase in intensity over time could be due to the slow formation kinetics of this species. Arsenic speciation analysis revealed no significant decomposition of As-GSH complexes in the cell matrix compared to those in the standards for the trials with 5 mM GSH. In fact, further stabilization of $\text{DMA}(\text{GS})$ in the cellular matrix compared to standards was observed (Fig. 3C). Hence, it was possible to verify that the chromatographic method employed does not effect on the equilibrium of the As species in the cell lysates.

These results have significant implications about the possible formation of As-GSH in cells and artifacts on As speciation so far performed in human cells in this field. Cells are rich in GSH and upon extraction its concentration is diluted, generating a false situation from which As-GSH complexes generally exist in cells. In order to obtain the true speciation pattern, extraction should be performed through mimicking the cell conditions particularly the GSH concentration. Using PBS at pH 7.4 that mimics cell pH and salinity is apparently not enough. To test this hypothesis further experimentation was performed.

3.4. Speciation analysis of $\text{DMA}(\text{GS})$ incubated cell lines

To identify the metabolites of $\text{DMA}(\text{GS})$ in cell lines, As speciation of $\text{DMA}(\text{GS})$ incubated cell lines was evaluated with or without maintaining the GSH concentration similar to the intracellular GSH. The concentration of intracellular GSH in the cell lines extracted with PBS was first determined and found to be $8.26 \pm 0.09 \mu\text{M}$. Since 1.5 mL PBS was used as extracting solvent and the cell volume in the centrifuge tube was initially around 3 to 8 μL , it was determined that 190 - 500 (mean of 350) fold dilution of the intracellular GSH was performed. Hence, the original concentration of intracellular GSH was estimated to be around 2.5 mM. Based on these results, extraction of the $\text{DMA}(\text{GS})$ incubated cell lines was performed using PBS (pH = 7.4) in the presence or absence of 2.5 mM GSH. The speciation analysis of the cell extracts revealed a drastic increase in $\text{DMA}(\text{GS})$ peak area in the presence of 2.5 mM GSH (Fig. 4). The relative intensity of $\text{DMA}(\text{GS})$ increased from 14 % without GSH to 89 % with GSH, while DMA^{V} decreased from 76 % to 11 %. These results confirmed that $\text{DMA}(\text{GS})$ was likely present in the cells in large amounts, however, it was decomposed and oxidized during extraction due to the dilution in intracellular GSH concentration. It is also worth mentioning that with the presence of 2.5 mM GSH in the extracts, the increase in $\text{DMA}(\text{GS})$ peak area was fast. Previously, when GSH was added to DMA^{III} standard, the formation of $\text{DMA}(\text{GS})$ was slow with a lower relative intensity in the standards compared to the cell matrix (Fig. 3C). Probably, the cell matrix is enhancing the reaction kinetics for the formation and stability of $\text{DMA}(\text{GS})$.

4. Conclusions

Extraction of arsenicals from cell lines was evaluated in terms of the use of extraction solvents and tools. The ultrasonic probe was capable of extracting the As species from the MM cell lines with high recoveries. Since the ultrasonic probe is very time efficient, its use is highly recommended for the preservation of As species identity. Among the extraction solvents tested, water, pepsin or trypsin provided the best stability for the As species. It should be noted that As-GSH complexes are highly unstable in the absence of excess GSH and samples should be analyzed immediately following extraction to prevent the variations in As species speciation. The effect of GSH at biologically relevant concentrations on the formation of As-GSH complexes is important to note; the presence of 5 mM GSH in the cellular extracts could prevent the decomposition of the As-GSH complexes at biological pH conditions. The results of As speciation in DMA(GS) incubated MM cell lines in the presence of 2.5 mM GSH (the concentration mimic to that in the MM cells) provide further evidence of the role GSH plays in the cellular metabolism of As and the identification of As-GSH complexes. This study indicates that in order to maintain the integrity of arsenicals present in cells, the selection of the extraction tools and solvents must be performed carefully. GSH should be added to the extractant at the concentration similar to that in the test cells.

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Fig. 1.

Extraction recoveries ($n \geq 3$) obtained through the use of different extraction tools. Water was used as extraction solvent. Vortexing was performed for 15 min., the ultrasonic bath was operated for 1 h while the ultrasonic probe was employed for 9 s.

Fig. 2. Stabilities of 1.33 μM of trivalent As species (As^{III} , MMA^{III} and DMA^{III}) and As-GSH complexes ($\text{As}(\text{GS})_3$, $\text{MMA}(\text{GS})_2$ and $\text{DMA}(\text{GS})$) in the presence of 2.5 mM GSH in water, chemicals and enzymes over a five day period. Samples were prepared and analyzed in triplicate using HPLC-ICP-MS.

Fig. 3. Effect of GSH concentration on stability of A- As(GS)₃, B- MMA(GS)₂ and C- DMA(GS). 1.33 μ M of As incubated with either 0.5 or 5mM GSH over 20 hours at room temperature and light in PBS buffer at pH = 7.4. Comparison of standard mixture against a cell matrix containing 1.0×10^6 cells broken down with ultrasonic probe at level 2 is demonstrated over a period of 5 days. Samples were prepared and analyzed in triplicate.

Fig. 4. Speciation chromatogram of DMA(GS) culture incubated cell lines obtained using HPLC-ICP-MS. Cells were extracted with PBS (pH 7.4) in the presence or absence of 2.5 mM GSH. The peaks in the chromatograms correspond to: 1- DMA^V, 2- unknown As, 3- DMA(GS)

Table 1
HPLC, ICP-MS and ESI-MS operating parameters

HPLC		
Gradient program	Formic acid (%)	Acetonitrile (%)
0 min.	95	5
10 min.	85	15
Equilibrate 5 min.	95	5
Injection volume (μL)	25 for ESI-MS	50 for ICP-MS
ICP-MS		
Rf power (W)	1350	
Plasma flow rate (L min. ⁻¹)	15.00	
Auxiliary gas flow (L min. ⁻¹)	1.20	
Nebulizer gas flow (L min. ⁻¹)	0.94-1.02	
Dwell time (ms)	600	
Lens Voltage (V)	7.25	
Acquisition time (min.)	15	
Post column injection time (min.)	13	
Post column injection duration (sec.)	25	
ESI-MS		
ISpray voltage (kV)	5	
Sheath gas (arb)	30	
Capillary voltage (V)	25	
Temperature (°C)	260	
Tube lens offset (V)	10	
Acquisition time (min)	15	