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Determination of multiple human arsenic metabolites employing high performance liquid chromatography inductively coupled plasma mass spectrometry

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Abstract

During the metabolism of different arsenic-containing compounds in human, a variety of metabolites are produced with significantly varying toxicities. Currently available analytical methods can only detect a limited number of human metabolites in biological samples during one run due to their diverse characteristics. In addition, co-elution of species is often unnoticeable with most detection techniques leading to inaccurate metabolic profiles and assessment of toxicity. A high performance liquid chromatography inductively coupled mass spectrometry (HPLC-ICP-MS) method was developed that can identify thirteen common arsenic metabolites possibly present in human with special attention dedicated to thiolated or thiol conjugated arsenicals. The thirteen species included in this study are arsenite (As^{III}), arsino-glutathione ($\text{As}(\text{GS})_3$), arsenate (As^{V}), monomethylarsonous acid (MMA^{III}), monomethylarsino-glutathione ($\text{MMA}^{\text{III}}(\text{GS})_2$), monomethylarsonic acid (MMA^{V}), dimethylarsinous acid (DMA^{III} (from $\text{DMA}^{\text{III}}\text{I}$)), S-(dimethylarsinic)cysteine ($\text{DMA}^{\text{III}}(\text{Cys})$), dimethylarsino-glutathione ($\text{DMA}^{\text{III}}(\text{GS})$), dimethylarsinic acid (DMA^{V}), dimethylmonothioarsinic acid (DMMTA^{V}), dimethyldithioarsinic acid (DMDTA^{V}), dimethylarsinothiyl glutathione ($\text{DMMTA}^{\text{V}}(\text{GS})$). The developed method was applied for the analysis of cancer cells that were incubated with Darinaparsin ($\text{DMA}^{\text{III}}(\text{GS})$), a novel chemotherapeutic agent for refractory malignancies, and the arsenic metabolic profile obtained was compared to results using a previously developed method. This method provides a useful analytical tool which is much needed in unequivocally identifying the arsenicals formed during the metabolism of environmental arsenic exposure or therapeutic arsenic administration.

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Keywords

High performance liquid chromatography inductively coupled mass spectrometry (HPLC-ICP-MS); arsenic speciation; arsenic metabolism in humans; dimethylarsinous glutathione

1. Introduction

Arsenic (As), through naturally occurring from the Earth's crust to groundwater and anthropogenic activities such as nonferrous metal mining and smelting, coal combustion, and pesticide application, has resulted in the contamination of water, soil, and food around the world [1-4]. The chronic toxicity of As exposure through drinking water and food (e.g., rice) poses serious health risks to millions of people [5-9]. Toxic effects of As include diabetes, anemia, diarrhea, and more importantly, the development of different types of cancers such as carcinomas of the skin, lung, bladder, kidney and liver [10-12].

Despite of its known toxicity and potential to cause cancer, As has been historically used for medicinal purposes to treat such diseases as ulcers, head lice, and plague [13-16]. Although the current use of medical As has been limited due to its high toxicity and carcinogenicity, some As compounds have been recently rediscovered for the treatment of certain cancers [13-14, 17]. For example, arsenic trioxide (As_2O_3 , ATO, Trisenox) was revived for the treatment of both newly diagnosed and relapsed acute promyelotic leukemia (APL) [13, 18]. S-dimethylarsino-glutathione (Darinaparsin, DAR) has been in clinical trials for hematological cancers and refractory solid tumors [19-22].

Both toxicity and anticancer activity of As are determined by the metabolism of As in human body. Arsenic metabolism is rather complicated, as the metabolic profile depends on the As species introduced, the route of administration, and the type of cells involved in As elimination. A wide variety of As species, including methylated arsenicals such as DMA^{V} , MMA^{V} , DMA^{III} , and MMA^{III} and As-glutathione (GSH) conjugates, have been observed during As metabolism (see Table 1 for the names, structures, and abbreviations of all thirteen arsenicals included in this study). In particular, a new type of arsenicals, thiolated As compounds including DMMTA^{V} , DMDTA^{V} and $\text{DMMTA}^{\text{V}}(\text{GS})$, has been recently detected in humans and/or mammals [23-28]. Although efforts have been made to reveal the pathways of As metabolism, much remains unclear about how these As species are formed and what role these arsenicals play in the toxicity and therapeutic efficacy of As [29-30].

In order to understand the metabolic pathways of As, the mode of action of As toxicity, and the mechanisms underlying As chemotherapy, we must have an analytical method that is capable of unequivocally identifying these arsenicals formed during the metabolism of environmental As exposure or therapeutic As administration. Speciation analysis of As metabolites is important, because each As species possesses varying toxicities. For example, MMA^{III} and DMA^{III} were shown to be more genotoxic than inorganic As (iAs^{III}) and more potent inhibitors of enzymes while DMMTA^{V} is more cytotoxic than DMA^{V} [23, 31-33]. Generally trivalent arsenicals are much more toxic than pentavalent ones, and are taken up more efficiently by cells than pentavalent ones [34-35].

Existing As speciation techniques usually employ high performance liquid chromatography (HPLC) separation on reverse phase or ion exchange column, coupled to an element specific detector such as inductively coupled plasma mass spectrometer (ICP-MS), atomic fluorescence spectrometer (AFS), or atomic absorption spectrometer (AAS) [36-40]. Unfortunately, currently available techniques have shortcomings and cannot meet the need of “full-spectrum” analysis of As metabolites. First, ion chromatography was found to decompose glutathione (GSH) complexes of As, failing to detect these As-GSH complexes [41-42]. Since As-GSH complexes are thought to be necessary intermediates for As methylation, their unequivocal identification is important to depict the pathways of As metabolism. Second, many current As speciation methods are unable to detect thiolated arsenicals such as DMMTA^V, DMDTA^V, and DMMTA^V(GS), and in particular cannot simultaneously determine methylated species (trivalent and pentavalent) and sulfur-containing arsenicals. It is necessary to identify thiolated arsenicals as these As species may play an important role in As metabolism, toxicity, and anticancer activity. Finally, current analytical methods usually are able to separate a limited number of As species and co-elution of species occurs during analysis. For example, many of the methods employing anion exchange columns could not distinguish MMA^{III} from MMA^V and DMA^{III} from DMA^V, providing incomplete information on the profile of As metabolism.

The objective of this study was to develop an analytical method using HPLC-ICP-MS that is capable of performing a “full-spectrum” analysis of As metabolites. The method was aimed to simultaneously determine the newly discovered thiolated or thiol conjugated As metabolites (e.g., DMMTA^V, DMDTA^V, and DMMTA^V(GS)) and highly toxic trivalent methylated As (DMA^{III} and MMA^{III}), in addition to the As species of traditional interest (e.g. inorganic As^V and As^{III} and organic DMA^V and MMA^V). Thirteen possible As metabolites, representing most of the As species reported so far during As metabolism, were included in this study. As the method was intended for analysis of human As metabolites, thiolated arsenicals, trivalent methylated As species, and As-GSH complexes were the primary targets of separation and detection. A variety of chemical and instrumental parameters were optimized for the method, with a focus on the key parameters influencing separation and detection of these thiolated arsenicals. The method was applied to the analysis of human cancer cells treated with Darinaparsin to demonstrate its application in determining human metabolites of this anticancer drug.

2. Materials and Methods

2.1. Reagents

Argon (Ar) purged double deionized water (DDIW) (18.2 MΩ·cm, Barnstead Nanopure Diamond) was used throughout the experiments. Acetonitrile (ACN) and methanol were purchased from Thermo Fisher Scientific, USA. Ammonium hydroxide (NH₄OH), sodium hydroxide (NaOH), potassium hydroxide (KOH), phosphoric acid (H₃PO₄), hydrochloric acid (HCl), and nitric acid (HNO₃) used for pH adjustments were also purchased from Thermo Fisher Scientific. Isopropyl alcohol was obtained from Fluka. Glycine, L-glutathione reduced, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich, USA. TFA and anhydrous citric acid were bought from Acros Organics. Formic acid and

acetic acid used to prepare formate and acetate buffers were purchased from Acros Organics and Thermo Fisher Scientific, respectively. Sulfur dioxide gas used for the synthesis of $\text{MMA}^{\text{III}}\text{I}_2$ and $\text{DMA}^{\text{III}}\text{I}$ was acquired from Specialty Gasses of America. Sodium metaarsenite (98%), sodium arsenate (99%), and cacodylic acid (98%) were from Aldrich. Monosodium acid methane arsonate sesquihydrate was obtained from Chem Service, PA, USA. Solid $\text{DMA}^{\text{III}}(\text{GS})$, obtained from Ziopharm Oncology, USA, was prepared in solution in our lab along with other arsenic standards used. All reagents used were of analytical grade or better.

Arsenical standards synthesized in house—Due to the lacking availability, the following arsenical standards were synthesized in the laboratory: $\text{MMA}^{\text{III}}\text{I}_2$, $\text{DMA}^{\text{III}}\text{I}$, $\text{DMA}^{\text{III}}(\text{Cys})$, $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MMA}^{\text{III}}(\text{GS})_2$, $\text{DMA}^{\text{III}}(\text{GS})$, DMMTA^{V} , DMDTA^{V} , and $\text{DMMTA}^{\text{V}}(\text{GS})$. The synthesis of these arsenicals was based on the previously reported procedures with some modifications if necessary, and the identity and purity (if it was possible to purify the arsenicals using currently available procedures) of the synthesized As standards were verified with NMR, HPLC-ESI-MS, and/or HPLC-ICP-MS. The detailed information for the synthesis of these As standards was provided in the Supplementary Information.

2.2 Instrumentation

A Perkin Elmer Series 200 HPLC system equipped with a temperature controlled column compartment was coupled to a Perkin Elmer Elan DRC-e ICPMS. The ICPMS was equipped with a cyclonic spray chamber and a Meinhard nebulizer and was used in the standard mode. ICPMS signal at m/z 75 for ^{75}As was monitored. Data was collected and processed using Elan v. 3.4 and Chromera v. 1.2 software (Perkin Elmer, USA).

2.3 Optimization of HPLC and ICP-MS parameters

Experiments were performed to optimize a variety of parameters, including HPLC column type and dimension, column temperature, mobile phase (composition, pH, and gradient program), and ICP-MS parameters such as nebulizer gas, RF power, analog stage, and lens voltages. It was observed that HPLC mobile phase and ICP-MS nebulizer gas were the key factors influencing the separation and detection of the As compounds of interest. Therefore the experiments were focused on these two parameters to find the most optimal conditions for method performance.

The optimization of mobile phase included selection of aqueous buffer and organic solvent and the gradient elution program. Considering the pK_a values of arsenicals, a number of buffers, including trifluoroacetate (TFA, $\text{pK}_a = 0.5$), formate ($\text{pK}_a = 3.8$), and acetate ($\text{pK}_a = 4.8$), were tested. The commonly used organic solvents, such as acetonitrile, methanol, and isopropyl alcohol, were test for separation of the As species of interest. The gradient elution program was optimized by changing the v/v % of the selected organic component from 0-20%.

Nebulizer gas was optimized for its composition and flow rate to investigate its effect on detection of the arsenic metabolites selected in this study. In order to address the issue of

plasma stability and instrument contamination, which was caused by high v/v % of organic solvent used here (see Results and Discussion), O₂ was added through a T-connector to the nebulizer Ar gas. The flow rate of Ar for nebulization was further optimized to investigate its effect on method performance, by varying the flow rate from 0.80 to 1.04 L min⁻¹.

2.4 Linearity, limit of detection (LOD), and limit of quantitation (LOQ)

The v/v % of organic solvent in mobile phase significantly changes the ionization characteristics in the plasma and thus the sensitivity of the method. Consequently, the linearity, LOQ, and LOD of the method were determined using two compounds, As^{III} and As^{III}(GS)₃, with the former eluting during 1% of organic solvent (ACN was selected, see Results and Discussion) while the latter at 15% of ACN. Linearity, LOQ, and LOD studies were performed under the optimized instrumental conditions.

2.5 Determination of metabolites of DMA^{III}(GS) in human cells

The developed method was used to analyze the metabolites of DMA^{III}(GS) in 8226/S multiple myeloma cell line (ATCC, Manassas, VA). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ on RPMI-1640 media, supplemented with 100 U mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin, 10% heat inactivated fetal bovine serum and 2 mM L-glutamine (all culture reagents from Cellgro, MediaTech, Herndon, VA). Following incubation the cells were harvested by centrifugation at 1000 rpm for 5 minutes, washed with PBS once, spun down again, then the pellets were frozen in liquid nitrogen and stored at -11 °C. Each cell pellet contained 5.0×10^6 cell counts. For the As exposure experiment, the cell pellets were thawed, spiked with 0.26 mM DMA^{III}(GS) in double deionized water (DDIW) and left for 10 minutes. The cells were then broken up with a sonication probe, and the sample was filtered through a 0.2 µm nylon syringe filter into an HPLC vial and analyzed. Unspiked cells used as control were prepared in the same way, but without spiking the cells with DMA^{III}(GS).

3 Results and Discussion

Since a number of As metabolites could exist in human cells and some of which (e.g., trivalent arsenicals) are redox sensitive, an efficient separation is required for their determination to maintain integrity of the analytes. We first compared three HPLC columns, namely ion exchange, C₁₈, and C₈, and found that C₈ column was the most promising for further optimization to achieve better separation of a variety of arsenicals. Further experiments were then performed to optimize the C₈ column-based HPLC-ICP-MS method, focusing on mobile phase and nebulizer gas as we observed they are critical in the performance of the method. Since lower temperatures help preserve the oxidation state of trivalent As species and prevent the degradation of As-glutathione complexes, concurring with previous studies [41], all experiments were conducted with the column kept at 10 °C.

3.1 Mobile phase

It was observed that a mixture of aqueous buffer and organic solvent was needed for the separation of the arsenic species of interest. For aqueous mobile phase, a number of buffers were evaluated, particularly such volatile buffers as trifluoroacetate (TFA, pK_a = 0.5),

formate ($pK_a = 3.8$), and acetate ($pK_a = 4.8$) [43]. The selection of these buffers was primarily based on pH. Ideally, the pH of the mobile phase should be chosen to be at least two units away from the analyte pK_a values in order to have all the analytes present in their neutral or fully ionized forms [43]. Considering that the species to be separated have a wide range of pK_a values (including unknown pK_a for some species) and that many of these species are more stable at lower pH values, the final pH range studied was set from 1.5 to 4.6 based on the available pK_a values [41]. In addition to pH, volatility was considered a factor for selection of aqueous mobile phase as the developed separation method is intended to be used not only with ICP-MS, but also with electrospray mass spectrometry (ESI-MS). HPLC-ESI-MS can be used for the unequivocal identification of the species in question. A comparison of the above 3 mobile phases yielded that the best separation can be achieved using 0.05% TFA as the aqueous mobile phase (data not shown).

For organic solvents in the mobile phase, ACN, methanol, and isopropyl alcohol were considered. It was found that ICP did not tolerate isopropyl alcohol well even at a concentration as low as 2-3 % v/v and isopropyl alcohol was not used after the initial trials. Methanol, though evaluated extensively to be used for separation, was not selected due to the decreased stability of certain arsenic species in methanol [42]. ACN was eventually selected, as it has higher eluotropic strength when used at the same volume percentage compared to methanol [43] achieving adequate separation within reasonable time. An additional benefit of acetonitrile addition to the mobile phase was the enhanced signal on ICP-MS detection (data not shown). This was not surprising, as it is known that the presence of organic solvents at 1-5 v/v% would cause signal enhancement of As and other elements with higher ionization potentials due to the modification of equilibrium in the plasma [44]. Since the degree of ionization of carbon is significantly larger than that of Ar, the introduction of organic solvents in the plasma would lead to the increased population of C^+ (ionization energy, E_i , 11.26 eV). The transfer of electron to C^+ from an element with lower E_i (such as ^{75}As which has an E_i of 9.81 eV) would increase the degree of ionization of that element, resulting in the signal enhancement.

After selection of aqueous and organic mobile phases, the gradient program was optimized by modifying the v/v % of organic component, as this v/v % is the principal factor that governs the retention of analytes in reversed-phase LC separations. It was found that the v/v % needed to be gradually increased and a relative large amount of ACN (20% v/v) at the late stage of separation was necessary to achieve the most optimal separation of all species within reasonable time. Fig. 1 illustrates the separation of major As compounds ($25 \mu g L^{-1}$ of As for all species) under the optimized HPLC gradient program and other conditions (Table 2), excluding As-GSH complexes which were separately discussed in section 3.3. The method was able to determine a number of As species, including As^V , As^{III} , MMA^V , DMA^V , MMA^{III} , and DMA^{III} , $DMMTA^V$, and $DMDTA^V$. The good separation for $DMMTA^V$, $DMDTA^V$, MMA^{III} , and DMA^{III} indicates the applicability of the method in determining thiolated and trivalent methylated human As metabolites. Peak overlapping and broadening could be observed for As^{III} , MMA^V , and DMA^V , but further effort was not made to better resolve these species, as the arsenicals of primary interest for this method were thiolated and trivalent methylated arsenicals. A broad peak was observed for DMA^{III} ,

but in human cells DMA^{III} is likely present in GSH complexed form which can be detected as a separate species (see section 3.3).

The flow rate of mobile phase was chosen at 0.5 mL min⁻¹ during the run, and the total run time was set to 30 min with the retention time of the last peak being around 23.4 min. The extra run time (between 26 to 30 min) can be used for the post column injection of a stable internal standard (e.g. As^V), if required, to correct for instrumental drifts. Prior to each run, there was a equilibration stage of 6 min when the mobile phase was 99:1 (v/v) of TFA:ACN at a flow rate of 1 mL min⁻¹ (Table 2). During the last ten minutes of the gradient program, the v/v % of ACN was decreased to 5% from 20% in the previous step. This change of ACN v/v % not only had no significant effect on the retention time and the resolution of the peaks eluted during this period, but also provided a way to reduce the usage of organic solvent and to protect the stability of plasma. If the run was finished with 20% ACN, the plasma would be frequently extinguished once the equilibration stage began when the mobile phase changed to 1% v/v ACN.

3.2 Nebulizer gas

From the optimization of mobile phase, it was seen that high ACN composition (up to 20% v/v) was needed for the separation of As species of interest. The usage of high fraction of organic solvent in the mobile phase caused several problems. First, high fraction of ACN extinguished the plasma frequently during the run. Second, due to the high organic composition in the mobile phase, carbon built up quickly on the ICP sampling interface and torch, necessitating the frequent cleanup or replacement of the torch, the injector, and the cones. Finally, high ACN v/v % in mobile phase produced severe baseline disturbance during gradient elution and caused appearance of ghost peaks, interfering with detection of As species. In order to solve these problems, we did further optimization on the method and found that nebulizer gas was the critical factor. We then optimized nebulizer gas, including composition and flow rate, to solve these problems.

For nebulizer gas composition, we introduced a small amount of O₂ to the nebulizer gas flow, aiming to burn the carbon from organic solvent to avoid carbon deposition on ICP hardware. A T-connector was employed to add 0.2 L min⁻¹ oxygen gas to the Ar nebulizer flow, and the O₂ flow rate was not further optimized, as the manufacturer-recommended 0.2 L min⁻¹ was found appropriate to prevent the instrument from carbon build up for a long time (e.g., months) and from plasma extinguishing.

During the experiments of introducing O₂ into the nebulizer gas flow, it was observed that the intensity of the ICP-MS signal was enhanced when the nebulizer gas was composed of O₂ and Ar than was Ar alone, with the total flow rate being the same (Fig. 2). For example, the signal for MMA^{III}(GS)₂, which was eluted at high ACN v/v %, increased by a factor of about 7 when the nebulizer gas was composed of 0.2 L min⁻¹ of O₂ and 0.84 L min⁻¹ of Ar, compared to 1.04 L min⁻¹ of Ar (Fig. 2A). Similarly, under the same experimental conditions, the signals for As^V and As^{III}, which were eluted at low ACN v/v %, increased when the mixture of O₂/Ar was used as nebulizer gas (Fig. 2B). This signal increase could be due to the introduction of O₂ and/or the difference in Ar flow rates (0.84 L min⁻¹ when O₂ was added versus 1.04 L min⁻¹ with Ar only).

Arsenic standards were run to assess the effect of Ar flow rate on instrumental signal. It was observed that lowering the nebulizer Ar gas flow increased the sensitivity of the instrument for As detection (Fig. 3). Fig. 3A demonstrates an example of the effect of Ar flow on the response of the instrument, with DMA^{III}(Cys) being detected on ICP-MS. The instrumental intensity for DMA^{III}(Cys) increased by a factor of 2 when the Ar flow decreased from 0.90 to 0.80 L min⁻¹ with O₂ present in the nebulizer gas at 0.20 L min⁻¹. This result is indicative of the signal enhancing effect of lower Ar flow rate, but cannot rule out the possibility that the signal enhancement was caused by the decrease in total flow rate of nebulizer gas (from 1.1 to 1.0 L min⁻¹).

Additional experiments were conducted to examine the effect of total flow rate of nebulizer gas (Ar + O₂) on instrumental signal, by detecting DMA^{III}(Cys) at a fixed Ar flow rate with and without the addition of O₂. It was observed that the ICP-MS signals were fairly similar when 0.2 L min⁻¹ of O₂ was added to 0.86 L min⁻¹ of Ar (making up a total flow of 1.06 L min⁻¹), compared to 0.86 L min⁻¹ of Ar alone being used as nebulizer gas (Fig. 3B). This result suggests that the Ar flow rate in the nebulizer gas remarkably affected the instrumental response, whereas O₂ had little effect even if the addition of O₂ increased the total flow rate of nebulizer gas.

Baseline disturbance from high ACN v/v % in the mobile phase was another obstacle affecting the performance of the method, and was found to be related to nebulizer gas. Blanks without As compounds were used to check the chromatographic baseline during gradient elution under different flow rates of nebulizer Ar gas. It was found that the ghost peaks showing at 17-25 min can be substantially decreased by increasing slightly the Ar nebulizer flow (Fig. 4). Increasing the Ar flow from 0.80 to 0.90 L min⁻¹ could reduce the baseline disturbance to an acceptable level. It appears that O₂ had no influence on baseline disturbance, as indicated by the similarity of baseline signals with or without O₂ present in the nebulizer gas when Ar was 0.8 L min⁻¹.

Increasing Ar flow rate reduced baseline disturbance, but at the same time decreased instrumental response. Therefore, the nebulizer Ar flow rate should be carefully optimized prior to analysis, to achieve adequate sensitivity while minimizing the interference from baseline disturbance. After testing Ar at 0.80-0.90 L min⁻¹ for instrumental sensitivity and baseline disturbance, the final Ar flow rate for sample analysis was set to 0.84 L min⁻¹, and the O₂ was 0.2 L min⁻¹ (Table 2). The enhanced signal intensity at lower nebulizer Ar flow rates could be due to more efficient conversion and atomization of sample aerosol, as previously reported [45]. As for O₂, it was observed that O₂ protected the instrument from carbon buildup and from plasma extinguishing, but did not affect signal intensity. Previous studies have reported inconsistent effects of O₂ on instrumental signal, with both suppressed and unchanged signal intensity being observed when O₂ was added to nebulizer gas, probably related to the specific plasma conditions [44, 46].

3.3 Analysis of As-GSH complexes

As-GSH complexes represent an important type of intermediates during As metabolism in human body, and thus the developed method was also intended for analysis of As-GSH complexes. For analysis of As-GSH complexes, the first consideration was about the

standards. The As-GSH complexes standards were prepared by mixing As standard solutions (As^{III} , MMA^{III} , and DMA^{III}) with excess GSH, and thus the standards of As-GSH complexes, e.g., $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MMA}^{\text{III}}(\text{GS})_2$, and $\text{DMA}^{\text{III}}(\text{GS})$, were always present in excess GSH. The standard solution of As-GSH complexes could not mixed with the non-GSH complexed As standards, because, once mixed, the non-GSH complexed arsenicals (free As^{III} , MMA^{III} , and DMA^{III}) would be complexed by GSH from the solution of As-GSH complexes. Therefore we prepared two sets of standards, one for As-GSH complexes and the other for non-GSH complexed arsenicals, and these two types of As standards need to be injected separately. For samples, when GSH and non-GSH complexed arsenicals are present in a sample, equilibrium will be established between the GSH complexes and their non-GSH counterparts depending on the reduced GSH concentration in the sample. When injected, the relative amounts of each arsenic species can be determined based on the two sets of As standards.

Fig. 5A shows a typical chromatogram with good separation for sulfur-containing As compounds ($25 \mu\text{g L}^{-1}$), including As-GSH complexes, such as $\text{As}^{\text{III}}(\text{GS})_3$, $\text{DMMTA}^{\text{V}}(\text{GS})$, $\text{MMA}^{\text{III}}(\text{GS})_2$, and $\text{DMA}^{\text{III}}(\text{GS})$, and thiolated arsenicals, such as DMDTA^{V} and DMMTA^{V} . An overlay of the chromatograms for non-GSH complexes As standards and As-GSH complexes shows the separation of major As metabolites of interest (Fig. 5B), suggesting that the developed method is indeed able to detect both sulfur-containing and non-sulfur-containing arsenicals if they are both present in the sample. In particular, the method was able to analyze thiolated arsenicals, trivalent methylated As species, and As-GSH complexes, including DMMTA^{V} , DMDTA^{V} , $\text{As}^{\text{III}}(\text{GS})_3$, $\text{DMMTA}^{\text{V}}(\text{GS})$, $\text{MMA}^{\text{III}}(\text{GS})_2$, and $\text{DMA}^{\text{III}}(\text{GS})$, suggesting the validity of the method in analyzing human As metabolites.

Another consideration for analyzing As-GSH complexes was related to specific form of DMA^{III} species. The DMA^{III} peak (#12 in the chromatogram, Fig. 5B) corresponded the injection of $\text{DMA}^{\text{III}}\text{I}$ synthesized in our lab, which could be hydrolyzed to $\text{DMA}^{\text{III}}(\text{OH})$ upon dilution in aqueous solutions. When a high concentration of $\text{DMA}^{\text{III}}(\text{GS})$ (#13 in the chromatogram, Fig. 5) was injected, it partially dissociated to DMA^{III} during the run, as shown by the fronting peak of $\text{DMA}^{\text{III}}(\text{GS})$ (Fig. 6). The decomposition of $\text{DMA}^{\text{III}}(\text{GS})$ to DMA^{III} during analysis, along with the lack of availability of pure DMA^{III} standard and DMA^{III} being extremely prone to oxidation during storage and sample preparation, make it nearly impossible to accurately determine the native DMA^{III} concentration in a sample. As the preparation of $\text{DMA}^{\text{III}}\text{I}$ is rather difficult, $\text{DMA}^{\text{III}}(\text{Cys})$ has been used as standard for DMA^{III} in a number of studies, which further complicated the issue related to identification and detection of the specific form of DMA^{III} species. By using the developed method, we observed that $\text{DMA}^{\text{III}}\text{I}$ (probably should be labeled as DMA^{III} as this species usually refers to the non-conjugated form) and $\text{DMA}^{\text{III}}(\text{Cys})$ gave two completely different peaks, with a very broad peak for DMA^{III} and a sharp one for $\text{DMA}^{\text{III}}(\text{Cys})$ (Fig. 6). Therefore, $\text{DMA}^{\text{III}}(\text{GS})$, $\text{DMA}^{\text{III}}(\text{Cys})$, and DMA^{III} (non-conjugated form) should be treated as three different As species and caution should be exercised to avoid confusion. We further demonstrated that the developed method was able to separate all these three forms of the trivalent dimethylated arsenical (Fig. 6), if they are present as As metabolites in a sample.

3.4 Linearity, LOQ and LOD

Linearity, LOQ, and LOD were determined using As^{III} and $\text{As}^{\text{III}}(\text{GS})_3$ standards under the optimized conditions, with a nebulizer flow consisting of $0.2 \text{ L min}^{-1} \text{ O}_2$ and $0.84 \text{ L min}^{-1} \text{ Ar}$ giving a total flow of 1.04 L min^{-1} . Because As^{III} and $\text{As}^{\text{III}}(\text{GS})_3$ were two peaks eluted at low and high acetonitrile v/v %, respectively, the results obtained for these two species are indicative of the method performance for all As species. The method was found to be linear between the tested range of 1.25 to $2500 \mu\text{g L}^{-1}$ for both As^{III} and $\text{As}^{\text{III}}(\text{GS})_3$ ($r^2 > 0.999$). Limit of detections of As^{III} and $\text{As}^{\text{III}}(\text{GS})_3$ were experimentally determined to be $1.25 \mu\text{g L}^{-1}$ with an average of signal to noise ratio (S/N) of 3 for three injections for both compounds (at $5 \mu\text{g L}^{-1}$). The limits of quantitation for As^{III} and $\text{As}^{\text{III}}(\text{GS})_3$ were set to be $2.5 \mu\text{g L}^{-1}$ for each, based on the low percent relative standard deviations of the peak areas (4.2 for As^{III} and 7.0 for $\text{As}^{\text{III}}(\text{GS})_3$, respectively).

3.5 Application of method

The analysis of the 8226/S multiple myeloma cells exposed to $\text{DMA}^{\text{III}}(\text{GS})$ was performed using the developed method, following the separate injection of non-GSH complexed and GSH-complexed As standards. The results showed that, in addition to the parent $\text{DMA}^{\text{III}}(\text{GS})$, its dissociation product, DMA^{III} , along with the further oxidation product, DMA^{V} , were detected (Fig. 7). The thiol-containing arsenical, DMMTA^{V} , was detected, and more importantly its GSH conjugate, $\text{DMMTA}^{\text{V}}(\text{GS})$, was found to be the major metabolite of $\text{DMA}^{\text{III}}(\text{GS})$ metabolism in the cells. These results suggest that thiol-containing arsenicals may play an important role in the metabolism of $\text{DMA}^{\text{III}}(\text{GS})$ and these thiolated arsenicals could be eventually related to the anticancer activity of $\text{DMA}^{\text{III}}(\text{GS})$. The simultaneous determination of thiol-containing and non-thiol-containing arsenicals (e.g., $\text{DMMTA}^{\text{V}}(\text{GS})$, DMMTA^{V} , and DMA^{III}) demonstrates the robustness of the developed method, as a combination of two HPLC-ICP-MS methods had to be used to run the same sample twice for the separation of the metabolites of interest in our previous studies [25]. Even running both previous methods (on two different columns) could not separate all possible As intermediates during Darinaparsin metabolism, as indicated by the missing of DMMTA^{V} in the previous studies. It was also observed that the method developed had better resolution of As species separation.

3.6 Potentials and challenges

The HPLC separation method developed in this study can be used for ICP-MS (as reported here) or ESI-MS detection, as the mobile phases used in the HPLC separation are compatible with ESI-MS. It is possible to use the separation method on ESI-MS for the identification of unknown As species, with the flow rate of mobile phase being adjusted to 0.5 mL/min during equilibration and the equilibration time being extended due to the reduced flow rate. Fortunately, these changes during equilibration stage will not affect chromatographic separation of As species. This dual suitability of the separation method for both ICP-MS and ESI-MS can help us in the unequivocal identification of As species. It should be noted that not all As species could be easily detected on ESI-MS, as some As species do not have high ionization efficiency and/or could get oxidized during the electrospray ionization.

When identifying and quantifying As metabolites, the unavailability of some species as stable high purity standards poses a significant problem. As^{III}, As^V, MMA^{III}, MMA^V, DMA^V, As^{III}(GS)₃, MMA^{III}(GS)₂, DMA^{III}(Cys), and DMA^{III}(GS) can be purchased or synthesized as high purity standards, but DMDTA^V, DMMTA^V, DMA^{III}, and DMMTA^V(GS) cannot, making the quantification of these species difficult. For example, DMA^{III} has the high potency of being oxidized to DMA^V without the presence of relatively high concentrations of GSH, so it is difficult to accurately quantify this species in the absence of GSH. However, it is also difficult to detect DMA^{III} in the presence of GSH, as in this case DMA^{III} is present in the form of DMA^{III}(GS) and not free DMA^{III}.

4 Conclusions

An HPLC-ICP-MS method was developed and optimized in an attempt to analyze for the above mentioned thirteen confirmed and plausible human As metabolites and intermediates of As metabolism in one chromatographic run. Results showed that while it is difficult to separate all these species due to their very different chemical properties, it is indeed achievable. The HPLC-ICP-MS technique described here is applicable to the analysis of these arsenic metabolites from cells as demonstrated with the incubation experiments of human cancer cells with DMA^{III}(GS). While ICP-MS is supposed to be element specific, significant baseline disturbance was observed due to high ACN v/v% present in the gradient elution program. Adjustment of Ar nebulizer flow rate could reduce the problem. Under optimum conditions, the detection limits were shown to be 1.25 µg L⁻¹ and the quantitation limit 2.5 µg L⁻¹. The method was found to be linear between the tested ranges of 1.25 to 2500 µg L⁻¹. This method could be also suitable for use on HPLC-ESI-MS for the identification of unknown As metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Development of an HPLC-ICP-MS method for the detection of 13 human arsenic metabolites.
- Analysis of multiple sulfur-containing arsenicals and glutathione complexes.
- Identification of arsenic metabolites in human cancer cells exposed to dimethylarsinous glutathione.

Fig. 1.

Typical chromatogram for separation and analysis of As compounds ($25 \mu\text{g L}^{-1}$) containing no glutathione using HPLC-ICP-MS. The compounds separated are as follows: 1. As^{V} , 2. DMDTA^{V} , 3. As^{III} , 4. MMA^{V} , 5. MMA^{III} , 6. DMMTA^{V} , 7. DMA^{V} , 12. DMA^{III} , * Baseline disturbance originating from high concentration acetonitrile gradient program.

Fig. 2.

Demonstration of signal enhancement due to changes in nebulizer gas composition and flow rate for (A) $\text{MMA}^{\text{III}}(\text{GS})_2$ eluting during high (20%) v/v% acetonitrile and (B) As^{V} and As^{III} eluting during low (1%) v/v% acetonitrile in the gradient program.

Fig. 3.

The effect of nebulizer gas on instrumental response demonstrated by (A) decreased sensitivity for DMA^{III}(Cys) detection with increasing Ar flow rate and (B) similar responses of DMA^{III}(Cys) at the same Ar flow rate regardless of the presence of oxygen. The effect of Ar flow rate on baseline disturbance is also observable in the figure.

Fig. 4.
Baseline disturbance vs nebulizer flow rate and composition for blank injection.

Fig. 5.

Separation of As metabolites demonstrated by (A) injection of glutathione complexes and thiolated arsenicals and (B) overlay of the chromatograms for injection of sulfur-containing and non-sulfur-containing arsenicals ($25 \mu\text{g L}^{-1}$). The compounds separated are as follows: 1. As^{V} , 2. DMDTA^{V} , 3. As^{III} , 4. MMA^{V} , 5. MMA^{III} , 6. DMMTA^{V} , 7. DMA^{V} , 8. $\text{As}^{\text{III}}(\text{GS})_3$, 9. $\text{DMMTA}^{\text{V}}(\text{GS})$, 10. $\text{MMA}^{\text{III}}(\text{GS})_2$, 12. DMA^{III} , 13. $\text{DMA}^{\text{III}}(\text{GS})$, * Baseline disturbance originating from high concentration acetonitrile gradient program.

Fig. 6. Resolution of DMA^{III}, DMA^{III}(Cys), and DMA^{III}(GS) demonstrated by (A) generation of DMA^{III} during high concentration of DMA^{III}(GS) injection, (B) separation of DMA^{III}(Cys) (#11) and DMA^{III}I (#12), and (C) separation of DMA^{III}(Cys) from As-GSH complexes.

Fig. 7. Results of human cells analysis using the developed method after incubation with darinaparsin. The compounds separated are as follows: 6. DMMTA^V, 7. DMA^V, 9. DMMTA^V(GS), 12. DMA^{III}, 13. DMA^{III}(GS)

Table 1

Names, abbreviations and structures of compounds of interest

Names	Most common		Structure	Occurrence in humans	pK _a [47]
		Abbreviations			
Arsenate, Arsenic acid		As ^V	$\begin{array}{c} \text{O} \\ \parallel \\ \text{HO} - \text{As} - \text{OH} \\ \\ \text{OH} \end{array}$	Urine [48-52] Fingernail [50] Blood plasma [50, 53]	2.3 6.7 11.6
Dimethyldithioarsinic acid Dimethylarsinodithioic acid		DMDTA ^V	$\begin{array}{c} \text{S} \\ \parallel \\ \text{CH}_3 - \text{As} - \text{CH}_3 \\ \\ \text{SH} \end{array}$	In mammals [24, 26]	
Arsenite, Arsenious acid		As ^{III}	$\begin{array}{c} \text{HO} - \text{As} - \text{OH} \\ \\ \text{OH} \end{array}$	Urine [48-52] Fingernail [50] Blood plasma [53]	9.2
Monomethylarsonic acid, Methylarsonic acid		MMA ^V MA ^V S ^V	$\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_3 - \text{As} - \text{OH} \\ \\ \text{OH} \end{array}$	Urine [48-52] Fingernail [50] Blood plasma [50, 53] Hepatocytes [54]	3.6 8.2
Monomethylarsonous acid, Methylarsonite		MMA ^{III} MA ^{III} S ^{III}	$\begin{array}{c} \text{CH}_3 - \text{As} - \text{OH} \\ \\ \text{OH} \end{array}$	Urine [48-52, 55] Hepatocytes [54]	

Most common		Structure	Occurrence in humans	pK _a [47]
Names	Abbreviations			
Dimethylmonothioarsinic acid, Dimethylarsinothioic acid, Thio-dimethylarsinate	DMMTA ^V Thio-DMA DMA ^V _S	$\begin{array}{c} \text{S} \\ \parallel \\ \text{CH}_3 - \text{As} - \text{CH}_3 \\ \\ \text{OH} \end{array}$	Urine [23] Red blood cells [27]	
Dimethylarsinic acid, Dimethylarsinate	DMA ^V DMA ^V _S	$\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_3 - \text{As} - \text{CH}_3 \\ \\ \text{OH} \end{array}$	Urine [48-52] Fingernail [50] Blood plasma [50, 53] Hepatocytes [54]	6.3
Arsino-glutathione	As ^{III} (GS) ₃ ATG	$\begin{array}{c} \text{GS} - \text{As} - \text{SG} \\ \\ \text{GS} \end{array}$	In mammals [56-57]	
Dimethylarsinothioyl glutathione	DMMTA ^V -GS DMATG DMA ^V _S -GS DMTA ^V (G S)	$\begin{array}{c} \text{S} \\ \parallel \\ \text{CH}_3 - \text{As} - \text{CH}_3 \\ \\ \text{SG} \end{array}$	[25]	
Monomethylarsino-glutathione, Monomethylarsonic diglutathione	MMA ^{III} (GS) ₂ MADG	$\begin{array}{c} \text{GS} - \text{As} - \text{CH}_3 \\ \\ \text{SG} \end{array}$	In mammals [56-57]	
S-(Dimethylarsenic) Cysteine	DMA ^{III} Cys	$\begin{array}{c} \text{CH}_3 - \text{As} - \text{CH}_3 \\ \\ \text{Cys} \end{array}$		

Most common			Structure	Occurrence in humans	pK _a [47]
Names	Abbreviations				
Dimethylarsinous acid, Dimethylarsinite	DMA ^{III} DMA _s ^{III}	$\begin{array}{c} \text{CH}_3 - \text{As} - \text{CH}_3 \\ \\ \text{OH} \end{array}$	Urine [48-52, 55] Fingernail [50] Hepatocytes [54]		
Dimethylarsinous glutathione, Dimethylarsinic glutathione, Dimethylarsino-glutathione, Darinaparsin, ZIO-101, DAR	DMA ^{III} (GS) DAR DMAG DMA _s ^{III} GS	$\begin{array}{c} \text{CH}_3 - \text{As} - \text{CH}_3 \\ \\ \text{SG} \end{array}$	[25]		

Table 2

A. Instrumental parameters after optimization

Parameter (unit)	Typical values
ICP-MS	
ICP-MS Nebulizer Ar Gas Flow (L min ⁻¹)	0.84-0.90
Nebulizer Oxygen Gas Flow (L min ⁻¹)	0.2
Auxiliary Gas Flow (L min ⁻¹)	1
Plasma Gas Flow (L min ⁻¹)	15
Lens voltage	9
ICP RF Power (W)	1350
Analog Stage Voltage	-1525
Pulse Stage Voltage	950
Quadrupole Rod Offset Std (QRO)	-2
Cell Rod Offset Std (CRO)	-7
Discriminator Threshold	40
Cell Path Voltage Std (CPV)	-20
HPLC	
Column	Waters Spherisorb 5 µm C8, 4.0×250 mm
Column temperature	10°C
Mobile phase temperature	Cooled in ice bath during analysis
Sample temperature	Room temperature
Mobile phase	0.05% TFA and acetonitrile gradient

B. Final HPLC gradient elution program						
Step	Step type	Step time (min)	Flow (mL min ⁻¹)	0.05% TFA	ACN %	Curve
0	Equilibration	6.0	1.0	99	1	N/A
1	Run	5.5	0.5	99	1	0.0
2	Run	3.5	0.5	87	13	1.0
3	Run	11.0	0.5	80	20	0.0

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B. Final HPLC gradient elution program					
Step	Step type	Step time (min)	Flow (mL min ⁻¹)	0.05% TFA	ACN %
4	Run	10.0	0.5	95	5
					Curve
					0.0