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***In vitro* Assays of Inorganic Arsenic Methylation**

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Abstract

Inorganic arsenic is extensively metabolized to produce mono-, di-, and trimethylated products. The formation of these metabolites produces a variety of intermediates that differ from inorganic arsenic in terms of patterns of distribution and retention and in toxic effects. In order to elucidate the pathway for arsenic methylation, it was necessary to develop a reliable *in vitro* assay system in which the formation of methylated metabolites could be monitored. Here, *in vitro* assay system that uses the postmicrosomal supernate from rat liver is used as the source of the enzymatic activity that catalyzes methylation reactions. This system can be used to study the requirements for methylation reactions (e.g., identifying the donor of methyl groups) and for screening of compounds as potential activators or inhibitors of arsenic methylation.

Keywords

arsenic; rat liver; postmicrosomal fraction; methylation reactions; methylated arsenicals

Basic Protocol

Protocol Title - Inorganic arsenic methylation in rat liver cytosol assay system

Introduction—This section describes the elements of an *in vitro* assay system in which the methylation of arsenicals can be studied. This is a simple assay system that uses the cytosolic fraction prepared from rat liver to provide the enzymatic machinery needed to catalyze the conversion of inorganic arsenic to methylated metabolites. Its value to investigators lies mainly in that it provides a relatively simple assay system in which the methylation of inorganic arsenic can be monitored and the effects of various additives on this activity can be determined. Indeed, an *in vitro* assay system based on rat liver cytosol as the source of methylating activity has been used to characterize methylation of various arsenicals (Styblo et al., 1995), to identify factors which affect *in vitro* methylation of inorganic arsenic (Styblo et al., 1996), and to study the association between metabolism of

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inorganic arsenic *in vitro* and binding of its methylated metabolites to cytosolic proteins (Styblo and Thomas, 1997). This approach could be applied to further studies of the linkage between the metabolism of arsenicals and the effects of the metabolites on cellular metabolism. A similar experimental approach could be applied to the study of the metabolism of other metalloids (e.g., tellurium) that are methylated by mammals.

Materials List

Adult male Fischer 344 rats (8 to 10 weeks old) - Charles River Laboratory, Raleigh, NC

Phenobarbital (or another appropriate anesthetic)

Trizma base - 2-amino-2-(hydroxymethyl)-1,3-propanediol

Trizma HCl - tris(hydroxymethyl)aminomethane hydrochloride

Sucrose

Glutathione

70 percent Isopropanol

S-Adenosylmethionine, p-toluenesulfonate salt

Arsenous (As^{III}) acid, sodium salt (iAs^{III})

Carrier-free sodium [^{73}As] arsenate – Los Alamos Meson Production Facility through Oak Ridge National Lab

Equipment

Refrigerated ultracentrifuge – Must be able to attain $135,000 \times g$

Glass mortar with loose fitting teflon pestle

Protocol Steps

Part 1 – Preparation of rat liver cytosolic fraction

1. Anesthetize donor rat with an appropriate anesthetic agent (e.g., phenobarbital). The fully sedated rat is secured in the dorsal recumbent position.

Investigators should consult with animal care committees and veterinarians for advice on animal husbandry and selection of anesthetic.

2. Thoroughly clean the ventral surface from xyphoid process to pubis with 70 percent isopropanol.

3. Make midline incision through dermis and underlying tissue. Reflect skin and secure it out of operative field.

Investigators should consult atlases of rat anatomy to become familiar with the anatomical features critical to successful liver perfusion.

4. Make a midline incision through abdominal wall, exposing the peritoneal membrane. Reflect the abdominal wall and secure it out of operative field.
5. Divide the peritoneal membrane, exposing the ventral aspect of the liver. Reflect the liver and secure it out of operative field.
6. Expose and isolate the inferior vena cava. Place a loose ligature around the inferior vena cava below the level of liver hilus.

7. Insert a 20 gauge butterfly type needle into the inferior vena cava at about the level of the renal artery and secure it by tightening the ligature.
8. Place a small clamp around inferior vena cava posterior to the needle.
9. Section the aorta anterior to liver (at the ventral margin of the diaphragm). Sectioning of these vessels permits one-pass perfusion of liver and causes death.
10. Perfuse liver *in situ* with 120 to 180 ml of ice-cold Buffer A.

Removal of residual blood from the liver is important in preparation of rat liver cytosol for use in assays. Hemoglobin derived from residual erythrocytes avidly binds DMAs and could influence rate and extent of metabolism in the assay system. A well-perfused rat liver rapidly changes color from dark red brown to light tan. Uniformity of color across all lobes of the perfused liver is evidence of successful perfusion.

11. Remove perfused liver *en bloc* and clean it of adherent connective tissue and blood vessels.
12. Mince perfused liver into ~ 5 mm blocks and rinse in ice cold Buffer A. Collect and weigh minced liver.

Part 2 – Preparation of rat cytosolic fraction

1. Prepare a 20% (w/v) homogenate by homogenizing minced liver in ice cold Buffer A in a glass mortar-teflon pestle homogenizer using a loose fitting pestle. Cool Buffer A in mortar by immersion in an ice bath before addition of tissue. Homogenize liver with about 10 to 12 strokes of homogenizer, cooling mortar and its contents on ice after every 3 or 4 strokes.

Critical to the success of this step is the inclusion of GSH in Buffers A and B. Omission of GSH from either buffer is associated with rapid loss of As methylating activity in rat liver cytosol.

2. Transfer homogenate to appropriate tubes for centrifugation. Spin homogenate at $135,000 \times g$ for 60 minutes at 4°C.

This separation yields a large pellet consisting of cellular debris and membranous components and a supernatant fraction consisting of postmicrosomal components of the cell, i.e., cytosolic proteins. Supernate usually includes a floating layer of lipids at the air-supernate interface. The lipid layer is removed with a Pasteur pipette that has been modified by introduction of a U-shaped curve in its tip. As shown in Figure 1, the U-shaped tip is carefully inserted through the lipid layer and overlying lipid material is removed by slow aspiration. This procedure can remove practically all floating lipid. Supernate is then carefully removed without disturbing material at the supernate-pellet interface.

3. Transfer supernate to a graduated cylinder cooled in an ice bath and determine its volume. Add an equal volume of Buffer B to this material and mix well. This yields rat liver cytosolic fraction for use in assays. Store rat liver cytosolic fraction at –65 to –80°C.

The liver cytosolic fraction can be stored for at least four weeks at –80°C. Aliquoting of the rat liver cytosol to appropriate volumes for experimental use avoids repeated thawing and freezing.

Part 3 - In vitro assays of the arsenic methylation activity in a reaction mixture

containing rat liver cytosolic fraction: The following procedure is designed for the use of

radiolabeled [^{73}As]-labeled arsenous acid, sodium salt, as the substrate. It is based on the protocol of Styblo and Thomas (1997) used in a study of the role of methylation of arsenicals in the binding of inorganic and methylated arsenicals to the cytosolic proteins of rat liver. As discussed below, assay conditions can be adapted for the use of other substrates (e.g., stable arsenicals) or to accommodate different experimental objectives.

1. Immediately before use thaw an aliquot of rat liver cytosolic fraction by incubation in a water bath at 37°C. After thawing, mix rat liver cytosolic fraction to insure homogeneity. Pipet 6 ml of rat liver cytosolic fraction into a small beaker cooled in an ice bath. To produce the reaction mixture, add 0.6 ml of the 10 mM AdoMet stock solution to the contents of the beaker and mix well. Store reaction mixture on ice until use.

In our experience, AdoMet must be added to the reaction mixture immediately before the initiation of an assay and spiking solutions of AdoMet must be prepared immediately before use. Earlier work on the methylation of arsenic in tissue homogenates or subcellular fractions added other factors (e.g., Mg^{2+} , methylcobalamin) that were thought to be necessary for methyl group transfer reactions. However, these factors are not required to support methylation activity in the assay system described here; AdoMet alone is sufficient to support the methylation of arsenicals by rat liver cytosol.

2. Pipet 0.6 ml of the reaction mixture to a capped 1.5 ml polypropylene tubes at 37°C for 5 minutes.
3. Start reactions by addition of the arsenical substrate. In this example, [^{73}As]-labeled sodium arsenite is added as the substrate.

*Sodium arsenite radiolabeled with ^{73}As was used in this procedure to track formation of methylated metabolites. Carrier-free sodium [^{73}As] arsenate (i.e., arsenic acid, sodium salt) is available through Isotope Business Office, Oak Ridge National Lab, Oak Ridge, TN 37831-6158 (www.ornl.gov/sci/isotopes/catalog.htm). Potential users should check on availability of the radionuclide. For the purposes of this assay, sodium [^{73}As] arsenate must be reduced to sodium [^{73}As] arsenite. A modification of the method of Reay and Asher (1977) for reduction of radiolabeled arsenate is described in the Support Protocol entitled “**Reduction of [^{73}As] arsenate to [^{73}As] arsenite for use as a radiolabeled substrate in in vitro assays of arsenic methylation**”.*

Based on the 0.6 ml volume of the reaction mixture used in this study between 60 and 80 μCi of [^{73}As]-labeled sodium arsenite was added to each assay. Stable arsenite can be added mixed with [^{73}As] sodium arsenite to attain any desired concentration in the reaction mixture. In Styblo and Thomas (1997) stable sodium arsenite was mixed with [^{73}As] sodium arsenite so that the final concentration of arsenic (as arsenite) in the reaction mixture was 1 μM .

Use of an unlabeled substrate (i.e., sodium arsenite) would not materially change the set up of this assay but would determine the selection of appropriate methods for use in detection and quantitation of reaction products. For example, in an early study using stable arsenicals as substrates and hydride generation-cryotrapping-atomic absorption spectrometry for detection and quantitation (Styblo et al., 1995), the volume of reaction mixtures was scaled to 1.9 ml to provide sufficient sample volume for analysis. Indeed, it is the flexibility of this assay system that makes it useful in studies of arsenic methylation

4. Place capped tubes in a water bath at the desired incubation temperature (usually 37°C).

5. Terminate reactions by transfer of tubes to an ice-cold water bath.
6. Process samples as described in **Chapter 5 “Analysis of Arsenical Metabolites in Biological Samples” Basic Protocol 1- Analysis of arsenical metabolites by thin layer chromatography** to liberate arsenicals bound to proteins and to reduce all arsenicals to the pentavalent oxidation state before separation and quantitation by thin layer chromatography.

Support Protocol

Protocol Title - Reduction of [^{73}As] arsenate to [^{73}As] arsenite for use as a radiolabeled substrate in *in vitro* assays of arsenic methylation

Introduction—As described above, the *in vitro* assay system for arsenic methylation has most commonly used a radiolabeled substrate to track the formation of radiolabeled products. This approach was dictated largely with the relative ease of analysis of radiolabeled products. Because arsenicals containing trivalent arsenic were shown to be the preferred substrate for methylation reactions occurring in rat liver cytosol, it was necessary to develop a method to reduce commercially available arsenate to arsenite. The following protocol describes the procedure use to produce [^{73}As] arsenite from [^{73}As] arsenate.

Materials List

Sodium metabisulfite - ACS reagent grade
 Sodium thiosulfate - ACS reagent grade
 Sulfuric acid - ACS reagent grade (98%)
 Carrier-free sodium [^{73}As] arsenate – Los Alamos Meson Production Facility through Oak Ridge National Lab

Protocol steps

1. Transfer an aliquot of the aqueous [^{73}As] arsenate stock to a small capped tube for reduction.

Investigators should consult with their institutional radiation safety office to assure the safety of this operation involving the manipulation of radioactive material.

2. Mix 2 volumes of the [^{73}As] arsenate stock with 1 volume of the reducing solution. Cap tube and let stand at room temperature for at least several hours. At the end of the reduction period, the radiolabeled material is stored at 4°C.

The volume of aqueous [^{73}As] arsenate stock reduced depends on the concentration of the radionuclide in the stock solution and the anticipated need for [^{73}As] arsenite.

Reagents and Solutions—Buffer A - 60 mM tris, 250 mM sucrose, 10 mM GSH, pH 7.6

Prepare 60 mM tris stock by mixing 5.86 g of trizma HCl and 2.76 g of trizma base in ~ 900 ml deionized water and stirring to dissolve. Then adjust to final volume of 1000 ml. To 900 ml of 60 mM tris stock add 87.56 g of sucrose and stir to dissolve. Adjust to final volume of 1000 ml by addition of 60 mM tris stock. To 900 ml of 60 mM tris/250 mM sucrose stock, add 3.07 g GSH and stir to dissolve. Adjust to final volume of 1000 ml by addition of 60 mM tris/250 mM sucrose stock. Check pH of final mix. At room temperature (~ 25°C), the pH should be 7.9. If necessary, adjust to this final value. Aliquot Buffer A into 25 or 50 ml

volumes and store at -20°C . This buffer is stable for several months with low temperature storage.

Buffer B - 100 mM tris, 10 mM GSH, pH 7.6

Prepare 100 mM tris stock by mixing 9.76 g of trizma HCl and 4.6 g of trizma base in ~ 900 ml deionized water and stirring to dissolve. Then adjust to final volume of 1000 ml. To 900 ml of 100 mM tris, add 3.07 g GSH and stir to dissolve. Adjust to final volume of 1000 ml by addition of 100 mM tris stock. Check pH of final mix. At room temperature ($\sim 25^{\circ}\text{C}$), the pH should be 7.9. If necessary, adjust to this final value. Aliquot Buffer B into 25 or 50 ml volumes and store at -20°C . This buffer is stable for several months with low temperature storage.

Mixing of trizma HCl and trizma base produces a tris buffer with a working range between pH 7 and 9. Trizma has a substantial temperature coefficient for pH; from 25°C to 37°C , the pH decreases an average of 0.025 pH units per $^{\circ}\text{C}$. Therefore, it is necessary to compensate for difference in the temperature at which the buffer is prepared (typically $\sim 25^{\circ}\text{C}$) and the temperature at which the buffer will be used (37°C). Most vendors of trizma base and trizma HCl provide tables for temperature compensation. Alternatively, mixtures of trizma HCl and trizma base that produce solutions of known molarity and pH are available from some vendors (e.g., Sigma). Using commercially prepared mixtures of trizma HCl and trizma base probably produces buffers that are more consistent in composition than solutions made at infrequent intervals in a laboratory

10 mM AdoMet stock solution: Weigh 26.1 mg of S-Adenosylmethionine, p-toluenesulfonate salt, and transfer to a small test tube. Add 3 ml of Buffer A and 3 ml of Buffer B to the test tube. Mix well to dissolve. Cap tube and store on ice. Make immediately before use and discard at day's end.

1% Sodium thiosulfate: Weigh 0.1 g of sodium thiosulfate and add to a 10 ml volumetric flask. Add deionized water to 10 ml final volume. Mix well to dissolve. Prepare this solution immediately before use and discard at day's end.

15 N Sulfuric acid: Add 4.076 ml of sulfuric acid (98%) to a 10 ml volumetric flask. Add deionized water to 10 ml final volume. This solution is stable for storage at room temperature.

Reducing Solution: Dissolve 0.28 g of sodium metabisulfite in 15 ml of deionized water. Mix well to dissolve and add 2 ml of 1% sodium thiosulfate. Add 0.25 ml of Sulfuric acid to this mixture. Prepare this solution immediately before use and discard at day's end.

Commentary

Background Information

The description of *in vitro* assays systems for use in the study of the methylation of arsenic can be found in earlier work (Hirata et al., 1989; Buchet and Lauwerys, 1985, 1987; Smith et al., 1992). These researchers used tissue homogenates or subcellular fractions as the source of an enzyme activity that would catalyze the formation of methylated arsenicals from inorganic arsenic. The inclusion of methylcobalamin and magnesium in the reaction mixtures reflected the then current idea that methylation of metals and metalloids in eukaryotes would use factors that had been shown to be involved in methylation of metals in bacterial systems. The inclusion of AdoMet in the assay system described here reflects recognition that this molecule is essentially the universal methyl group donor in eukaryotes.

Use of the rat as the source of liver for preparation of the cytosolic fraction during development of this assay was fortuitous. Rat liver is a rich source of the enzymatic activity that catalyzes methylation of arsenicals. In contrast, mouse liver contains much lower levels of this activity and is not a good source of starting material for this assay (Stybło and Thomas, unpublished observations).

This assay system has been used primarily with radiolabeled arsenic as the substrate for methylation assays. The radiolabel precursor and products can be resolved and quantified using thin layer chromatography (TLC) are described in Chapter 5 **Analysis of Arsenical Metabolites in Biological Samples**. An alternative approach uses stable arsenicals as substrates for the assay. In this case, one can use one of many methods for separation and quantitation of the precursor and products, including hydride generation-atomic absorption spectrometry.

Critical Parameters

In vitro assays—Two factors require special attention in the use of this *in vitro* assay system. First, rat liver must be well perfused *in situ* to remove residual erythrocytes. Removal of erythrocytes from donor rat liver is critical because rat hemoglobin displays an unusually high affinity for the binding of DMAs^{III} (Lu et al., 2004, 2007). If hemoglobin derived from residual erythrocytes is present in the assay system, it will avidly bind DMAs^{III} and may alter the overall kinetics of arsenic methylation in this system. For *in vitro* studies designed to evaluate the binding of arsenicals to endogenous proteins of the liver (Stybło and Thomas, 1997), the presence of residual hemoglobin is likely to affect the pattern and extent of binding of inorganic arsenic and its methylated metabolites. Hence, careful attention to the removal of blood from the donor liver is a critical step in assay preparation.

The second factor that is critical to the success of this *in vitro* assay system is inclusion of GSH in Buffer A and Buffer B used in preparation of the cytosolic fraction of rat liver. Omission of GSH from the buffers leads to rapid loss of As methylation activity during preparation of the cytosolic fraction and its storage at low temperature. Subsequent work on purification of the arsenic methyltransferase activity from rat liver and with recombinant rat As3mt and human AS3MT shows a critical role for GSH in the maintenance of the activity of this enzyme during its purification from rat liver (Lin et al., 2002). Notably, GSH is not essential for the catalytic activity of human AS3MT but does stimulate its activity in the presence of other physiological reductants (e.g., thioredoxin, glutaredoxin, lipoic acid) (Waters et al., 2004).

Reduction and use of [⁷³As] arsenite—Chemical reduction of arsenate to arsenic can be readily accomplished using the reducing mixture described by Reay and Asher (1977). In the original method, the reduced material was subjected to ion exchange chromatography on a mixed AG resin bed to separate arsenite for residual arsenate. However, chromatographic separation was omitted from preparation of material for use as substrate in the *in vitro* assay described here because the radionuclide in column eluate was too dilute to be useful in these assays.

Troubleshooting

Table 1 lists problems commonly encountered in the *in vitro* assay system, the likely causes of these problems, and potential solutions.

The stability of the radionuclide [⁷³As] arsenite produced by chemical reduction is a major concern. The presence of contaminating metals (e.g., Fe and Al) in the stock solution of

sodium [^{73}As] arsenate is thought to affect the rate of oxidation of [^{73}As] arsenite. Oxidation to [^{73}As] arsenate markedly reduces the rate at which it is enzymatically converted to methylated arsenicals. Oxidation can be monitored by use of thin layer chromatography which separates arsenate from arsenite. Conditions for thin layer chromatographic separation of arsenate and arsenite are presented in **Chapter 5 “Analysis of Arsenical Metabolites in Biological Samples” Basic Protocol 1- Analysis of arsenical metabolites by thin layer chromatography.**

Anticipated Results

Because rat liver is a rich source of the enzymatic activity that catalyses the conversion of inorganic arsenic to methylated products, an *in vitro* assay using rat liver cytosol is relatively easy to prepare. Examples of the time course and extent of conversion of arsenate or arsenite to methylated and dimethylated species are shown in Figure 2. Here, iAs^{III} is rapidly converted to methylated and dimethylated species. As expected, the predominant metabolite formed in this assay system is DMAs. In contrast, iAs^{V} as a substrate is associated with lower rates of conversion to methylated products and a loss of methylation activity as the incubation time increases beyond about 30 minutes. The relatively lower activity of the assay system when iAs^{V} is used as substrate probably reflects the preferential activity of arsenic methyltransferase for substrates containing trivalent arsenic.

Time Considerations

Preparation of rat liver cytosol including preparation of buffers, *in situ* perfusion of the liver, preparation of liver homogenate and of a cytosolic fraction can be performed in two to four hours. Set up of the *in vitro* assay system using rat liver cytosol as the source of arsenic methyltransferase activity takes one to two hours, including time for preparation of reagents that must be freshly prepared immediately before use (e.g., AdoMet). Reduction of arsenate is usually performed overnight. Monitoring of the extent of conversion to arsenate to arsenite by thin layer chromatography will require a minimum of eight hours for chromatography and detection of the separated forms.

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Figure 1.

Use of curved tip Pasteur pipette for removal of floating lipid layer from supernatant fraction produced by ultracentrifugation of rat liver homogenate.

Figure 2.

Time course for conversion of arsenite (iAs^{III}) or arsenate (iAs^V) to methylated (MAs) or dimethylated (DMAs) species in reaction mixtures containing rat liver cytosol in 80 mM tris, 125 mM sucrose, 10 mM GSH, pH 7.6, containing 1 mM AdoMet and 1 μ M [^{73}As]-labeled arsenite or arsenate. Reaction mixtures incubated at 37°C for up to 90 minutes. Mean $\pm$ standard deviation are shown (n=3). Abbreviations: iAs^{III} , arsenite; iAs^V , arsenate; MAs, methylated arsenicals; DMAs, dimethylated arsenicals.

Table 1

Troubleshooting guide for inorganic arsenic methylation in rat liver cytosol assay system

Problem	Cause	Solution
Donor liver is not cleared of blood	Inadequate perfusion	Reposition cannula in inferior vena cava to improve perfusion.
Turbid cytosolic fraction	Inadequate removal of lipid layer before harvesting of cytosol	Remove lipid layer with curved tip Pasteur pipette before harvesting of cytosol.
Low methylation activity in <i>in vitro</i> assay system	Loss of methylation activity in cytosolic fraction	Examine composition of assay system. Assure presence of GSH in all buffers used for preparation of cytosolic fraction and in assay system. Solutions containing AdoMet used in this assay are not stable for storage and should be prepared immediately before use.