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Journal of Chromatography B, 677 (1996) 161–166

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Liberation and analysis of protein-bound arsenicals

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Received 17 October 1995; accepted 31 October 1995

Abstract

Protein-bound arsenicals were liberated from binding sites on liver cytosolic proteins by exposure to 0.1 *M* CuCl at pH 1. This method released greater than 90% of the arsenicals associated with biological matrices. Ultrafiltrates of CuCl-treated cytosols were subjected to thin-layer chromatography to speciate and quantify inorganic and methylated arsenicals. For rat liver cytosol in an in vitro methylation assay and for liver and kidney cytosols from arsenite-treated mice, most inorganic arsenic was protein bound. Appreciable fractions of the organoarsenical metabolites present in these cytosols were also protein bound. Therefore, CuCl treatment of cytosols releases protein-bound arsenicals, permitting more accurate estimates of the pattern and extent of arsenic methylation in vitro and in vivo.

Keywords: Arsenicals; Proteins

1. Introduction

In many species, inorganic arsenic (iAs) is metabolized to mono-, di- and possibly trimethylated species [1]. Because inorganic arsenate (iAs^{V}) and arsenite (iAs^{III}) are more acutely toxic than pentavalent methylarsonate (MAs^{V}) and dimethylarsinate ($DMAs^{V}$), the methylation of iAs is generally considered a mechanism of detoxification [2]. Several analytical schemes have been used to study the methylation of iAs in vivo and in vitro. However, these schemes were designed to speciate As in urine or in protein-free extracts prepared by ultrafiltration or by acid precipitation from tissues [3–8]. Recent studies indicate that analytical methods that rely on the quantitation of arsenicals in deproteinized samples may misrepresent the actual pattern and extent of As metabolism in vivo and in vitro [9]. In this report, the method of Reinke et al. [10] has been adapted to liberate arsenicals from proteins. Arsenicals liberated by this approach can be separated from other cellular constituents by ultrafiltration and speciated by previously described thin-layer chromatographic methods [11].

2. Experimental

2.1. Radiolabelled compounds

Carrier-free arsenic acid [^{73}As]iAs^{V} was purchased

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0378-4347/96/$15.00
SSDI 0378-4347(95)00490-4

from Los Alamos Meson Production Facility (Los Alamos, NM, USA). Disodium [^{14}C]MAs^{V} (10 mCi/mmol) and sodium [^{14}C]DMAs^{V} (11.2 mCi/mmol) were obtained from ICN Radiochemicals (Irvine, CA, USA). Radiolabelled trivalent arsenicals (iAs^{III}, MAs^{III} and DMAs^{III}) were prepared from the corresponding pentavalent species by reduction with metabisulfite-thiosulfate reagent (MTR) [9,12].

2.2. Preparation and treatment of biological samples labelled with ^{73}As

Preparation of rat liver cytosol for in vitro assay has been previously described [9]. The assay mixture contained cytosol (ca. 15 mg protein/ml), 60 m*M* Tris (pH 7.6), 10 m*M* glutathione, 1 m*M* *S*-adenosylmethionine and a radiolabelled arsenical (1 μCi for ^{73}As, 1 nCi for ^{14}C). Final volume of the mixture was 51 μl. Incubations were performed at 37°C and were terminated by transfer to an ice bath. In some experiments, sodium arsenite (Sigma) was added as carrier. To examine the binding of different radiolabelled arsenicals to cytosolic proteins in the absence of methylation [8], samples were incubated at 0°C for 3 h. Two adult female B6C3F1 mice (Charles River Breeding Laboratories) were injected intravenously with 50 μCi of carrier-free [^{73}As]iAs^{III} in physiological saline (4 ml/kg). Thirty minutes after injection, the mice were killed by cardiac puncture under light CO_2 anesthesia. Liver and kidney cytosols were prepared by centrifugation (145 000 *g* for 35 min at 4°C) of 20% (w/v) tissue homogenates in 20 m*M* Tris, 250 m*M* sucrose buffer, pH 7.6. To liberate protein-bound arsenicals, samples of the in vitro assay mixture that were incubated with radiolabelled arsenicals or of liver or kidney cytosol prepared from mice that received [^{73}As]iAs^{III} were mixed (1:1, v/v) with a solution of 0.2 *M* CuCl (Fisher, Fair Lawn, NJ, USA) in 0.2 *M* HCl and maintained at 37°C for 3 or 5 h. All samples were cooled to 0°C before ultrafiltration.

2.3. Identification of ^{73}As-labelled metabolites

CuCl-treated and untreated samples were ultrafiltered using Microcon microconcentrators (Amicon, Beverly, MA, USA) with 10 kDa cutoff that were centrifuged at 12 000 *g* for 12 min at 4°C. Protein retentates from untreated samples were washed twice with 25 μl of 0.1 *M* Tris buffer, pH 7.6; retentates from CuCl-treated samples were washed twice with 25 μl or 50 μl of 0.2 *M* CuCl. Each wash was followed by centrifugation at 12 000 *g* for 12 min at room temperature. Radioactivities of ultrafiltrates and protein retentates (protein and ultrafiltration apparatus) were measured with a Minaxi γ 5000 counter or a Tri-Carb 2200CA liquid scintillation analyzer (Packard, Downers Grove, IL, USA). In some experiments, protein was removed from the ultrafiltration apparatus by centrifugation at 12 000 *g* for 5 min at room temperature and was radioassayed separately. To improve chromatographic resolution, aliquots of the combined ultrafiltrate and washes were oxidized with 10% H_2O_2 (Sigma) for 30 min at room temperature to convert all arsenicals to pentavalency [11]. Oxidized samples were analyzed by TLC on 20×20 cm Baker-flex cellulose PEI-F plates with a 254-nm fluorescent indicator developed with an acetone–acetic acid–water (2:1:1) solvent system [9]. Radiolabelled arsenical standards prepared in liver cytosol ultrafiltrate with or without addition of CuCl were separated in parallel with samples. An isopropanol–acetic acid–water (10:1:5) mobile phase [11] was also used to evaluate the chromatographic behavior of arsenical standards prepared in presence of excess CuCl. Radioactive compounds separated by TLC were detected and quantified with an Ambis 4000 imaging detector (Scanalytics, Billerica, MA, USA). Student's two-sided *t*-test was used to compare results of untreated and CuCl-treated samples.

3. Results

3.1. Effect of CuCl treatment on binding of trivalent and pentavalent arsenicals to liver cytosolic proteins

Incubation mixtures containing radiolabelled trivalent or pentavalent arsenicals were incubated at 0°C (i.e., in absence of enzymatic methylation) for 3 h and then ultrafiltered without further treatment or subjected to CuCl treatment before ultrafiltration.

Table 1
Effect of CuCl or HCl treatment on the percentages of arsenicals bound to cytosolic proteins or retained on filters after ultrafiltration

Arsenical	Untreated		0.1 *M* CuCl–0.1 *M* HCl		0.1 *M* HCl	
	Protein-bound	Residual[a]	Protein-bound	Residual	Protein-bound	Residual
[^{73}As]iAs^{V}	17.34±0.75	4.45±0.33	8.78±2.88[b]	3.20±0.55[b]	67.78±2.55[b]	1.80±0.23[b]
[^{73}As]iAs^{III}	55.40±3.58	4.48±1.08	8.48±1.77[b]	3.15±0.26	86.60±0.95[b]	1.50±0.55[b]
[^{14}C]MAs^{V}	20.15±1.61	5.50±1.27	8.58±3.21[b]	4.08±0.91	29.78±1.86[b]	2.68±0.39[b]
[^{14}C]MAs^{III}	32.05±2.26	11.13±1.97	4.30±1.44[b]	3.00±0.52[b]	51.13±11.07[b]	14.83±10.44
[^{14}C]DMAs^{V}	17.50±0.61	4.90±0.65	3.83±0.88[b]	2.53±0.21[b]	8.23±0.97[b]	3.45±0.99
[^{14}C]DMAs^{III}	27.78±1.91	5.70±0.66	5.20±1.41[b]	2.45±0.44[b]	29.73±5.42	11.20±4.16

Percentage of the recovered radioactivity after incubation at 0°C for 3 h ($\bar{x}$±S.D., n=4).
[a] The residual fraction is the percentage of the recovered radioactivity retained on the ultrafiltration apparatus after removal of the protein retentate.
[b] Significantly different (p:<0.05) *from corresponding value for untreated sample.*

Table 1 shows the percentage of the radiolabel (^{73}As or ^{14}C) that was associated with proteins or with the ultrafiltration apparatus after ultrafiltration. The latter component was designated as the residual fraction. In untreated samples, percentages of protein-bound radionuclide ranged from 17.34% (iAs^{V}) to 55.40% (iAs^{III}) of the total radioactivity and were always higher for trivalent arsenicals than for pentavalent arsenicals. For all arsenicals, the percentage of the total radionuclide in CuCl-treated incubation mixtures that was protein bound was always significantly lower (p<0.05) than in untreated incubation mixtures, accounting for 3.8 to 8.8% of the total radioactivity. Treatment of samples with 0.1 *M* HCl alone increased the amount of protein-bound radiolabel for most arsenicals tested. For untreated samples, the residual radioactivity ranged from 4.5 to 11.1%. Residual radioactivity after CuCl treatment ranged from 2.5 to 4.1%. Recovery of radioactivity after CuCl treatment and ultrafiltration ranged from 94% to 104% as compared with untreated samples.

3.2. TLC separation of arsenicals in presence of CuCl

To examine the chromatographic behavior of arsenicals in presence of the excess CuCl, radiolabelled standard arsenicals prepared in liver cytosol ultrafiltrate and treated with CuCl were separated in parallel with untreated arsenicals using both solvent systems. Relative mobilities (R_F) were determined for each arsenical (Table 2). In both solvent systems, Cu migrated as a blue spot with an R_F of 0.08 to 0.1. R_F values for iAs^{V} and MAs^{V} in CuCl-treated ultrafiltrates increased in both solvent systems. The acetone–acetic acid–water solvent system provided better separation of arsenicals in the presence of

Table 2
Effect of CuCl treatment on relative mobilities (R_F) of arsenicals separated in liver cytosol ultrafiltrate

Arsenical	Isopropanol–acetic acid–water		Acetone–acetic acid–water	
	Untreated	CuCl-treated	Untreated	CuCl-treated
iAs^{V}	0.01	0.15	0.02	0.16
iAs^{III}	0.48	0.49	0.33	0.34
MAs^{V}	0.33	0.38	0.49	0.53
DMAs^{V}	0.75	0.74	0.91	0.92

Radiolabelled standard arsenicals were prepared in ultrafiltrate of rat liver cytosol. R_F was calculated from migration of peak radioactivity. All samples except that containing iAs^{III} were oxidized with H_2O_2 before TLC separation.

CuCl and was used for speciation of arsenicals from the in vitro and in vivo experiments.

3.3. Analysis of protein-bound As metabolites generated in vitro from carrier-free [^{73}As]iAs^{III}

Following incubation at 37°C for 60 min, the pattern of metabolites produced in vitro from carrier-free [^{73}As]iAs^{III} was examined in ultrafiltrates and retentates (protein and ultrafiltration apparatus). For the zero-time interval, the incubation mixture was prepared in an ice bath and immediately ultrafiltered with or without CuCl treatment. The percentage of ^{73}As in retentate ranged from 52.5% to 70.5% in untreated samples and from 9.1% to 10.8% in samples treated with CuCl (Fig. 1a). Following conversion of arsenicals to pentavalency by oxidation with H_2O_2 [11], both untreated and CuCl-treated samples were separated by TLC. The percentage of iAs in both untreated and CuCl-treated samples decreased during a 60-min incubation (Fig. 1b); MAs, an intermediate metabolite, peaked between 5 and 15 min (Fig. 1c) and the percentage of DMAs, the major terminal product of As methylation in vivo, increased continuously after 5 min of incubation (Fig. 1d). For most time intervals, the amounts of iAs and its metabolites in ultrafiltrates from CuCl-treated samples were significantly higher than those found in ultrafiltrates of untreated samples. This suggested that a considerable fraction (up to 60%) of each arsenical was bound to cytosolic proteins and could be released by CuCl treatment. The amount of protein-bound arsenicals could be determined as a difference between the amounts of arsenicals found in ultrafiltrates from CuCl-treated and untreated samples. The higher amount of MAs in untreated samples at the zero time interval (Fig. 1b) may be explained by additional methylation of iAs^{III} during sample processing and ultrafiltration; in CuCl-treated samples, conversion of iAs^{III} was inhibited by low pH and high CuCl concentration. When compared with recovery from untreated samples, the average recovery of the radioactivity after CuCl treatment and ultrafiltration was 97.1±4.1% (mean±S.D., n=20).

The capacity of CuCl treatment to release protein-bound As was determined using the in vitro assay

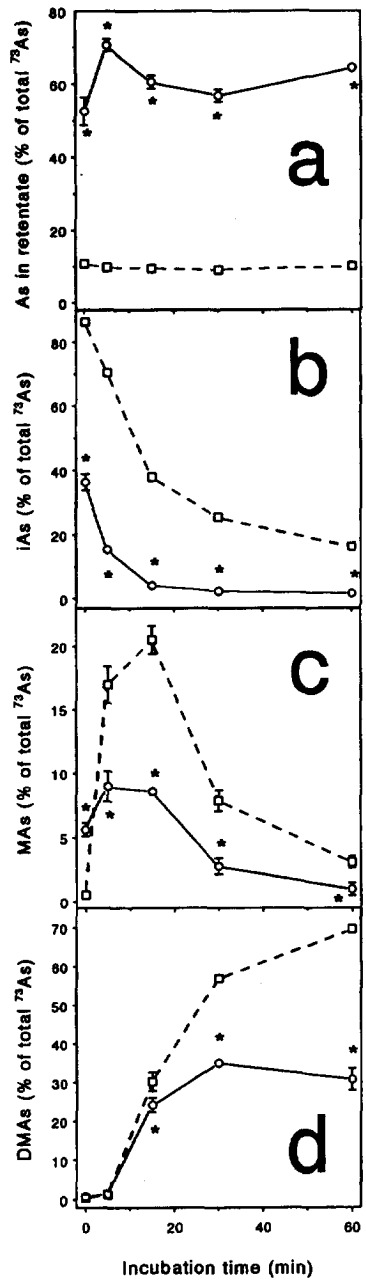

Fig. 1. Radiolabelled arsenicals in rat liver cytosol incubated at 37°C with carrier-free [^{73}As]iAs^{III} for 60 min; effect of CuCl treatment: ^{73}As in the whole retentate (protein and filter) (a), iAs (b), MAs (c) and DMAs (d) in 10 kDa ultrafiltrate ($\bar{x}$±S.D., n=4). □=untreated cytosol, ○=cytosol treated with CuCl. *= Statistically significant difference (p:<0.05) between untreated and CuCl-treated samples.

Table 3
Speciation of arsenicals from untreated and CuCl-treated liver and kidney cytosols prepared from [^{73}As]iAs^{III}-treated mice

Arsenical	Liver cytosol		Kidney cytosol	
	Untreated	CuCl-treated	Untreated	CuCl-treated
iAs	0.5±0.25[a]	5.5±2.08[b]	0.6±0.37	12.7±5.45[b]
MAs	0.3±0.25	37.5±2.23[b]	0.2±0.15	8.4±1.68[b]
DMAs	22.2±1.43	46.6±2.33[b]	55.9±0.54	66.6±3.64[b]
Retentate-bound As	77.3±0.87	11.8±0.95[b]	43.3±0.32	10.4±1.21[b]

Cytosols prepared from liver and kidneys of mice 30 min after intravenous injection of [^{73}As]iAs^{III}.
[a] Results expressed as percentages of recovered radioactivity ($\bar{x}$±S.D., n=3).
[b] Mean value is significantly different (p:<0.05) *from corresponding mean value for untreated sample.*

mixture that was incubated at 37°C for 15 or 60 min with 0.1, 1, 10 or 100 μM [^{73}As]iAs^{III}. The binding of radiolabel to the retentate from CuCl-treated samples did not exceed 10% of total radioactivity at either time point (data not shown). To liberate at least 90% of ^{73}As from proteins, the time of the CuCl-treatment was extended from 3 to 5 h for assay mixtures that contained 10 or 100 μM [^{73}As]iAs^{III} and the volume of 0.2 M CuCl used for wash of the retentate after ultrafiltration was increased from 25 to 50 μl.

3.4. Analysis of protein-bound As metabolites in mouse liver and kidney after [^{73}As]iAs^{III} treatment

Binding of arsenicals to proteins was examined in liver and kidney cytosol isolated from mice 30 min after i.v. injection of carrier-free [^{73}As]iAs^{III}. About 77% of the total ^{73}As was retentate bound in untreated liver cytosol; about 44% was retentate bound in kidney cytosol. CuCl treatment decreased the percentage of retained radionuclide to less than 12% in either sample (Table 3). DMAs was the major or only metabolite detected in ultrafiltrates from untreated cytosols. However, in CuCl-treated samples, significant amounts of iAs and MAs were found. This suggested that during in vivo metabolism of iAs these arsenicals were mostly bound to cytosolic proteins. A portion of the protein-bound DMAs was also released by CuCl treatment.

4. Discussion

Arsenicals have been shown to bind to a variety of cytosolic proteins and macromolecular constituents of tissues in both methylating [3,4,13,14] and non-methylating [5,6] animal species. Although the protein-bound fraction of As has been suggested to be primarily iAs^{III} [4], conclusive evidence about the chemical form of As associated with proteins has been lacking. Recently, parallel analyses by hydride generation-atomic absorption spectrophotometry and TLC of the pattern of metabolites produced in vitro in a rat liver cytosol-containing assay mixture showed that about one half of the methylated metabolites was protein bound [9]. These arsenicals could not be liberated by precipitation of proteins with 5% trichloroacetic acid (TCA) [8] In preliminary studies, treatment of rat liver cytosol with thiol-reactive agents (N-ethylmaleimide, 5,5′-dithio-bis(2-nitrobenzoic acid), β-mercaptoethanol) or with chelators (dithiothreitol, 2,3-dimercaptopropanol) failed to release most protein-bound arsenicals. An alternative strategy to liberate protein-bound arsenicals was suggested by the work of Reinke et al. [10] who analyzed iAs in fish, shellfish and lobster tissues by treatment with 0.2 M CuCl at low pH with subsequent extraction of iAs into benzene. In a preliminary evaluation, this method failed to extract organoarsenicals from an assay mixture that contained liver cytosol. However, treatment with CuCl released about 90% of all protein-bound arsenicals. Liberated arsenicals could be separated from proteins by ultrafiltration and arsenicals in the ultrafiltrate could be analyzed by previously described TLC procedures [9,11]. CuCl treatment was equally effective for the biologically significant pentavalent and trivalent arsenicals (radiolabelled trimethylated arsenicals were not available). As noted above, neither precipi-

tation of cytosolic proteins with TCA nor treatment of cytosol with HCl quantitatively released protein-bound arsenicals. The present work demonstrates that significant binding of inorganic and organic arsenicals occurs both in vivo and in vitro and that treatment with CuCl liberates arsenicals for speciation. Binding of arsenicals to protein could play an important role in As metabolism and toxicity. Additional studies to characterize the interaction between arsenicals and specific proteins will contribute to our understanding of the fate of this metalloid in biological systems.

Acknowledgments

We thank Karen Herbin-Davis, Brenda Edwards and Carol Mitchell for technical assistance. M.S. is a visiting scientist supported by Training Grant T901915 of the US Environmental Protection Agency/University of North Carolina Toxicology Research Program with the Curriculum in Toxicology, University of North Carolina at Chapel Hill. This manuscript has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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