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On-column formation of arsenic–glutathione species detected by size-exclusion chromatography in conjunction with arsenic-specific detectors[1]

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Abstract

The 'retention analysis method', which is based on size-exclusion chromatography (SEC) in conjunction with an arsenic-specific detector (graphite furnace atomic absorption spectrometer, GFAAS), was used to study the effect of pH (range 2.0–10.0), temperature (4, 25 and 37°C), and the concentration of glutathione in the mobile phase (0.5–7.5 m*M*) on the formation of arsenic–glutathione species after injection of sodium arsenite using phosphate-buffered saline solutions as mobile phases. The formation of arsenic–GSH species was facilitated by low temperatures (4°C), pH 6.0–8.0 and high concentrations of glutathione (7.5 m*M*) in the mobile phase. Simulating the physicochemical parameters found inside human red blood cells (~3.0 m*M* glutathione, 37°C, pH 7.4) and hepatocytes (~7.5 m*M* glutathione, 37°C, pH 7.4), SEC–GFAAS provided evidence for the formation of arsenic–glutathione species under these conditions. In addition, the 'chelating agent', sodium DL-2,3-dimercapto-1-propanesulfonate (1.0 and 2.0 m*M*) was demonstrated to bind arsenous acid stronger in the presence of glutathione (7.5 m*M*) under these conditions (PBS buffer, pH 7.4, 37°C).

Keywords: Arsenite; Glutathione

1. Introduction

The tripeptide glutathione (GSH) (L-γ-glutamyl-L-cysteinyl-glycine), which is present in a large variety of mammalian cells at concentrations ranging from 0.1 to 10 m*M*, is the most prevalent intracellular thiol in mammals [1]. Two human cell types have particularly high intracellular GSH concentrations: red blood cells (~3.0 m*M* GSH) [2] and hepatocytes (~7.5 m*M* GSH) [3]. Although other thiols, such as L-cysteine, ergothioneine and hemoglobin can also be present in certain mammalian cells, their concentrations are often negligible compared to the concentration of GSH. In hepatocytes, for instance, the concentration of L-cysteine is 0.2–0.5 m*M* and,

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hence, an order of magnitude lower than that of GSH [4].

Because of the generally high affinity of thiols for metal ions, such as Zn^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, CH_3Hg^+, Cu^{2+}, Fe^{2+}, Cr^{3+}, Pt^{2+}, Ni^{2+}, Co^{2+} and Ag^+, and for metalloid compounds, such as arsenous acid, numerous metal/metalloid–GSH complexes have been detected intracellularly by noninvasive ^1H spin-echo Fourier transform (SEFT)–NMR spectroscopy [5,6]. In addition, matrix assisted laser desorption/ionization mass spectroscopy (MALDI) has been utilized to detect arsenic/antimony–trypanothione adducts [7]. Because metal–GSH complexes are involved in the uptake and excretion of several metal ions in mammals [8,9], the detection of metal–GSH complexes is of high interest for understanding the metabolism of trace metals/metalloids. Conversely, metal/metalloid–GSH complexes can seriously affect the cellular metabolism of GSH [10–12].

Chromatographic techniques in conjunction with radio or element-specific detectors have also been employed to detect metal–GSH complexes in biological fluids [13–15]. However, the main difficulty using this technique is that the metal, complexed on a certain binding site of GSH, can be released easily, depending on the equilibrium constant [5,16]. Consequently, labile metal/metalloid–GSH complexes cannot be detected directly by conventional chromatographic techniques because they dissociate during the chromatographic process. This undesirable situation can be remedied by the 'retention analysis method', a chromatographic method based on size-exclusion chromatography (SEC), which was introduced in 1980 to study reversible associations between drugs, such as warfarin or furosemide, and human serum albumin under simulated physiological conditions [17]. Generally, an increase of the albumin concentration in the mobile phase causes a decrease in the retention time of the drug [18,19], provided that the drug–albumin associate formed is excluded from the size-exclusion matrix. Hence, any reduction in the retention time of a drug when albumin is added to the mobile phase indicates the formation of a drug–albumin associate.

This work examines the formation of arsenic–GSH species in phosphate-buffered saline (PBS) buffers applying the 'retention analysis method' using SEC (Sephadex G-10) in conjunction with graphite furnace atomic absorption spectrometry (GFAAS) or radiodetection of ^{73}As as the arsenic-specific detector. The influence of the temperature, the pH, the concentration of GSH and the presence of sodium DL-2,3-dimercapto-1-propanesulfonate (DMPS) in the PBS buffer on the formation of arsenic–GSH species was studied.

2. Experimental

2.1. Chemicals

$NiSO_4 \cdot 6H_2O$ and $NaAsO_2$, both of p.a. quality, NaCl, $Na_2HPO_4 \cdot 7H_2O$, KCl, KH_2PO_4, glutathione (GSH) >98% and Nesslers reagent were purchased from Merck (Darmstadt, Germany). Sodium DL-2,3-dimercapto-1-propansulfonate (DMPS) (95%) was purchased from Aldrich (Milwaukee, WI, USA), 5,5′-Dithiobis(2-nitro-benzoic acid) (DTNB) was purchased from Fluka (Buchs, Switzerland) and Sephadex G-10 was purchased from Pharmacia (Uppsala, Sweden). A solution of $^{73}As^V$ in conc. hydrochloric acid with a specific activity of 1.62 mCi/ml was purchased from Los Alamos Laboratory (Los Alamos, NM, USA). All other chemicals were of the highest purity.

2.2. Size-exclusion chromatography

A Pharmacia thermostatable XK-26 gel-chromatography column (70×2.6 cm) was filled with Sephadex G-10 to a final height of 63 cm. Similarly, a Pharmacia thermostatable XK-16 gel-chromatography column (70×1.6 cm) was filled with Sephadex G-10 to a final height of 60 cm. The temperature of both columns was kept at the desired temperature using a Lauda RC6 thermostat. The flow-rate was maintained either at 0.5 or 0.6 ml/min with a peristaltic pump. The PBS buffer was prepared by dissolving 40.0 g of NaCl, 13.6 g of $Na_2HPO_4 \cdot 7H_2O$, 1.0 g of KCl and 1.2 g of KH_2PO_4 in triply distilled water and the volume was adjusted to the 5-l mark. The PBS buffer was degassed with a water aspirator for 10 min before use. After the addition of either GSH or GSH and DMPS to the degassed PBS buffer, the pH was adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 7.4, 8.0, 9.0 or 10.0 by the dropwise addition of either conc. HCl or 2.0 *M* NaOH using

an Orion SA 720 pH meter. To prevent the oxidation of GSH by oxygen from air during storage, the PBS buffers containing GSH and/or DMPS were prepared freshly for each chromatographic run. The PBS buffers with pH>7.4 were continuously flushed with nitrogen during the whole chromatographic process to prevent oxidation of GSH. The Sephadex G-10 column with a diameter of 2.6 cm was equilibrated with at least 200 ml and the G-10 column with a diameter of 1.6 cm was equilibrated with at least 100 ml of mobile phase before an aqueous solution of sodium arsenite was injected.

With the 2.6 cm-diameter Sephadex G-10 column, 5-ml fractions were collected, and with the 1.6 cm-diameter Sephadex G-10 column, 2-ml fractions were collected, using a FRAC 300 fraction collector (Pharmacia). The exclusion volume, which was determined by injection of 0.3 ml of an aqueous solution of blue dextran, was 115 ml (fraction 23) for the 2.6 cm-diameter column and 40 ml (fraction 20) for the 1.6 cm-diameter column. The 2.6 cm-diameter Sephadex G-10 column (fractionation range up to a molecular mass of 700) was roughly calibrated with oxidized glutathione, GSSG (molecular mass, 612), GSH (molecular mass, 307), glycine (molecular mass, 75) and NH_4^+ ions (molecular mass, 17). GSSG was detected in the individual fractions by UV detection at 212 nm. GSH was detected visually in the fractions after the addition of 100 μl of a methanolic DTNB solution (200 mg of DTNB in 50 ml of methanol). NH_4^+ was also visually detected in the fractions after the addition of 0.5 ml of Nesslers reagent to each fraction. Glycine was detected visually after the addition of 100 μl of a methanolic ninhydrin solution (100 mg of ninhydrin in 5.0 ml of methanol) to each fraction.

Aliquots (0.3 ml) of a sodium arsenite stock solution (100 mg As/l, 1.3 m*M*) or aliquots (0.22 ml) of a solution of radiolabeled $Na^{73}AsO_2$ (0.29 μCi/ml), both containing 30 μg of total arsenic, were injected onto both columns and fractions were collected. Every chromatogram was carried out in duplicate.

2.3. Graphite furnace atomic absorption spectrometric determination of arsenic

A Hitachi Z-9000 Zeeman graphite furnace atomic absorption spectrometer (GFAAS) equipped with an arsenic hollow cathode lamp (S&J Juniper, Essex, UK), operated at 10 mA, was used to determine arsenic (193.7 nm) in the collected fractions. Graphite cuvettes (Ringsdorff Werke, Bonn, Germany) of the highest purity graphite (type RWO, shape RWO 521) and argon (99.999%) were used. Direct determination of arsenic in PBS buffer by GFAAS was impossible because of the high background absorption signal produced by the high salt concentration of the PBS buffer (~10 g/l) during atomization. Hence, the PBS buffer was diluted 1:10 (v/v) with triply distilled water to allow background correction of the arsenic signal. Because nickel salts enhance the GFAAS signal for arsenic and, therefore, increase the signal-to-noise ratio [20], nickel sulfate was used as a coanalyte. To 1.0 ml of the collected fraction (PBS buffer), 80 μl of a 2.0-*M* nickel sulfate solution were added and the obtained solution was filled to the 10-ml mark with triply distilled water. Aliquots of this solution (20 μl) were subsequently injected into the GFAAS, dried at temperatures rising from 50 to 200°C within 5 s, kept at 200°C for 20 s, ashed at 300°C for 5 s, and atomized at 2600°C for 5 s. The cuvette was then cleaned at 3000°C for 3 s. The calibration curve for arsenic was linear up to 0.3 AU and the detection limit (5 s) for arsenic was 70 μg As/l in PBS buffer. The recovery of arsenic in the fractions was always better than 80%.

2.4. Radiodetection of ^{73}As

An aliquot (20 μl) of the purchased $^{73}As^{V}$ solution was reduced to $^{73}As^{III}$ according to Reay and Asher [21]. After the addition of 'cold' sodium arsenite and sodium arsenate, the obtained solution was passed over a QAE Sephadex A 25 column (30×1.6 cm) using PBS buffer as the mobile phase. Residual $^{73}As^{V}$ was strongly retained by the column, whereas $^{73}As^{III}$ eluted in a single peak. The peak fractions corresponding to $^{73}As^{III}$ were pooled. The concentration of the radiolabeled $^{73}As^{III}$ solution was 136 μg As/ml and corresponded to 53 200 cpm. Aliquots (0.22 ml) of this solution were injected onto the 1.6 cm-diameter Sephadex G-10 column. Radiolabeled arsenic was detected in the individual fractions by counting each whole fraction (2 ml) for 60 s in a gamma counter (1282 Compugamma universal

gamma counter, LKB Wallac, MD, USA). The recovery of ^{73}As in the fractions was always >87%.

3. Results and discussion

Although considerable knowledge about the mammalian metabolism of arsenous acid has been accumulated, strikingly little is known about the molecular form of arsenic inside mammalian cells. In hepatocytes, the major site of arsenous acid biotransformation, the most prevalent low-molecular-mass thiol is GSH. Because arsenous acid has a high affinity for thiols [22,23], the intracellular reaction between arsenous acid and GSH could yield arsenic–GSH species. Arsenous acid with a pK_1 of 9.2 is present as undissociated $As(OH)_3$ at pH 7.4, the pH inside mammalian liver cells [24]. At this pH, GSH has both carboxyl groups completely deprotonated ($pK_{COOH\ 1}$=2.1; $pK_{COOH\ 2}$=3.5), the amino group protonated (pK_{NH2}=9.6), and 2.8% of the sulfhydryl groups ionized [25]. Because arsenous acid has three OH groups, it could in principle react with one, two or three GSH molecules, giving rise to $GS–As(OH)_2$ (molecular mass, 415), $(GS)_2As–OH$ (molecular mass, 704) or $(GS)_3As$ (molecular mass, 993). Scott et al. [26] titrated a solution of arsenous acid in 0.5 *M* potassium phosphate buffer solution (pH 7.1 in D_2O) with a solution of GSH and monitored the methine (Cys a) and methylene (Cys b) protons of glutathione by ^1H-NMR, compared to an aqueous GSH solution. Because of a large chemical shift of the methylene protons (Cys b) and a smaller shift of the methine protons (Cys a) upon addition to the arsenous acid, they concluded that binding of arsenous acid to the thiol group of GSH had occurred. Because signals corresponding to excess GSH were detected only after the molar ratio of GSH–arsenous acid had reached 3.5, they postulated that the stoichiometry of the formed arsenic–GSH species was $(GS)_3As$ (Eq. (1); x=3).

$$As(OH)_3 + xGSH \underset{x=1,2,3}{\overset{H_2O}{\rightleftharpoons}} (GS)_x - As(OH)_{3-x} + xH_2O \quad (1)$$

We used a chromatographic technique, i.e. the ‘retention analysis method’, to study the reaction between arsenous acid and GSH in PBS buffers using SEC coupled off-line to either GFAAS or the radiodetection of ^{73}As. Because any attempts to characterize the detected product(s) of the reactions between arsenous acid and GSH, and between arsenous acid, GSH and DMPS, by mass spectroscopy fractions failed (presumably because of the PBS buffer matrix and the large excess of GSH), the term ‘arsenic–GSH species’ will be used in the following discussion. The term ‘arsenic–GSH species’ does not provide information about how many GSH molecules are bound to the arsenic atom and may even refer to mixtures of molecules with one, two or three GSH molecules bound to the arsenic atom.

3.1. Influence of the GSH concentration, temperature and the pH of the PBS buffer on the formation of arsenic–GSH species

3.1.1. Influence of the GSH concentration in the PBS buffer at 4°C

Separate injections of blue dextran, GSSG, GSH, NH_4^+ ions and glycine showed that the column separates these molecules presumably according to their molecular mass (Fig. 1a). The smallest molecule injected onto the column, the NH_4^+ ion, eluted in fractions 41–47 (glycine also eluted in this fraction range). Surprisingly, arsenous acid, with a molecular mass of 126 (which should elute before the NH_4^+ ion and should, hence, be present in fractions prior to number 40) was detected in fractions 53–66 (Fig. 1a). This suggests that, apart from the size-exclusion mechanism, another, as yet unspecified, chemical interaction occurs between arsenous acid and the Sephadex G-10 matrix. Sephadex is a bead-formed gel prepared by crosslinking dextran with epichlorhydrin. The free hydroxyl groups of the dextran in the Sephadex G-10 gel probably interact with the OH groups of arsenous acid via hydrogen bonds and, hence, retard the migration of arsenous acid through the column. Two of the three possible products of the reaction of arsenous acid with GSH, namely $GS–As(OH)_2$ and $(GS)_2As–OH$, also have OH groups that could interact with the hydroxyl groups of dextran, as arsenous acid does with three hydroxyl groups. Because these interactions may also occur, the exact molecular masses of the de-

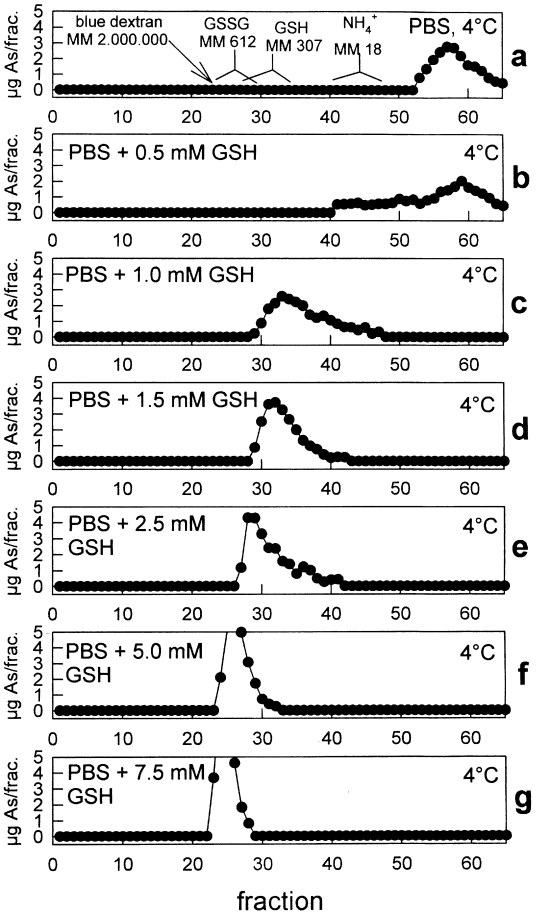


Fig. 1. Chromatograms obtained at 4°C after the injection of 30 µg of arsenic, as sodium arsenite. Column, Sephadex G-10 (63× 2.6 cm); mobile phase, PBS buffer (151 m*M*, pH 7.4) and PBS buffers with GSH concentrations ranging between 0.5 and 7.5 m*M*, adjusted to pH 7.4; injection volume, 0.3 ml; flow-rate, 0.6 ml/min; fraction volume, 5.0 ml; arsenic-specific detector, GFAAS at 193.7 nm. Abbreviations: GSSG, oxidized glutathione; MM, molecular mass; Gly, glycine.

tected arsenic–GSH species (Fig. 1b–g) cannot be deduced.

When arsenous acid was chromatographed with a 0.5-m*M* solution of GSH in PBS buffer (adjusted to pH 7.4), arsenic was detected in fractions 41–66 (Fig. 1b). The major arsenic peak (fractions 53–66) corresponds to unchanged arsenous acid, because it covers the same fraction range as found for arsenous acid with PBS buffer alone (Fig. 1a). However, arsenic, at low concentrations, was also present in fractions 41–53 (Fig. 1b). This experimental fact can be rationalized by the presence of an arsenic–GSH species with a larger molecular mass than that of arsenous acid. After the injection of arsenous acid onto the column, a portion of the arsenous acid very likely forms an arsenic–GSH species of larger molecular mass than arsenous acid, which migrates faster than arsenous acid because it is excluded from the gel. This should cause the arsenic signal to tail towards smaller fraction numbers, as observed. Probably because of hydrolysis, the arsenic–GSH species then fall apart, and arsenous acid once again reacts with GSH to form arsenic–GSH species, and so on. Hence, the formation of arsenic–GSH species followed by rapid hydrolysis could explain the observed peak shape in Fig. 1b.

Chromatography of arsenite with 1.0 or 1.5 m*M* solutions of GSH in PBS buffer brought about a more pronounced shift in the elution of arsenic towards the exclusion volume (Fig. 1c–d). With the 1.0 m*M* solution of GSH in PBS buffer, the major arsenic peak covered fractions 29–47 (peak maximum: fraction 33). However, the peak still showed considerable tailing (Fig. 1c). With the 1.5 m*M* solution of GSH in PBS buffer, arsenic eluted in a distinct band (fractions 29–42; peak maximum: fraction 32) and exhibited only minor tailing (Fig. 1d).

With 2.5 m*M* GSH in the PBS buffer, arsenic eluted in fractions 27–41 (peak maximum: fraction 28) (Fig. 1e). This indicates that an even larger molecular mass arsenic–GSH species is formed under these conditions, compared to those found in the 1.0 and 1.5 m*M* solutions of GSH in PBS buffer.

A further increase of the GSH concentration in the PBS buffer, to 5.0 and 7.5 m*M*, shifted the elution of arsenic to the exclusion volume (fraction 23) (Fig. 1f–g). The chromatograms obtained with 5.0 and 7.5 m*M* GSH are very similar. With the 7.5 m*M* GSH PBS buffer, arsenic eluted in a narrow band covering fractions 23–28 (Fig. 1g). Because Sephadex G–10 fractionates molecules up to a molecular mass of 700, which corresponds to fraction 23, the arsenic–GSH species detected under these conditions may be tentatively characterized with a molecular mass of $\geq$700 [$(GS)_3As$ with a molecular mass of 994 and/or $(GS)_2As–OH$ with a molecular mass of 704].

These results clearly indicate that an increase in

the concentration of GSH in the mobile phase shifts the elution of arsenic from the small-molecular-mass region towards the large-molecular-mass region. This can be rationalized by the formation of arsenic–GSH species that have a larger molecular mass than arsenous acid and are, hence, excluded from the gel. An increase of the GSH concentration of the mobile phase consequently shifts the chemical equilibrium in Eq. (1) to the right.

3.1.2. Influence of the temperature of the PBS buffer at 2.5, 5.0 and 7.5 mM GSH

The effect of temperature on the formation of arsenic–GSH species was investigated at physiologically relevant concentrations of GSH in PBS buffer. An increase of the temperature from 4 to 37°C did not change the elution volume of blue dextran, GSSG, GSH and NH_4^+ injected onto the column. Hence, the observed retention shifts cannot be caused by an altered pore size of the Sephadex G-10 matrix.

3.1.2.1. 2.5 *mM GSH in PBS buffer*. With a 2.5-m*M* solution of GSH in PBS buffer, arsenous acid was chromatographed on a Sephadex G-10 column at 4, 25 and 37°C (Fig. 2a–d). At 4°C, arsenic was detected in a band covering fractions 27–41, indicating that arsenic–GSH species had been formed under these conditions (Fig. 2a).

At 25°C, arsenic eluted in fractions 27–38 and fractions 49–53 (Fig. 2b). Both peaks contained similar amounts of total arsenic. The first band corresponds to the same fraction range as observed at 4°C (Fig. 2a).

At 37°C, arsenic eluted in a single band covering fractions 52–59 (Fig. 2c). Because arsenic was detected in fractions 57–66 when arsenous acid was chromatographed with PBS buffer alone at 37°C (Fig. 2d), a weak association of arsenous acid and GSH must occur under these conditions. Because these conditions resemble the physicochemical conditions in human red blood cells (PBS buffer, ~3.0 m*M* GSH, pH 7.4, 37°C), a weak association of arsenous acid with GSH possibly also occurs in these cells in vivo.

This finding is in accord with results obtained by ^{1}H-spin echo NMR with rabbit red blood cells (~2.8 m*M* GSH) [6]. Intracellular binding of arsenous acid

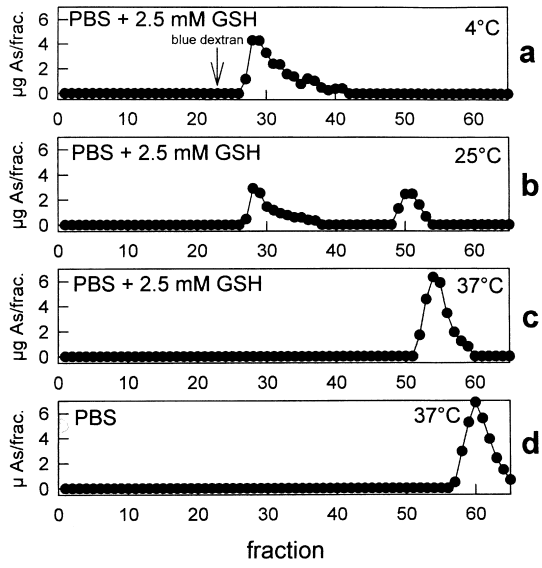


Fig. 2. Chromatograms obtained after the injection of 30 µg of arsenic, as arsenous acid, at 4, 25 and 37°C. Column, Sephadex G-10 (63×2.6 cm); mobile phase: 2.5 m*M* GSH in PBS buffer (151 m*M*), adjusted to pH 7.4, and PBS buffer (151 m*M*, pH 7.4); injection volume: 0.3 ml; flow-rate, 0.6 ml/min; fraction volume, 5.0 ml; arsenic-specific detector, GFAAS at 193.7 nm.

to GSH was observed after the addition of arsenous acid to the extracellular medium. Exposure of red blood cells to ^{14}C-labeled phenyldichloroarsine resulted in the intracellular formation of a 1:2 adduct with intracellular GSH [27]. Evidence for the formation of mixed complexes of arsenous acid with GSH and hemoglobin in red blood cells has also been reported [28].

3.1.2.2. 5.0 *mM GSH in PBS buffer*. The chromatograms obtained with arsenous acid on a Sephadex G-10 column and with a PBS buffer containing 5.0 m*M* GSH at 4, 25 and 37°C are shown in Fig. 3a–c. At 4°C, arsenic was detected in fractions 24–32, with the highest arsenic concentration being detected in fraction 26 (Fig. 3a).

Chromatography of arsenous acid at 25°C resulted in the elution of arsenic in a double peak (Fig. 3b). The first peak (fractions 25–37) showed considerable tailing and had a second, relatively sharp peak superimposed on its long retention end (fractions 37–42).

At 37°C, arsenic eluted in two sharp, distinct

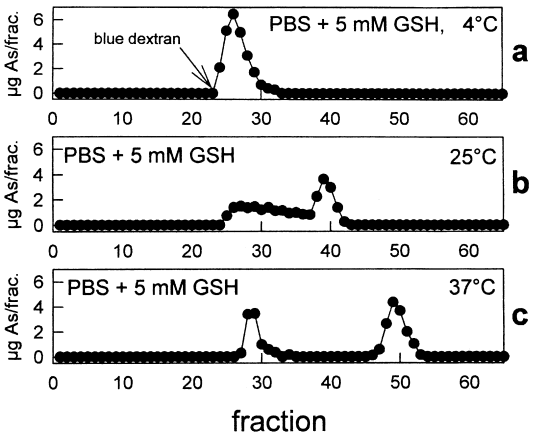


Fig. 3. Chromatograms obtained after injection of 30 µg of arsenic, as arsenous acid, at 4, 25 and 37°C. Column, Sephadex G-10 (6.3×2.6 cm); mobile phase, 5.0 m*M* GSH in PBS buffer (151 m*M*), adjusted to pH 7.4; injection volume, 0.3 ml; flow-rate, 0.6 ml/min; fraction volume, 5.0 ml; arsenic-specific detector, GFAAS at 193.7 nm.

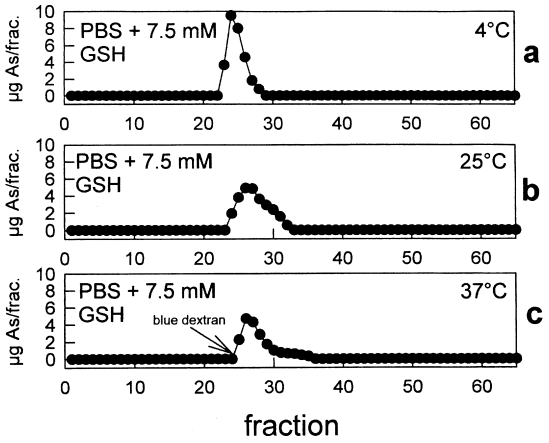


Fig. 4. Chromatograms obtained after injection of 30 µg of arsenic, as arsenous acid, at 4, 25 and 37°C. Column, Sephadex G-10 (6.3×2.6 cm); mobile phase, PBS buffer (151 m*M*) with 7.5 m*M* GSH, adjusted to pH 7.4; injection volume, 0.3 ml; flow-rate, 0.6 ml/min; fraction volume, 5.0 ml; arsenic-specific detector, GFAAS at 193.7 nm.

peaks covering fractions 27–32 and 47–52, respectively (Fig. 3c).

3.1.2.3. 7.5 *mM GSH in the PBS buffer*. The chromatograms obtained with arsenous acid on a Sephadex G-10 column using a PBS buffer containing 7.5 m*M* GSH at 4, 25 and 37°C are shown in Fig. 4a–c. At 4°C, arsenic eluted in a sharp peak covering fractions 23–28 (Fig. 4a). At 25°C, arsenic eluted in a broader peak than at 4°C, covering fractions 24–32 (Fig. 4b). When arsenous acid was chromatographed at 37°C, arsenic was detected in fractions 25–35 (Fig. 4c). Because these conditions (7.5 m*M* GSH, pH 7.4, 37°C) resemble those of human hepatocytes, our results suggest that arsenous acid possibly binds to GSH in vivo.

These results indicate that the equilibrium in Eq. (1) is strongly affected by temperature changes in the range 4–37°C. An increase in temperature shifts the equilibrium to the left. Hence, arsenic–GSH species are rather labile at ambient temperature, a finding that is in accord with other reports [23,27,29]. The detection of arsenic–GSH species at simulated physiological conditions (37°C, pH 7.4, 2.5–7.5 m*M* GSH in PBS buffer) substantiates indirect experimental evidence for the formation of arsenic–GSH species in the liver of animals exposed to arsenous acid [29–31]. These reports also claim that arsenic–GSH species are involved in the excretion of arsenous acid across the liver/bile barrier, which is mediated by ATP-dependent glutathione S-conjugate (GS-X) pumps located at the canalicular site of hepatocytes plasma membranes [32]. Because these GS-X pumps generally exhibit a broad substrate specificity towards different types of glutathione S-conjugates [32,33], arsenic-GSH species could also be substrates for these pumps. The first direct experimental evidence that arsenic-GSH species are the active species pumped across the liver/bile barrier has been reported by Müller et al. [34], who showed that, in TR^- rats, i.e. transgenic rats with inactive GS-X pumps in their hepatocytes, the excretion of arsenous acid and GSH is abolished. In addition, Dey et al. [35] demonstrated an ATP-dependent arsenic–GSH transport system in membrane vesicles of *Leishmania tarentolae*. Hence, arsenic–GSH species are involved in the excretion of arsenous acid and may also be involved in drug resistance.

3.1.3. Influence of the pH of the PBS buffer

To determine the influence of pH on the on-column formation of arsenic–GSH species, arsenous acid was chromatographed on a 1.6-cm-diameter

Sephadex G-10 column with PBS buffers containing 5.0 m*M* GSH and with the pH adjusted to values between 2.0 and 10.0 at 4°C. The chromatograms are summarized in Fig. 5.

At pH values of 2.0 and 3.0, arsenic was detected in fractions 21–64 (peak maximum: fraction 53) (Fig. 5a). The major arsenic peak (fractions 48–64, 86% of total As) covers the same fraction range as arsenous acid with PBS buffer alone (fraction 43–65) and, hence, corresponds to unchanged arsenous acid. However, a small fraction of the arsenic (14%) elutes in the fraction range of arsenic–GSH species.

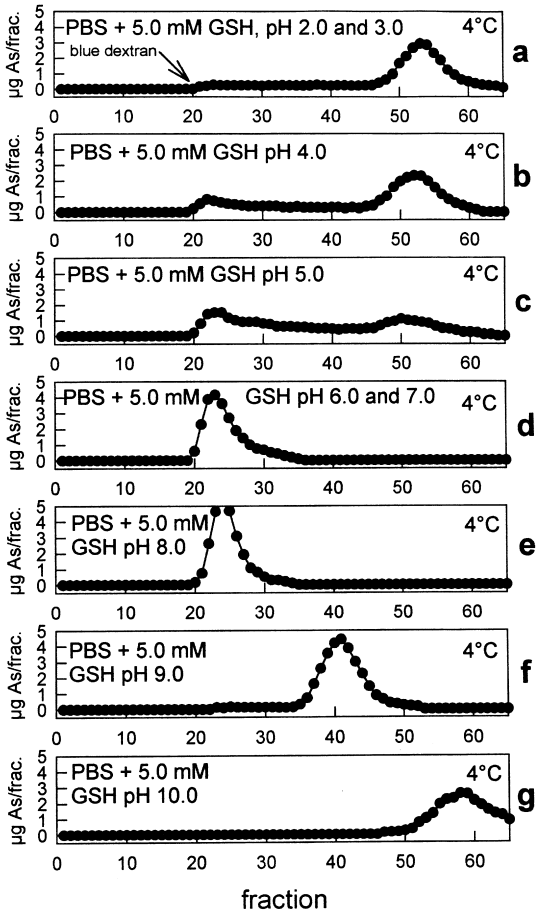


Fig. 5. Chromatograms obtained at 4°C after injection of 30 μg of arsenic, as ^{73}As labeled arsenous acid, at pH values between 2.0 and 10.0. Column, Sephadex G-10 (62×1.6 cm); mobile phase, 5.0 m*M* GSH in PBS buffer (151 m*M*), adjusted to the desired pH; injection volume, 0.22 ml; flow-rate, 0.5 ml/min; fraction volume, 2.0 ml; arsenic-specific detection of ^{73}As by gamma-counting, 5 μg As corresponds to 1950 cpm.

At pH 4.0, arsenic eluted in fractions 20–61 (peak maximum: fraction 52) (Fig. 5b). The major arsenic peak (fraction 46–61) contained 65% of the total arsenic. The arsenic that eluted in fractions 20–45 indicates the presence of arsenic–GSH species.

At pH 5.0, arsenic covered the fraction range 20–63 (peak maximum: fraction 23) (Fig. 5c). The major arsenic peak (fraction 20–44) accounted for 63% of the total arsenic and had a minor arsenic peak sitting on its long retention end (fractions 45–63).

Chromatography of arsenous acid at pH values of 6.0, 7.0 and 8.0 produced only one arsenic band that eluted in fractions 20–38 (Fig. 5d–e). Because this peak accounts for more than 90% of the injected dose, and because no arsenic was detected in larger fraction numbers, arsenic is present entirely as an arsenic–GSH species at these pH values.

A further increase of the pH to 9.0 shifted the elution of arsenic to fractions 35–52 (Fig. 5f). At pH 10, arsenic was quantitatively recovered in fractions 49–65 (Fig. 5g), which corresponds to the fraction range found for the elution of arsenous acid with PBS buffer at this pH alone (data not shown).

All attempts to chromatograph the arsenic–GSH species, prepared by mixing 30 μg of radiolabeled As^{III} (0.22 ml) with a stoichiometric amount of GSH (3 mol equivalents), with PBS buffers, pH 2.0 and 7.0, failed to detect an arsenic peak in the fraction range corresponding to that for arsenic–GSH species (fractions 20–40). Hence, arsenic–GSH species are labile and fall apart during the chromatographic process.

In the observed pH range, i.e. pH 2.0 and 10.0, arsenic–GSH species were detected in the pH range between 2.0 and 8.0. These findings are generally in accord with results by Delnomdedieu et al. [36], who reported arsenic–GSH species to be stable between pH 1.5 and 7.5. They used the ^{13}C-chemical shift of the methylene (Cys b) and methine (Cys a) carbon atoms as a measure of the binding of arsenous acid to GSH. However, in our experiments, the detection of only arsenic–GSH species (without free arsenous acid) was restricted to the pH range between 6.0 and 8.0. This apparent contradiction of Delnomdedieu's data may be because ^{13}C-NMR provides a static

picture of the solution chemistry, whereas the results obtained by SEC in conjunction with radiodetection of ^{73}As provide a dynamic picture of the chemical reactions involved. Hence, our experiments indicate that a decrease in the pH of the mobile phase from pH 7.0 to 2.0 shifts the equilibrium in Eq. (1) to the left.

3.2. Influence of sodium DL-2,3-dimercapto-1-propanesulfonate on the formation of arsenic–GSH species

The 'retention analysis method' can be ideally applied to the study of competitive interactions between arsenous acid, monothiols (GSH) and dithiols (DMPS). The 'chelating agent', DMPS, is an orally effective drug for mobilizing heavy metals, such as mercury and arsenic, from the bodies of patients suffering from arsenic/mercury intoxication [37,38]. We therefore studied the effect of DMPS on the formation of arsenic–GSH species under simulated physiological conditions (PBS buffer, pH 7.4, 37°C).

After the injection of arsenous acid onto a Sephadex G-10 column equilibrated with a PBS buffer containing 7.5 m*M* GSH at 37°C, arsenic eluted in fractions 25–35 (Fig. 6a), suggesting the presence of arsenic–GSH species. When arsenous acid was chromatographed with a PBS buffer containing 7.5 m*M* GSH and 1.0 m*M* DMPS (pH 7.4, 37°C), arsenic was detected in fractions 40–50 (Fig. 6b). Arsenic eluted in fractions 44–53 when a PBS buffer containing 7.5 m*M* GSH and 2.0 m*M* DMPS (pH 7.4, 37°C) was used to chromatograph arsenous acid (Fig. 6c). With 1.0 m*M* DMPS in PBS buffer alone, arsenic eluted in fractions 71–90 (Fig. 6d). Because arsenous acid eluted in fractions 56–64 at 37°C with PBS buffer alone (data not shown), an interaction between the formed arsenic–DMPS adduct and the free hydroxyl groups of the Sephadex G-10 gel does occur.

Compared to the elution of arsenic with PBS buffer with GSH alone (7.5 m*M*), the addition of DMPS (1.0 and 2.0 m*M*) to this buffer shifted the elution of arsenic to higher fraction numbers (Fig. 6a–c). This shift can be rationalized by the formation of an arsenic–DMPS adduct that is more stable than the arsenic–GSH species under these conditions.

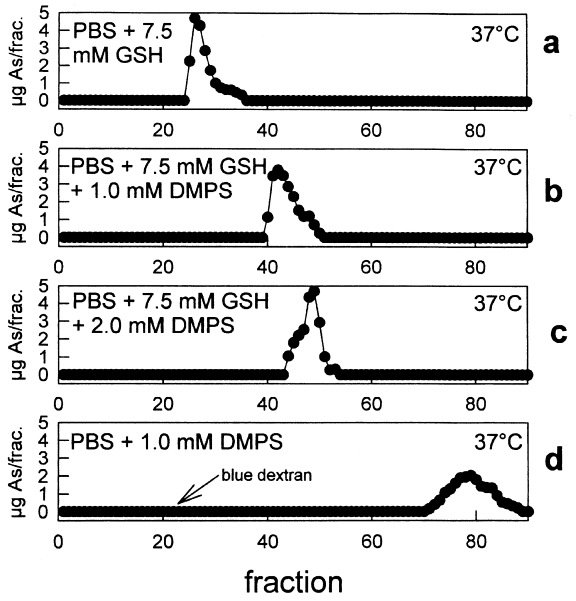
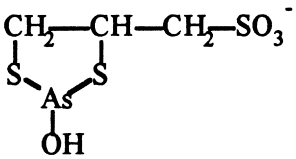


Fig. 6. Chromatograms obtained at 37°C after injection of 30 µg of arsenic, as arsenous acid, using PBS buffers containing GSH and/or DMPS. Column, Sephadex G-10 (62×2.6 cm); injection volume, 0.3 ml; flow-rate, 0.6 ml/min; fraction volume, 5.0 ml; arsenic-specific detector, GFAAS at 193.7 nm.

The observed shift in the retention of arsenic after the addition of DMPS may be caused by a smaller molecular mass of the formed arsenic–DMPS adduct. According to Dill et al. [39], who reported a six-membered heteroadduct between lipoic acid (6,8-dithiooctanoic acid) and phenyldichlorarsine, a five-membered heteroadduct between DMPS and arsenous acid is most likely formed in solution:



This adduct has recently been detected in aqueous solution (data not shown) and has a molecular mass of 277. This is considerably smaller than the molecular mass of $(GS)_3As$ (994) and, thus, could account for the observed retention shift from the large to the small molecular mass fraction range.

Because of the observed interaction of the arsenic–DMPS adduct with the Sephadex G-10 column

with PBS buffer alone (Fig. 6d), the observed retention shift of arsenous acid upon addition of DMPS could also be explained by this interaction. Which of these two mechanisms (probably both) caused the observed retention shift cannot be deduced on the basis of these observations. In either case, however, stronger binding of arsenous acid to DMPS than to GSH is evident. This experimental finding is in good agreement with results reported elsewhere [40,41]. Experiments are underway to purify and to characterize an arsenic–DMPS adduct.

3.3. Advantages and disadvantages of SEC–GFAAS

SEC–GFAAS offers several advantages compared to noninvasive ^{1}H SEFT–NMR spectroscopy for studying metal/metalloid–peptide interactions. For instance, SEC–GFAAS can be used to simulate reactions at physiological temperatures and physiological metal/metalloid concentrations, which may be a problem with ^{1}H SEFT–NMR spectroscopy because of linebroadening phenomena at high temperatures and a lack of sensitivity. Like ^{1}H SEFT–NMR spectroscopy, SEC–GFAAS allows one to study competitive interactions concerning molecules dissolved in the mobile phase (GSH and/or DMPS).

However, the utilization of SEC–GFAAS has also some inherent drawbacks. Because the detection of the metal/metalloid–peptide species is performed with an excess of the peptide (GSH) in the mobile phase, no complex formation constant for the metal/metalloid–peptide species can be calculated. In addition, the excess peptide (GSH) may also drastically impede the molecular characterization of the metal/metalloid–peptide complex formed by e.g. MALDI–TOF. Unknown interactions between the metal/metalloid and the Sephadex gel matrix do occur and SEC–GFAAS does not provide information about the specific binding site on the peptide (GSH).

4. Conclusion

SEC in conjunction with GFAAS and using the ‘retention analysis method’ proved to be a useful tool for studying the dynamic on-column formation of arsenic–GSH species in PBS buffer under various physicochemical conditions. The formation of arsenic–GSH species was facilitated at high concentrations of GSH (7.5 m*M*) in the mobile phase and low temperatures in the observed temperature range (4–37°C). At 4°C, the formation of arsenic–GSH species was detected at pH values of between 2.0 and 8.0, with arsenic–GSH species being the only species formed between pH values of 6.0 and 8.0. These results, together with the inability to detect arsenic–GSH species in the corresponding fraction range after injection of a mixture of arsenous acid (30 μg As) and GSH (370 μg) with PBS buffers without GSH in the pH range 2.0–7.0 at 4°C clearly indicate that arsenic–GSH species are labile.

Simulating the intracellular physiological parameters found inside human red blood cells (2.5 m*M* GSH, pH 7.4, 37°C) and hepatocytes (7.5 m*M* GSH, pH 7.4, 37°C) with the mobile phase demonstrated that arsenic–GSH species can be formed under these conditions. Together with NMR investigations, which demonstrated that arsenic binds to GSH via the sulfhydryl group [5], these results suggest that arsenic–sulfur single bonds can be formed under physiological conditions. Investigations into the competitive interaction between arsenous acid, GSH (7.5 m*M*) and the chelating agent DMPS (1.0 m*M*) revealed stronger binding of arsenous acid to the dithiol, DMPS, than to the monothiol, GSH, under simulated physiological conditions.

Our results regarding the formation of arsenic–GSH species generally substantiate those obtained by ^{1}H-NMR. Hence, SEC–GFAAS is an alternative analytical technique for studying labile associations between metal/metalloid ions and peptides (GSH) and chelating agents (DMPS). The simulation of these reactions at physiologically relevant metal/metalloid concentrations by SEC–GFAAS, together with noninvasive ^{1}H SEFT–NMR spectroscopy, providing information about the specific binding site, may be useful for investigating metal/metalloid–peptide interactions generally.

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