APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. **14**, 355–363 (2000)

Simultaneous multielement-specific detection of a novel glutathione–arsenic–selenium ion $[(GS)_2AsSe]^-$ by ICP AES after micellar size-exclusion chromatography

J. Gailer,[1]* S. Madden,[2] M. F. Burke,[2] M. B. Denton[2] and H. V. Aposhian[3]
[1]Department of Nutritional Sciences, University of Arizona, 309 Shantz Building, PO Box 210038, Tucson, AZ 85721, USA
[2]Department of Chemistry, University of Arizona, Tucson, AZ 85721, USA
[3]Department of Molecular and Cellular Biology, University of Arizona, Life Sciences South Building, Tucson, AZ 85721, USA

In order to confirm the solution structure of $[(GS)_2AsSe]^-$ (GS = glutathione), we have investigated the retention behaviour of a $[(GS)_2AsSe]^-$/oxidized glutathione (GSSG) mixture on a Sephadex G-25 (SF) column with Tris buffers (0.1 mol dm^{-3}, pH 8.0) containing various surfactants at concentrations above the critical micellar concentration (CMC): hexadecyltrimethlammonium bromide (HDTAB; 30, 40 and 50 mmol dm^{-3}); dodecyltrimethylammonium bromide (DDTAB; 50 mmol dm^{-3}); and sodium lauryl sulfate (SLS; 50 mmol dm^{-3}). An inductively coupled plasma atomic emission spectrometer (ICP AES) provided simultaneous on-line detection of arsenic, selenium and sulfur in the column effluent. The chromatographic retention behaviour was used to investigate the association of both compounds with the positively charged micelles (HDTAB and DDTAB mobile phases). The relative strength of association with the micelles provided insight into the effective negative charge on $[(GS)_2AsSe]^-$ and GSSG. The chromatograms obtained with 50 mmol dm^{-3} HDTAB indicated that two glutathione molecules are associated with the elution of an arsenic–selenium compound. Combined, these chromatographic data strongly support the spectroscopically derived solution structure of $[(GS)_2AsSe]^-$. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: glutathione–arsenic–selenium ion; chromatography; micelles

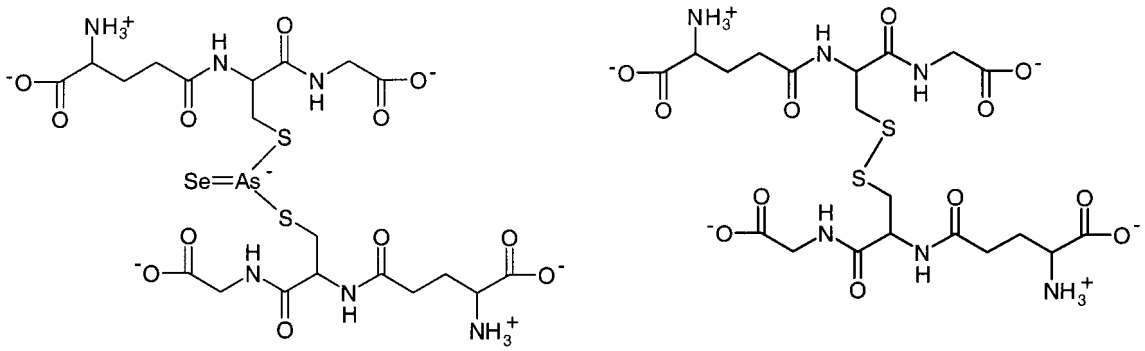
* Correspondence to: J. Gailer, Department of Nutritional Sciences, University of Arizona, 309 Shantz Building, PO Box 210038, Tucson, AZ 85721-003, USA.

Received 15 September 1999; accepted 1 December 1999

INTRODUCTION

Arsenic and selenium are widely dispersed in a large variety of geological formations in the crust of the Earth, where—owing to their high affinity for sulfur—both elements are predominantly associated with sulfidic ores.[1,2] Weathering processes dissolve the primary arsenic and selenium minerals to arsenite, arsenate, selenite and selenate, the most common oxidation states of these metalloids in fresh water.[3,4] Through the consumption of drinking water and food, man unwittingly ingests a variety of inorganic and organic arsenic and selenium compounds, among which both arsenite and selenite are teratogenic[5] and toxic.

Experiments concerned with the simultaneous exposure of rats to arsenite and selenite, however, revealed a striking mutual detoxification of these metalloid compounds.[6] The most recent investigations aimed at the elucidation of the molecular basis underlying this antagonistic interaction in rabbits revealed, that after the intravenous (i.v.) injection of sodium arsenite and sodium selenite, a novel arsenic- and selenium-containing compound was excreted in bile.[7] This compound, which contained equimolar amounts of arsenic and selenium, exhibited an X-ray absorption spectrum that was essentially identical to that of a synthetic species in solution, the structure of which was identified spectroscopically [by X-ray absorption spectroscopy (XAS), Raman spectrometry and ^{77}Se NMR]



1 **2**

Figure 1 The $[(GS)_2AsSe]^-$ ion (**1**) and GSSG (**2**).

as $[(GS)_2AsSe]^-$.[7] This compound can be synthesized chemically by addition of an aqueous solution containing equimolar amounts of sodium arsenite and sodium selenite to a solution containing $\geq$8 mol equiv. GSH adjusted to pH 7.4.[7] In the reaction mixture oxidized glutathione (GSSG) was identified by Raman spectroscopy,[7] suggesting a redox reaction according to Eqn [1].

$$As(OH)_3 + HSeO_3^- + 8GSH \rightarrow (GS)_2As^-Se + 3GSSG + 6H_2O \quad (1)$$

Unfortunately, however, no direct chemical evidence for the negative charge on the arsenic atom of $[(GS)_2AsSe]^-$ could be obtained by the techniques employed. Thus, experimental proof of this negative charge and proof that two glutathione (GSH) molecules are attached to the arsenic–selenium compound would unequivocally confirm the EXAFS/Raman/^{77}Se NMR-derived molecular structure.

Chromatographic separation methods coupled on-line to element-specific detectors, such as inductively coupled plasma mass spectrometers (ICP MS) or inductively coupled plasma atomic emission spectrometers (ICP AES) are widely used to identify metal/metalloid compounds in environmental and biological samples.[8] In addition, investigations of the pH-dependent retention behaviour on reversed-phase and anion-exchange HPLC columns can provide very useful information about the protonation/deprotonation behaviour of arsenic compounds whose solution chemistry is not well understood.[9,10] Similarly, an appropriately chosen chromatographic technique should provide proof of the additional negative charge on $[(GS)_2AsSe]^-$ as compared with GSSG. The additional proof of two GSH molecules being attached to the arsenic–selenium moiety of $[(GS)_2AsSe]^-$ would require a chromatographic separation of $[(GS)_2AsSe]^-$ from the by-product, GSSG (Eqn [1]).

Although $[(GS)_2AsSe]^-$ (M_r 767) is larger than GSSG (M_r 612), the overall volumes of the two molecules are probably rather similar because the arsenic–selenium structural element will not contribute substantially to the overall volume of $[(GS)_2AsSe]^-$. Thus, a separation according to size seems impossible, bearing in mind the low resolution of Sephadex-based size-exclusion columns. Furthermore, both $[(GS)_2AsSe]^-$ and GSSG contain two GSH groups per molecule. This is likely to result in a very similar solution chemistry, further hampering a chromatographic separation of these two compounds. One significant difference between $[(GS)_2AsSe]^-$ and GSSG is the additional negative charge on the former (Fig. 1).

Hence, one potential separation strategy could target the difference in net electrostatic charge per molecule, for example with anion-exchange chromatography. Micellar chromatography, however, has also been used successfully to separate a large variety of anions[11–15] and several reviews have been published since its recognition as a very useful analytical tool.[16–20] Because micellar chromatography could be of potential use to separate $[(GS)_2AsSe]^-$ from GSSG, we investigated the effect of increasing amounts of hexadecyltrimethylammonium bromide (HDTAB),

dodecyltrimethylammonium bromide (DDTAB) and sodium lauryl sulfate (SLS) in the mobile phase on the chromatographic retention behaviour of a $[(GS)_2AsSe]^-$ /GSSG mixture on a size-exclusion column, using an ICP AES as the on-line simultaneous arsenic-, selenium- and sulfur-specific detector.

EXPERIMENTAL

Chemicals

Sodium selenite ($Na_2SeO_3{\cdot}5H_2O$; >97%) and hexadecyltrimethylammonium bromide (HDTAB; >99%) were purchased from Fluka (Buchs, Switzerland) and sodium arsenite ($NaAsO_2$; >99%) from GFS Chemicals (Columbus, OH, USA). Glutathione (>98%), dodecyltrimethylammonium bromide (DDTAB; 99%) and sodium lauryl sulfate (>99%) were obtained from Sigma (St Louis, MO, USA). Sodium hydroxide was purchased from MCB Reagents (Cincinnati, OH, USA) and conc. hydrochloric acid was obtained from Fischer Chemicals (Pittsburgh, PA, USA). Tris buffer (0.1 mol dm^{-3}, pH 8.0) and all other solutions were prepared from triply distilled water.

Synthesis of $[(GS)_2AsSe]^-$

A solution containing equimolar amounts of As(III) and Se(IV) was prepared by dissolving $NaAsO_2$ (239 mg, 1.853 mmol) and $Na_2SeO_3{\cdot}5H_2O$ (491 mg, 1.870 mmol) in 0.6 cm^3 Tris buffer and diluting to 1.0 cm^3 with additional buffer. GSH (205 mg, 0.670 mmol) was dissolved in 0.6 cm^3 Tris buffer, the pH was adjusted to 7.5 (4.0 mol dm^{-3} NaOH) and the solution was diluted to 1.0 cm^3 with additional buffer. After incubation of the GSH solution (0.670 mmol; 9 mol equiv. to protect the easily oxidizable product from air[7]) for 20 min at 37 °C, an aliquot of the As (III)/Se(IV) solution (40 μl; 0.074 mmol each of As and Se) was added. After the initial formation of a red–brown precipitate, thorough mixing resulted in a clear solution of pH 8.6.

Chromatography

A Beckman 110 B Solvent Delivery Module HPLC pump in conjunction with a Rheodyne six-port injection valve (200-μl loop) was used throughout the study. A Pharmacia HR 10/30 column (i.d. 1.0 cm) was filled with Sephadex G-25 (SF), which has a dry-bed diameter of 10–40 μm and separates peptides and globular proteins in the M_r range of 1000–5000 Da. After at least 100 cm^3 of Tris buffer at a flow rate of 1.0 cm^3 min^{-1} had been pumped over the column, the final bed height was 29.5 cm. The column was subsequently equilibrated with at least 60 cm^3 of the corresponding mobile phase before any injections were made. The column exit was connected to a Meinhard TR-30-K2 concentric glass nebulizer with the minimum length of polyethylene tubing (120 mm × 0.1 mm). The flow rate was maintained at 1.0 cm^3 min^{-1}, which had been previously determined to give a maximum ICP AES emission signal with this nebulizer/spray chamber combination when the nebulizer was operated at its rated pressure of 30 psi (206 kPa). The concentrations of the surfactant in the 0.1 mol dm^{-3} Tris buffer (pH 8.0) were held well above the reported critical micellar concentrations (CMC) at 25 °C: SDS 8.1 mmol dm^{-3}, HDTAB 0.92 mmol dm^{-3},[18] DDTAB 15 mmol dm^{-3}.[21] Solutions of 30, 40 and 50 mmol dm^{-3} HDTAB and 50 mmol dm^{-3} solutions of DDTAB and SLS in Tris buffer (0.1 mol dm^{-3}, pH 8.0) were prepared by dissolving the appropriate amounts of the surfactant in Tris buffer (0.1 mol dm^{-3}, pH 8.0). The addition of the surfactant to the buffer did not change the pH noticeably (PHM 220 Lab pH-meter; Radiometer, Copenhagen, Denmark).

The exclusion volume was determined by injection of distilled water onto the column using 50 mmol dm^{-3} SLS as the mobile phase and sulfur-specific detection. A dip in the on-line sulfur signal of the chromatogram corresponded to the exclusion volume and was 8.1 cm^3. The inclusion volume, which was determined by injection of an aqueous 50 mg dm^{-3} Mg^{2+} solution followed by magnesium-specific detection by ICP AES at the 285.213-nm line, was 17.9 cm^3. The inclusion volume minus the exclusion volume defines the chromatographic window of 9.8 cm^3. Using 50 mmol dm^{-3} in 0.1 mol dm^{-3} Tris buffer as the mobile phase, the column was calibrated with aqueous solutions adjusted to pH 8.0 of $NaAsO_2$, GSSG, Na_2SeO_3 and GSH containing the same amount of arsenic, selenium or sulfur as the 200 μl of the injected $[(GS)_2AsSe]^-$/GSSG mixture. The retention times were 23.3 min (arsenite), 12.6 min (GSSG), 15.9 min (selenite) and 15.0 min (GSH), respectively. The strong retention of arsenite on the Sephadex G-25 (SF) stationary phase was in accord

with previous findings which revealed an unspecified chemical interaction between arsenite and a Sephadex G-10 stationary phase.[22] Shifts in retention times of the arsenic, selenium or sulfur peaks with increasing concentrations of HDTAB in the mobile phase are given in minutes as well as relative to the corresponding peak in the chromatogram obtained with the 30 mmol dm^{-3} HDTAB mobile phase (given as a percentage of the chromatographic window).

Experimental proof that $[(GS)_2AsSe]^-$ eluted from the column intact was obtained by XAS analysis of two fractions collected from the column effluent. After injection of the $[(GS)_2AsSe]^-$/GSSG mixture [injection volume 200 μl; flow rate 1.0 cm^3 min^{-1}; mobile phase 50 mmol dm^{-3} HDTAB in 0.1 mol dm^{-3} Tris buffer (pH 8.0)], fractions were collected (fraction **I**, 8.5–9.7 min; fraction **II**, 9.7–10.7 min). After the addition of an aliquot (0.6 cm^3) of each fraction to 0.4 cm^3 glycerol and thorough mixing, the obtained solutions were transferred to Lucite sample holders, frozen in liquid nitrogen and subsequently analysed by arsenic and selenium K-edge XAS at the Stanford Synchrotron Radiation Laboratory. Fitting results were obtained by comparison with previously reported arsenic and selenium K-edge XAS data.[7]

ICP AES

Arsenic, selenium and sulfur-specific detection was achieved with a Thermo Jarrel Ash (Franklin, MA, USA) IRIS HR radial view ICP AES at 228.812 nm (order 147), 196.090 nm (order 172) and 182.034 nm (185), respectively. Data were taken from several emission lines for each element, but the above-mentioned lines were chosen for convenient display without off-line scaling. ThermoSPEC/CID software (version 2.10.04) provided the necessary time-scan functions, and the multitasking controller allowed the processing of one atomic emission line every 0.02 s. Custom software allowed for variable dt derivatives of the integral signal as well as various forms of filtering. The nebulization gas was maintained at a flow of 2.0 dm^3 min^{-1} and at a pressure of 30 psi, the plasma forward power was kept at 1150 W, and the CID temperature was cooled to -85 °C. The recovery of arsenic, selenium and sulfur after the injection of the $[(GS)_2AsSe]^-$/GSSG mixture using 50 mmol dm^{-3} HDTAB (in 0.1 mol dm^{-3} Tris-buffer at pH 8.0) was 89 ± 2, 86 ± 4 and 98 ± 2%, respectively. The column effluent eluting between 6 and 25 min was collected, made up to 25 cm^3 in a volumetric flask with 50 mmol dm^{-3} HDTAB in 0.1 mol dm^{-3} Tris (pH 8.0) and quantified by ICP AES using calibration curves of 20 and 40 ppm of arsenic, selenium and sulfur in 50 mmol dm^{-3} HDTAB in 0.1 mol dm^{-3} Tris (pH 8.0).

RESULTS AND DISCUSSION

The first deliberate application of the unique properties of micelles in chromatographic separations using size-exclusion chromatography on Sephadex G-100 was reported in 1977.[15] Subsequently, many advantages of the use of micelles in chromatographic separations, such as their unique and modifiable selectivity, have been recognized.[19] A more straightforward approach than Sephadex G-100 for micellar separations involves the use of small-pore stationary phases, such as Sephadex G-25.[21] On a Sephadex G-25 column, compounds with an M_r greater than 5000 are excluded from the gel. Since the molecular weight of micellar HDTAB exceeds 22 kDa,[18] the aggregated surfactant is thus excluded from the Sephadex G-25 gel.[21] If a Sephadex G-25 column is equilibrated with a mobile phase containing HDTAB at a concentration well above the CMC of 0.92 mmol dm^{-3},[17] the movement of any compound with an M_r smaller than 5000 will depend on its partition coefficient between the bulk aqueous phase and the micellar pseudo-phase.[21] A small compound that associates with the micelles will consequently tend to elute more rapidly (with the micelle) with increasing concentrations of the surfactant.[21] Hence, the behaviour of a small compound on a size-exclusion column is determined mostly by its interaction with the micelle.[21]

Because $[(GS)_2AsSe]^-$ is chemically unstable below pH 7.0,[7] only mobile phases with pH $\geq$8.0 were investigated. At pH 8.0, GSH has both carboxyl groups completely deprotonated ($pK_{COOH,1} = 2.1$; $pK_{COOH,2} = 3.5$) and the amino group protonated ($pK_{NH_2} = 9.6$).[23] Hence, at pH 8.0 $[(GS)_2AsSe]^-$ (if the EXAFS/Raman/^{77}Se NMR-derived structure is correct) should have an overall net charge of -3, whereas GSSG has a net charge of -2.

Several attempts to separate $[(GS)_2AsSe]^-$ from GSSG on an anion-exchange column (DEAE Sephadex A-25, 1.0 cm $\times$ 29.2 cm) with PBS buffers (pH 7.4 and pH 10.0 at a flow rate of

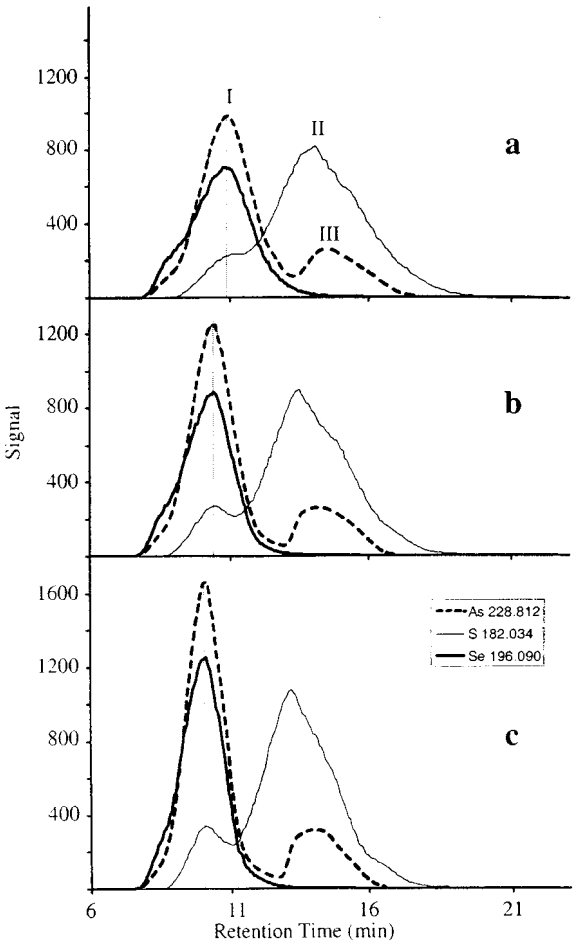


Figure 2 Separation of $[(GS)_2AsSe]^-$ (peak I) and oxidized glutathione (peak II) with (a) 30 mmol dm^{-3}, (b) 40 mmol dm^{-3} and (c) 50 mmol dm^{-3} HDTAB in Tris buffer (0.1 mol dm^{-3}, pH 8.0). Peak III is an unidentified arsenic-containing breakdown product of I. Column, Sephadex G-25 (SF) (1.0 cm × 29.5 cm); flow rate, 1.0 cm^3 min^{-1}; detector, ICP AES (arsenic 228.812 nm, selenium 196.090 nm, sulphur 182.034 nm); loop, 200 μl; the solution injected contained $[(GS)_2AsSe]^-$ (71 mmol dm^{-3}), GSSG (213 mmol dm^{-3}) and GSH (71 mmol dm^{-3}).

1.0 cm^3 min^{-1}) and Tris buffers (0.1 mol dm^{-3}, pH 8.0 and pH 10.0 at a flow rate of 1.0 cm^3 min^{-1}) were unsuccessful because $[(GS)_2AsSe]^-$ was irreversibly bound to the column (or decomposed), resulting in a brown coloration on the column head. With a Sephadex G-10 column (1.0 cm × 29.0 cm) and PBS buffers of pH 7.4 and 10.0 the arsenic-, selenium- and sulfur-specific chromatograms obtained after the injection of the $[(GS)_2AsSe]^-$/GSSG mixture showed a single peak with the same retention time for arsenic, selenium and sulfur (data not shown). Hence, $[(GS)_2AsSe]^-$ had passed through the column intact, but had not been separated from GSSG. The size difference of 145 Da was clearly too small to result in significant separation of these two compounds on this particular stationary phase.

Influence of the HDTAB concentration on the retention behaviour of $[(GS)_2AsSe]^-$ on Sephadex G-25 (SF)

Because $[(GS)_2AsSe]^-$ could be passed through Sephadex-based size-exclusion columns intact and because of the different net negative charges on $[(GS)_2AsSe]^-$ (−3) and GSSG (−2), the influence of a surfactant with a single positive charge (HDTAB) on the separation of the two compounds was investigated. HDTAB was added in 10 mmol dm^{-3} increments to the Tris buffer (0.1 mol dm^{-3}, pH 8.0) and the arsenic-, selenium- and sulfur-specific chromatograms were measured after the injection of the $[(GS)_2AsSe]^-$/GSSG mixture (Fig. 2a–c). We noted that an orange band formed immediately after the injection of the $[(GS)_2AsSe]^-$/GSSG mixture onto the column. This orange band subsequently moved through the entire column. The orange colour is hypothesized to be caused by elemental selenium, indicating some decomposition of $[(GS)_2AsSe]^-$ on the column head.

Because an increase of the HDTAB concentration in the mobile phase from 30 to 50 mmol dm^{-3} is likely to affect the efficiency of the nebulizer and thus the mass transfer of arsenic/selenium/sulfur into the inductively coupled plasma, the total areas obtained for each chromatogram (and for each element) were measured. Although the same total amount of arsenic/selenium/sulfur was injected with the 30, the 40 and the 50 mmol dm^{-3} HDTAB mobile phases, the signal areas obtained corresponded to the proportions 1:1.03:1.18, suggesting an increase in mass transfer with increasing surfactant concentration in the mobile phase (Fig. 2a–c).

All arsenic-specific chromatograms obtained with 30, 40 and 50 mmol dm^{-3} HDTAB in the mobile phase showed a large arsenic peak (Fig. 2a–c, peak I) eluting first, followed by a smaller one (Fig. 2a–c, peak III). With the 30 mmol dm^{-3} HDTAB mobile phase, the two arsenic peaks had retention times of 10.9 min and 14.5 min (Fig. 2a).

An increase of the HDTAB concentration in the mobile phase to 40 mmol dm^{-3} brought about a 5.1% decrease in the retention time of the first arsenic peak to 10.4 min (Fig. 2b). Further increase of the HDTAB concentration in the mobile phase to 50 mmol dm^{-3} decreased the retention time of the first arsenic peak by 9.2% to 10.0 min (Fig. 2c). Concomitantly with a decrease in the retention time of arsenic peak I with increasing concentrations of HDTAB in the mobile phase, the peak noticeably narrowed while maintaining the same approximate area ratio when considering the above-noted increase in mass transfer with increasing HDTAB concentrations in the mobile phase.

All selenium-specific chromatograms displayed a single selenium peak (Fig. 2a–c, peak I) which had a shoulder located on the short retention end that diminished in magnitude with increasing HDTAB concentrations in the mobile phase (Fig. 2a–c). The retention time of selenium peak I decreased from 10.8 min with the 30 mmol dm^{-3} mobile phase to 10.0 min with the 50 mmol dm^{-3} mobile phase (9.2%; Fig. 2a–c) and narrowed in a similar fashion to arsenic peak I. The fact that selenium peak I always co-eluted with arsenic peak I (Fig. 2a–c) suggests that both peaks correspond to the elution of $[(GS)_2AsSe]^-$.

The sulfur-specific chromatogram obtained with the 30 mmol dm^{-3} HDTAB mobile phase contained a large sulfur peak with a retention time of 14.0 min (Fig. 2a, peak II) overlapping significantly with a smaller sulfur peak which had a retention time of 11.0 min (Fig. 2a, peak I). An increase in the HDTAB concentration to 40 mmol dm^{-3} in the mobile phase decreased the retention time of sulfur peak I to 10.4 min (6.1%), whereas the retention time of sulfur peak II (measured at the maximum signal height) decreased in a less pronounced manner to 13.5 min (5.1%; Fig. 2b). The chromatogram obtained with 50 mmol dm^{-3} HDTAB in the mobile phase showed two sulfur peaks with retention times of 10.1 min (9.2%) and 13.2 min (8.2%), respectively (Fig. 2c). Because the retention time of the smaller sulfur peak was always identical with that of the arsenic peak I and selenium peak I (Fig. 2a–c), we identified this peak as $[(GS)_2AsSe]^-$. To identify peak I unequivocally as $[(GS)_2AsSe]^-$, peak I was collected and analysed by arsenic and selenium K-edge XAS. Fitting results indicated that 70% (fraction I) and 79% (fraction II) of the selenium was present as $[(GS)_2AsSe]^-$. The remaining selenium could be identified as elemental α-selenium. This is consistent with the fact that the As/Se molar ratio for a collected fraction of peak I (whole peak, compared with a standard solution containing As/Se 1:1 in a solution of the same composition as the mobile phase) is 1:1.3 rather than 1:1, since the selenium breakdown product (elemental α-selenium) co-eluted with $[(GS)_2AsSe]^-$. The selenium breakdown product (elemental α-selenium) also associates with the micelles and is probably responsible for the leading shoulder on selenium peak I (Fig. 2a–c). Incidentally, the observed orange colour is probably due to elemental α-selenium. Arsenic K-edge X-ray absorption spectrometry of fraction II showed that 97% of the arsenic was present as $[(GS)_2AsSe]^-$ and that 3% were present as arsenite. Hence, X-ray absorption spectroscopy demonstrates that partial decomposition of $[(GS)_2AsSe]^-$ occurred during the chromatographic separation. The arsenic breakdown product eluted as peak III.

Sulphur peak II was identified as GSSG by comparison with the retention time obtained for a GSSG standard. Because the peak-area integration of the two significantly overlapping sulfur peaks was likely to result in artifacts, the signal height ratio of both sulfur peaks (both displayed a nearly symmetrical peak shape) was determined at the signal maximum. The signal height ratio between the smaller and the bigger sulfur peaks was 1:3 or a multiple of that, e.g. 2:6 (the same ratio was found for the 30 and 40 mmol dm^{-3} HDTAB mobile phase). This signal height ratio is in accord with the expected sulfur ratio for $[(GS)_2AsSe]^-$ and GSSG of 2:6 according to Eqn [1][7]. Because $[(GS)_2AsSe]^-$ was synthesized with an excess of 1 mol equiv. of GSH to prevent the oxidation of $[(GS)_2AsSe]^-$ by exposure to air (see the Experimental section), it seems that one sulfur equivalent is missing. However, the significant tailing of sulfur peak II that is evident upon closer investigation of Fig. 2c very probably corresponds to the unreacted GSH molecule (GSH is smaller than GSSG and thus is eluted with a longer retention time; see the Experimental section). Since the small overlapping GSH peak in this tailing region does not contribute to the overall signal height of the GSSG peak, the 2:6 sulfur ratio obtained agrees well with the expected sulfur ratio of 2:6 between $[(GS)_2AsSe]^-$ and GSSG. Furthermore, these data strongly support the EXAFS/Raman/^{77}Se NMR-derived result of two GSH molecules being bound to the arsenic–selenium species.

Similarly to the decrease in elution volume of nucleosides (on the same stationary phase) with

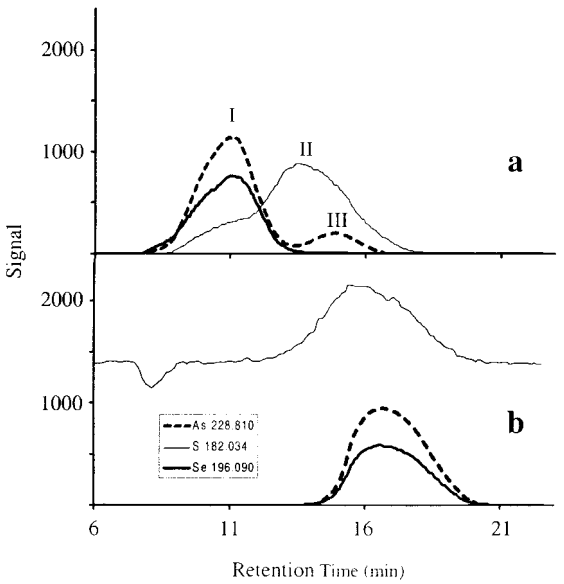


Figure 3 Separation of (a) $[(GS)_2AsSe]^-$ and (b) oxidized glutathione with 50 mmol dm^{-3} DDTAB (a) and (b) 50 mmol dm^{-3} SLS, in Tris buffer (0.1 mol dm^{-3}, pH 8.0). Peak III is an unidentified arsenic-containing breakdown product of I. Column, Sephadex G-25 (SF) (1.0 cm × 29.5 cm); flow rate, 1.0 cm^3 min^{-1}; detector; ICP AES (arsenic 228.812 nm, selenium 196.090 nm, sulphur 182.034 nm); loop, 200 μl; the solution injected contained $[(GS)_2AsSe]^-$ (71 mmol dm^{-3}), GSSG (213 mmol dm^{-3}) and GSH (71 mmol dm^{-3}).

increasing concentrations of sodium dodecanoate in the mobile phase,[11] the retention time of both $[(GS)_2AsSe]^-$ and GSSG decreased with an increase in the concentration of HDTAB (and hence the number of micelles per volume) in the mobile phase (Fig. 2a–c). This retention behaviour suggests that both compounds associate with the positively charged HDTAB micelles. Consequently, the observed separation of $[(GS)_2AsSe]^-$ and GSSG cannot be due to a saturation of the micelle surface with the $[(GS)_2AsSe]^-$ molecules followed by the elution of the unbound GSSG. The fact that $[(GS)_2AsSe]^-$ always eluted earlier than GSSG further suggests stronger association of $[(GS)_2AsSe]^-$ with the HDTAB micelles. This stronger association can be explained by additional negative charge on the $[(GS)_2AsSe]^-$ molecule as compared with GSSG.

Combined, these chromatographic data demonstrate that two GSH molecules are associated with the arsenic–selenium solution species and that $[(GS)_2AsSe]^-$ has additional negative charge compared with GSSG at pH 8.0. Thus, our chromatographic results strongly support the EXAFS/Raman/^{77}Se NMR-derived structure of the synthetic arsenic—selenium solution species, $[(GS)_2AsSe]^-$.

Influence of 50 mmol dm^{-3} DDTAB and SLS on the retention behaviour of $[(GS)_2AsSe]^-$ on Sephadex G-25 (SF)

The arsenic-, selenium- and sulfur-specific chromatograms obtained for the $[(GS)_2AsSe]^-$/GSSG mixture with a mobile phase consisting of 50 mmol dm^{-3} DDTAB in Tris buffer (0.1 mol dm^{-3}, pH 8.0) is shown in Fig. 3(a). As with the HDTAB mobile phases, an orange band was observed on the column head after the injection of the $[(GS)_2AsSe]^-$/GSSG mixture. The arsenic-, selenium- and sulfur-specific chromatograms obtained were very similar to those obtained for the 30 mmol dm^{-3} HDTAB mobile phase (Fig. 3a vs Fig. 2a). Arsenic peak I had a retention time of 11.0 min, which was identical with the retention time of selenium peak I (Fig. 3a). The sulfur-specific chromatogram consisted of a small peak with a retention time of approximately 11.0 min that had a much bigger peak (II) on its long retention end with a retention time of 13.6 min (Fig. 3a). Since the retention time of the small sulfur peak was identical with that of arsenic and selenium peak I, this sulfur peak must correspond to $[(GS)_2AsSe]^-$. Sulphur peak II consequently corresponds to GSSG. The fact that $[(GS)_2AsSe]^-$ eluted before GSSG (as with HDTAB in the mobile phase) demonstrates again that the former interacts more strongly than GSSG with the positively charged DDTAB micelles.

If indeed GSSG and $[(GS)_2AsSe]^-$ associate with the positively charged micelles in the mobile phase, a mobile phase containing the same concentration of a surfactant with a negative charge on the micelle surface should result in arsenic-, selenium- and sulfur-specific chromatograms with increased retention times for all three elements (since no interaction with micelles should occur). In addition, no separation of $[(GS)_2AsSe]^-$ and GSSG should be observed.

When a 50 mmol dm^{-3} solution of SLS in Tris buffer (0.1 mol dm^{-3}, pH 8.0) was used as the mobile phase to chromatograph the $[(GS)_2AsSe]^-$/GSSG mixture, no orange band formed on the

column after the injection of the $[(GS)_2AsSe]^-$/GSSG mixture. The arsenic- and selenium-specific chromatogram showed single peaks with an identical retention time of 16.6 min (Fig. 3b). Because the mobile phase contained sulfur, the sulfur baseline was always elevated and showed a single, rather broad sulfur peak at a retention time of 16.2 min. This implies that $[(GS)_2AsSe]^-$ was not separated from GSSG. The dip in the sulfur-specific chromatogram corresponds to the void volume of the column, since the $[(GS)_2AsSe]^-$/GSSG mixture was prepared in Tris buffer which did not contain sulfur. The significantly increased retention time of $[(GS)_2AsSe]^-$ with the SLS mobile phase (16.2 min), as compared with the DDTAB mobile phase (11.0 min) at the same concentration (corresponding to a difference of 57%; Fig. 3a,b), hence provides indirect evidence for the association of $[(GS)_2AsSe]^-$ and GSSG with the DDTAB and the HDTAB micelles.

Because the additional negative charge on $[(GS)_2AsSe]^-$ (compared with GSSG) is accessible for interactions with positively charged micelles (Fig. 2a–c and 3a), the negative charge is likely to be located on the surface of the molecule. In view of the fact that $[(GS)_2AsSe]^-$ was recently detected in bile of rabbits that had been previously injected i.v. with sodium arsenite and sodium selenite (the sulfur donor has not yet been unequivocally identified as GSH),[7] the location of additional negative charge on the surface of the $[(GS)_2AsSe]^-$ ion may be relevant to the export of this novel compound from liver to bile. It is well known that a large variety of glutathione *S*-conjugates (GS-X) are excreted from liver to bile via ATP-dependent GS-X export pumps at the canalicular site of hepatocyte plasma membranes.[24] Thus, $[(GS)_2AsSe]^-$ may also be excreted through these pumps, and the accessible additional negative charge on the surface may be specifically involved in molecular recognition as a substrate by the GS-X export pumps.

CONCLUSION

Investigations of the chromatographic retention behaviour of a $[(GS)_2AsSe]^-$/GSSG mixture on a Sephadex G-25 (SF) column with a Tris buffer (0.1 mol dm^{-3}, pH 8.0) containing various surfactants (HDTAB at 30, 40 and 50 mmol dm^{-3}; DDTAB at 50 mmol dm^{-3}; SLS at 50 mmol dm^{-3}), with ICP AES as the on-line simultaneous multi-element-specific detector, showed that both $[(GS)_2AsSe]^-$ and GSSG interact with micelles with a positive surface charge (HDTAB, DDTAB) but not with micelles having a negative surface charge (SLS). $[(GS)_2AsSe]^-$ could be separated from GSSG with 50 mmol dm^{-3} HDTAB in Tris buffer. Based on the comparative migration behaviour of $[(GS)_2AsSe]^-$ and GSSG, our results indicate the presence of additional negative charge on $[(GS)_2AsSe]^-$. In addition, the sulfur-specific chromatogram obtained with the 50 mmol dm^{-3} HDTAB mobile phase suggested that two glutathione molecules are indeed associated with $[(GS)_2AsSe]^-$. These chromatographic data hence confirm the EXAFS/Raman/^{77}Se NMR-derived structure of $[(GS)_2AsSe]^-$.

Acknowledgements Graham N. George and Ingrid J. Pickering from the Stanford Synchrotron Radiation Laboratory are gratefully acknowledged for measuring the arsenic and selenium K-edge X-ray absorption spectra and for data-fitting analyses. Mike Pilon of Thermo Jarrel Ash Corporation (Franklin, MA) is acknowledged for instrumental and source code assistance.

REFERENCES

1. Boyle RW, Jonasson IR. *J. Geochem. Explor*. 1973; **2**: 251.
2. Berrow ML, Ure AM. *Occurrence and Distribution of Selenium*, in Ihnat M (ed.), CRC Press, Boca Raton, FL, 1989; 213.
3. Conde JE, Alaejos MS. *Chem. Rev.* 1997; **97**: 1979.
4. Cullen WR, Reimer KJ. *Chem. Rev.* 1989; **89**: 713.
5. Keen CL, in *Toxicology of Metals*, Chang LW, (ed.), CRC Lewis Publishers, Boca Raton, FL, 1996; 977.
6. Moxon AL. *Science* 1938; **88**: 81.
7. Gailer J, George GN, Pickering IJ, Prince RC, Ringwald SC, Pemberton JE, Glass RS, Younis HS, DeYoung DW, Aposhian HV. *J. Am. Chem. Soc.* in press.
8. Shibata Y, Morita M, Fuwa K. *Adv. Biophys*. 1992; **28**: 31.
9. Gailer J, Irgolic KJ. *J. Chromatogr. A* 1996; **730**: 219.
10. Gailer J, Madden S, Cullen WR, Denton MB. *Appl. Organomet. Chem.* 1999; **13**: 837.
11. Nagyvary J, Harvey JA, Nome F, Armstrong D, Fendler JH. *Precambrian Res.* 1976; **3**: 509.
12. Armstrong DW, Seguin R, Fendler JH. *J. Mol. Evol.* 1977; **10**: 241.
13. Maley F, Guarino DU. *Biochem. Biophys. Res. Commun.* 1977; **77**: 1425.
14. Okada T, Shimizu H. *J. Chromatogr. A* 1995; **706**: 37.
15. Armstrong DW, Fendler JH. *Biochim. Biophys. Acta* 1977; **478**: 75.
16. Cline Love LJ, Habarta JG, Dorsey JG. *Anal. Chem.* 1984; **56**: 1132A.
17. Mullins FGP. in *Ordered Media in Chemical Separations*,

Hinze WL, Armstrong DW (eds), American Chemical Society, Washington DC, 1987; Chapter 4, 115.
18. Khaledi MG. *Trends Anal. Chem.* 1988; **7**: 293.
19. McIntire GL. *Crit. Rev. Anal. Chem* 1990; **21**: 257.
20. Okada T. *J. Chromatogr. A.* 1997; **780**: 343.
21. Armstrong DW. *Sep. Purif. Methods* 1985; **14**: 213.
22. Gailer J, Lindner W. *J. Chromatogr. B* 1998; **716**: 83.
23. Rabenstein DL. *J. Am. Chem. Soc.* 1973; **95**: 2797.
24. Ishikawa T. *Trends Biochem. Sci.* 1992; **17**: 463.

