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Speciation Analysis AOC

Identification of $[(GS)_2AsSe]^-$ in rabbit bile by size-exclusion chromatography and simultaneous multielement-specific detection by inductively coupled plasma atomic emission spectroscopy

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An arsenic–selenium metabolite that exhibited the same arsenic and selenium X-ray absorption near-edge spectra as the synthetic seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ was recently detected in rabbit bile within 25 min after intravenous injection of rabbits with sodium selenite and sodium arsenite. X-ray absorption spectroscopy did not (and cannot) conclusively identify the sulfur-donor in the *in vivo* sample. After similar treatment of rabbits, we analyzed the collected bile samples by size-exclusion chromatography (SEC) using inductively coupled plasma atomic emission spectroscopy (ICP-AES) to monitor arsenic, selenium and sulfur simultaneously. The bulk of arsenic and selenium eluted in a single peak, the intensity of which was greatly increased upon spiking of the bile samples with synthethic $[(GS)_2AsSe]^-$. Hence, we identify $[(GS)_2AsSe]^-$ as the major metabolite in bile after exposure of rabbits to selenite and arsenite. The reported SEC–ICP-AES method is the first chromatographic procedure to identify this biochemically important metabolite in biological fluids and is thus a true alternative to X-ray absorption spectroscopy, which is not available to many chemists.

KEYWORDS: seleno-bis(*S*-glutathionyl) arsinium ion; speciation; bile

Both arsenic and selenium are widely distributed in many geological formations of the Earth's crust.[1,2] Uncontaminated soils, for instance, typically contain 5.0–14.3 mg of arsenic and 0.5–5.5 mg of selenium per kilogram of soil.[3] Hydrolysis, along with biotic/abiotic oxidation and/or reduction reactions, eventually releases potentially toxic arsenite, arsenate, selenite and selenate from soils to natural waters.[4–6] Apart from these natural sources, human activities, such as fossil fuel combustion[7–9] and nonferrous metal production,[7,10] also contaminate freshwater resources, resulting in an accelerated accumulation of these toxic metalloid compounds in the human food chain.[7–9] Humans, therefore, are unwittingly exposed to toxic arsenite and arsenate, predominantly *via* the ingestion of drinking water,[5,11] and to potentially toxic selenium compounds, mostly *via* food.[8,12] The unintended consequence of a 'safe-water' program in Bangladesh has resulted in the pollution of the alluvial Ganges aquifers used for public water supplies with inorganic arsenic (arsenite + arsenate) on a massive scale.[11]

When administered individually, arsenite and selenite are teratogenic and highly toxic in animals.[12] Quite unexpectedly, however, the simultaneous ingestion of arsenite along with toxic dietary selenium (seleniferous wheat or sodium selenite) prevented the characteristic symptoms of selenium poisoning in rats.[13,14] The most recent investigation aimed at an elucidation of the underlying molecular mechanism in mammals has revealed the biliary excretion of a previously

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unknown arsenic- and selenium-containing metabolite in rabbits. This metabolite contained equimolar arsenic and selenium and exhibited arsenic and selenium X-ray absorption near-edge spectra that were essentially identical to those of a synthetic species in solution.[15] The structure of the synthetic species could be elucidated by extended X-ray absorption fine structure (EXAFS) analysis, ^{77}Se NMR spectroscopy and Raman spectroscopy as the seleno-bis (*S*-glutathionyl)arsinium ion, $[(GS)_2AsSe]^-$ [note that the charge in this schematic representation does not include the charges introduced by the carboxyl/amine groups on glutathione (GSH)].[15] The structure of this compound was then confirmed experimentally by micellar size-exclusion chromatography (SEC) with simultaneous arsenic-, selenium- and sulfur-specific detection by inductively coupled plasma atomic emission spectroscopy (ICP-AES).[16] In addition, SEC-ICP-AES was recently used to detect a mercury-selenium and sulfur-containing compound after separation from its byproducts.[17] Accordingly, SEC followed by simultaneous arsenic-, selenium- and sulfur-specific detection by ICP-AES has the potential to become an alternative method to synchrotron-radiation-based X-ray absorption spectroscopy to detect and identify $[(GS)_2AsSe]^-$ in biological samples. We therefore treated rabbits with selenite and arsenite (as previously reported)[15] and subsequently analyzed the collected bile samples by SEC–ICP-AES. The bile samples were then spiked with synthethic $[(GS)_2AsSe]^-$ and rechromatographed.

EXPERIMENTAL

Chemicals

Reduced GSH (>98%) was purchased from Sigma (St Louis, MO, USA). $NaAsO_2$ (>99 %) was purchased from GFS Chemicals (Columbus, OH, USA) and $Na_2SeO_3{\cdot}5H_2O$ (>97%) from Fluka (Buchs, Switzerland). NaOH was purchased from MCB Reagents (Cincinatti, OH, USA) and HCl (36.5–38%) from J. T. Baker (Phillipsburg, NJ, USA). The mobile phase (0.1 mol dm^{-3} Tris-buffer, pH 7.5) was prepared with doubly distilled water. Solutions (0.02 mol dm^{-3}) of each sodium selenite and sodium arsenite were prepared in PBS-buffer (prepared from dry powder pouches) and subsequently adjusted to pH 7.4 by dropwise addition of HCl.

New Zealand white rabbit experiment

Two male New Zealand white rabbits (2–3 kg) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA) and maintained for 1 week on a 'hi-fiber' rabbit diet (7015 Harlaw Tekland, Madison, WI, USA). The animals were prepared for the experiment as reported previously.[15] 2 min after the injection of aqueous sodium selenite (0.63 mg of selenium per kilogram body weight), aqueous sodium arsenite (0.60 mg of arsenic per kilogram body weight) was injected and bile was collected for 25 min into ice-cold polyethylene tubes. The bile flow was approximately 50 mg kg^{-1} min^{-1} and the pH of the bile was 7.7. After gently mixing the samples they were immediately analyzed by SEC–ICP-AES.

Chromatography

A Beckman 110 B solvent delivery module high-performance liquid chromatography (HPLC) pump in conjunction with a Rheodyne six-port injection valve (400 µl loop) was used. All separations were performed at 4 °C. A prepacked Pharmacia Superdex Peptide HR 10/30 column (I.D. 1.0 cm; length: 30 cm; the spherical beads are a composite of cross-linked agarose and dextran; average particle size 13 µm; pH stability 1–14), which fractionates molecules in the range between M_r 100 and 7000 was equilibrated with at least 100 cm^3 of degassed Tris-buffer (0.1 mol dm^{-3}, pH 7.5). The column exit was connected to a Meinhard TR-30-K2 concentric glass nebulizer with the minimum length of polyethylene tubing. 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). Arsenic-, selenium- and sulfur-specific detection was achieved with a Thermo Jarrel Ash (Franklin, MA, USA) IRIS HR radial view ICP-atomic emission spectrometer at 189.042 nm (order 178), 196.090 nm (order 172) and 180.731 nm (order 186) respectively. Because of the long retention times and the limited time window in the time-scan mode of the ThermoSPEC/CID software (version 2.10.04), data accumulation was initiated 9.0 min after injection. After chromatographic analysis of the two bile samples from the rabbit experiment, an aliquot of each bile sample (1.0 cm^3) was spiked with 20 µl of a solution of $[(GS)_2AsSe]^-$, prepared as previously reported,[16] and rechromatographed. The dead volume of the column was determined with blue dextran and was 7.5 cm^3.

RESULTS AND DISCUSSION

Using X-ray absorption spectroscopy, we have previously reported on a novel arsenic–selenium compound in bile of rabbits that had been injected with aqueous selenite and arsenite.[15] The similarity between the arsenic and selenium X-ray absorption near-edge spectra of the collected bile samples with those obtained from the synthethic species $[(GS)_2AsSe]^-$ suggested the presence of a structurally similar compound, $[(RS)_2AsSe]^-$ (R being a low molecular weight intracellular thiol). The sulfur donor could not be identified by X-ray absorption spectroscopy because this technique cannot distinguish between sulfur atoms from e.g. cysteine or GSH. Since GSH is the most prevalent intracellular thiol in mammalian hepatocytes,[18] and therefore the most likely sulfur-donor in $[(RS)_2AsSe]^-$, an alternative method must be employed to unequivocally identify the sulfur donor in the bile species.

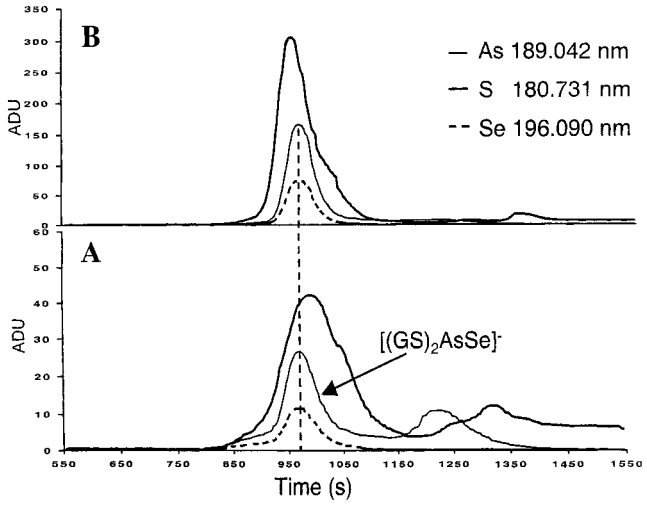

Figure 1. Identification of $[(GS)_2AsSe]^-$ in rabbit bile by SEC (column: Pharmacia Superdex Peptide HR 10/30; temperature: 4 °C; mobile phase: 0.1 mol dm^{-3} Tris buffer pH 7.5; flow rate: 1.0 cm^3 min^{-1}) and arsenic-, selenium- and sulfur-specific detection by ICP-AES (adu: analog to digital units). (**A**) Rabbit bile. (**B**) Rabbit bile spiked with synthethic $[(GS)_2AsSe]^-$; note the different *y*-scale. (The chromatograms obtained from the second animal were similar.)

Today, HPLC coupled on-line to element-specific detectors, such as an ICP mass spectrometer (MS) can be routinely used to identify and quantify trace metalloid compounds in environmental samples.[19–21] Even in the presence of a substantial matrix, the compound of interest can usually be identified by the addition of an internal standard ('spiking') followed by re-chromatography.[19,20] ICP-AES can also be used as an element-specific detector.[22] In addition, its simultaneous multielement-specific detection capability makes it perfectly suited to detect compounds that contain two or more metals/metalloids.[16,17]

In order to identify $[(GS)_2AsSe]^-$ in rabbit bile, we analyzed bile collected from rabbits that had been treated with selenite and arsenite as reported[15] by SEC–ICP-AES. Figure 1a shows the corresponding arsenic-, selenium- and sulfur-specific chromatogram. The bulk of arsenic and selenium eluted simultaneously with a retention time of 970 s, suggesting a single compound that contained these elements. This is in accord with the shape of the arsenic peak (peak height: 26 adu; approximately 60% of total arsenic) and the selenium peak (peak height: 11 adu), which also indicate the presence of a single compound. Conversely, the retention time of the sulfur peak was 985 s; the peak was much broader than the arsenic and selenium peak and also showed a distinct shoulder at the long retention end. These features imply the elution of several sulfur-containing compounds, which is to be expected, since bile usually contains numerous sulfur compounds, such as the taurine conjugates of bile acids (e.g. cholic and chenodeoxycholic acid).[23] Thus, the approximately 15 s difference between the arsenic/selenium peak maximum and the sulfur peak maximum must be attributed to the elution of a sulfur-containing compound that is more abundant in bile than the detected arsenic-, selenium- and sulfur-containing metabolite. The arsenic-specific chromatogram revealed a second, minor arsenic peak with a retention time of 1220 s (peak height: 11 adu; approximately 35% of total arsenic). Based on the retention time obtained for an arsenite standard, we tentatively identify this peak as arsenite. These data are in good accord with previous data, which showed that approximately 40% of arsenic in bile from similarly treated rabbits was present as a decomposition product of $[(GS)_2AsSe]^-$, most likely $(GS)_3As$ (the other decomposition product, α-selenium, was probably retained on the column).[15] Subsequent hydrolysis of $(GS)_3As$ to arsenous acid has been demonstrated under similar chromatographic conditions[24] and could, therefore, explain the presence of free arsenite in the bile samples. The broad sulfur peak with a peak maximum of 1325 s could not be identified (Fig. 1a). Nevertheless, the elution of this sulfur peak indicates the inclusion volume of the employed size-exclusion column and clearly demonstrates that the arsenic-, selenium- and sulfur-containing peak did not elute in the inclusion volume.

Because bile contains large amounts of matrix constituents (*e.g.* human bile contains up to 3% solids, such as bile salts and inorganic matter),[23] we identified the biliary arsenic–selenium compound by the addition of synthetic $[(GS)_2AsSe]^-$ followed by re-chromatography. Figure 1b shows the corresponding arsenic-, selenium- and sulfur-specific chromatogram obtained from the spiked bile sample. A single peak containing arsenic (peak height: 161 adu), selenium (peak height: 73 adu) and sulfur eluted with a retention time of 970 s and was followed by the elution of an extremely broad arsenic peak and a somewhat broad sulfur peak. The net increase in peak intensity of arsenic and selenium after spiking was 135 adu and 62 adu respectively. The quotient of these readings was 2.2 and is comparable to the quotient of 2.3 obtained from the peak intensities of arsenic and selenium in the unspiked bile sample. Based on these data, we identify the arsenic to selenium molar ratio in unspiked bile as 1:1. Because the addition of synthetic $[(GS)_2AsSe]^-$ increased the peak height of the major arsenic- and selenium-containing peak in the unspiked bile sample, we unequivocally identify GSH as the sulfur donor in $[(RS)_2AsSe]^-$ and identify $[(GS)_2AsSe]^-$ as the major metabolite in bile of rabbits that had been treated with selenite and arsenite.

The conjugation of a large variety of exogenous substances, such as polycyclic hydrocarbons, aromatic amines and halogenated phthaleins to GSH *via* a thio-ether linkage followed by the biliary excretion of the conjugate is a well known detoxification pathway in mammals.[23] Since many

GSH conjugates are excreted from hepatocytes to bile *via* ATP-dependent GS-X (X = xenobiotic) export pumps located at the canalicular site of hepatocyte plasma membranes,[25] [(GS)$_2$AsSe]$^-$ may be similarly excreted by these pumps.

CONCLUSION

After treatment of rabbits with selenite and arsenite, an arsenic- and selenium-containing metabolite containing the structural element [(RS)$_2$AsSe]$^-$ was recently detected in rabbit bile by X-ray absorption spectroscopy.[15] In the present work we used an alternative method to unequivocally identify the sulfur donor in this biochemically important metabolite. Analysis of bile samples from rabbits that had been treated with selenite and arsenite by SEC–ICP-AES followed by spiking with synthethic [(GS)$_2$AsSe]$^-$ allowed us to identify GSH as the sulfur donor. Thus, the biliary excretion of [(GS)$_2$AsSe]$^-$ becomes an important excretory pathway when rabbits are simultaneously exposed to selenite and arsenite. Since ICP-AES is available in many laboratories, the method developed can also be used as an alternative method to X-ray absorption spectroscopy to identify [(GS)$_2$AsSe]$^-$ in biological samples, such as plasma and urine. Accordingly, the SEC–ICP-AES method developed will greatly facilitate further studies into the toxicology of arsenite and selenite, which, since the discovery of [(GS)$_2$AsSe]$^-$, should no longer be investigated individually.

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REFERENCES

1. Boyle RW and Jonasson IR. *J. Geochem. Explor.* 1973; **2**: 251.
2. Berrow ML and Ure AM. In *Occurence and Distribution of Selenium*, Ihnat M (ed.). CRC Press: Boca Raton, FL, 1989; 213.
3. Zhu B and Tabatabai MA. *Soil Sci. Soc. Am. J.* 1995; **59**: 1564.
4. Conde JE and Alaejos MS. *Chem. Rev.* 1997; **97**: 1979.
5. Cullen WR and Reimer KJ. *Chem. Rev.* 1989; **89**: 713.
6. Lahermo P, Alfthan G and Wang D. *J. Environ. Pathol. Toxicol. Oncol.* 1998; **17**: 205.
7. Nriagu JO and Pacyna JM. *Nature* 1988; **333**: 134.
8. Yang G, Wang S, Zhou R and Sun S. *Am. J. Clin. Nutr.* 1983; **37**: 872.
9. Finkelman RB, Belkin HE and Zheng B. *Proc. Natl. Acad. Sci. U.S.A.* 1999; **96**: 3427.
10. Nriagu JO and Wong HK. *Nature* 1983; **301**: 55.
11. Nickson R, McArthur J, Burgess W, Ahmed KM, Ravenscroft P and Rahman M. *Nature* 1998; **395**: 338.
12. Keen CL. In *Toxicology of Metals*, Chang LW (ed.). CRC Lewis Publishers: Boca Raton, FL, 1996; 977.
13. Moxon AL. *Science* 1938; **88**: 81.
14. Dubois KP, Moxon AL and Olson OE. *J. Nutr.* 1940; **19**: 477.
15. Gailer J, George GN, Pickering IJ, Prince RC, Ringwald SC, Pemberton JE, Glass RS, Younis HS, DeYoung DW and Aposhian HV. *J. Am. Chem. Soc.* 2000; **122**: 4637.
16. Gailer J, Madden S, Burke MF, Denton MB and Aposhian HV. *Appl. Organomet. Chem.* 2000; **14**: 355.
17. Gailer J, George GN, Pickering IJ, Madden S, Prince RC, Yu EY, Denton MB, Younis HS and Aposhian HV. *Chem. Res. Toxicol.* 2000; **13**: 1135.
18. Bellomo G, Vairetti M, Stivala L, Mirabelli F, Richelmi P and Orrenius S. *Proc. Natl. Acad. Sci. U.S.A.* 1992; **89**: 4412.
19. Byrne AR, Sleikovec Z, Stijve T, Fay L, Gössler W, Gailer J and Irgolic KJ. *Appl. Organomet. Chem.* 1995; **9**: 305.
20. Gailer J, Francesconi KA, Edmonds JS and Irgolic KJ. *Appl. Organomet. Chem.* 1995; **9**: 341.
21. Francesconi KA, Gailer J, Edmonds JS, Gössler W and Irgolic KJ. *Comp. Biochem. Physiol. C* 1999; **122**: 131.
22. Gailer J, Madden S, Cullen WR and Denton MB. *Appl. Organomet. Chem.* 1999; **13**: 837.
23. Smith RL. In *The Excretory Function of Bile*, Smith RL (ed.). Chapman and Hall: London, 1973; 12, 61.
24. Gailer J and Lindner W. *J. Chromatogr. B* 1998; **716**: 83.
25. Ishikawa T. *Trends Biochem. Sci.* 1992; **17**: 463.

