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DETERMINATION OF ^{75}Se-LABELLED SELENITE AND METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE RADIOACTIVITY DETECTION

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SUMMARY

A reversed-phase ion-pair high-performance liquid chromatographic method with on-line radioactivity detection for the simultaneous determination of ^{75}Se-labelled selenite and metabolites has been developed. With this system a good resolution of various radiolabelled selenium complexes can be achieved. The detection limit of the radioactivity detector (signal-to-noise ratio = 3) is 49 pg of selenium (specific activity of 3 GBq/mg selenium) per millilitre of urine or plasma ultrafiltrate. The detector response is independent of both the chemical structure of the selenium complexes and the matrix composition of the samples. This method may serve as a reference system for other high-performance liquid chromatographic systems with less specific and sensitive detectors.

INTRODUCTION

Several important toxic effects of selenium have been described [1,2]. Selenite is considered to be more toxic than organic selenium complexes, such as selenomethionine and selenocysteine [1]. However, selenium is an essential trace element [3], and selenium deficiency has been associated with several diseases [4,5], including an increased risk on certain cancers. In addition, sev-

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eral important beneficial effects of selenite have been reported [6–9]. Selenite reduces the toxic effects of heavy metals such as cadmium and mercury [7,8]. Recently, it has been shown that administration of sodium selenite selectively reduces the nephrotoxicity of the antitumour drug cisplatin without reducing its antitumour activity [9]. The molecular mechanisms of these protective effects of selenite are unknown. It is also not known whether selenite itself or one of its metabolites is responsible for these effects. The chemical formulae of selenite and some possible metabolites are shown in Fig. 1. Studies on the metabolism of selenite and on the molecular mechanisms of its biochemical effects are seriously hampered by the lack of suitable analytical tools for the determination of various selenium compounds in complex biological matrices.

Several high-performance liquid chromatographic (HPLC) methods [10–16] have been developed to study the metabolism of selenite, but their application is limited by several disadvantages. The resolving power of most chromatographic systems appears to be insufficient to achieve a good separation of complexes of selenite with endogenous compounds such as N-acetylcysteine, cysteine and glutathione. In addition, most of the on-line detection methods applied require extensive sample pretreatments, notably fluorimetric detection with pre- or post-column derivatization with 2,3-diaminonaphthalene [10,11], fluorimetric detection with pre-column derivatization with penicillamine–7-fluoro-4-nitrobenz-2,1,3-oxadiazole [12] and inductively coupled plasma atomic emission spectrometric detection [13]. These sample pretreatments are not only laborious, but also destroy the information concerning the number and chemical structures of the selenium compounds present in the sample. HPLC in combination with neutron activation analysis, reported to be suitable for the determination of selenite in concentrations of 10–40 ng/ml in plasma ultrafiltrate [14], has the major disadvantage that detection has to be performed off-line. This detection method is impractical for routine applications, because of its time-consuming and laborious character.

Apparently there still exists a great need for a sensitive and selective analytical method, enabling the determination of selenite and metabolites in biological fluids. A promising approach is the application of HPLC with on-line radioactivity detection. The isotope ^{75}Se is especially suitable for this purpose, since it has a long half-life (2880 h) [17].

Selenite can form compounds that may be cationic, anionic or neutral. Differently charged compounds can be separated on chemically bonded or solvent-

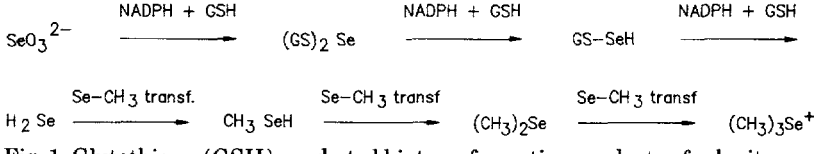

Fig. 1. Glutathione (GSH)-mediated biotransformation products of selenite.

generated ion-exchange columns. The primary aim of the present study was to develop a selective and sensitive HPLC method, with a good resolving power for ^{75}Se-labelled selenium compounds and on-line radioactivity detection, and further to evaluate the applicability of this method to studies on the metabolism and biochemical effects of selenite.

EXPERIMENTAL

Chemicals

^{75}Se-labelled selenite with a specific activity of 3 GBq/mg selenium was obtained from Dupont de Nemours (Dreieich, F.R.G.). The radionuclide ^{75}Se decays with a half-life of 2880 h to stable ^{75}As [17]. It emits various γ-lines, with most of the radioactivity signal between 120 and 480 keV. This allows measurement of ^{75}Se via liquid scintillation counting.

Chemicals for preparing the mobile phase of the chromatographic system were of HPLC grade. Other chemicals were of analytical grade.

Chromatography

The liquid chromatograph consisted of an LKB 2150 HPLC pump and a Rheograde 7125 high-pressure injection valve. Gradient elution was performed with an LKB 2152 LC controller and an LKB 11300 Ultrograd mixer driver (LKB-Pharmacia, Woerden, The Netherlands). Separations were performed on a 100 mm $\times$ 3.0 mm I.D. column, packed with Spherisorb ODS, particle size 5 μm (Chrompack, Middelburg, The Netherlands). Several mobile phases and gradient programmes were evaluated. The flow-rate was 0.25 ml/min and the injection volume 20 μl.

Detection

The detector consisted of a Berthold LB 506 C HPLC monitor and a Bernhard 1. Stadler E.100.1 scintillator pump (Betron Scientific, Rotterdam, The Netherlands). The LB 506 C HPLC monitor contained an LB 506 C radioactivity flow-through monitor with a computer (Motorola 6809 cpu) for data reduction and a personal computer for data handling. Measurements of radioactivity were made with the admixture method. A liquid scintillator, Lumagel (Lumac, Landgraaf, The Netherlands), was aspirated with the scintillator pump and mixed continuously with the HPLC eluate, and the resulting mixture was passed through a flow-through cell, arranged between the cathodes of two photomultipliers operating in coincidence to reduce the background. Cells of different volumes (1.0, 0.5 and 0.2 ml) were tested. Data processing, including peak-area measurements, was performed with the LB 506C HPLC software.

In vitro experiments

^{75}Se-Labelled sodium selenite (1 m*M*) was incubated in distilled water with several thiols in a selenite-to-thiol molar ratio of 1:4 at 4°C for 30 min. The thiols used were glutathione (GSH), N-acetyl-L-cysteine (NAC), L-methionine (METH) and L-cysteine (CYS). HPLC analysis of each reaction mixture was performed immediately after the incubation.

Animal experiments

Male Wistar rats (200 g) were obtained from the Central Institute for the Breeding of Laboratory Animals [Harlan Sprague–Dawley (CPB/HSD), Zeist, The Netherlands], and fed water and standard laboratory chow ad libitum. The rats were treated intravenously with solutions of ^{75}Se-labelled sodium selenite, 2 mg of selenium per kg, in physiological saline. At selected intervals blood and urine were collected. Blood was obtained through heart puncture under diethyl ether anesthesia and immediately centrifuged for 5 min at 1000 *g* to obtain plasma. These plasma samples were immediately ultrafiltered over YMT filters in an Amicon MPS-1 micropartition system (Amicon, Oosterhout, The Netherlands) for 20 min at 2000 *g*. Ultrafiltrates were immediately injected into the HPLC column. Urine samples were obtained under diethyl ether anesthesia, filtered over 0.45-μm Millipore filters (Nihon Millipore Kogyo, Yonezawa, Japan) and stored in liquid nitrogen. Urine samples were analysed within 2 h and were shown to be stable for at least 24 h.

RESULTS AND DISCUSSION

Chromatography

The primary aim of the present study was to develop a selective and sensitive HPLC method, with on-line radioactivity detection, for the separation of biologically important ^{75}Se-labelled selenium complexes and further to evaluate the applicability of this method to studies on the metabolism and on the molecular mechanisms of the biochemical effects of selenite. In order to achieve optimal resolution of selenite–GSH and selenite–NAC complexes, a reversed-phase ion-pair HPLC system was used. The best results were ultimately obtained with a gradient system, consisting of the following two mobile phases: (I) 5 m*M* sodium dodecyl sulphate (SDS), 10 m*M* sodium phosphate buffer (pH 2.6); (II) 5 m*M* SDS, 25% 2-propanol, 60 m*M* sodium phosphate buffer (pH 2.6). The following gradient programme was used: a linear gradient from 100% I to 100% II in 45 min; 10 min at 100% II; a linear gradient from 100% II to 100% I in 5 min; 10 min at 100% I. The chromatographic conditions were evaluated with in vitro incubation mixtures of selenite and several biologically relevant thiols, notably GSH, CYS, METH and NAC. Fig. 2 clearly illustrates the resolving power of this chromatographic system. Within 1 h, various reaction products of selenite could be separated in a single run.

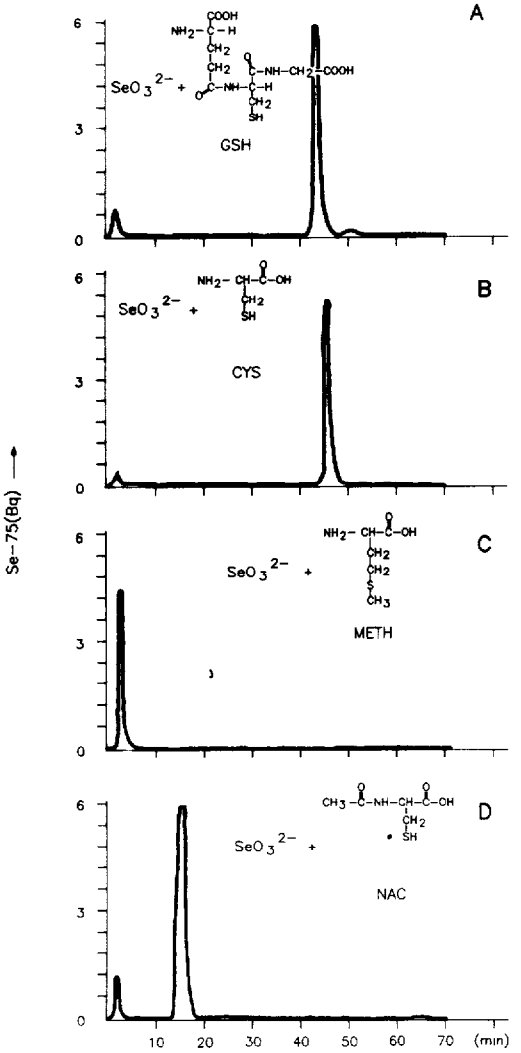

Fig. 2. Radiochromatograms of several mixtures of selenium complexes, obtained after incubation of ^{75}Se-labelled selenite at 4 °C for 30 min with (A) glutathione (GSH), (B) cysteine (CYS), (C) methionine (METH) and (D) N-acetylcysteine (NAC). Incubations were performed at a molar ratio of 1 4 (selenite/thiol). Chromatographic conditions are described in the text.

To study the possibility of reactions between the selenium compounds and constituents of the eluents, the reaction mixtures were diluted (1:1) with water or eluent II. After standing for 1 h at room temperature, radiochromatograms were recorded again. There were no significant differences between the radio-

chromatograms of the two types of mixture. Apparently, the analyses were not significantly disturbed by reactions between selenium complexes and constituents of the mobile phases during the chromatographic run.

The identities of the various selenium compounds, shown in Fig. 2, remain to be established. GSH possesses nucleophilic groups, capable of substituting one or more oxygen atoms in selenite. Incubation of selenite with GSH in an aqueous solution resulted in a mixture of one major and two minor selenium complexes, which could be separated from each other with our HPLC system (Fig. 2). The major complex (retention time 44 min) was spectrometrically identified as selenodiglutathione (GS–Se–SG) as described by Ganther [18]. NAC and CYS may form similar complexes, selenotrisulphides, with selenite. Careful control of pH during the chromatographic run is necessary as selenotrisulphides are relatively unstable, especially in alkaline solutions.

The recovery of selenium from urine, spiked with $1 \cdot 10^{-8}$ *M* ^{75}Se-labelled selenite, was determined as $95 \pm 5\%$ ($n=3$). For plasma ultrafiltrate samples spiked with ^{75}Se-labelled selenite the recovery was also found to be $95 \pm 5\%$ ($n=3$). Recoveries of selenite–thiol incubation mixtures and of urine samples and plasma ultrafiltrate samples of rats treated with selenite were all at least 95%. These results demonstrate that this chromatographic system is suitable for the resolution of various selenium complexes in several matrices.

Detection

The sensitivity of the radiodetector is dependent on the residence time in the measuring cell, the efficiency and background of the detector and the specific activity of the radioactive compound. Basically, two types of measuring cell can be distinguished: cells with solid scintillator beads and flow-through cells designed for liquid scintillation counting. Cells with beads are not suitable for the determination of ^{75}Se-labelled compounds, because they are easily contaminated, owing to adsorption of selenium on the beads. Therefore, and because liquid scintillation measurements of ^{75}Se are more sensitive, the measurements were made with liquid scintillation counting in a flow-through cell. With this method, the possibility of contamination is virtually excluded.

Optimal conditions for sensitivity and resolution were determined by repeated injections of selenite–NAC and selenite–GSH incubation mixtures, using various cell volumes and various discriminator settings. The best results were obtained with a Z-1000-4 flow-through cell with a volume of 1 ml. The optimal conditions are summarized in Table I. The necessity of correction for differences in quenching in the eluents I and II was studied by measuring the radioactivity of ^{75}Se-labelled selenite in both eluents with a liquid scintillation counter. No differences were observed and therefore no quench corrections were necessary.

Under optimal conditions, the efficiency, the detection limit, the linearity of detector response and the reproducibility of the measurements were determined.

The efficiency of the Z-1000-4 flow-through cell was established as $18 \pm 0.3\%$

TABLE I

OPERATING CONDITIONS FOR THE LB 506C ON-LINE RADIOACTIVITY HPLC MONITOR

Dwell time	13 s
Low energy window, upper level	600
Measuring cell	Z-1000-4
Cell volume	1.00 ml
Flow-rate	1.55 ml/min
Efficiency	constant
Scintillator	Lumagel
Scintillator flow	1.3 ml/min
Detection limit	3 Bq

($n=3$) by comparing the measurements of ca. 24 000 Bq ^{75}Se-labelled selenite, with those measured on a germanium–lithium detector, calibrated with a solid disc point source (QCD.1) from Amersham. The efficiency of the Berthold HPLC detector was found to be lower than that of a standard liquid scintillation counter (efficiency 51%), owing to the smaller volume, the geometry and the composition of the tube of the flow-through cell.

The absolute detection limit, defined as a signal-to-noise ratio of 3, was found to be as low as 3 Bq, i.e. 1 pg of selenium (specific activity 3 GBq/mg). This allowed the determination of selenite concentrations of 49 pg/ml in urine and plasma ultrafiltrate. This detection limit is much lower than those of other on-line HPLC detectors used for the determination of selenium (Table II). In principle, the detection limit may be improved by using selenium with a higher specific activity or by off-line counting in a liquid scintillation counter.

Calibration curves of standard solutions of known amounts of selenite in physiological saline were found to be linear in the range $1.65 \cdot 10^{-9}$ M–$6.2 \cdot 10^{-6}$ M corresponding to 8–30 000 Bq ($r=0.997$, $n=15$). The lower detection limit was determined by the background and the counting time, the upper by the dead time. Standard solutions of $1 \cdot 10^{-8}$ M selenite in 0.9% sodium chloride could be determined with a precision of $\pm 2.2\%$ (standard error of the mean; $n=5$).

It is important to note that the response of the radioactivity detector described in this paper is independent of the chemical structure and the valency state of the selenium compounds, in contrast to the response obtained with other detectors, such as the inductively coupled plasma atomic emission spectrometric detector [13] and fluorimetric detectors [10–12]. Obviously, this is an important advantage when one is interested in the simultaneous quantitative determination of different selenium compounds. The response of the radioactivity detector is also independent of the matrix. This is again in contrast to the response obtained with other detectors, such as the fluorimetric detector

TABLE II

COMPARISON OF THE DETECTION LIMITS OF SEVERAL ON-LINE HPLC DETECTORS FOR THE DETERMINATION OF SELENIUM

HPLC detection	Sample	Detection limit[a]		Ref.
		ng/ml	ng	
Pre-column derivatization with 2,3-diaminonaphthalene (DAN) (spectrofluorimetric)	Serum		0.05	10
Post-column derivatization with DAN (spectrofluorimetric)	Water	0.17		11
Inductively coupled plasma atomic emission spectrometry	Water	7000		13
Pre-column derivatization with penicillamine–7-fluoro-4-nitrobenz-2,1,3-oxadiazole (spectrofluorimetric)	Serum	5		12
^{75}Se Radioactivity (on-line)	Urine, plasma ultrafiltrate	0.049[b]	0.001	This study

[a]Concentration or absolute amount of selenium injected (signal-to-noise ratio = 3).
[b]For a specific activity of 3041 MBq/mg selenium: this detection limit is independent of both the chemical structures of the selenium complexes and the matrix composition.

[10–12], which suffers from considerable background signal originating from biological matrices. With our radioactivity detector, no destructive sample pretreatment is required: therefore, the chemical structures of the selenium compounds to be analysed remain intact, in contrast with the fluorimetric detector. Our HPLC method requires no or only minor sample pretreatment: plasma ultrafiltrate samples can directly be injected into the column and urine samples require only a simple filtration step prior to injection. Other HPLC methods [10–12,14] require extensive sample pretreatments, which are laborious and time-consuming.

Animal studies

Plasma ultrafiltrate samples and urine samples, obtained from male Wistar rats 10 min or 2 h after treatment with sodium selenite (2 mg of selenium per kg), were analysed (Fig. 3A). In the plasma ultrafiltrate samples one radiolabelled selenium compound was detected: this compound (retention time 2.5 min) was identified as unchanged selenite on the basis of co-elution with authentic reference compound.

In urine samples, several radiolabelled selenium compounds were detected; one was selenite (Fig. 3B), but the chemical structures of the others are unknown. Trimethylselenonium, previously identified in the urine of mice treated

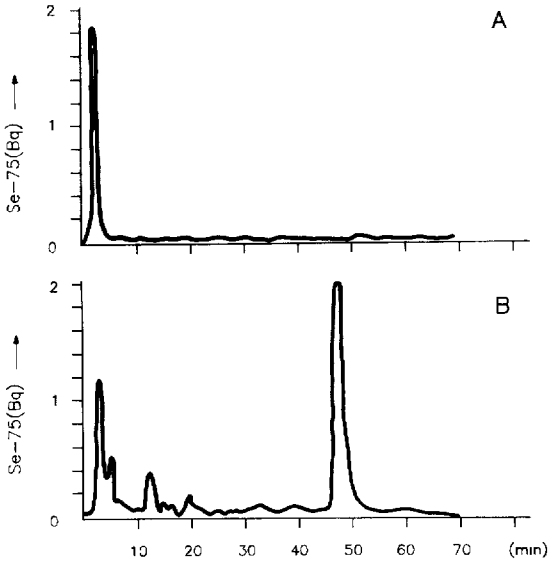

Fig. 3. Radiochromatograms of plasma ultrafiltrate (A) and urine samples (B) from Wistar rats obtained 30 min (A) and 2 h (B) after intravenous treatment with selenite (2 mg/kg). Chromatographic conditions are described in the text. In the chromatogram of the plasma sample (A), one selenium complex was observed. This complex (retention time 2.5 min) was identified as selenite In the urine chromatogram (B), several selenium complexes were observed, including selenite.

with sodium selenite [16], might also be present in the urine of rats treated with selenite.

The HPLC method described in this paper enables the determination of several selenium metabolites in urine in a single run. Blotcky et al. [14] have described an HPLC method for the determination of selenium metabolites in urine. Their method, however, requires two chromatographic runs and several different sample pretreatments for the analysis of one urine sample consisting of a mixture of selenium compounds. The HPLC methods described by Kraus et al. [15] and Hoffman and McConnell [16] is only suitable for the separation and determination of cationic selenium compounds.

CONCLUSIONS

The resolving power of the HPLC method described in this paper enables the determination of various selenium compounds in one run. HPLC with on-line radioactivity detection is superior to HPLC with other detectors for selenium compounds, in terms of selectivity and sensitivity. The signal response of the radioactivity detector, which measures ^{75}Se radioactivity, is independent of both the chemical structure of the selenium compounds and the matrix com-

position of the samples. Therefore, this method is an excellent reference method for other, less specific and sensitive methods for the detection of selenium compounds. Animal experiments demonstrated that our HPLC method might be a powerful research tool in studies of the metabolism of selenite and might open new perspectives in elucidating the molecular mechanisms of the biochemical effects of selenite and other selenium compounds.

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