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Column-switching system for selenium speciation by coupling reversed-phase and ion-exchange high-performance liquid chromatography with microwave-assisted digestion–hydride generation–atomic fluorescence spectrometry

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Abstract

Speciation of selenocysteine (SeCys), selenomethionine (SeMet), selenoethionine (SeET), selenite ($Se^{(IV)}$) and selenate ($Se^{(VI)}$) has been accomplished using high-performance liquid chromatography, with the aid of an anion exchange column and a reversed-phase column, both connected through a six-port switching valve. On-line microwave-assisted digestion and hydride generation steps were performed prior to the atomic fluorescence detection. The elution of the seleno amino acids was accomplished in the reversed-phased column using water as mobile phase. Selenite and selenate were separated in the anion exchange column, using gradient elution with an acetate buffer. The separation of the five selenium compounds took place in 15 min. The detection limits obtained ranged between 0.6 and 0.9 μg l^{-1}. Values of $r>0.998$ were obtained for linear fit graphs. A commercial available urine sample was analyzed, in which SeCys and $Se^{(IV)}$ were quantified. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Column switching; Atomic fluorescence detection; Detection, LC; Organoselenium compounds; Selenium; Amino acids

1. Introduction

Speciation of selenium is a difficult but necessary task, due to the simultaneous essential and toxic character of this element. The concentration range in which selenium intake is estimated as adequate is narrow, between 0.1 and 0.3 mg kg^{-1} (body mass) and outside this range deficiency or toxicity occurs [1,2]. The nutritional, bioavailability, toxicity and cancer chemoprotective activity of selenium have been found to be species-dependent [3]. The selenium consumed by humans in foods and supplements exists in a number of different organic and inorganic forms, which include selenomethionine, selenocysteine, selenite and selenate. The way in which they are handled in the body can be studied by observing the urinary excretions [4]. Levels of $Se^{(IV)}$ between 98 and 423 μg l^{-1} have been reported in non-spiked urine samples, $Se^{(VI)}$ not being detected [5]. Other selenium compounds found in biological tissues include seleno amino acids [6,7] and selenium-containing proteins [8–10].

In speciation studies, the distinction between selenite and selenate has been performed by hydride generation (HG) considering that $Se^{(IV)}$ forms a

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volatile hydride, whereas $Se^{(VI)}$ does not. For the quantification of $Se^{(VI)}$, its reduction to $Se^{(IV)}$ has been achieved by means of microwave-assisted digestion (MAD) [11], the use of a reducing agent with heating [12] or UV irradiation [13].

High-performance liquid chromatography (HPLC) has been coupled to specific atomic detectors for the speciation of non-volatile selenium compounds. The separation of the inorganic selenium species, selenite and selenate, has been performed using anion-exchange stationary phases [14] or a reversed-phase column with ion-pair agents [15,16]. Ion-exchange resins have been also used for the preconcentration of inorganic selenium in aqueous samples [17,18].

The speciation of seleno amino acids has been achieved either by HPLC or gas chromatography (GC). This latter technique is not widely extended, because it involves necessarily a pre-derivatization step in order to produce volatile derivatives, and provides poor detection limits [19]. Optionally, pre-derivatization can also be used before HPLC separation to convert the seleno amino acids into fluorescent derivatives to improve the detection limits [20,21].

Simultaneous determination of inorganic selenium and seleno amino acids is a more complicated task, due to the inherent differences in physicochemical characteristics of these species. HPLC using phosphate buffer as mobile phase and a stationary phase consisting of octadecyl and anion exchange groups (ODS/amino) [22] has been reported for this purpose, although in our experience, this packing lacks stability. Another interesting approach is based on the use of vesicular mobile phases of didodecyldimethylammonium bromide (DDAB) in a C_{18} reversed-phase column modified with DDAB molecules. However, this approach produces the overlapping of selenite with the amino acids when analyzing urine samples, due to the ionic strength of the sample [23]. Other authors have successfully used a strong anion-exchange column for organic and inorganic selenium speciation, with a chromatographic system that needed more than 30 min for the separation of four selenium compounds [24]. Recently, an attempt to separate 23 selenium species was undertaken, using perfluorinated carboxylic acid ion-pairing agents, but failed in the separation of selenite and selenate [25].

In this work, we attempt the separation of selenite, selenate, selenocysteine, selemethionine and selenoethione using a reversed-phase column for the separation of the three seleno amino acids and an ion-exchange column for the separation of the inorganic compounds. A column switching valve allowed the selection of the mobile phase and the connection of the outlet of the columns to the detector. Different column setups and mobile phases were tested during the experiments. Due to the fact that the levels of selenium compounds in environmental samples are usually low, sensitive detection was achieved by atomic fluorescence spectrometry (AFS), which can provide detection limits lower than the $\mu g\ l^{-1}$ [26,27]. On-line MAD and HG were introduced as intermediate steps to produce volatile hydrides before detection. Various parameters affecting coupled HPLC–MAD–HG–AFS were optimized, including the column selection and the system setup.

2. Experimental

2.1. Instrumentation

The chromatographic system used consisted of Varian 9012 quaternary pump with a Rheodyne 7125 six-port valve for sample injection, fitted with a 200-μl loop. For the separation of the amino acids the following reversed-phase columns were tried: Nucleosil C_{18} (150×4 mm, 5 μm) and (250×4 mm, 5 μm) were purchased from Supelco (Sigma–Aldrich, Gillingham, UK) and Teknocroma (Barcelona, Spain) and Tracer Excel (150×4.6 mm, 5 μm) purchased also from Teknocroma. For the separation of the inorganic selenium, two anion-exchange guard columns were tried: SAX (20×4 mm, 5 μm) from Supelco and PRP X-100 (20×4.1 mm, 10 μm) from Hamilton (Reno, NV, USA). A Supelco six-port switching valve was used for the connection of the columns. Solutions added at the HG step were pumped by means of two Gilson Minipulse-3 peristaltic pumps. A Moulinex Y 55 domestic microwave was used for the MAD. Argon was used as carrier gas, being dried with a hygroscopic membrane (Perma Pure Products, Farmingdale, NJ, USA). The detection of the selenium hydrides was performed

using an atomic fluorescence detector PSA Excalibur 10.33 (PS Analytical, Kent, UK). A boosted-discharge hollow cathode lamp (Photron, Victoria, Australia) was used as Se radiation source. The analog output signal was treated by a computer equipped with chromatographic software.

2.2. Reagents

Stock solutions of 1000 mg l^{-1} (as Se) were prepared for Na_2SeO_3, Na_2SeO_4, seleno-DL-cysteine, seleno-DL-methionine and seleno-DL-ethionine, which were purchased from Sigma (Gillingham, UK) and Aldrich (Milwaukee, WI, USA). Stock solutions were stored in the dark at 4°C. Diluted working solutions were prepared daily. Water obtained from a Milli-Q Gradient System (Millipore, Bedford, MA, USA) was used to prepared the different aqueous solutions. Concentrated HBr, HNO_3, $HClO_4$ (Merck, Darmstadt, Germany), $KBrO_3$, $NaBH_4$, KCH_3COO (Panreac, Barcelona, Spain) were of analytical grade. C_{18} cartridges (600 mg) for sample clean-up were obtained from Waters.

2.3. Selenium speciation procedure

A schematic diagram of the instrumental coupling is depicted in Fig. 1. In position 1 of the switching valve, 200-μl sample of solution were injected into the anion-exchange column, using water as mobile phase, at a flow-rate of 1.2 ml min^{-1}. Selenite and selenate are retained in the anion-exchange column, whereas the three seleno amino acids eluted at the dead volume and entered the reversed-phase column. At the column outlet a redox reagent was added on-line, consisting of 15 m*M* $KBrO_3$ (flow-rate, 0.6 ml min^{-1}) and 47% HBr (flow-rate, 1.2 ml min^{-1}). Destruction of the organic portion of the seleno amino acids and reduction to selenite ($Se^{(IV)}$) was achieved in a 6-m long PTFE loop placed inside a domestic microwave oven operated at 150 W. An ice-bath cooled loop was placed after the microwave treatment. Hydride generation was carried out by adding 1 ml min^{-1} of 1.5% (w/v) $NaBH_4$ in 1% (w/v) NaOH. An argon flow of 100 ml min^{-1} carried the hydrides to the gas–liquid separator, and a second one of 200 ml min^{-1} transported them to the AFS system. A hydrogen flow of 60 ml min^{-1} was added at the gas–liquid separator in order to maintain a hydrogen diffusion flame. The primary current of the boosted-discharge hollow cathode lamp was set at 20.0 mA and the boosted current at 25.0 mA.

After the detection of the three seleno amino acids, the column switching valve is changed to position 2, and the mobile phase changed to 0.4% (w/v) potassium acetate. The elution order of the inorganic compounds is selenite followed by selenate. After the detection of the last peak, the anion exchange column is cleaned during 3 min with 0.01% (v/v) HNO_3 and another 3 min with water. Then the switching valve is changed to position 1 for the next analysis.

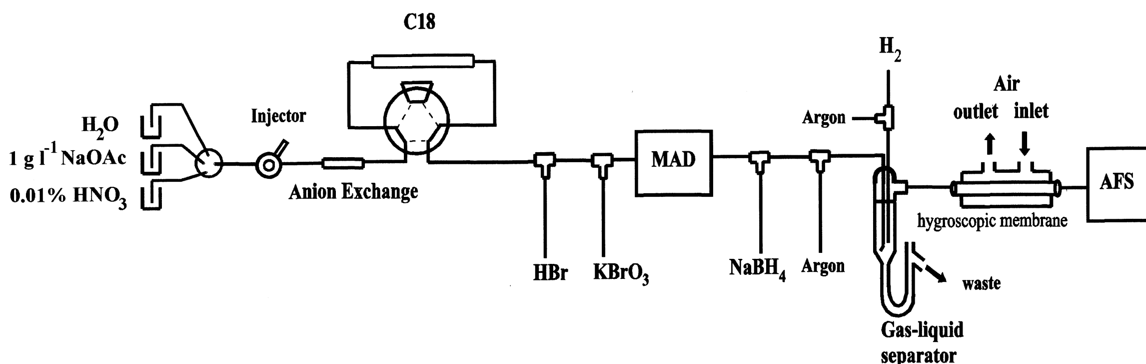

Fig. 1. Scheme of coupled HPLC–MAD–HG–AFS.

2.4. Total selenium analysis

The lyophilized urine sample was dissolved with 4 ml of HNO_3 and transferred to a Kjeldahl flask. After 1 h of predigestion at room temperature, 1 ml of $HClO_4$ was added and heated until the appearance of white fumes. The sample was evaporated almost to dryness, transferred to a volumetric flask to a final volume of 10 ml with water. Total selenium determinations were performed by flow injection (FI)–MAD–HG–AFS, using a manual valve for sample introduction.

3. Results and discussion

Two chromatographic approaches were studied separately: one for the separation of the seleno amino acids by a reversed-phase mechanism, and a second one for the separation of selenite and selenate by anion exchange chromatography. Afterwards, both columns were connected by a switching valve, placing the anion exchange column before the reversed-phase one.

3.1. Reversed-phase column selection

The separation of the seleno amino acids was attempted using a reversed-phase column assuming a partition mechanism between the stationary phase and the alkyl chain of the analytes.

Two Nucleosil C_{18} columns (4 mm I.D., 5 μm particle size), 150 and 250 mm long, were tried, as well as a Tracer Excel column (150×4.6 mm I.D., 5 μm particle size). Water was employed as mobile phase for the three columns, at a flow-rate of 1.0 ml min^{-1}. The seleno amino acids were eluted in the following order: selenocysteine (SeCys)<selenomethionine (SeMet)<selenoethionine (SeEt). Retention times are summarized in Table 1. The addition of methanol to the mobile phase to increase the eluent strength of the mobile phase was discarded, due to the distortion that it produced in the baseline of the AFS signal. The Nucleosil columns showed lack of reproducibility in the results, when using different commercial stock columns. Hence, the Tracer Excel column was chosen for the following experiments. Retention times were shortened increasing the flow-rate to 1.2 ml min^{-1}.

Table 1
Retention times (min) for selenium compounds using reversed-phase columns

Column	SeCys	SeMet	SeEt
Nucleosil (250×4.1 mm, 5 μm)[a]	2.5	5.0	11.1
Nucleosil (150×4.1 mm, 5 μm)[a]	2.1	3.4	6.5
Excel (150×4.6 mm, 5 μm)[a]	2.4	5.2	12.5
Excel (150×4.6 mm, 5 μm)[b]	2.2	4.6	10.8

[a] Flow-rate, 1.0 ml min^{-1}
[b] Flow-rate, 1.2 ml min^{-1}

3.2. Anion-exchange column selection

In order to obtain an adequate separation of the studied selenium species, the anion-exchange column, placed before the reversed-phase column, has to fulfil two conditions: firstly, it must not interact with the seleno amino acids, which should be eluted in the solvent front. This has to be so, in order not to interfere with the separation mechanism of the reversed-phase column placed next. Secondly, the inorganic selenium species must be completely retained in the anion-exchange column using water as mobile phase while the selenoaminoacids are separated in the C_{18} column. Once the three seleno amino acids have been detected, the inorganic species are eluted from the anion exchange column by changing the water to another mobile phase.

In a previous work [26] we reported the separation of selenite and selenate using a Hamilton PRP X-100 column (250×4.1 mm, 10 μm) and potassium acetate and potassium sulphate buffers as mobile phases. The elution of the inorganic selenium species was 15 min. This time, added to the 10 min needed at the reversed-phase column, yields a total time for the analysis of 25 min. In order to minimize the time for chromatographic separation of selenite and selenate, two guard columns were considered: the polymeric-based Hamilton PRP-X100 guard column (20×4.1 mm, 10 μm) and the silica-based Supelco SAX (20×4.6 mm, 5 μm). Using the first one, SeCys and SeEt were partially retained and resulted in broad peaks. Moreover, SeMet was completely retained. Better results were obtained with the SAX guard column. In this second case, the three seleno

amino acids eluted at the dead volume of the column with water as mobile phase, and no distortion of the peaks was observed, and the two inorganic compounds were not eluted after 10 min. A suitable mobile phase for the elution of selenite and selenate was then investigated for the SAX guard column. The two species were eluted using 2 g l^{-1} potassium acetate as mobile phase. A progressive decrease in the retention times for $Se^{(IV)}$ and selenate ($Se^{(VI)}$) was observed after prolonged use, until both peaks overlapped. This drawback was overcome by including a regeneration cycle after each chromatographic run, in order to restore the efficiency of the column. The process consisted of pumping a 0.01% (v/v) HNO_3 solution through the column during 3 min and then flushing with water for another 3 min. A gradient elution was then optimized for selenite and selenate separation. First, by pumping 0.4 g l^{-1}acetate, selenite was eluted. Then, the salt content was increased to 1 g l^{-1} acetate for the elution of selenate. The retention time for selenite was 3.1 min and for selenate 5.5 min.

3.3. Column switching valve

The column switching valve allows two configurations of the system. In the first position of the valve, water, used as mobile phase, passes first through the anion-exchange column and then through the reversed-phase column. The sample is then injected. Selenite and selenate are retained in the anion-exchange column, but the seleno amino acids, which eluted in the dead volume, are transported to the reversed-phase column. In this second column, they are separated and the flow of the eluent is carried on-line to the microwave treatment and hydride generation steps for the final detection by AFS. Once they have been detected, the switching valve is manually rotated to the second position. In this new configuration the reversed-phase column remains in a by-pass, and the mobile phase is pumped only through the anion exchange column. The mobile phase is then changed from water to acetate, following the gradient already described, allowing the elution and detection of selenite and selenate. After the regeneration of the anion exchange column, the valve is turned to the initial position and a new analysis can be performed. The total time needed for the complete chromatographic separation of the studied selenium species was 15 min. Retention times for SeCys, SeMet, SeEt, $Se^{(IV)}$ and $Se^{(VI)}$ were 2.3, 4.6, 10.6, 12.7 and 14.5 min, respectively. A chromatogram obtained with coupled HPLC–MAD–HG–AFS for 10 μg l^{-1} (as Se) of each compounds is depicted in Fig. 2.

3.4. Features of the method

Linear ranges (n=7) for the five selenium compounds were calculated for concentrations ranges between 1 and 50 μg l^{-1}. In all cases linear regression coefficients resulted in values of $r>0.998$. Repeatability, calculated after five consecutive injections of either 5 or 50 μg l^{-1}solution, resulted in values between 3 and 7% RSD. Detection limits, calculated as three times the standard deviation at the origin of the calibration graph, divided by the sensitivity, were 0.6, 0.8, 0.9, 0.8 and 0.6 μg l^{-1} for SeCys, SeMet, SeEt, $Se^{(IV)}$ and $Se^{(VI)}$, respectively.

3.5. Analysis of urine samples

A lyophilized urine sample Seronorm Trace Elements Urine Lot 403125 (Nycomed, Oslo, Norway) was analyzed with the proposed instrumental coupling. The sample was reconstituted adding 5 ml of distilled water and letting it stand during 30 min. Due to the organic content of the sample, that could affect the C_{18} column, it was cleaned using a C_{18} Sep Cartridge 600 mg. A previous experience was performed to evaluate if there were losses of the studied seleno compounds in the cartridge. Five ml of 10 μg l^{-1} (as Se) standard solution containing the five studied species were passed through the cartridge. Water was added for their elution and the volume of elution was investigated, which resulted in a value of 5 ml. Selenite and selenate were completely eluted in the first 2 ml, and the three seleno amino acids in the following 3 ml. The recoveries obtained were between 94 and 97% for the organic species and the inorganic ones, thus indicating the feasibility of the clean-up procedure, as has been previously reported [25]. For the analysis of the urine sample, 5 ml of sample followed by 5 ml of water were passed

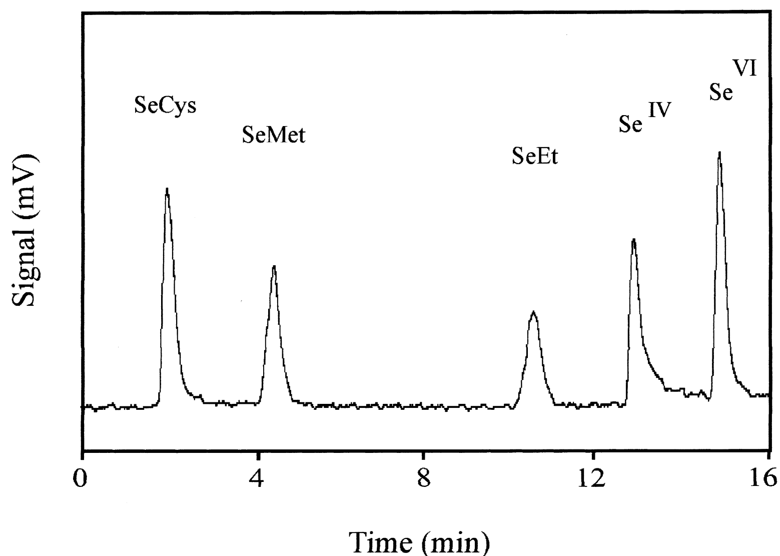

Fig. 2. HPLC–MAD–HG–AFS trace for selenium standards, each at 10 μg l^{-1} (as Se).

through the cartridge. A chromatogram corresponding to the analysis of one sample is depicted in Fig. 3. The results of three sample analyses allowed the quantification of SeCys (2.6±0.4 μg l^{-1}) and $Se^{(IV)}$ (1.1±0.2 μg l^{-1}). The urine sample was spiked before the clean-up step at 5 μg l^{-1} level and the recoveries for the selenium species ranged between 91 and 95%. A possible peak corresponding to SeEt was also detected, although its concentration was too low for its quantification. Because it is an artificial reagent and that it is not a usual urinary constituent, its identity is not guaranteed. Total selenium determinations were made replacing the HPLC separation by a manual valve.The total

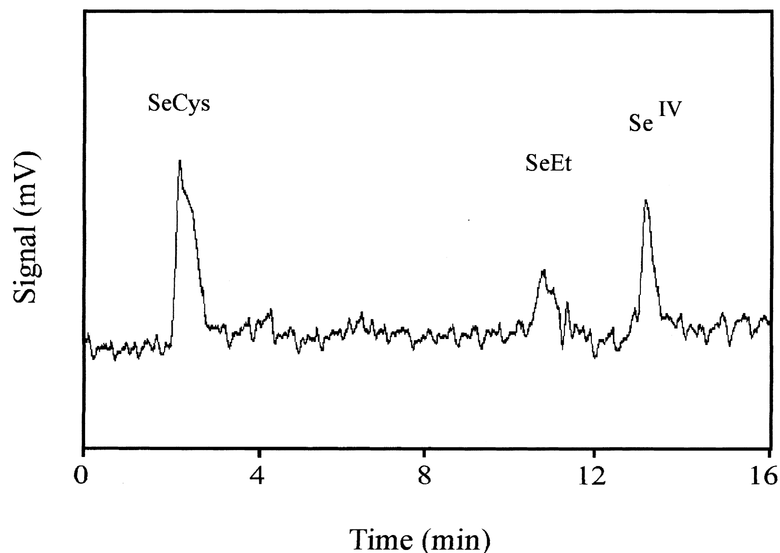

Fig. 3. HPLC–MAD–HG–AFS trace for selenium species in a lyophilized human urine.

selenium content of the lyophilized urine, obtained after acid digestion of three separated samples, was 25.4±0.8 μg l^{-1}. This value was comparable to 22.6±1.0 μg l^{-1}, obtained by direct analysis of the sample without digestion. The discrepancy between the total value and the results obtained after speciation could be attributed to other organic compounds which could be retained in the columns.

4. Conclusions

The chromatographic approach based on the use of two columns (reversed-phase and anion exchange) connected by a switching valves provides a fast system for the separation of SeCys, SeMet, SeEt, $Se^{(IV)}$ and $Se^{(VI)}$ in 15 min. The AFS detection provides also good sensitivity, which is rather important in the speciation studies, in which low detection limits are usually required. This hyphenated technique provided also good repeatability, which makes coupled HPLC–MAD–HG–AFS an interesting possibility for the study of environmental samples.

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