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Quantitative determination of selenium metabolites in human urine by LC-DRC-ICP-MS†

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Two selenium metabolites, *Se*-methylseleno-*N*-acetylgalactosamine (SeGal-N-Ac) and *Se*-methylselenogalactosamine (SeGal-NH_2), were quantified in human urine by LC-DRC-ICP-MS. Urine samples were analysed after 1 + 1 dilution in a reversed phase chromatographic system using an eluent consisting of 200 mM ammonium acetate and 5% methanol with a pH of 9.25 and quantified by standard addition. Samples were collected from 8 volunteers before and after 5 days ingestion of 100 μg Se day^{-1} in form of selenized yeast. The average concentration of (SeGal-NH_2) before and after selenium intake was 1.4 and 1.9 μg Se L^{-1}, respectively, while the average concentration of Se-Gal-N-Ac increased from 2.6 to 11.6 μg Se L^{-1} before and after selenium consumption. Detection limits calculated on basis of three times the standard deviation on peak areas of 2 μg Se L^{-1} solutions were 0.1 μg Se L^{-1} for SeGal-NH_2 and 0.2 μg Se L^{-1} for SeGal-N-Ac based on peak areas and monitoring ^{80}Se. The precision expressed as the relative standard deviation ($n = 6$) at the 2 μg Se L^{-1} level was 3.1 and 1.7% for SeGal-NH_2 and SeGal-N-Ac, respectively, while the corresponding values were 1.0 and 0.7% at the 10 μg Se L^{-1} level. Linearity in urine matrix was examined in the range 0.5–100 μg Se L^{-1} and correlation coefficients better than 0.999 were obtained. As the cationic compound SeGal-NH_3^+ may be confounded with the trimethylselenonium ion ($TMSe^+$), urine samples were also analysed in a cation exchange chromatographic system in which SeGal-NH_3^+ was separated from the trimethylselenonium ion. None of the samples contained $TMSe^+$ in detectable amounts. Three sample introduction systems were compared—a microconcentric nebuliser in combination with a cyclonic spray chamber (MCN), a direct injection nebuliser (DIN) and an ultrasonic nebuliser (USN). The MCN was most suitable for this purpose.

Introduction

Selenium is an essential trace element and associated with several adverse health effects if the organism is depleted of the element.[1] Especially the possible cancer protective effect of selenium has attracted attention to the element in recent years.[2] The cancer protective effect was more pronounced in groups with low selenium levels in plasma. Hence, supplementation with selenium of populations living in areas deficient in selenium, *e.g.* Europe, could be worth consideration. However, selenium has a narrow therapeutic index and the metabolism of selenium is still not totally elucidated. This will be necessary prior to raising the recommended daily intake of selenium.

A general and widely accepted model of selenium metabolism was described by Ganther[3] and Ip.[4] According to this model the general excretion route of selenium was supposed to be successive methylation of hydrogen selenide to the trimethylselenonium ion ($TMSe^+$) that is excreted in the urine. This model, however, was based on experiments with rats supplemented with toxic doses of selenium.

When the hyphenated analytical techniques with element specific detection were introduced, several reports on selenium speciation analysis in biological material appeared. Reviews on selenium speciation analysis have been given by Lobinski *et al.*[5] and Uden.[6] In addition to $TMSe^+$, several other selenium compounds were reported to be present in urine based on co-elution with available standards.

When the analytical challenge changed from separation and detection of selenium compounds by ICP-MS to identification by soft ionisation mass spectrometry, the picture changed. The first identified selenium compounds in human urine by electrospray ionisation mass spectrometry (ESI-MS) were selenomethionine and selenocystamine.[7] The major metabolite of urine was first identified by Ogra *et al.*[8] in rat urine and shortly after in human urine.[9] This metabolite appeared to be a selenosugar, *Se*-methylseleno-*N*-acetylgalactosamine (SeGal-N-Ac). Shortly afterwards, the corresponding glucose form of the sugar, *Se*-methylseleno-*N*-acetylglucosamine was identified by ESI-MS as a metabolite in urine with a high content of selenium[10] and the de-acetylated form, *Se*-methylselenogalactosamine (SeGal-NH_2) was identified as a common metabolite in basal human urine.[10] A few minor metabolites in human urine have not yet been identified, but $TMSe^+$ has until now not been identified by ESI-MS. Several previous investigations have dealt with the quantitative determination of $TMSe^+$ in human urine; a critical review on these determinations and selenium metabolites in urine in general has recently been given by Francesconi and Pannier.[11]

As the recently identified metabolite SeGal-NH_2 is a cationic compound at physiological pH, it could raise the question if this metabolite previously has been mistaken for $TMSe^+$.

As part of a larger project with the ultimate aim of elucidating the human metabolism of selenium, the purpose of this work was to quantify the identified selenosugars and $TMSe^+$ in

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human urine with reference to future experiments on metabolism of different selenium compounds.

Experimental

Apparatus

The HPLC system was a G1376A capillary pump, a G1313A autosampler and a G1379A degasser controlled by ChemStation software, all from the Agilent 1100 series (Agilent Technologies, Waldbron, Germany).

The flow rate was 500 μL min^{-1} when the 4 mm i.d. column was used, 200 μL min^{-1} when 2 mm i.d. columns were used, and 50 μL min^{-1} when 1 mm i.d., columns were used. The corresponding sample injection volumes were 50 μL, 12 μL and 3 μL, respectively.

Reversed phase chromatography: The columns were two Luna C18(2), 100 mm × 2 mm (or 1 mm) i.d., 3 μm particle size in series (Phenomenex, Aschaltenburg, Germany).

Cation exchange chromatography: The column was a Dionex IonPac CS5 250 × 4 mm i.d. and a CG5 50 × 4 mm i.d., 9 μm particle size (Dionex, Sunnyvale, CA, USA). The ICP-MS instrument was a PE SCIEX Elan DRC-e (PerkinElmer, Norwalk, CT, USA).

The sample introduction systems were a MicroMist AR30-1-F02 glass concentric nebulizer and a cyclonic spraychamber (Glass Expansion, Romainmotier, Switzerland); a direct injection nebuliser that has previously been described[12] or a U6000 AT^{+} ultrasonic nebuliser (Cetac Technologies Inc., Omaha, NB, USA).

Sampler and skimmer cones were of platinum. The plasma and auxiliary argon flow rates were 15 and 1.1 L min^{-1}, respectively. The nebulisation argon gas flow rate was 0.95 L min^{-1} for the microconcentric nebuliser and 0.2 L min^{-1} for the direct injection nebuliser. The data acquisition parameters were: dwell time: 300 ms, sweeps per reading: 1, readings per replicate: 750. The ^{78}Se, ^{80}Se and ^{82}Se isotopes were monitored.

The DRC was optimised to the following parameters: RPa: 0, RPq: 0.25, cell gas: 0.45 mL min^{-1}, QRO: −6.5 V, CRO: −1.3 V, CPV: −16 V. The cell gas was methane (Air Liquide, Malmö, Sweden).

To achieve maximum sensitivity, the instrument was optimised with respect to the nebuliser gas flow rate, ion lens voltage and rf power when aspirating each eluent spiked with selenite. Peak areas were calculated by TotalChrom (Perkin Elmer)

Reagents

All reagents were of analytical-reagent grade. Purified water, obtained from a Milli-Q de-ionisation unit (Millipore, Bedford, MA, USA) was used throughout.

Stock standard solutions of 10 mg Se L^{-1} were prepared in water from *Se*-methylseleno-*N*-acetylgalactosamine (synthesized at Dept. of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences), *Se*-methylselenogalactosamine (synthesized according to ref. 10) and trimethylselenonium iodide (synthesized according to ref. 13). The stock standard solutions were standardized against a 1.001 g Se L^{-1} PE pure atomic spectroscopy standard (PerkinElmer). Working standards were prepared daily in water.

Eluent for reversed phase chromatography: 200 mM ammonium acetate (Sigma-Aldrich Chemicals, Steinheim , Germany) +5% methanol (VWR international Aps., Albertslund, Denmark), pH adjusted to 9.25 with ammonia (Merck, Darmstadt, Germany).

Eluent for cation exchange chromatography: 10 mM oxalic acid (Sigma), 20 mM potassium sulfate (Merck), and 2% methanol, pH 2.5.

Urine samples

Urine samples were collected from 8 adult volunteers, before and after consumption of tablets containing selenized yeast (Selenoprecise, PharmaNord, Vejle, Denmark). Each individual consumed 100 μg Se for 4 days followed by 200 μg Se on the fifth day. Urine samples were delivered within three hours after selenium consumption on the last day.

Urine samples were stored at −18 °C until analysis. Samples were filtered through a 0.45 μm cellulose acetate filter (Frisenette aps., Ebeltoft, Denmark) prior to analysis, diluted 1 + 1 with water and quantified by means of three standard additions.

The reference material Seronorm™ Trace Elements in Urine (Sero AS, Billingstad, Norway, Lot No. 2525) was analysed for the selenosugars.

Results and discussion

Comparison of sample introduction systems

It has previously been shown that the ultrasonic nebuliser (USN) shows better sensitivity compared to the cross-flow nebuliser for some selenium compounds. However, the sensitivity was dependent of the compound structure.[14,15] As the structures of the selenosugars are very similar it was examined if ultrasonic nebulisation resulted in equal and improved sensitivity of these sugars. Flow injection of the selenosugars in the eluent for reversed phase chromatography showed that the sensitivity of SeGal-NH_2 was more than three times better than for SeGal-N-Ac. Furthermore, a large wash out time of the nebuliser was observed resulting in tailing of the FIA peaks. The signal did not reach the baseline within 3 min of wash out. Hence, this sample introduction system was not suitable for quantification by chromatography.

When comparing the direct injection nebuliser (DIN) and the microconcentric nebuliser (MCN), sensitivity and detection limits were equal for the two systems. As the methanol concentration in the eluent was only 5%, this could easily be handled by the MCN and there was no need for the low flow rates of the DIN that makes this nebuliser superior regarding introduction of organic solvents into the plasma. Hence, the MCN in combination with the cyclonic spray chamber was applied for the analysis.

Optimisation of the DRC instrument

Besides nebuliser gas flow rate, RF power and lens voltage, the dynamic reaction cell (DRC) was optimised with regards to the lower and upper rejection parameters, RPa and RPq, respectively, the quadrupole rod offset (QRO), the cell rod offset (CRO), the cell path voltage (CPV) and cell gas flow rate. Only the RPq and cell gas flow parameters were of major importance. The RPq value showed minimum background when optimised on 0.25. This value is different from the default value for selenium and the values reported by others.[16] Hence this parameter is instrument dependent.

The ^{78}Se, ^{80}Se and ^{82}Se signals of the selenosugars in aqueous solution are shown in Fig. 1. The background on ^{80}Se was about 300 cps, while the background on the ^{78}Se signal was reduced from 12 000 cps to about 500 cps compared to regular ICP-MS without a DRC due to removal of argon based interferences by reaction with the methane gas. These backgrounds are high compared to backgrounds reported by others,[16] however reducing the background further resulted in a relatively large loss of sensitivity.

Linearity was obtained in the concentration range 0.5–100 μg Se L^{-1} in aqueous solution as well as urine diluted 1 + 1 ($r > 0.999$). Detection limits were calculated on basis of three times the standard deviations on peak areas of 2 μg Se L^{-1} solutions. The precision was determined at two concentration

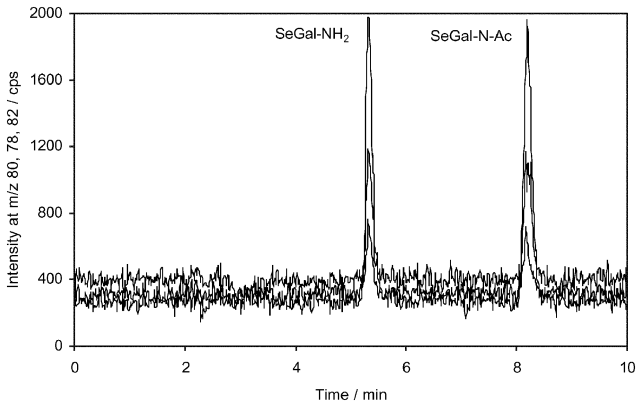

Fig. 1 Chromatogram of an aqueous solution of SeGal-NH_2 and SeGal-N-Ac in concentrations of 0.5 μg Se L^{-1}. Column: Luna C18 2 × 100 mm × 2 mm i.d., eluent: 200 mM ammonium acetate, pH 9.25 + 5% methanol, flow rate 200 μL min^{-1}, injection volume 12 μL.

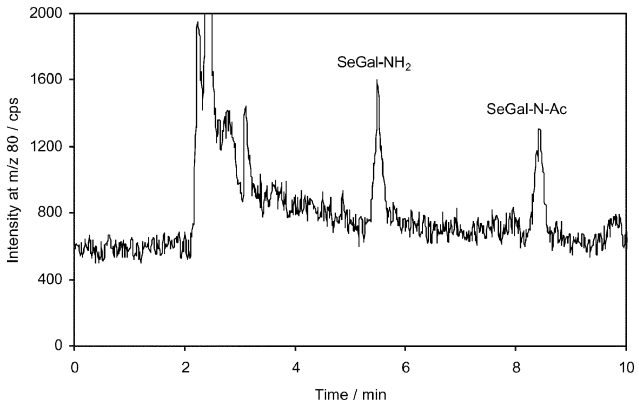

Fig. 2 Chromatogram of a basal urine sample diluted 1 + 1 with water. The concentrations of SeGal-NH_2 and SeGal-N-Ac are 0.41 and 0.35 μg Se L^{-1}, respectively. Chromatographic conditions as in Fig. 1.

levels. The detection limits and the precisions are shown in Table 1. It appears that both the detection limits and the precisions were better for ^{80}Se than for ^{78}Se and ^{82}Se. Hence, the ^{80}Se isotope was used for quantitative determinations. However, the differences in detection limits based on monitoring the different isotopes were not equal to the differences in abundance of the isotopes. Hence, the precision of the peak evaluation of the chromatography is of paramount importance compared to instrument sensitivity.

Chromatographic systems and quantification of selenosugars

The reversed phase chromatographic system consisting of ammonium acetate at pH 9.25 was chosen as SeGal-NH_2 at this pH is partly de-protonated as the neutral amine and hence is better retained on the reversed phase column. This system was not suitable for analysis of $TMSe^+$ as this compound eluted in the void volume.

A chromatogram of a urine sample containing basal amounts of the selenosugars is shown in Fig. 2. It appears that the retention times in urine were similar to the retention times in aqueous solution (Fig. 1). In Fig. 3(A) and (B), examples of chromatograms of urine samples before and after selenium consumption are shown. It appears that both sugars were present in all samples and in most samples SeGal-N-Ac increased relatively more than the amine after consumption of selenium. Hence, selenium is mainly excreted *via* the acetylated form of the aminosugar. However, in one sample the increase in concentration of the two sugars was comparable. This shows the variation in metabolism among individuals and caution should be taken not to draw conclusions on selenium metabolism from experiments on single individuals.

The concentrations of the two selenosugars in urine before and after selenium consumption are given in Table 2. The mean values before selenium consumption were 1.4 ± 0.7 and 2.6 ± 2.3 μg Se L^{-1} for SeGal-NH_2 and SeGal-N-Ac, respectively, while the corresponding mean values after selenium consumption were 1.9 ± 2.2 and 11.6 ± 6.2 μg Se L^{-1}, respectively. This is in accordance with earlier results that show that the latter is the main metabolite in urine after selenium consumption.[9]

A chromatogram of the reference material Seronorm™ trace elements in urine is shown in Fig. 4. It appears that the reference material only contained traces of the selenosugars corresponding to 1.1 μg Se L^{-1} of each sugar, corresponding to 1.6% of the total amount. However, this freeze-dried material has probably deteriorated during preparation or storage.

The recovery on the column determined as the total peak area of the chromatogram related to the total peak area of a flow injection was 90 ± 3% ($n = 3$), which is acceptable taking the uncertainty on peak area integration of the chromatograms into account.

Table 1 Figures of merit

Species	Parameter	^{78}Se	^{80}Se	^{82}Se
SeGal-NH_2	DL, μg L^{-1}	0.3	0.1	0.4
	%RSD at 2 μg Se L^{-1}	4.3	3.1	2.1
	%RSD at 10 μg Se L^{-1}	1.3	1.0	1.7
SeGal-N-Ac	DL, μg L^{-1}	0.4	0.2	0.5
	%RSD at 2 μg Se L^{-1}	5.8	1.7	4.9
	%RSD at 10 μg Se L^{-1}	1.6	0.7	2.3

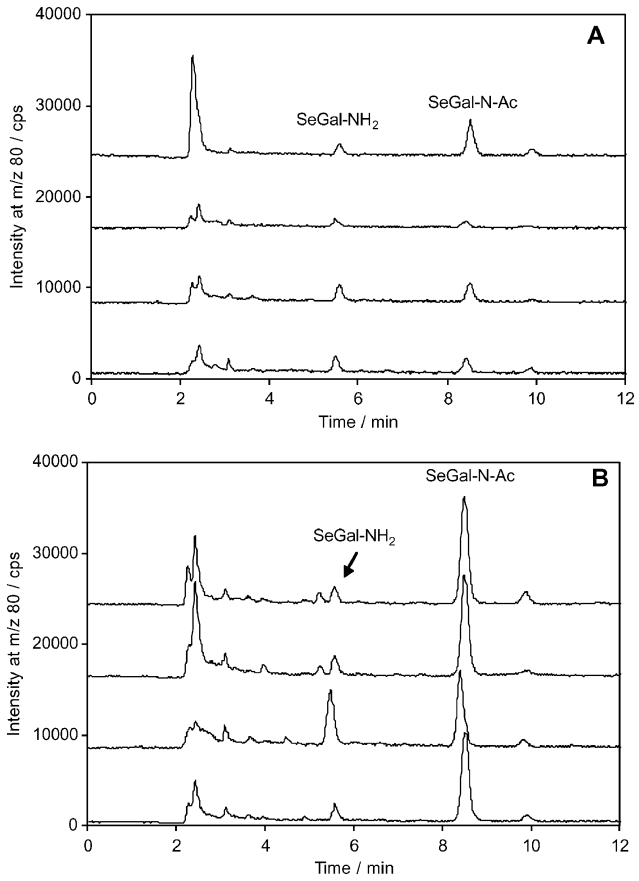

Fig. 3 Examples of chromatograms of urine samples diluted 1 + 1 with water. Chromatographic conditions as in Fig. 1. A: Samples before selenium supplementation. B: Samples after 5 days selenium supplementation. Each sample is offset by 8000 cps.

Table 2 SeGal-NH_2 and SeGal-N-Ac before and after ingestion of selenium supplements

	SeGal-NH_2/μg Se L^{-1}		SeGal-N-Ac/μg Se L^{-1}	
Volunteer	Before	After	Before	After
a	2.0	2.6	1.8	18.9
b	2.2	6.8	4.3	8.0
c	0.8	0.7	2.6	16.1
d	0.7	1.4	1.1	14.8
e	0.8	0.6	1.2	5.2
f	0.8	0.7	0.9	10.7
g	2.1	1.9	7.6	17.3
h	2.0	0.2	1.1	1.8

Cation exchange chromatography—is $TMSe^+$ present in urine samples?

As the recently identified selenosugar SeGal-NH_2 has cationic properties below pH about 9, it was considered whether this compound could have been mistaken for the cationic trimethylselenonium ion in previous investigations.[17]

A chromatogram of aqueous standards of the selenosugars and $TMSe^+$ is shown in Fig. 5(A). It appears that $TMSe^+$ was well separated from the aminosugar and the neutral sugar was eluted in the front. A chromatogram of a urine sample spiked with $TMSe^+$ is shown in Fig. 5(B). It appears that there was no co-elution with $TMSe^+$. $TMSe^+$ was not identified in any of the urine samples. Naturally, this is no evidence that $TMSe^+$ is not present in urine. However, it should be considered if previously published reports on the presence of this metabolite are in error.

Sample pre-concentration

As the basal selenium concentration in urine sample is low, it was attempted to pre-concentrate urine samples prior to quantification. Previously used protocols for pre-concentration of urine pools for qualitative identification seemed suitable for this purpose.[18] The pre-concentration procedure involved evaporation in nitrogen atmosphere, extraction with cold methanol, centrifugation, and evaporation of the supernatant whereafter the residue was dissolved in the mobile phase. This would result in a pre-concentration factor of 10. The recoveries for this procedure ($n = 6$) were 77 ± 11 and 76 ± 14% for SeGal-NH_2 and SeGal-N-Ac, respectively. The size and variation of this recovery was not considered satisfactory for quantitative determinations. Furthermore, new compounds appeared after the pre-concentration procedure—probably an oxidation product of the sugar. This is shown in Fig. 6, from which it is seen that a new compound eluted at 5.3 min, just before the Se-Gal-NH_2 peak. It has been suggested that the oxidation product of the SeGal-N-Ac is methaneseleninic acid,[19] however, this is not the case here, as methaneseleninic acid is eluted in the void volume, and does not co-elute with the oxidation product in this system. It was thus decided not to pre-concentrate samples as accuracy and precision of the quantification was jeopardised and no advantage was obtained compared to direct measurements.

In conclusion, a procedure for quantitative determination of the selenosugars has been presented. This can be a valuable tool in future experiments to elucidate the human metabolism of different selenium compounds.

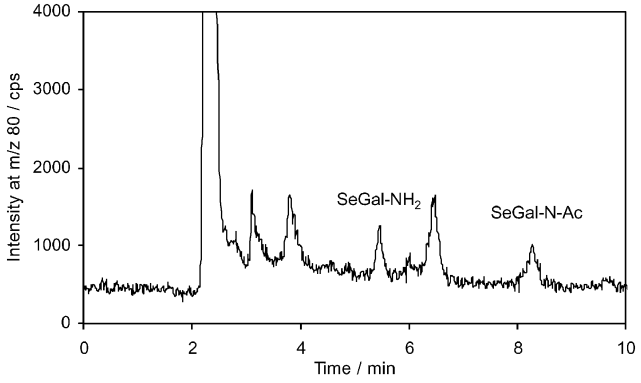

Fig. 4 Chromatogram of the reference material Seronorm™ trace elements in urine. Chromatographic conditions as in Fig. 1.

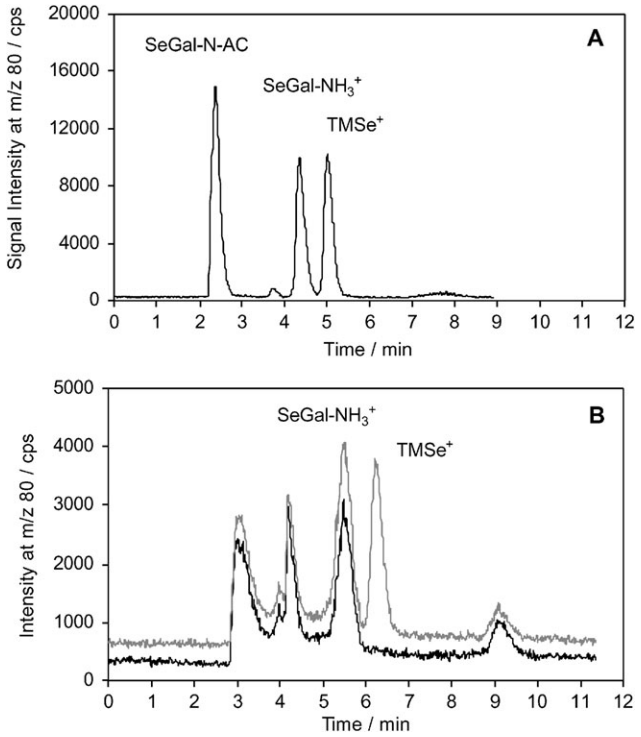

Fig. 5 (A) Chromatogram of aqueous solution of standards in concentrations of 10 μg Se L^{-1}. (B) Chromatogram of urine sample diluted 1 + 1 with water—black line, and a urine sample diluted 1 + 1 with 4 μg Se L^{-1} as $TMSe^+$—grey line (offset by 300 cps). Column: Dionex IonPac CS5 250 mm × 4 mm i.d. + CGS 50 mm × 4 mm i.d., eluent: 10 mM oxalic acid + 20 mM potassium sulfate +2% methanol, flow rate: 0.5 mL min^{-1}, injection volume 50 μL.

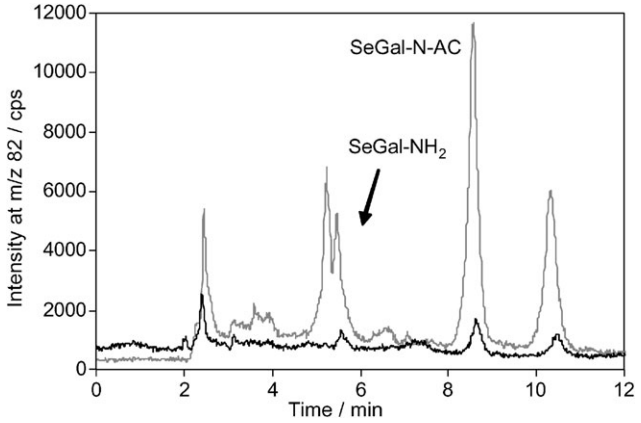

Fig. 6 Chromatograms of basal urine sample before preconcentration—black line, and after preconcentration—grey line. Chromatographic conditions as in Fig. 1.

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