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Separation and identification of Se-methylselenogalactosamine—a new metabolite in basal human urine—by HPLC-ICP-MS and CE-nano-ESI-(MS)^2

Lars Bendahl and Bente Gammelgaard*

Department of Analytical Chemistry, The Danish University of Pharmaceutical Sciences,
Universitetsparken 2, DK-2100 Copenhagen, Denmark. E-mail: bg@dfuni.dk;
Fax: +45 3530 6010

Received 4th May 2004, Accepted 9th June 2004
First published as an Advance Article on the web 19th July 2004

Three minor metabolites were isolated from human urine. Two of these were identified by nano electrospray ionisation mass spectrometry (nESI-MS) as Se-methylseleno-*N*-acetylglucosamine and Se-methylselenogalactosamine, respectively. A human urine pool was lyophilised and reconstituted in methanol prior to fractionation by preparative reversed phase HPLC. In addition to the major urinary metabolite, Se-methylseleno-*N*-acetylgalactosamine, more than seven minor metabolites were separated by this system and detected by ICP-MS. Three of the metabolite fractions were isolated, re-chromatographed in the reversed phase system and further purified in different separation systems before analysis by nESI-MS. By CE-nESI-MS analysis of one of the fractions, the characteristic selenium pattern was recognized around *m*/*z* 285 and (MS)^2 fragmentation resulted in a fragments at *m*/*z* 267, 173 and 155, respectively. It was not possible to identify this selenium compound on basis of the available data. The selenium compound in the second fraction showed co-elution with a Se-methylseleno-*N*-acetylglucosamine standard. The identity of this compound was verified by nESI-MS after further purification by size exclusion chromatography. The third fraction was further purified by ion-pair and anion exchange chromatography, reconstituted and subjected to CE-nESI-MS. The *m*/*z* of the compound was 258 and (MS)^2 resulted in a fragment at *m*/*z* 162, corresponding to loss of methylselenium. This indicated that the structure of the compound was Se-methylselenogalactosamine. To verify the identity of the compound, the Se-methylselenogalactosamine and the Se-methylselenoglucosamine were prepared by hydrolysis of the corresponding *N*-acetylhexosamines. The mass spectra of these standards were identical and also identical to the mass spectra of the purified urine compound. The urine selenium compound co-eluted with Se-methylselenogalactosamine in a reversed phase chromatographic system able to separate Se-methylselenogalactosamine and Se-methylselenoglucosamine. Analysis of basal urine samples from volunteers who had not been supplemented with selenium showed the presence of Se-methylselenogalactosamine when only traces of the metabolite Se-methylseleno-*N*-acetylgalactosamine, which is the major metabolite in urine after selenium supplementation was present. Hence, this new metabolite may be the main metabolite in basal urine.

Introduction

The analytical challenges in selenium speciation analysis have, in recent years, changed from separation and detection of selenium compounds by hyphenated techniques to identification of selenium compounds by soft ionisation mass spectrometry in biological samples.

The main obstacles are the complicated matrices of biological samples combined with, at least for human biological material, the very low concentrations.

The average selenium concentration in human urine is about 30 µg L^{-1}, although much larger values can be found in populations living in areas with selenium rich soil.[1] As the detection limits for most known selenium compounds are about three orders of magnitude higher in organic mass spectrometry as compared with ICP-MS, preconcentration and purification of samples is mandatory prior to identification by soft ionisation mass spectrometry. However, these procedures should be as gentle as possible and performed cautiously to prevent any change of structure of the original compounds in the sample during the procedures.

Selenium metabolism

The majority of the present knowledge on selenium metabolism is based on experiments on rats. In particular, the experiments based on administration of ^{82}Se-enriched inorganic selenium to rats, reported by Suzuki and co-workers, has contributed to this knowledge. Their results are based on the determination of ^{82}Se levels in organs and body fluids by size exclusion chromatography and ICP-MS detection. According to their results, intravenously injected selenite is taken up by the red blood cells (RBCs),[2,3] reduced by glutathion (GSH) to selenide, bound selectively to albumin and transported to the liver. In the liver, selenide is incorporated in selenoprotein P, which is excreted, transported to the kidneys where it is degraded and used for synthesis of extracellular glutathion peroxidase (GPx).[4] Selenate was not taken up by RBCs but was retained in the bloodstream until it entered the liver or was excreted directly in urine. The metabolic products of selenite and selenate were not distinguishable.[3] In the liver, selenium was distributed between two compounds, Se-methylseleno-*N*-acetylgalactosamine, the major urinary metabolite,[5] and a compound in which the methyl group on the selenium was exchanged with glutathione. It was suggested that this compound was a precursor of the major urinary metabolite.[6]

Selenium metabolites in urine

According to previous models of selenium metabolism,[7] the main excretory selenium product in urine is the trimethylselenonium ion (TMSe). However, new studies involving

DOI: 10.1039/b406589a

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identification of selenium compounds in urine by soft ionisation mass spectrometry have shown that the main urinary selenium metabolite is Se-methylseleno-*N*-acetylgalactosamine. This metabolite has been identified in rat urine after supplementation with selenite[5] or selenomethionine,[8] as well as in human urine after supplementation with selenium enriched yeast.[9]

Cao *et al.*[10] used ESI-MS in the multiple reaction monitoring mode after fractionation with reversed phase chromatography to identify two metabolites in a urine sample, selenomethionine and selenocystamine. These compounds were found in a human sample after supplementation with selenomethionine.

Based on co-elution with standards, the presence of TMSe,[11–13] selenite[14,15] and selenate[16] in human urine has been suggested. Also, the two volatile compounds dimethyldiselenium and dimethylsulfurselenium have been detected in human urine by GC-ICP-MS.[17] Recently, the presence of Se-methyl-*N*-acetylglucosamine in human urine was suggested on basis of co-migration with a standard using CE-ICP-MS.[18] However, none of these compounds have yet been identified by soft ionisation mass spectrometry.

The aim of this study was to separate and purify selenium metabolites from human urine prior to identification by mass spectrometry.

Experimental

ICP-MS

The ICP-MS instrument was a PE SCIEX Elan 6000 (PerkinElmer, Norwalk, CT, USA) equipped with a MicroMist AR30-1-F-02 glass concentric nebulizer and a cyclonic spraychamber (Glass Expansion, Romainmotier, Switzerland). The spraychamber was cooled to −4 °C when the eluent contained more than 5% methanol.

Sampler and skimmer cones were made of platinum. The plasma and auxilliary argon flow rates were 14 and 1.2 l min^{-1}, respectively. The nebulization argon flow and rf power were optimized in each eluent. The data acquisition parameters were: dwell time, 500 ms; sweeps per reading, 1; readings per replicate, between 400 and 700. The ^{78}Se and ^{82}Se isotopes were monitored.

nESI-MS

Nano electrospray ionisation mass spectrometry was performed on a Finnigan MAT LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA, USA) using the following settings: spray voltage, +3.2 kV; capillary temperature, 200 °C.

Samples were either introduced by direct infusion at a flow rate of 200 nL min^{-1} or by CE using a laboratory made nano-ESI-MS interface.[19]

CE-nESI-MS

The high voltage power supply for capillary electrophoresis consisted of a Spellman, Type CZE2000 (Spellman High Voltage Electronics Corp., Hauppange, NY, USA) and a laboratory-made control unit.

Chromatography

The Agilent 1100 series HPLC system consisted of a G1376A capillary pump, a G1313A autosampler, G1314A wavelength detector, a G1316A column compartment and a G1379A de-gasser controlled by the ChemStation software, all from the Agilent 1100 series (Agilent Technologies, Waldbron, Germany). The UV detector was operated at 254 nm. The pump for preparative chromatography was a Jasco 880-PU (Jasco, Gross-Umstadt, Germany) equipped with a Rheodyne 7125 injection valve (Rheodyne, CA, USA).

Chromatographic separations

Preparative chromatography was performed on a Luna C18(2) column, pore size 100 Å, 250 mm × 15 mm id (Phenomenex, Aschaltenburg, Germany) protected by a SecurityGuard Cartridge C18, 10 mm × 10 mm, in a SecurityGuard Cartridge Holder (Phenomenex). The flow rate was 10 ml min^{-1} and the injection volume was 1000 µl.

Size exclusion chromatography was performed on a Shodex Asahipak GS-320 HQ column, 300 mm × 7.6 mm id. The column temperature was kept constant at 40 °C. The flow rate was 600 µl min^{-1} and the injection volume was 100 µl.

Analytical chromatography was performed on two Luna C18(2) columns in series, 100 mm × 2.0 mm id, 3 µm particle size (Phenomenex), with a flow rate of 200 µl min^{-1} and an injection volume of 12 µl.

Eluent for reversed phase chromatography: 200 mM ammonium acetate + 5% methanol (LabScan Analytical Sciences, Dublin, Ireland).

Eluent for ion-pair chromatography: 10 mM heptafluorobutanoic acid (HFBA) (Sigma) + 20% methanol.

Eluent for size exclusion chromatography: 0.1% formic acid in 3% methanol.

Eluent for separation of Se-methylselenohexosamines: 0.1% formic acid + 5% methanol.

Electrophoretic separations

Separations were performed on a 363 µm od fused silica capillaries coated externally with poly(imide), 65 cm × 50 µm id (Poly Micro Technologies, Phoenix, AZ, USA) coated internally with the cationic polymer polydiallyldimethylammonium chloride, MW 4–500000 (Sigma). Buffer, 300 mM formic acid. Run voltage, −30 kV; hydrostatic injection at 20 mbar for 10 s.

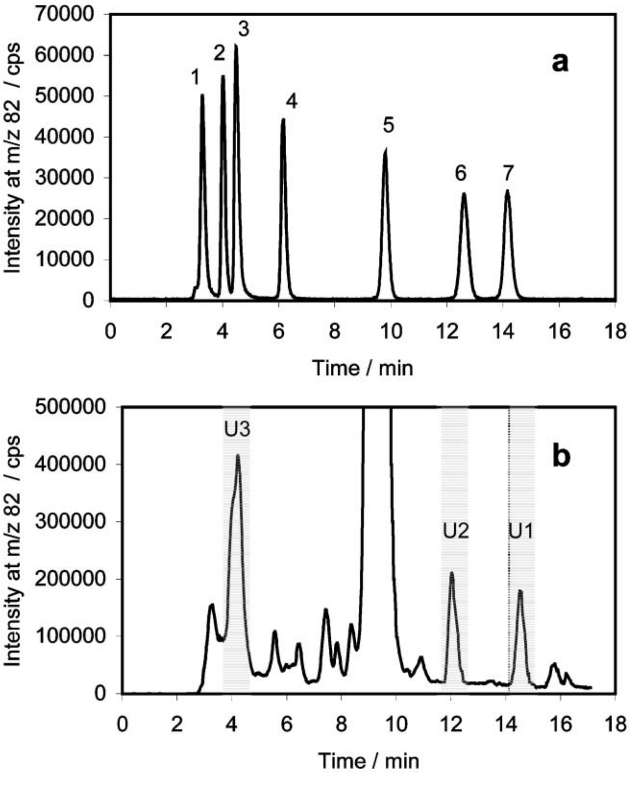

Fig. 1 a, Separation of 7 selenium standards, each in concentrations of 100 µg Se L^{-1}. 1, Trimethylselenonium ion; 2, Se-methylselenocysteine; 3, selenocystamine; 4, selenomethionine; 5, Se-methylseleno-*N*-acetylgalactosamine; 6, Se-methylseleno-*N*-acetylglucosamine; 7, selenoethionine. b, Lyophilized, methanol extracted and reconstituted urine pool. Column, Luna C18(2), 250 × 15 mm id; eluent, 200 mM ammonium acetate + 5% methanol.

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 selenomethionine (Sigma), Se-methylselenocysteine (Sigma), selenocystamine (Sigma), selenoethionine (Sigma), trimethylselenonium iodide (synthesized according to ref. 20), Se-methylselenomethionine (synthesized according to ref. 21), Se-methyl-*N*-acetylglucosamine (synthesized in a modified version according to tef. 12) and Se-methyl-*N*-acetylgalactosamine (synthesized at the Department of Medical Chemistry, The Danish University of Pharmaceutical Sciences). The stock

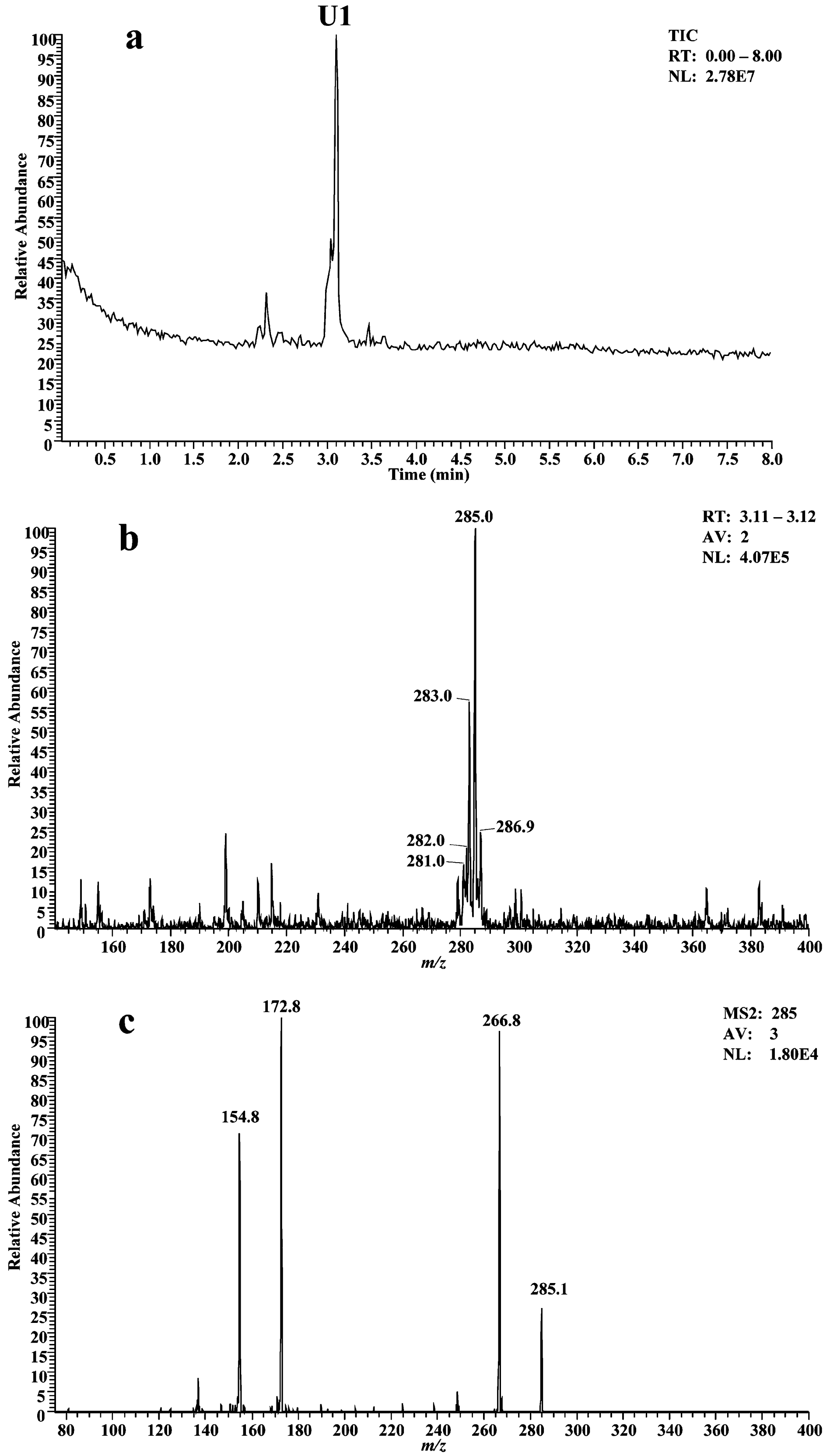

Fig. 2 CE-nESI-MS of fraction U1. a, Total ion current. b, Full scan spectrum of the peak with migration time 3.1 min. C, CE-nESI-(MS)^2 of *m/z* 285.0.

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.

Se-methylselenogalactosamine and Se-methylselenoglucosamine standards: 1 ml solutions of Se-methylseleno-*N*-acetylgalactosamine and Se-methylseleno-*N*-acetylglucosamine, respectively, containing *ca.* 100 mg Se L^{-1}, were each added to 1 ml of 5 M potassium hydroxide and heated on a boiling water bath for one hour. The reaction mixtures were neutralized in an ice-water bath by slow addition of concentrated perchloric acid and the precipitated potassium perchlorate was removed by centrifugation. The de-acylated selenosugars were subsequently purified by preparative chromatography. The overall yields were in the range 25–50%.

Urine pool. Urine samples from a volunteer supplied with a single dose of selenium in the form of selenized yeast were collected during a day. Samples with high contents of selenium were pooled, lyophilised and extracted with methanol by sonication. The methanol extract was evaporated to dryness on a rotary evaporator (Büchi Rotavapor, Büchi Laboratoriums Technik AG, Flawil, Switzerland) by heating to 45 °C and the residue was reconstituted in the eluent for preparative chromatography.

Procedure. Fractions from the preparative chromatography were lyophilised, dissolved in eluent and re-chromatographed on the preparative column. The resulting fractions were lyophilised and reconstituted in a solution of 0.1% formic acid in 30% methanol for analysis by nESI-MS. Fraction U1 was introduced to the CE-nESI-MS system without further purification.

Fraction U2 was further purified by size exclusion chromatography prior to nESI-MS identification by direct infusion.

Fraction U3 was further purified by preparative ion-pair chromatography, followed by ion-exchange on a SAX solid phase extraction cartridge to remove the HFBA anion. The cartridge was conditioned with 1 M ammonium formate, washed with 0.1% formic acid, prior to application of the sample, and eluted with 0.1% formic acid in 30% methanol. The eluate was analysed by CE-nESI-MS.

Results and discussion

Urine samples from a volunteer who had been supplied with selenium in the form of selenized yeast were collected for 24 hours and analysed for their selenium content. Samples with high selenium concentrations were pooled. After immediate lyophilisation, reconstitution in methanol by sonication, evaporation and reconstitution in eluent, the pooled sample

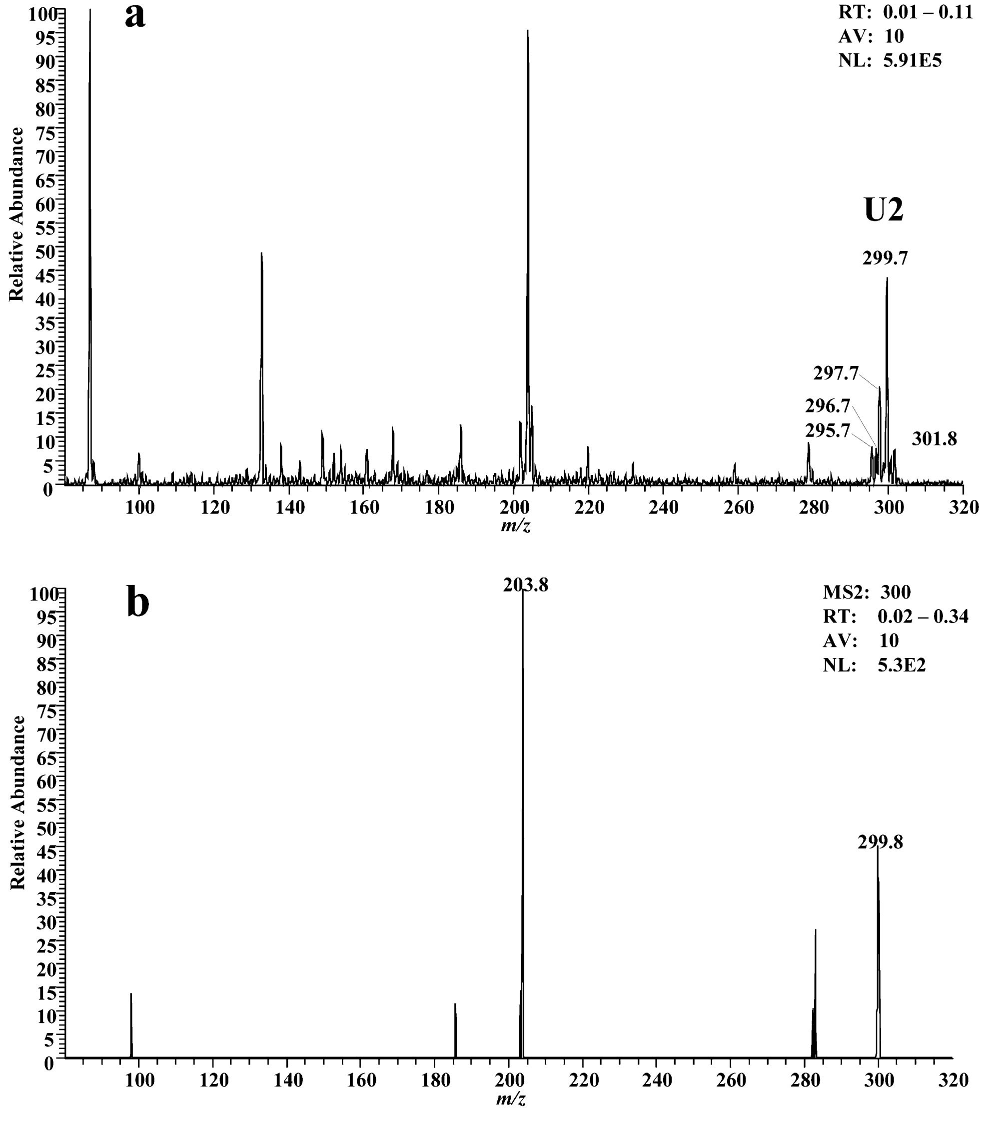

Fig. 3 nESI-MS of fraction U2. a, Full scan spectrum of fraction U2; b, (MS)^2 of *m*/*z* 299.8

was analysed by reversed phase chromatography. Chromatograms from analysis on the preparative column of available standards (a) and the urine pool after methanol extraction and reconstitution (b) are shown in Fig. 1. The eluent flow of the preparative column was split with a T-piece in order to monitor the separation by ICP-MS.

The chromatographic recovery of selenium was determined by comparing the total areas resulting from flow injection and chromatographic introduction of a urine sample. The recovery, calculated on basis of ^{82}Se, was 88.4 ± 7.5% (RSD, $n = 3$). Hence, neither known nor unknown selenium compounds were retained in significant amounts on the column.

The main peak in the chromatogram of the urine sample has previously been identified as methylseleno-*N*-acetylgalcatosamine and it is the main selenium metabolite of human urine after selenium supplementation.[9] Several other metabolites can be seen—these are, however, present in much lower concentrations than the main metabolite. The fractions assigned U1, U2 and U3 were collected for further purification and identification.

Fraction U1 had the longest retention time and visual inspection of the lyophilised fraction revealed that the content of solids was low compared with the solid content of the other fractions. Hence, an attempt was made to analyse this fraction directly by nESI-MS after re-chromatography on the preparative column. However, no selenium isotope pattern was identified. Instead, the sample was analysed by CE-nESI-MS using a modified version of a previously described interface.[19] The total ion current (TIC) of the electrophoretic separation, the mass spectrum of the selenium-containing peak and the (MS)^2 of the ^{80}Se-containing peak are shown in Fig. 2. It appears from the TIC that although the fraction was relatively pure, it had to be separated from co-eluting compounds to avoid ion suppression. A distinct selenium isotope pattern was observed in the peak migrating at 3.1 min with the *m*/*z* 285.0 corresponding to ^{80}Se (49.8%), *m*/*z* 283.0 corresponding to ^{78}Se (23.5%), and *m*/*z* 286.9 corresponding to ^{82}Se (9.2%), respectively. Fragmentation of the $[M+H]^+$ ion at *m*/*z* 285.0 resulted in signals at *m*/*z* 266.8, *m*/*z* 172.8 and *m*/*z* 154.8, respectively. Fragmentation of *m*/*z* 283.0 resulted in signals at *m*/*z* 264.8, 172.8 and 154.8, respectively (not shown). Hence, the compound most probably contains selenium and the first fragments at *m*/*z* 266.8 and 264.8, respectively, contain selenium and are probably results of loss of water $[MH^+-H_2O]$. The fragment at *m*/*z* 172.8 has lost selenium, and the fragment at *m*/*z* 154.8 corresponds to loss of water $[172.8-H_2O]$.

The *m*/*z* of this unknown compound is only 15 mass units lower than the *m*/*z* of the main metabolite Se-methylseleno-*N*-acetylgalactosamine, hence it would be obvious to conclude that this unknown compound corresponded to a de-methylated product of this sugar. However, fragmentation did not result in the fragment at *m*/*z* 204 that is characteristic of *N*-acetylhexosamines.[22] Furthermore, the *m*/*z* of the parent ion of the unknown compound is an even number, which indicates that the ion contains an even number of nitrogen atoms. The compound eluted later than the selenosugars in the reversed phase system, hence it is probably more lipophilic than the sugars. However, it was not possible to identify the compound on the basis of the available data.

The compound eluting at 12 min (fraction U2) co-eluted with Se-methylseleno-*N*-acetylglucosamine, as previously described.[18] This fraction was further purified by size exclusion chromatography prior to identification by nESI-MS. The mass spectrum and the (MS)^2 spectrum of this fraction is shown in Fig. 3, while the similar spectra of the synthesised Se-methylseleno-*N*-acetylglucosamine standard are shown in Fig. 4. It appears that the mass spectra of the compound isolated from human urine and the standard are identical. The $[M+H]^+$ ion at *m*/*z* 299.8 results in the fragment at *m*/*z* 203.9, which is characteristic for *N*-acetylhexosamines and corresponds to $[M-CH_3Se]^+$. The fragment at *m*/*z* 186.0 corresponds to $[204-H_2O]^+$. These mass spectra are, of course, identical to the mass spectra of the major urinary metabolite Se-methylseleno-*N*-acetylgalactosamine, as the only difference between these two compounds is the spatial orientation of one hydroxy group.[9] However, as the urinary compound co-elutes with the standard in a chromatographic system and co-migrates with the standard in an electrophoretic system, as previously described,[18] and the mass of the compound has now been determined, the identity of this compound is now verified.

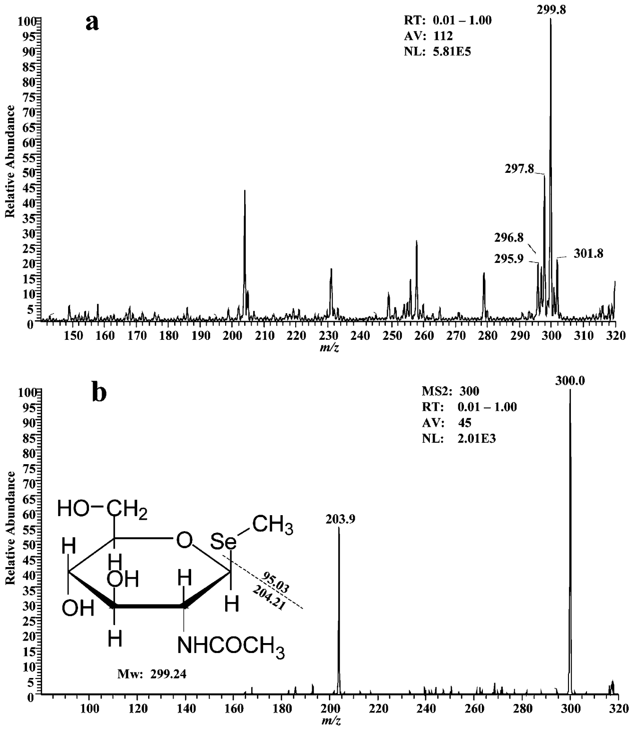

Fig. 4 nESI-MS of a 5 mg Se L^{-1} solution of a Se-methylseleno-*N*-acetylglucosamine standard in 50% acetonitrile + 1% formic acid. a, Full scan spectrum; b, (MS)^2 of *m*/*z* 299.8

Fraction U3, collected around 4 min (Fig. 1), contained large amounts of salts and other impurities as it eluted close to the void volume. It was not possible to analyse this fraction directly on CE-nESI-MS after the preparative chromatography as the high ionic strength jeopardized the electrophoretic separation. Hence, this fraction was further purified by preparative ion-pair chromatography followed by anion exchange on a strong anion exchange cartridge. The total ion current of the electrophoretic separation together with the mass spectrum and the (MS)^2 spectrum of this fraction is shown in Fig. 5. It appears from the TIC that, although the urine fraction had been purified in three orthogonal chromatographic systems, several impurities were still remaining and separation with CE prior to the MS detection was necessary.

The mass spectrum of the peak migrating at 5 min shows a distinct selenium pattern with *m*/*z* of $[M+H]^+$ at 257.8 corresponding to the ^{80}Se isotope. The uneven *m*/*z* of the parent ion indicates the presence of an uneven number of nitrogen atoms. The fragmentation shows only one fragment at *m*/*z* 161.9 corresponding to $[M-CH_3Se]^+$. The *m*/*z* of this compound corresponds to the structure of Se-methylselenogalactosamine, the de-acylated form of the main metabolite.

To verify the identity of this new compound, standards of the Se-methylselenogalactosamine and Se-methylselenoglucosamine were prepared by alkaline hydrolysis of the corresponding *N*-acetylhexosamines. The standards were purified by preparative chromatography and analysed by nESI-MS. The spectra of the two standards were identical. The mass spectrum and (MS)^2 spectrum of the Se-methylselenogalactosamine

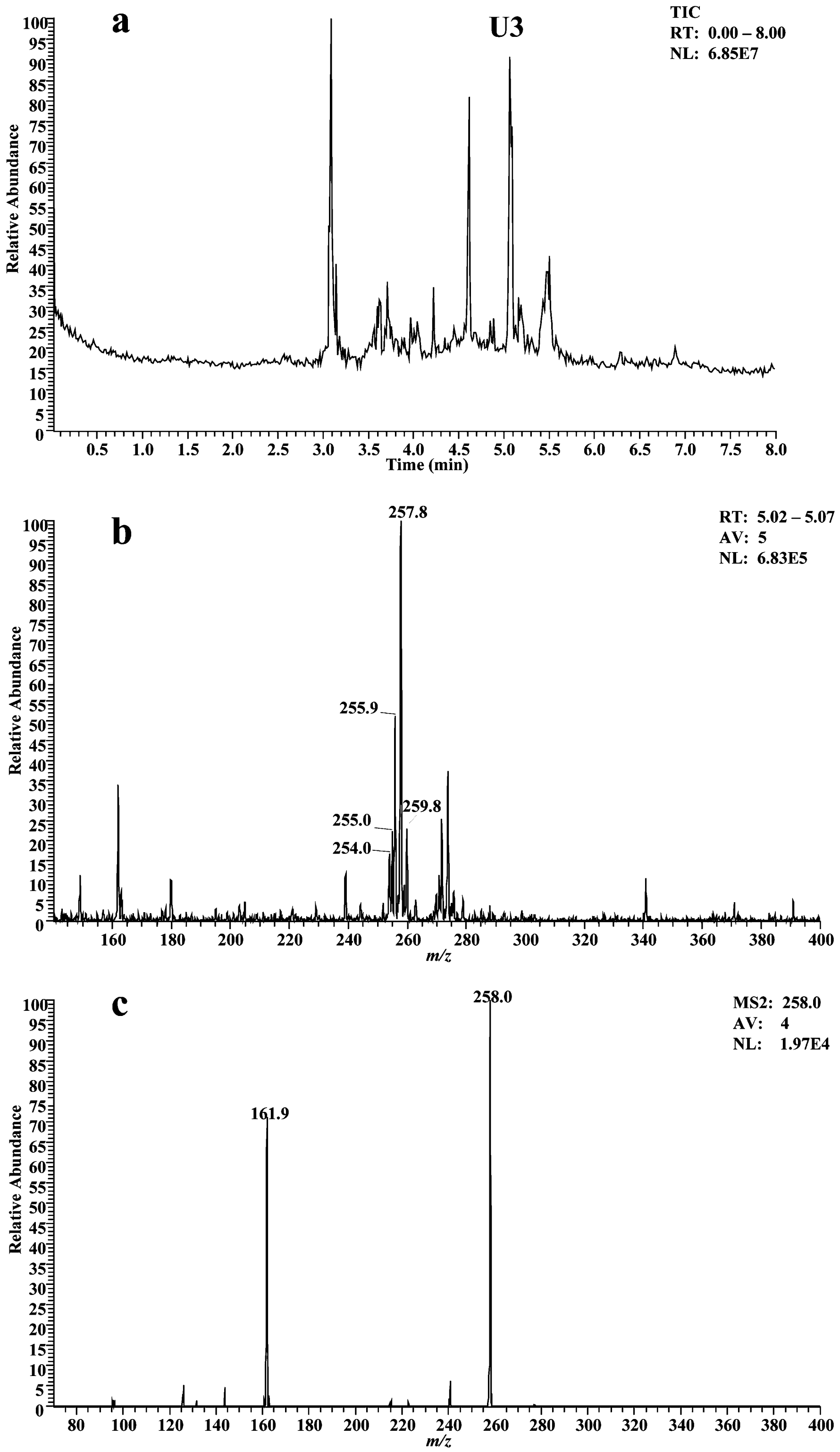

Fig. 5 CE-nESI-MS of fraction U3 after further purification. a, Total ion current; b, full scan spectrum of the peak with migration time 5.0 min; c, CE-nESI-(MS)^2 of *m*/*z* 257.8.

standard is shown in Fig. 6. It appears that the mass spectra of the selenium compound isolated from human urine (Fig. 5) and the synthetic standards are identical.

As the mass spectra of the galactosamine and glucosamine forms are identical, the purified fraction U3 was chromatographed and spiked with a mixture of these two standards. This is shown in Fig. 7. It appears that the purified urine fraction co-eluted with Se-methylselenogalactosamine. Thus, the identity of the selenium compound in fraction U3 is verified.

To verify that the selenium compound was present in the original urine samples, and not a degradation product of the main metabolite produced during the fractionation, urine samples from a volunteer was collected before supplementation with selenium and during the excretion process of the next 24 hours. The result is shown in Fig. 8. It appears that the metabolite was present in the same concentration before as well as during the excretion of the ingested selenium, which indicates that the presence of this metabolite is not dependent on the concentration of Se-methylseleno-*N*-acetylgalactosamine. The first peak in the chromatograms does not contain

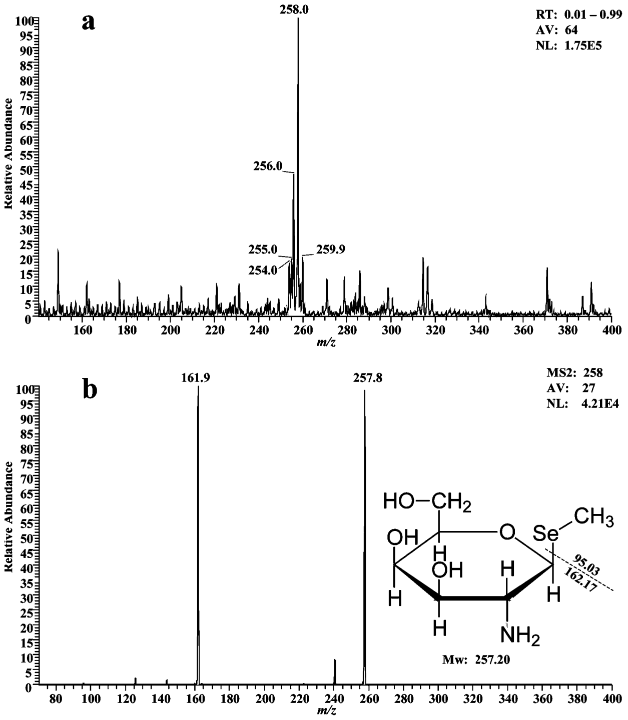

Fig. 6 nESI-MS of a 2.5 mg Se L^{-1} solution of a Se-methylselenogalactosamine standard in 0.1% formic acid + 5% methanol. a, Full scan spectrum; b, (MS)^2 of *m*/*z* 258.0

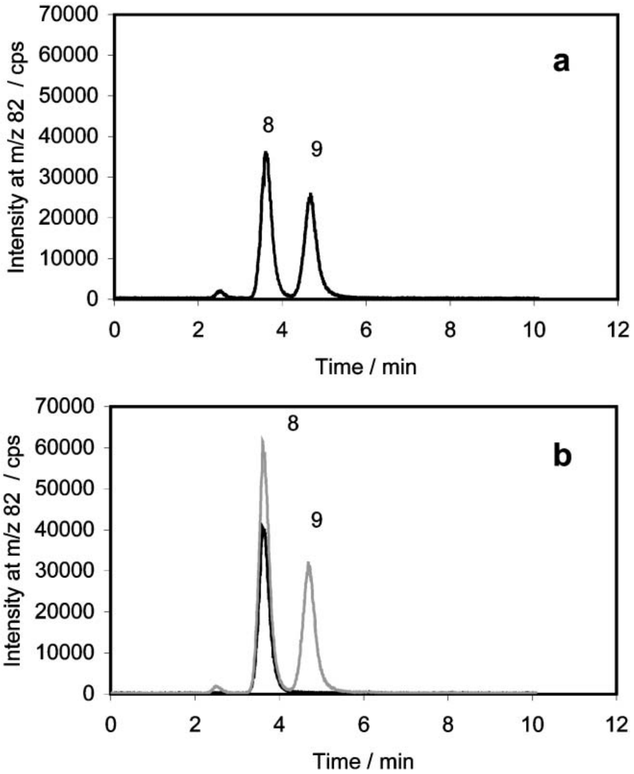

Fig. 7 a, Chromatogram of standards of 8, Se-methylselenogalactosamine, and 9, Se-methylselenoglucosamine, each in a concentration of 100 µg Se L^{-1}. b, Chromatogram of purified fraction U3. Black line, purified fraction A, grey line, purified fraction U3 spiked with standards each in concentration of 100 µg Se L^{-1}. Column: 2 × Luna C18(2), 100 mm × 2 mm. Eluent: 0.1% formic acid + 5% methanol.

selenium but is an interferent, probably $^{1}H^{81}Br$ originating from bromide in the urine sample. The isotopic ratios in the selenium metabolite peak of the three selenium isotopes ^{77}Se,

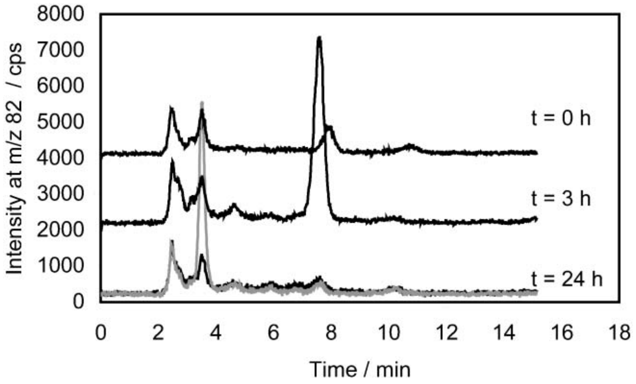

Fig. 8 Chromatograms of urine samples from a volunteer before selenium supplementation (*t* = 0 h), 3 hours (*t* = 3 h) and 24 hours (*t* = 24 h) after selenium supplementation, respectively. The first peak is an interference. Black line, urine sample, grey line, urine sample (*t* = 0 h) spiked with 10 µg Se L^{-1} Se-methylselenogalactosamine. Column: 2 × Luna C18(2), 100 mm × 2 mm; eluent: 200 mM ammonium acetate + 5% methanol.

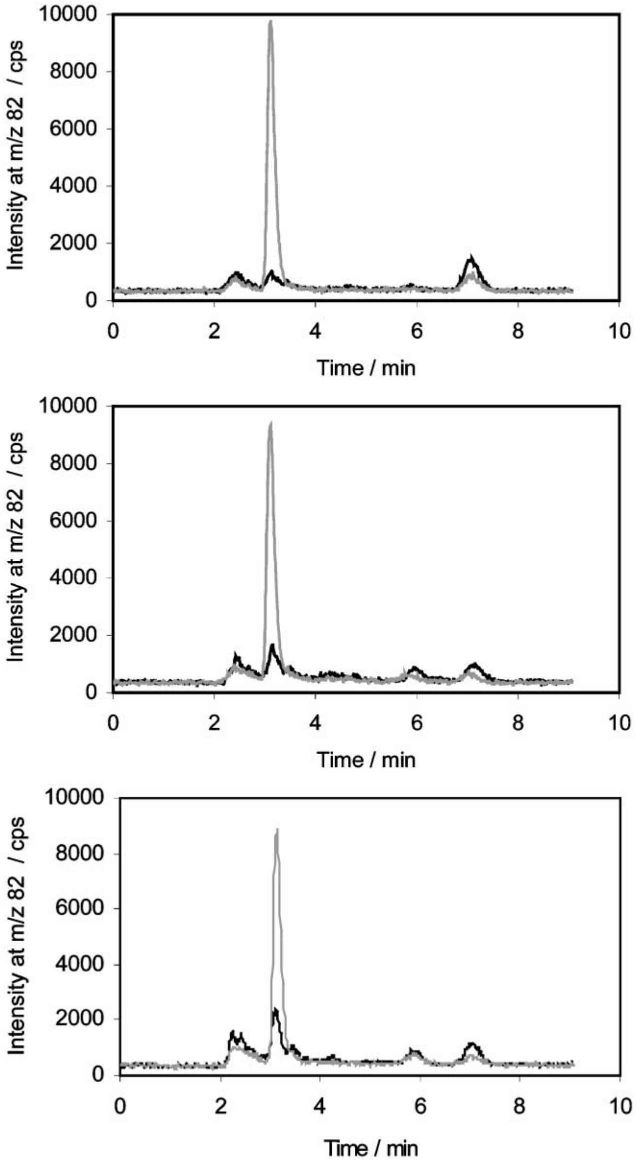

Fig. 9 Chromatograms of basal urine samples from three volunteers, who were not supplemented with selenium. Black line, urine sample, grey line, urine sample diluted 1 + 1 with 25 µg Se L^{-1} as Se-methylselenogalactosamine. Column: 2 × Luna C18(2), 100 mm × 2 mm; eluent: 200 mM ammonium acetate + 5% methanol.

^{78}Se and ^{82}Se, which were routinely monitored, corresponded to the natural abundance. Hence, the metabolite peak was not interfered by the presence of salts. Besides, no other selenium compounds were detected during the chromatographic purification steps.

Urine samples from volunteers who had not been supplemented with selenium, were collected and analysed shortly after delivery. Examples of chromatograms of these samples as well as spiked samples are shown in Fig. 9. It appears that although the samples are very low in selenium concentration and only traces of Se-methylseleno-*N*-acetylgalactosamine are present, all samples show co-elution with the synthesized Se-methylselenogalactosamine. Hence, according to these results, this new metabolite is present in basal urine from individuals who have not been supplemented with selenium.

In conclusion, three minor metabolites were isolated and purified from human urine. When analysed by CE-nESI-MS, one of the metabolites showed the characteristic selenium pattern at *m*/*z* 285, but it was not possible to determine the structure on the basis of the available data. One of the metabolites was identified as Se-methylseleno-*N*-acetylglucosamine by nESI-MS. The third metabolite was identified by CE-nESI-MS as Se-methylselenogalactosamine and appeared to be one of the main metabolites in basal human urine from individuals who have not been supplied with selenium.

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