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Determination of selenium stable isotopes by gas chromatography–mass spectrometry with negative chemical ionisation

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Abstract

A gas chromatography mass spectrometric method using negative chemical ionisation was developed for the determination of stable isotopes of selenium for evaluation of selenium absorption and retention from foods in humans. The method involves an acid digestion to convert all selenium into selenite, which subsequently reacts with 4-nitro-*o*-phenylene-diamine to form a volatile piazselenole. The piazselenole, after extraction into an organic solvent, was analysed for its isotopic selenium composition by gas chromatography mass spectrometry. Negative chemical ionisation is reported for the first time for the determination of selenium stable isotopes and its analytical characteristics were compared to those of electron impact mass spectrometric ionisation, classically used for the determination of selenium. The negative chemical ionisation technique allowed accurate determination of total selenium by isotope dilution and of selenium isotope ratios in biological samples. The repeatability for total selenium and for stable isotope ratios was good (R.S.D.≤10%) within the range of 50 to 250 ng selenium. The detection limit for the investigated selenium isotopes was approximately 1 pg (signal to noise ratio at 3). The applicability of the developed stable isotope methodology was demonstrated by the determination of the selenium absorption and retention from foods in a pilot study using one human adult.

Keywords: Stable isotopes; Selenium

1. Introduction

Selenium (Se) is recognised as an essential trace element for humans and dietary recommendations were established [1,2]. Se status is determined by dietary Se intake and its bioavailability [3]. Population groups on restricted diets, such as infants and patients on total parenteral nutrition, and people living in low Se areas were reported to be at increased risk for low dietary Se intake [4,5]. The bioavailability of Se, influenced by the chemical form in which Se is present in the diet, is reported to be generally high (40–90%) [3]. Se bioavailability can be estimated by assessing absorption and retention of Se stable isotopes [6]. Stable isotopes offer the possibility to evaluate Se absorption and retention in all population groups without major constraints [6]. These studies require analytical techniques which allow precise and accurate determination of stable isotope ratios at low levels of total selenium.

Stable isotopes of Se were determined by neutron activation analysis, inductively coupled plasma mass spectrometry (ICP-MS), and gas chromatography mass spectrometry (GC–MS) [7–11]. GC–MS, probably the most widely used technique, employs

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selective derivatisation of selenite, with an aromatic *o*-diamine to form in acidic medium a volatile piazselenole. The piazselenole is extracted into an organic solvent and analysed by GC–MS. The analysis of stable isotopes of Se by GC–MS with electron impact ionisation (EI) was first described by Reamer and Veillon [7] using 4-nitro-*o*-phenylenediamine (NPD) as derivatizing agent. This and analogous methods were used to determine total Se by isotope dilution or to measure isotopic ratios of Se [8–11].

This article describes for the first time the determination of stable isotopes of Se in biological samples by GC–MS using negative chemical ionisation (NCI). The analytical characteristics of this mass spectrometric detection technique are discussed. The methodology was validated for the assessment of Se absorption and retention in a pilot study with one human volunteer.

2. Experimental

2.1. Instrumentation

Gas chromatographic separations were performed on a Hewlett–Packard 5890 gas chromatograph (Hewlett-Packard, Geneva, Switzerland) equipped with a HP-7673 autosampler and coupled to a Finnigan MAT SSQ-7000 mass spectrometer (Finnigan MAT, Bremen, Germany) for both NCI and EI or to a HP-5988 mass spectrometer for EI.

A Tecator Digestion System 40/1016 with an Autostep 1012 controller (Perstorp Analytical, Höganas, Sweden) was used for acid digestion of the biological samples.

Total Se was quantified by continuous flow hydride generation (HG) atomic absorption spectrometry (AAS) according to the manufacturer's guidelines [16] with a Varian AA-975 atomic absorption spectrometer equipped with a VGA-76 hydride generation device and a PSC-55 autosampler (Varian, Mulgrave, Australia), and by Zeeman graphite furnace (GF) AAS [14] using a Varian 400 spectrometer equipped with a graphite tube atomizer and autosampler.

A Speedvac A110 vacuum concentration system (Savant Instruments, Farmingdale, USA) was used for the concentration of the digests and for the evaporation of organic solvents according to the manufacturer's guidelines.

Faecal samples were freeze-dried with a Lyolab B lyophiliser (LSL Secfroid, Lausanne, Switzerland) according to the manufacturer's recommendations.

2.2. Chemicals and reagents

All chemicals were of analytical reagent grade (Merck, Darmstadt, Germany) unless otherwise stated. Nitric and hydrochloric acid were further purified by a sub-boiling distillation.

A sodium selenite standard stock solution 1000 mg Se l^{-1} (Merck, Darmstadt, Germany) was used to prepare calibration standards in 1.0 *M* HCl. Elemental Se enriched with ^{74}Se, ^{76}Se or ^{82}Se was purchased from Isotec (St-Quentin, France). Aliquots (~1 mg) of the enriched Se powders (Table 1) were dissolved in nitric acid (~1 ml) [13]. The solutions were then diluted to 10 ml with 0.1 *M* HNO_3 to yield solutions of approximately 100 μg of ^{74}Se, ^{76}Se or ^{82}Se per ml.

A 0.4% (m V^{-1}) NPD solution in 0.1 *M* HCl was shaken for 2 h on a mechanical shaker and extracted with three portions of cyclohexane (50 ml each) for

Table 1
Isotopic composition of natural Se[a] and isotopically enriched Se[b]

Selenium	Isotopic composition (%)					
	^{74}Se	^{76}Se	^{77}Se	^{78}Se	^{80}Se	^{82}Se
Natural	0.89	9.36	7.63	23.78	49.61	8.73
^{74}Se-enriched	98.2	1.78	<0.1	0.01	0.01	0.01
^{76}Se-enriched	1.2	98.5	0.2	0.03	0.01	0.06
^{82}Se-enriched	0.06	0.62	0.56	1.76	4.8	92.2

[a]Data from De Bièvre et al. [12]; [b]Data provided by supplier.

purification. The NPD solution was found to be stable for at least 2 months when stored in the dark at 4°C.

A neutralising solution was prepared by dissolving 1 mg of bromocresol purple in 100 ml of 7 *M* ammonium hydroxide containing 20 millimoles of EDTA (Fluka AG, Buch, Switzerland).

Chromatographic separations were performed on a fused-silica capillary column DB-5 (30 m×0.32 mm ID, 0.25 μm film thickness, J&W Scientific, MSP-Friedli, Koeniz, Switzerland).

2.3. Human pilot study

A healthy 36 year old man was recruited after passing a medical examination to evaluate the stable isotope methodology in a pilot study. He maintained his normal life style and dietary habits and did not take any medication or dietary supplements. Dietary supplements were discontinued one week before starting the study. The subject was requested to avoid the consumption of seafood, offal meat and mushrooms from two days before until the end of the study.

After an overnight fast, the subject consumed 500 ml of a milk based formula labeled with ^{74}Se-selenite and ^{76}Se-selenate as breakfast on two consecutive days. Both selenite and selenate were added at a dose of 40 μg Se. After consumption of the formula the subject was not allowed to drink or eat until lunchtime. Complete faecal and urinary collection started after the consumption of the first labelled formula and was continued for ten days. Faecal material was collected as individual stools, whereas urine was voided and collected every 4 h (8 h during the night) for the first 4 days and as 24 h urinary collections for the remaining period of the study. A 24 h urine was collected on the day prior to the consumption of the Se labeled formula. A faecal marker (brilliant blue) was ingested by the subject the evening (22 h) before consuming the first formula and on day 7 at lunchtime (12 h). All excreta labelled with date and hour of collection were frozen.

NIST 1577a Bovine Liver and NIST 2670 Freeze-dried Urine (NIST, Gaithersburg, USA) were used to establish the accuracy of the method for the determination of total Se by isotope dilution GC–MS (^{82}Se-spike). Urine samples were stored at −20°C. Faecal samples were frozen, freeze-dried, ground and homogenised prior to storage in a dessicator.

2.4. Preparation and GC–MS of piazselenole

The derivatisation of selenium to a volatile piazselenole was carried out according to [8]. Aliquots of freeze-dried, powdered faeces (100–500 mg) or of urine (3–5 ml) were weighed into borosilicate digestion tubes. Concentrated HNO_3 (3.0 ml), 70% perchloric acid (1.5 ml) and three glass beads were added to each tube. The tubes were kept at 70°C for 2 h, 120°C for 2 h, 140°C for 2 h, 180°C for 2 h and 210°C for 4 h. The digests were allowed to cool to room temperature. Two ml of hydrochloric acid (32%) were added to each tube. The tubes were then heated for 30 min at 100°C to reduce selenate to selenite. The digests were transferred into Pyrex tubes. The digestion tubes rinsed with 0.1 *M* HCl, and the rinses poored into the Pyrex tubes. The Pyrex tubes were kept in the Speedvac system for 12 h to reduce the digests in each tube to approximately 0.5 ml.

The concentrated digests were neutralised against bromocresol purple by careful addition of the neutralising solution. Then 0.1 *M* HCl (5.0 ml) and the NPD solution (0.5 ml) were added. This mixture was heated for 15 min at 60°C. The piazselenole was extracted into chloroform (5.0 ml) by shaking the two phases for 15 min. The chloroform layer, quantitatively transferred into Teflon tubes, was evaporated to dryness in the Speedvac system (approx. 15 min). The residue was dissolved in dichloromethane (1000 μl for NCI and 50 μl for EI). The dichloromethane solution was transferred into GC-vials and stored in the dark at −20°C until analysis. The piazselenole was stable for at least two months under these conditions.

The solutions of piazselenole in dichloromethane were subjected to gas chromatography (DB-5 J. and W. fused capillary column 30 m×0.32 mm ID, film thickness 0.25 μm; helium as carrier gas at a pressure of 70 kPa; splitless injection of 5 μl at 250°C; oven temperature program 60°C for 1 min, ramp of by 30°C min^{-1} for 5 min to 200°C, 30°C min^{-1}. for 2 min to 300°C; interface temperature (280°C). The mass spectrometers were operated

for NCI with an electron energy of 150 eV and isobutane as reagent gas and for EI with an electron energy of 70 eV. The source temperature was set at 200°C for NCI and at 250°C for EI. For both ionisation modes the signals at m/z 223, 225, 229, and 231 were monitored (piazselenole with ^{74}Se, ^{76}Se, ^{80}Se, and ^{82}Se). The isotope ratios ^{n}Se/^{80}Se (n=74, 76, 82) were calculated with inclusion of the contributions (~1%) of ^{13}C, ^{15}N and ^{18}O.

Total Se was determined by isotope dilution using a ^{82}Se-spike. The sample was accurately weighed into the digestion tube and an aliquot of the ^{82}Se-spike solution (1 mg ^{82}Se l^{-1}), containing an amount of Se comparable to that of the faeces or urine aliquot was added. The mixture was digested as described.

Total Se in faeces or urine was calculated according to Eq. (1).

$$\mathrm{Se}_{\mathrm{natural}} = \mathrm{Se}_{\mathrm{spike}}(0.9088R - 0.0468)/(0.496 - 0.0878R) \quad (1)$$

R: experimental ^{80}Se/^{82}Se from piazselenole; $\mathrm{Se}_{\mathrm{natural}}$: mass of Se in the aliquot of faeces/urine; $\mathrm{Se}_{\mathrm{spike}}$: mass of total Se added with ^{82}Se spike; coefficients: are the isotopic abundances from Table 1.

Total Se in samples enriched with three isotopes ^{74}Se, ^{76}Se, ^{82}Se was calculated according to the triple spike method [15].

3. Results and discussion

3.1. Extraction procedure

Evaluation of the extraction and derivatisation conditions demonstrated that the Se recovery was neither influenced by the organic extraction solvent (toluene, chloroform, dichloromethane) nor by the derivatisation (concentration of the NPD solution (0.4% vs. 1.5%); reaction conditions (40°C-30 min vs. 60°C-15 min)) or by the extraction conditions (mixing: 30 min vs. 15 min). Therefore the shorter extraction procedure (reaction: 60°C, 15 min; extraction: 15 min) was selected using a 0.4% NPD solution and chloroform as organic solvent. A 0.4% NPD solution was selected, offering the advantage to inject less unreacted NPD on the GC-column, which most likely increases the column life. Chloroform as organic solvent allows an easier separation of the aqueous and organic phase when compared to the other solvents tested. Under these conditions 85 to 95% of Se was recovered in the organic phase, which is comparable to literature data for a single organic solvent extraction [8]. In view of the present application, the obtained Se recovery after a single extraction was considered sufficient.

3.2. Analytical characteristics

To evaluate the analytical performance of the GC–MS method for isotope ratio and total Se determination, repeatability, accuracy of total Se determination and isotope ratio enrichment and determination limit were established for NCI and compared to EI. Typical GC–MS chromatograms for NCI and EI are shown in Fig. 1. The NCI technique allows a higher specificity compared to the EI technique, because only compounds which contain electron capturing groups, such as nitro-groups, halogen atoms or conjugated double bonds, will be detected [17]. The presence of the nitro-group in the

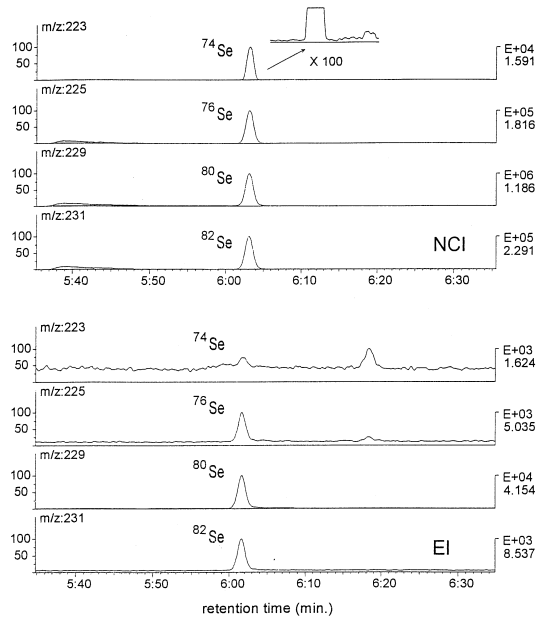

Fig. 1. Chromatograms recorded in NCI and EI for a faecal sample (~75 ng faecal Se+75 ng ^{82}Se-spike).

piazselenole enables this compound to be detected after electron capture by the NCI technique. Because of this specificity there are no interfering peaks in the NCI chromatograms even for the ion at m/z 223 having a relative intensity of 0.89%.

3.2.1. Accuracy of total Se determination by isotope dilution

The accuracy of the total Se determination, measured by the isotope dilution technique using a ^{82}Se-spike, was evaluated with two standard reference materials (SRM). As shown in Table 2, both methods provided results, which were in agreement with the certified Se levels of the SRM's. The precision (R.S.D. $\leq$10%) was good compared to other analytical methods for Se determination [18].

The accuracy of total Se determined by isotope dilution (^{82}Se-spike) in urine and faecal samples enriched with stable isotopes was evaluated through comparison with results obtained for total Se by hydride generation AAS. As shown in Table 3 results obtained by both isotope dilution GC–MS methods are similar to those found by HGAAS.

3.2.2. Repeatability of natural isotope ratio measurements

The repeatability of the ratios ^{74}Se/^{80}Se, ^{76}Se/^{80}Se and ^{82}Se/^{80}Se, measured by the two GC–MS techniques for aqueous standards and urine, are summarised in Table 4. Isotopic ratios determined by NCI were less repeatable (R.S.D. $\leq$10%) than in EI (R.S.D.$<$5%). For the less abundant ^{74}Se isotope R.S.D. was higher for both techniques.

3.2.3. Isotope enrichment

Accurate determination of isotope enrichments is indispensable for stable isotope techniques used for total Se determination and for human absorption studies. Isotope enrichment curves were prepared with the ^{74}Se, ^{76}Se and ^{82}Se stock solutions. Total Se for extraction into chloroform was kept constant at 250 ng, with an isotopic enrichment varying from 5% to 100% ($n=8$). Because enrichments $<$30% are of concern for biological samples, five measurement points were selected in this range. As shown in Table 5, calculated and measured enrichments correlated well for both NCI and EI mode.

Table 3
Accuracy of total Se determination by the isotope dilution GC–MS: Comparison with hydride generation AAS

Biological sample	Se concentration by	
	GC–MS[a]	HGAAS[d]
Urine	(28.5±0.9) μg l^{-1}[b]	(27.6±1.2) μg l^{-1}
	(27.3±1.2) μg l^{-1}[c]	
Faeces	(629±34) μg kg^{-1}[b]	(597±38) μg kg^{-1}
	(608±43) μg kg^{-1}[c]	

[a]GC–MS conditions: see Section 2; Mean±S.D. for six replicates; [b]NCI mode; [c]EI mode; [d]HGAAS conditions: Section 2; Mean±S.D. for six replicates.

3.2.4. Detection limit

For measurement of isotopic ratios, the smallest Se level which can be determined with reasonable certainty depends on the selected isotope. Because the limiting factor for isotopic measurement of Se is the determination of the ^{74}Se isotope accounting for 0.89% of isotopic cluster, the signal to noise ratio obtained with the ion at m/z 223 for various amounts of Se injected was determined. A signal to noise ratio of 3 was obtained in EI after injection of 10 ng of Se corresponding to 90 pg of ^{74}Se. NCI allows to determine approximately 1 pg of ^{74}Se with a similar signal to noise ratio.

Table 2
Accuracy of total Se analysis by the isotope dilution GC–MS: Evaluation against Standard Reference Materials (SRM)

SRM	Se concentration experimental[a]	Certified
NIST 1577a	(736±54) μg kg^{-1}[b]	(710±70) μg kg^{-1}
Bovine liver	(756±66) μg kg^{-1}[c]	
NIST 2670	(28.9±1.1) μg l^{-1}[b]	(30±8) μg l^{-1}
Freeze-dried urine (normal level)	(29.9±0.1) μg l^{-1}[c]	

[a]GC–MS conditions: see Section 2; Mean±S.D. for six replicates; [b]NCl mode; [c]EI mode.

Table 4
Repeatability of stable isotope ratio measurements[a]

Sample[b]	$^{74}Se/^{80}Se$	$^{76}Se/^{80}Se$	$^{82}Se/^{80}Se$
Negative Chemical Ionisation			
Se standards:			
250 ng Se (0.5 ng)	0.0213 (8.0%)	0.2043 (3.2%)	0.1922 (2.8%)
100 ng Se (0.2 ng)	0.0191 (10.5%)	0.1897 (4.3%)	0.1974 (0.7%)
Urine (~0.5 ng)	0.0201 (4.0%)	0.1819 (1.0%)	0.1911 (1.1%)
Electron Impact			
Se standards:			
500 ng Se (20 ng)	0.0172 (1.2%)	0.1863 (0.6%)	0.1829 (0.5%)
250 ng Se (10 ng)	0.0160 (5.0%)	0.1844 (1.1%)	0.1818 (0.7%)
Urine (~10 ng)	0.0185 (2.1%)	0.1835 (2.3%)	0.1813 (1.4%)
Theoretical ratio	0.0179	0.1887	0.1760

[a]Experimental conditions according to Table 3 ($n=6$); R.S.D. in parentheses; [b]Se standards/Urine: extracted according to experimental conditions after acid digestion; amount of injected Se is given between brackets.

3.3. Human pilot study

To illustrate the applicability of the method, the Se isotopic enrichment was measured in the faeces and the urine of an adult healthy male volunteer who consumed a milk-based formula containing 40 μg of ^{74}Se-selenite or ^{76}Se-selenate. The results indicated that both absorption and urinary excretion were higher for ^{76}Se-selenate than for ^{74}Se-selenite (Table 6). Urinary excretion of the ^{76}Se-selenate was rapid (>90% of oral dose within 4 h of administration), but was considerably slower for the ^{74}Se-selenite label (see Fig. 2). The overall Se retention however was comparable for both ^{74}Se-selenite and ^{76}Se-selenate labels. The absorption and urinary excretion data obtained by stable isotope methodology for the adult volunteer were comparable to data reported earlier by Robinson and Thomson [19] using natural non-enriched selenate and selenite tracers in a classical balance approach. These results indicate, that the NCI method was able to determine faecal and urinary Se stable isotope enrichments which allow faecal and urinary Se excretion to be determined.

Table 5
Accuracy of isotopic enrichment determination by GC–MS

Isotope	$^{x}Se_{theor.} = a\,^{x}Se_{meas.} + b$[a]	Correlation
Negative Chemical Ionisation		
^{74}Se	$a=0.996$; $b=-0.005$	1.000
^{76}Se	$a=0.969$; $b=-0.001$	1.000
^{82}Se	$a=1.132$; $b=-0.031$	1.000
Electron Impact		
^{74}Se	$a=0.991$; $b=0.641$	0.999
^{76}Se	$a=1.013$; $b=0.231$	0.999
^{82}Se	$a=1.01$; $b=0.034$	0.999

[a]Relation between theoretical $^{x}Se_{theor.}$ and measured $^{x}Se_{meas.}$ isotopic abundance determined for five isotopic enrichment (six replicates); a = slope; b = intercept; GC–MS conditions: according to Section 2 for 250 ng of total Se (injected amount: 0.5 ng for NCI and 10 ng Se for EI).

4. Conclusions

A method for the determination of total Se and of Se isotope ratios was developed to evaluate the absorption and retention of Se from foods by man using stable isotope methodology. The method involved a mineralisation to convert all selenium into selenite and the reaction of selenite with 4-nitro-*o*-phenylenediamine to form a volatile piazselenole in acidic medium. The piazselenole, after extraction

Table 6
Absorption and retention data for pilot study[a]

Se-label	% Absorbed	% Re-excreted	% Retention
^{74}Se-selenite	47.3	10.3	37.0
^{76}Se-selenate	90.8	54.8	36.0

[a]The applicability of this method to measure Se absorption and retention was tested by NCI GC–MS determination of Se isotopic enrichments in the feces and the urine of a adult male healthy volunteer who absorbed a milk-based formula containing ^{74}Se-selenite or ^{76}-Se-selenate; Data are expressed in percent of Se refering to an administered dose of 40 μg Se.

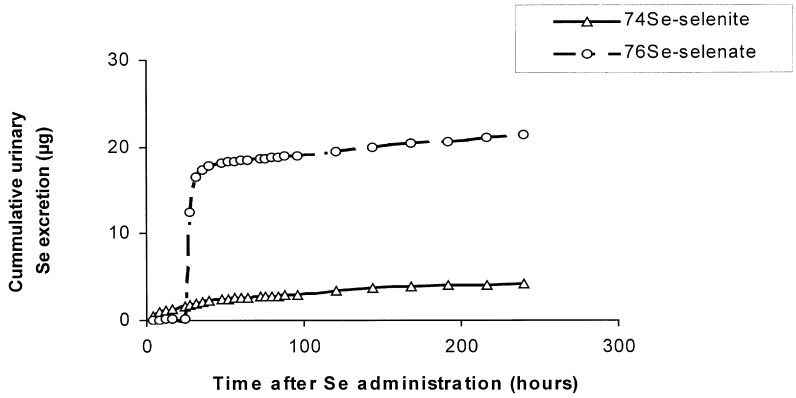

Fig. 2. Urinary excretion pattern of ^{74}Se and ^{76}Se in an adult male volunteer after a single oral dose administration of 40 µg Se as ^{74}Se-selenite or as ^{76}Se-selenate. ^{76}Se-selenate was consumed 24 h after ^{74}Se-selenite was administered.

chloroform, is analysed for its isotopic Se composition by GC–MS working in NCI.

Analytical characteristics of the NCI method were determined and compared to the classical EI method, demonstrating the ability of the NCI technique to accurately determine total Se and Se isotope ratios in biological samples. The detection limit for any selenium isotope was found to be 1 pg for NCI and 90 pg for EI. Repeatability (R.S.D.≤10%) and accuracy for total Se were comparable for both methods. Repeatability of stable isotope ratio measurements for NCI was at R.S.D.≤10%.

The application of the stable isotope methodology for determining the absorption and retention of Se from foods by man was evaluated in a pilot study with an adult male consuming a Se stable isotope labeled food. Data from this study showed, that the stable isotope technique is adequate for measuring Se stable isotope enrichments in biological samples. Therefore this technique can be applied to determine absorption and retention of Se in humans, and due to its lower detection limit compared to EI, NCI is preferential when the sample size is small (e.g. biological samples from infants).

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