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Selenium and its compounds – Determination of selenite and selenate in urine by anion-exchange chromatography-ICP-MS

Biomonitoring Method – Translation of the German version from 2022

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method. This analytical method is used to determine selenite and selenate in urine, which are separated by anion-exchange chromatography and subsequently detected by inductively coupled plasma-mass spectrometry (ICP-MS). Calibration is performed using standard solutions, which are prepared in pooled urine, processed, and analysed analogously to the samples. The analytical method allows for the sensitive and precise determination of selenite and selenate without interferences by other selenium species. The low limits of quantitation enable the determination of very low urinary concentrations of selenite or selenate. However, selenite and selenate do not normally contribute substantially to total selenium content in urine.

1 Characteristics of the method

Matrix	Urine
Analytical principle	High-performance liquid chromatography with inductively coupled plasma-mass spectrometry (HPLC-ICP-MS)

Parameter and corresponding hazardous substances

Hazardous substances	CAS No.	Parameter	CAS No.
Selenium and its compounds	7782-49-2	Selenite	14124-67-5
		Selenate	14124-68-6

Reliability data

Selenite (Se(IV))

Within-day precision:	Standard deviation (rel.)	s_w = 5.3% or 4.5%
	Prognostic range	u = 13.6% or 11.6%
	at a concentration of 0.77 µg or 7.46 µg per litre of urine (as selenium) and n = 6 determinations	
Day-to-day precision [a]:	Standard deviation (rel.)	s_w = 8.1%, 11.8%, or 10.2%
	Prognostic range	u = 20.8%, 30.3%, or 26.2%
	at a concentration of 0.24 µg, 2.92 µg, or 10.7 µg per litre of urine (as selenium) and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 111%
	at a spiked concentration of 2.0 µg per litre of urine (as selenium) and n = 10 determinations	
Detection limit:	0.19 µg per litre of urine (as selenium)	
Quantitation limit:	0.57 µg per litre of urine (as selenium)	

[a] Day-to-day precision data were collected by the external verifier of the method.

Selenate (Se(VI))

Within-day precision:	Standard deviation (rel.)	s_w = 6.6% or 3.9%
	Prognostic range	u = 17.0% or 10.0%
	at a concentration of 0.78 µg or 7.69 µg per litre of urine (as selenium) and n = 6 determinations	
Day-to-day precision [a]:	Standard deviation (rel.)	s_w = 5.6%, 10.3%, or 9.6%
	Prognostic range	u = 14.4%, 26.5%, or 24.7%
	at a concentration of 0.928 µg, 3.37 µg, or 9.16 µg per litre of urine (as selenium) and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 115%
	at a spiked concentration of 2.0 µg per litre of urine (as selenium) and n = 10 determinations	
Detection limit:	0.10 µg per litre of urine (as selenium)	
Quantitation limit:	0.30 µg per litre of urine (as selenium)	

[a] Day-to-day precision data were collected by the external verifier of the method.

2 General information on selenium and its compounds

Selenium (Se; relative atomic mass: 78.97; atomic number: 34) is an element that is found ubiquitously in the environment and which occurs most frequently in compounds in the oxidation states –II, +IV, and +VI. The most important producers of selenium are the USA, Canada, and Japan, which altogether generate about 70% of world production. About 45% of selenium is then used in electrical engineering, 20% in the production of pigments, 8% in the chemical industry, and about 27% in the ceramics and glass industries as well as for other industrial purposes (Butterman and Brown 2004; RÖMPP-Redaktion and Hartwig 2006). Each year, 1000 to 10 000 tonnes of selenium are either produced in or imported into the European Economic Area (ECHA 2022).

Selenium is an essential trace element for humans. In the form of selenocysteine, it is incorporated into the structure of more than 25 different enzymes such as glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases (Lu and Holmgren 2009). As such, selenium plays a major role in a number of physiological processes such as protection against oxidative stress, redox-regulated signalling pathways, and the synthesis of thyroid hormones (Rayman 2000, 2012). Aside from occupational exposure, selenium intake occurs primarily via the diet, whereby a daily intake of 70 µg and 60 µg is recommended for men and women, respectively (D-A-CH 2016). The tolerable upper intake level for selenium lies at 300 µg per day (SCF 2006).

Employees in the workplace are primarily exposed to elementary selenium and inorganic selenium compounds, whereby the selenium compounds are primarily absorbed in the form of water-soluble inorganic compounds via oral, inhalation, and dermal routes. In contrast, the non-occupationally exposed general population ingests mainly organic selenium compounds, like selenomethionine and selenocysteine, via the diet (WHO 1987). The absorption rates for inorganic selenites and selenates lie between 62–84% and 92–94%, respectively, and those for organic selenium compounds lie between 75–95% (Rettenmeier 2019).

Selenium is eliminated biphasically with average half-lives of 2.4 ± 0.3 and 162 ± 9 days. Inorganic selenites or selenates are excreted more rapidly than organic selenium compounds (such as selenomethionine), which can be explained by the incorporation of selenomethionine in proteins (RKI 2006).

The evaluation of essential selenium supply as well as any excessive selenium exposure is usually carried out by examining selenium levels in blood plasma. There are only a few studies on renal selenium excretion following occupational exposure (Göen and Greiner 2018), most of which consist of determining total selenium content in employee urine (Table 1).

Tab. 1 Selenium concentrations in urine following occupational exposure

Collective (Sample number n)	Sample matrix	Selenium (mean ± SD (range))		References
		Workers	Controls	
Selenium-processing plant, Germany (20; 20 controls)	Urine (post-shift)	107 µg/g creatinine (16–816 µg/g creatinine) [a]	23 µg/g creatinine (12–50 µg/g creatinine) [a]	Göen et al. 2015
Selenium-processing plant, Germany (14; 18 controls)	Urine (pre-shift)	50.6 µg/g creatinine (20.7–253 µg/g creatinine) [a]	18.7 µg/g creatinine (9.20–40.6 µg/g creatinine) [a]	Greiner et al. 2020
	Urine (post-shift)	71.8 µg/g creatinine (22.1–340 µg/g creatinine) [a]		
Selenium-rectifier manufacture, England (1517 samples from 200–300 workers; 793 controls)	Urine	84 µg/l	34 ± 24 µg/l (0–150 µg/l)	Glover 1967
Copper refinery, Canada (20)	Urine	92.9 ± 42.8 µg/l (34.0–190 µg/l)	74.6 ± 25.3 µg/l (26.7–118 µg/l)	Rajotte et al. 1996
Steel production, Taiwan (23; 23 controls)	Urine	67.7 ± 27.4 µg/l (24.1–114 µg/l)	33.2 ± 12.9 µg/l (13.0–58.9 µg/l)	Horng et al. 1999

[a] Median (range)

For selenium and its inorganic compounds, the Commission has derived a biological tolerance value (BAT value) of 150 µg selenium/l plasma and a maximum workplace concentration (MAK value) of 0.02 mg selenium/m^3 E (as selenium). In addition, selenium and its inorganic compounds have been classified as Category 3 carcinogens and are designated with an "H," as they are readily absorbed through the skin. Details on the toxicological evaluation can be found in the corresponding MAK documentation published by the Commission (DFG 2021; Hartwig 2014, 2015). Furthermore, the Commission has derived a biological reference value (BAR) of 30 µg selenium/g creatinine for the urine matrix (Greiner et al. 2021). Only German and Western European studies were used to derive this BAR, as the varying selenium concentrations in soil substantially influence the selenium concentrations in food products and, in turn, selenium intake (Combs 2001).

The absorbed inorganic and organic selenium compounds are reduced to selenide, whereby this reaction is glutathione-dependent for selenites and selenates. Selenide is the central metabolic selenium species (see Figure 1) and serves as a common source for the synthesis of selenoproteins and selenosugars (Birringer et al. 2002; Fairweather-Tait et al. 2011; Navarro-Alarcon and Cabrera-Vique 2008; Ohta and Suzuki 2008). Excess selenide can be eliminated via three metabolic pathways. The most important elimination products of selenium are the selenium-containing sugars (Francesconi and Pannier 2004; Kuehnelt et al. 2005). So far, the three compounds methyl-2-acetamido-2-deoxy-1-seleno-*β*-*D*-galactopyranoside (SeSug 1), methyl-2-acetamido-2-deoxy-1-seleno-*β*-*D*-glucopyranoside (SeSug 2), and methyl-2-amino-2-deoxy-1-seleno-*β*-*D*-galactopyranoside (SeSug 3) have been detected in human urine, with SeSug 1 being the most prominent selenosugar (Hildebrand et al. 2020; Jäger et al. 2013). Another metabolic pathway involves methyltransferases, which convert the intermediate selenide to methylated compounds such as monomethylselenol, dimethylselenide, and the trimethylselenonium ion (TMSe) (Kremer et al. 2005; Ohta and Suzuki 2008). Dimethylselenide is excreted via the skin (Ganther 1986) and lungs (Jiang et al. 1983), and TMSe is excreted renally (Kuehnelt et al. 2006). The excretion of TMSe via the kidneys is subject to major interindividual variations ranging from trace concentrations up to the primary elimination product (Gammelgaard and Jøns 2000; Kuehnelt et al. 2006; Lu and Holmgren 2009). In a study in Germany, TMSe was either not or only marginally detectable in 80% of the study participants, but was found to be the main metabolite in the remaining 20% (Jäger et al. 2013). For the selenium species selenate, it has been shown that a large proportion of orally ingested selenate is excreted renally in its unmetabolised form, and is thus not available to the central selenide pool (Gammelgaard et al. 2012; Jäger et al. 2016). A portion of selenium is also excreted via the biliary tract and is subject to enterohepatic circulation (RKI 2006).

The individual selenium compounds (species), which are absorbed by the body or formed metabolically, may differ considerably in terms of their physical properties as well as their toxicological potential (Nuttall 2006). The determining factor for bioavailability and for biochemical or toxic effects is the type and amount of the selenium species, which is not reflected by the total concentration of selenium in urine (Cornelis et al. 1993; Kiss and Odani 2007; Lund 1990; Michalke 2002 a, b). Therefore, an analytical differentiation of the relevant selenium species found in urine is imperative for the evaluation of both occupational and non-occupational exposure.

The "Analyses in Biological Materials" working group developed and validated three methods allowing for the quantification of a total of eleven selenium species which utilise various liquid-chromatographic separation mechanisms (I: anion-exchange chromatography; II: reversed-phase chromatography; III: cation-exchange chromatography). Due to these different liquid-chromatographic separation mechanisms, the three submethods are published separately. Figure 2 shows the structures of the selenium species which can be determined with the three submethods.

The determination of selenite and selenate is performed using anion-exchange chromatography and is described as part of the hereby presented Submethod I.

Data on selenite and selenate concentrations in urine following occupational exposure have not been published. The published concentrations of these species have been determined using urine samples either from the non-occupationally exposed general population or after supplementation with selenium-containing preparations.

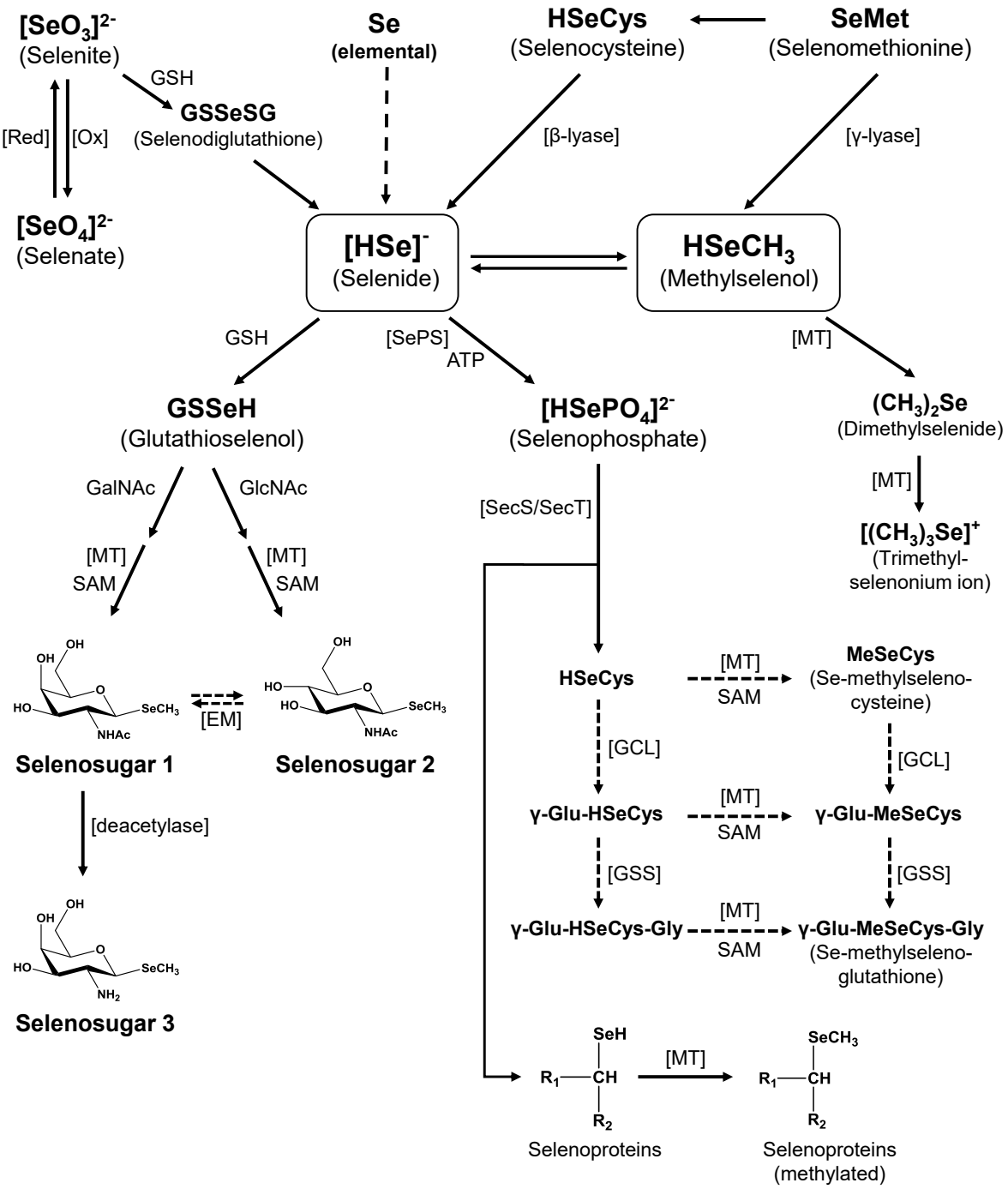

Fig. 1 Metabolism scheme of selenium according to Navarro-Alarcon and Cabrera-Vique (2008) as well as Fairweather-Tait et al. (2011). EM: epimerase; GalNAc: *N*-acetylgalactosamine; GlcNAc: *N*-acetylglucosamine; GCL: glutamate–cysteine ligase; GSH: glutathione; GSS: glutathione synthetase; MT: methyltransferase; SAM: *S*-adenosyl methionine; SecS/SecT: *L*-seryl-tRNASec selenium transferase; SePS: selenophosphate synthetase

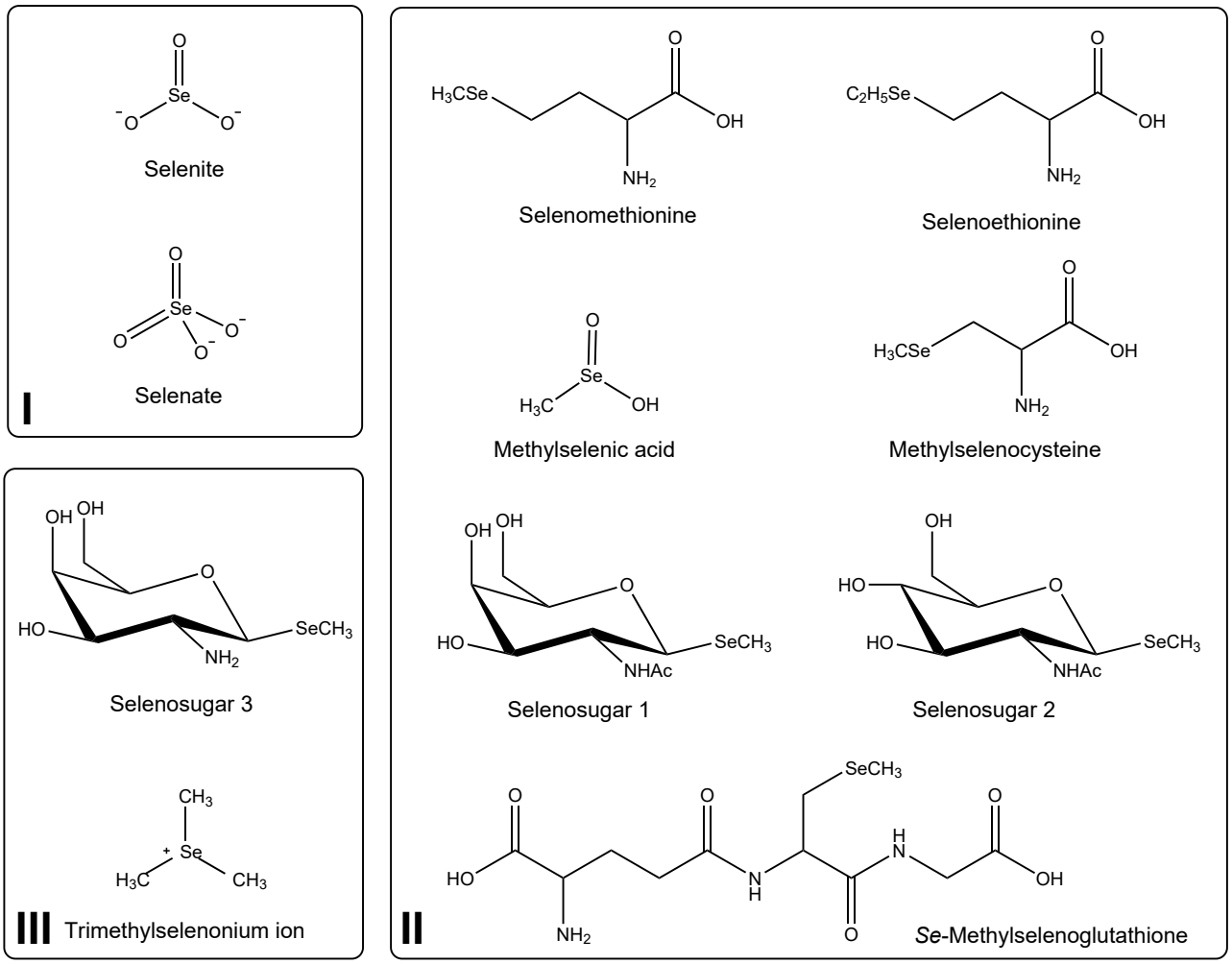

Fig. 2 Structures of the selenium species which can be determined with the three submethods

In more recent studies, selenite concentrations were found which ranged from below the quantitation limit (Lajin et al. 2016) up to 7.1 µg Se/l (Gammelgaard and Jøns 2000). In urine samples from Germany, selenite could not be detected in either the general population or after supplementation (Jäger et al. 2013, 2016) (see Section 11.4). Published selenate concentrations in urine ranged from below the detection limit (Gammelgaard and Jøns 2000; Zheng et al. 2002) to over 80 µg Se/l (Eichler et al. 2015). In urine samples from the German general population (n = 47), selenate background levels of 0.19–0.82 µg Se/l (19% of the samples > LOD) were determined (Jäger et al. 2013). After supplementation with sodium selenite, selenate excretions of 1.2 ± 0.4 µg Se/24 h were found (Jäger et al. 2016).

3 General principles

The analytical method described herein is used to determine selenite and selenate in urine. The urine samples are diluted with eluent, and the analytes are separated by anion-exchange chromatography and subsequently detected by ICP-MS. Calibration is performed using standard solutions, which are prepared in pooled urine, processed, and analysed analogously to the samples.

4 Equipment, chemicals, and solutions

4.1 Equipment

- HPLC system (e.g. Agilent 1200 series with a binary pump (G1312A) and an autosampler (G1329A), Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Inductively coupled plasma-mass spectrometer with collision/reaction cell (e.g. Agilent 7500cx, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Analytical column: Hamilton PRP-X100 PEEK (150 × 4.6 mm; 5 µm) with PRP-X100 PEEK guard column (8 × 3 mm; 10 µm) (e.g. Hamilton Bonaduz AG, Bonaduz, Switzerland)
- Ultrasonic bath (e.g. VWR International GmbH, Darmstadt, Germany)
- Vortex mixer (e.g. Heidolph Instruments GmbH & Co. KG, Schwabach, Germany)
- pH meter (e.g. VWR International GmbH, Darmstadt, Germany)
- Precision balance (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Cellulose filter, pore size of 0.45 µm, membrane of mixed cellulose esters (e.g. Merck KGaA, Darmstadt, Germany)
- 0.7-ml snap-top polypropylene microvials (e.g. VWR International GmbH, Darmstadt, Germany)
- 1.5-ml vials (e.g. Eppendorf AG, Hamburg, Germany)
- 1000-ml beakers (e.g. VWR International GmbH, Darmstadt, Germany)
- 10-, 100-, and 1000-ml volumetric flasks (e.g. VWR International GmbH, Darmstadt, Germany)
- 100-ml measuring cylinders (e.g. VWR International GmbH, Darmstadt, Germany)
- Urine cups (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Various pipettes and Multipettes® with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade.

- Formic acid 98–100% (e.g. No. 100264, Merck KGaA, Darmstadt, Germany)
- Diammonium oxalate monohydrate (e.g. No. 101192, Merck KGaA, Darmstadt, Germany)
- Methanol ≥ 99.9% (e.g. No. 34860, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Milli-Q® Direct Water Purification System, Merck KGaA, Darmstadt, Germany)
- Pooled urine from individuals who are neither occupationally exposed to selenium nor ingest selenium via nutritional supplements; refrigerated for 48 h
- Argon 5.0 (Linde GmbH, Pullach, Germany)
- Hydrogen 5.0 (Linde GmbH, Pullach, Germany)

4.3 Reference standards

- Sodium selenite pentahydrate ≥ 99% (e.g. No. 15670510, Fisher Scientific GmbH, Schwerte, Germany)

- Sodium selenate ≥ 95% (e.g. No. S0882, Merck KGaA, Darmstadt, Germany)
- Selenium ICP standard Certipur®, SeO_2 in 2–3% HNO_3, 1000 mg Se/l (e.g. No. 1.70350, Merck KGaA, Darmstadt, Germany)

4.4 Solutions

- Formic acid (2.5%)
  Approximately 50 ml of ultra-pure water are placed into a 100-ml volumetric flask. After adding 2.5 ml of formic acid, the flask is made up to the mark with ultra-pure water.
- Diammonium oxalate solution (100 mmol/l)
  1.42 g of diammonium oxalate monohydrate are weighed out into a 100-ml volumetric flask and dissolved in ultra-pure water. The flask is then made up to the mark with ultra-pure water.
- Diammonium oxalate solution (10 mmol/l) with 2% methanol (pH = 5.0)
  Approximately 800 ml of ultra-pure water are placed into a 1000-ml beaker, and 100 ml of diammonium oxalate solution (100 mmol/l) as well as 20 ml of methanol are added. The pH value is adjusted to pH = 5.0 using 2.5% formic acid. The solution is subsequently transferred into a 1000-ml volumetric flask, which is then made up to the mark with ultra-pure water.
  The eluent is freshly prepared for each analytical run, filtered prior to use, and degassed in an ultrasonic bath for five minutes.

4.5 Calibration standards

Due to the instability of selenite (see Section 11.4), the stock, working, and spiking solutions, which are used for the preparation of the calibration standards, are prepared in pooled urine which has been refrigerated for 48 h.

- Stock solutions (1000 mg Se/l)
  33 mg of sodium selenite pentahydrate (263 g/mol) and 24 mg of sodium selenate (188.9 g/mol) are each weighed into a 10-ml volumetric flask. The flasks are then made up to the mark with ultra-pure water.
  The solutions thus prepared contain about 1 g Se/l. In order to determine the exact selenium concentration, the stock solutions are measured against an ICP single-element standard to correct for any potential weighing errors.
- Working solutions (10 mg Se/l)
  Depending on the measured concentrations of the stock solutions, about 1000 µl of each stock solution are pipetted into 100-ml volumetric flasks. The flasks are made up to the mark with ultra-pure water.
- Spiking solution I (1 mg Se/l)
  100 µl of each working solution are pipetted into a 1.5-ml vial. Subsequently, 800 µl of ultra-pure water are added by pipetting and the solution is thoroughly mixed.
- Spiking solution II (0.1 mg Se/l)
  100 µl of spiking solution I are mixed with 900 µl of ultra-pure water in a 1.5-ml vial.

The stock and working solutions of the analytes are stored at –18 °C. Especially with regard to selenite, the method user must ensure the stability of the prepared solutions in cases of long-term storage.

For the preparation of the calibration standards, spiking solutions I and II are brought to a final volume of 10 ml with pooled urine, according to the pipetting scheme given in Table 2. The spiking and calibration solutions are freshly prepared for each analytical run. The calibration standards are processed analogously to the samples as described in Section 5.2.

Tab. 2 Pipetting scheme for the preparation of calibration solutions for the determination of selenite and selenate in urine

Calibration standard	Spiking solution I [µl]	Spiking solution II [µl]	Pooled urine [ml]	Concentration [µg Se/l]
0	–	–		0
1	–	20		0.2
2	–	50		0.5
3	–	200	ad 10	2
4	50	–		5
5	100	–		10
6	250	–		25

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine samples are collected in urine cups. Due to the instability of selenite (see Section 11.4), the samples should be processed and analysed – if possible – directly after specimen collection. If this is not possible, the urine samples should be stored at –18 °C directly after specimen collection and are to be analysed as soon as possible. In this case, the method user must take measures to ensure the stability of selenite in the samples.

5.2 Sample preparation

If necessary, the urine samples are brought to room temperature and thoroughly mixed. 300 µl of the sample are transferred into a 0.7-ml polypropylene microvial. The sample is mixed with 300 µl of eluent and subsequently thoroughly mixed for ten seconds on the vortex mixer. Of the sample thus diluted, 50 µl are applied for analysis.

6 Operational parameters

Analytical determination was performed on an HPLC system with inductively coupled plasma-mass spectrometry (HPLC-ICP-MS).

6.1 Liquid chromatography

Analytical column:	Hamilton PRP-X100 PEEK (150 × 4.6 mm; 5 µm) with PRP-X100 PEEK guard column (8 × 3 mm; 10 µm)
Separation principle:	Anion-exchange chromatography
Eluent:	Diammonium oxalate solution (10 mmol/l) with 2% methanol (pH = 5)
Flow rate:	0–6 min: 0.8 ml/min
	6–18 min: 1.2 ml/min
Injection volume:	50 µl
Runtime:	18 min

All given parameters must be optimised in accordance with the manufacturer's specifications.

6.2 Inductively coupled plasma-mass spectrometry

Rf power:	1500 W
Nebuliser:	MikroMist
Spray chamber:	Scott Quartz
Spray-chamber temperature:	10 °C
Carrier gas:	0.9 l argon/min
Make-up gas:	0.2 l argon/min
Sampling/skimmer cone:	Nickel
Reaction-/collision gas:	H_2
Reaction-/collision-gas flow rate:	3.5 ml/min
Analysis mode:	Spectrum Multi Tune
Number of measurements per mass trace (repetition):	1
Parameter-specific settings:	see Table 3

The device-specific parameters must be determined and set individually by the user for the ICP-MS system utilised. The parameters listed in this section have been determined and optimised for the instrument configuration used for method development. The selected mass traces as well as the retention times are summarised in Table 3. The mass trace ^{78}Se is used for quantification.

Tab. 3 Retention times and parameter-specific settings for the determination of selenite and selenate in urine

Analyte	Retention time [min]	Analysed mass trace	Measurement time per mass trace [s]
Selenite	3.9	^{78}Se	0.5
		^{82}Se	0.3
		^{77}Se	0.3
Selenate	12.0	^{78}Se	0.5
		^{82}Se	0.3
		^{77}Se	0.3

7 Analytical determination

50 µl of each sample prepared as described in Section 5.2 are injected into the HPLC-ICP-MS system. Identification of selenite and selenate is based on their individual retention times. The retention times given in Table 3 can only serve as a point of reference. The user must ensure proper separation performance of the HPLC column used and the resulting retention behaviour of the analytes. A reagent blank (ultra-pure water instead of a urine sample) is included in each analytical run. Figure 3 shows a representative chromatogram of a urine sample spiked with selenite and selenate.

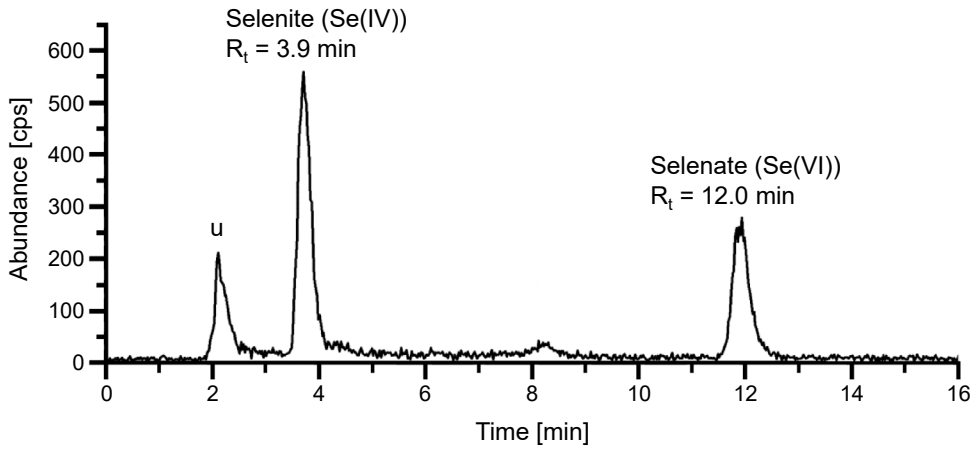

Fig. 3 Chromatogram of a urine sample spiked with selenite (5 µg Se/l) and selenate (5 µg Se/l); u = unknown metabolite

8 Calibration

The calibration standards (see Section 4.5) are processed analogously to the urine samples (see Section 5.2) and analysed. Calibration curves are obtained by plotting the peak areas of the analytes against the concentration of the corresponding calibration standard. The calibration curves for selenite and selenate are linear under the described conditions from 0.25–25.0 µg/l (as selenium). Figure 4 shows representative calibration curves for the determination of selenite and selenate in urine.

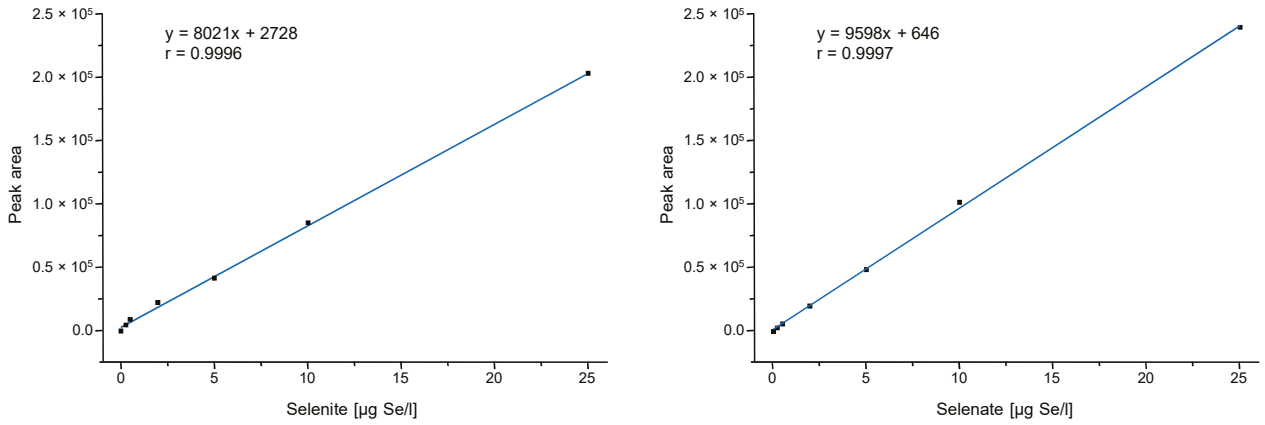

Fig. 4 Calibration curves for the determination of selenite und selenate in urine

9 Calculation of the analytical results

The analyte concentration of a sample in µg/l urine (as selenium) can be calculated by inserting the peak area of the corresponding analyte into the calibration function of the respective analytical run. It may be necessary to adjust the calibration range to the expected range of concentrations. If the measured result lies above the calibration range, the sample in question is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

For quality assurance, two quality-control samples with different concentrations are processed and analysed analogously to the samples as part of each analytical run. As reference material is not currently commercially available, it must be prepared in the in-house laboratory using pooled urine from individuals who are neither occupationally exposed to selenium nor ingest selenium via nutritional supplements. The pooled urine is spiked with 0.75 µg/l (as selenium; Q_{low} – low quality-control material) or 7.5 µg/l (as selenium; Q_{high} – high quality-control material) of each species. Prior to the preparation of the quality-control materials, the pooled urine should be refrigerated for 48 h at 4 °C in order to ensure the stability of the spiked selenite. The quality-control materials thus prepared are aliquoted to 300 µl and frozen at –18 °C until use.

The nominal values and tolerance ranges of the quality-control materials are determined in a pre-analytical period (one analysis of each control material on ten different days) (Bader et al. 2010).

11 Evaluation of the method

The reliability of this method was confirmed by comprehensive validation as well as by replication and verification in a second, independent laboratory.

11.1 Precision

Within-day precision

For the determination of within-day precision, the Q_{low} and Q_{high} quality-control materials were processed and analysed six times in parallel on one day. The within-day precision data thus obtained are summarised in Table 4.

Tab. 4 Within-day precision for the determination of selenite and selenate in urine (n = 6)

Analyte	Spiked concentration [µg Se/l]	Measured concentration [µg Se/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Selenite	0.75	0.77	5.3	13.6
	7.5	7.46	4.5	11.6
Selenate	0.75	0.78	6.6	17.0
	7.5	7.69	3.9	10.0

Day-to-day precision

The day-to-day precision data for the determination of selenite and selenate were collected by the external verifier of the method. To this end, unspiked pooled urine as well as pooled urine, which was spiked with 2.5 µg/l or 10 µg/l of selenite and selenate (as selenium), was processed and analysed on six different days. The data thus obtained are presented in Table 5.

Tab. 5 Day-to-day precision for the determination of selenite and selenate in urine (n = 6)

Analyte	Spiked concentration [µg Se/l]	Measured concentration [µg Se/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Selenite	0.0	0.24	8.1	20.8
	2.5	2.92	11.8	30.3
	10.0	10.7	10.2	26.2
Selenate	0.0	0.93	5.6	14.4
	2.5	3.37	10.3	26.5
	10.0	9.16	9.6	24.7

11.2 Accuracy

To check the accuracy of the method, individual urines (creatinine concentrations in the range of 0.25–2.21 g/l) from ten persons were each spiked with 2 µg/l (selenite and selenate, as selenium), processed, and analysed. In addition, the unspiked urines were processed and analysed. Relative recovery was calculated using the concentrations ascertained from the spiked urines, subtracting any background values of the analytes which arose in the unspiked urines. The relative recovery rates thus obtained are presented in Table 6.

Tab. 6 Mean relative recovery rates for the determination of selenite and selenate in ten individual urines

Analyte	Spiked concentration [µg Se/l]	Mean rel. recovery r [%]	Range [%]
Selenite	2.0	111	105–118
Selenate	2.0	115	103–125

11.3 Limits of detection and quantitation

The limits of detection and quantitation were determined in accordance with DIN 32645 (DIN 2008). To this end, an equidistant 10-point calibration was established, processed, and analysed in conjunction with a blank value (unspiked pooled urine). In accordance with DIN 32645, the limits of detection and quantitation were calculated based on the standard deviations of the calibration curves thus obtained. Table 7 shows the limits of detection and quantitation ascertained for selenite and selenate.

Tab. 7 Limits of detection and quantitation for the determination of selenite and selenate in urine (n = 3)

Analyte	Limit of detection [µg Se/l]	Limit of quantitation [µg Se/l]
Selenite	0.19	0.57
Selenate	0.10	0.30

11.4 Sources of error

During method development, it became evident that the selenite concentration in some freshly voided urine samples decreased significantly within a few hours. By repeated measurements of these fresh urine samples, which had been spiked with selenite (25 µg/l as selenium), the peak areas of selenite declined by 60% after a five-hour period of storage at room temperature without any new peaks from degradation products arising in the chromatogram. The total selenium concentration in the stored samples remained constant. When pooled urine, which had been previously refrigerated for 48 hours at 4 °C, was spiked and analysed after storage at room temperature, no degradation of selenite was observed (see Figures 5 and 6).

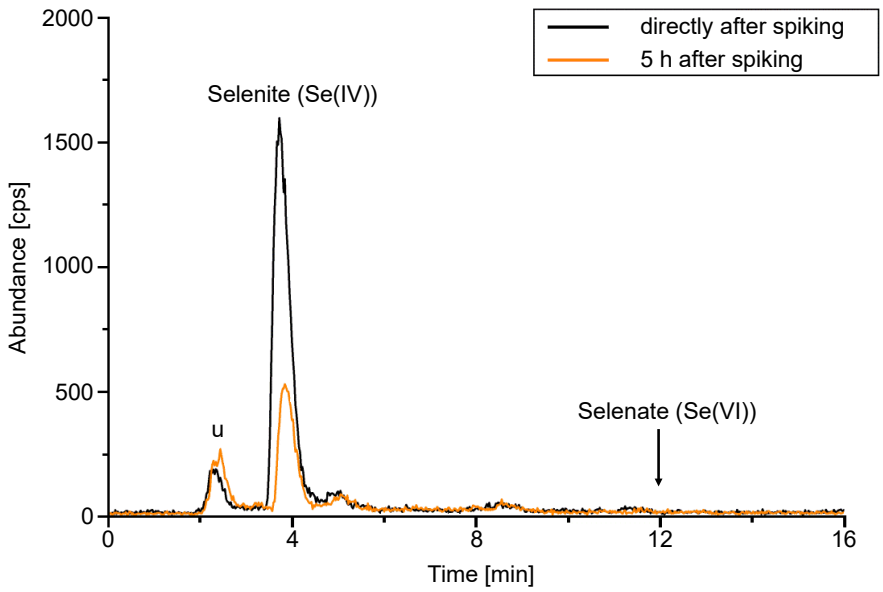

Fig. 5 Chromatogram of a urine sample spiked with selenite (25 µg Se/l) (black: directly after spiking; orange: measurement after the sample had been stored in the autosampler for 5 hours at room temperature; u = unknown selenium species from background exposure)

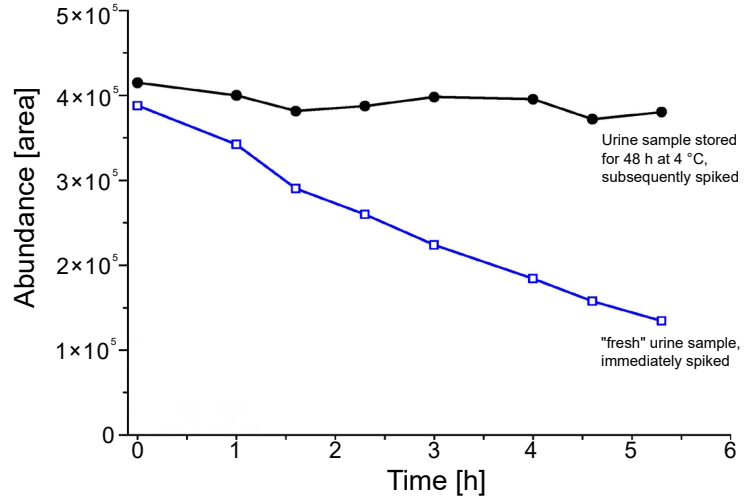

Fig. 6 Peak areas of selenite during sample storage at room temperature (fresh urine sample or a urine sample previously refrigerated for 48 h, each spiked with selenite (25 µg Se/l))

The instability of selenite in urine samples has also been described by other working groups (Eichler et al. 2015; Gammelgaard and Jøns 2000; Gómez et al. 1998). A 5% loss within 24 hours in pooled urine which had been spiked with selenite (20 µg Se/l) was observed by Gammelgaard and Jøns (2000). A 30% loss in urine spiked with 100 and 200 µg Se/l as selenite within five hours was found by Gómez et al. (1998). The stability of various selenium species in urine under different storage conditions was investigated by Eichler et al. (2015). While selenate was stable even at room temperature over a storage period of ten weeks, the added selenite had almost completely dissipated after three days at room temperature, at −5 °C, and at −35 °C. A detailed storage study revealed that urine samples used for selenite determination

should be frozen directly following sample collection and analysed within twelve hours. At room temperature, urine samples must be analysed within three hours when selenite is to be determined.

A possible explanation for the instability of selenite may be that selenite is reduced to elementary selenium by reduction agents found in the urine matrix, such as ascorbic acid (Biswas et al. 2011; Jäger 2014), and is therefore not detectable with the biomonitoring methods used in each case. Gómez et al. (1998), however, assume that organic colloids are formed in the urinary matrix over time which adsorb selenite and are retained on the HPLC column.

For the user of this method, this means that, in urine samples in which selenite is to be determined, analysis should take place within three hours of specimen collection. The developers of this method have not investigated the influence of freezing and thawing of the urine samples or of possible additives to stabilise urinary selenite concentration.

12 Discussion of the method

The method allows for the selective and sensitive determination of selenite and selenate in human urine. The diammonium oxalate solution (10 mmol/l) with 2% methanol and a pH value of 5.0, which is used as the eluent, allows the separation of selenite and selenate within 18 minutes without interferences by other selenium species. The applied procedure is characterised by relatively easy sample preparation, as the samples are simply diluted 1:1 with eluent and are subsequently directly analysed. The limits of quantitation of selenite and selenate are 0.57 µg and 0.30 µg per litre of urine (as selenium), respectively, and are therefore sufficient to determine even low urinary concentrations of selenite or selenate.

Investigations suggest that both selenite and selenate only contribute substantially to total selenium content in urine samples following specific selenite or selenate exposure.

Instruments used Agilent 1200 series HPLC system with a binary pump (G1312A) and an autosampler (G1329A) (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany) as well as Agilent 7500cx inductively coupled plasma-mass spectrometer with collision/reaction cell (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

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