Quantification of SeMet and SeCys in Biological Fluids and Tissues by Liquid Chromatography Coupled to Inductively Coupled Plasma Mass Spectrometry (HPLC-ICP MS)

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Véronique VACCHINA(1*), Katarzyna BIERLA(2), Joanna SZPUNAR(2) and Ryszard LOBINSKI(2)

(1) UT2A, Hélioparc Pau-Pyérénées, 2 avenue du president Angot, 64000 Pau, France

(2) IPREM-LCABIE, Hélioparc Pau-Pyrénées, 2 avenue du président Angot, 64000 Pau, France

(*) corresponding author : veronique.vacchina@univ-pau.fr

Running Head : Selenoamino-acids determination in biological samples by HPLC - ICP MS

Abstract

Selenium (Se) is an element readily absorbed during the intestinal tract and distributed in the body. In biological fluids, tissues and animal products, Se is known to be present mainly in the form of a selenoamino-acid (selenomethionine (SeMet) or selenocysteine (SeCys)). Both amino-acids have different biological activity which justifies their discrimination. Here, we describe the method allowing the simultaneous determination of SeMet and SeCys in blood/plasma, animal tissues, milk and eggs by two-dimensional Liquid Chromatography coupled to Inductively Coupled Plasma Mass Spectrometry (2D HPLC – ICP MS).

Keywords

selenomethionine – selenocysteine – HPLC - ICP MS – biological fluids and tissues

1. Introduction

Selenium (Se) is an element readily adsorbed by the intestinal tract [1] and distributed via the blood into different organs (offal tissues, edible tissues…) or animal products when appropriate (milk, eggs…). Most of the Se present in these samples is known to be proteins bound [2] and to occur in the form of two selenoamino-acids. One of them, the selenocysteine (SeCys), is considered biologically active and forms the active center of selenoproteins like glutathione-peroxydases, thioredoxin reductases and selenoprotein P. The other one, the selenomethionine (SeMet), is supposed to be randomly incorporated into methionine-containing proteins. The difference in function of these amino-acids demands accurate methods for their routine determination.

The HPLC – ICP MS coupling has been predominantly used for speciation analysis of low-molecular weight selenospecies [3]. In particular, the quantification of SeMet by HPLC –

ICP MS following proteolytic digestion to release the free amino-acids has been addressed in different samples [4]. But the determination of SeCys is more complicate due to the instability of this species.

The aim of the paper is therefore to describe the analytical methods allowing the simultaneous determination of both SeMet and SeCys in biological fluids, tissues and animal products by two-dimensional Liquid Chromatography coupled to Inductively Coupled Plasma Mass Spectrometry (2D HPLC – ICP MS). The methods are summarized Figure 1 and are described separately elsewhere [5-8].

2. Materials

Prepare all solutions using ultrapure water (18.2 MΩ.cm) and analytical grade reagents. Prepare and store all reagents at room temperature (unless stated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1. Reagents

1. 100 mM Tris-HCl (pH 7.5) solution : dissolve 12 g of Tris (99.9 %) in 1 L of ultrapure water and adjust the pH to 7.5 by dropwise addition of HCl.
2. 6 M urea solution : dissolve 3.6 g of urea (99%) in 10 mL of 100 mM Tris-HCl buffer.
3. 7 M urea solution : dissolve 4.2 g of urea (99%) in 10 mL of 100 mM Tris-HCl buffer.
4. 0.2 M dithiothreitol (DTT) solution : dissolve 0.31 g of DTT (≥ 99%) in 10 mL of 100 mM Tris-HCl buffer.
5. 0.5 M DTT solution : dissolve 0.78 g of DTT (≥ 99%) in 10 mL of 100 mM Tris-HCl buffer.
6. 0.5 M iodoacetamide (IAM) solution : dissolve 0.9 g of IAM (≥ 99%) in 10 mL of ultrapure water.

7. 2% protease solution : dissolve 0.2 g of protease (type XIV) in 10 mL of 100 mM Tris-HCl buffer.

8. 1% lipase solution : dissolve 0.1 g of lipase (type VII) in 10 mL of 100 mM Tris-HCl buffer.

9. 100 mM ammonium acetate solution : dissolve 7.7 g of ammonium acetate (> 98%) in 1 L of ultrapure water and adjust the pH to 7.5 by dropwise addition of NH_4OH.

10. Methanol, HPLC grade.

11. 30% ammonium hydroxyde (NH_4OH) (Instra).

12. 0.1% heptafluorobutyric acid (HFBA) solution : add 100 µL of HFBA (HPLC grade) in 1 L of ultrapure water or methanol.

13. 37% hydrochloric acid (HCl) (Instra).

14. 1000 µg Se/mL selenomethionine (SeMet) : dissolve 25 mg of SeMet (≥ 99%) in 10 mL of ultrapure water.

15. 10 µg Se/mL SeMet: dilute 50 µL of the 1000 µg Se/mL SeMet solution in 4.95 mL of ultrapure water.

16. Acetone, HPLC grade (only for milk samples).

17. Cyclohexane, HPLC grade (only for milk samples).

2.2. Instrumentation

1. freezer.

2. grinder (only for tissue samples).

3. sieve (only for tissue samples).

4. analytical grade balance.

5. adjustable volume pipettes.

6. plastic tubes.

7. bottles fitting with the HPLC system.

8. elliptic table.

9. sonication probe (only for tissue samples).

10. shaking water bath.

11. centrifuge.

12. ultracentrifuge (only for tissue samples).

13. freeze-drier.

14. fraction collector.

15. proofer (only for milk samples).

16. Superdex HPLC column (300 x 10 mm) (Amersham Biosciences).

17. Altima C8 HPLC column (150 x 4,6 mm x 5 µm) (Alltech)

18. binary HPLC pump coupled to an ICP MS equipped with a collision/reaction cell filled with H_2 and with an organic kit (*see* **Notes 1, 2 and 3**).

3. Methods

Carry out all procedures at room temperature unless stated otherwise.

3.1. Samples storage and pre-treatment

1. For blood samples, a Se-free anti-coagulant has to be added at the time of the sampling in the sampling tube.

2. Fresh tissues and fluids samples have to be stored at -20°C.

3. All the samples have to be freeze-dried prior analysis. For tissues, an additional step of grinding and sieving has to be performed.

3.2. Protocol for defatting

This is only applicable for milk and egg samples.

1. Take around 2.5 g of sample in a plastic tube.

2. Add 10 mL of cyclohexane.

3. Shake the tube on an elliptic table during 10 min.

4. Centrifuge (1000 g, 3 min).

5. Remove the supernatant.

6. Repeat steps 2 to 4 two times on the residue.

7. Dry the residue in a proofer at 37°C during 17 h.

3.3. Protocol for proteins precipitation

This is only applicable for milk samples.

1. Add 10 mL of ultrapure water on the preliminary residue.

2. Add 30 mL acetone.

3. Shake 10 min on an elliptic table.

4. Centrifuge (1000 g, 5 min).

5. Remove the supernatant.

6. Dry the residue in a proofer at 37°C during 17 h.

3.4. Protocol for extraction

This is applicable for all the matrices. The mass/volume uptakes and buffer/salts concentrations are given Table 1.

1. Precisely weight 100 mg (80 mg in the case of tissue samples) of each sample in a plastic tube. Include at least one tube devoted for a blank. One sample should be weighed in 4 different tubes (**see Note 4**).

2. Add 850 µL of ultrapure water (this is only applicable for blood and plasma samples).

3. Add the urea solution (see **Note 5**).

4. For tissue samples, sonicate the solution (amplitude 20%, pulse on 1, pulse off 1) keeping the tube in ice. Each sample should be sonicated two times not consecutive (see **Note 6**). Ultra-centrifuge (20 min, 7500 g, 4°C) and transfer the supernatant into another plastic tube. For the other matrices than tissues, shake the tube 1 h on an elliptic table.

5. Add the DTT solution (DTT-1 in Table 1) (see **Note 7**).

6. Shake 1 h on an elliptic table.

7. Add the IAM solution (see **Note 8**).

8. Shake 1 h on an elliptic table away from light.

9. Add the DTT solution (DTT-2 in Table 1) (see **Note 9**).

10. Shake 1 h on an elliptic table.

11. Add the Tris buffer (see **Note 10**).

12. Proceed to the addition of the SeMet standards in the tubes devoted for that. The volume given in Table 1 corresponds to the lowest spike. The intermediate one should be twice this volume and the highest one should be three times this volume.

13. Add the proteolytic solution (see **Note 11**).

14. Shake 17 h at 37°C.

15. Freeze-dry the solution.

16. Dissolve the dried material in 1 mL of ultrapure water.

17. Centrifuge the solution (9500 rpm, 10 min) and put the supernatant in an HPLC vial.

3.5. Purification of the extract by size-exclusion chromatography

(see **Note 12**).

1. Check the chromatographic profile on one sample (see **Note 13**). The chromatographic conditions are given Table 2.

2. For all the samples, collect the low molecular weight fraction starting around 1 min before the first peak and ending 1 min after the last peak (see **Note 15**).

3. Each maximum 8 samples, inject the 0.2 M DTT solution without collecting fractions to clean the column.

4. Freeze-dry the fraction collected.

5. Dissolve the freeze-dried material in 500 µL of ultrapure water and put the solution in an HPLC vial.

6. Add 10 µL of the 0.2 M DTT solution.

3.6. Analysis by RP HPLC – ICP MS

1. The chromatographic conditions are detailed in Table 3.

2. The sequence of analysis should be as follow : the blanks, the standard addition curve in the rising order of the concentrations and the samples.

3.7. Calculations

The validation of the standard addition curve should be made according to the rules in force in the laboratory.

1. The concentrations in the raw sample are calculated according the following equation :

$$C_i = \frac{A_i}{m \times S}$$

with : C_i : concentration for the species i

A_i : area of the peak of the species i

S : slope of the standard addition curve (see **Notes 16 and 17**)

D : dilution factor when appropriate

m : sample uptake

2. In the absence of Certified Reference Material for selenoamino-acids quantification,

validation of the analysis by checking the sum SeMet + SeCys measured with the total Se content in the same sample.

4. Notes

1. The on-line coupling between in HPLC and ICP MS allows performing the speciation of non-volatile species without preliminary chemical treatment except extraction. As most of the mobile phases and flow rates used in HPLC are convenient with the ICP MS, the exit of the HPLC column is directly connected to the ICP MS. The species of interest are separated on the HPLC column and reach step by step the ICP MS where they are detected. The identification of the species is made on the basis of their retention time.

2. When the ICP MS is coupled to a reversed-phase column, the interface should be adapted because of the high content of organic solvent in the mobile phase that would, otherwise, switch off the plasma. This amendment consists in cooling the spray chamber, decreasing the inner diameter of the injector, adding O_2 and replacing the Ni cones by Pt ones. This adapted interface is called “organic kit”.

3. To break the polyatomic interferences generated in the plasma, the use of an ICP MS equipped with a collision/reaction cell is now generalized. In the case of Se, even if different collision/reaction gas may be used, the most frequently used one remains H_2 [9].

4. In these three tubes, rising concentrations of SeMet will be spiked later on to build a calibration curve.

5. Urea is a commonly used chaotropic agent added in order to help the solubilization of the proteins.

6. The two sonication steps must not be consecutive in order to avoid too high increase of the temperature in the sample.

7. The Se-H group of the SeCys can easily form bridges with other Se-H or S-H group present

in the sample. DTT is a commonly used reducing agent added to break the Se-Se and Se-S bridges.

8. When SeCys is not protected by Se-Se or Se-S bridges, it undergoes an oxidation into selenoxide followed by syn-β-elemination of selenic acid [10]. A derivatization reaction, e.g. with IAM, can prevent this process [11].

9. This fresh DTT is added to destroy the excess of IAM.

10. Tris buffer is added in order to decrease concentration of urea to the appropriate level for proteolytic digestion.

11. Proteases are enzymes able to break unspecifically the bonds between amino-acids and therefore release the free amino-acids.

12. The raw extracts cannot be directly analyzed by RP HPLC – ICP MS. Indeed, because of a strong matrix effect, the chromatograms obtained this way are not usable. Prior to the selenoamino-acids quantification, the extracts need to be purified. This can be achieved by size-exclusion chromatography. This purification consists of collecting the low molecular weight fraction (that contains the selenoamino-acids) at the exit of the size-exclusion column and therefore simplifying the matrix. Beforehand, the chromatographic profile should have been checked to evaluate the fraction time range to collect. This cleaning approach by size-exclusion presents two main advantages : (1) even if the resolution on this kind of column is low, strong matrices can be injected on them; (2) the separation is made mainly as a function of the size, the big compounds, such as proteins, eluting in or close to the void of the column, and the low molecular weight compounds, such as amino-acids, eluting later. The size-exclusion profile allows, besides determining the fraction time range to collect, checking the proper completeness proteins digestion and the release of the amino-acids reflected by the absence of peaks in or close to the void and the presence of peaks in the low molecular weight fraction of the chromatograms.

13. It’s more reliable to check the profile on one of the spiked samples.

14. In case of doubt, ^{78}Se remains the most reliable isotope.

15. For long series of samples, the retention times may move a little bit. It’s better to maintain a safety margin of 1 min at the beginning and the end of the fraction collected.

16. In the absence of standard of SeCys, the SeCys quantification is performed with the slope obtained for the SeMet.

17. To calculate the slope, consider the amount of SeMet in ng in the solution injected on the reversed-phase column.

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Figure captions

Figure 1 : summary diagram of the analytical method allowing the determination of selenoamino-acids in biological fluids and tissues and animal products by 2D HPLC – ICP MS

Table captions

Table 1 : concentrations and volumes of the reactants as a function the matrix (expressed as concentration / volume)

Table 2 : HPLC and ICP conditions for the coupling with the size-exclusion column

Table 3 : HPLC and ICP conditions for the coupling with the reversed-phase column

Table 1

	Blood/plasma	Tissues	Milk	Eggs
urea	6 M / 300 µL	7 M / 2 mL	7 M / 2 mL	7 M / 4 mL
DTT-1	0.2 M / 30 µL	0.2 M / 60 µL	0.5 M / 80 µL	0.5 M / 80 µL
IAM	0.5 M / 40 µL	0.5 M / 100 µL	0.5 M / 320 µL	0.5 M / 320 µL
DTT-2	0.2 M / 375 µL	0.2 M / 300 µL	0.5 M / 480 µL	0.5 M / 450 µL
Tris	N/A	100 mM / 11 mL	100 mM / 11 mL	100 mM / 20 mL
SeMet	10 mg/L / 5 µL	10 mg/L / 5 µL	10 mg/L / 5 µL	10 mg/L / 10 µL
protease+lipase	2%+1% / 900 µL	2%+N/A / 1 mL	2%+N/A / 1 mL	2%+N/A / 1 mL

Table 2

HPLC	
Column	Superdex
Mobile phase	100 mM ammonium acetate (pH 7.5)
Gradient	Isocratic
Volume injected	100 µL
Flow rate	0.7 mL/min
Duration	1 h
ICP MS	
Parameters	Value
Rf power	1500 W
Isotopes monitored	^{76}Se, ^{77}Se, ^{78}Se (see **Note 14**)
Integration time	0.15 sec

Collision/reaction cell	Filled with H_2
Carrier gas flow, lens voltage…	Optimized daily
Organic kit	No

Table 3

HPLC	
Column	Altima C_8
Mobile phase	A : 0.1% of HFBA in water
	B : 0.1% of HFBA in methanol
Gradient	0 – 5 min : 5% of B
	5 – 30 min : up to 40% of B
	30 – 40 min : 40% of B
	40 – 42 min : down 5% of B
	42 – 48 min : 5% of B
Volume injected	100 µL
Flow rate	0.9 mL/min
ICP MS	
Parameters	Value
Rf power	1500 W
Isotopes monitored	^{76}Se, ^{77}Se, ^{78}Se (see **Note 14**)
Integration time	0.15 sec
Collision/reaction cell	Filled with H_2
Carrier gas flow, lens voltage…	Optimized daily
Organic kit	Yes

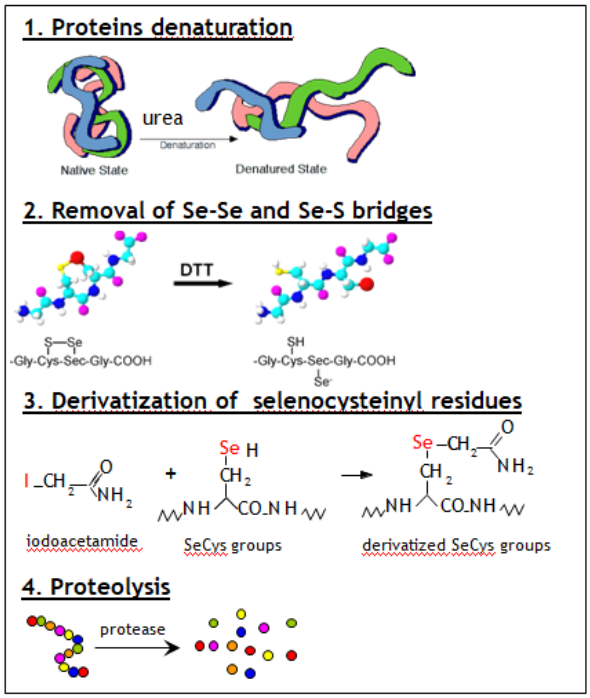
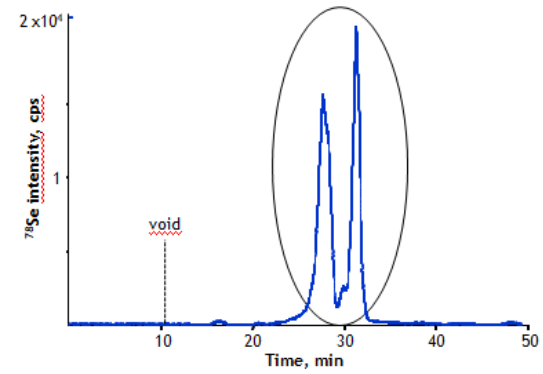


Figure 1

5. Size-exclusion purification of the proteolysate



6. Quantification by RP HPLC - ICP MS