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Direct determination of seleno-amino acids in biological tissues by anion-exchange separation and electrochemical detection

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Abstract

Several studies have described the determination of selenium in protein extracts from tissues of marine or terrestrial animals, but have not identified the different chemical forms of selenium that are present. Selenium may be present as seleno-amino acids. Selenocysteine, for example, is a normal component of glutathione peroxidase, an antioxidant enzyme which may behave like other antioxidants, such as vitamin E, protecting the tissues against methylmercury toxicity.

The present study illustrates a method for the characterization of seleno-amino acids, such as selenocysteine and selenomethionine, in proteins extracted from the liver of marine mammals. The mechanism of detoxification of methylmercury, which involves seleno-compounds, is identified.

The analytical determination was carried out using high-performance anion-exchange chromatography coupled with integrated pulsed amperometric detection (HPAEC–IPAD). This method allows the direct determination of underivatized amino acids, eliminating the procedure of pre- or postcolumn derivatization.

The chromatographic separation was carried out on an anion-exchange column using a quaternary gradient elution. In order to optimize this method, interferences of amino acids and the influence of pH and ionic strength on the separation and electrochemical detection were studied. The IPAD response for the direct detection of amino acids is optimum at pH > 11. The detection limit ($S/N = 3$) for selenocysteine was found to be 450 μg/l.

The application of this method for the identification of seleno-amino acids in protein hydrolysates is also shown.

1. Introduction

1.1. Selenium metabolism in marine animals

Selenium has been recognized to be an essential nutrient element in certain species, but relatively high levels result in toxic effects. The biochemical cycle of this element is relatively complex because of the involvement of both inorganic and organometallic species, such as seleno-amino acids

Different studies on marine mammals showed that selenium is strictly correlated to the detoxification processes of methylmercury in the liver [1,2]. When the level of methylmercury increases above a certain threshold (i.e. 100 μg/g Hg wet weight) demethylation starts, which causes the formation of compounds with low toxicity, with a 1:1 Se/Hg molar ratio [3]. The structure of these compounds has not yet been completely determined.

Several studies have isolated selenium in protein extracts from muscle and liver tissues of marine animals [4–7], but have not identified the

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chemical forms of selenium present. Selenium compounds in some marine fish have also been found to be associated with lipid material and have properties similar to lipoproteins.

Possible chemical transformations of selenium in marine animals have been reviewed by Maher et al. [4], but on this argument various interpretations still exist. Selenium-dependent glutathione peroxidase has been isolated in marine animals indicating that some selenium may be present as selenocysteine, as a normal component of glutathione peroxidase [8]. The identity of organoselenium compounds in the aquatic environment has not been fully established. In marine bacteria and plankton, selenium is predominantly found in protein as seleno-amino acids [9–11]. Selenomethionine has been isolated from the hydrophilic fulvate fraction of soil [12] and from protein of marine algae [13]

Alternatively, selenium may be in a non-protein moiety tightly held to protein, but not covalently bound. For example, it has been shown that selenium can readily form selenotrisulphides with thiols such as cysteine, glutathione, etc. [14]. These can then be incorporated and stabilized within protein structures. Chemical isolation of these types of compounds during extraction would prove difficult as destabilization results in the precipitation of elemental selenium. Many analytical procedures for selenium determination in different matrices exist. In recent years, in addition to the methods for the determination of the total selenium content, methods for the determination of different forms of selenium in water, soil, and biological matrices have been developed. Speciation of selenium is important in order to understand its biological cycle and to define the diffusion and the toxicity of this element in the ecosystem.

1.2. Chromatographic analysis

Progress in the determination of amino acids can be attributed to the technological advances in liquid chromatography and chromatographic detectors. Separations of amino acids in liquid chromatography are readily achieved by using reversed-phase stationary phases [15] or ion exchangers [16,17], and for the separation of complex mixtures, gradient elution is essential.

Sensitive photometric detection requires some kind of pre- or postcolumn derivatization. *o*-Phthalaldehyde (OPA) [18,19], a fluorescent derivatizing agent, offers excellent sensitivity, but the derivatives of OPA are not stable, OPA does not form adducts with secondary amino acids (e.g. proline and hydroxyproline), and the thiol group of amino acids may compete with the mercapto propionic acid used in the postcolumn reaction. Ninhydrin has been widely employed to enhance UV–Vis detection, but the reaction requires temperature control (135°C) and a postcolumn device is prone to plug. Furthermore, sensitivity for this reagent is lower than that for OPA. Recently HPLC analysis with the fluorescent reagent *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid has been utilized for the determination of selenocysteine in plasma at the μM level [20].

Detection methodology that does not require derivatization is preferred for ease and convenience, thus a selective detection, such as electrochemical detection has gained prominence in liquid chromatography as a sensitive and selective detection technique. Among the different electrochemical detection techniques, integrated pulsed amperometric detection (IPAD) has proven to be a selective and sensitive technique for the determination of amino acids without derivatization [21–23]. Amino acids can be detected directly at gold electrodes in an alkaline medium. The Au electrode is preferred over Pt in order to minimize the interference from dissolved oxygen [24].

IPAD, equipped with a Au electrode, is only selective for compounds containing oxidizable functional groups such as hydroxyl, amine and sulphide. IPAD is preferred over PAD in order to minimize baseline offset and drift during gradient pH elution.

A review has recently appeared on the different analytical methods used for speciation of selenium compounds [25]. Two different ana-

lytical approaches are reported in the literature for the determination of seleno-amino acids: gas chromatography and ion-exchange chromatography. The gas chromatographic methods require a precolumn preparation of volatile derivatives of seleno-amino acids [26–28], then the compounds are separated and identified by GC–MS. Various derivatizing reagents have been studied such as bis-(trimethylsilyl)acetamide, *N*-methyl - *N* - (*tert.* - butyldimethylsilyl)trifluoro - acetamide and cyanogen bromide. Ion-exchange chromatography separates seleno-amino acids from other amino acids [29–31], but one main problem is the oxidative destruction of these amino acids due to the protein hydrolysis procedure used in sample preparation [32]. Selenocysteine cannot be determined accurately under the classical hydrolysis conditions because of decomposition.

The most widely used hydrolysis procedure is gas-phase hydrolysis. Proteins are subjected to 6 *M* HCl vapour in the presence of 0.5% phenol for 24–72 h at 110°C. These conditions are known to destroy tryptophan and convert glutamine and asparagine to their acidic analogues. Furthermore, cysteine and cystine are at least partially converted to cysteic acid.

This paper shows the application of IPAD following gradient elution ion chromatography for the direct and simultaneous detection of selenocysteine and selenomethionine.

IPAD parameters, such as applied potentials and duration as well as the choice of reference electrode, were determined in order to optimize sensitivity for selenocysteine and selenomethionine detection. A gradient procedure, that incorporated a change in pH for the resolution of all the amino acids of interest, was developed. Furthermore, an innovative technique for protein hydrolysis, based on microwave irradiation [33,34], is shown. This technique overcomes the drawbacks of classical acidic hydrolysis of proteins, thus leading to a high recovery of seleno-amino acids.

This technique was used for the determination of the seleno-amino acid composition in protein hydrolysates of dolphin liver.

2. Experimental

2.1. Instrumentation

A metal-free modified Model 4000i quaternary gradient liquid chromatograph (Dionex, Sunnyvale, CA, USA) equipped with a PED-2 electrochemical detector (Dionex) was used to analyse amino acids. Data manipulation and the operation of all the components in the system were controlled by AI-450 chromatographic software (Dionex) interfaced via an advanced computer interface ACI-2 (Dionex) to a 80486-based computer (Olivetti, Ivrea, Italy). A modified MSD-81D microwave digester (CEM Co., Matthews, NC, USA) was used for protein hydrolysis. For mercury determination in dolphin liver, homogenized samples were digested under pressure in a Teflon vessel with a 1:1 (v/v) concentrated H_2SO_4–HNO_3 mixture for 4 h at 160°C. Mercury was determined by the cold vapour atomic absorption spectrophotometry using a modified 1100 B spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

Total selenium was determined by graphite furnace atomic absorption spectrophotometry (GF-AAS) using a modified Model 3030 Zeeman spectrophotometer (Perkin Elmer). Before analysis, liver samples were digested under pressure in a Teflon vessel with concentrated HNO_3 for 4 h at 160°C. Methylmercury was determined by gas chromatography [35] using a modified Mega Model 5600 gas chromatograph (Carlo Erba, Rodano, Italy) equipped with a ^{63}Ni electron capture detector.

2.2. Reagents and standards

Sodium tetraborate, sodium acetate, sodium hydroxide, 50% solution low carbonate, hydrochloric acid, nitric acid, sulphuric acid, and phenol were of analytical reagent grade (Novachimica, Milano, Italy); amino acid standard solution, selenomethionine and selenocystine were obtained from Sigma (St. Louis, MO, USA). By reducing selenocystine with threo-1,4-dimercapto-2,3-butanediol (dithiothrei-

tol or DTT) (Aldrich, Milwaukee, WI, USA) selenocysteine was obtained. The reduction was carried out at pH 8.2–8.5 for 30 min at room temperature.

All reagents were prepared daily with ultra-pure deionized water (<0.1 μS at 25°C) obtained using a Milli-Q system (Millipore, Milford, MA, USA). Working standard solutions were prepared by serial dilution of stock solutions. In the case of seleno-amino acids the standard solution was prepared in 1 *M* HCl.

2.3. Microwave heating-gas phase hydrolysis of proteins

The biological tissue (dolphin liver) was homogenized; to 1 g of homogenate, 5.5 ml of water, 1 g of NaCl and 1.5 ml of concentrated HCl was added. The sample was sonicated for 30 min and centrifuged at 6000 *g* for 10 min.

The precipitate containing the protein fraction was then separated, washed with 0.1 *M* HCl and submitted to acidic digestion. Sample digest was cleaned up on a AG-50 resin and analysed by IC–IPAD. Atomic absorption analysis demonstrated that most of the selenium (>95%) was in the protein fraction.

For protein hydrolysis, 100 mg of purified liver proteins were placed in a reaction vessel with 10 ml 6 *M* HCl and 0.5% phenol solution, added in order to protect the easily oxidizable amino acids [36]. Teflon reaction vessels containing a sample, were then evacuated and purged with N_2. Pressure was set to 55 p.s.i. resulting in a temperature of 150°C. Hydrolysis was performed with microwave irradiation at 645 W for 25 min using a microwave digestion system. The resulting solution was then filtered through a 0.45-μm filter and diluted 1:1 with eluent A (see Table 1) prior to injection.

2.4. Chromatography of amino acids

Separation was carried out on a 250 × 4 mm I.D. AminoPac PA1 pellicular anion-exchange column (Dionex) at a flow-rate of 1 ml/min at room temperature. Injection loop was 50 μl.

Amino acids were separated with a sodium hydroxide, sodium borate and sodium acetate linear quaternary gradient system shown in Table 1. All eluents and the postcolumn reagent were prepared daily with ultra-pure deionized water. A 300 m*M* sodium hydroxide solution, used at 0.8 ml/min as a postcolumn addition, showed the tendency to reduce baseline shifts that occurred with sodium hydroxide gradient and to increase sensitivity. Fig. 1 shows a chromatogram of a standard solution of amino acids.

Table 1
Gradient conditions for separation of amino acids

Time (min)	A (%)	B (%)	C (%)	D (%)	Valve position
0.0	100	0	0	0	Load
4.0	100	0	0	0	Load
4.1	0	0	0	100	Load
13.9	0	0	0	100	Load
14.0	100	0	0	0	Load
25.8	100	0	0	0	Load
26.0	100	0	0	0	Inject
36.0	100	0	0	0	Inject
40.0	0	100	0	0	Inject
46.0	0	100	0	0	Inject
46.1	0	90	10	0	Inject
56.0	0	0	100	0	Load
60.0	0	0	100	0	Load
66.0	0	0	100	0	Load

Eluent A, 0.023 *M* sodium hydroxide–0.007 *M* sodium tetraborate. Eluent B, 0.08 *M* sodium hydroxide–0.023 *M* sodium tetraborate. Eluent C, 0.65 *M* sodium acetate. Eluent D, 1 *M* sodium hydroxide–0.3 m*M* sodium tetraborate. Flow-rate = 1 ml/min.

2.5. IPAD detection

Detection was effected by using integrated pulsed amperometry with a gold working electrode and a standard Ag/AgCl reference electrode: the pH electrode could better compensate the baseline drift, but it is prone to degradation in mid-term life. The working pulse potentials and duration, and the integration time, that were used throughout the detection of amino acids are shown in Table 2. The NH_3 groups were oxidized at +0.35 V; the reaction products are removed at +0.9 V, while the electrode is cleaned at a negative potential of −0.9 V.

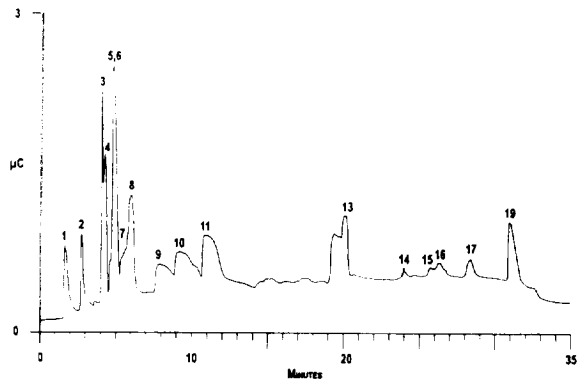

Fig. 1. Gradient elution of amino acids standard. Peaks: 1 = Arg; 2 = Lys; 3 = Thr; 4 = Ala; 5 = Gly; 6 = Ser; 7 = Val; 8 = Pro; 9 = Ileu; 10 = Leu; 11 = Met; 13 = His; 14 = Phe; 15 = Glu; 16 = Asp; 17 = Cys; 19 = Tyr. Chromatographic conditions as shown in Table 1. Column, AminoPac PA1. Detection, IPAD conditions as in Table 2.

3. Results and discussion

For the chromatographic determination of seleno-amino acids a separation on an anion-exchange column based on a quaternary gradient system, as shown in Table 1, was chosen. The separation on the AminoPac PA1 column is effectuated by the relative difference in the dissociation constants of the amino, carboxylic and R groups of each amino acid. Fig. 1 shows a typical example of chromatographic separation of a standard solution of amino acids. The detection protocol of the IPAD is shown in Table 2 and is relative to amino group detection. During the amperometric detection, the electrode current is continuously integrated during the cycle in which the electrode is oxidized and then reduced to its original state. Amines and sulphur compounds that have an unbalanced electron pair on their N and S atoms can be adsorbed at the oxide-free Au electrode surface when $E < 0.1$ V and anodically detected by oxide-catalysed reactions during the positive scan.

The advantage of using integrated amperometry compared to pulsed amperometry is the reduction in baseline drift due to the elimination of the charge caused by oxide formation and its reduction. The same gradient and detection system were also used for the seleno-amino acid determination. Experimental results show a good separation of the selenomethionine and selenocystine peaks from their corresponding sulphur analogues. As shown by the experimental results, the substitution of a sulphur atom with selenium increases the retention time of the corresponding amino acids, which can be easily identified because those zones of the chromatogram are relatively free from interfering peaks. Fig. 2 shows a chromatogram of a standard solution of methionine, selenomethionine,

Table 2
Time/potential used for IPAD detection of seleno-amino acids

Time (s)	Potential (V)
0.00	−0.10
0.20	−0.10
0.30	0.35
0.40	0.35
0.50	−0.10
0.70	−0.10
0.71	0.90
0.90	0.90
0.91	−0.90
1.00	−0.90

Integration time from 0.20 to 0.70 s. Reference electrode A–AgCl.

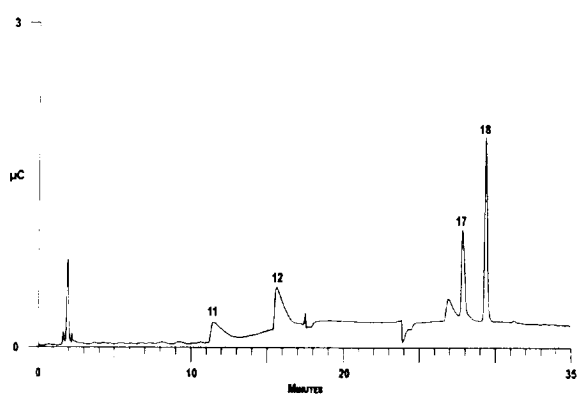

Fig. 2. Gradient elution of amino acids and seleno-amino acids standard. Peaks: 11 = Met; 12 = SeMet; 17 = Cys; 18 = SeCys. Chromatographic conditions as shown in Table 1. Column, AminoPac PA1. Detection, IPAD conditions as in Table 2.

cysteine, and selenocysteine (1 μg of each injected); because of the asymmetric peak shapes, peak areas were chosen for quantitation.

The anion exchange at alkaline pH is evidenced by the good separation of the seleno-amino acids from other amino acids. The baseline drift during the gradient results from changes in pH and ionic strength. In order to reduce the drift, a postcolumn addition of 300 m*M* NaOH at 0.5 ml/min was made. Detection limits for the determination of selenomethionine and selenocysteine were found to be 620 μg/l and 450 μg/l, respectively. The IPAD response shows good linearity for both selenomethionine and selenocysteine up to 10 mg/l. Quantitative analysis was carried out, using the standard addition method and measuring peak area. The reproducibilities of the peak areas were calculated by performing five replicate analyses of standard solutions of 1 mg/l of both selenomethionine and selenocysteine. The R.S.D.s for the peak areas were 4.7 and 8.1% for selenocysteine and selenomethionine, respectively.

This technique was used for the determination of seleno-amino acids in protein hydrolysate of dolphin liver.

Selenium, in fact, is considered to play a key role in reducing the toxic manifestations of mercury in rats [37,38]. Published studies suggest that mercury and selenium concentration may be correlated in man [39] and some marine organisms [40]. For instance, there is a significant correlation between selenium and mercury in black marlin liver [41], tuna tissues [42] and marine mammals [2,43].

Table 3 shows the mercury, methylmercury and selenium concentrations in the liver of 16 dolphin specimens (*Stenella coeruleoalba*) found beached along the coast of Apulia (southern Italy). Fig. 3 shows the behaviour of mercury and selenium concentration in liver. It is evident that a strict correlation exists between mercury and selenium accumulation. Because the presence of mercury in dolphin is mainly due to methylmercury intake in the diet, the low percentage of methylmercury in the liver demonstrates that a detoxification process exists which

Table 3
Mercury, methylmercury (HgMet), selenium and Se/Hg′ molar ratio in dolphin liver

Specimen	Hg (μg/g)[a]	HgMet		Se (μg/g)[a]	Se/Hg′
		(μg/g)[a]	(%)		
16	374.5	6.8	1.8	165.0	1.13
13	351.9	10.0	2.8	158.5	1.17
9	263.3	8.0	3.0	132.8	1.32
12	242.0	7.0	2.9	104.0	1.12
5	216.7	5.4	2.5	92.4	1.11
2	206.3	12.2	5.9	85.4	1.12
1	206.2	9.5	4.6	90.2	1.16
15	183.1	7.9	4.3	92.3	1.34
7	168.0	4.6	2.7	91.8	1.43
4	160.3	3.4	2.1	87.4	1.42
6	156.3	10.6	6.7	86.4	1.49
14	107.4	6.3	5.9	48.6	1.23
10	12.3	1.9	15.4	7.8	1.90
11	3.2	0.9	28.1	4.8	5.53
8	2.3	1.2	52.2	4.4	10.13
3	1.9	1.1	57.9	4.3	13.68

[a] μg/g wet weight.
$Hg' = Hg_{tot} - HgMet$.

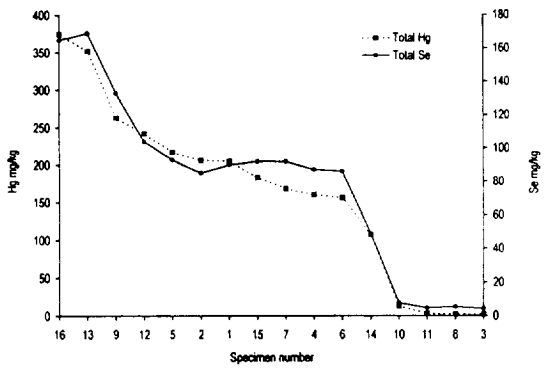

Fig. 3. Correlation between total mercury and total selenium concentration in liver of different dolphins (*Stenella coeruleoalba*).

involves the demethylation of methylmercury. Where concentrations of methylmercury are lower than 10%, the atomic ratio Se/Hg′, where $Hg' = Hg_{tot} - HgMet$, is approximately 1. This has also been observed by other researchers [1,2] and their conclusion was to consider the final product of methylmercury demethylation as a Hg–Se compound (tiemannite) which is insoluble and less toxic [1].

Recently, Palmisano et al. [3] suggested that very stable complexes such as Hg–selenoprotein could play an important role in mercury detoxification. The role of selenium in this process has not been completely explained. Demethylation is activated in the liver only when a threshold value of the methylmercury concentration is reached [3]. With these premises, the determination of seleno-amino acids in the protein fraction of dolphin liver is important in order to understand both the demethylation process of methylmercury and the selenium compound structure.

The analytical method proposed in the present work was applied to the determination of seleno-amino acids in hydrolysed samples of dolphin liver.

Hydrolysis was carried out in a microwave digester (the conditions have been described above) to reduce the risk of oxidative decomposition of sulphur-amino acids. In the same way, acidic hydrolysis tests on standard solutions of selenomethionine and selenocysteine showed a recovery better than 90%.

Fig. 4a shows a typical chromatogram of a hydrolysate (liver of dolphin No. 16) where a Ag–AgCl reference electrode was used. The gradient drift due to the change in pH and ionic strength of the eluent was partially compensated by the postcolumn addition of NaOH (300 m*M*). This sample contains a seleno-amino acid concentration close to the detection limit; the concentration of selenocysteine, calculated by the standard addition method, is 5 nmol/ml of hydrolysate. As shown in Fig. 4b, the selenocysteine peak was confirmed by the addition of selenocysteine standard solution (80 μg of SeCys was added to 100 mg of liver proteins). A substantial amount of the total selenium (>40% average value) was present in liver proteins such as selenocysteine.

This preliminary result seems to confirm the hypothesis that selenium is present in liver proteins such as seleno-amino acids. The main function of these proteins may be the binding of mercury, in Se–Hg bonds, in order to lower its toxicity.

Experimental work is progressing and we are also investigating hyphenated techniques such as IC–ICP or IC–ICP–MS in order to have a more

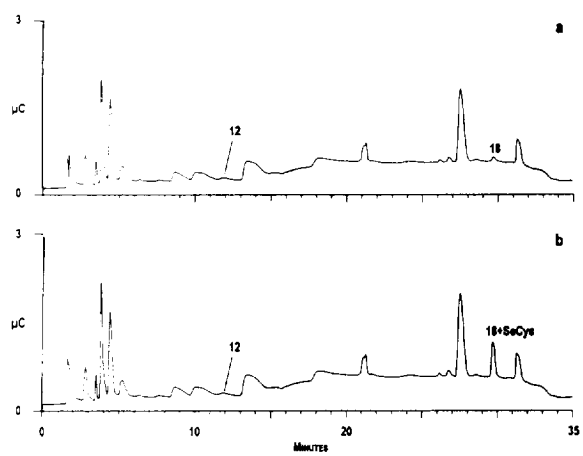

Fig. 4. (a) Gradient elution of protein hydrolysate of dolphin liver. Peaks: 12 = SeMet; 18 = SeCys. Chromatographic conditions as shown in Table 1. Column, AminoPac PA1. Detection, IPAD conditions as in Table 2. (b) Same sample as (a) spiked with selenocysteine standard solution (80 μg of SeCys was added to 100 mg of liver protein).

accurate detection system coupled with the chromatographic separation for reducing the number of peaks and to increase the selectivity of the method.

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