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Short Communication

Separation of selenium analogues of sulphur-containing amino acids by high-performance liquid chromatography and high-resolution gas chromatography

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Abstract

The historically conditioned adaptation of living organisms to chemically corresponding elements is influenced in nature by anthropogenic activities in many regions, the selenium–sulphur pair being one example of such a case. The separation of selenomethionine, selenoethionine and selenocystine was studied by HPLC and high-resolution GC. Ion-exchange chromatography followed by temperature-programmed GC gives the possibility of the analytical separation of trace amounts of selenomethionine in a complex mixture of common amino acids. Diastereoisomers of selenocystine were identified by HPLC in the AccQ-Tag mode.

1. Introduction

The anthropogenic influence in nature often causes specific variations of chemically related elements, which are able to change considerably the existing biological equilibrium based on the historically conditioned adaptation of living organisms to the ratio of these elements in the environment. Extensive industrial and agricultural activities carried out in different regions lead to substantial changes in the natural ratios of chemically corresponding elements, e.g., sulphur–selenium, phosphorus–arsenic and iodine–bromine.

Recently, considerable attention has been paid to selenium as an element with a considerable biogenic function [1–3], since the recommended daily intake of selenium for a healthy human being is about 50–80 μg whereas a daily intake of more than ca. 800 μg is considered to be toxic. Selenium has a higher redox activity than sulphur, which positively influences the ability of certain enzymes, e.g., glutathione peroxidase, to scavenge unwanted oxygen-derived free radicals, which play a role in mutagenesis during the growth of living matter.

Although the determination of the absolute selenium content in biological materials by means of hydride (SeH_2) generation has been studied previously [4,5], it is more significant to speciate the selenium in the different chemical forms in which it occurs in food and which serve

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as intermediates in the biosynthesis of selenoprotein P and/or glutathione peroxidase [6]. The most relevant results have been obtained by the electrophoretic localization of selenium in individual protein fragments [6]. Wolf and coworkers tested ion-exchange chromatography for the separation and preparation of selenomethione in a complex of common amino acids. They observed co-elution of selenomethionine with isoleucine and leucine and subsequently used a complicated chemical reaction with cyanogen bromide [7] and a microbiological assay [8] for the identification of selenomethionine. Owing to the relatively low resolving power of the system they used [7], in this work we investigated the potential of the separation of several selenium analogues of sulphur-containing amino acids by HPLC and high-resolution (HR) GC.

2. Experimental

2.1. Chemicals

The selenoamino acids seleno-D,L-ethionine (S-3750, Lot 116F-0171) and seleno-D,L-cystine (S-3625, Lot 108F-0321) were pure products from Sigma (St. Louis, MO, USA) and seleno-L-methionine (batch No. 561505, Lot 074491) was a pure product from Calbiochem (La Jolla, CA, USA).

2.2. Liquid chromatography

Chemicals for the Pico-Tag and AccQ-Tag system were purchased from Waters, Division of Millipore (Milford, MA, USA) according to their prescription.

The Waters Pico-Tag amino acid analysis system and procedure as described in the Waters operators' manual No. 88140 (July 1990, 9/86, 2/86, 5/84, revision 4) was used. The Waters Pico-Tag system is an automated gradient elution liquid chromatograph designed for the determination of amino acids by precolumn derivatization followed by reversed-phase HPLC. The precolumn derivatization is based on the reaction of phenyl isothiocyanate to produce phenylthiocarbamoylamino acids. The total run time is 20 min.

The AccQ-Tag system is described in the Waters manual No. WAT052874, rev. 0, April 1993. The method is based on precolumn derivatization according to the method of Cohen and Michaud [9]. The gradient conditions were changed in order to be able to separate glutamine, asparagine, ornithine and tryptophan together with the amino acids present in acid protein hydrolysates. The gradient procedure is as follows. Mobile phase A contains 70 m*M* sodium acetate solution adjusted to pH 5 with phosphoric acid and mobile phase component B consists of 50% mobile phase A and 50% acetonitrile. The gradient profile is 98% A and 2% B at the start, then changed from 98 to 88% A in 26 min followed by a decrease to 54% A in 23 min. At that point, the column is purged with 100% B and re-equilibrated with 98% A for the next injection. The total run time is 65 min.

2.3. Gas chromatography

Derivatization was performed according to Ref. [10]. The selenoamino acids are mixed with ethyl chloroformate ester in aqueous solution [water–ethanol–pyridine (60:32:8)], after which relevant ethoxycarbonyl amino acid esters (EtOxC-AAEtEst) are formed in a few seconds. After extraction with chloroform, GC separation was performed [11] on a DB-1701 column (J & W Scientific, Folson, CA, USA) (5 m × 0.18 mm I.D., 0.4 μm thick film of 7% phenyl–7% cyanopropylpolydimethylsiloxane). A Shimadzu GC 14A gas chromatograph controlled by a Shimadzu C-R 18 Chromatopak was used with flame ionization detection using a hydrogen overpressure of 0.75 atm and temperature programming at 20°C/min from 120 to 300°C followed by an isothermal run for 5 min at 300°C.

3. Results and discussion

The retention data measured by the applied HRGC and HPLC systems are given in Table 1.

Table 1
Retention times (min) of the derivatized amino acids as measured by GC and HPLC

Derivatives of		GC	Pico-Tag	AccQ-Tag	IC [8]
Abbreviation[a]	Full name				
Ala	Alanine	0.55	8.81	27.65	
Gly	Clycine	0.68	6.97	18.42	
AAba	α-Aminobutyric acid	1.10	–	35.96	
Val	Valine	1.23	12.21	40.78	
Leu	Leucine	1.55	14.89	46.29	33.64
Ile	Isoleucine	1.59	14.57	46.00	32.46
Pro	Proline	1.85	9.14	33.30	
Thr	Threonine	1.99	8.59	25.03	
Ser	Serine	2.11	6.36	15.86	
Glu	Glutamic acid	2.11	3.47	17.56	
Asn	Asparagine	2.28	6.00	16.40	
Asp	Aspartic acid	2.66	3.17	14.25	
Met	Methionine	3.05	12.77	41.41	30.20
Hyp	Hydroxyproline	3.47	–	–	
Phe	Phenylalanine	3.55	16.32	47.47	
CysH	Cysteine	3.87	13.72	34.00	
I.S.[b]	Chlorophenylalanine	4.49	–	–	
Gln	Glutamine	4.92	6.66	19.64	
Orn	Ornithine	5.13	–	43.32	
Lys	Lysine	5.52	18.18	44.99	
His	Histidine	5.82	7.52	22.90	
Tyr	Tyrosine	6.24	11.18	40.33	
Trp	Tryptophan	7.02	16.75	48.77	
Cys	Cystine	7.88	–	38.80	32.40
NH_3	Ammonia	–	–	24.04	
Arg	Arginine	–	8.23	28.31	
SeMet	Selenomethionine	3.36	13.80	43.49	33.09
SeEt	Selenoethionine	3.63	16.20	47.41	
$SeCys_1$	Selenocystine (1)	8.38	13.80	40.30	
$SeCys_2$	Selenocystine (2)	8.38	14.15	40.73	

[a] Used in Figs. 1 and 2.
[b] Internal standard.

To evaluate the separation possibilites and resolution problems with the studied seleno analogues of sulphur-containing amino acids in mixtures with naturally occurring amino acids, it is illustrative to compare the chromatograms of pure selenoamino acids and common amino acids under identical conditions obtained by GC and LC.

Fig. 1 shows the results of independent runs with an equimolar test mixture of common amino acids and selenoamino acids as EtOxC-AAEtEst separated by temperature-programmed GC. It is evident that selenomethionine is well resolved from methionine and hydroxyproline and that selenoethionine is well separated from phenylalanine and cysteine. The selenocystine derivative forms a single peak eluting far behind the cystine peak.

In Fig. 2, liquid chromatograms of relevant derivatives of the investigated selenoamino acids are depicted for both the (A) Pico-Tag and (B) AccQ-Tag protocols. For selenomethionine, 89% of the total recorded peak areas, measured as the absorbance at 254 nm of the phenylthiocarbamoyl derivative, was found in the main peak. Similarly, the selenoethionine

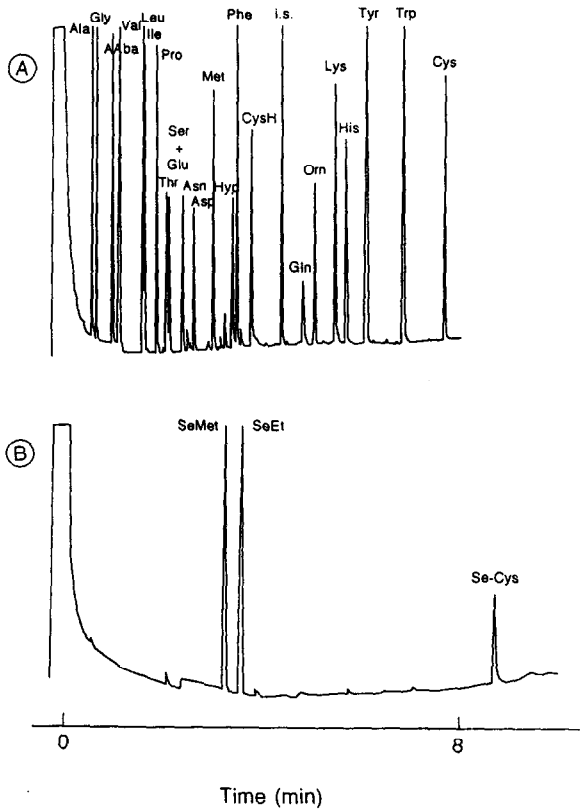

Fig. 1. GC separation of (A) a test mixture of common amino acids and (B) a mixture of the studied selenoamino acids, all as EtOxC-AAEtEst under identical conditions.

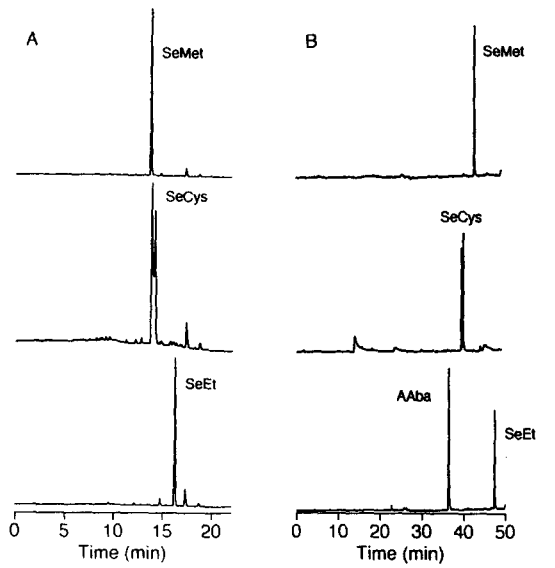

Fig. 2. Liquid chromatograms of selenoamino acid derivatives obtained using (A) the Pico-Tag and (B) the AccQ-Tag (B) systems.

peak represents 78% and the double peak of selenocystine 85% of the total peak areas. It is remarkable that selenomethionine has the same retention as the first peak of selenocystine, in contrast to the large retention distance between selenomethionine and selenoethionine, which differ by only one CH_2 group.

To rule out of presence of selenomethionine in the selenocystine preparation, the samples were also analysed using the AccQ-Tag protocol. Virtually baseline resolution for the selenocystine double peak was obtained and selenomethionine was well separated from selenocystine. Here it appears that valine (see Table 1) co-elutes with the second peak of the selenocystine doublet, but as this amino acid is well resolved from selenocystine in the Pico-Tag chromatogram (see Table 1), the double peak is either due to another impurity or represents two different derivatives of selenocystine. Interestingly, GC reveals only a single peak and does not show the presence of valine or selenomethionine, in agreement with the results obtained by HPLC. Cystine and selenocystine have two optically active centres, giving rise to four possible diastereoisomers, DD, DL, LD and LL. It is clear that an achiral system is able to distinguish only between DD and LL diastereoisomers. A similar separation has been observed earlier for cystine diastereoisomers [12]. It is possible that the full separation of all isomers can be achieved by chiral chromatography.

The small differences in the peak heights of impurities in the HPLC traces are caused by the different detection modes used in the Pico-Tag (absorbance) and AccQ-Tag (fluorescence) protocols.

4. Conclusions

It can be concluded that optimal detection of trace amounts of selenomethionine in the presence of a large excess of the common amino

acids can be achieved in the following way. The sample is subjected to an ion-exchange separation according to Ref. [8] and the cysteine–isoleucine–leucine peak cluster (32–35 min) is collected and, after derivatization with chloroformate, subjected to GC. Temperature-programmed GC results in complete separation of the components of the mixture with the other amino acids eluting either well before (1.6 min) or after (3.85 min) the selenomethionine peak (3.38 min), thus permitting the detection of trace amounts of selenomethionine. A quantitative evaluation of the detection limit of trace amounts of selenomethionine among the excess of common amino acids based on the investigation of real samples will be published later.

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