Journal of Chromatography A, 659 (1994) 481–485
Elsevier Science B.V., Amsterdam

CHROM. 25 683

Short Communication

Determination of selenocyst(e)amine, selenocyst(e)ine and selenomethionine by gas chromatography with flame photometric detection

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(First received September 6th, 1993; revised manuscript received October 25th, 1993)

ABSTRACT

A selective and sensitive method for the determination of selenocyst(e)amine, selenocyst(e)ine and selenomethionine (Se-Met) by gas chromatography (GC) was developed. Selenocystamine and selenocystine were reduced to selenocysteamine (Se-CYE) and selenocysteine (Se-Cys), respectively, by adding sodium tetrahydroborate prior to derivatization. Se-CYE, Se-Cys and Se-Met were converted into N,Se-isopropoxycarbonyl (isoPOC), N,Se-isoPOC methyl ester and N-isoPOC methyl ester derivatives, respectively, and determined by GC with flame photometric detection (FPD) using a DB-17 capillary column. These derivatives were sufficiently volatile and stable, giving single and symmetrical peaks, and provided an excellent FPD response. The detection limits of Se-CYE, Se-Cys and Se-Met were *ca.* 1.4, 1.0 and 1.4 pmol injected, respectively. The calibration graphs for these selenium compounds were linear in the range 1–20 nmol and sufficiently reproducible for quantification.

INTRODUCTION

Selenocyst(e)amine, selenocyst(e)ine and selenomethionine (Se-Met) have been found in the free form in onion [1] and seleniferous cabbage [2], and as integral moieties in several selenium-containing proteins such as plasma selenoprotein P [3], mammalian glutathione peroxidase [4] and bacterial glycine reductase [5], formate dehydrogenase [6] and acetoacetyl-CoA thiolase [7]. However, the physiological significance and the origin of these selenium compounds are not fully understood.

The identification and determination of Se-cyst(e)amine, Se-cyst(e)ine and Se-Met have been carried out by thin-layer chromatography [1,2], with an amino acid analyser [3–10] and by gas chromatography (GC) [11]. However, these methods lack the sensitivity or specificity required for determining these selenium compounds in biological samples.

It has been demonstrated in our laboratory that a variety of amines [12–14] and amino acids [15–17] can be successfully analysed by GC as their N-alkyloxycarbonyl and N-alkyloxycarbonyl methyl ester derivatives, respectively, which are conveniently prepared by a simple procedure involving alkyloxycarbonylation with alkyl chloroformate in an aqueous medium followed by esterification with diazomethane. In this work, a selective and sensitive method for determining selenocyst(e)amine, selenocyst(e)ine

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0021-9673/94/$07.00
SSDI 0021-9673(93)E1158-V

and Se-Met by GC with flame photometeric detection (FPD) was investigated.

EXPERIMENTAL

Reagents

Selenocystamine, seleno-DL-cystine and seleno-DL-methionine (Se-Met) were purchased from Sigma (St. Louis, MO, USA), and were dissolved in 0.05 *M* hydrochloric acid in order to obtain a stock solution at a concentration of 1 m*M*. Bis(*p*-methoxyphenyl) selenoxide (BMPS) (Tokyo Kasei Kogyo, Tokyo, Japan) as an internal standard (I.S.) was dissolved in ethyl acetate at 50 μM. Sodium tetrahydroborate (Nacalai Tesque, Kyoto, Japan) was used as a 100 mg/ml solution in 0.1 *M* sodium hydroxide. Isopropyl chloroformate (isoPCF) was obtained from Wako (Osaka, Japan). Hydrogen chloride in methanol (HCl–MeOH) obtained from Tokyo Kasei Kogyo was diluted with methanol at a concentration of 1 *M*. Peroxide-free diethyl ether was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytical-reagent grade.

Gas chromatography

GC analysis was carried out with a Shimadzu Model 12A gas chromatograph equipped with hydrogen flame ionization detection (FID) and flame photometric detection (FPD) and with a 477-nm interference filter (GL Sciences, Tokyo, Japan) inserted in the optical path. A fused-silica capillary column (15 m × 0.53 mm I.D., film thickness 1.0 μm) of cross-linked DB-17 (J&W Scientific, Folsom, CA, USA) was used. The operating conditions were as follows: column temperature, programmed at 10°C/min from 170 to 270°C; injector and detector temperatures, 280°C; nitrogen flow-rate, 12 ml/min.

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett-Packard Model 5890A gas chromatograph was operated in conjunction with a VG Analytical Model 70-SE mass spectrometer and a VG-250J mass data system. The GC column was of the same type as used for GC analysis, with an ionizing voltage of 40 eV, an ion-source temperature of 250°C and a helium flow-rate of 10 ml/min.

Derivatization procedure

An aliquot of the sample solution containing 1–20 nmol of selenocysteamine (Se-CYE), selenocysteine (Se-Cys) and Se-Met was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. To this solution were added 0.05 ml of 0.5 *M* sodium hydroxide and 0.2 ml of 100 mg/ml sodium tetrahydroborate solution, and the mixture was incubated at 100°C for 10 min. After cooling, 0.05 ml of isoPCF was added and the mixture was shaken up and down at 3000 rpm for 10 min at room temperature. The reaction mixture was saturated with sodium chloride and acidified to pH 1–2 with 2 *M* hydrochloric acid, 0.1 ml of 50 μM BMPS (I.S.) was added and then the mixture was extracted with 3 ml of peroxide-free diethyl ether. After the ethereal extract had been evaporated to dryness at 80°C, to the residue was added 0.2 ml of 1 *M* HCl–MeOH and the mixture was incubated at 80°C for 10 min. After the residual solvent had been evaporated to dryness at 80°C under a stream of dry air, the residue was dissolved in 0.1 ml of ethyl acetate and 1 μl of this solution was injected into the gas chromatograph. The derivatization process for Se-Cys is summarized in Fig. 1.

RESULTS AND DISCUSSION

We investigated a simple and rapid derivatization method (outlined in Fig. 1) using isoPCF as a derivatizing reagent for amino and selenohydryl functions and HCl–MeOH for the carboxyl function. Selenocystamine and selenocystine were reduced to Se-CYE and Se-Cys, respectively, by adding sodium tetrahydroborate prior to derivatization. The reduction was accomplished within 10 min at 100°C by using 20 mg of sodium tetrahydroborate in aqueous alkaline media. Se-CYE, Se-Cys and Se-Met could be successfully converted into N,Se-isoPOC, N,Se-isoPOC methyl ester and N-isoPOC methyl ester derivatives, respectively, by essentially the same procedure as that used in the derivatization of amines [12–14] and amino acids [15–17],

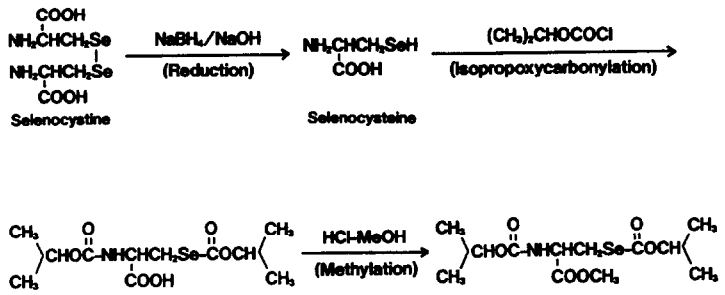

Fig. 1. Process for derivatization of selenocyst(e)ine.

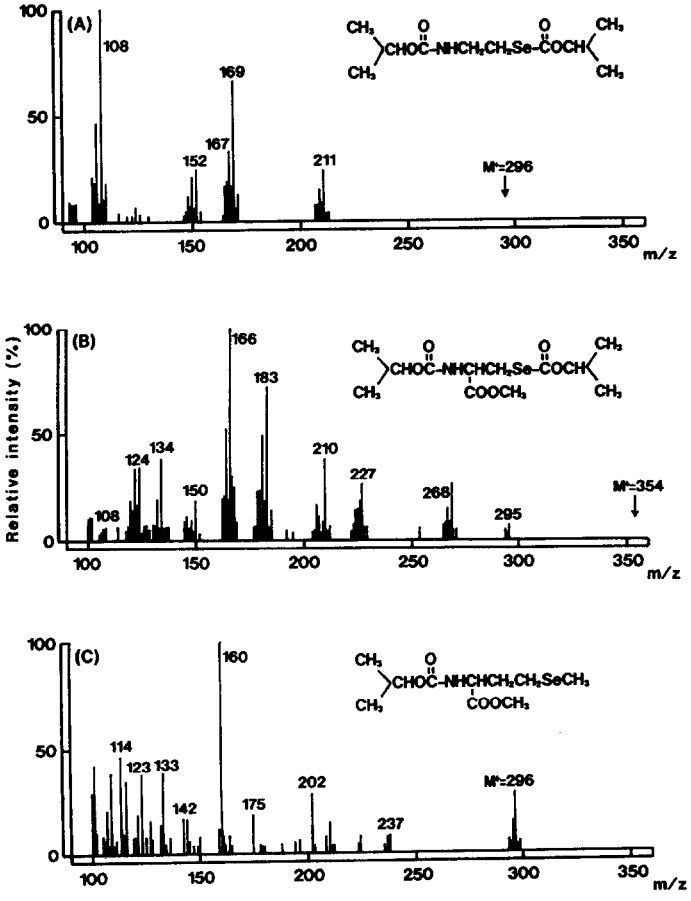

Fig. 2. Mass spectra of (A) N,Se-isoPOC selenocysteamine, (B) N,Se-selenocysteine methyl ester and (C) N-isoPOC selenomethionine methyl ester.

except for the use of HCl–MeOH instead of diazomethane. The isopropoxycarbonylation of the amino and selenohydryl functions of these compounds was accomplished within 5 min in aqueous alkaline media by shaking at room temperature. Peroxide-free diethyl ether was used as an extraction solvent for N,Se-isoPOC or N-isoPOC derivatives of these compounds in order to prevent the oxidation to the selenoxide and selenone derivatives [5]. The N,Se-isoPOC derivatives were quantitatively extracted into diethyl ether in acidic media. Although the methylation of the carboxyl function of the isoPOC derivatives with diazomethane was simple [15–17], the interfering peaks originating in the diazomethane generating reagents were observed under our GC–FPD conditions. Therefore, HCl–MeOH was used as a methylation reagent. The methylation with HCl–MeOH was completed within 5 min at 80°C, and no interfering peak was observed. The derivative preparation including reduction with sodium tetrahydroborate was accomplished within 40 min, and several samples could be treated simultaneously.

The structures of the derivatives of Se-CYE, Se-Cys and Se-Met were confirmed by GC–MS. The mass spectra of these derivatives are shown in Fig. 2. Although a molecular ion peak (M^+) was not observed for Se-CYE and Se-Cys derivatives, prominent fragment ion peaks at $M^+ - 59$ [$(CH_3)_2CHO$ or $COOCH_3$], $M^+ - 86$ [$2(CH_3)_2CH$], m/z 166 [$SeCOOCH(CH_3)_2$] and m/z 108 (CH_3CH_2Se) were observed. On the other hand, the M^+ with postulated m/z 296 and the prominent fragment ion peaks at $M^+ - 59$ and $M^+ - 94$ (CH_3Se) were observed for the Se-Met derivative. These peaks were useful for structure elucidation. These derivatives were found to be very stable under normal laboratory conditions, and no decomposition was observed during GC analysis.

The FPD responses of various selenium com-

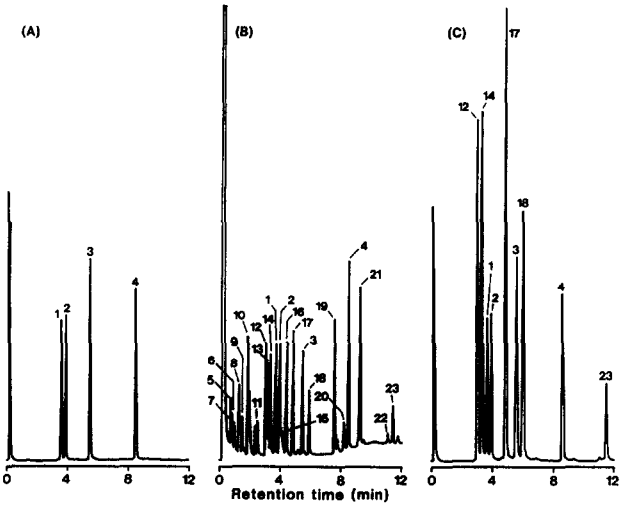

Fig. 3. Gas chromatograms obtained from selenium and non-selenium compounds. (A) Selenium compounds (containing 5 nmol of each compound); (B) selenium + non-selenium compounds (containing 100 nmol of each compound); (C) selenium + non-selenium compounds (containing 5 nmol of each compound). The derivatized samples were analyzed by (A and C) GC–FPD and (B) GC–FID. GC conditions are given under Experimental. Attenuation: GC–FPD, 10×32; GC–FID, $10^2 \times 4$. Peaks: 1 = Se-CYE; 2 = Se-Met; 3 = Se-Cys; 4 = BMPS (I.S.); 5 = alanine; 6 = glycine; 7 = valine; 8 = leucine; 9 = isoleucine + serine; 10 = proline + threonine; 11 = aspartic acid; 12 = cysteamine; 13 = glutamic acid; 14 = methionine; 15 = hydroxyproline; 16 = phenylalanine; 17 = cysteine; 18 = homocysteine; 19 = lysine; 20 = histidine; 21 = tyrosine; 22 = tryptophan; 23 = cystathionine.

pounds have been examined by Flinn and Aue [18]. Maximum responses for selenium compounds were found with dominant emissions between 450 and 500 nm. Therefore, we used a 477-nm interference filter for GC–FPD. The flow conditions for maximum response, established with the derivatives of Se-CYE, Se-Cys and Se-Met, were 20 ml/min of hydrogen and 29 ml/min of air. As shown in Fig. 3A, each derivative was eluted as a single and symmetrical peak, and provided an excellent FPD response; the minimum detectable amounts of Se-CYE, Se-Cys and Se-Met, giving a signal three times higher than the noise under our instrumental conditions, were *ca.* 1.4, 1.0 and 1.4 pmol injected, respectively. The GC–FPD system described here was over 20 times more sensitive than the GC–FID system. On the other hand, other amines and amino acids were also derivatized and detected with FID (Fig. 3B), but these compounds were not detected with FPD except for sulphur compounds (Fig. 3C). Although the sulphur compounds provided the same FPD response under our instrumental conditions, Se-CYE, Se-Cys and Se-Met were completely separated from biological sulphur compounds such as cysteamine, methionine, cysteine, homocysteine and cystathionine.

In order to test the linearity of the calibration graph, various amounts of Se-CYE, Se-Cys and Se-Met ranging from 1 to 20 nmol were derivatized and aliquots representing 10–200 pmol were injected. In each instance a linear relationship was obtained from both logarithmic plots, and the regression lines for Se-CYE, Se-Cys and Se-Met were $\log y = 1.754 \log x - 1.320$ ($r = 0.9995$, $n = 15$), $\log y = 1.814 \log x - 1.186$ ($r = 0.9999$, $n = 15$) and $\log y = 1.841 \log x - 1.347$ ($r = 0.9992$, $n = 15$), respectively, where y is the peak-height ratio and x is the amount of selenium compounds.

These experiments conclusively demonstrated that the method proposed is suitable for the selective and sensitive determination of Se-CYE, Se-Cys and Se-Met. Further investigations on the application of this method to biological samples are in progress.

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