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Short communication

Determination of total plasma homocysteine and related aminothiols by gas chromatography with flame photometric detection

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Abstract

A selective and sensitive method for the determination of total homocysteine (Hcy) and related aminothiols, such as cysteine (Cys) and cysteinylglycine (CysGly), in plasma samples by gas chromatography (GC) has been developed. After reduction of the sample with sodium borohydride, the liberated Hcy and other aminothiols were converted to their *N,S*-diisopropoxycarbonyl methyl ester derivatives and measured by GC with flame photometric detection using a DB-17 capillary column. The calibration curves were linear over the range 0.5–10 nmol for Hcy and CysGly, and over the range 5–100 nmol for Cys, and the correlation coefficients were above 0.996. Using this method, total plasma Hcy, Cys and CysGly could be directly analysed without prior clean-up of the sample and without any interference from coexisting substances. Overall recoveries of Hcy and other aminothiols added to plasma samples were 95–106%. Analytical results for the determination of total plasma Hcy, Cys and CysGly from normal subjects are presented.

1. Introduction

Homocysteine (Hcy) is an important intermediate in the transsulphuration and remethylation pathways [1] of methionine metabolism. The plasma concentration of Hcy is markedly increased in patients with the inborn defect called homocystinuria [2], and during vitamin B_{12} [3–6] and folate deficiency [3,6,7]. Furthermore, a moderate increase of plasma Hcy has been reported in renal failure [8,9], psoriasis [10], leukemia [11] or solid tumors [12]. In addition, recent evidence suggests that total plasma Hcy may be used as an independent risk factor for premature vascular disease [13,14]. Thus, the plasma Hcy level is useful as a clinical marker. Possible applications of the measurement of Hcy include not only risk assessment of vascular disease and evaluation of nutritional status, but also monitoring of drug efficacy, renal status, and cancer prognosis. In freshly prepared plasma from healthy subjects, the major fraction is protein-bound and most free Hcy exists as the Hcy–Cys mixed disulphide and thus determination of total (free reduced and oxidized plus protein-bound) Hcy is preferable in a clinical setting [15].

The determination of total plasma Hcy has been carried out by amino acid analysis, gas chromatography–mass spectrometry (GC–MS)

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and high-performance liquid chromatography (HPLC). However, amino acid analysis [16] is non-selective for sulphur amino acids and lacks sensitivity, while the GC–MS method based on the conversion into *tert.*-butyldimethylsilyl derivative [17] requires expensive equipment and anhydrous derivatization conditions. Although HPLC analyses based on precolumn fluorescence derivatization with 4-(aminosulphonyl)- or ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulphonate [18–20], monobromobimane [21–23] and *o*-phthaldialdehyde [24], or based on postcolumn derivatization with 4,4'-dithiopyridine [25] and electrochemical detection [26] were highly sensitive, some of these methods lack specificity and require clean-up of the sample to remove excess reagent and coexisting substances. Methods and clinical applications for the determination of total Hcy in plasma or serum have been reviewed in detail by Ueland et al. [27] and Hagan [28].

Recently, we have developed a selective and sensitive method for the determination of sulphur amino acids by gas chromatography with flame photometric detection (GC-FPD), in which these compounds were analysed as their *N*,(*S*)-isopropoxycarbonyl (isoPOC) methyl ester derivatives [29], and demonstrated that free sulphur amino acids in small urine samples could be accurately and precisely determined without any influence from other coexisting substances. This paper reports the extension of this work to the determination of total Hcy and related aminothiols in plasma samples.

2. Experimental

2.1. Reagents

D,L-Homocysteine (Hcy), L-cysteine (Cys), D,L-homocystine, L-cystine and L-methionine were purchased from Nacalai Tesque (Kyoto, Japan). Cysteinylglycine (CysGly) and *S*-2-aminoethyl-L-cysteine, used as internal standards (I.S.), were purchased from Sigma (St. Louis, MO, USA). Each compound was dissolved in 0.05 *M* hydrochloric acid to make a stock solution with a concentration of 2 m*M* and stored at 4°C. The working standard solutions were made up freshly as required by dilution of the stock solution with 0.01 *M* hydrochloric acid. Sodium borohydride ($NaBH_4$, Nacalai Tesque) was used as a 100 mg/ml solution in 0.1 *M* sodium hydroxide. Isopropyl chloroformate (isoPCF) was obtained from Wako Pure Chemicals Industries (Osaka, Japan). Hydrogen chloride in methanol (HCl–MeOH) obtained from Tokyo Kasei Kogyo (Tokyo, Japan) 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.

2.2. Preparation of samples

Venous blood samples from healthy volunteers were collected in ethylenediamine tetraacetate (5 m*M*). After centrifugation at 2000 *g* for 5 min, the plasma layer was carefully collected. The plasma sample was processed immediately or stored at −20°C until used. To obtain total Hcy, Cys and CysGly, plasma samples were chemically reduced with $NaBH_4$. To 0.1 ml of plasma sample was added 0.1 ml of 20 μM *S*-2-aminoethyl-L-cysteine (I.S.) and 0.2 ml of 100 mg/ml $NaBH_4$, and then total volume was made up to 1 ml with distilled water. After adding 1 drop of *n*-hexanol, the mixture was incubated at 100°C for 10 min. After cooling, this reaction mixture was used for derivatization.

2.3. Derivatization procedure

To the $NaBH_4$-reduced sample was added 0.05 ml of isoPCF, and then the mixture was shaken at 300 rpm (up and down) for 5 min at room temperature. The reaction mixture was extracted with 3 ml of peroxide-free diethyl ether in order to remove excess of reagent, the ethereal extract being discarded. The aqueous layer was acidified to pH 1–2 with 2 *M* hydrochloric acid and saturated with sodium chloride, and then the mixture was extracted twice with 3 ml of peroxide-free diethyl ether. After the ethereal extracts were evaporated to dryness at 80°C, 0.2 ml of 1

M HCl–MeOH was added to the residue and the mixture was incubated at 80°C for 10 min. After the residual solvent was evaporated to dryness at 80°C under a stream of dry air, the residue was dissolved in 0.1–0.2 ml of ethyl acetate and 0.2–0.5 μl of this solution was injected onto the gas chromatograph.

2.4. Gas chromatography

GC analysis was carried out with a Shimadzu 12A gas chromatograph equipped with a flame photometric detector (S-filter). A fused-silica capillary column (15 m × 0.53 mm I.D., 1.0 μm film thickness) of crosslinked DB-17 (J and W, Folsom, CA, USA) was used. The operating conditions were as follows: column temperature, programmed at 10°C/min from 160 to 270°C; injection and detector temperature, 280°C; nitrogen flow-rate, 10 ml/min. The peak heights of aminothiols and the I.S. were measured and the peak-height ratios against the I.S. were calculated to construct calibration curves.

3. Results and discussion

Knowledge of the species of Hcy circulating in vivo is important for evaluating the hyperhomocysteinemia; some effects of elevated Hcy levels may result from secondary effects on other aminothiols such as Cys and CysGly. In practice the relationship between Hcy and other aminothiols was evaluated during hyperhomocysteinemia [5,22,23,25,30,31]. Therefore, we investigated the simultaneous determination of Hcy, Cys and CysGly. In order to determine the total concentration of these aminothiols, disulphides in the sample must be reduced to the corresponding thiols and then derivatized with an appropriate reagent for GC analysis. $NaBH_4$ efficiently converts all Hcy present as homocystine and mixed disulphides with other low-molecular-mass thiols or protein-bound Hcy to the thiol form [21–23,26]. In order to determine the reduction conditions for the disulphide forms of Cys, Hcy and CysGly, standard homocystine, cystine and plasma sample were reduced with $NaBH_4$. The reduction of these samples was accomplished within 10 min at 100°C by using 20 mg $NaBH_4$ in 0.2 ml of 0.1 *M* sodium hydroxide. Subsequently, the sample treated with $NaBH_4$ could be directly derivatized after cooling, and Cys, Hcy and CysGly could be rapidly and quantitatively converted to *N*,*S*-isoPOC methyl ester derivatives as previously described [29].

As shown in Fig. 1A, Cys, Hcy and CysGly were separately eluted as single and symmetrical peaks. Oxidation of aminothiols to disulphides was not observed under the analytical conditions used. The derivatives gave excellent FPD responses and the minimum detectable amounts of Cys, Hcy and CysGly at a signal-to-noise ratio of 3 under the instrumental conditions employed were ca. 0.5, 0.5 and 0.7 pmol as injected amounts, respectively. In order to test the linearity of the calibration curve, various amounts ranging form 0.5 to 10 nmol for Hcy

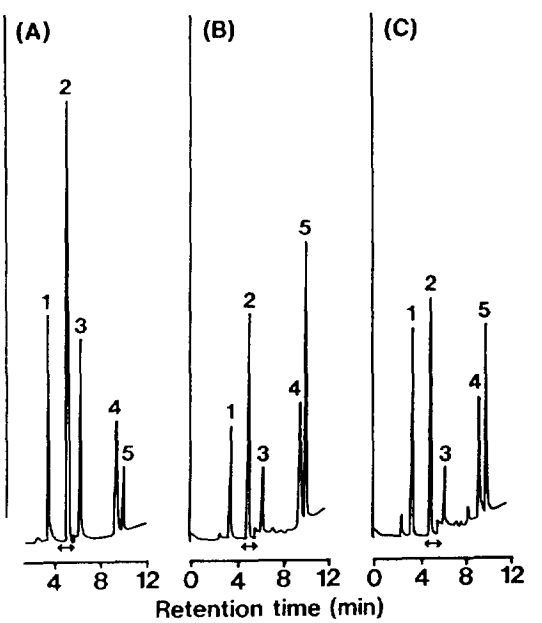

Fig. 1. Gas chromatograms obtained from standard and plasma samples. (A) Standard (containing 20 nmol of cysteine and 2 nmol of other aminothiols), (B) and (C) plasma (0.1 ml). GC conditions are given in Experimental. The double arrow on the time scale indicates the time during which the recorder response was reduced to one sixteenth. Peaks: 1 = methionine, 2 = cysteine, 3 = homocysteine, 4 = *S*-2-aminoethyl-L-cysteine(I.S.), 5 = cysteinylglycine.

Table 1
Recoveries of homocysteine and other aminothiols added to plasma samples

Sample	Aminothiol [a] added (nmol/ml)	Concentration found [b] (nmol/ml)		Recovery (%)
		Non-addition	Addition	
A	Cys (100)	201.4 ± 17.8	297.2 ± 30.7	95.8
	Hcy (10)	4.3 ± 0.4	13.8 ± 0.6	95.0
	CysGly (10)	40.7 ± 2.7	51.1 ± 2.3	104.0
B	Cys (100)	171.0 ± 5.7	266.6 ± 26.6	95.6
	Hcy (10)	10.5 ± 0.6	20.8 ± 0.8	103.0
	CysGly (10)	34.3 ± 3.3	44.9 ± 1.7	106.0

[a] Cys = cysteine; Hcy = homocysteine; CysGly = cysteinylglycine.
[b] Mean ± S.D. ($n = 4$).

and CysGly and form 5 to 100 nmol for Cys were derivatized in the mixture, and aliquots representing 5–100 pmol of Hcy and CysGly and 50–1000 pmol of Cys were injected onto the GC-FPD system. In each case, a linear relationship was obtained in double logarithmic plots, and the correlation coefficients were above 0.996.

The developed method was successfully applied to plasma samples without any pretreatment. Hcy in plasma is stable for 4 days at room temperature and for several years when kept frozen, and repeated freezing–thawing cycles did not affect the plasma concentration [27]. Formation of gas and foaming during the $NaBH_4$ reduction of the plasma samples could be reduced by adding one drop of *n*-hexanol, a surface-active agent. Fig. 1B,C show typical chromatograms obtained from 0.1 ml of plasma samples. Methionine was also detected in these samples. The recorder response was reduced to one sixteenth between ca. 4.4 and 5.6 min after sample injection because of the large difference in concentration between Cys and the other aminothiols. Cys, Hcy and CysGly in plasma could be analysed without any interference from coexisting substances. As shown in Table 1, the overall recoveries of Cys, Hcy and CysGly added

Table 2
Total plasma cysteine, homocysteine and cysteinylglycine in normal subjects

Subject	Age	Total concentration [a] (nmol/ml)		
		Cysteine	Homocysteine	Cysteinylglycine
Male				
1	22	130.0 ± 8.2	11.0 ± 0.5	47.4 ± 3.8
2	23	134.8 ± 11.8	11.6 ± 0.6	43.6 ± 0.5
3	25	127.8 ± 8.7	11.1 ± 0.4	51.8 ± 3.2
4	39	135.5 ± 12.6	10.1 ± 0.8	82.1 ± 4.4
Female				
5	21	124.8 ± 12.5	9.2 ± 0.4	48.6 ± 1.3
6	22	126.9 ± 4.1	10.6 ± 0.2	38.4 ± 0.6
7	23	153.0 ± 13.2	11.1 ± 0.8	40.2 ± 0.4
8	23	165.3 ± 12.2	13.1 ± 0.9	65.5 ± 1.7

[a] Mean ± S.D. ($n = 4$).

to plasma samples were 95–106%, and the relative standard deviations were 3.3–10.3% ($n = 4$). The quantitation limit of total Cys, Hcy and CysGly in plasma sample was ca. 2 nmol/ml. The intra-assay C.V.s for the aminothiols were 1.0–10.0% ($n = 4$) and inter-assay C.V. in a plasma control sample over a period of 4 days was 5.5–14.5% ($n = 4$). Table 2 shows the results obtained from healthy volunteers. Total Cys, Hcy and CysGly in plasma were 137.3 ± 14.4, 11.0 ± 1.1 and 52.2 ± 14.7 nmol/ml, respectively, in 8 subjects, and no statistical differences were found between male and female.

4. Conclusions

A convenient and reliable method for the determination of total Hcy and related aminothiols in plasma samples has been established. The method is selective and sensitive, and plasma samples can be analysed without prior clean-up and without any interference from other coexisting substances. We believe that this method provides a useful tool in biochemical and clinical research.

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