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A Modified Method for the Determination of Plasma Total Homocysteine by High Performance Liquid Chromatography

Abstract: We have modified a high-performance liquid chromatography (HPLC) procedure for homocysteine assay using 7-fluoro-2, 1, 3-benzoxadiazole-4-sulfonamide (ABD-F) pre-column derivatization. In this study, the thiol compounds were liberated from plasma proteins by reduction with 2-mercaptoethanol (BME) and derivatized with a thiol-specific fluorogenic marker, ABD-F. The derivatives were separated by reversed-phase HPLC on a Hypersil C 18 column (4.6 x 150 mm; 5 μ) using isocratic elution with 0.1 M phosphate buffer pH 6.0 containing 8% methanol and fluorescence detection. Excitation and emission wavelengths were 386 and 516 nm. The peak of homocysteine was eluted at 413 ± 25 s. Mean within-day and between-day precisions were determined to be 1.96 and 4.34% CV, respectively. The method was linear up to 100 μmol/L and proved to be sensitive with a detection limit of 1 μmol/L for homocysteine.

In plasma samples from healthy adult subjects, the concentration of homocysteine was found to be higher in men than in women (11.69 ± 2.9 versus 9.45 ± 1.78 μmol/L, p = 0.002). The method is simple, sensitive, and reproducible and allows a rapid determination of total homocysteine in human plasma under routine conditions.

Key Words: Homocysteine, High performance liquid chromatography, ABD-F, Sulfamoylbenzofurazan, Mercaptoethanol

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Introduction

Homocysteine (Hcy) is a sulfur-containing aminoacid. Circulating forms of Hcy include homocysteine itself, also called the reduced form (1%), the oxidized form of Hcy (5-10%), homocysteine-cysteine mixed disulfide (5-10%) and protein-bound homocysteine (80-90%) (1). Total Hcy is defined as the sum of all homocysteine species in plasma, including free and protein-bound forms.

The determination of Hcy in plasma is useful both in the diagnosis and follow-up of folate and cobalamine deficencies and the rare inborn errors causing homocystinuria (2, 3). In addition, hyperhomocysteinemia is an independent risk factor for premature cardiovascular diasease (4). Thus, the accurate determination of plasma concentrations of Hcy is essential for understanding the role of Hcy in the pathogenesis of related diseases.

The increasing interest in measuring total Hcy in plasma has led to the development of several methods of quantitation (5-7). A widely used technique is reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection (FD) (5, 8-13). The determination of total Hcy in plasma requires the reduction of the disulfide bond between Hcy and other thiols or albumin. The selection of reductant depends on the separation and detection system used. Sulfhydryl-containing reducing agents such as dithioerythritol, dithioreitol, and mercaptoethanol liberate Hcy from various disulfides (10). Sodium or potassium borohydride (11) and tri-n-butylphosphine (8, 12) have also been utilized as the reducing agent.

Hcy formed during the reduction step can be reoxidized before derivatization or detection. Thiol oxidation is inhibited by adding EDTA to the reaction mixture. Precolumn derivatization with fluorogenic reagents for thiols followed by HPLC has become increasingly popular (5). Commonly used fluorogenic reagents include monobromobimane (mBrB) (11), ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonate (SBD-F) (9) and 7-fluoro-2, 1, 3-benzoxadiazole-4-sulfonamide (ABD-F) (8). SBD-F and ABD-F are halogenated sulfamoylbenzofurazans that have been used to determine thiols, including Hcy (14). ABD-F reacts quantitatively with thiols, including Hcy, at 50 °C at pH 8.0–9.5 for 5–10 min (15).

In this study, we describe a simple method for the rapid determination of total plasma Hcy by using the simultaneous mercaptoethanol reduction of disulfide bonds and derivatization of sulfhydryl groups with ABD-F reagent followed by HPLC-FD. Besides describing the method, we also report on its use to determine differences between the concentrations of total Hcy in the plasma of healthy adults and patients.

Materials and Methods

Reagents

DL-Homocysteine, L-cysteine (Cys), cysteinylglycine (CysGly), glutathione (GSH), and 7-fluoro-2, 1, 3-benz-oxadiazole-4-sulfonamide (ABD-F) were obtained from Sigma (St. Louis, MO, U.S.A.). 2-Mercaptoethanol (BME), sodium tetraborate, EDTA disodium salt, trichloroacetic acid (TCA), HPLC-grade methanol, Na_2HPO_4 and KH_2PO_4 were from Merck (Darmstadt, Germany). HPLC-grade water was produced by a Milli-Q water purification system from Millipore (Elga, Germany).

Standard Solutions

DL-Homocysteine (0.5 mmol/L), cysteinylglycine (0.5 mmol/L), glutathione (1 mmol/L) and L-cysteine (2 mmol/L) were dissolved in 0.1 mol/L HCl. These stock solutions were further diluted in borate buffer (0.05 mmol/L, pH 8.0, containing 2 mmol/L Na_2EDTA) to known concentrations.

Instrumentation

Separation and quantification were performed with a Hewlett Packard 1050 HPLC-FD equipped with a Phenomenex (CA, U.S.A.) Hypersil C 18 reverse-phase analytical column (4.6 x 150 mm, 5 μm particles) protected by a Phenomenex (CA, U.S.A.) Hypersil C 18 guard column (4.6 x 50 mm, 5 μm particles). Fluorescence intensities were measured with excitation at 386 nm and emission at 516 nm. Peaks were quantified by reference to a calibration curve constructed by triplicate analysis of Hcy standards (0, 2.5, 5, 10, 15, 25, 50 and 100 μmol/L) and plotting peak height versus Hcy concentration. The linear regression equation obtained from the calibration curve was used to calculate the concentration of Hcy in unknown plasma samples from healthy donors and patients. Calibration curves for Cys, CysGly and GSH were also established similarly.

Sample Collection and Subjects

Plasma was used to determine total Hcy and other thiols. Blood was obtained by venipuncture and collected into evacuated tubes containing EDTA. The tubes were placed on ice and the blood cells were removed without delay by centrifugation for 10 min at 2000 x g (11). Plasma was stored at –40 °C until analysis. Control blood samples were collected from fasting, apparently healthy adult volunteers (25 men and 21 women, aged 25–47 years). We also studied 10 male patients with premature cardiovascular disease diagnosed by coronary angiography, 11 male patients with hypogonadism and six patients with Behçet's disease. Two of the hypogonadic patients were receiving testosterone replacement therapy.

Method

One hundred microliters of plasma or standards was mixed with 20 μL of 0.03 mol/L borate buffer, pH 8.0, containing 4 mmol/L Na_2EDTA. Ten microliters of 2-mercaptoethanol (0.1 mol/L dissolved in 0.05 mol/L sodium tetraborate) was added. The mixture was incubated at room temperature for 30 min to accomplish the reduction of Hcy and the mixed disulfide (cysteine-homocysteine) as well as the release of protein-bound homocysteine and other plasma thiols. This method therefore measures total (free plus protein-bound) plasma Hcy levels. After reduction, 50 μL of ABD-F reagent (23 mmol/L dissolved in 0.05 mol/L borate buffer, pH 8.0, containing 4 mmol/L Na_2EDTA) was added. The mixture was incubated for 10 min at 50 °C to accomplish the complete derivatization of Hcy and other plasma thiols. Deproteinization was achieved by the addition of 20% (w/v) TCA (40 μL) at 2 °C and centrifugation at 23 000 g. for 10 min at 2 °C. The clear supernatant (150 μL) was stored at 4 °C for HPLC analysis on the same day.

All samples were analyzed on a Hewlett Packard 1050 HPLC-FD system. The mobile phase was 0.1 mol/L phosphate buffer, pH 6.0, containing 8% (v/v) methanol. HPLC was performed under isocratic conditions at a flow rate of 1.6 mL/min. Twenty microliters of sample were used for each injection. The column effluent was monitored by a fluorescence detector for 14 min. Excitation and emission wavelengths were at 386 and 516 nm, respectively.

To investigate the recovery and precision of the assay, Hcy was added to the plasma pool so as to increase its concentration by 5 and 10 µmol/L (S5 and S10). These samples were stored at –40 °C until analysis. The concentrations in the plasma pool (P) and in the plasma pools with added standards (PS5 and PS10) were then determined in 10 replicates and analytical recovery was calculated as follows:

Recovery (%) = 100 . (PS – P) / S

Statistical Analysis

The statistical analyses were performed by using SPSS version 9.0 for Windows. All results were expressed as means ± standard deviation (SD). Mann-Whitney U and Kruskal-Wallis nonparametric tests were used to assess overall differences between the healthy (control) group and the different groups of and patients, with $p < 0.05$ being considered statistically significant.

Results

As shown in Figure 1, the four thiols were separated with near-baseline resolution using the standard solutions. The retention times of Cys, Hcy, CysGly and GSH were 299 ± 20, 413 ± 25, 530 ± 20 and 600 ± 30 s, respectively. The retention times were also identical in the HPLC chromatograms of healthy subjects (Figure 2) and patients (Figure 3) using plasma samples.

Determination of total Hcy in plasma requires the reduction of the disulfide bond between Hcy and other thiols before the derivatization step. After testing with different concentrations of BME as a reducing agent, we decided to use 0.1 mol/L BME for the optimum reduction of the thiols. BME was found to be effective for reduction of the thiols over pH 7.0 and was compatible with ABD-F reagent in this pH range.
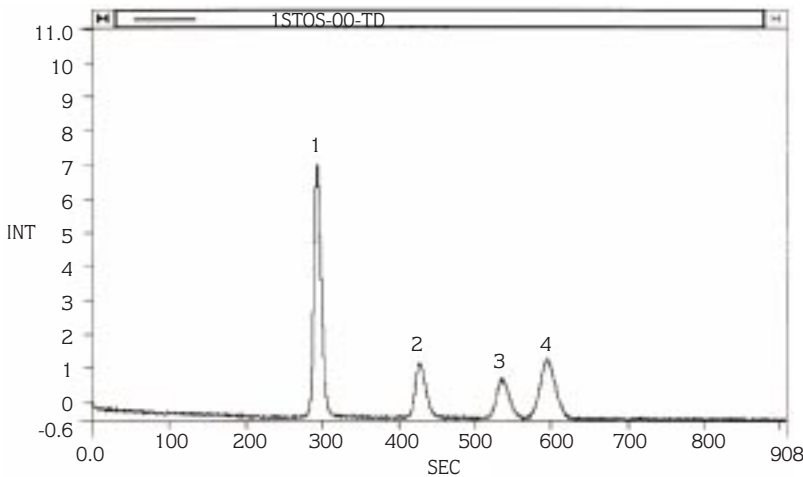

Figure 1. HPLC chromatogram of 100 µmol/L cysteine and 5 µmol/L homocysteine, cysteinylglycine, and glutathione standards. Peaks: 1 = Cys, 2 = Hcy, 3 = CysGly, 4 = GSH.
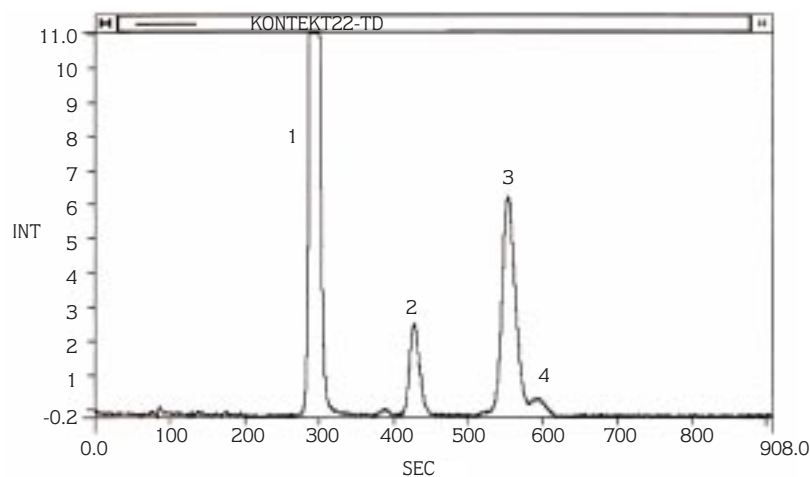

Figure 2. HPLC chromatogram of the thiols in total plasma from a healthy subject. Peaks: 1 = Cys, 2 = Hcy, 3 = CysGly, 4 = GSH.
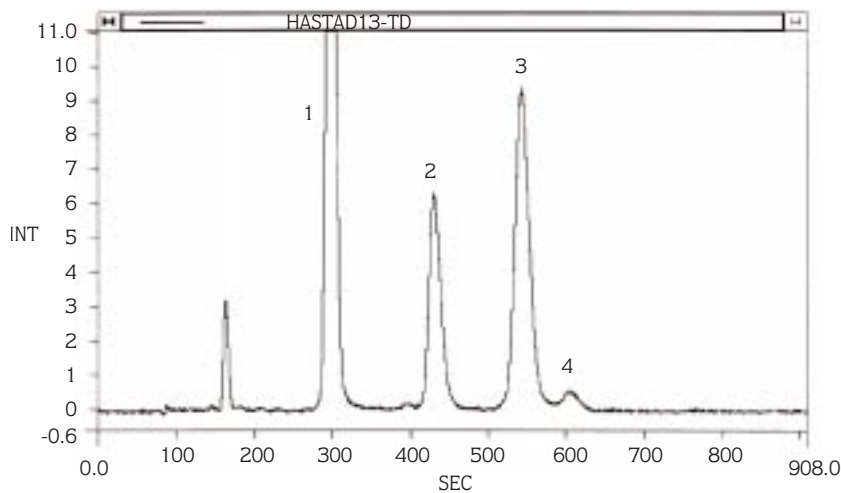
Figure 3. HPLC chromatogram of the thiols in total plasma from a patient with premature cardiovascular disease. Peaks: 1 = Cys, 2 = Hcy, 3 = CysGly, 4 = GSH.

Calibration curves were obtained by assaying known concentrations of standards. The standard curve for Hcy was linear over a concentration range of 0–100 µmol/L; higher concentrations were not tested as they were not encountered in normal populations. Linear regression analysis of the results gave y = 0.0285 + 0.3027 x (r = 0.9998). The curves for Cys, CysGly and GSH were also linear: y = -1.9012 + 0.1304 x (r = 0.9958), y = 0.2058 + 0.2238 x (r = 0.9947) and y = 0.8616 + 0.2028 x (r = 0.9922) (data not shown). The detection limit of the method for Hcy was 1 µmol/L.

Performance parameters for the determination of plasma total Hcy levels, as calculated from the repeat analysis of three plasma samples, are summarized in Table 1. The mean recovery of Hcy added to a plasma pool to final concentrations of 5.0 and 10.0 µmol/L was 97.4%. The within- and between-day coefficients of variation (CV) for Hcy were <5%.

Total plasma Hcy was determined by the current method in healthy subjects and patients (Table 2). The mean (±SD) total plasma Hcy in 25 healthy men was 11.69 (±2.32); the corresponding value in healthy women was 9.45 (±1.78) µmol/L. The difference between the mean value for middle aged healthy males and females was significant at p < 0.001.

The concentrations of total Hcy in plasma were significantly higher in patients with premature atherosclerotic vascular disease (p = 0.0026) and Behçet's disease (p = 0.035) than in healthy subjects. The differences between patients with hypogonadism and healthy subjects were not statistically significant (p =

0.17), but the plasma concentrations of total Hcy in two hypogonadic patients receiving hormonal replacement therapy were found to be about two-fold higher than those of healthy subjects.

Discussion

Hyperhomocysteinemia is a possible risk factor for premature vascular disease, and is independent of other factors such as hypertension, diabetes, smoking, and plasma cholesterol. In coronary artery disease, the ratio for mean plasma Hcy in patients vs. controls was 1.2 to 1.8 (1, 13, 16, 17). Thus, methods with high precision and sensitivity are required to detect a minimal rise in total Hcy concentrations. Here we describe a simple and rapid method for the determination of total Hcy by using HPLC-FD.

Of the various thiol-specific derivatization reagents (8, 9, 13-15, 18), we chose ABD-F as a suitable reagent because mBrB gives rise to several reagent peaks that may interfere with Hcy determination and SBD-F requires more drastic conditions (pH 9.5, and 60 °C for 1 h). In addition, the short reaction time of ABD-F helps to prevent the reoxidation of Hcy. The derivatization of Hcy and other thiols with the ABD-F reagent proceeds optimally above pH 7.0 (14, 15). In our study, BME was used together with ABD-F and was compatible with it in this pH range. This eliminated the necessity of adjusting pH for the mixture. As this pH range is also suitable for plasma samples, the extensive dilution of plasma was therefore prevented.

Table 1. Precision and recovery of the assay.

Sample[a]	Recovery		Within-day (n = 10)	Between-day (n = 10)
	Added, µmol/L	Recovered, %	Mean ± SD (CV%)[b]	Mean ± SD (CV%)[b]
PS 10	10.0	98.9 ± 1.8	19.04 ± 0.36 (1.91)	18.81 ± 0.86 (4.58)
PS 5	5.0	95.9 ± 4.5	13.52 ± 0.27 (2.01)	13.62 ± 0.60 (4.42)
P	0.0	---	8.89 ± 0.17 (1.96)	8.74 ± 0.35 (4.04)

[a] described in the text.
[b] mean and standard deviation are expressed as µmol/L.

Table 2. Comparison of plasma total Hcy levels in patients and healthy subjects.

	Healthy subjects		Patients		
	Men	Women	Atherosclerosis	Hypogonadism	Behçet's Disease
n	25	21	10	11	6
Age (mean)	35	33	40	26	30
Hcy (mean ± SD)[a]	11.69 ± 2.32*	9.45 ± 1.78	18.93 ± 9.0 **	13.20 ± 1.95	14.44 ± 3.72***

[a] mean and standard deviation are expressed as µmol/L.
* $p < 0.001$, ** $p < 0.005$, *** $p < 0.05$.

We used 20% TCA for removing plasma proteins after the reduction and derivatization steps. The time of deproteinization (pre- versus post-derivatization) was found not to affect the results of the Hcy assay. The sample preparation procedure involved minimal dilution (2.2-fold) of the plasma sample. This increased the sensitivity of our method. We tested different mobile phases to achieve the optimum elution of the thiols.

The mean ± SD within- and between-day CVs% for Hcy were determined to be 1.96 ± 0.27 and 4.43 ± 0.6, respectively. These values are similar to those obtained with other widely used methods (1, 13). Significantly higher concentrations of total Hcy were found in plasma from men ($p = 0.002$). These findings are therefore consistent with most other studies (11, 19). We also found plasma Hcy levels to be 1.2- to 1.8-fold higher in patients with premature cardiovascular disease as compared to healthy subjects. This moderate increase in plasma Hcy above normal is statistically associated with premature cardiovascular disease (1). The plasma concentrations of total Hcy in hypogonadic patients tended to be elevated compared with those in healthy subjects, but the difference was significant only in two hypogonadic patients receiving hormonal replacement therapy. Therefore, we believe that studies on patients with hypogonadism will help to explain the relations between sex hormones and plasma Hcy levels. There are studies related to the high plasma Hcy levels in patients with autoimmune disease (13, 20). We also found significantly high plasma Hcy levels in patients with Behçet's disease.

In conclusion we have developed a HPLC method for measuring total homocysteine in plasma, using ABD-F and BME as a fluoregenic marker and reducing agent, respectively. The method is simple, sensitive and suitable for routine use in the clinical laboratory.

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