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Short communication

Determination of plasma and serum homocysteine by high-performance liquid chromatography with fluorescence detection

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Abstract

Determination of homocysteine in plasma or serum is becoming an important diagnostic procedure. Accurate, rapid and low cost methods for measuring homocysteine are therefore required. We have improved an HPLC method and made it suitable for clinical application. The total homocysteine in plasma consists of free homocysteine (i.e., reduced plus oxidized homocysteine in the non-protein fraction of plasma) and protein-bound homocysteine. The method consists of the following steps: reduction of the sample with tri-*n*-butylphosphine, precipitation of proteins with trichloroacetic acid (10%) and derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. The derivatives are separated by reversed-phase high-performance liquid chromatography followed by fluorescence detection. The concentrations (mean±S.D.) of total homocysteine in plasma from 77 normal subjects, 44 male and 33 female adults, were 8.4±2.15 and 7.1±1.18 μmol/l, respectively. Serum concentrations were 8.8±2.6 μmol/l in males and 7.6±1.5 μmol/l in females.

Keywords: Derivatization, LC; Homocysteine; Amino acids

1. Introduction

Homocysteine is a sulfur amino acid with a free sulfhydryl group. It was first discovered by du Vigneaud et al. in 1932 as a product of demethylation of methionine [1]. Methionine is converted to *S*-adenosylmethionine, which represents the major source of methyl groups. The resulting *S*-adenosylhomocysteine is hydrolyzed to homocysteine. Homocysteine can be remethylated to methionine by 5-methyltetrahydrofolate in a vitamin B_{12}-dependent reaction. Alternatively homocysteine can be converted to cysteine with cystathionine as an intermediate. Both enzymes of this conversion, cystathionine β-synthase and γ-cystathionase, are vitamin B_6 dependent. This metabolic pathway of methionine shows that decreased levels of vitamin B_6, vitamin B_{12} or folate cause an accumulation of homocysteine. Markedly elevated plasma levels of homocysteine are found in subjects with homocysteinuria [2,14]. Numerous studies have indicated that a milder degree of hyperhomocysteinemia is also associated with an increased risk of developing occlusive vascular diseases [3–5] and that homocysteine is a potent inducer of atherosclerosis [6,7]. Genetic factors, such as heterozygosity for homocysteinuria and the thermolabile variant of methylenetetrahydrofolate reductase, or nutritional factors, such as deficiency of folate or cobalamin, partly seem to explain the etiology of the hyperhomocysteinemia in vivo: 70–80% of homocysteine is protein bound, probably to albumin; 20–30%

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exists as free symmetric or mostly asymmetric mixed disulfides; free reduced homocysteine exists in only trace amounts [8]. Since Kang et al. [9] reported the presence of homocysteine bound to plasma proteins in normal individuals, total (i.e., free plus protein-bound) homocysteine in normal human plasma has been measured by different methods. The determination of free, acid-soluble homocysteine gives variable results. In contrast, total homocysteine seems to be stable for years in samples stored in

Table 1
Construction and evaluation of assays for total HCYS in plasma and serum (after [24])

Reduction	Derivatization	Separation	Detection	Advantages	Disadvantages	Refs.
2-Mercapto-ethanol	*S*-Carboxy-methylation with iodoacetic acid	Ion exchange (amino acid analysis)	Ninhydrin reaction	Equipment available, reliable assay of the amino acids. Yes autoinjection	Laborious sample preparation, low sensitivity, low analysis time and low sample output	[15]
DTE	Enzymic conversion to *S*-adenosyl-homocysteine	HPLC	Absorbance at 254 nm or scintillation counting	Specific, sensitive autoinjection	Laborious sample preparation, sensitive to enzyme inactivity or denaturation, low range, low sample output	[16]
2-Mercapto-ethanol	*tert*-Butyldimethilsilyl derivatization	Capillary GC	Mass spectrometry (single ion monitoring)	Specific, simultaneous determination of cysteine and methionine, autoinjection	Laborious sample clean-up and preparation, instrument not available in most routine laboratories	[17]
Tri-*n*-butylphosphine	SBD-F	HPLC	Fluorescence	Specific, sensitive, measures other thiols, autoinjection	Long incubation (90 min) and heating (60°C), toxic reduction agents	[12,18]
DTE	Enzymic conversion to *S*-adenosyl-homocysteine	TLC	Scintillation counting	Specific, sensitive, inexpensive equipment	Laborious sample preparation, scintillation counter necessary, sensitive to enzyme inactivity or denaturation, low range	[19]
Sodium borohydride	None	HPLC	Electrochemical detection	Specific, sensitive, measures other thiols, no derivatization, autoinjection	Careful maintenance of flow-cell and reference, electrode required	[20,21]
Sodium borohydride	Monobromo-bimane	HPLC	Fluorescence	Fairly specific, sensitive, precise, fully automated, high sample-autoput, measures other thiols	Requires expensive equipment	[22]
Potassium borohydride	Monobromo-bimane	HPLC	Fluorescence	Fairly specific, sensitive, measures other thiols	Sample clean-up	[13]
DTT	None	Ion exchange (amino acid analyzer)	Ninhydrin reaction	Equipment available, autoinjection, measures methionine	Low sensitivity	[23]

GC, gas chromatography; SBD-F, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate; DTE, dithioerythreitol; DTT, dithiothreitol; TLC, thin-layer chromatography.

closed vials at −20°C. In the clinical setting and in studies based on stored samples, determination of total homocysteine is recommended [10]. Since 1982, several methods for the determination of total homocysteine in plasma or serum have been described. Most assays can be divided into four steps: (a) reduction of oxidized homocysteine species to thiol; (b) precolumn derivatization; (c) chromatographic separation; and (d) detection of the homocysteine derivative. The construction of these assays and evaluation of their performance are summarized in Table 1. A fluorogenic thiol-specific reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) has been developed for the measurement of biologically important thiols [11]. This paper describes a simple but sensitive and selective HPLC method for the determination of total homocysteine, cysteine, cysteinyl-glycine and glutathione in plasma or serum using SBD-F. It is suitable for routine determination of plasma/serum homocysteine levels and well within the capabilities of the average clinical pathology laboratory.

2. Experimental

2.1. Material

D,L-Homocysteine, L-cysteine, and tri-*n*-butylphosphine were obtained from Sigma (St. Louis, MO, USA). Cysteinyl-glycine was from Serva (Westbury, NY, USA). Glutathione was bought from Boehringer (Mannheim, Germany), while SBD-F was supplied by Wako (Dusseldorf, Germany). All other reagents were obtained from Merck (Darmstadt, Germany).

2.2. Sample preparation

Plasma samples were obtained from about 3 ml of whole blood, which were collected in a Venoject tube containing EDTA, then immediately cooled on ice and centrifuged as soon as possible at 3000 rpm (2000 *g*) for 10 min at room temperature. Serum was prepared from whole blood which was left to clot for 30 min at room temperature, then centrifuged. Specimens were stored at 4°C for analysis on the same day. Storage for longer periods was at −20°C.

Five hundred microlitres of plasma/serum (sample or calibration material) was mixed with 50 μl of 100 ml/l tri-*n*-butylphosphine in dimethylformamide, and the reduction allowed to proceed for 30 min at 4°C. Samples were then mixed with 500 μl of trichloroacetic acid (TCA) (10%), containing 1 mmol/l EDTA, left at room temperature for 10 min, then centrifuged at 13 000 rpm (15 500 *g*) for 10 min. Two hundred microlitres were taken from the middle of the supernatant and mixed with 400 μl of 125 mmol/l potassium borate, pH 10.5, containing 4 mmol/l EDTA. Ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate solution (200 μl) (1.0 g/l of 125 mmol/l potassium borate, pH 9.5) were added and the mixture incubated at 60°C for 60 min. After cooling in an ice bath, the samples were ready for HPLC; 20 μl of derivatized sample were used for each injection.

2.3. Apparatus

Separation and quantification were performed with a Hewlett-Packard system (HP 1090 LC, fluorescence detector HP 1046-A and autosampler 79847-A) equipped with a Merck LiChrospher 100 RP-18 column (125×4 mm, 5 μm particles). The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. The detection signal was recorded and the peak areas quantified and processed with a Pascal work-station (series 9000).

2.4. Chromatographic conditions

We have not used the gradient procedures described by Araki and Sako [12], but an isocratic system, which was run for 10 min at a flow-rate of 1.00 ml/min. Although our column and HPLC system differs from theirs and we observe shorter retention times, the procedures work satisfactorily.

Buffer: 0.1 mol/l acetate buffer, pH 4.0, containing 20 ml/l methanol (prepared from 0.1 mol/l acetic acid and 0.1 mol/l sodium acetate, 600:1500). The buffer was filtered through a 0.45-μm Millipore filter.

2.5. Calibration and calculations

Standards of homocysteine, cysteine, cysteinyl-

glycine and glutathione were carried throught the sample preparation procedure with or without TCA. The concentration of plasma homocysteine was automatically calculated by the integrator from a two-point calibration line obtained by triplicate analysis of two different solutions containing SBD-F-homocysteine as external standard.

The significance of the differences between means was assessed by the Student's *t*-test. Relative standard deviations (R.S.D.s) were calculated as standard deviations expressed as a percentage of mean values.

3. Results and discussion

Detection conditions were optimized for homocysteine; fluorescence output for cysteine and cysteinyl-glycine was thus often too high to allow quantification. However, it is clear that these thiols may also be determined if a lower sensitivity adjustment on the fluorescence detector is used. Glutathione levels in plasma were relatively low, and since this compound was not of interest in our clinical studies, we routinely used a HPLC analysis time of 8 min. The use of isocratic elution simplified the HPLC procedure; this renders the method suitable for large population studies. We were able to confirm the excellent features of SBD-F as analytical reagent for biological thiols as described by Imai et al. [11]. SBD-F exhibited no fluorescence, neither were fluorescent degradation products formed during the derivatization procedure. This constitutes a major advantage of this product with regards to, i.e., monobromobimane, which has been reported to show background fluorescence and to form fluorescent degradation products [13]. Also, the high selectivity of SBD-F results in specific labeling of thiols, thus allowing isocratic elution to separate SBD-homocysteine from other SBD-thiols. We investigated the effect of incubation of whole blood at room temperature before separation of plasma from cells. A fresh blood sample left at room temperature without centrifugation will show a considerable increase in homocysteine concentration. This increase in homocysteine concentration can be eliminated by centrifugation followed by pipetting of plasma. After centrifugation and pipetting the concentrations of homocysteine in the sample are stable, even at room temperature, for at least 24 h. Therefore, the blood samples must be cooled or centrifuged immediately after blood sampling. We stored plasma/serum samples at −20°C for 3 months

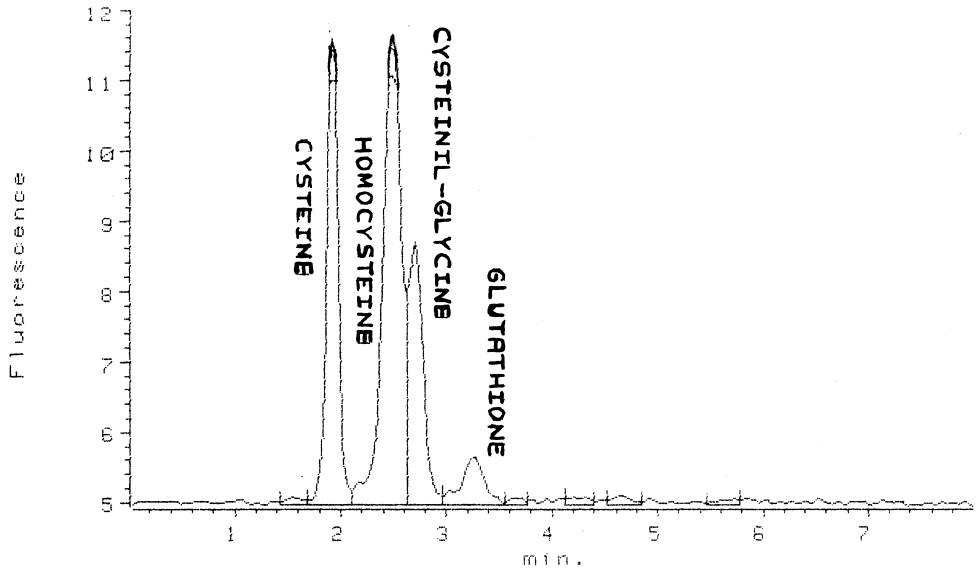

Fig. 1. Chromatograms obtained from the analysis of total homocysteine (t-hcy) in a plasma sample of patient affected by methylmalonic acidemia with homocysteine, (t-hcy 215 μmol/l).

without any measurable change in the homocysteine concentrations. The samples are also very stable after derivatization with SBD-F. They can be stored at 4°C for several weeks, in a cold and dark place until use. Whether it is preferable to use plasma or serum is very dependent on the laboratory routines.

Total plasma homocysteine was determined in 77 healthy subjects. The means±S.D. concentration of total homocysteine in 44 male adults (mean age, 51.6; range 30–72) and 33 female adults (mean age 50 years; range 25–83) were 8.4±2.15 μmol/l (range 4.1–12.7) and 7.1±1.18 μmol/l (range 4.12–11.48), respectively.

Serum concentrations were 8.8±2.6 μmol/l in males and 7.6±1.5 μmol/l in females. No significant difference were found comparing plasma and serum samples.

The homocysteine range has been reported to differ between men and women, but also to be age-dependent. In general, men seem to have higher concentrations of homocysteine than women, while older people have higher concentrations than younger people. The method described herein represents an improvement of previously published procedures. Total homocysteine, but also cysteine, cysteinyl-glycine and glutathione, are quickly measured using commercially available reagents. Finally, the plasma homocysteine assay is useful for detection of inborn errors of metabolism (Fig. 1) and well within the capabilities of the average clinical pathology laboratory. The relative simplicity and stability of this assay should facilitate screening of large populations who may be at risk for vascular diseases.

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