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Short communication

Plasma total homocysteine quantification: an improvement of the classical high-performance liquid chromatographic method with fluorescence detection of the thiol-SBD derivatives

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Abstract

A rapid, shorter, isocratic high-performance liquid chromatographic method is described for the determination of plasma total homocysteine. In this method the sample preparation was modified for reduction of the time of the thiolic reduction from 30 at room temperature to 10 min at 37 °C with tris-(2-carboxyethyl) phosphine (TCEP), reduction the time of derivatization from 60 to 10 min at 60 °C and elution of the SBD-thiols derivative by a shorter HPLC-column which is commercially available. The SBD–homocysteine derivative was eluted at 3.7 min. The method was equally precise and faster for quantification of tHcy in plasma as other previously described method and should be very useful for epidemiological studies in which large numbers of samples have to be analyzed

Keywords: Homocysteine

1. Introduction

Several studies have revealed that elevated levels of total homocysteine (tHcy) in plasma in fasting state is associated with an increased risk for atherosclerotic and tromboembolic vascular disease [1–4]. This non-essential, sulfur-containing amino acid is formed as an intermediate, during the essential amino acid methionine metabolism, which is at the intersection of two main metabolic pathways: remethylation and trans-sulfuration, regulated by methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS) [5].

In the last decade the growing interest in so called ''emergent risk factors'' has led to the development of several methods for the determination of tHcy [6]. They can be classified in two groups: first, the chromatographic methods, including gas chromatography–mass spectrometry (GC–MS), and ion-exchange chromatography, and high-performance liquid chromatography with fluorescence (HPLC–FD) or electrochemical detection (HPLC–ED). Second, the Immunoassay methods: these including Enzyme Immuno Assay (EIA), and Fluorescence Polarization Immuno Assay (FPIA). Although all of them include treatment of whole plasma/serum with a reductant, the principles for separation and detection vary markedly among the methods [7].

The HPLC methods for thiols determinations have

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been reviewed [8,14]. The absence of chromophore for sensitive detection is a problem, which were met with these analytes. However, two approaches are possible in order to resolve this point: (a) direct detection using electrochemistry, either amperometry on gold/mercury amalgamed [15] electrodes or coulometry on porous graphite electrodes [16]; (b) Derivatization coupled with UV or espectrofluorometric detection. The derivatization reactions either pre-column or post-column involve different thiol selective reagents: monobromobimane [17], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [18], *N*-(1-pyrenyl)maleimide [19], ortho-phthalaldehyde (OPA) [20], (for review see Ref. [14])

The reversed-phase HPLC with fluorescence detection after derivatization of plasma thiols with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) is the most widely used method to determine total plasma amino thiols (cysteine, cysteinylglycine and homocysteine) [8]. It is based on a procedure originally described by Toyo'ka et al. [9], which was later optimized by Araki y Sako [10]. The time required for sample preparation (thiolic reduction, deproteinization and pre-column derivatization with SBD-F) and for thiol derivatives separation is nearly 2 h per sample. We have modified the method of Araki y Sako (named Method A in this paper) by using tris-(2-carboxyethyl) phosphine (TCEP), as the reducing agent [11], and cysteamine hydrochloride as internal standard [12] and isocratic separation of SBD-thiols derivatives [13]. We have also decreased the time for thiolic reduction to 10 min at 37 °C with derivatization for 10 min at 60 °C and isocratic separation of the fluorescent adducts on a C_{18} Column (53 mm×7 mm I.D., 3 μm).

2. Experimental

2.1. Chemicals

Tris-(2-carboxyethyl) phosphine (TCEP) was obtained from Molecular Probes (Eugene, Oregon USA), SBD-F was supplied by Wako Chemicals (Richmond, VA, USA), Acetonitrile HPLC grade was obtained from Aldrich Chemical Co. (Wilwauke, WI, USA), and Glutathione was bought from Calbiochem (San Diego, California, USA). L-homocystine; L-Cysteine; Cysteamine Hydrochloride (2-mercaptoethylamine); and all other reagents were obtained from Sigma Chemical (St Louis, MO, USA)

2.2. Sample preparation

Plasma Samples from healthy adults (volunteer blood donors) were obtained from whole blood colleted into evacuated EDTA Tubes (Vacutainer Tubes, Becton Dickinson Co, USA). The plasma was separated by centrifugation at 4 °C within 30 min after venipuncture and stored at −70 °C until used. Blood samples used in the present study were obtained during the course of an epidemiological protocol focused on emergent risk factors for cardiovascular disease in the Venezuelan population. This protocol has been approved by the Committee of Bioethics of the Instituto Venezolano de Investigaciones Científicas (IVIC).

One-hundred μl of plasma or homocysteine calibrator (final concentrations as homocysteine = 5, 10, 12.5, 15, 17.5, 20, 40, 80 μ*M*), 50 μl of cysteamine (30 μ*M*), and 15 μl of 100 g/l TCEP, was incubated 30 min at room temperature (Method A) or 10 min at 37 °C (Method B) to reduce and release protein-bound thiols. In both methods, the deproteinization was achieved by addition of 150 μl of trichloroacetic acid 100 g/l containing 2 m*M* EDTA followed by centrifugation (14 000 *g* for 10 min). The following additions were made to 50 μl of the supernatant: NaOH (10 μl, 1.55 mol/l), buffer borate (125 μl, 0.125 mol/l, pH 9.5, containing 4 mmol/l EDTA), and SBD-F (50 μl, 1 g/l), and incubated for 60 min at 60 °C (Method A) or for 10 min at 60 °C (Method B). After terminating the reaction, the solution was cooled at 4 °C for 15 min. The reaction mixture was filtered through a 0.2-μm Alltech filter (Alltech Associates, Inc. Deerfield, IL, USA). An aliquot of 20 μl was subjected to the HPLC analysis.

L-Homocystine hydrochloride and Cysteamine hydrochloride diluted in 5 m*M* HCl were used as Homocysteine calibrator and internal standard respectively.

2.3. Apparatus and chromatographic conditions

HPLC was performed on a Shimadzu LC-6A system with two pumps and an SCL-6B system

controller (Shimadzu, Kyoto, Japan). Samples were injected through a Rheodyne 7125i injection valve fitted with a 20-μl sample loop (Rheodyne, Cotati, CA, USA). The thiol-SBD derivatives separation was eluted isocratically at room temperature with an Supelcosil™ LC-18-DB analytical column (150×4.6 mm I.D., 3 μm particle size) (SUPELCO, Inc., Bellefonte, PA, USA) for Method A or a Platinum EPS C_{18} analytical column (53×7 mm I.D., 3 μm particles size) (Alltech Associates, Inc. Deerfield, IL, USA) for Method B, protected in both cases by a Supelguard™ LC-18-DB guard column (SUPELCO Inc., Bellefonte, PA, USA). The mobile phase in both methods was 0.1 mol/l KH_2PO_4, pH 2.0, containing 40 ml/l acetonitrile at a flow-rate of 1 ml/min (Method A) or 1.5 ml/min (Method B).

Fluorescence signals (excitation 385 nm, emission 515 nm) were measured using Shimadzu RF-551 spectrofluorometric detector (Shimadzu, Kyoto, Japan). The detector signal was recorded, and the peak area was quantified with a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan).

3. Results and discussions

Fig. 1 show the HPLC pattern of a plasma sample, to which cysteamine was added as internal standard.The five peaks were identified as cysteine, cysteamine, cysteinylglycine, homocysteine, and glutation, respectively, by comparison of the retention times with those corresponding to the pure compounds run separately and confirmed by co-elution when plasma was spiked with these compounds. The chromatographic time for all SBD-thiols derivatives was 12 min shorter in Method B. The retention time for SDB–homocysteine was reduced from 8.16 to 3.74 min. Glutathione levels in plasma were relatively low, and since this compound was not of interest in our epidemiological studies, we routinely used a HPLC analysis time of 5 min.

The range of recovery of SBD–homocysteine in both methods was from 94 to 104%. The coefficient of variation (C.V.) maximum within-batch was 3% and between-batch was 6% for both methods. Fluorescence of SBD–homocysteine was linear over a concentration range 5–80 μmol/l; higher concentrations were no tested, as they were not encountered in normal population.

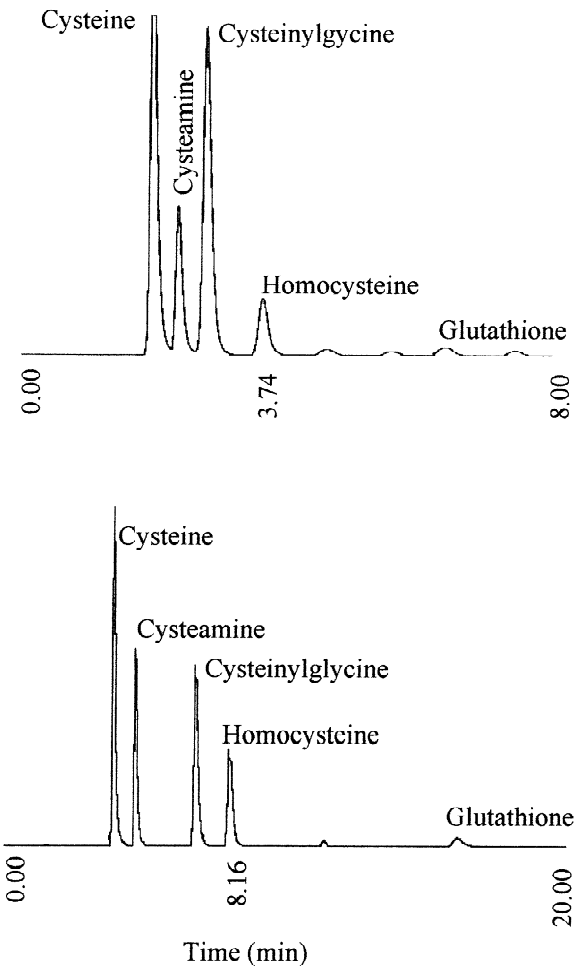

Fig. 1. Chromatograms for human SBD-thiols plasma sample. (Homocysteine 8.6 μ*M*). Method B (top) and Method A (bottom).

The comparison of the Hcy:cysteamine peak area ratio obtained from 12 standard curves yielded the following equation: Method B=(0.979±0.004)× Method A; $S_{y/x}$=0.0181; r=0.999. When the Hcy:I.S. peak area ratio from plasma samples were considered the equation was Method B= (0.9874±0.1132)×Method A; $S_{y/x}$=0.0539; r= 0.961, n=82 (Fig. 2). The tHcy concentrations obtained from both methods were not statistically different (P=0.804, Mann–Whitney Rank Sum Test)

The optimization of reaction parameters was carried out with a pooled plasma. The differences in the mean values of Hcy:cysteamine peak area ratio obtained when a thiolic reduction was carried out for 30 min at room temperature and those obtained when

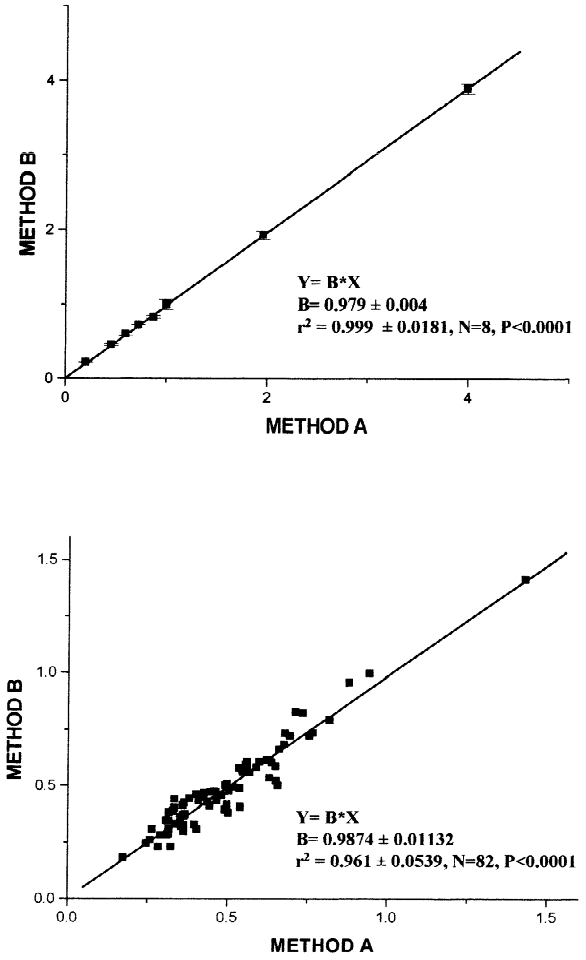

Fig. 2. The Hcy:cysteamine peaks area ratio mean (95% CI, error bars) from 12 standard curves (top). The Hcy:cysteamine peaks area ratio from 82 human plasma samples (bottom).

the thiolic reduction was carried out for 10, 20, and 30 min at 37 °C, independently of the derivatization time at 60 °C, were not statistically significant different (see Table 1, ANOVA, $P=0.628$, alpha=0.05). Based on these results, we chose 10 min at 37 °C for thiolic reduction with TCEP and 10 min for pre-column derivatization at 60 °C with SBD-F to shorten the analysis time.

The observed results indicated that Method B provide an equally precise and faster quantification of tHcy in plasma as Method A. The modification described (Method B) should be very useful for epidemiological studies in which large numbers of samples have to be analyzed.

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Table 1
Optimization of reaction parameters

Derivitization time (min)	Reduction Time (at room temperature)			Reduction Time (at 37 °C)		
	10 min	20 min	30 min	10 min	20 min	30 min
10	0.385[a] (0.373–0.397)[b] 4.34[c]	0.425 (0.408–0.442) 5.48	0.470 (0.458–0.482) 3.49	0.464 (0.460–0.468) 1.05	0.467 (0.458–0.476) 2.55	0.468 (0.459–0.477) 2.60
20	0.394 (0.386–0.402) 2.73	0.424 (0.408–0.440) 5.18	0.466 (0.453–0.479) 3.74	0.464 (0.458–0.470) 1.82	0.466 (0.459–0.473) 1.99	0.471 (0.463–0.479) 2.41
40	0.406 (0.403–0.409) 1.11	0.436 (0.433–0.439) 1.04	0.476 (0.466–0.486) 2.89	0.467 (0.460–0.474) 1.98	0.469 (0.460–0.478) 2.74	0.478 (0.468–0.488) 2.86
60	0.406 (0.400–0.412) 2.04	0.421 (0.411–0.431) 3.30	0.474 (0.464–0.484) 2.96	0.470 (0.463–0.467) 1.99	0.472 (0.465–0.479) 2.06	0.473 (0.468–0.478) 1.36

[a] The mean of the Hcy:cysteamine peak area ratios from 10 replicate runs of a pooled plasma.
[b] Confidence interval.
[c] Coefficient of variation as a percentage.

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