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High-performance liquid chromatographic method for measuring total plasma homocysteine levels

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Abstract

We have modified a high-performance liquid chromatographic (HPLC) procedure based on SBD-F (ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate) pre-column derivatization to obtain an assay that is useful for routine clinical total plasma homocysteine (tHcy) analysis. The introduction of easily handled sodium borohydride instead of the traditional tri-*n*-butylphosphine in dimethylformamide as a reductant and a 14-min run-time using basic isocratic HPLC equipment are the more notable advantages. The addition of mercaptopropionylglycine as an internal standard contributed to improvements in the reproducibility of the assay, yielding within- and between-run precisions of 1.9 and 4% (C.V.), respectively. Reference values for fasting tHcy were 7.65±2.3 and 8.9±2.4 μmol/l, while post-methionine load gave tHcy levels of 19.9±5.5 and 26.8±5.5 μmol/l, for women and men, respectively (n=40).

Keywords: Derivatization, LC; Homocysteine; Methionine; Amino acids

1. Introduction

In the last two decades, various studies have pointed out that moderate and persistent hyperhomocysteinemia is implicated in the development of atherosclerosis, which is responsible for 50% of all mortality and morbidity in western countries [1–4]. Considering that the most traditional risk factors for heart disease and stroke, such as plasma lipids, cigarette smoking, hypertension, obesity and diabetes, only account for 50% of cardiovascular disease [5,6], one can understand the reason why total plasma homocysteine (tHcy) measurement is included in the list of tests for investigating the causes of atherosclerosis and thrombosis. Several assay methods for tHcy have been developed, with few of them employing gas chromatography–mass spectrometry (GC–MS) procedures [7,8], and most of them being based on high-performance liquid chromatography (HPLC) [9–16] or, very recently, on an immunoenzymatic technique [17]. The latter method, together with the HPLC methods proposed in 1993 by Fiskerstand et al. [10] and, more recently, by Pastore et al. [11], were, until now, the only fully automated tests available for determining plasma tHcy. Nevertheless, both of them involve an expensive investment: the immunoassay, notable for its easy handling, necessitates the purchase of a kit, while the HPLC procedures, based on monobromo-

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bimane derivatization [10,11], are automated by the use of a programmable sample processor.

Among the HPLC procedures for tHcy analysis in plasma, methods based on a thiol-specific SBD-F (ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate) reaction appear attractive for routine use in clinical chemistry laboratories. This derivatization yields stable adducts and very clean chromatograms in comparison with the monobromobimane reaction, which involves the formation of interfering fluorescence byproducts. From the original method of Araki and Sako [12], several modifications have been proposed. In 1991, Ubbink et al. [13] shortened the analysis time to 6 min using an isocratic run, and Vester and Rasmussen [14] introduced mercaptopropionylglycine as an internal standard, using a 20-min gradient run. Very recently, new papers have proposed further improvements: the use of the mucolytic agent *N*-acetylcysteine as an internal standard, associated with a 13.5-min isocratic run [16], or a short isocratic run at 35°C, without internal standard [15]. All of these methods are based on tri-*n*-butylphosphine (TBP) in dimethylformamide reduction, a reagent which must be prepared under nitrogen, under controlled conditions [18].

Here, we describe an improved SBD-F procedure based on sodium borohydride ($NaBH_4$) reduction and a 14-min isocratic run, including elution of the internal standard. This simple and relatively inexpensive assay is able to support an elevated daily workload, thus being useful for routine applications.

2. Experimental

2.1. Chemicals

Mercaptopropionylglycine and ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) were purchased from Sigma (St. Louis, MO, USA). L-Homocystine, D,L-homocysteine (Hcy) and sodium borohydride ($NaBH_4$) were from Fluka (Buchs, Switzerland). All chemicals and solvents were of analytical-reagent grade and were obtained from BDH (Poole, UK). Doubly distilled water and solvents were filtered through 0.45 μm filters from Millipore (Bedford, MA, USA).

2.2. Reagents and solutions

$NaBH_4$ (3 mol/l) was prepared in 0.1 *M* NaOH, then mixed with dimethylsulphoxide (DMSO; 2:1, v/v) and stored at 4°C [19]. The derivatizing solution was made by dissolving 1 mg of SBD-F in 1 ml of 0.2 mol/l boric acid, pH 9.5, containing 4 mmol/l EDTA. The concentrations of the homocystine working solutions were 400, 200, 100, 50 and 25 μmol/l. The concentration of mercaptopropionylglycine used as the internal standard (I.S.) was 7.5 μmol/l. Homocystine and I.S. were dissolved in 5 mmol/l HCl and 0.1 mol/l sodium borate buffer, pH 9.5, containing 2 mmol/l EDTA, respectively. The procedure was carried out as follows: A 100-μl volume of plasma was mixed with 50 μl of I.S. and 20 μl of $NaBH_4$ in an eppendorf tube and incubated at 50°C for 30 min. Then, 100 μl of 0.6 mol/l perchloric acid in 1 mmol/l EDTA were added. After centrifugation at 11 600 *g* for 5 min, 100 μl of supernatant were mixed with 200 μl of 0.2 mol/l borate buffer, pH 9.5, 20 μl of 2 mol/l sodium hydroxide (NaOH) and 50 μl of SBD-F solution. The samples were mixed and then incubated at 60°C for 1 h. A 20-μl aliquot was later injected into the HPLC system.

2.3. Chromatographic conditions

The System Gold from Beckman (Palo Alto, CA, USA) consisted of a monopump model 116 with a solvent selector valve that allows the use of four different solvents in sequence. The chromatograph was on line with an autosampler model 507 connected through an analog interface model 406 with a Shimadzu model RF 551 fluorimetric detector (Kyoto, Japan) set to $\lambda_{ex}=385$ nm and $\lambda_{em}=515$ nm. The Beckman Ultrasphere ODS column (150×4.6 mm I.D., 5 μm) was protected by a Merck LiChrospher 100 RP-18 guard-column (40×4 mm I.D., 5 μm) (Darmstadt, Germany). The HPLC mobile phase consisted of two buffers: (A) 0.2 *M* sodium acetate, pH 4.0 containing 2% methanol and (B) 0.2 *M* sodium acetate, pH 4.0 containing 30% methanol. The column was equilibrated with 100% buffer A at 1.2 ml/min and, 0.2 min after the analysis started, the selector valve was switched to 100% eluent B. At 4 min, the flow-rate was increased to 1.4 ml/min over 0.5 min and at 4.5 min, the valve switched back

to 100% eluent A. At 12 min, the flow-rate was decreased to 1.2 ml/min in 0.5 min and at 14 min, the HPLC apparatus was ready for the next injection.

2.4. Plasma sample preparation

The control group consisted of 40 females and 40 males (33.0±10 years, mean±SD), recruited on a voluntary basis from the hospital staff. Venous blood specimens were taken in the morning from fasting donors just before the oral administration of L-methionine (0.1 g/kg body weight) in 200 ml of orange juice, and 8 h after methionine intake. Blood, collected in sterile vacutainer tubes containing sodium citrate (Becton Dickinson, Rutherford, NJ, USA), were centrifuged at 1500 *g* for 15 min, and plasma samples were immediately frozen and stored at −20°C.

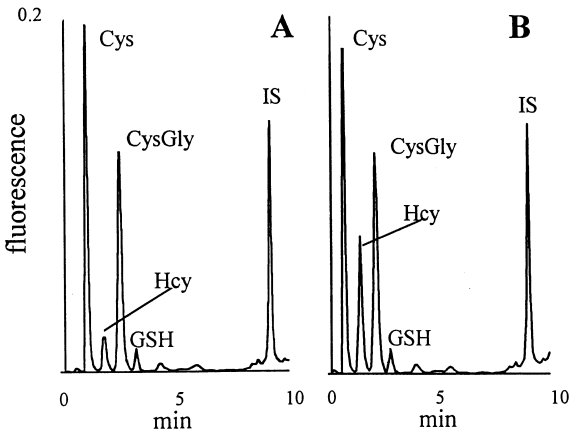

Fig. 1. Chromatograms of (A) plasma sample with a tHcy concentration of 7.5 μmol/l; (B) plasma sample supplemented with 20 μmol/l standard Hcy. The separation was carried out on a Beckman Ultrasphere ODS column (150×4.6 mm I.D.; 5 μm), as reported in Section 2.3 (Cys=cysteine, Hcy=homocysteine, CysGly=cysteinylglycine, GSH=glutathione, I.S.=mercaptopropionylglycine).

3. Results

To cleave the protein-bound sulphur-containing amino acids and to maintain the thiols in the reduced form, we employed the same $NaBH_4$ molar concentration (3 mol/l) that was used previously in our laboratory for tHcy determination using *o*-phthalaldehyde (OPA) precolumn derivatization [19].

We optimized the HPLC analysis, which allowed the separation of cysteine, homocysteine, cysteinyl–glycine, glutathione and the I.S. in only 10 min (Fig. 1). The retention times were 2.67 min (C.V.% 0.8) and 9.36 min (C.V.% 0.3) for Hcy and I.S., respectively. By exploiting the possibility to switch the valve on two delivery solvents, we achieved this performance by using a basic single pump HPLC and, in spite of the flow changes during the analysis, the column still showed well resolved peaks after 700 runs. We also tested a Beckman Ultrasphere XL C_{18} column (70×4.6 mm I.D., 3 μm), using only buffer A as the mobile phase at a flow-rate of 0.7 ml/min. Elution of the five peaks was complete in only 8.4 min (Fig. 2) and, since re-equilibration was unnecessary, a new run was performed every 10 min. Under these conditions, however, the glutathione peak was not baseline-separated from the cysteinylglycine peak and, in addition to the higher cost of the column, no data about its performance to support a large working load are available.

In accordance with other procedures employing

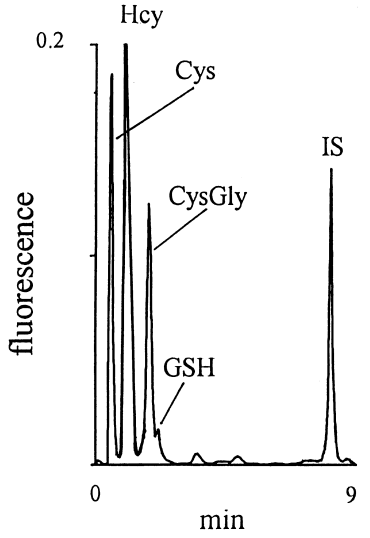

Fig. 2. Chromatogram of a plasma sample with pathological levels of tHcy (82.5 μmol/l). The separation was carried out on a Beckman Ultrasphere XL C_{18} column (70×4.6 mm I.D.; 3 μm). Conditions were as reported in Section 3 (Cys=cysteine, Hcy=homocysteine, CysGly=cysteinylglycine, GSH=glutathione, I.S.=mercaptopropionylglycine).

SBD-F [13–16], we utilized borate buffer/EDTA as the diluent in sample processing but we lowered the concentration from ≅2.0 mol/l to 0.2 mol/l. The high concentration of this salt, in fact, caused occlusions along the narrow steel tubes of the HPLC equipment. To reinstate the optimal pH (≅9.5) necessary for the derivatization [20], 20 μl of 2 *M* NaOH were added.

Fixed volumes (10 μl) of working homocystine solutions were added to 100 μl of pooled plasma, to attain final concentrations of 5, 10, 20, 40 and 80 μmol/l Hcy, and the ratio of fluorescence intensities of SBD–Hcy–I.S. was plotted as a function of Hcy concentration. Linear regression analysis yielded $y=0.026\ (\pm 0.000435)x+0.195\ (\pm 0.0164)$ (±SE) with a correlation coefficient $r=0.999$, $S_{x/y}=0.0292$. We obtained a similar equation by adding Hcy instead of homocystine (range 0–80 μmol/l): $y=0.026\ (\pm 0.00033)x+0.18\ (\pm 0.0137)$ (±SE), $r=0.999$, $S_{x/y}=0.021$. Nevertheless, as already suggested by Dudman et al. [21], we preferred to use homocystine in place of Hcy because of its chemical stability, high purity and the possibility of checking the reduction step. Calibration lines in the same range (0–80 μmol/l) prepared by replacing plasma with a buffer (0.1 *M* sodium borate buffer, pH 9.5, 2 mmol/l EDTA), showed a different slope: $y=0.0213\ (\pm 0.000123)x+0.00571\ (\pm 0.00465)$ (±SE), $r=0.999$, $S_{x/y}=0.0083$.

The within-run reproducibility of the method was determined by successively injecting aliquots ($n=7$) of two plasma samples with low and high tHcy concentrations (6.9 and 27.5 μmol/l, respectively). The C.V. values were 1.9 and 3.8%, respectively. The between-run reproducibility was determined by analysing the same plasma samples for seven consecutive days and the C.V. values found were 4.0 and 4.7%, respectively. The analytical recovery of the method was tested by adding known Hcy concentrations (0–80 μmol/l) to five independent aliquots of a plasma sample before the reduction step. Results are reported in Table 1. The detection limit (LOD) of the method (at a signal-to-noise ratio of three) for Hcy in plasma was 0.1 μmol/l, while at a signal-to-noise ratio of ten, the lowest limit for reliable quantification (LOQ) was 0.85 μmol/l, corresponding to 1.7 pmol injected ($n=7$).

By applying this procedure, we found the following reference values for fasting tHcy plasma levels: 7.65±2.36 and 8.9±2.14 μmol/l for females ($n=40$) and males ($n=40$), respectively ($p=0.0072$, Student's *t*-test). Eight hours after oral administration of methionine, the normal ranges found in relation to sex were: 19.9±5.5 and 26.8±5.49 μmol/l, respectively ($p=0.0001$, Student's *t*-test).

Table 1
Recovery of homocysteine added to plasma

Hcy added (μmol/l)	Hcy found (μmol/l)	Recovery (%)
0	6.41±0.2	–
5	11.23±0.7	96.4±4.0
10	16.83±0.8	101.4±6.3
20	26.0±0.57	98.0±4.9
40	46.9±0.71	100.9±2.1
80	86.6±0.36	100.2±1.9

Different amounts of homocysteine were added to 100 μl aliquots of plasma before $NaBH_4$ reduction.
Values are expressed as means±SD of five independent plasma samples.

4. Discussion

In relation to the increased demand for tHcy analysis in our laboratory, we decided to modify the original Araki and Sako procedure [12] in order to obtain a method that was suitable for routine clinical application. The first notable modification was the use of $NaBH_4$ as a reducing agent before SBD-F derivatization instead of TBP in dimethylformamide, which is commonly combined with this labelling agent [12–16]. Moreover, because of its routine use, we used isocratic HPLC analysis that only lasted 14 min, in spite of the introduction of mercaptopropionylglycine as an internal standard.

Most of the procedures based on SBD-F derivatization [12–16] employed TBP/dimethylformamide solution as a reductant, which requires safety measures to avoid potential hazards. Recently, Gilfix et al. [18] proposed the routing use of tris(2-carboxyl-ethyl)phosphine (TCEP) in aqueous solution. We preferred to replace the aforementioned named phosphines by $NaBH_4$ because of our past experience with this reducing agent for plasma tHcy analysis by OPA [19].

As previously utilized by Vester and Rasmussen

[14], we also employed mercaptopropionylglycine as an I.S. We preferred this molecule to the alternative, *N*-acetylcysteine, used by Durand et al. [16], as the latter thiol may be present in plasma of patients under mucolytic therapies or during the treatment of acetaminophen overdose. The presence of an I.S. contributed to improvements in the precision of the assay by correcting possible manual errors due to sample handling. To keep the analysis times short, immediately after injection, the solvent selector valve was switched to a solvent containing a high percentage of methanol for 4 min (see Section 2). The two percentages of methanol (2 and 30%) represented the best compromise for obtaining acceptable retention times for the I.S. and a good resolution of the early eluting peaks. Under these conditions, the I.S. eluted at 9.5 min vs. 15.6 min using the method of Vester and Rasmussen [14], and the separation was carried out at room temperature instead of 35°C, as described by Feussner et al. [15], with a basic and relatively inexpensive HPLC apparatus.

The difference (20%) found between slopes of the calibration lines performed in buffer and in plasma indicated the notable influence of the matrix on thiol derivatization. A similar behaviour was observed by Vester and Rasmussen [14] and by Durand et al. [16] who employed mercaptopropionylglycine and *N*-acetylcysteine as internal standards, respectively. Recently, Kuo et al. [22] showed that the matrix influence is less evident when employing 2-mercaptoethylamine instead of mercaptopropionylglycine, thus allowing the use of aqueous calibrators. As already hypothesized, this important and very interesting matrix effect could be due to some species present in the biological matrix that catalyzes the derivatization reaction. A similar behaviour was observed when we carried out Hcy quantification in urine samples (data not shown).

The stability of the SBD–Hcy adduct in the dark, compared to the OPA–Hcy complex, which is quite unstable, represents a notable advantage, which allows storage of the labelled samples for at least one week at 4°C [20,21]. The sample output was ≅70 samples in 24 h, including all pre-analytical steps, which is lower than that of the immunoassay procedure already proposed (20 samples/h) [17], but high enough to match our laboratory needs and is compensated for by the more convenient cost per sample.

We discriminated between reference values on the basis of sex because male tHcy plasma levels are higher then female [9,23–25]. The average concentrations found for the control subjects agreed with the tHcy plasma levels reported by other groups that used SBD-F: 9.8±1.6 and 6.8±1.2 μmol/l [14], 9.16±1.84 and 7.23±1.56 μmol/l [16] (mean±SD), 8.5 and 7.2 μmol/l (median values) [22] for male and female groups, respectively. Similar ranges were described also using different derivatizing agents, such as monobromobimane: 9.26±1.88 and 7.85±2.29 μmol/l [9], 9.2±2.0 and 6.7±0.5 μmol/l [11] (male and female, respectively), or ninhydrin: 9.3±2.3 μmol/l [26]. Data on tHcy plasma levels after methionine intake were reported only by Candito et al. [26] who used an amino acid analyzer and found an average concentration of 29.5±5.5 μmol/l in a control group of 20 subjects 6 h after the oral methionine load.

Due to the utilization of open and flexible HPLC equipment and the performance of simple and short chromatographic analysis, this method appears robust and easy to handle and is therefore suitable for routine application in clinical chemistry laboratories.

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