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Journal of Chromatography A, 846 (1999) 93–100

JOURNAL OF CHROMATOGRAPHY A

Automated high-performance liquid chromatographic method for the determination of homocysteine in plasma samples

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Abstract

Plasma homocysteine determination is essential for the diagnosis of inborn errors of metabolism of sulfur amino acids and is achieving considerable importance as a possible risk marker in vascular occlusive pathology. The aim of this study was therefore to develop a fast and sensitive method to assay total and free homocysteine and total and free cysteine in plasma samples, using an automated precolumn sample pretreatment including reduction with 2-mercaptoethanol, carboxymethylation of free thiols and derivation with *o*-phthalaldehyde. The chromatographic separation was accomplished in 7 min, the within-run and between-run R.S.D.s were all less than 4.3%, the response was linear in the range 0.4–150 μ*M* for homocysteine and 4–1000 μ*M* for cysteine and the mean recoveries were higher than 96%. Moreover, with minimal modification, the method allowed the evaluation of methionine, another important marker of transsulfuration and remethylation defects. The method was applied to the diagnosis of inborn errors involving sulfur amino acids metabolism and to detect mild hyperhomocysteinemia.

Keywords: Homocysteine; Cysteine; Methionine; Amino acids

1. Introduction

Homocysteine (Hcy) is a sulfur amino acid involved in two important metabolic pathways: the methionine cycle, in which Hcy is a key substrate in the recycling of intracellular folates and in the catabolism of choline and betaine, and the transsulfuration pathway that leads to the formation of the important metabolites, cystathionine, cysteine and glutathione [1].

Quantitative determination of plasma Hcy is important for the diagnosis of genetic defects of the enzymes involved in Hcy metabolism. The homozygous deficiency of cystathionine β-synthase (CBS) (EC 4.2.1.22), a pyridoxal phosphate-dependent enzyme, methylenetetrahydrofolate reductase (EC 1.7.99.5), a folate-dependent enzyme, and methionine synthase (EC 2.1.1.13), a vitamin B_{12}-dependent enzyme, are the main causes of the inborn errors of homocystinuria [1–4]. An increased plasmatic Hcy concentration is present in folate and cobalamin deficiency and in renal failure [5].

A large number of prospective and case–control studies, different in sample size, types of patients and control subjects, evaluation of other risk factors and statistical techniques, showed that mild elevation of plasma Hcy concentration is an important risk factor for atherosclerotic vascular disease and for arterial and venous thromboembolism [5–8]. An increase of 5 μ*M* in the Hcy concentration is associated with an increased risk of 1.5–1.9 times for coronary artery

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0021-9673/99/$ – see front matter
PII: S0021-9673(98)01091-7

and cerebrovascular disease [9]. These values indicate that small differences in the concentration might be of clinical relevance. Moreover, it was reported that an elevated plasma Hcy level, is in 27% of the patients due to a mutation of the gene for thermolabile methylenetetrahydrofolate reductase, present in mothers of children with neuronal tube defects, showing that impaired Hcy and folate metabolism is a risk factor for spina bifida [10,11]. Therefore, the importance of a reliable and automated method for the determination of plasma Hcy is evident.

Several techniques, based on manual processing of samples and cumbersome precolumn derivatization using different label agents, followed by reversed-phase HPLC in association with fluorescence detection, have been described [12–22]. Furthermore, an automated procedure using monobromobimane (mBrB) as labelling agent in combination with sodium tetraborate NaBH4 as reducing agent was reported [23].

The aim of this work has been to develop an automated method for the determination of total Hcy, including free, mixed disulfides and protein-bound Hcy (tHcy), total Cys (tCys), non-protein-bound Hcy (free Hcy) and non-protein-bound Cys (free Cys) in plasma samples using reduction with 2-mercaptoethanol (MCE), carboxymethylation and derivatization with *o*-phthalaldehyde (OPA). The method has been applied to estimation of the reference values in the laboratory, to the diagnosis of inborn errors involving Hcy and Cys metabolism, and to detect mild hyperhomocysteinemia.

2. Experimental

2.1. Materials

Homocystine and cystine were obtained from ICN (Costa Masa, CA, USA). L-Amino acid standard solution containing 17 amino acids in 0.1 *M* HCl at the concentration of 2.5 m*M*, amino acid and iodoacetic acid crystalline salt were obtained from Sigma (St. Louis, MO, USA). OPA was purchased from Pierce (Oud Beijerland, The Netherlands), 2-mercaptoethanol (2-MCE), tetrahydrofuran (THF) and triethylamine (TEA) from Fluka (Buchs, Switzerland) and potassium tetrahydroborate, sodium hydroxide, sodium acetate trihydrate, glacial acetic acid, methanol and acetonitrile (HPLC gradient grade) from Merck (Darmstadt, Germany). Phosphate buffer saline solution (PBS) 1× was purchased from HyClone (Cramlington, NE, USA). All aqueous solutions were made up using highly purified water produced by a Millipore Milli-RO/Milli-Q system (Bedford, MA, USA). Millipore Ultrafree-MC 10 000 mass cut-off filters were used to remove the proteins from plasma samples.

A Hewlett-Packard HP 1090 M HPLC apparatus (Waldbronn, Germany), including a 90-position cooled autosampler, DR5 solvent delivery system, heated column compartment, HPLC ChemStation and a programmable fluorescence detector HP1046A, was used. The column was a TSK gel ODS 100×4.6 mm I.D., 2 μm (TosoHaas, Montgomeryville, PA, USA), in conjunction with a Hewlett-Packard ODS Hypersil 20×4 mm I.D. guard column.

2.2. Method

After an overnight fast, blood was collected in ice-kept heparin containing vacutainer tubes and centrifuged within 30 min from venipuncture to avoid cell production of Hcy that can lead to falsely increased plasma Hcy concentration [9]. Plasma aliquots were stored frozen at −70°C for the determination of tHcy and tCys. To determine the free fraction of Hcy and Cys, proteins must be immediately removed from fresh plasma samples: 50 μl of PBS, pH 7.4, were added to 50 μl of plasma and ultrafiltered using Ultrafree-MC filters at 5000 *g* for 20 min. To determine total plasmatic concentration of Hcy and Cys, 50 μl of 0.1% (v/v) MCE in PBS were added to 50 μl of plasma to cleave the disulfide bonds to proteins and ultrafiltered using Ultrafree-MC filters at 5000 *g* for 20 min. The filtrates obtained were subjected to a completely automatic procedure for reduction of S–S bonds using 0.5% MCE in borate buffer 1 *M*, pH 10.5, carboxymethylation with iodoacetic acid 50 mg/ml in borate buffer 1 *M*, pH 10.5, and derivation using OPA 20 mg/ml in borate buffer containing 10% of methanol and 2% of MCE. The steps of the injection programme are shown in Table 1. Sample and reagents vials were

Table 1
Injection programme

Function	Amount (μl)	Vial	Reagent
Draw	8	From vial *x*	Sample
Draw	6	From vial 0	2-MCE
Eject	14	In vial *x*+1 (reaction vial)	
Mix	12×3 cycles	In reaction vial	
Draw	0	From vial 100 (wash)	H_2O
Draw	14	From vial 1	Iodoacetic acid
Eject	14	In vial *x*+1	
Mix	20×3 cycles	In reaction vial	
Draw	0	From vial 100	H_2O
Draw	4	From vial 2	OPA–2-MCE
Eject	4	In vial *x*+1 (reaction vial)	
Mix	20×3 cycles	In reaction vial	
Draw	0	From vial 100	H_2O
Draw	5	From vial 3	Acetic acid
Eject	5	From vial *x*+1 (reaction vial)	
Mix	20×3 cycles	In reaction vial	
Draw	18.5	From vial *x*+1 (reaction vial)	
Inject			

stored in a cooled rack (4°C). The separation of Cys and Hcy was obtained in 7 min using a flow-rate of 0.8 ml/min and the gradient elution reported in Table 2. Mobile phase A was 20 m*M* sodium acetate buffer, pH 7.2, containing 0.040% of TEA and 0.4% of THF; mobile phase B was 20% made of 100 m*M* sodium acetate buffer, pH 7.2, 40% of acetonitrile and 40% of methanol. The excitation and emission wavelengths were, respectively, 340 and 450 nm. Since Cys and Hcy in plasma have different concentrations, to obtain comparable signals different attenuations were used, changing the Pmt gain of fluorescence detector from 9 to 12 at 6 min after injection.

Table 2
Elution gradient

Time (min)	*B* (%)	Flow-rate (ml/min)
0	0	0.8
1	12	0.8
5	20	0.8
7	30	0.8
8	100	0.8
9	100	0.8
10	0	0.8

Mobile phase A: 20 m*M* sodium acetate buffer, pH 7.2, 0.04% TEA and 0.4% THF. Mobile phase B: 20% 100 m*M* sodium acetate buffer, pH 7.2, 40% acetonitrile and 40% methanol.

3. Results

OPA–2-MCE is not a thiospecific labelling agent but reacts with primary amino groups forming highly fluorescent isoindoles, so that the determination of the sulfur amino acid Hcy and Cys in plasma samples requires a good separation. Under the conditions described, the separation of Hcy and Cys was achieved in 7 min (13 min including column regeneration). A chromatogram obtained from a standard solution containing 5 μ*M* of Hcy and 50 μ*M* of Cys is shown in Fig. 1A. The chromatogram obtained from the analysis of tHcy and tCys in a plasma sample is shown in Fig. 1B, and the chromatogram obtained from the analysis of free Hcy and free Cys in a plasma sample is shown in Fig. 1C.

With minimal modification of mobile phase and elution gradient, the method allows the separation of other important metabolites, including Met, another important marker of transsulfuration and remethylation defects (Fig. 2). Further research to validate this widening of the method is in progress.

The linear correlation between peak areas and concentrations was assessed in the range 0.4–150 μ*M* for Hcy and 4–1000 μ*M* for Cys. The correlation coefficients were both 0.99997.

The detection limit was 0.8 pmol for Hcy and 2.4 pmol for Cys with a signal-to-noise ratio of 3.

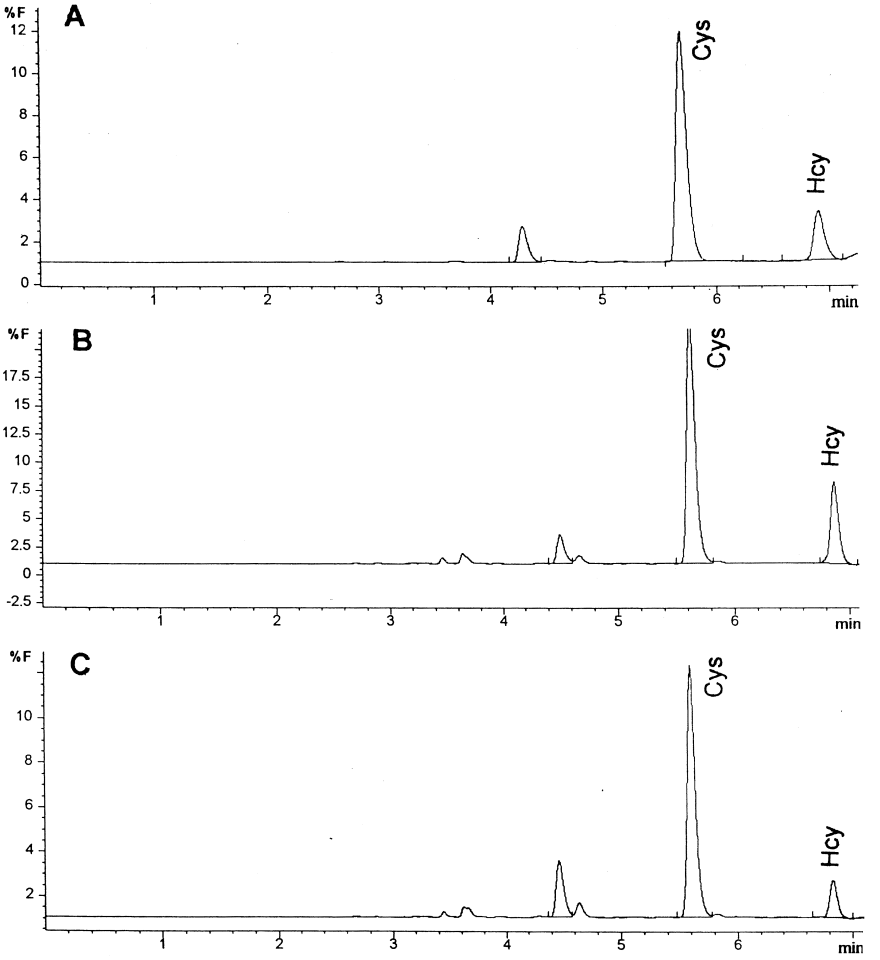

Fig. 1. Chromatogram (A) of a 5 μ*M* Hcy and 50 μ*M* Cys standard solution, and chromatograms obtained (B) from the analysis of tHcy (5.9 μ*M*) and tCys (162 μ*M*) and (C) from the analysis of free Hcy (1.5 μ*M*) and free Cys (82 μ*M*) in a plasma sample.

The precision of the method was assessed using nine consecutive analyses of a standard solution at a concentration of 6 μ*M* of Hcy and 300 μ*M* of Cys. The area R.S.D.s were 1.5% for Hcy and 1.7% for Cys (Table 3). The within-run precision was evaluated performing nine analyses of a plasma sample in the same day. The R.S.D.s were 3.8% for plasma tHcy (mean, 8.7 μ*M*), 3.8% for free Hcy (mean, 2.3 μ*M*), 3.6% for tCys (mean, 333 μ*M*) and 4.3% for free Cys (mean, 170 μ*M*). The between-run precision was assessed performing 16 analyses of aliquots of a single plasma sample stored at −70°C, on different days within a 40-day period. The between-run R.S.D.s were 4.2% for tHcy and 3.9% for tCys (Table 3).

The recovery was evaluated adding different amounts of a Hcy and Cys standard solution to aliquots of the same plasma sample, in order to obtain four different concentrations (2–4–8–10 μ*M* for Hcy and 100–200–400–500 μ*M* for Cys). The mean recovery of tHcy was 98% and of tCys was 96%.

In order to determine the physiological plasma levels of tHcy and tCys, 46 samples from healthy fasting subjects (23–56 years old, mean 34±8 years, 19 premenopausal females and 27 males) were

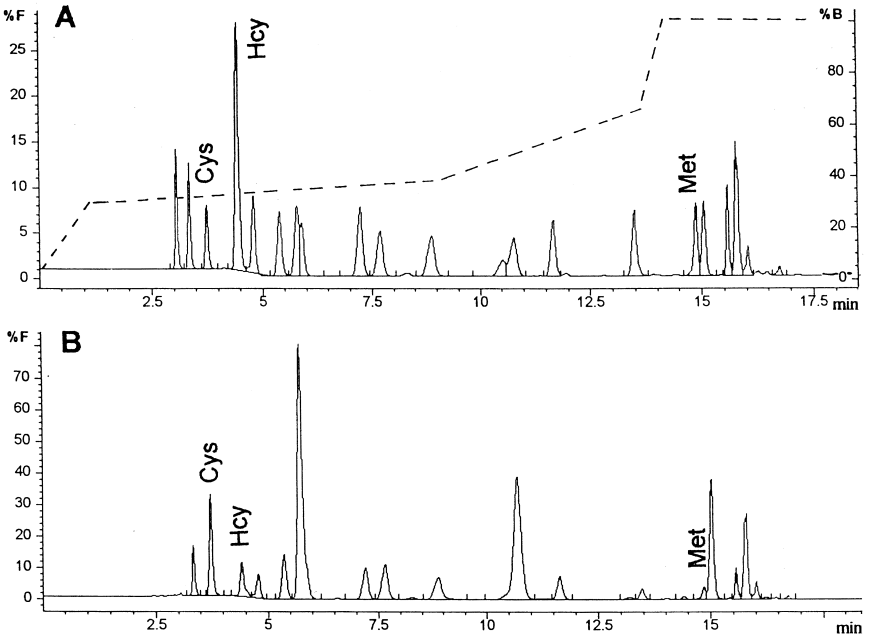

Fig. 2. Chromatogram (A) of a standard solution containing 23 amino acids including cysteine, homocysteine and methionine at the concentration of 50 μ*M* each. Gradient profile is shown. (B) Chromatogram of a plasma sample. The mobile phase A was 20 m*M* sodium acetate buffer, pH 7.2, 0.04% TEA and 0.4% THF; mobile phase B was 20% 100 m*M* sodium acetate buffer, pH 7.2, 20% acetonitrile and 60% methanol.

Table 3

	Repeatability of peak areas		Within-run				Between-run	
	Hcy	Cys	tHcy (μ*M*)	tCys (μ*M*)	Free Hcy (μ*M*)	Free Cys (μ*M*)	tHcy (μ*M*)	tCys (μ*M*)
	35.0	149	9.0	335	2.3	165	9.7	372
	35.0	151	9.0	348	2.4	174	8.9	359
	34.5	143	9.0	349	2.5	165	10	339
	34.5	151	8.5	310	2.3	167	9.6	358
	34.7	148	8.5	329	2.3	185	9.4	329
	34.5	149	8.1	324	2.4	162	9.1	344
	36.0	148	8.6	332	2.3	171	9.8	361
	35.5	151	8.8	340	2.5	177	8.9	355
	34.4	151	9.1	331	2.5	165	9.2	364
Mean	34.9	149	8.7	333	2.4	170	9.4	353
S.D	0.5	2.6	0.3	12	0.1	7.4	0.4	13
R.S.D. (%)	1.5	1.7	3.8	3.6	3.8	4.3	4.2	3.8

The precision of the method was evaluated analysing a Hcy 6 μ*M* and Cys 300 μ*M* standard solution, in the table are reported areas (%F×second) of Hcy and Cys peaks. The within-run precision was assessed analysing aliquots of a plasma sample nine consecutive times in 1 day, and between-run precision was assessed analysing nine aliquots of a single plasma sample stored at −70°C in different days within a 20-day period. Reported in the table are the concentrations of tHcy, tCys, free Cys, free Hcy for within-run precision and the concentration of tHcy and tCys for the between-run precision.

analysed. The mean value for tHcy was 7.7±1.9 μ*M* (range 4.6–14.8 μ*M*), the mean value for tCys was 168±28 μ*M* (range 113–228 μ*M*), which were in agreement with literature values [24–26].

The method was applied to the diagnosis of inborn errors involving Hcy metabolism and to detect mild hyperhomocysteinemia. Fig. 3 shows chromatograms of plasma samples from a patient affected by CBS

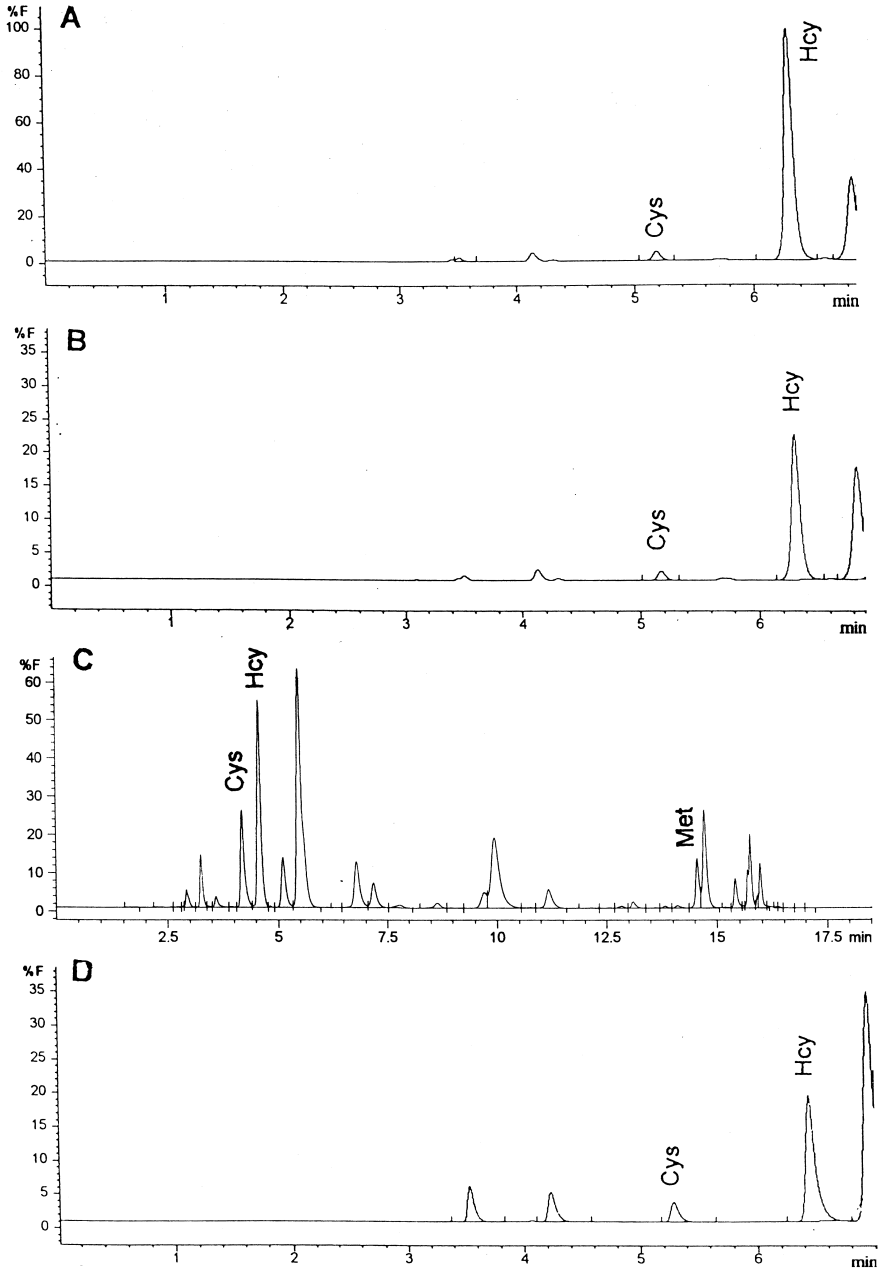

Fig. 3. Chromatograms obtained from the analysis of tHcy (A), free Hcy (B) and Met (C) in a plasma sample of a patient affected by CBS deficiency (tHcy 188 μ*M*, tCys 54 μ*M*, free Hcy 40 μ*M*, free Cys 21 μ*M* and Met 120 μ*M*) and from the analysis of tHcy (D) in a plasma sample of a patient showing mild hyperhomocysteinemia (tHcy 36 μ*M*, tCys 42 μ*M*).

deficiency and a patient showing mild hyperhomocysteinemia.

4. Discussion

The described method allows to improve sensitivity and to obtain a great simplification by automation and the contemporary determination of tHcy and tCys, as well as the determination of free Hcy and Cys.

For the determination of Hcy, the reduction of disulfides using MCE and blockage of thiols using iodoacetic acid before the OPA reaction, has been shown to be a reliable method [17]. To establish the optimum conditions for a completely automated procedure, we tested different concentrations of reagent solutions and different reaction times. Furthermore, to prevent the reoxidation of free thiols and reformation of disulfide forms, a reduction step using MCE was added before the reaction with iodoacetic acid.

Even if the high sensitivity of the method allows the determination of free Hcy, for routine applications the evaluation of total plasma Hcy is recommended. Whereas the determination of free Hcy requires the immediate removal of proteins of the sample to prevent the increase of protein-bound Hcy with consequent loss of free fraction, total Hcy concentration can be obtained using samples simply stored frozen [5].

The codetermination of Hcy and Cys is important for clinical diagnostic purposes; the ratio of tHcy/tCys concentrations is the best metabolic discrimination for the determination of heterozygotes for CBS deficiency [27]. Furthermore, we have shown that the method is easily extendible, with minimal modification to the mobile phase and gradient elution, to the determination of other important metabolites, such as Met. The advantage of contemporary determination of Hcy and Met is evident in monitoring the Met load test, which is useful to delineate the at-risk population for cardiovascular disease [28]. Plasma Hcy, Cys and Met are key metabolites in the first diagnosis of both transsulfuration and remethylation defects, and the evaluation of the concentration of these metabolites is important to distinguish these two disorders, and for the follow-up of patients [29].

The present method shows high precision, sensitivity and minimal sample handling, and can be easily applied to routine analysis in the clinical laboratory for the diagnosis of inborn errors of sulfur amino acid metabolism, as well as for the screening of congenital predisposition of vascular diseases.

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