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High-performance liquid chromatographic method with fluorescence detection for the determination of total homocyst(e)ine in plasma

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ABSTRACT

A high-performance liquid chromatographic method for the determination of total plasma homocyst(e)ine [H(e)] after reduction with sodium tetrahydroborate and precolumn derivatization with *o*-phthaldialdehyde is described. The analyses, carried out on a reversed-phase C_{18} column, were based on spectrofluorimetric detection. The sensitivity was 1 pmol per injection and the intra- and inter-assay relative standard deviations were 1.8% and 5%, respectively. The plasma H(e) concentration determined in 40 healthy volunteers (20–60 years old) was 12.4 ± 2.9 μM (mean ± S.D.), in good agreement with reference values.

INTRODUCTION

Homocysteine [HCys] is a thiol-containing amino acid produced during the transsulphuration pathway of methionine [1,2]. In the last decade attention has been focused on a possible role of H(e) metabolism in the pathogenesis of atherosclerosis. The possibility that mild impairments of homocyst(e)ine [H(e)] levels may be a risk factor for vascular diseases in some patients has been suggested by studies on homocystinuria. This disease, due to cystathionine β-synthase (EC 4.2.1.22) deficiency in the homozygous form, or to defects in the remethylation of HCys to methionine for vitamin B_{12} or folate deficiency, was shown to be frequently associated with several vascular diseases in infancy and childhood [1–7]. Experimental studies [8,9] demonstrated that the abnormal accumulation of this amino acid in tissues and blood has injurious effects on the endothelial cells, inducing vascular damage.

One of the major problems encountered in studies on the potential atherogenic role of H(e) was the development of an accurate and simple assay, able to screen, in a normal population, subjects having a congenital predisposition to occlusive vascular disease. Several approaches to the analysis of H(e) have been described, including gas chromatography–mass spectrometry (GC–MS) [10], radioenzymic assay [11] and high-performance liquid chromatography (HPLC). The last technique, which is the most widely applied, may be combined with different detectors such as post-column ninhydrin derivatization and spectrophotometric detection in the visible range [12–14], electrochemical detection [15–18] or precolumn derivatization and spectrofluorimetric detection [19–23].

The most recently described precolumn labelling agent is monobromobimane (mBrB), a fluorogenic thiol-specific reagent [19–22]. However this derivatization, characterized by the formation of interfering adducts, requires different clean-up steps to remove the mBrB hydrolysis products [20,22]. In view of this fact we tested *o*-phthaldialdehyde (OPA) as derivatizing agent, already used previously for amino acid analyses [24,25]. The OPA fluorophore rapidly links the primary amino groups of amino acids, giving derivatized products that could be detected with high sensitivity, and was previous-

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ly found to be suitable for the determination of particular amino acids present in very low concentrations in the urinary matrix [25].

This paper describes a simple and sensitive HPLC method with OPA precolumn derivatization for the determination of total homocyst(e)ine in plasma.

EXPERIMENTAL

Chemicals

Homocysteine, OPA, sodium tetrahydroborate ($NaBH_4$) and 2-mercaptoethanol (2-MCE) were obtained from Fluka (Buchs, Switzerland) and homocysteic acid from Sigma (St. Louis, MO, USA). Iodoacetic acid, propionic acid and dimethyl sulphoxide (DMSO) were purchased from Janssen Chimica (Beerse, Belgium). All chemicals and solvents were of analytical-reagent grade and were obtained from BDH (Poole, UK). Doubly distilled water and solvents were filtered, prior to use, through a 0.45- or 0.22-μm filter Millipore (Bedford, MA, USA).

Reagents and solutions

$NaBH_4$ (3 *M*) was dissolved in NaOH (0.1 *M*), then mixed with DMSO (2:1, v/v) and stored in a glass vial at 4°C. The solution was prepared freshly every week. Iodoacetic acid solution (9.3 mg/ml) was prepared every day in boric acid (0.1 *M*, pH 9.5). The derivatization solution was made by dissolving 5 mg of OPA in 100 μl of methanol and then adding 0.9 ml of sodium borate buffer (400 m*M*, pH 9.5) and 3 μl of 2-MCE. The concentrations of homocysteine and homocysteic acid standard solutions in 0.1 *M* HCl were 0.4 and 1.2 m*M*, respectively. For the preparation of stock sodium propionate buffer, propionic acid (15.68 ml) and anhydrous disodium hydrogenphosphate (49.6 g) were dissolved in water with stirring. The solution was titrated exactly to pH 6.5 with a few drops of NaOH (2 *M*), diluted to 1 l with water and stored at room temperature.

Apparatus

The HPLC System Gold (Beckman, Palo Alto, CA, USA) consisted of a Model 126 pump connected through a Model 406 analog interface with a Model LS-3 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA). The spectrofluorimeter was set at a fixed scale of 10 and operated at excitation and emission wavelengths of 230 and 417 nm, respectively.

Chromatographic conditions

The Beckman Ultraspere ODS analytical (5 μm) column (250 × 4.6 mm I.D.) was protected by a Brownlee (Santa Clara, CA, USA) Spheri 5-ODS (5 μm) guard column (30 × 4.6 mm I.D.). The mobile phase consisted of two eluents: A [water–stock sodium propionate buffer–acetonitrile (60:30:10)] and B [water–acetonitrile–methanol (45:30:25)]. The flowrate was 1.4 ml/min. The analysis was carried out with 100% solvent A. After the elution of HCys (about 22 min), the proportion of solvent B was increased to 90% over 2 min and held for 10 min, then decreased to 0% in 2 min and after 5 min the HPLC system was ready for next run. The total analysis time was 35 min.

Derivatization procedure

For the determination of homocyst(e)inemia, the reduction and cleavage of protein-bound HCys were performed by adding to 200 μl of plasma 300 μl of water, 300 μl of 9 *M* urea (pH 9.0), 10 μl of homocysteic acid solution as internal standard, 30 μl of *n*-amyl alcohol antifoaming agent and 45 μl of $NaBH_4$–DMSO solution. The samples were incubated at 50°C for 30 min and deproteinized with 500 μl of 6% perchloric acid. After centrifugation, 500 μl of the supernatant were mixed with 100 μl of 40 m*M* iodoacetic acid and 100 μl 3 *M* NaOH and then 200 μl were derivatized with 100 μl of of OPA solution. After exactly 1 min, because of the instability of the derivatives [25], 20 μl were injected into the column.

Plasma sample preparation

Whole blood specimens were collected in sterile Vacutainer tubes containing sodium citrate. After centrifugation at 1500 *g* for 15 min, the plasma samples were immediately frozen and stored at −20°C until analysis.

Plasma samples were not reduced with $NaBH_4$ prior to the derivatization step, to quantify the non-protein-bound homocysteine.

RESULTS

Sample preparation

In order to obtain the maximum yield of the OPA–H(e) derivative, the optimum $NaBH_4$ concentration was initially determined. The reducing agent, in fact, is necessary for cleavage of the protein-bound sulphur-containing amino acids and to keep the thiols in a reduced form. Testing various $NaBH_4$ concentrations (34–408 mM in the reduction step) with normal (15 μM) and above-normal (35 μM) H(e) plasma samples, a plateau in the yield of H(e) between 103 and 204 mM (Fig. 1) was observed. When concentrated homocystine solution (200 μM homocysteine equivalents) was used, the plateau was reached with 204 mM $NaBH_4$. To avoid the formation of interfering adducts $NABH_4$ solution was usually prepared freshly every week.

Different excitation and emission wavelengths were chosen, compared to our previous work on amino acid analysis [24,25], because the HCys–OPA derivative shows a higher fluorescence intensity. Under these conditions a very large interfering peak in the initial part of the chromatogram due to 5-sulphosalicylic acid was observed, so we preferred to use 6% $HClO_4$ for deproteinization.

An essential condition for the formation of a thio-substituted isoindole is the reinstatement of a basic pH (pH > 9) [26], so addition of 3 M NaOH is necessary to buffer the acidity of the samples.

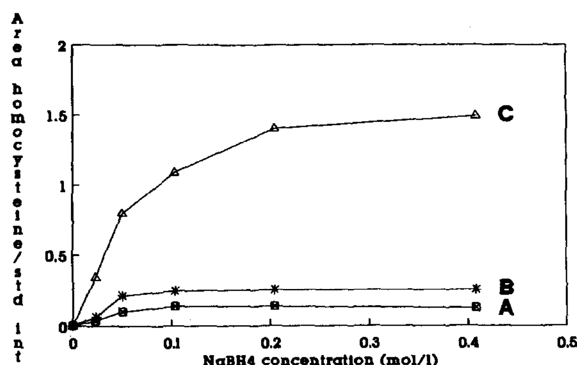

Fig. 1. Influence of $NaBH_4$ in homocyst(e)ine determination. Increased concentrations of $NaBH_4$ were used to analyse A = a normal plasma sample (15 μM); B = a plasma sample containing an above normal concentration of H(e) (35 μM); C = homocystine standard solution (200 μM homocysteine equivalents). The concentration of $NaBH_4$ on the abscissa refers to the amount present in the reduction step.

To stabilize OPA derivatives, which can produce non-fluorescent adducts by intermolecular sulphur–oxygen rearrangement [27], 2-MCE was added to OPA solution. The low volume of 2-MCE used (3:1000, v/v) in the last step of sample derivatization precluded its possible contribution to a reduction of plasma disulphide bonds, allowing the application of this method also for the determination of free homocysteine (Fig. 2D).

To prevent the subsequent reoxidation of the thiols and above all to strengthen the weak OPA–H(e) bond, the reaction mixture, immediately after deproteinization, was treated with iodoacetic acid (50 mM) for S-carboxymethylation.

HPLC analysis

The determination of HCys was initially performed by an isocratic run, using a mixture of 50 mM disodium hydrogenphosphate buffer (pH 7.4) and acetonotrile (89:11) as described by Cooper and Turnell [28] for plasma cystine analysis. As HCys eluted very close to the surrounding peaks, to improve the separation, different percentages of acetonitrile (ranging from 8% to 12%) and various pH values and ionic strengths were tested (data not shown).

The best resolution of HCys was observed by using a buffer (pH 6.5) containing sodium hydrogenphosphate (105 mM), propionic acid (75 mM) and 10% of acetonitrile. Applying these conditions, HCys eluted at 22 min and was well separated from the asparagine and serine peaks (Fig. 2).

Linearity, precision and sensitivity

Quantitative assay was performed by means of calibration graphs, obtained from an aqueous solution of homocysteine in the range 0–320 μM. The relative fluorescence intensities of OPA–homocysteine/internal standard were plotted as a function of homocysteine concentration (Fig. 3). The response of the detector was linear in the tested range and linear regression analysis yielded $y = 0.0061x + 0.030$ with a correlation coefficient of 0.999. When increased amounts of HCys were added to normal plasma samples, and immediately processed according to the present method, a linear relationship ($r = 0.999$, $y = 0.007x + 0.17$) was observed between the peak area of OPA–H(e) and the concentration of HCys added to human plasma (Fig. 3).

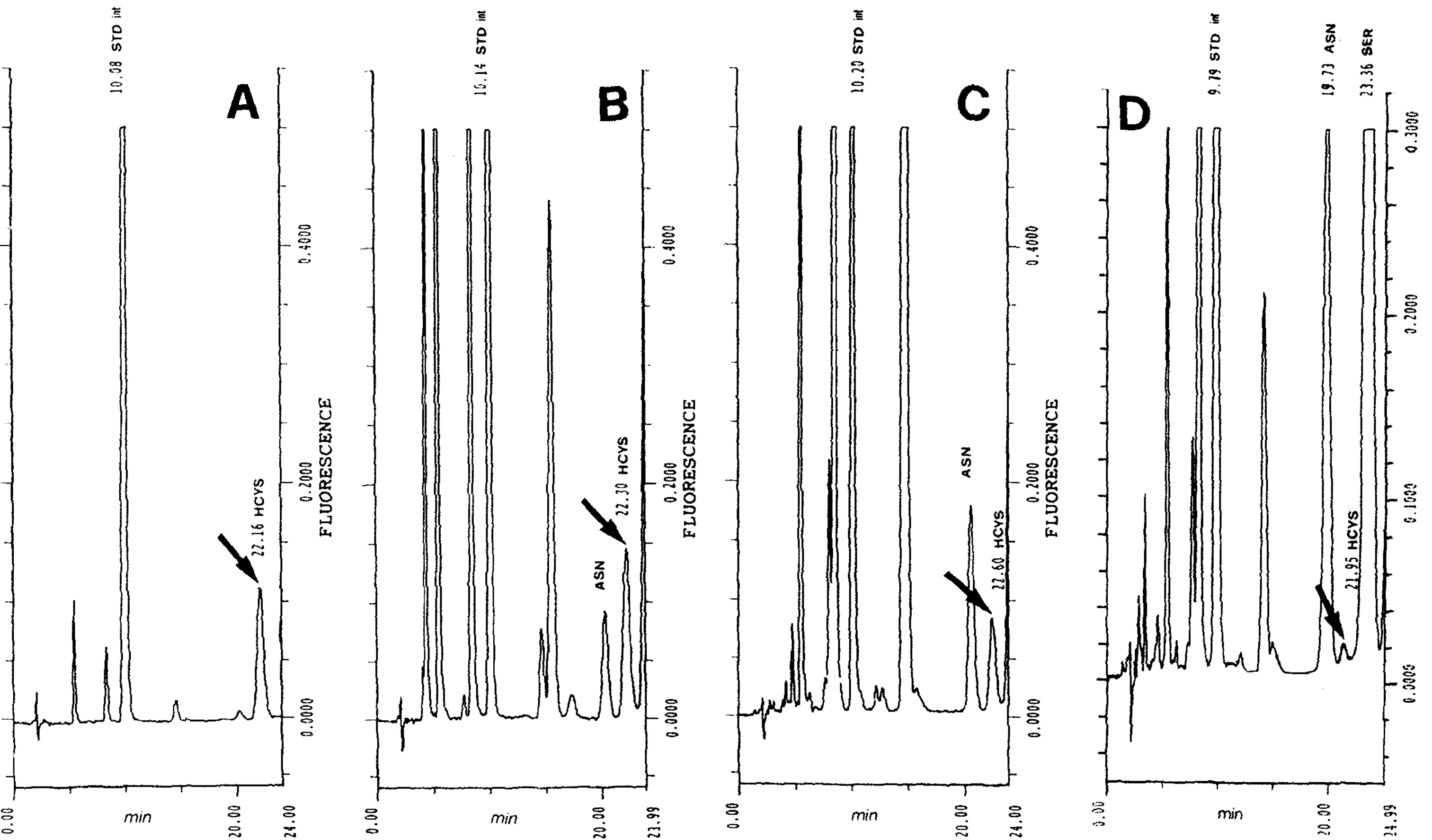

Fig. 2. HPLC determination of plasma homocyst(e)ine. Eluent, acetonitrile–sodium propionate buffer–water (10:30:60). The analysis was carried out on a Beckman Ultrasphere C_{18} (5 μm) column (250 × 4.6 mm I.D.). Flow-rate 1.4 ml/min. Excitation at 230 nm and emission at 418 nm. Fixed scale = 10. (A) Chromatogram of a solution of homocysteine (20 μM) with internal standard homocysteic acid. (B) Partial reproduction of the chromatogram of a Sigma standard physiological amino acid mixture [26] enriched with homocysteine standard. (C) Analysis of a reduced plasma sample. (D) Analysis of a plasma sample not reduced with $NaBH_4$. Fixed scale of detector = 20.

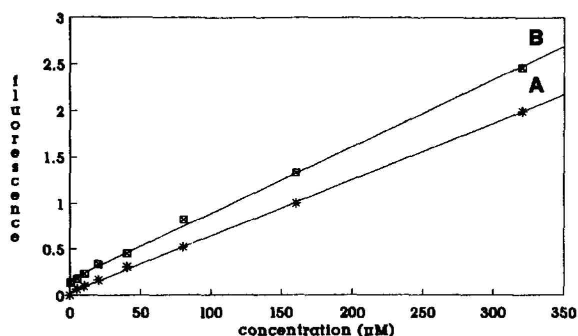

Fig. 3. Linearity of the assay for OPA–homocyst(e)ine in (A) a standard mixture and (B) a normal plasma sample. The integrated peak areas, normalized to the internal standard, were plotted *versus* the amount of homocysteine standard added.

The within-day reproducibility of the assay for total H(e) was determined by injecting aliquots (n = 8) of the same plasma sample, and the between-day reproducibility was calculated from the analysis of the same plasma sample for seven consecutive days. The relative standard deviations (R.S.D.) were 1.8% and 5%, respectively.

To determine the recovery of the method, different amounts of HCys (1–64 nmol) were added to 200-μl aliquots of the same pooled plasma. The average recovery was 94 ± 17% (mean ± S.D.). The lower limit of sensitivity was 1 pmol injected, equivalent to a plasma concentration of 0.7 μM.

Influence of anticoagulants

In order to study the possible interference of anticoagulants on plasma H(e) determination, whole blood specimens, withdrawn by venipuncture from five healthy donors, were collected in different Vacutainer tubes containing EDTA, heparin and sodium citrate. The mean levels found were 12.24 ± 0.70, 11.86 ± 1.11 and 11.84 ± 1.36 μM for EDTA, heparin and sodium citrate, respectively, showing no statistical differences among the three different treatments ($p > 0.05$, Student's t-test). We adopted sodium citrate as anti-coagulant, as it is commonly used in the coagulation laboratory of our Institute.

Plasma reference values

In order to define the physiological plasma H(e) levels, we determined this amino acid in plasma samples withdrawn by venipuncture from 40 fasting healthy subjects (20–60 years old). The mean H(e) concentration was 12.4 ± 2.9 μM (S.D.), which was consistent with literature values [2,10,18,21].

DISCUSSION

A sensitive and reproducible assay for total plasma homocyst(e)ine with OPA derivatization and HPLC analysis has been developed. As HCys, in normal human plasma, is predominantly bound with proteins (particularly to albumin) [11], we employed $NaBH_4$ as a disulphide reducing agent. After deproteinization with $HClO_4$, plasma samples were treated with 50 mM iodoacetic acid for S-carboxymethylation. The blocking of the sulphydryl groups, in fact, involves the formation of a very stable fluorescent isoindole product [28], providing reproducible analytical values.

The OPA reaction is highly pH dependent and at pH < 9.0 no reaction occurs [26], therefore the addition of 3 M NaOH after the deproteinization step is necessary to ensure a high derivatization yield (more than 94%).

The OPA derivatization method shows a very good sensitivity (1 pmol per injection), similar to other HPLC procedures [17,20,21]. No problems of interference from the reducing agent and a great saving of time over mBrB derivatization [21] were observed. In addition, the fluorescence intensity of the OPA–HCys derivative is sufficiently high to determine the free form also. H(e) determination was carried out on a commercial reversed-phase column at room temperature without requiring the utilization of special and expensive chromatographic apparatus or column heating [20]. The use of an internal standard with respect to other procedures [20,21,23] contributed to improving the precision of the method.

The average concentration of total plasma H(e) determined with our method was 12.4 ± 2.9 μM (n = 40). This value was in good agreement with H(e) levels obtained applying different other analytical techniques including GC–MS (7–22 μM) [10], a reference analyser (11.5 ± 0.9 μM) [2] or HPLC in combination with either mBrB derivatization (16.15 ± 5.4 μM) [21] or electrochemical detection (10.0 ± 3.2 μM) [18].

These data confirm the validity of this specific and reproducible method, allowing its accurate ap-

plication in clinical research. The evaluation of the potential atherogenic properties of H(e), made possible by the present method, will be investigated by an accurate definition of normal plasma H(e) range and by the determination of H(e) levels in patients afflicted with different cardiovascular diseases. This study will allow us to evaluate the potential atherogenic properties of H(e).

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