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Short Communication

Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum

JOHAN B. UBBINK*, W. J. HAYWARD VERMAAK and SIEGBERT BISSBORT

Department of Chemical Pathology, Faculty of Medicine, University of Pretoria, P.O. Box 2034, Pretoria 0001 (South Africa)

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ABSTRACT

A rapid, isocratic high-performance liquid chromatographic (HPLC) method is described for the determination of total homocysteine levels in human serum. Prior to reversed-phase HPLC analysis, the serum thiols were derivatized with SBD-F (ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate), a thiol-specific fluorogenic probe which is commercially available. Retention of SBD-homocysteine was sensitive to pH, and a mobile phase pH of 2.1 ensured baseline separation of serum thiols within 6 min. The method is simple, sensitive, reproducible (between-run coefficient of variation of 6.6%) and very suitable for routine determination of serum homocysteine levels in a clinical pathology laboratory.

INTRODUCTION

Recent reports have implicated elevated serum homocysteine levels as a risk factor for premature vascular diseases [1–5]. Prospective studies and intervention trails to scrutinize the possible role of moderate homocysteinaemia in vascular disease have, however, not yet been done, possibly due to difficult analytical techniques to measure circulating homocysteine levels.

Published methods to quantify serum homocysteine concentrations include amino acid analyzers [6,7] or high-performance liquid chromatography (HPLC) coupled with electrochemical detection [3,8,9]; both techniques are cumbersome and only a limited amount of blood samples could be analyzed daily. Serum homocysteine levels may also be assayed by condensation with [^{14}C]adenosine to S-adenosylhomocysteine, a reaction catalyzed by S-adenosyl-L-homocysteine hydrolase [10–13]. The radiolabeled product, S-adenosylhomocysteine, is subsequently isolated by thin-layer chromatography or HPLC. This method is laborious and commercial sources of S-adenosylhomocysteine hydrolase are expensive, implying that the method is unsuitable for large clinical studies.

Labeling of plasma thiols with a thiol-specific fluorogenic reagent, followed by

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HPLC and fluorescence detection, have been used to quantify circulating homocysteine levels [14–19]. At least two thiol-specific reagents, monobromobimane and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), are commercially available. Assay of the homocysteine-bimane conjugate either requires prior removal of excess monobromobimane [16], or column switching by a two-dimensional HPLC system to avoid monobromobimane interference in the assay [17]. Furthermore, binary solvent programmes are required to separate homocysteine from other serum thiols and monobromobimane degradation products [16,17]. Methods based on monobromobimane derivatization are therefore difficult to implement in an ordinary pathology laboratory. Thiols derivatized with SBD-F were reported to show high fluorescence and excellent stability [18–20]. SBD-F itself is non-fluorescent, and no fluorescent by-products are formed during the derivatization procedure [18,20]. Using reversed-phase HPLC and a gradient elution programme, Araki and Sako [19] reported separation of plasma thiols within 20 min. We now report a rapid, isocratic HPLC method to separate plasma SBD-derivatized thiols within 6 min. This method has recently been applied in clinical trials [21] and is also suitable for routine homocysteine quantification in pathology laboratories.

EXPERIMENTAL

Materials

D,L-Homocysteine, L-cysteine, γ-glutamyltransferase, and tri-*n*-butylphosphine were obtained from Sigma (St. Louis, MO, U.S.A.). Glutathione was bought from Boehringer (Mannheim, Germany) while SBD-F was supplied by Wako (Düsseldorf, Germany). All other reagents were obtained from Merck (Darmstadt, Germany). Cysteinylglycine was prepared by enzymatic hydrolysis of glutathione by γ-glutamyltransferase according to the method of Hanes *et al.* [22].

Sample preparation

Serum or plasma thiols were derivatized with SBD-F essentially according to the method of Araki and Sako [19], except that smaller volumes were used to economize the use of reagents. In short, 30 μl of a 10% solution of tri-*n*-butylphosphine in dimethylformamide were added to 0.3 ml of serum or standard. The mixture was incubated at 4°C for 30 min to accomplish reduction of homocystine and the mixed disulphide (cysteine–homocysteine) as well as the release of protein-bound homocysteine. This method therefore measures total (free plus protein-bound) serum homocysteine levels. Subsequently, 0.3 ml of 10% trichloroacetic acid (containing 1 mmol/l EDTA) was added. After centrifugation, 100 μl of the clear supernatent were added to a mixture of 20 μl of 1.55 mol/l sodium hydroxide, 250 μl of a 0.125 mol/l borate buffer (pH 9.5) containing 4 mmol/l EDTA and 100 μl of SBD-F solution (1 mg/ml dissolved in borate buffer). The

mixture was incubated for 1 h at 60°C to accomplish complete derivatization of homocysteine and other plasma thiols. A 20-μl aliquot was subsequently used for HPLC analysis.

High-performance liquid chromatography

A Waters Model 510 (Milford, MA, U.S.A). HPLC pump, coupled to a Spectra-Physics (San Jose, CA, U.S.A.) SP 8780 XR autosampler, was fitted with a Supelco (Bellefonte, PA, U.S.A.) LC-18-DB analytical column (150 mm × 4.6 mm I.D.). To protect the analytical column, a Whatman (Clifton, NJ, U.S.A.) reversed-phase guard column was fitted between the analytical column and the autosampler, while a Whatman Solvecon pre-column was installed between the autosampler and the pump. Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a Perkin Elmer (Beaconsfield, U.K.) LS 4 fluorescence spectrohotometer coupled to a Spectra-Physics Model 4290 integrator. We used a 0.1 mol/l potassium dihydrogenphosphate buffer (pH 2.1, adjusted with ortophosphoric acid) containing 4% acetonitrile as mobile phase with a flow-rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Fig. 1 illustrates the determination of plasma homocysteine levels after derivatization with SBD-F. Detection conditions were optimized for homocysteine; fluorescence output for cysteine and cysteinylglycine 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 5 min. The autosampler required 2.5 min to prepare the next sample for injection, leaving thus ample time for glutathione to elute from the column. The use of isocratic elution simplified the HPLC procedure; this renders the method suitable for large population studies.

The retention time of SBD-homocysteine was very sensitive to changes in pH (Fig. 2). When the mobile phase was adjusted to pH 2.25, SBD-homocysteine was only partially resolved from SBD-cysteinylglycine (Fig. 2B). With a mobile phase pH of 2.4, SBD-homocysteine and SBD-cysteinylglycine co-eluted from the analytical column (Fig. 2C). The shorter retention times for SBD-homocysteine with higher mobile phase pH presumably relates to the dissociation of the carboxyl proton (pK 2.2) from SBD-homocysteine. A mobile phase pH of 2.1 ensures that more than 50% of SBD-homocysteine carboxyl groups are protonated, resulting in a longer retention time of this compound and complete resolution from SBD-cysteinylglycine.

Performance parameters for the determination of serum homocysteine levels, as calculated from repeat analysis of two serum samples, are summarized in Table

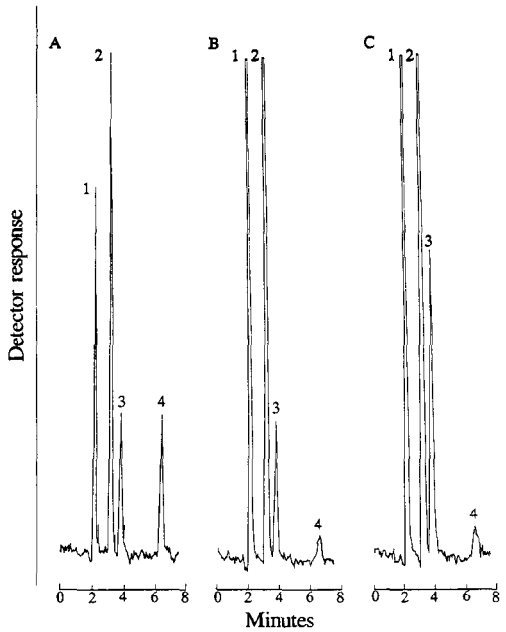

Fig. 1. Determination of serum total homocysteine levels after derivatization with SBD-F. (A) Standard, containing cysteine (40 μmol/l), cysteinylglycine, homocysteine (10 μmol/l) and glutathionine (20 μmol/l). (B) Serum sample from a normal, apparently healthy person. (C) The same sample, to which homocysteine (10 μmol/l) was added. Peaks: SBD derivatives of 1 = cysteine, 2= cysteinylglycine, 3 = homocysteine and 4 = glutathione.

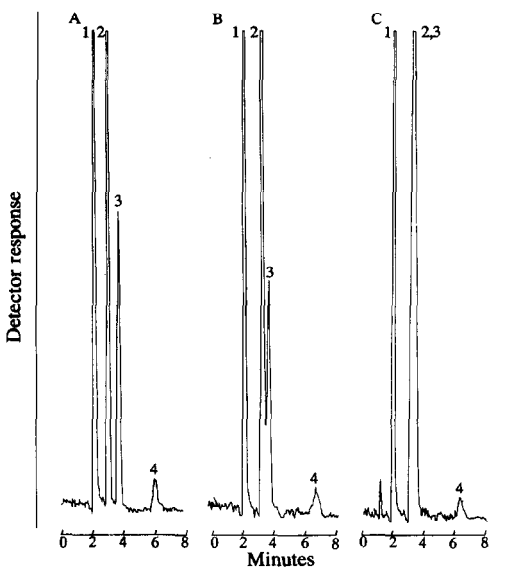

Fig. 2. Effect of mobile phase pH on the separation of SBD-derivatized thiols. The mobile phase pH was (A) 2.10, (B) 2.25 and (C) 2.40, respectively. Peak identification: refer to Fig. 1.

TABLE I

PERFORMANCE STATISTICS OF THE HOMOCYSTEINE ASSAY

Serum sample	Concentration range[a] (μmol/l)	Precision (coefficient of variation, %)	
		Within-run (n = 12)	Between-run (n = 12)
A	7.60–8.85	4.5	6.6
B	17.37–19.67	3.9	5.9

[a] Concentration range refers to the minimum and maximum estimates of homocysteine concentration obtained during twelve consecutive days.

I. Recovery of homocysteine, added to different serum samples (n = 10) to a final concentration of 10 μmol/l, was 104.3% (S.D. = 5.4). Fluorescence of SBD-homocysteine was linear over a concentration range of 2–70 μmol/l; higher concentrations were not tested as they were not encountered in normal populations.

We were able to confirm the excellent features of SBD-F as analytical reagent for biological thiols as described by Imai *et al.* [20]. SBD-F exhibited no fluorescence, neithere 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 [16,17]. These problems necessitate advanced chromatography to allow homocysteine quantification as a homocysteine-bimane conjugate [16,17]. In contrast, the high selectivity of SBD-F results in specific labeling of thiols, thus allowing isocratic elution to separate SBD-homocysteine from other SBD-thiols.

It is well known that established and important risk factors (elevated serum cholesterol levels, hypertension, tobacco consumption) can only explain 50% of coronary heart disease incidence [23]. In a recent study, which showed that 66% of patients with severe atherosclerosis had no evidence of hypercholesterolaemia, diabetes or hypertension, measurement of circulating homocysteine levels together with the blood lipid profile is recommended in persons with atherosclerosis [24]. The method presented in this paper is simple, suitable for routine determinations of serum homocysteine levels and well within the capabilities of the average clinical pathology laboratory.

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