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Short Communication

Modification of a high-performance liquid chromatographic method for assay of homocysteine in human plasma

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ABSTRACT

A modification of a previously published method for analysis of total homocysteine in human serum is presented. The modification was implemented to allow use of a different derivatizing agent (*i.e.*, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonamide) which reacts much faster than the original derivatizing reagent and at a lower temperature. Shorter reaction time and lower temperature lead to less destruction of some biological thiols. In order to retain an isocratic mobile phase with the new derivatizing agent, a different concentration of acetonitrile was found that affords a 7–8 min retention time.

INTRODUCTION

Over the last several years, a number of methods for the analysis of homocysteine in human samples have been published [1–6]. Most methods are difficult enough to perform to preclude their use in large-scale surveys. A recent publication of Ubbink *et al.* [6] describes a rapid high-performance liquid chromatographic (HPLC) assay for total homocysteine levels in human serum. We modified the method to incorporate the more rapidly reacting fluorogenic reagent 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonamide (ABDF) [7], which reacts six times faster than the reagent used by Ubbink, *et al.* [6], *i.e.*, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBDF). The reaction is also complete at a lower temperature (50 *versus* 60°C) when using the ABDF compound. When using the mobile phase of Ubbink *et al.* [6], the derivatizing agent ABDF resulted in an increase in the retention time of ABD-homocysteine to 21 min. Subsequent work with varying concentrations of acetonitrile in the mobile phase resulted in a concentration of acetonitrile (10%) which gave a satisfactory retention time of 7.2 min for ABD-homocysteine, with baseline separation from cysteinylglycine and glutathione. We determined plasma levels of homocysteine with this modified method in normal human controls whose plasma folate and vitamin B_{12} levels were known.

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EXPERIMENTAL

Chemicals

D,L-Homocysteine, tri-*n*-butylphosphine, SBDF, ABDF, and L-homocysteine thiolactone were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade.

Sample preparation

Plasma samples were prepared for derivatization according to the method of Ubbink *et al.* [6], which is a modification of the method of Araki and Sako [8]. Plasma samples were obtained from human blood drawn into sterile EDTA tubes and were treated as follows: to 300 μl of plasma or standard were added 30 μl of a 10% solution of tri-*n*-butylphosphine in dimethylformamide. After thorough mixing this mixture was chilled at 4°C for 30 min during which time the biological thiols were reduced and deconjugated from the plasma proteins. After reduction, 300 μl of 10% trichloroacetic acid which contained 1 mmol/l EDTA were added to the mixture and the samples were mixed on a vortex mixer for 1 min. The samples were centrifuged at 1700 *g* for 10 min. To 100 μl of the supernant was added a mixture of 20 μl of a 1.55 mol/l NaOH solution; 250 μl of 0.125 mol/l borate buffer, pH 8.0, containing 4 mmol/l EDTA; 20 μl of ABDF solution (1 mg/ml in borate buffer). A 10-min incubation at 50°C was used to derivatize the thiols. After cooling to room temperature, the samples were centrifuged at 1700 *g* and the clear supernatant was used for analysis.

High-performance liquid chromatography

An Isco (Lincoln, NE, USA) Model 2350 HPLC pump, coupled to a Hitachi (Danbury, CT, USA) AS-2000 autosampler, was connected to a Beckman Ultrasphere ODS 5-μm analytical column (250 mm × 4.6 mm I.D.). An Adsorbosphere C_{18}, 5-μm guard column (Alltech) was used in front of the analytical column. An Hitachi Model F1000 fluorescence spectrophotometer was connected to an Hewlett-Packard (Avondale, PA, USA) Model HP3394 integrator. The fluorescence spectrophotometer was operated at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The mobile phase, pumped at 1.5 ml/min, consisted of 0.1 mol/l potassium dihydrogenphosphate (adjusted to pH 2.1 with orthophosphoric acid) containing various concentrations of acetonitrile.

RESULTS AND DISCUSSION

Our early work with SBDF gave us reference points with which to compare the results obtained with ABDF. Standards of known homocysteine concentration were prepared which were then used as sample in the *Sample preparation* section outlined above. ABDF was used as the derivatizing agent and the incubation period was 10 min at 50°C. In order to reduce the retention time of ABD-homocysteine, different batches of mobile phase were prepared, each containing a different concentration of acetonitrile, from 4 to 12%, all at pH 2.1. Subsequently, 10% acetonitrile was chosen as it resulted in a retention time of 7.2 min, which is very close to the retention time of SBD-homocysteine in the method of Ubbink *et al.* [6]. Subsequent work with human plasma has shown that this mobile phase plus the new derivatizing agent produces satisfactory chromatograms with clear separation of homocysteine from cysteinylglycine and glutathione. A further extension of our work was to confirm that the concentration of ABDF in the derivatizing step, which is approximately sixteen times that of the concentration of the total thiols present in human serum [9], was an appropriate concentration for total reaction. No changes were noted in chromatograms of control plasma derivatized with 20 μg as opposed to 100 μg of fluorophore.

Fig. 1 shows a typical chromatogram of (a) a homocysteine standard and (b) a normal human plasma. The cysteine, cysteinylglycine and glutathione peaks are integrated and the information is available if needed.

Table I summarizes the precision of the modified method. Data were obtained from repeated analysis of two plasma samples over a period of three weeks. In a recovery study of eight plasma samples, the mean recovery was 102%.

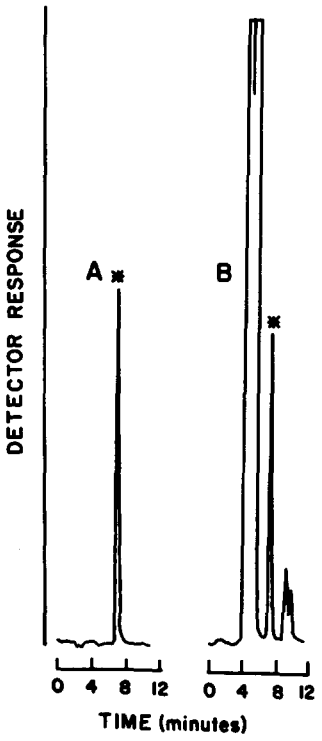

Fig. 1. Illustration of chromatograms of homocysteine after derivatization with ABDF. (A) Standard, containing homocysteine (15 μmol/l). (B) Plasma sample from human control sample.

Table II illustrates the descriptive statistics of determinations of plasma homocysteine in 23 normal human control samples. This control group contains only plasma samples that were analyzed for folic acid and vitamin B_{12} and shown to have normal values for these nutrients. The plasma folic acid and vitamin B_{12} means were 24.9 nmol/l and 377.6 pmol/l, respectively.

TABLE I

PRECISION OF THE MODIFIED HOMOCYSTEINE ASSAY

Plasma sample	Concentration range[a] (μmol/l)	Coefficient of variation between run (n = 13) (%)
S.M.	8.4–9.52	4.58
H.V.	6.71–8.7	6.71

[a] Concentration range refers to the minimum and maximum levels of homocysteine in that plasma sample when analyzed thirteen times over a three-week period.

TABLE II

DESCRIPTIVE STATISTICS OF THE MODIFIED HOMOCYSTEINE ASSAY

Twenty-three samples were analyzed for homocysteine from known normal human controls. Each of these samples was also analyzed for folic acid and B_{12} content and was found to be normal or above normal for that nutrient.

Sample size (n)	Mean (nmol/ml)	Range (nmol/ml)	S.D. (nmol/ml)
23	8.86	5.91–12.45	1.62

In light of recent interest in the relationship of homocysteine to atherosclerosis and coronary heart disease [10], it is essential that a facile, reliable assay be devised for homocysteine. The present method is fast, convenient, and requires no elaborate pre- or post-assay work. While the difference in reaction temperature between the present work and that of Ubbink *et al.* [6] (50 *versus* 60°C) is small, it becomes significant when the incubation times are compared (10 *versus* 60 min), particularly when one considers that thiols are easily oxidized in an alkaline solution [11]. Mild conditions, *i.e.*, lower pH (8.0 *versus* 9.5), lower temperature and reduced reaction time, become important parameters of the derivatization reaction [7].

This paper is the first to report homocysteine levels in human samples obtained from subjects who are known to be replete in both folate and vitamin B_{12}, using this thiol-specific compound. The relative simplicity and stability of this assay should faciltate screening of large populations who may be at risk for vascular disease.

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