

A Modified HPLC method for routine laboratory measurement of total homocystine.

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Abstract

There is evidence that mild to moderate elevation of total homocysteine due mainly to vitamin B₁₂ and folate deficiency or Methylene tetrahydrofolate reductase gene polymorphism is associated with an increased risk of occlusive vascular disease, thrombosis and stroke etc. Different methods of assaying total homocysteine (tHcy) have been reported in the past few decades. However, most of the HPLC methods reported in determination of tHcy cannot be directly used under local laboratory conditions due to many reasons. Therefore, a HPLC method with fluorescence detection was developed with some modifications of the method described by Pfeiffer et. al. (1999) using cystamine dihydrochloride as the internal standard for routine determination of tHcy in plasma under local conditions.

Key Words : Plasma total homocysteine, Chromatography, Fluorescence detection, Internal standard

1. Introduction

Homocystine is a sulphur containing amino acid whose metabolism stands at the intersection of two pathways: remethylation to primary amino acid Methionine which requires folate and vitamin B₁₂ (or betaine in an alternative reaction); and transulfuration to cystathionine which requires another B vitamin pyridoxal -5' phosphate (vitamin B₆). Hyperhomocysteinaemia, a condition which has come to the forefront as a risk factor for vascular disease, arises from the disruption of Homocysteine metabolism. Severe hyperhomocysteinaemia is due to rare genetic defects of enzymes involved in the metabolic pathway while mild to moderate hyperhomocysteinaemia seen in fasting conditions is due to mild impairment of the

methylation pathway [i. e. folate/ B₁₂ deficiencies or Methylene tetrahydrofoalate reductase (MTHFR) thermolability due to MTHFR gene polymorphism]. Post-methionine load hyperhomocysteinaemia may be due to heterozygous cystathionine β-synthase defect or vitamin B₆ deficiency¹. Thus total fasting homocysteine levels are taken as a good indicator of vitamin B₁₂ and folate status.

Mild to moderate hyperhomocysteinaemia is related to pregnancy complications², neural tube defects, stroke³, occlusive peripheral arterial disease⁴ etc. Elevated total homocysteine has been confirmed as a risk factor for ischaemic heart disease and other vascular disorders⁵.

Thus homocysteine measurement has become a novel way of assaying folic acid and vitamin B₁₂ deficiencies and risk assessment in many diseases, particularly ischaemic heart disease.

Several methods of measuring plasma total homocysteine have been described⁶⁻⁹. One of the most widely used methods of total plasma homocysteine (and other aminothiols) measurement is reversed phase High Performance Liquid Chromatography (HPLC) with fluorescence detection after derivatization of plasma amino thiols with ammonium 7- fluorobenzo-2oxa-1,3 diazole sulfonate (SBD-F)^{8,9}. Plasma amino thiols exist as free reduced and oxidized compounds or as protein bound ones and one of the most critical steps in the sample processing is the reduction of disulfide bonds before derivatization so that total homocysteine in plasma is measured. For this step trialkylphosphines are used and tri-butyl phosphine (TBP) and *tris* (2-carboxyethyl) phosphine (TCEP) are the most widely used. TBP has an irritaitng odour, is poorly water soluble and it has to be dissolved in toxic dimethylformamide, where as TCEP is less toxic, soluble in aqueous solutions and stable¹⁰. Thus recently researchers have preferred TCEP over TBP as the reductant in the analysis of aminothiols in human plasma.

One of the methods using the above was described by Pfeieffer *et. al.* in 1999 and this method has several advantages such as being a rapid, user friendly assay which uses a stable and non-hazardous reducing agent in TCEP, and rapid isocratic separation of aminothiols of interest using a mobile phase of mild pH using only 50µl of plasma¹¹.

The intention of our study was to determine the suitability of the above method for our laboratory condition (and to modify it if necessary) and to identify a suitable internal standard to run with the method so that it could be used in routine laboratory use and also in a clinical case control study on hyperhomocysteinaemia and ischaemic heart disease in Sri Lanka.

2. Material and methods

Chemicals and Reagents

All chemicals used in the study were of analytical or HPLC grade. L-homocysteine, cystamine dihydrochloride, N-(2-mercaptopropionyl) glycine, Phosphate-buffered saline (PBS ; pH 7.4), TCEP, SBD-F and EDTA were purchased from Sigma-Aldrich Co. U.S.A. Acetic acid and Methanol were purchased from Fischer scientific U.K. and all the other chemicals were from BDH U.K.

Blood samples :

Blood sample were obtained from the participants who have undergone fasting for 12 hours. 2 mL of Blood was collected by venipuncture into pre-chilled K3 EDTA containing tubes (Vacutainer[®] brand) and immediately placed in ice. Plasma was separated by centrifuging at 3000rpm for 10 minutes and assayed immediately or stored for a maximum of 1 month at -20^oC pending analysis. Blood specimens were collected from participants at Colombo South Teaching Hospital, Kalubowila, Sri Lanka after obtaining informed written consent at the point of recruitment to the study.

Preparation of samples for Hcy assay:

Plasma sample (50 μ L) was mixed with 25 μ L of internal standard [IS; Cystamine dihydrochloride 100 μ mol/L or N-(2-mercaptopropionyl) glycine 200 μ mol/L dissolved in phosphate buffered saline (PBS, pH 7.4)] and 25 μ L of PBS, pH 7.4 and incubated with 10 μ L of 100/gL TCEP for 30 minutes at room temperature to reduce disulfides and release protein bound thiols. 90 μ L of 100 g/L trichloro acetic acid containing 1 mmol/L EDTA was added for deproteinization. Precipitated proteins were removed by centrifugation at 1300rpm for 10 minutes and 50 μ L of the supernatant was added to vial containing 10 μ L of 1.55 mol/L NaOH; 125 μ L of 0.125 mol/L borate buffer containing 4mmol/L EDTA,pH 9.5; and 50 μ L of g/L SBD-F in the borate buffer. The sample was incubated for 30 minutes at 60^oC. After deproteinization, the samples were cooled and protected from direct light until injection into the column.

Apparatus and chromatographic conditions:

HPLC was carried out on a Waters 515 solvent delivery pump and a Waters 474 scanning fluorescence detector (Waters technologies corp. U.S.A.). Chromatograms were obtained by way of a chromject * CH1 integrator from Thermo Electra Corporation U.S.A. Aminothiols were separated on a Supelco* LC18-DB analytical column 150x 4.6 mm ID packed with 3 μ m particles with a

Supelguard[®] LC18-DB 2cm guard column (Sigma-Aldrich U.S.A.) 10 μ L of prepared sample was injected into the column and 0.1 mol/L acetic acid-acetate buffer (pH 5.5), containing 30 ml/L methanol was used as the mobile phase at a flow rate of 0.7 ml/min. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. All analyses were performed at room temperature.

Validation of assay method:

L-homocystine calibrators (0-150 μ mol/L free thiol) were prepared by adding known L-homocystine concentrations to PBS (pH 7.4). Initially the two internal standards, Cystamine dihydrochloride and N-(2-mercaptopropionyl) Glycine were run separately and the selected internal standard was then added to all samples.

3. Results

Comparison of the internal standards :

When N-(2-mercaptopropionyl) glycine was used as the internal standard the retention time for the IS was 25 minutes, where as when cystamine dihydrochloride was used the retention time was much shorter at 10 minutes. Therefore it was decided to use cystamine dihydrochloride as the internal standard in consequent tHcy assays.

Performance characteristics of the assay method:

The analyses of standard samples of homocystine prepared in PBS, pH 7.4 produced a linear curve up to 150 μ mol/L with a coefficient correlation of 0.994 and a 0.25 μ mol/L lower limit of detection for homocysteine.

Chromatogram pattern:

According to the chromatographic condition used for tHcy assay, it was found that homocysteine and the internal standard were not clearly baseline separated from other aminothiols (Fig. 1).

Lowering the methanol concentration of the mobile phase stepwise from 30 mL/L to 12 mL/L methanol in acetic acid-acetate buffer (pH 5.5), showed that with the decrease in the level of methanol a good baseline separation of the aminothiols and the internal standard could be achieved (Fig.2).

According to the present modified method, no interfering peaks were observed between the peaks for the aminothiols and the internal standard. It was also observed that the plasma sample without the internal standard showed no cystamine peak.

Furthermore no significant changes were observed in the retention times of tHcy even after storing the plasma sample at -20^oC for 3 months (Table 1). This is further

confirmed by analysis of 442 samples, using the above method for homocysteine analysis with excellent reproducibility, in a 2 year long study started in 2002, using young Sri Lankan ischaemic heart disease patients (manuscript in preparation).

4. Discussion

Results of the present study have revealed that cystamine dihydrochloride appeared to be a better internal standard when compared to N-(2-mercapto-propionyl) glycine as the retention time of cystamine dihydrochloride is less, thus saving on time and the amount of solvents when samples are run. Cystamine also has the advantage that it overcomes the matrix effect of plasma when comparing with calibrators prepared in PBS or water¹¹.

Under the chromatographic conditions described, calibration curves for homocysteine was linear up to 150 $\mu\text{mol/L}$. Hyperhomocysteinaemia is graded according to the fasting plasma levels as mild to moderate (15-30 $\mu\text{mol/L}$), intermediate (31-100 $\mu\text{mol/L}$), and severe ($> 100 \mu\text{mol/L}$)¹². Thus this method is able to differentiate between normal and the hyperhomocysteinaemic persons as well as grading them according to the severity of hyperhomocysteinaemia.

The present assay method was modified by having the column at room temperature (28- 32°C) thus negating the difficult task of maintaining a controlled column temperature with the apparatus available when compared with the assay method described by Pfeiffer *et. al*, (1999) whose assay method was carried out at an exact column temperature of 29°C. However it was found that baseline separation of the thiols and the internal standard with the present assay method was not optimal at 30mL/L methanol in acetic acid-acetate buffer (pH 5.5). This improved as the methanol concentration was decreased gradually and we were able to obtain a good chromatogram with base line separation at a methanol concentration of 12 mL/L. These results suggest that methanol at 12 mL/L in acetic acid-acetate buffer is a better way of getting good chromatograms in conditions described above.

The assay performance characteristics (coefficient of variation) of homocysteine and internal standard which appeared to be consistent within and between run assays (Table 1) indicate that the test method could be reproduced with ease over a longer period of time. Thus, the present assay method is an acceptable effective way of assaying tHcy.

In conclusion, the modified method described for homocysteine analysis based on the method described by Pfeiffer *et. al* (1999) could be used as a reliable and reproducible method for routine determination of plasma homocysteine.

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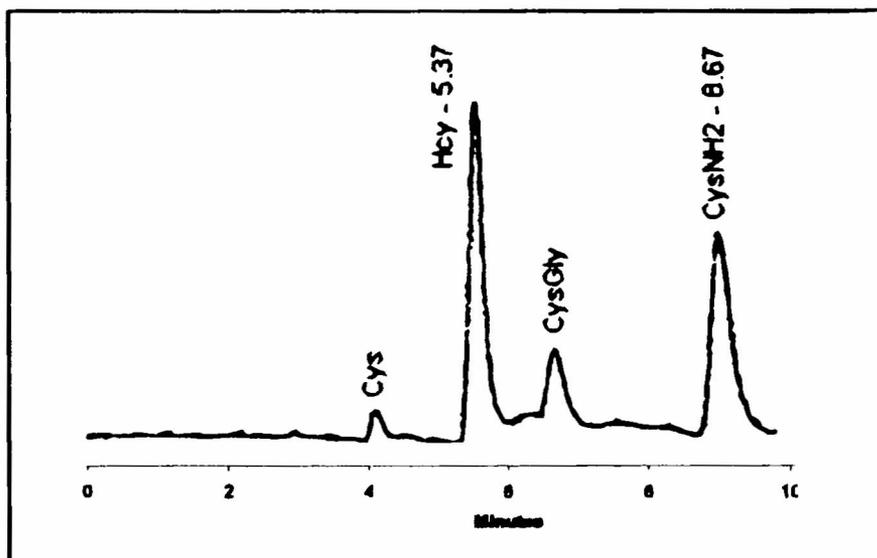


Figure 1. Chromatogram of a representative plasma sample measured at a methanol concentration of 30 mL/L.

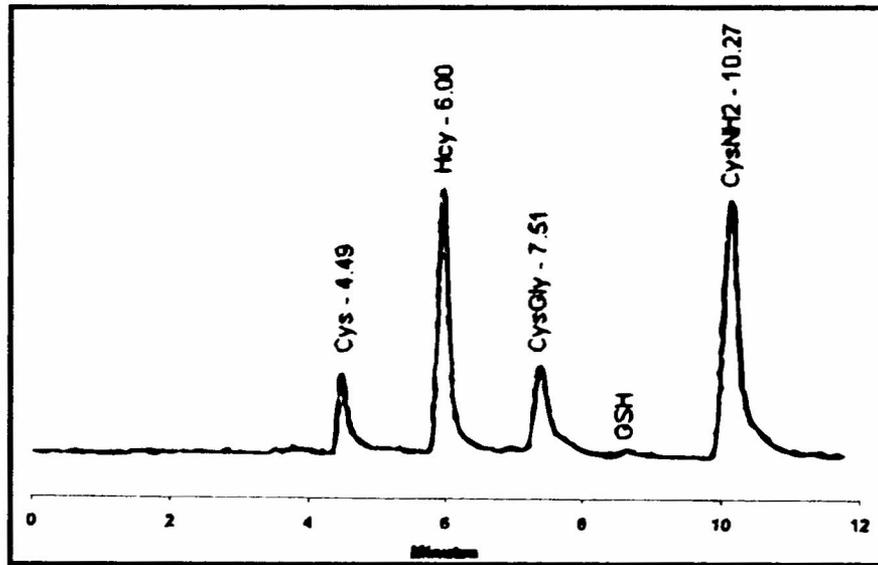


Figure 2. Chromatogram of a representative plasma sample measured at a methanol concentration of 12 mL/L.

Table 1. Results of comparison of retention times for tHcy and internal standard after 3 Months

	Total Homocysteine		Internal standard	
	Beginning	3 months later	Beginning	3 months later
Mean	5.34	5.92	8.60	10.18
SD	0.10	0.15	0.18	0.24
CV	1.96	2.49	2.07	2.32