STANDARDIZATION OF HOMOCYSTEINE ON HPLC USING DTT AS REDUCTANT

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ABSTRACT

Determination of plasma total homocysteine by high –pressure liquid chromatography (HPLC) usually requires reduction of protein bound or free homocysteine -disulphides into thiols by a reducing agent and the liberated thiols are then derivatized by a fluorescent marker. In this study we have standardized the HPLC method for homocysteine measurement using dithiothreitol (DTT) as reductant.

The results of plasma total homocysteine values obtained by HPLC were compared with IMx method. The difference between the two means was statistically insignificant [$P=0.616847$ (two tail)] Linear regression analysis showed strong correlation between the two methods ($r=0.983$). Using this method we have analyzed 132 controls and 130 Coronary Heart Disease (CHD) patients for plasma total homocysteine, wherein, the mean plasma total homocysteine levels were 10.51 ± 8.36 and 11.51 ± 10.06 $\mu\text{mol/L}$ respectively. Our research study suggests that DTT method is a simple and inexpensive assay for homocysteine determination in human plasma for research application.

KEY WORDS:

Homocysteine, HPLC, DTT

INTRODUCTION

High homocysteine (Hcy) level has emerged as an important parameter in pathological conditions, such as atherosclerosis and thrombosis. The known reasons for an increased level of plasma total homocysteine (tHcy) are inherited genetic defects of Cystathionine β Synthase (CBS) and Methylenetetrahydrofolate Reductase (MTHFR) or acquired causes, such as deficiency of folic acid, vitamin B6 and B12. Total Hcy is composed of free and protein – bound forms of Hcy. Within person variability of plasma Hcy concentrations in adults is relatively low. A single measurement characterizes the average concentration in plasma over atleast one-month (1).

The increasing requirement for total Hcy measurement has led to the development of various measurement assays. High Pressure Liquid

Chromatography (HPLC) method with fluorometric detection, (2,3) gas-chromatography – mass spectrometric (GS-MS) technique (4) and recently an automated fluorescence polarization immunoassay for the measurement of total Hcy in plasma or serum using Abbott IMx analyzer has been described (5). Studies comparing Abbott IMx with HPLC methods have also been documented (6,7).

A frequently used method for tHcy measurement is reversed- phase HPLC with fluorescent detection after derivatization of plasma Hcy with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) (2,3,8). Plasma Hcy exist as free reduced and oxidized compounds or as protein- bound ones.

One of the most critical steps in the sample-processing procedure is the reduction of disulfide bonds before derivatization (9). Among the reductants widely used are tri-n-butylphosphines (TBP), tris (2-carboxylethyl) phosphine (TCEP) and sodium borohydride (NaBH_4).

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Here we describe modified SBD-F procedure adapted from that of Fermo et al (10) where we have used DTT as a reducing agent and cysteamine dihydrochloride as an internal standard (I.S). Cysteamine dihydrochloride has sufficient chemical similarities to Hcy to make it suitable as an I.S. L-Homocystine (Hcy-Hcy) was preferred to be used as standard instead of DL-Hcy because of its chemical stability, and to check for the reduction step.

MATERIALS AND METHODS

Twelve-hour fasting venous blood samples were collected into citrated tubes and were kept on ice. The tubes were centrifuged within an hour and plasma was separated and kept at -70°C till further analysis.

The procedure was carried out as follows: A 200 μl of plasma was mixed with 100 μl of I.S (30 $\mu\text{mol/L}$) and 200 μl of 1 $\mu\text{mol/L}$ DTT in an Eppendorf tube and was incubated at $50^{\circ}\text{C}/60\text{mins}$. Deproteinization was performed by adding 200 μl of 0.6 mol/L perchloric acid in 1mmol/L EDTA and it was centrifuged at 8000 rpm/20 mins. The supernatant was filtered through Millipore filter. To 100 μl of filtered supernatant 200 μl of 0.2 mol/L borate buffer containing 4 $\mu\text{mol/L}$ EDTA, pH 9.5, 20 μl of 2mol/L sodium hydroxide and 50 μl of SBD-F solution was added. The samples were mixed and incubated at $60^{\circ}\text{C}/1\text{hr}$. A 20 μl aliquot was injected into the HPLC system.

Chromatographic conditions: The HPLC system from Waters consisted of Waters 600s controller, Waters 626 pump, Waters 474 scanning Fluorescence detector which was set at 385 nm and 515 nm as excitation & emission wavelength respectively. The HPLC mobile phase consisted of only one buffer, 0.1mol/L sodium acetate pH 5.5 containing 30% of methanol. The column used was AccQ.Tag (Waters) dimensions 3.9 x 150mm, particle size 4 μm , packing material-silica base bonded with C_{18} . The column was equilibrated with 100% buffer at 1ml/min. The analysis was carried out at the same flow rate. The run was for 6 minutes.

The principles of IMx procedure: Bound Hcy (oxidized form) is reduced to free Hcy and the free Hcy is enzymatically converted to S-adenosyl-L-homocysteine (SAH). Hcy and mixed disulfide and protein-bound forms of Hcy in the sample are reduced to form free Hcy by the use of dithiothreitol (DTT). Total free Hcy is converted to SAH by the

use of SAH hydrolase and excess adenosine mouse monoclonal anti - SAH antibody then recognizes SAH. SAH and labeled Fluorescein Tracer compete for the sites on the monoclonal antibody molecule. The intensity of polarized fluorescent light is measured by the FPIA optical assembly.

RESULTS AND DISCUSSION

The peak area generated for 10 $\mu\text{mol/L}$ Hcy-Hcy reduced with 0.2 ml of 1mmol/L DTT was equivalent to peak area generated by 20 $\mu\text{mol/L}$ Hcy confirming that Hcy-Hcy is quantitatively reduced and derivatized.

Forty samples were simultaneously analyzed for Hcy by HPLC as well as by Abbott enzyme immunoassay (Abbott IMx analyzer). The mean levels were 9.25 ± 2.92 and 9.61 ± 2.62 $\mu\text{mol/L}$ respectively. There was no statistically significant difference between the means of the two sample sets [$P=0.616841$ (two tail)]. A regression analysis was carried out of HPLC values over IMx values. It gave regression equation as $\text{HPLC} = -1.49 + 1.12$ (IMx) and correlation coefficient as 0.9830. High correlation coefficient coupled with a regression coefficient of 1.12 (95% CI 1.04 – 1.20) is suggestive of the agreement between the two methods (Figs. 1, 2). Using this method we have further analyzed 132 controls and 130 CHD patients by this method which showed mean plasma total Hcy levels to be 10.51 ± 8.36 and 11.51 ± 10.06 $\mu\text{mol/L}$ respectively.

The reduction of the disulfide bonds between Hcy and plasma proteins and the reduction of Hcy disulfides probably represent one of the most crucial steps in the determination of the total plasma Hcy. The efficiency of the reduction step may significantly influence the performance of the entire method.

Most of the procedures based on SBD-F derivatization (2,3) employed TBP / dimethyl-formamide solution as reductant, which requires safety measures to avoid potential hazards. In 1997, Gilfix et al.(11) introduced a non hazardous water – soluble reductant for determination of tHcy :tris (2-carboxyl-ethyl) phosphine (TCEP). Fermo et al. (10) described the use NaBH_4 , however this reagent cause formation of gas and foaming during the reaction.

The method highlights the use of DTT as a reducing agent before SBD-F derivatization.

It has been previously reported that DTT and other

sulphydryl containing reductants form adducts with thiol specific reagents and therefore may consume derivatization reagent (12). But in our study no such problems were observed as complete reduction of Hcy-Hcy to Hcy was obtained. The concentration of the reducing agent and incubation time was optimum for the method. The TCEP method (11) has been widely used and has been adopted by Pfeiffer et al (13). We found the DTT method works well at our lab. In conclusion, we suggest that DTT could ideally be used as a reductant in protocols

with SBD-F as derivatizing reagent for Hcy determination in human plasma on HPLC. The DTT method is a simple and relatively inexpensive assay, thus being useful for research application.

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Fig. 1: Regression lines for the Abbott IMx versus homocysteine measurement by high –pressure liquid chromatography (HPLC).Regression equation is $HPLC = -1.49 + 1.12 (IMx)$

Fig. 1

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