

*Advances in Basic, Laboratory and Clinical Aspects of Thromboembolic Diseases****METHODOLOGICAL ASPECTS OF TOTAL PLASMA HOMOCYSTEINE MEASUREMENT**

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In the last decade homocysteine (Hcy) has been proposed as an independent risk factor in the development of premature occlusive vascular diseases. One of the major problems encountered in studies on the potential atherogenic role of Hcy has been the development of an accurate and simple assay capable of screening, in a normal population, subjects having a congenital predisposition to occlusive vascular pathologies.

Several approaches have been described in the literature for measuring total plasma homocysteine (tHcy), which is defined as the sum of free and protein-bound homocysteine, homocystine and homocysteine-cysteine mixed disulfide. These approaches involve the use of radioenzymic¹ or immunoassay,² gas chromatography-mass spectrometry (GC-MS)³ and high-performance liquid chromatography (HPLC) procedures. This last technique, which is the one most widely applied, may be combined with different detectors such as a) post-column ninhydrin derivatization and spectrophotometric detection in the visible range (amino acid analyzer),⁴ b) electrochemical detection,^{5,6} or c) pre-column derivatization with different labelling agents such as monobromobimane,⁷ *o*-phthalaldehyde (OPA)^{8,9} or halogensulfonylbenzofurazans (ABD-F and SBD-F)^{10,11} and spectrofluorimetric detection.

In this presentation we compare and evaluate the methodologies currently available for measuring tHcy in plasma or serum, taking into consideration their feasibility as routine methods in the clinical chemistry laboratory.

Since analysis of tHcy requires the use of reducing agents for cleavage of the protein-bound sulphur-containing amino acids and maintenance of the thiols in a reduced form, aspects of sample collection and preparation that represent sources of variation within and between methods will be also described.

Pre-analytical steps*Collection of serum/plasma samples*

Differences in sample handling can cause significantly different results in Hcy measurement. During whole blood storage at room temperature Hcy concentrations tend to increase over time due to the protracted production and release of Hcy by blood cells.^{3,12} This process is slowed down when blood samples are left on ice. There are no problems for serum or plasma samples because the Hcy in these biological fluids is stable for at least four days at room temperature, for several weeks at 4°C and for years at -20°C.¹³

Reduction reactions

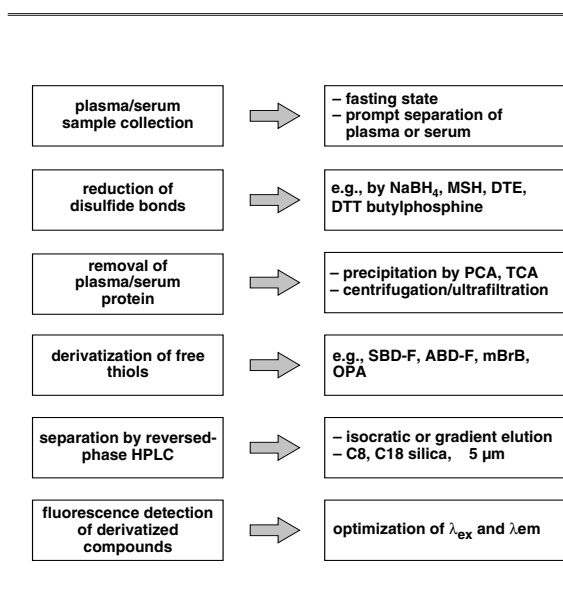
Usually in clinical laboratories total plasma/serum homocysteine (tHcy) is determined. This quantification requires reduction of the disulfide bonds between Hcy and other thiols or blood proteins. The selection of reducing agent depends on the separation and detection system used. Concerning sulphhydryl-containing reducing agents such as dithioerythritol, dithiothreitol and 2-mercaptoethanol, these can react with thiol-specific derivatization agents to produce fluorescent material which represents a possible source of interference during HPLC analysis.¹⁴ Sodium and potassium borohydride are very potent reductants. Reduction takes a few minutes at high concentration (1.4 mol/L)⁷ but, at lower concentrations (~40-100 mmol/L), it requires up to 30 min or heating.^{8,15} The drawback is the formation of gas during the reaction and to avoid sample foaming, surface active agents such as octanol or amyl alcohol are added. Tri-*n*-butylphosphine does not react with thiol-specific derivatization agents nor does it produce gas during the reduction step; however, this substance is an irritant and strict safety measures are required to avoid potential hazards.

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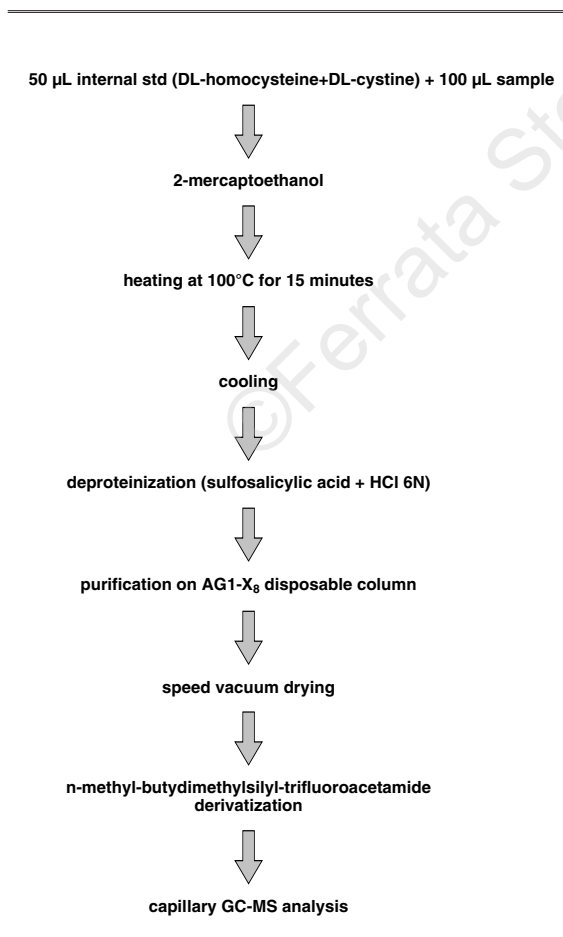
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Flow chart 1. Pre-column HPLC derivatization methods.



Flow chart 2. GC-MS sample processing steps.



Derivatization reactions

All procedures proposed to determine plasma or serum tHcy, apart from Abbott immunoassay and HPLC analysis based on electrochemical detection, require Hcy derivatization. Most biological amino acids, including Hcy, lack structural properties necessary for the production of signals compatible with the most frequently used HPLC detection systems such as spectro-photometric and -fluorometric detectors. These drawbacks are by-passed using the derivatization reaction: before the detection step amino acids react with labelling agents which enhance fluorescence or UV/visible signals. For Hcy, pre-column and post-column derivatization reagents may be used. The first group includes the halogensulfonylbenzofurazans (ABD-F, SBD-F), monobromobimane and *o*-phthalaldehyde (OPA), and all these labelling reagents produce a fluorescence complex with Hcy.⁷⁻¹¹ The steps involved in this treatment are reported in flow chart #1, where the first three passages are common to all other assays proposed for this determination. In the second class, the analysis is usually performed on a conventional amino acid analyzer with ninhydrin as labelling reagent.⁴

Methods

Immunoenzymic assay

Last year Abbott laboratories set up a totally automated immunoassay for tHcy plasma determination based on fluorescence polarization (FPIA) detection, a useful technique for measuring small molecules.² For this analysis Abbott laboratories proposed an indirect approach based on the high selective conversion of homocysteine to S-adenosylhomocysteine in the presence of S-adenosylhomocysteine hydrolase enzyme.¹⁶ Abbott laboratories, in fact, produced monoclonal antibodies able to recognize S-adenosylhomocysteine and its fluoresced analog. All the steps in this procedure are reported in Figure 1.

Since the assay is new, until now no direct experience has been available concerning its feasibility as a routine method, but obviously the most attractive feature is its complete automation.

Gas chromatography-mass spectrometry

In 1987 Stabler *et al.* proposed a new capillary GC-MS technique for quantifying tHcy.³ For this determination known amounts of Hcy and cysteine, which contain stable deuterated isotopes, serve as internal standards and to correct the variable recovery through the several steps that characterize sample processing (flow chart #2). Using semiautomated pipetting equipment, 320 samples can be assayed in 8h. The disadvantages of this approach are: the lack of total automation, considering the different steps involved in sample preparation; the

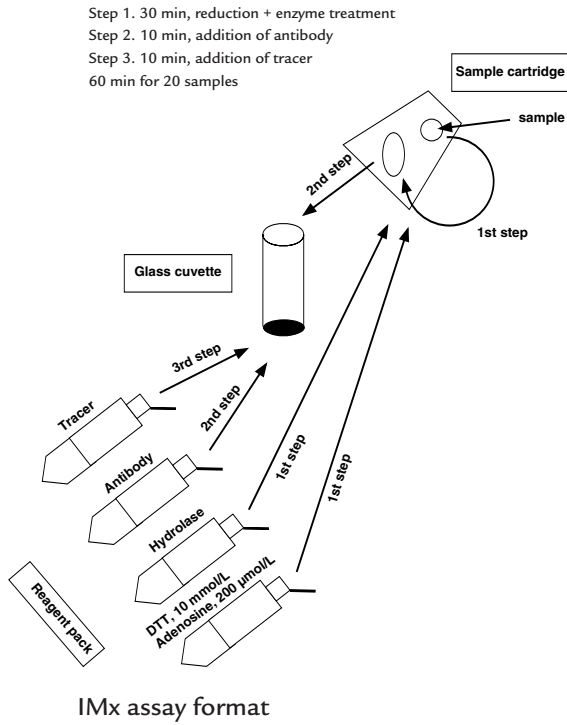


Figure 1. Immunoassay steps performed on an IMx Abbott analyzer (modified from *Clinical Chemistry* 1995; 41:995).



Figure 3. Amino acid analyzer plasma Hcy determination (4) (a: valine, b: homocysteine, c: methionine).

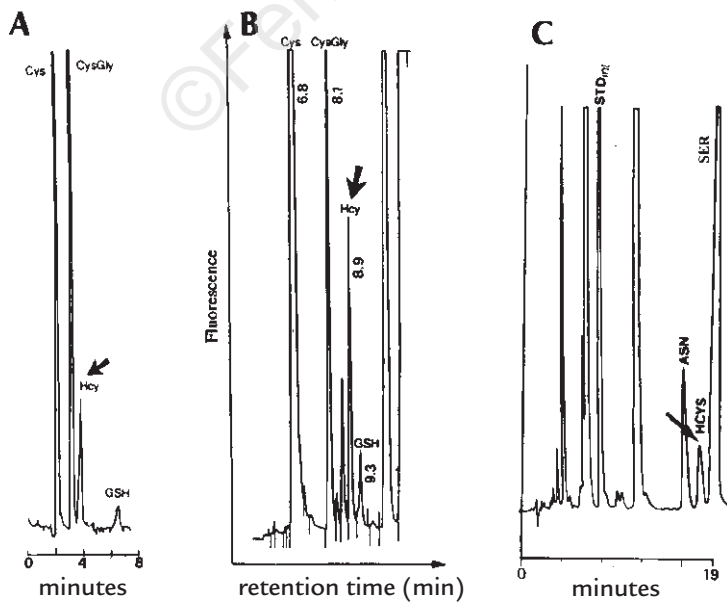


Figure 2. Plasma Hcy determination by HPLC pre-column derivatization methods and fluorescence detection. A) SBD-F (11), B) monobromobimane (7) and C) OPA (8). (Cys: cysteine, CysGly: cysteinylglycine, GSH: glutathione, ASN: asparagine, SER: serine)

high cost of the equipment and a well-experienced staff. The most notable advantages are its high specificity and sensitivity, which also allow Hcy urine analysis, and the codetermination, using the appropriate internal standards, of cysteine, methionine and cystathionine.

HPLC

ABD-F and SBD-F. (4-aminosulfonyl- and ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate) react specifically with the sulfhydryl group of Hcy, developing a fluorescent product. The method using SBD-F was first setup by Araki and Sako¹⁰ and later optimized by Ubbink¹¹ who obtained baseline separation of cysteine, cysteinylglycine, homocysteine and glutathione, within 8 min using a simple isocratic run (Figure 2a).

Long derivatization time (1h at 60°C), the use of tri-*n*-butylphosphine as reductant and possible dissolution of the silica matrix resulting from the low pH of the mobile phase (the use of a guard-column is recommended) represent the main disadvantages of this procedure. The stability of the homocysteine-SBD adduct and the very fine resolution of chromatographic separation without interfering peaks are the most impressive advantages.

Monobromobimane. Like SBD-F, monobromobimane is also a fluorogenic thiol-specific reagent. The derivatization reaction occurs rapidly at room temperature and sodium borohydride is used as reducing agent.⁷ However, this labelling reagent produces fluorescent subproducts during the derivatization reaction which can interfere during HPLC analysis (Figure 2b). To minimize these drawbacks thiol Sepharose treatment and solid-phase extraction are required to pre-purify the samples before HPLC analysis.¹⁴ These clean-up steps to remove fluorescent degradation products are the most remarkable drawbacks, while instantaneous derivatization and automation represent the most attractive features.

OPA. Unlike thiol-specific reagents, OPA reacts with primary amino groups forming fluorescent isoindole derivatives. To prevent the subsequent reoxidation of the sulfhydryl groups of Hcy which are not involved in the derivatization reaction, samples were *s*-carboxymethylated using iodoacetic acid after the deproteinization step.^{8,9} Concerning HPLC analysis, since OPA reacts with all amino acids present in the serum/plasma samples, the resulting chromatogram can be quite complex (Figure 2c). In any case, the Hcy peak is well separated from the close amino acids by using simple isocratic runs. Despite these characteristics, the assay is suitable for automation thanks to the instantaneous derivatization reaction.

Amino acid analyzer. In this analysis the derivatization reaction occurs after the HPLC run and, like OPA, ninhydrin also reacts with amino groups to

produce a blue product that is detected in the visible range. Dithiothreitol and 2-mercaptoethanol are used as reductants. Since Hcy is directly chromatographed, the amino acid is protected against reoxidation by the sulphosalicylic acid used for deproteinization, by the presence of reducing agents in the sample and by the low pH of the mobile phase.⁴

A complex chromatogram (Figure 3) and time consuming analysis (the separation lasts about 26 min plus 16 min for column regeneration) are the drawbacks to this approach. The most attractive features are: complete automation, easy availability since the analyzer is often part of hospital laboratory equipment and, in this case, the staff does not require a training period.

Electrochemical detection. In this approach, after HPLC analysis Hcy is revealed by an electrochemical detector. This detection is based on the oxidation-reduction reactions of the eluted compounds and on the measurement of the electrical stream developed. Homocysteine, thanks to the presence of sulfhydryl groups, appears to be suitable for this kind of investigation. The procedure was optimized by Malinow *et al.*⁵ and Hcy elutes after only 4.3 min (Figure 4). The weakness of this procedure involves the detection system because careful maintenance

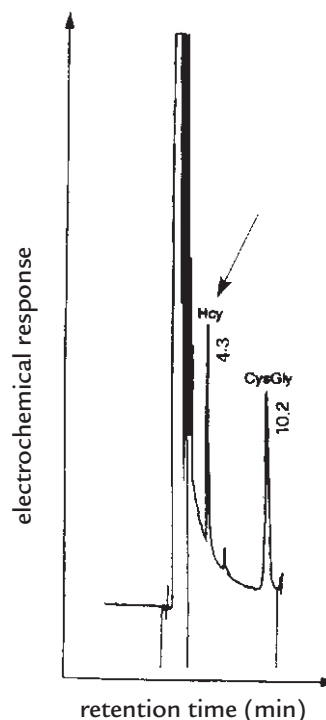


Figure 4: Plasma Hcy determination by HPLC coupled with an electrochemical detector (5) (CysGly: cysteinylglycine).

of this equipment is required to avoid contamination of the flow-cell and deterioration of the gold-mercury electrode. Automation and a very short analysis time, which allows the processing of a considerable number of samples daily, are the major advantages.

Radioenzymic assay. This procedure was set up at first to determine tissue homocysteine¹⁷ and was later applied to biological fluids. For this analysis as well as the Abbott immunoassay biochemical approach, based on the conversion of homocysteine to s-adenosylhomocysteine, represented an attractive alternative. In this assay Hcy was incubated with ¹⁴C-adenosine and the resulting s-adenosylhomocysteine was quantified on HPLC coupled with scintillation counting.¹

Various drawbacks, including several manual steps, a narrow assay range due to the consumption of ¹⁴C-adenosine and the use of radioactive material, have limited its application.

The advantages of this approach are its high sensitivity and a reduction of costs and a simpler procedure when the HPLC system is replaced by less expensive equipment such as thin layer chromatography or paper chromatography.¹⁸

Discussion

The reference value range for tHcy differs from one laboratory to another and this variability may be related to the procedures adopted or differences in sample processing. In any case values between 5 and 15 $\mu\text{mol/L}$ are usually considered normal.¹⁶ In relation to the increasing interest in plasma/serum tHcy determination, several new HPLC methods have been proposed and old techniques have been refined. When homocysteine measurement is introduced in a laboratory the choice of method depends on different parameters such as personnel and instrumental resources available, the training of the technical staff, the number of samples to be analyzed, special interests concerning the codetermination of other metabolites and, obviously, the cost. Immunoassay equipment and amino acid analyzers are often part of the hospital laboratory instrumentation. These procedures are completely automated and the staff is usually well experienced in the use of both.

Concerning pre-column derivatization methods,

these procedures are recommended when, especially in research laboratories, open HPLC instrumentation is already present. This last technique is not expensive in comparison to immunoassay and it can be automated to fit one's own requirements, as described by different authors.

At the moment the HPLC procedures used for this measurement are evolving and improving to yield shorter chromatographic runs, to increase specificity, sensitivity and, due to full automation, to reduce sources of error and manual handling.

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