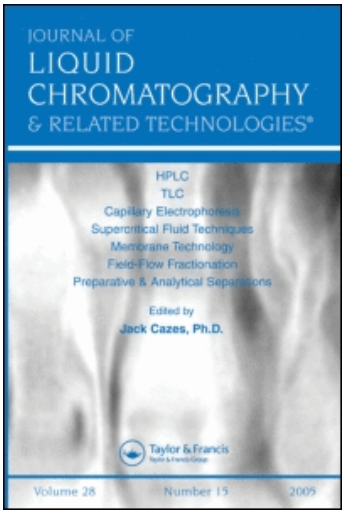

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DETERMINATION OF PLASMA HOMOCYSTEINE BY HPLC WITH FLUORESCENCE DETECTION: A SURVEY OF CURRENT METHODS

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ABSTRACT

Homocysteine is a sulfur-containing amino acid whose utility as a marker in the clinical setting is being extensively explored. Persons with extremely high blood levels of homocysteine, resulting from an enzyme deficiency in the homocysteine trans-sulfuration pathway (homocystinurics) exhibit high mortality rates at an early age due to venous thromboembolism. Moreover, recent research indicates that even moderately elevated plasma homocysteine concentrations may be an independent risk factor for premature vascular disease. In addition to vascular disease risk assessment, the monitoring of plasma homocysteine levels has application in the evaluation of folate and vitamin B-12 status, determination of drug efficacy, along with the estimation of renal status and relative tumor burden. The current method of choice for homocysteine analysis is high performance liquid chromatography (HPLC) using pre-column derivatization prior to reverse phase separation and fluorescence detection. This article discusses a set of current papers that use this particular method for homocysteine analysis in plasma or serum. The difficulties encountered in homocysteine analysis by this technique are discussed, with particular attention paid to sample collection and preparation aspects representing sources of variation within and between methods.

The views expressed in this material are those of the author, and do not reflect the official policy or position of the U.S. Government, the Department of Defense, or the Department of the Air Force.

INTRODUCTION AND BACKGROUND

Homocysteine is a sulfur-containing amino acid sitting at a biochemical crossroads, essential for both the trans-sulfuration and remethylation pathways (1). Figure 1 depicts the

general scheme of homocysteine metabolism, showing its importance in the production of cysteine, and its prominence in the folate and vitamin B-12 dependent production of S-adenosylmethionine, which is needed for numerous methyl transfer reactions. This structurally simple amino acid has generated much interest since its discovery in 1932 (1). Enzyme defects resulting in extremely elevated homocysteine in urine (homocystinuria) were identified in the early 1960's (2). The occurrence of homocystinuria in the homozygous form is rare, with an incidence worldwide of about 1 in 335,000 (3). Homocystinuria is attributed to a deficiency of cystathionine β-synthase (EC 4.2.1.22) (3), the enzyme responsible for the condensation of homocysteine and serine to form cystathionine (Figure 1). Less commonly, defects in 5,10-methylenetetrahydrofolate reductase (E.C. 1.1.1.68) or enzymes essential for methyl-B-12 synthesis may lead to homocystinuria (1) (Figure 1). The homozygous form of this congenital disorder is characterized by symptoms such as premature atherosclerosis, thromboembolism, mental retardation, skeletal abnormalities and optic lens defects (1,3). The resulting mortality rate in these patients is quite high, ranging from 20-75% before age 30 (4). Heterozygous homocysteinemia is a more common form of the disease, with reported frequencies in the general population that range from 1 in 290 to 1 in 70 (1,3). Although serum/plasma levels of homocysteine are much lower in heterozygotes, studies indicate that moderately elevated serum homocysteine levels may be an independent risk factor for vascular disease (5-9). These and other investigations have created a wave of renewed research interest into the potentially toxic effects of elevated levels of homocysteine.

Histopathologic examination of vessels from homocysteinemics reveals arteriosclerotic lesions in most medium and large sized arteries (1,5). More specifically, homocysteinemia is directly associated with plaque formation from increased deposition of collagen, calcium, and proteoglycosaminoglycans, and elastin degeneration within the arterial wall (10). Additionally, homocysteine has been shown to promote the detachment of human arterial endothelial cells in culture, although at higher concentrations than observed *in vivo* (11). Theories have been proposed linking endothelial cell damage to homocysteine, including the production of free radicals (4). However, the exact mechanism by which homocysteine exerts its toxic effects and concrete proof of a link between vascular disease and homocysteine are still being sought (11,12).

Other studies indicate that homocysteine levels are also elevated in subjects with nutritional deficiencies of folate, vitamin B-12, and possibly vitamin B-6 (4,13,14). The use of

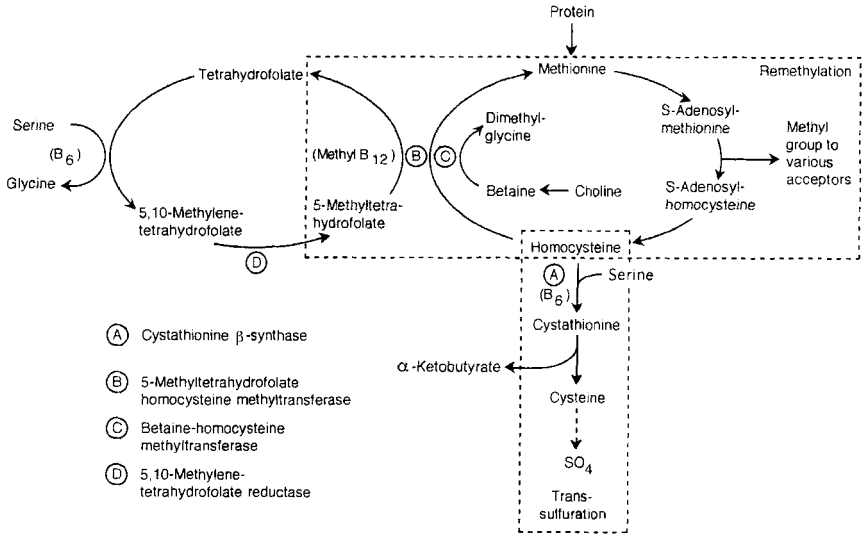

FIGURE 1. Outline of homocysteine biochemistry. Dashed lines outline the remethylation and trans-sulfuration pathways.

serum homocysteine concentrations to assess vitamin B-12 and folate status is increasing in the clinical setting (14). Some investigators recommend monitoring methylmalonic acid as well when evaluating possible vitamin B-12 deficiency. Like homocysteine, methylmalonate is associated with a vitamin B-12 dependent pathway (15). Additionally, increased serum homocysteine levels have been reported in association with zinc deficiency, chronic renal failure, liver disease, cancer and psoriasis (1). Drug therapies with methotrexate, azaribine, penicillamine and anticonvulsants such as carbamazepine likewise appear to alter homocysteine blood levels (1).

The numerous conditions that alter blood homocysteine levels point to the potential usefulness of this amino acid as a clinical marker. Possible applications of homocysteine measurement include not only risk assessment of vascular disease and nutritional status evaluation, but also monitoring drug efficacy, renal status, and relative tumor burden. Conversely, the relative lack of specificity of this test alone could lead to false interpretation of results when a particular application is examined. Therefore, further research into methods for homocysteine analysis and correlation of the resulting data to pathologic conditions is needed.

The majority of published methods dealing with the analysis of homocysteine in serum/plasma employ high performance liquid chromatography (HPLC). Although amino acid analysis by liquid chromatography is commonplace in some settings, homocysteine (and related thiols) pose special challenges.

PRE-ANALYTICAL CONSIDERATIONS

Chemistry of Homocysteine

The analysis of amino acids by HPLC can be quite perplexing, with the chemical nature of homocysteine further complicating the process of method development. Most biologically relevant amino acids, including homocysteine, lack the structural properties necessary for the production of signals compatible with common HPLC detection methods such as UV absorbance and fluorescence. Aside from the aromatic amino acids phenylalanine, tyrosine, and tryptophan, with light absorbance in the 250-280 nm range, most amino acids absorb light at a shorter wavelength (around 190 nm). Detection at this wavelength would be insufficient for clinical purposes due to the numerous compounds with similar absorbance characteristics (16) and the shorter wavelength UV absorbance of many commonly used solvents for HPLC mobile phases. Therefore, the analyst must resort to derivatization for signal enhancement if fluorescence and/or UV/VIS detection methods are employed. The most commonly used homocysteine derivatives will be discussed later in this article. Besides the lack of a suitable chromophore, homocysteine has a reactive thiol group separated from the alpha carbon by an ethylene spacer.

The thiol group of homocysteine is easily oxidized to form disulfide bonds with other sulfhydryls. This results in about 70% of serum/plasma homocysteine being protein bound, most likely to albumin via disulfide linkages. The remaining homocysteine, representing the free fraction, exists primarily as a homocysteine-cysteine mixed disulfide (17). Due to the increase in protein-bound homocysteine at the expense of the free fraction over time upon frozen storage of plasma samples, measurement of total (protein-bound + free) plasma homocysteine is of greatest clinical utility. To determine total homocysteine in serum or plasma, a mild reducing agent is used to break these disulfide bonds and generate free thiols. Although disulfide reduction may seem a solution to this problem, the free thiols may become reoxidized *in vitro* (18). Care must be taken to protect the free thiols and prevent the reformation of disulfide forms of homocysteine.

Thus, the unique chemical nature of biological thiols such as homocysteine, and the added complexity of the serum/plasma matrix in which it is distributed demand that special cautions be taken when collecting samples for analysis.

Collection of Serum/Plasma Samples

An example average value for fasting plasma homocysteine is 8.07 ± 2.41 nmol/mL (18), although reference values for serum/plasma homocysteine tend to vary widely between laboratories, reportedly by more than 100% (1). Real differences in the populations under investigation might partially explain this wide variation, although lack of standardization in sample collection and analytical procedures are also significant contributors (18). For instance, homocysteine concentrations tend to increase in plasma derived from uncentrifuged whole blood samples over time. This increase has been attributed to the continued production and export of homocysteine by erythrocytes (18,19). When collecting plasma samples, the choice of anticoagulant may also influence homocysteine concentrations as well. Whole blood samples left uncentrifuged at ambient temperature for 8 hours showed a 69.7% increase in basal plasma homocysteine when EDTA was chosen as the anticoagulant, compared to 26.7% when sodium fluoride containing tubes were used for venipuncture. Therefore, regardless of the choice of anticoagulant, collected plasma samples should be immediately centrifuged to prevent falsely elevated homocysteine values (18). In studies requiring serum, whole blood samples should be allowed to clot at room temperature no longer than one hour prior to refrigerated centrifugation (20).

Experiments examining the intake of food prior to sampling revealed that baseline homocysteine levels decrease over time after a breakfast meal, with a return to pre-prandial levels after approximately 8 h (18). A possible explanation given for this suprising finding is an increase in homocysteine remethylation following a meal to support methylation reactions essential for intermediary metabolism. As a result, homocysteine export from tissues could be lowered, leading to decreased plasma concentrations (18). Drawing patient samples in a fasting state may therefore serve as another measure of standardization.

HPLC ANALYSIS OF HOMOCYSTEINE

There are several methods for the analysis of homocysteine in serum/plasma. In addition to HPLC methods (e.g. 17,20-28), radioenzymatic (29,30) and gas chromatographic-mass

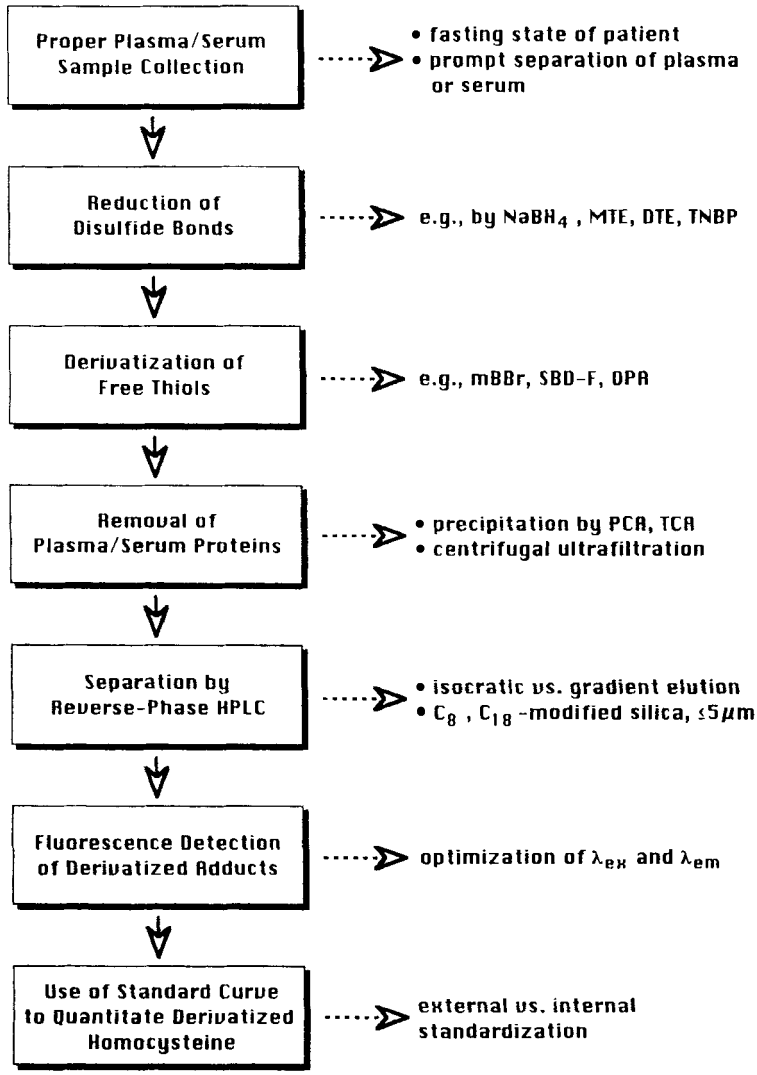

FIGURE 2. Generalized scheme for the determination of total plasma homocysteine by HPLC. TCA, trichloroacetic acid; PCA, perchloric acid; other abbreviations are given in the text.

spectrometric (31) techniques have been developed. A survey of HPLC methods for homocysteine analysis shows three main strategies: 1) pre-column derivatization of homocysteine followed by reverse-phase separation and fluorescence detection, 2) chromatographic separation of free thiols and electrochemical detection, and 3) ion-exchange separation followed by post-column derivatization and fluorescence detection. These strategies are also used for the detection and quantitation of amino acids in general; however, homocysteine analysis usually employs more specific derivatizing reagents and an additional reduction step during sample preparation to generate free thiols. The most widely used method is pre-column derivatization followed by reverse phase separation and fluorescence detection. Figure 2 provides a general outline of this technique from sample collection to quantification. The following discussion will focus on three aspects of the sample preparation and analysis process: 1) reduction of disulfide bonds, 2) derivatization of homocysteine, and 3) external vs. internal standardization.

Generation of Free Thiols

Analysis of total plasma homocysteine requires the reduction of homocysteine disulfides formed through linkages with albumin and other thiols, such as cysteine. Chemical reducing agents commonly used in preparing plasma/serum samples for analysis are sodium borohydride ($NaBH_4$), 2-mercaptoethanol (MTE), dithioerythritol (DTE), and tri-*n*-butylphosphine (TNBP). Each of these reagents has reported advantages and disadvantages. Sodium borohydride has been used successfully for the production of free homocysteine in plasma (17,20,27,28). Frothing and rapid degradation may be a problem with buffered solutions of $NaBH_4$ (20,26), but there is no risk of $NaBH_4$-homocysteine adducts being formed, as when MTE is used as the reducing agent (26,31). Excess $NaBH_4$ is destroyed upon the addition of acid, such as perchlorate, which also serves to precipitate serum proteins (20,26). Refsum *et al* report $NaBH_4$ solutions stable for several days when stored in 0.1 M NaOH mixed 2:1 with DMSO for use in an fully automated assay for total homocysteine (17,28).

As mentioned, MTE has been shown to initially rapidly reduce disulfides, only to later react with free thiols, forming MTE-homocysteine mixed disulfides (26,31). Since the rate of formation of the MTE-homocysteine product was found to be slow, with > 90% of total homocysteine remaining as free thiols after 1 h, the use of MTE as a reducing agent was deemed

quite suitable for rapid sample preparation procedures (26). Like MTE, DTE is an excellent reagent for the rapid reduction of disulfides to free thiols (29). DTE performed well as a reducing agent prior to an ion-exchange separation and post-column derivatization of homocysteine after proper concentration adjustment to prevent reoxidation during chromatography (22). Another group reported the presence of fluorescent contaminants after sample disulfide reduction with DTE. The resulting peaks represented a possible source of interference during HPLC analysis, possessing retention times similar to homocysteine (26). TNBP is also useful in the production of free homocysteine for derivatization. TNBP is usually used for disulfide reduction as a 10% solution in dimethylformamide, with no reported problems for plasma/serum homocysteine analysis (24,25).

As an alternative to chemical reduction methods, Rabenstein and Yamashita reported the HPLC determination of homocysteine in human plasma and urine using electrochemical detection (23). Dual Hg/Au amalgam electrodes were employed in this procedure; the upstream electrode reduced the disulfides to thiols that were detected by a downstream electrode (23). Aside from this dual electrode electrochemical detection approach, other HPLC methods require derivatization and/or blocking of the free sulfhydryls following disulfide reduction. The sequence of sample preparation steps can vary, with protein precipitation occuring before or after derivatization.

Derivatization of Homocysteine

Numerous reagents are available for the derivatization and subsequent HPLC analysis of amino acids. Three reagents commonly used for homocysteine determination are monobrombimane (mBBr), ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), and *o*-phthaldialdehyde (OPA). These reagents and their generalized reactions with thiols are shown in Figure 3. Both mBBr and SBD-F are considered thiol-specific fluorogenic probes. The derivatization of thiols with mBBr and SBD-F represent classical aliphatic and aromatic nucleophilic substitution reactions. The resulting mB-homocysteine and SBD-homocysteine adducts are highly fluorescent, allowing detection in the picomolar range (20,25). Monobromobimane possesses some background fluorescence and interference is possible from mBBr fluorescent degradation products (17,20,28,33,34).

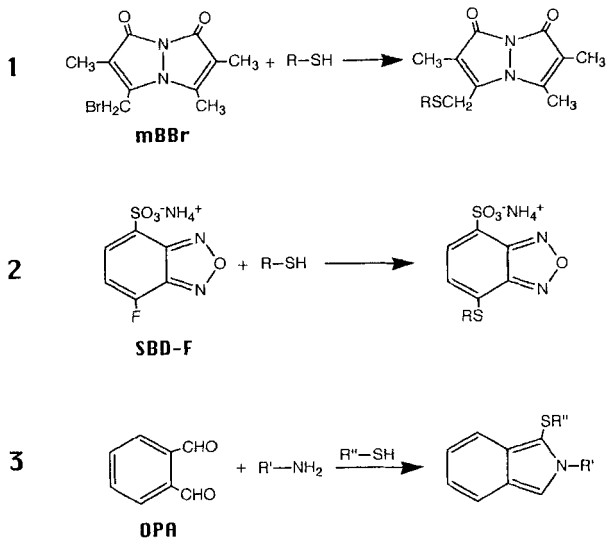

FIGURE 3. Reactions of commonly encountered fluorescent derivatizing reagents for homocysteine analysis. mBBr, monobromobimane; SBD-F, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate; OPA, *o*-phthaldialdehyde; R = $-CH_2CH_2CH(NH_2)COOH$; R' = $-CH(COOH)CH_2CH_2SCOCH_3$; R" = $-CH_2CH_2OH$.

Low pH buffers and organic modifiers such as acetonitrile and methanol are commonly used for the elution of mBBr and SBD-F derivatized thiols. Columns of varying length (125 -250 mm) and standard bore (4 - 4.6 mm) are satisfactory for separation of the fluorescent adducts. The stationary phase is normally a standard brand of C_{18} modified silica, 5 μm average particle size. Isocratic elution is often sufficient for SBD-homocysteine adducts, while gradient conditions are normally employed for the separtion of homocysteine-mB from other thiols (17,20,28). We have found isocratic elution with 0.025% acetic acid and acetonitrile adequate for the separation of mBBr derivatized thiols when used in conjunction with a final gradient to elute excess mBBr (35). To minimize possible interference from excess mBBr, one method utilized Sepharose-SH beads and solid phase extraction columns to remove excess mBBr and partially purify thiol-mB conjugates before chromatography (20). Stock solutions of mBBr and SBD-F can be prepared in acetonitrile and borate buffer, respectively (20,24,25). Fluorescence signals obtained with a particular detector and mobile phase should be optimized by running excitation and emission

scans to determine the proper λ_{ex} and λ_{em} values. At pH 8.0, the fluorescent thioethers of mBBr have λ_{ex} 375 nm, and λ_{em} 450 nm, while for SBD-thiol conjugates in alkaline media the values are λ_{ex} 380 nm and λ_{em} 515 nm (32).

Like mBBr and SBD-F, OPA forms highly fluorescent adducts with amino acids. Unlike the thiol-specific reagents, OPA reacts with primary amino groups forming fluorescent isoindole derivatives (Figure 3). This cyclization reaction requires a thiol such as MTE for completion. It is evident from the OPA reaction that problems may arise when thiol-containing amino acids (i.e. homocysteine) are the subject of analysis. Due to the non-specific derivatization of all primary amino acids by OPA, the resulting chromatograms may be quite complex, requiring careful refinement of gradient conditions to achieve adequate separation. Secondly, free thiols liberated by disulfide reduction could participate in the OPA cyclization, yielding unwanted products. To prevent these possible side reactions, the thiol groups must be protected prior to derivatization. Iodoacetic acid was successfully used to block homocysteine thiols by acetylation subsequent to OPA derivatization (26,27). This blocking of the sulfhydryl group of homocysteine by S-carboxymethylation results in a stable isoindole and reproducible results (27). Another possible drawback to the use of OPA in homocysteine analysis is reagent instability due to oxidation of the thiol catalyst (e.g. MTE) needed to drive the reaction to completion (36).

The conditions required for the mBBr and OPA derivatization reactions make both compounds suitable to automated analysis. With both reagents, product formation is complete in under 10 min at room temperature (17,26-28). Conversely, the excellent adduct stability and increased specificity for thiols characteristic of SBD-F are countered by the elevated temperature and longer time needed for derivatization (60 min at 60 °C) (21,24,25).

External vs. Internal Standardization

Sample preparation procedures for the analysis of homocysteine in plasma/serum can be quite involved. Although some laboratories prepare calibration curves for HPLC quantitation using homocysteine peak areas alone as the ordinate values, others use the internal standard method in an attempt to reduce intra- and inter-assay variation. Two related groups prepared *calibration curves* from true external standards by adding known amounts of homocysteine to water (17,28). These fully automated methods utilized mBBr as the derivatizing agent and were

very precise, with between-run and within-run CV's of 3.0% or less (17,28). Jacobsen, *et al* spiked "blank" plasma (or serum) from a collected pool with increasing concentrations of homocysteine. (20). These enriched samples were then manually processed along with sample "blanks" and unknowns by $NaBH_4$ reduction and mBBr derivatization. The homocysteine concentration of the pool plasma was determined and subtracted from the enriched samples to prepare the standard curve. Intra- and inter-assay CV's of < 5% were calculated for this method (20). A procedure using TNBP reduction and SBD-F derivatization of spiked plasma standards and samples yielded good, although somewhat higher, within-run and between-run CV's for medium and high control levels (within-run = 4.5% and 3.9%; between run = 6.6% and 5.9%) (24).

The use of an internal standard in HPLC has distinct advantages, such as minimizing the contributions of sample preparation and injection variations to the final result, hopefully improving precision. When choosing an internal standard it is desirable for the candidate compound to possess chemical and physical properties similar to the analyte in question. The internal standard should be carried through any extractions, derivatizations, etc. in a fashion similar to the compound of interest, such as homocysteine. Chromatographically, the internal standard should elute near, but not interfere with the homocysteine peak. Several thiols have been used as internal standards in homocysteine analysis. Toyo-Oka and coworkers (20) used N-acetylcysteine, although thiols in rat tissues such as kidney, liver, and spleen were the subject of analysis, rather than plasma/serum thiols. SBD adducts of homocysteine and N-acetylcysteine eluted approximately 8.5 min apart using a 0.15 M phosphoric acid:acetonitrile gradient (21). Structurally, N-acetylcysteine has one less methylene group than homocysteine, with the primary amine in an amide bond, thus reducing the basicity of the compound. Mercaptopropionylglycine was chosen by Vester and Rasmussen as an internal standard (25). The retention times of SBD-homocysteine and SBD-mercapto-propionylglycine were 3.36 and 15.68, respectively. Although the retention time was quite high for the internal standard, within-run and between run CV's of 2.3% and 3.0% were reported. Besides serving as a comparison for homocysteine peak size, this internal standard was useful as a derivatization control. Upon deletion of mercaptopropionylglycine from the assay, the method imprecision nearly doubled (25). Using pre-column derivatization with OPA, Hyland and Bottiglieri (26) and Fermo, *et al* (27) utilized

homocysteic acid as an internal standard. The OPA-homocysteic acid adduct in these assays eluted approximately 5 min prior (26) and 12 min prior (27) to OPA-homocysteine during 10 min and 24 min runs. Precision between these two methods varied greatly. Intra- and inter-assay CV's were 6.07% and 8.97% (26), in comparison with 1.8% and 5.0% (27). A notable difference between these procedures was the method of protein removal. While Fermo, *et al* used standard acid precipitation by the addition of 6% perchloric acid (27), Hyland and Bottiglieri performed centrifugal ultrafiltration using a 10,000 molecular weight cut-off filter (26).

It is obvious that internal standardization does not necessarily result in greater precision of homocysteine measurement. The main objections to the use of an internal standard are the different reactivities of homocysteine and the internal standard during derivatization and longer analysis times due to late elution of derivatized standard.

The source of homocysteine for spiking standards used in preparing calibration plots can also vary. For instance, Jacobsen, *et al* (20) performed a base hydrolysis of homocysteine thiolactone to yield L-homocysteine. The amount of free thiol in solution was then determined by titration with Ellman's reagent prior to preparation of the standards (20). Other sources include D,L-homocysteine (24-27), and reduction of homocystine, the disulfide dimer of homocysteine (17,28). Agreeing upon the form, source, and preparation of homocysteine standard solutions may aid in producing more reproducible quality results.

Concluding Comments

While the clinical utility of serum/plasma homocysteine levels for some purposes is still being scrutinized, homocysteine analysis has now been added to the lists of in-house laboratory tests at many large medical centers. Concurrently, the HPLC procedures used for its measurement continue to evolve and improve, yielding shorter chromatographic runs, increased specificity and sensitivity, along with reduced technician time and error due to automation. The interested analyst is encouraged to perform a thorough search of the recent literature when considering homocysteine analysis to arrive at a method matched to a laboratory's needs, sample volume, and budget. Regardless of the method chosen, awareness of potential problems and alternative techniques in sample collection and preparation, chromatographic separation, and calibration may save time and money when complications arise.

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