CLIN. CHEM. 39/9, 1764–1779 (1993)

Total Homocysteine in Plasma or Serum: Methods and Clinical Applications

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Total homocysteine is defined as the sum of all homocysteine species in plasma/serum, including free and protein-bound forms. In the present review, we compare and evaluate several techniques for the determination of total homocysteine. Because these assays include the conversion of all forms into a single species by reduction, the redistribution between free and protein-bound homocysteine through disulfide interchange does not affect the results, and total homocysteine can be measured in stored samples. Total homocysteine in whole blood increases at room temperature because of a continuous production and release of homocysteine from blood cells, but artificial increase is low if the blood sample is centrifuged within 1 h of collection or placed on ice. Different methods correlate well, and values between 5 and 15 μmol/L in fasting subjects are considered normal. Total homocysteine in serum/plasma is increased markedly in patients with cobalamin or folate deficiency, and decreases only when they are treated with the deficient vitamin. Total homocysteine is therefore of value for the diagnosis and follow-up of these deficiency states and may compensate for weaknesses of the traditional laboratory tests. In addition, total homocysteine is an independent risk factor for premature cardiovascular diseases. These disorders justify introduction of the total homocysteine assay in the routine clinical chemistry laboratory.

Indexing Terms: *cobalamin, folate deficiencies · sample handling · reference values · thiol compounds · heart disease · enzyme metabolism · amino acids · nutritional status*

Homocysteine (Hcy) determination was introduced into laboratory diagnosis in 1962 when the first patients with the inborn error homocystinuria were described (*1*, *2*).[6] These patients excrete large amounts of homocystine into the urine, and the blood concentrations become extremely high (*3*). The high concentrations could be determined by simple chemical tests (*4*) or by amino acid analysis.

Hcy in the acid-soluble fraction of plasma/serum (free Hcy) was detected in healthy subjects with the second-generation amino acid analyzers that became available in the middle 1970s (*5*, *6*). The first clinical studies on the relation between moderately increased plasma Hcy and increased risk for cardiovascular disease, published in the late 1970s and early 1980s, were based on this methodology (*7–9*).

Progress in Hcy research during the last 7 years has been greatly facilitated by the introduction of improved techniques for measuring Hcy in plasma and serum (*10–19*). These methods measure total Hcy, which is the sum of protein-bound and free Hcy. The main advantage is that stored samples can be analyzed because total Hcy is not altered when samples are kept frozen, even for years (B. Israelsson et al., 1992, unpublished). Total Hcy is measured in all studies of Hcy as a marker of vitamin deficiency states (*20*, *21*) and in most studies of Hcy and cardiovascular disease (*22*).

To date, ~20 clinical studies involving >1800 patients and an equal number of control subjects have demonstrated that a moderate increase of serum/plasma Hcy is an independent risk factor for premature cardiovascular disease. Above-normal plasma Hcy has been found in ~30% of the patients with premature cardiovascular disease who lack the traditional risk factors (*22–24*).

Since 1985, the value of plasma/serum Hcy determination in the diagnosis and follow-up of folate or cobalamin deficiencies has been established. These deficiency states are probably the most frequently encountered causes of marked increases of serum/plasma Hcy (*25–28*).

Here we review the methodologies for measuring total Hcy in plasma/serum and their feasibility as routine methods in the clinical chemistry laboratory. In the last part of the article, we evaluate total Hcy as a marker of human disease, with emphasis on folate and cobalamin deficiencies.

Biochemistry

Intracellular formation, metabolism, and release of Hcy into the extracellular compartment determine the concentration of Hcy in extracellular media (e.g., plasma/serum), which in turn is the basis for measuring

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[6] Nonstandard abbreviations: ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate; GC-MS, gas chromatography–mass spectrometry; Hcy, homocysteine; mBrB, monobromobimane; and SBD-F, ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate.

Received December 14, 1992; accepted March 24, 1993.

plasma/serum Hcy as an extracellular marker for human disease. In discussing these processes we will emphasize those that may affect the concentrations of extracellular Hcy.

Enzymes involved. Hcy is formed as a product of the adenosylhomocysteinase (*S*-adenosylhomocysteine hydrolase; EC 3.3.1.1) reaction, which is responsible for the removal of *S*-adenosylhomocysteine, a product of *S*-adenosylmethionine-dependent transmethylation (*29*).

Intracellular Hcy is either remethylated to methionine, converted to cystathionine, or exported from the cells. The first reaction is catalyzed by the enzyme 5-methyltetrahydrofolate–Hcy methyltransferase (methionine synthase; EC 2.1.1.13). This enzyme is ubiquitously distributed in mammalian cells. It requires cobalamin as cofactor and catalyzes a reaction in which Hcy remethylation is coupled to the conversion of 5-methyltetrahydrofolate to tetrahydrofolate; it thereby operates at a point of convergence of folate metabolism and the transmethylation/transsulfuration pathway. An alternative route of Hcy remethylation is confined to the liver: In this reaction, catalyzed by betaine–Hcy methyltransferase (EC 2.1.1.5), betaine serves as methyl donor (*30, 31*).

The vitamin B_6-dependent enzyme cystathionine β-synthase (EC 4.2.1.22) catalyzes the condensation of Hcy with serine to form cystathionine. The reaction is irreversible under physiological conditions, and from this point on Hcy is committed to the transsulfuration pathway. Cystathionine is further cleaved to cysteine and α-ketobutyrate, catalyzed by another vitamin B_6-dependent enzyme (γ-cystathionase; EC 4.4.1.1); this reaction completes the transsulfuration pathway (*31*).

Enzyme regulation and Hcy distribution between pathways. Hcy is metabolized by either catabolizing enzymes or methionine-conserving enzymes; the distribution between these competing pathways is determined by the K_m for Hcy, the regulatory effect of *S*-adenosylmethionine, and the enzyme concentrations. The differential K_m and metabolite regulation are processes that are put into immediate action in response to variable methionine availability, whereas up-regulation of enzyme synthesis is a slow adaptive process (*31*).

Cystathionine β-synthase and γ-cystathionase are Hcy-catabolizing enzymes, for which K_m values are >1 mmol/L. Cystathionine β-synthase is activated by *S*-adenosylmethionine, and the concentrations of both enzymes increase in response to excess dietary methionine. These properties ensure both immediate and long-term drainage of excess Hcy via the transsulfuration pathway (*31*).

The Hcy-remethylating enzymes, 5-methyltetrahydrofolate–Hcy methyltransferase and betaine–Hcy methyltransferase, have low K_m values for Hcy (<0.1 mmol/L). The activity is (directly or indirectly) inhibited by *S*-adenosylmethionine, and increased dietary methionine decreases the activity of the former enzyme. These properties favor methionine conservation at low Hcy concentrations (*31*).

Hcy export. The release of Hcy into the extracellular medium represents the third route of cellular Hcy disposal and is particularly important in relation to the plasma concentration of Hcy. Studies with isolated cells show that Hcy export into the extracellular medium reflects an imbalance between Hcy production and metabolism (*32, 33*). When cells are cultured in the presence of excess methionine, Hcy export from most cell lines is enhanced (*33*); this phenomenon resembles the response to the methionine loading test. Pharmacological (*34*) and cell genetic studies (B. Christensen et al., unpublished) show that the activity of methionine synthase is critical for Hcy export at low methionine concentrations, whereas cystathionine β-synthase activity influences the export at high methionine concentrations. The clinical corollary is that methionine synthase activity determines the concentration of fasting plasma Hcy, whereas a defect in cystathionine β-synthase results in an abnormal response to methionine loading. This model is supported by clinical data showing that fasting plasma Hcy is markedly increased in patients with folate (*25, 26, 35*) or cobalamin deficiency (*21, 25, 35*) but is usually normal in vitamin B_6 deficiency (*36*). Cobalamin-deficient patients and one patient with methylenetetrahydrofolate reductase deficiency had normal increases in plasma Hcy after methionine loading (*37*), whereas patients who were homozygotes (*3, 38*) or obligate heterozygotes (*39, 40*) for cystathionine β-synthase deficiency showed an abnormal response.

Different Forms of Hcy in Human Plasma: Implications for Hcy Determination

In the first studies of plasma Hcy in healthy subjects (*5, 41*) and in some early clinical studies (*20*), Hcy was measured in the acid-soluble fraction of plasma as Hcy–cysteine mixed disulfide. However, a major fraction of Hcy in serum/plasma is associated with plasma protein(s) (*42*), and probably forms a protein–Hcy mixed disulfide with albumin (*10*). This fraction represents ~70% of total Hcy in human plasma/serum from healthy subjects (*10*). Only trace amounts of reduced Hcy (*11, 43, 44*) and the disulfide homocystine (*45*) have been demonstrated. The sum of all Hcy species in plasma (free plus protein-bound) is referred to as total Hcy.

The protein binding of Hcy in plasma has some unique features. Both experimental (*46, 47*) and clinical studies (*48*) demonstrate the presence in plasma of binding sites for aminothiols, which interact preferentially with Hcy. In contrast to cysteine, binding of Hcy to plasma proteins seems to be saturable, with a maximal capacity of ~140 µmol/L (*48*). This implies that the free fraction of Hcy increases more than protein-bound Hcy does when total Hcy is markedly increased. During the brief hyperhomocysteinemia induced by methionine loading, only a moderate, transient increase in the free/bound ratio occurs. This indicates that a rapid equilibrium exists between free and protein-bound fractions in vivo (*20*).

The association of Hcy with plasma proteins has important implications for Hcy measurements. Free Hcy becomes progressively bound to plasma protein(s) ex vivo. Such redistribution takes place within 24 h at room temperature. Also, most Hcy in stored plasma is

protein-bound. Reliable measurement of free Hcy requires immediate acid treatment and centrifugation of plasma (*49*). This is impractical in the clinical setting, and determination of free Hcy has largely been abandoned.

General Considerations of Methodology

Notes on chemicals. Some batches of DL-Hcy contain large amounts (about 30%) of impurities, whereas L-homocystine from several suppliers seems to be pure. *S*-Adenosylhomocysteine hydrolase from rabbit erythrocytes used for the radioenzymic assays is occasionally available from Sigma Chemical Co. (St. Louis, MO) or can be prepared from liver of the mouse (*50*) or rat. Any adenosine deaminase present in the enzyme preparation is inhibited by 2′-deoxycoformycin (Pentostatin; Parke-Davis Research Labs., Ann Arbor, MI). Deuterated Hcy used as internal standard in the gas chromatography–mass spectrometry (GC-MS) assay (*12*) is available from C\D\N Isotopes (Vaùdrevìl, Quebec, Canada), as are stable isotope-labeled forms of other metabolites that can be assayed with this technique. Monobromobimane (mBrB) contains fluorescent impurities, the amount of which varies from one batch to another. This reagent is occasionally available from Calbiochem–Behring Diagnostics (La Jolla, CA), but can regularly be supplied by Molecular Probes (Eugene, OR); the product of Molecular Probes is also of relatively high purity. 4-(Aminosulfonyl)- and ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (ABD-F and SBD-F, respectively) are products of Wako (German Branch, Neuss, Germany).

Reduction. Determination of total Hcy in plasma/serum requires the reduction of the disulfide bond between Hcy and other thiols or albumin. Reductants must be prepared immediately before use, and attention should be paid to stability during automated unattended sample processing. The selection of reductant depends on the separation and detection system used. Sulfhydryl-containing reducing agents such as dithioerythritol, dithiothreitol, and mercaptoethanol liberate Hcy from various disulfides. These reductants form adducts with thiol-specific reagents and therefore may consume derivatization reagent (*51*). Sodium or potassium borohydride is a potent reductant. The reduction takes a few minutes at high concentrations (1.4 mol/L) (*14*), but requires up to 30 min (*15*) or heating (*52*) at lower concentrations (40–100 mmol/L). Formation of gas and foaming during the reaction may impose practical problems, especially with regard to automatization, but this can be overcome by adding a surface-active agent, e.g., octanol. Tri-*n*-butylphosphine does not react with thiol-specific reagents nor does it form gas during reaction; however, it consumes fluorogenic reagent (mBrB) both at 50 °C and at room temperature (*53*) and is an irritant with an unpleasant odor.

Reoxidation. Hcy formed during the reduction step can be reoxidized before derivatization or detection, and variable reoxidation is a source of erratic results. Thiol oxidation is catalyzed by many transition metals (*54*); inclusion of EDTA in the reaction mixture chelates divalent metals and inhibits oxidation. Hcy is also kept in its reduced state by the presence of other thiols or by resupplementation of reductant (*43*). In the GC-MS assay, inclusion of deuterium-labeled Hcy as the internal standard corrects for reoxidation (*12*). Using a different sulfur compound as the internal standard does not seem feasible because the rate of oxidation of the internal standard may differ from that of Hcy (*54*).

Derivatization and detection. All methods except those based on electrochemical detection depend on derivatization of Hcy. Precolumn derivatization with fluorogenic reagents for thiols followed by HPLC has become increasingly popular (*55*). Useful reagents must form Hcy adducts with sufficient fluorescent yield to measure Hcy at picomolar concentrations or less. Furthermore, the ideal reagent should be nonfluorescent, contain no fluorescence impurities, and react rapidly and specifically with Hcy and other thiols to form stable products. No reagent meets all these requirements, but among the fluorogenic reagents available, bimanes and halogenosulfonylbenzofurazans have been found practical (Figure 1).

mBrB couples rapidly with thiols at pH 8.0 at room temperature to produce a highly fluorescent thioether (*56*). One drawback is that the reagent itself, the hydrolysis products, and the impurities are fluorescent (*51*). These materials give rise to several reagent peaks that may interfere with Hcy determination (*14*). Removal of excess reagent is included in some methods (*15, 56*).

SBD-F and ABD-F are halogenosulfonylbenzofurazans that have been used for determination of thiols, including Hcy (*57*). ABD-F reacts quantitatively with thiols, including Hcy, at 50 °C at pH 8.0–9.5 for 5–10 min (*58*), whereas the less-reactive SBD-F requires more drastic conditions (pH 9.5 and 60 °C for 1 h). SBD-F is not reactive towards

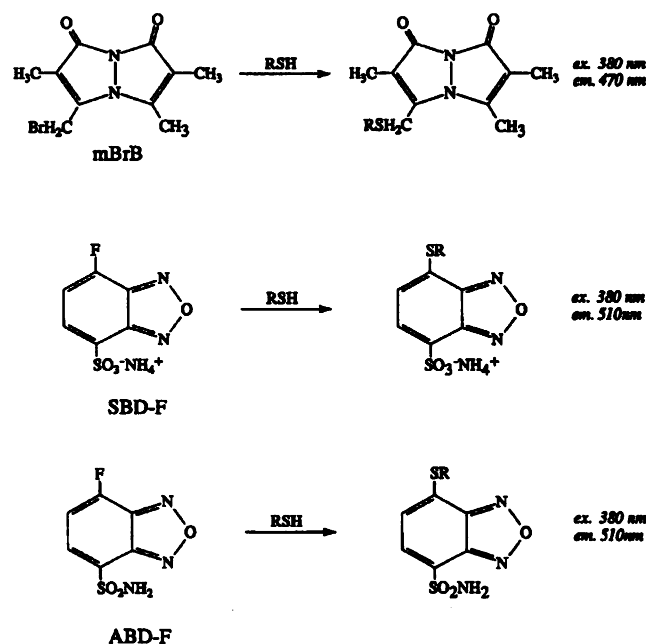

Fig. 1. Fluorogenic thiol(RSH)-specific reagents for precolumn derivatization of Hcy

The excitation and emission wavelengths for the derivatives are listed

amines (*59*), whereas ABD-F also derivatizes amines but with a low fluorescence yield (*57*).

The low reactivity of SBD-F may cause some problems because of Hcy reoxidation, and assays based on this agent may be difficult to automatize. However, unreacted benzofurazans are not fluorescent, the thiol adducts are stable, and no fluorescent hydrolysis products are formed. Their use results in clean chromatograms with no reagent peaks.

The formation of *tert*-butyldimethylsilyl derivatives for GC-MS (*12*) is not affected by traces of moisture, and these derivatives are stable at room temperature for many weeks.

Automatization and sample output. A high degree of automatization is advantageous, and high sample output is required for methods used in routine laboratory diagnosis.

Most of the present methods for total Hcy determination involve manual processing of the samples before chromatography. A fully automated procedure carried out in a sequential manner implies no centrifugation or filtration of sample, and the reduction and derivatization of Hcy must be rapid. The combined use of $NaBH_4$ and mBrB fulfills these criteria and has been successfully implemented in a fully automated procedure (*14*), as will be described below.

Development of an automated procedure based on derivatization with a halogenosulfonylbenzofurazan is possible and should be undertaken. The long reaction time and heating required because of the low reactivity of SBD-F may cause problems, whereas ABD-F may be a suitable reagent. Assay procedures based on electrochemical detection can also be fully automated as long as attention is paid to detector stability and maintenance.

Sample output depends on sample processing and chromatographic retention time. Manual processing of a large number of samples followed by a rapid chromatographic step gives a high sample output, as demonstrated for GC-MS (*12*) and for assays based on electrochemical detection (*13*) or fluorescence detection with use of SBD-F (*18*). The long analysis time of conventional amino acid analyzers, including analyzers optimized to detect Hcy (*16*), restricts sample output.

Codetermination of other metabolites. Some assays measure metabolites in addition to Hcy; this may provide useful data for interpretation of the results. Methylmalonic acid and 2-methylcitric acid in serum are specific markers for cobalamin deficiency and may serve to differentiate hyperhomocysteinemia caused by cobalamin deficiency from that due to other causes (*21, 60*). The concentration of cysteine in serum shows a complex relation to Hcy, both in homocystinurics (*48, 61, 62*) and healthy persons (*44, 63*); in one recent study, the ratio between Hcy and cysteine correlated better with serum folate than did plasma Hcy itself (*64*). Because methionine intake causes an increase in methionine concentration in blood, which is followed by a transient hyperhomocysteinemia, knowledge of the methionine concentration may be particularly useful when evaluating the results of a methionine loading test (*20*). Simultaneous determination of cystathionine, betaine, and *N,N*-dimethylglycine may provide additional information on metabolic alterations caused by folate and cobalamin deficiency and those caused by inborn errors of metabolism (*65, 66*). Measurement of *N*-methylglycine may provide a specific marker for folate deficiency (*66*).

Methods

Radioenzymic Assays

Total Hcy in human plasma was first determined with a radioenzymatic assay based on the conversion of Hcy to *S*-adenosylhomocysteine in the presence of [^{14}C]adenosine and *S*-adenosylhomocysteine hydrolase. Dithioerythritol was used as reductant, and radioactive *S*-adenosylhomocysteine was quantified by HPLC and scintillation counting (*10*). This assay can also be carried out with unlabeled adenosine and ultraviolet detection at 254 nm.

In two modified assays, *S*-adenosylhomocysteine is isolated and quantified by thin-layer chromatography (*67*) or paper chromatography (*17*) instead of HPLC.

The radioenzymic techniques are sensitive and specific and can also measure Hcy in urine. The modifications based on thin-layer chromatography or paper chromatography require inexpensive equipment and can be established in any laboratory regardless of resources available. The disadvantages are that tedious manual processes, e.g., enzyme incubation, protein precipitation, neutralization, and centrifugation, are required. Another drawback is the limited range of this assay, owing to the consumption of radioactive adenosine present in the assay mixture.

Gas Chromatography–Mass Spectrometry

The GC-MS method for total Hcy developed by Stabler et al. (*12*) has been modified recently (*65*) and is much simpler than the initial version. The steps before GC-MS are addition of deuterated internal standards in a single pipetting, addition of the reductant dithiothreitol and NaOH in a second pipetting, heating at 40 °C for 30 min, fractionation of sample on a disposable anion-exchange column, drying, and derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide. The *tert*-butyldimethylsilyl derivatives are separated and quantified by capillary GC-MS in the selected-ion monitoring mode, with use of a benchtop GC-MS equipped with an autosampler. The variable recovery through these steps and the reoxidation of Hcy during sample processing are corrected for by including deuterated Hcy as internal standard, which results in a highly precise assay. Using semiautomated pipetting equipment, a single technician can process 320 samples in 8 h. The capacity of the automated chromatograph is about 160 derivatized samples per 24 h. Figure 2 shows a selected-ion monitoring trace of Hcy and the internal standard in serum.

The lack of total automation and the relatively high cost of the equipment are the major disadvantages of

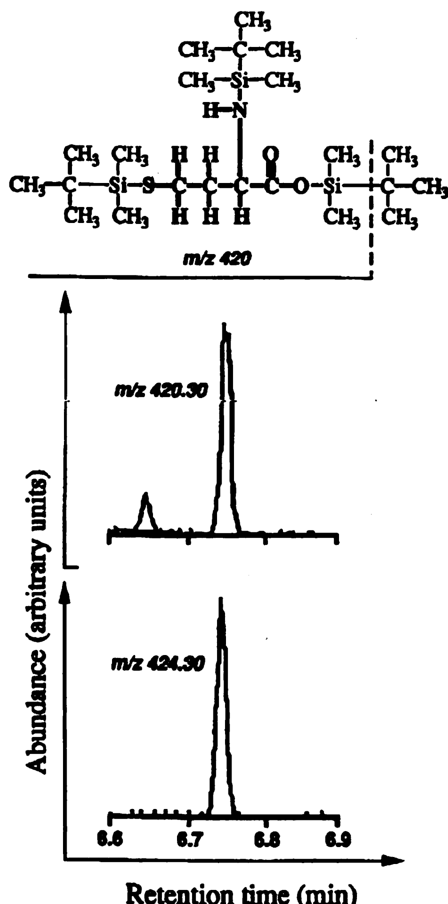

Fig. 2. Determination of Hcy in human serum by GC-MS

The structure of the *tert*-butyldimethylsilyl derivative of Hcy and the site of major fragmentation during mass spectroscopy are shown. The Hcy part of the formula is shown in **bold** type. The *upper trace* is a selected-ion monitoring recording for the ion *m/z* 420.3, which represents endogenous Hcy in serum (6.3 μmol/L). The *lower trace* is a selected-ion monitoring recording for the ion *m/z* 424.3, representing the internal standard, which contains four deuterium atoms. Details are given in references *12* and *65*

this method. Attractive features are high sensitivity and specificity, codetermination of cysteine, methionine, cystathionine, *N,N*-dimethylglycine, and *N*-methylglycine (*65, 66*), and the fact that the assay has been verified for urine samples (*12*). With a slight modification, methylmalonic acid (*60*), 2-methylcitric acid (*60*), and betaine (*66*) in serum can also be determined.

Precolumn Derivatization, HPLC, and Fluorescence Detection

In the method of Araki and Sako (*11*), Hcy is derivatized with SBD-F, and the Hcy, cysteine, and cysteinylglycine adducts are separated and quantified by gradient elution of a reversed-phase column within 12 min. This method has been improved by Ubbink et al. (*18*), who obtained baseline separation of these adducts within 6 min by isocratic elution on a reversed-phase column at pH 2.1. Their system ensures high sample output, but the low pH of the mobile phase increases dissolution of the silica matrix. A silica saturation column mounted in front of the injector or a guard column may reduce this process, and is recommended. Another modification has been constructed by Vester and Rasmussen (*19*): They eluted the SBD-F adducts with a methanol gradient and obtained high precision of the assay by including mercaptopropionylglycine as internal standard, which probably corrects for inaccurate composition of the assay mixture or variable injection volume. However, this compound elutes after 15 min, and significantly prolongs the run time.

The SBD-F-based methods are sensitive and specific, and there are no interfering reagent peaks in the chromatogram (Figure 3). Disadvantages include the low reactivity of SBD-F and the long reaction time and high temperature (60 °C) required. The derivatization is carried out manually, but the stability of the adducts allows autoinjection.

mBrB has been used for determining Hcy in plasma (*14, 15*). $NaBH_4$ is used as a reductant, and a high concentration is required to obtain maximal fluorescent yield (*53*). The presence of dithioerythritol during the reduction and derivatization increases the linearity of the assay in the lower range, probably by preventing reoxidation (*14*).

Excess fluorescent mBrB has been removed by treating the sample with thiol Sepharose (*15, 56*). Sufficient resolution of the Hcy adduct is also obtained after sample clean-up by solid-phase extraction (*15*) or column switching (*14*). However, thiol Sepharose treatment and solid-phase extraction are tedious procedures. Column switching requires two solvent-delivery systems, and

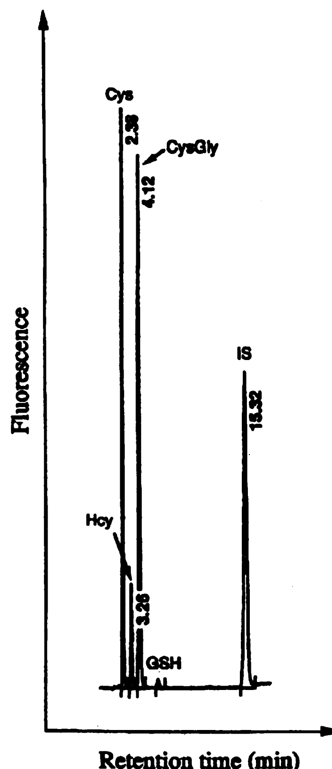

Fig. 3. Determination of Hcy and other thiols in human plasma by automated precolumn derivatization with SBD-F

The plasma contained ~10 μmol/L Hcy. Details of the chromatographic conditions are described in reference *19*. Cys, cysteine; CysGly, cysteinylglycine; GSH, glutathione; IS, internal standard (mercaptopropionylglycine)

the pressure surge during switching causes column deterioration and short column life.

A fully automated method for the determination of plasma Hcy based on $NaBH_4$ reduction and derivatization with mBrB was developed by Refsum et al. (*14*). To increase the reliability of this method, an improved procedure has been worked out (*49*) in which the column switching is omitted by taking advantage of the marked pH dependence of the retention of several mBrB derivatives in reversed-phase chromatography. Baseline separation of Hcy, cysteine, and cysteinylglycine is obtained by eluting the column with an ammonium formate buffer adjusted to pH 3.65 (*49*) (Figure 4).

Total plasma Hcy has recently been assayed with modifications of the method currently used for amino acid determination, which is based on precolumn derivatization with *o*-phthaldialdehyde followed by HPLC and fluorescence detection (*52, 68*). Two assays have been published, and in both, plasma is treated with a reductant, Hcy is carboxymethylated with iodoacetate before derivatization with *o*-phthaldialdehyde, and homocysteic acid is used as internal standard. The major differences between these assays are the reductant used and the retention times (10 and 22 min) for Hcy. When 2-mercaptoethanol is used, it reacts with iodoacetate, which must be added in excess (*68*). However, $NaBH_4$ might increase the complexity of the sample-handling procedure of these assays, particularly the generation of excessive frothing (*68*). Both techniques include several manual steps (reduction, deproteinization, carboxymethylation, pH adjustment, derivatization), and are not suitable for automation. The performance of the assays is satisfactory. The widespread experience with the *o*-phthaldialdehyde techniques and the possible determination of other aminothiols and amino acids (including cysteine and cysteinylglycine) are attractive features of these methods (*52, 68*).

HPLC and Electrochemical Detection

HPLC coupled to an electrochemical detector is well suited for the determination of biological thiols (*45, 69*), including Hcy (*70, 71*). This technique has been refined for assay of total Hcy in plasma by Malinow et al. (*13*), who modified the method of Smolin and Sneider (*72*). $NaBH_4$ is used as a reductant, and no derivatization of sample is required. Thiols in the column effluent are detected by a single gold–mercury electrode, which affords great specificity towards sulfhydryl components. This assay has been optimized for the determination of Hcy, which elutes at about 4.3 min (Figure 5), and samples can be assayed every 10 min (*13*). The chromatographic system can be modified to resolve cysteine, Hcy, and cysteinylglycine (*72*). Thus, the electrochemical assay offers several attractive features: simple sample processing, specificity, autoinjection, short run time, large sample throughput, and the possibility of determination of other thiols (e.g., cysteine).

One major weakness with the electrochemical assay is related to contamination of the flow cell and deterioration

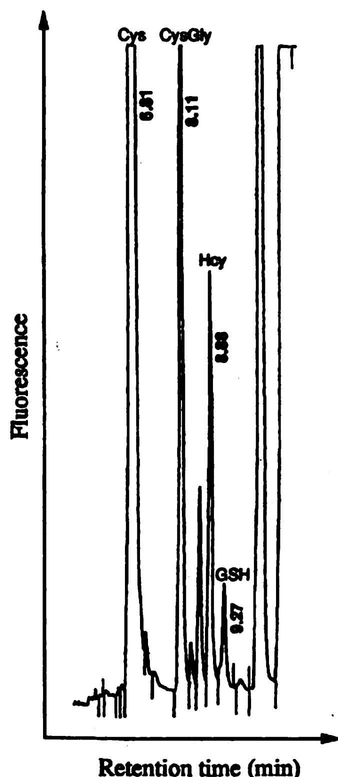

Fig. 4. Determination of Hcy and other thiols in human plasma by automated precolumn derivatization with mBrB

The plasma contained ~10 µmol/L Hcy. The column is eluted with a mobile phase adjusted to pH 3.65. Other details of the chromatographic conditions are described in reference *49*. Abbreviations as in Fig. 3

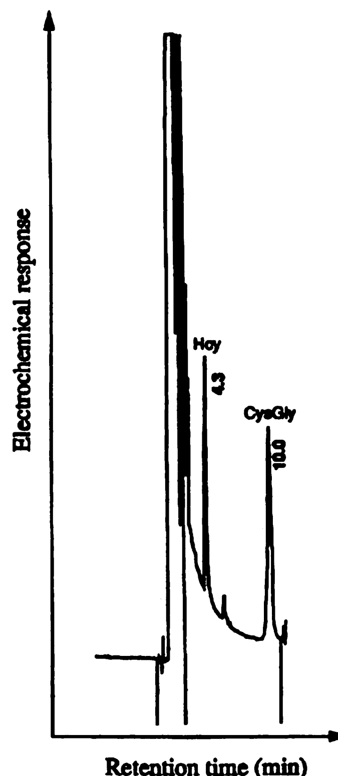

Fig. 5. Determination of Hcy in human plasma by HPLC and electrochemical detection

The plasma contained ~10 µmol/L Hcy. Details of the chromatographic conditions are described in reference *13*. CysGly, cysteinylglycine

of the gold–mercury electrode. Stability of this assay depends on careful maintenance of the detector system.

Amino Acid Analyzer, Postcolumn Derivatization

Determination of total Hcy with a conventional amino acid analyzer requires the conversion of the disulfide forms into reduced Hcy. 2-Mercaptoethanol (*73*) and dithiothreitol (*16*) have been used as reducing agents. Reduced Hcy has been *S*-carboxymethylated by using iodoacetic acid (*73*). Alternatively, Hcy has been chromatographed directly; under those conditions, Hcy is protected against reoxidation by the sulfosalicylic acid (*74*) used for protein precipitation, by the presence of reducing agent in the sample, and by the low pH of the mobile phase.

Hcy is eluted with a standard program for physiological fluids (*75*), which also permits quantification of cysteine, methionine, and cystathionine (Figure 6). The elution program is interrupted after the elution of cystathionine and a 16-min column regeneration period follows.

The determination of Hcy by a conventional amino acid analyzer has been optimized by Andersson et al. (*16*). The samples are treated with dithiothreitol (40 mmol/L plasma) and Hcy is analyzed in its reduced form. Lack of homocystine disulfide indicates that no reoxidation takes place before or during the chromatography. The method also measures methionine and cystathionine, which elute close to Hcy (*16*). The retention time of Hcy (25.6 min) and the time for column regeneration limit sample capacity, which is low compared with the HPLC assays. The construction and performance of the optimized method are shown in Table 1.

The sensitivity limit (~1 μmol/L in plasma) is less than that of other methods, but is sufficient for the determination of Hcy in 0.5 mL of plasma; the imprecision is 5–6% (*16*). A drawback is the nonselectivity for sulfur amino acids. In plasma samples frozen at −20 °C for 10 years, ninhydrin-positive material interfered with the Hcy peak, and the measured Hcy values were about half of those obtained with a specific HPLC method (B. Israelsson et al., 1992, manuscript submitted). This may prohibit using the amino acid analyzer for determination of Hcy in stored samples in prospective studies. However, in plasmas stored at −70 °C for several years, no interfering peaks have been observed.

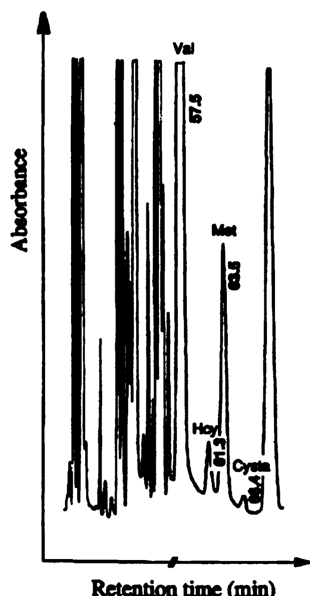

Fig. 6. Determination of Hcy in human plasma with an amino acid analyzer

The plasma contained ~10 μmol/L Hcy. The chromatographic conditions are in accordance with the standard program of the manufacturer (Biotronic LC 5000; Biotronic GmbH, Munich, Germany) for amino acids in physiological fluids. Hcy, homocysteine; Cysta, cystathionine; Met, methionine; Val, valine

The sample preparation is simple, and the technical staff often is well experienced in operating an amino acid analyzer, which is part of the routine instrumentation in many hospital laboratories. These facts justify the use of the ninhydrin-based Hcy assay in the routine laboratory.

Andersson et al. (*76*) recently described a method for plasma Hcy that also measures cysteine, cysteinylglycine, glutathione, and glutamylcysteine. The assay involved reduction of sample with dithiothreitol, separation of the thiols by HPLC, and postcolumn derivatization with 4,4′-dithiodipyridine and colorimetric detection at 324 nm. The method is characterized by high precision. The baseline fluctuation in this method reflects differences in absorbance between reagent and mobile phase. Therefore, the baseline noise is minimized by cooling the reagent reservoir to avoid hydrolysis and by delivering the postcolumn reagent with low pulsation flow.

Correlation Studies

Satisfactory correlation exists between the concentrations of plasma Hcy obtained by different methods. Values obtained with an automated mBrB assay (y) (*14*) have been compared with those obtained (x) with a radioenzymic assay (*10*) ($y = 1.009x - 1.05$, n = 160, $r = 0.989$) (*14*), an amino acid analyzer (*16*) ($y = 0.85x + 1.88$, n = 133, $r = 0.975$) (*14*), and electrochemical detection (*13*) ($y = 0.85x + 1.11$, $r = 0.93$). A correlation study has also been performed between values obtained with the electrochemical detection/HPLC assay (x) of Malinow et al. (*13*) and with an amino acid analyzer (*16*) ($y = 0.99x - 0.69$, n = 133, $r = 0.95$) (*77*).

Published methods for the determination of total Hcy are listed in Table 2, and the performance and practicalities of five methods are compared in Table 1.

Sample Collection and Stability

Effect of Food Intake

The data on possible diurnal variation in plasma Hcy and its relation to normal food intake are sparse and inconsistent. Malinow et al. (*13*) found no difference in total Hcy concentrations in fasting and nonfasting plasma from 13 subjects; Ubbink et al. (*78*) found a small but significant decrease in total plasma Hcy 2–4 h after consumption of a normal breakfast with the values returning to normal after 8 h. Ubbink et al. suggested that the postprandial decline in plasma Hcy could be

Table 1. Characteristics, Instrument Requirements, and Performance of Five Methods for Determination of Total Homocysteine in Plasma

Method (and reference)	Assay characteristics				Instrument		Capacity (number/24 h)	Other metabolites determined	CV for Hcy, %
	Sample vol, mL	Step	Time, min	Automated	Type	Price (US $)			
GC-MS (*12, 65*)	0.1	Pipetting		−	Vacuum-drying centrifuge	7 000	160	Cystathionine	2[a]–5[b]
		Reduction	30	−				Methionine	
		Solid-phase extraction	5	−	GC-MS	56 000		Cysteine	
		Drying	90	−				*N*-Methylglycine	
		Derivatization, heating	30	−				*N,N*-Dimethylglycine	
		Injection interval	7	+				Betaine[c]	
								Methylmalonic acid[c]	
								2-Methylcitric acid[c]	
Precolumn derivatization with SBD-F, HPLC, and fluorescence detection (*19*)	0.15	Pipetting		−	HPLC pump	15 000	90	Cysteine	3[b]
		Reduction	30	−	Autoinjector	15 000		Cysteinylglycine	
		Deproteinization	20	−	Detector	9 000			
		Derivatization, heating	60	−	Integrator	5 000			
		Injection interval	15	+					
Precolumn derivatization with mBrB, HPLC, and fluorescence detection (*49*)	0.1	Pipetting		+	HPLC pump	15 000	80	Cysteine	3[b]
		Reduction	3	+	Gilson 232 sample injector	17 000		Cysteinylglycine	
		Derivatization	3	+					
		Injection interval	18	+	Detector	9 000			
					Integrator	5 000			
HPLC and electrochemical detection (*13*)	0.2	Pipetting		−	HPLC pump	5 000	90	Cysteinylglycine	3–11[b]
		Reduction	30	−	Autoinjector	15 000			
		Deproteinization	20	−	Detector	7 000			
		Injection interval	10	+	Integrator	5 000			
Amino acid analyzer (*16*)	0.5	Pipetting		−	Amino acid analyzer	65 000	24	Methionine	5[a]
		Reduction	60	−				Other amino acids	
		Deproteinization	30	−					
		Injection interval	60	+					

[a] Intraassay CV.
[b] Interassay CV.
[c] Slight modifications are required for the codetermination of these metabolites (see references in the text).

Table 2. Construction of Assays for Total Hcy in Plasma/Serum

Reduction agent	Derivatization	Separation principle	Detection	Ref.
Radioenzymatic assays				
Dithioerythritol	Enzymic conversion to *S*-adenosylhomocysteine	HPLC	Absorbance at 254 nm or scintillation counting	*10*
Dithioerythritol	Enzymic conversion to *S*-adenosylhomocysteine	Thin-layer chromatography	Scintillation counting	*17, 67*
Gas chromatography–mass spectrometry				
Dithiothreitol	*tert*-Butyldimethylsilyl derivatization	Capillary GC	Mass spectrometry (single-ion monitoring)	*12, 65*
Precolumn derivatization, HPLC, and fluorescence detection				
Tri-*n*-butylphosphine	SBD-F	HPLC	Fluorescence	*11, 19, 18*
Sodium borohydride	mBrB	HPLC	Fluorescence	*14, 43, 49*
Potassium borohydride	mBrB	HPLC	Fluorescence	*15*
Potassium borohydride	Iodoacetic acid, *o*-phthaldialdehyde	HPLC	Fluorescence	*52*
2-Mercaptoethanol	Iodoacetic acid, *o*-phthaldialdehyde	HPLC	Fluorescence	*68*
HPLC and electrochemical detection				
Sodium borohydride	None	HPLC	Electrochemical detection	*13, 46*
Amino acid analyzer, postcolumn derivatization				
2-Mercaptoethanol	*S*-Carboxymethylation with iodoacetic acid	Ion exchange (amino acid analyzer)	Ninhydrin reaction	*73*
Dithiothreitol	None	Ion exchange (amino acid analyzer)	Ninhydrin reaction	*16*
Dithiothreitol	None	HPLC	Reaction with 4,4′-dithiodipyridine	*76*

attributed to an increased remethylation rate induced by the other nutrients in the meal.

In a study of 11 students, plasma Hcy increased slowly, reaching a maximum increase of about 15% by 6–8 h after dinner (a steak) (A.B. Guttormsen et al., unpublished results). Such a slow Hcy response to a meal is possibly due to the time it takes to degrade food proteins into free amino acids. After dinner, plasma methionine peaked after 3–4 h. In contrast, after peroral methionine loading, maximum plasma methionine is usually reached within 1–2 h (*44, 79*), and peak plasma Hcy is usually measured after 4–8 h (*20, 44, 79, 80*). Thus, both after a meal and after a peroral methionine load there is a time difference of about 3–4 h between the maximal plasma concentrations for methionine and Hcy.

These data indicate that intake of food may affect the concentration of plasma Hcy and that its effect, although small, may persist for several hours.

Stability of Hcy in Whole Blood

Total Hcy in serum or plasma increases when the separation of serum or plasma from the blood cells is delayed (*12, 19, 49, 78, 81, 82*). Within 1 h of storage of whole blood at room temperature, the plasma and serum Hcy concentrations increase by ~10% (*19, 49, 78, 82*). Stabler et al. (*12*) showed that storage of whole blood at room temperature for 4 and 24 h led to increases in serum Hcy of 35% and 75%, respectively. The increase is of similar magnitude (*12, 19, 49*), or greater (*78, 82*) in EDTA plasma. These data explain why serum may have a higher Hcy content than does optimally prepared plasma (*19*).

During storage of whole blood, the rate of release of Hcy from the blood cells is almost constant, independent of the plasma Hcy concentration (*49*). Thus, the Hcy release from blood cells may cause particularly large errors in the samples with low (i.e., normal) plasma Hcy content, whereas the percent increase is moderate at higher concentrations (*49*). Similarly, after a peroral methionine load, the percentage of increase during storage of whole blood was less in samples with high plasma Hcy (*49*).

The blood cells are the source of the Hcy increase during storage, because the Hcy concentration is stable in plasma or serum (*19, 49, 78*). Recently, Andersson et al. showed that the erythrocytes are the major contributors to the artificial increase (*82*). Because hemolysis does not change or reduce the concentration of Hcy in plasma (*49, 78*), the increase in plasma Hcy is presumably due to continuous production of Hcy in the erythrocytes, which is then released to the extracellular compartment. These data are consistent with the finding of low intracellular Hcy in most cells (*83*).

An artificial increase in total Hcy is avoided when blood is put on ice immediately after collection, or when plasma or serum is prepared within 1 h after collection (*12, 19, 49, 78, 81, 82*).

Plasma Hcy is to a large extent bound to albumin (*10*). Because of postural hemodilution, serum albumin averages 9% more in the blood collected from subjects who are standing than from those in the supine position (*84*). The position may, therefore, affect the measured plasma Hcy values, but this has not been evaluated.

Stability of Hcy in Plasma

Because Hcy in plasma or serum is stable for days when kept at room temperature (*19, 49, 78*), transport of unfrozen samples to the laboratory is feasible. Repeated freezing and thawing procedures (*12, 19, 78, 81*) do not affect the plasma or serum Hcy concentration; neither does freezing for a period of several months (*19, 85*). Recently, Hcy concentrations were investigated in samples kept frozen for an average of 10 years and in fresh samples from the same individuals (B. Israelsson et al., submitted). Included in the study were patients (n = 28) who had been healthy at the first investigation, but later suffered a stroke or cardiac infarct, and matched controls (n = 48). In both groups, the concentration of Hcy in plasma was significantly correlated between stored and fresh samples. Plasma Hcy was somewhat higher in the fresh samples than in the stored ones, but this may be related to the observed effect of the subjects' age on the plasma concentration of Hcy (*77*). Not only is plasma or serum Hcy stable in the frozen state for years, but also the fasting Hcy concentration remains fairly constant within most individuals for years (B. Israelsson et al., submitted).

The stability of total Hcy in whole blood and plasma/serum under various conditions is summarized in Table 3.

Reference Values

The normal values for total Hcy differ somewhat from one laboratory to another (Table 4), but values between 5 and 15 μmol/L are usually considered as normal. The variability may be related to different methodologies (Table 2), differences in sample processing, or the selection of subjects who are under the influence of various factors that affect the concentration of plasma/serum Hcy.

Total plasma Hcy seems to be dependent on age, gender, and, in women, possibly menopausal status (*77*). The large differences in cysteine–Hcy disulfide concentrations between pre- and postmenopausal women reported earlier (*6, 86–88*) have not been found when measuring total Hcy. Total plasma Hcy is decreased by 30–50% during pregnancy and is normalized within 2–4 days postpartum (*89, 90*).

Figure 7 shows the frequency distribution curve for total plasma Hcy in a screened population, showing a skewed distribution of values.

Table 3. Stability of Total Hcy in Whole Blood and Plasma/Serum

Sample	Storage conditions	Stability
Whole blood	Room temp.	<1 h
	0–2 °C	4–12 h
Plasma/serum	Room temp.	At least 4 days
	0–2 °C	Several weeks
	−20 °C	Years

Data from references *12, 19, 49, 78, 82*, and unpublished results.

Table 4. Normal Values for Total Hcy in Human Plasma/Serum

Subjects[a]	Age, years[b]	n	Hcy fraction	Value, μmol/L[c]	Ref.
M	25–55	18	Free	2.27 ± 0.48	*10*
			Bound	6.51 ± 1.35	
F	25–55	16	Free	1.95 ± 0.56	
			Bound	7.29 ± 2.62	
M	<30	5	Bound=total[d]	6.82 ± 1.28	*126*
	30–39	14		8.92 ± 2.32	
	40–49	25		9.44 ± 2.00	
	50–59	26		8.84 ± 2.02	
	60–69	23		8.06 ± 2.32	
F	<30	9	Bound=total[d]	7.50 ± 2.02	
	30–39	8		7.26 ± 1.64	
	40–49	24		7.00 ± 1.94	
	50–59	30		8.82 ± 3.82	
	60–69	38		9.20 ± 3.62	
M+F	18–65	50	Total	5.4–16.2[e]	*21*
	19–55	99	Total	5.0–13.9[f]	*104*
F[g]	62.9 ± 10.8	45	Total	5.8 ± 0.9	*127*
M[g]			Total	8.1 ± 3.2	
F[h]	63.3 ± 10.0	45	Total	7.8 ± 1.4	
M[h]			Total	10.9 ± 4.6	
M	20–39	26	Free	2.1 ± 0.7	*77*
			Total	9.7 ± 2.4	
	40–49	15	Free	2.5 ± 0.9	
			Total	10.4 ± 2.6	
	50–69	33	Free	2.8 ± 0.7	
			Total	11.7 ± 2.4	
F	20–39	24	Free	2.0 ± 0.9	
			Total	8.9 ± 3.4	
	40–49	22	Free	2.0 ± 1.0	
			Total	9.8 ± 3.0	
	50–69[i]	37	Free	2.1 ± 0.8	
			Total	10.0 ± 2.8	
M	56.0 ± 11.3	92	Total	11.26 ± 3.71	*118*
F	63.4 ± 11.5	167	Total	10.15 ± 4.99	
M+F	22–46	25	Total	8.01 ± 1.76	*128*
M	25–60	8	Total	9.8 ± 1.6	*19*
F	25–60	8	Total	6.8 ± 1.2	
M		195	Total	13.3 ± 4.7	*129*
M[j]		52	Total	9.7 ± 3.3	
M+F	20–60	40	Total	12.4 ± 2.9	*52*
M+F	1–38	16	Total	9.1 ± 3.0	*68*

[a] M, male; F, female.
[b] Range, or mean ± SD.
[c] Hcy equivalents, mean ± SD, except as noted.
[d] Bound=total Hcy in stored samples, because free Hcy becomes associated with plasma protein(s).
[e] Mean ± 2 SD after log normalization to correct for skewness towards higher values. The samples were allowed to stand at room temperature for 1–4 h before centrifugation.
[f] Same as [e] except that samples were centrifuged within 1 h of collection.
[g] Normotensive.
[h] Hypertensive.
[i] Postmenopausal.
[j] Black tribe.

Total Hcy in Laboratory Diagnosis

Cobalamin Deficiency

The classical view of cobalamin (vitamin B_{12}) deficiency is that it usually develops at age >60 years; usually presents with megaloblastic anemia, macrocytosis, and hypersegmented neutrophils; and is often accompanied by neurological abnormalities. In these cases, the hematological picture was taken as an important diagnostic feature (*91*). The neurological abnormalities have long been considered to be confined to advanced deficiency, and to occur only rarely in the absence of anemia or other hematological findings. Recent clinical research has demonstrated that the classical view is wrong and that cobalamin deficiency exists in a large number of patients with subtle biochemical changes and no hematological abnormalities (*92–94*); moreover, neuropsychiatric disorders due to cobalamin deficiency commonly occur in the absence of anemia and macrocytosis (*93*).

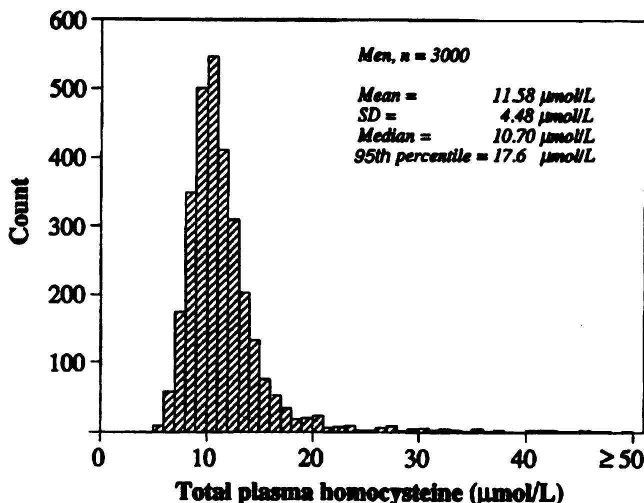

Fig. 7. Frequency distribution curve for total plasma Hcy screened in 3000 men, ages 40–42 years

This population was part of a survey conducted in the county of Hordaland, Norway, by the National Health Screening Service in collaboration with the University of Bergen. The samples were assayed with the method described in reference *49*

The established diagnostic tests for cobalamin deficiency have some disadvantages. Serum cobalamin assays do not completely discriminate deficient patients from normal persons because, by definition, 2.5% of normal subjects have low values. Furthermore, 5–10% of patients with clinically confirmed cobalamin deficiency have normal values for serum cobalamin (*93, 95*). Other procedures, e.g., bone marrow examination and the classic Schilling test, may also give false results (*21, 94*). Many cobalamin-deficient patients fail to absorb protein-bound cobalamin, but their absorption of the free vitamin is normal (*96*). The deoxyuridine suppression test is too cumbersome to be included in routine laboratory diagnosis (*97, 98*).

Concentrations of total Hcy in plasma/serum are increased in most patients with cobalamin deficiency (*21, 25, 28, 35, 93, 94, 99*). In a large population of patients, some of whom have classical cobalamin deficiency, serum cobalamin and plasma Hcy concentrations display a hyperbolic relation (Figure 8). The plasma Hcy content increases abruptly when serum cobalamin approaches values that are below normal (<130 pmol/L), but a negative correlation is observed in the normal to

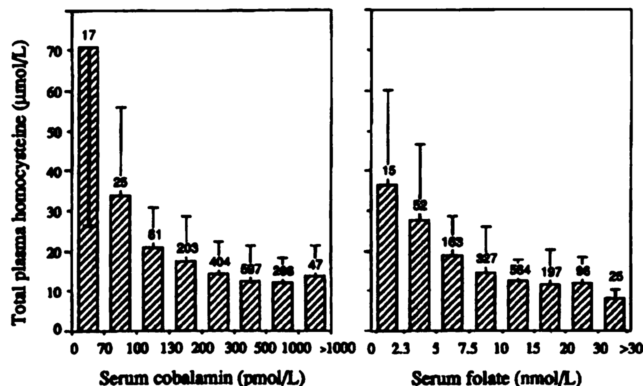

Fig. 8. The relation between total Hcy and serum cobalamin or serum folate

Total plasma Hcy, serum cobalamin, and (or) serum folate concentrations were determined in 1600 patients admitted to Haukeland University hospital. The patients were divided into groups according to their cobalamin or folate values. Each group is defined by the vitamin concentrations given at the base of each column; the column height (and *bars*) indicate the corresponding mean (± SD) Hcy concentrations for that group. The numbers at the *tops* of the columns indicate the number of patients in each group

low-normal range for cobalamin (130–300 pmol/L). This observation may suggest that having serum cobalamin in the normal range does not ensure optimal cobalamin-dependent Hcy remethylation in tissues.

Compared with serum methylmalonic acid, plasma/serum Hcy is not a specific marker for cobalamin deficiency: The plasma concentration of Hcy also increases markedly in other conditions, including folate deficiency, which causes indistinguishable hematological abnormalities. The specificity for both tests can be increased by measuring the metabolite concentrations before and after treatment with cobalamin or folic acid, because the concentration of Hcy and methylmalonic acid decreases rapidly towards normal (within 14 days) only after administration of the vitamin that is lacking (*21*).

Diagnosis of the correct vitamin deficiency is very important: If a cobalamin-deficient patient is mistakenly treated with folate, the hematological abnormalities may improve or completely correct, but the neuropsychiatric abnormalities will continue to progress (*21, 100*).

Clinical studies show that plasma/serum Hcy is a sensitive marker of cobalamin deficiency (*101*). Hcy and (or) serum methylmalonic acid concentrations were increased in 94% of 86 consecutive cobalamin-responsive patients, many of whom lacked one or more of the classic hematological or laboratory abnormalities (*94*). Furthermore, hematological relapse in patients on infrequent cobalamin maintenance therapy was associated with increased metabolite concentrations in 95% of the cases, whereas the serum cobalamin was low in only 69%. Serum methylmalonic acid was somewhat more sensitive than plasma/serum Hcy in this study (*95*).

The sensitivity of plasma/serum Hcy in comparison with other diagnostic tests for cobalamin deficiency is an important issue. This question is related to the ability of plasma/serum Hcy to reveal an early deficiency state. Total plasma/serum Hcy is a responsive marker that increases rapidly after perturbation of the intracellular cobalamin function. This is demonstrated in patients being anesthetized with nitrous oxide, which oxidizes cobal(I)amin, a cofactor for the enzyme methionine synthase; after 90 min of nitrous oxide exposure, a significant increase in total Hcy is observed (*102*).

Subtle or atypical cobalamin deficiency seems to be at least as common as the classical deficiency (*103*) and appears to occur often in the geriatric population (*104, 105*). Its nature is still being defined, and the importance of increased metabolites requires additional investigation. Nevertheless, determination of plasma/serum Hcy is both a sensitive indicator of cobalamin depletion and an objective measure of therapeutic response.

Folate Deficiency

Folate deficiency is often nutritional and usually develops in predisposed subjects such as alcoholics, pregnant women, and patients with generalized malabsorption. Classical signs are macro-ovalocytosis, anemia, and hypersegmented neutrophils (*98, 100*).

Plasma/serum Hcy is markedly increased in folate-deficient patients (*25, 26, 35, 101*). In 19 patients with subnormal concentrations of serum folate (<2 µg/L), 137 subjects with low-normal concentrations (2–3.9 µg/L), and 44 subjects with normal concentrations (4–17.9 µg/L), serum Hcy was negatively correlated with the serum folate concentration. In 84% of the patients with subnormal serum folate, Hcy was 2 SD greater than the normal mean, and up to 70 µmol/L. Of particular interest was the observation that more than half of the patients with low-normal serum folate had increased serum Hcy (*26*). A similar picture is shown for the relation between plasma Hcy and serum folate in 1620 patients (Figure 8). This suggests that folate intake that results in a serum folate content in the lower normal range is not necessarily sufficient for optimal remethylation of Hcy and that a relative deficiency of tissue folate may exist under these conditions (*26*). Data from an experimental model involving rats fed a folate-deficient diet show that low-normal and subnormal concentrations of serum folate cause a two- to fourfold increase in serum Hcy (*106*).

The possibility that subjects with no clinical or laboratory signs of folate deficiency may actually be deficient in intracellular folate is supported by a decrease in plasma Hcy after administration of high doses of folic acid. The effect of folic acid was observed both in hyperhomocysteinemic postmenopausal women (*88*) and in apparently healthy subjects (*85*).

An increase in plasma Hcy reflects intracellular folate deficiency or impaired function of folate-dependent reactions. The responsiveness of this factor is demonstrated by the observation that plasma Hcy increases markedly within hours after administration of the antifolate drug methotrexate (*80, 107–109*). Furthermore, the specificity of plasma Hcy as an indicator of folate deficiency is increased by the simultaneous determination of serum methylmalonic acid and by the reduction

of plasma Hcy in folate-deficient subjects within weeks after administration of folic acid but not after cobalamin (*21*).

Plasma Hcy as an indicator of folate deficiency may compensate for some defects of the conventional laboratory test. Serum folate does not discriminate between a transitory reduction in folate intake and chronic folate deficiency associated with depletion of tissue folate (*110*). Furthermore, both serum and erythrocyte folate may be normal in patients with alcoholism and megaloblastic anemia (*111*). In addition, assay of red cell folate has many technical pitfalls: Both the interlaboratory variation and the coefficient of variation have been high (*112*). The deoxyuridine suppression test has been regarded as a more reliable method than measurement of either serum or red cell folate for detecting folate deficiency (*113*), but the complexity of the procedure has prevented widespread use of this technique.

The combined use of serum cobalamin, folate, total Hcy, and methylmalonic acid concentrations currently provides the maximal utility in diagnosing and distinguishing between cobalamin and folate deficiency. Any of the appropriate tests can give normal results in some patients with clinically confirmed cobalamin or folate deficiency (*94, 95*), but the use of the panel makes it possible to make or exclude the diagnosis of cobalamin or folate deficiency in virtually every patient (*101*). Nevertheless, a logistic regression analysis evaluating each of the four tests would be of interest.

Homocystinuria

Homocystinuria is a group of inherited disorders characterized by excretion of large amounts of homocystine into the urine. The most common form is cystathionine β-synthase deficiency, which has a prevalence of 1:200 000 worldwide. Rare forms are caused by various defects of Hcy remethylation, including 5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20) deficiency and errors of cobalamin metabolism. Common clinical signs in these diverse conditions are mental retardation and premature vascular disease (*114*). Homocystinuria has been the subject of several recent review articles (*3, 22, 114*).

Determination of Hcy in plasma and homocystine in urine are laboratory tests for the diagnosis of various forms of homocystinuria. In patients with cystathionine β-synthase deficiency, increases of plasma Hcy up to 500 μmol/L have been reported (*3*). These patients also have large amounts of methionine in plasma and low or low-normal concentrations of cystathionine (*65*)—important biochemical features that serve to distinguish these patients from patients with inborn errors of Hcy remethylation. Patients with 5,10-methylenetetrahydrofolate reductase deficiency or impaired methylcobalamin synthesis have high concentrations of Hcy in plasma and urine, normal or subnormal concentrations of plasma methionine, and high concentrations of serum or plasma cystathionine (*65*). Measurement of methylmalonic acid and 2-methylcitric acid (*60*) in plasma or urine may differentiate reductase deficiency (with normal concentrations of methylmalonic acid and 2-methylcitric acid) from defects in cobalamin metabolism (in which these acid concentrations are increased) (*3*).

Measurement of metabolites in plasma and urine may point to a particular defect of Hcy metabolism, but confirmation of the diagnosis requires determination of enzyme activities in cultured fibroblasts. This laborious technique has been established in only a few laboratories (*3*).

Premature Cardiovascular Disease

Studies involving ~1800 patients and a comparable number of controls show a statistically significant correlation between hyperhomocysteinemia and premature cardiovascular disease (*22*). The overall increase in plasma Hcy in populations with early cardiovascular disease is ~30% over that in healthy subjects, but shows some variability related to the site of the vascular lesions. In coronary artery disease, the ratio for mean plasma Hcy in patients vs controls was 1.2 to 1.3; in peripheral and cerebrovascular disease, this ratio varied from 1.5 to 1.8 (*24*). This distribution of statistical means is related to the observation that the incidence of hyperhomocyst(e)inemia is highest in cerebrovascular disease [~40% (*115, 116*)], intermediate in peripheral arterial disease [~25% (*117*)], and lowest in coronary heart disease [~15% (*118*)].

Hyperhomocysteinemia is a possible risk factor for premature vascular disease, and is independent of other factors such as hypertension, diabetes, smoking, and plasma cholesterol (*20, 22*). A causative role of Hcy in atherogenesis has not been established. A prospective study showing a relationship between plasma Hcy and risk of myocardial infarction has been published recently (*119*), and another population-based prospective study has been completed (E. Arnesen et al., manuscript submitted). These studies confirm that Hcy is a strong risk factor for myocardial infarction, particularly in the younger age group. However, no intervention studies, which are critical for the question of the role of Hcy in atherogenesis, have been conducted.

We propose the tentative recommendation that plasma Hcy should be determined in patients with premature (age <55 years) cardiovascular disease, especially in patients who lack other risk factors. The results from ongoing epidemiological and proposed intervention studies may broaden this recommendation in the future.

Other Diseases and Drugs

Plasma Hcy is increased in patients with renal failure. This increase is moderate to marked (up to 50 μmol/L) and is positively correlated to serum creatinine (*20, 120*). A moderate increase of plasma Hcy is observed in most patients with psoriasis (*81*) and in some patients with leukemia (*109*) or solid tumors (*107*). Plasma concentrations are low in some patients with Down syndrome (*20, 121*) and in most patients with hyperthyroidism (Lien et al., unpublished).

Some drugs affect the plasma Hcy concentration (for a recent review, see *122*). The antifolate drug methotrex-

ate, at doses of 25 mg (*80*) to 8 g/m^2 (*108, 109*), and nitrous oxide anesthesia (*30, 102*) induce rapid and transient increases in plasma Hcy. Preliminary data suggest that the antiepileptic drugs phenytoin and carbamazepine induce hyperhomocysteinemia, probably due to interference with folate functions. The cholesterol-lowering regimen of colestipol plus niacin also causes a significant hyperhomocysteinemia (*123*), possibly through interference with folate absorption. Drugs acting as vitamin B_6 antagonists [isoniazide, cycloserine, hydralazine, penicillamine, phenelzine, and procarbazine (*124*)] would be expected to increase plasma Hcy, as demonstrated for the banned drug azauridine (*124*). High doses of folic acid (5 mg daily) cause variable decreases in high plasma Hcy concentrations in patients with renal failure but also in healthy subjects without overt folate deficiency (*84, 125*). The first doses of leucovorin (70 mg/m^2), administered to modulate the effect of 5-fluorouracil, induced a marked decrease in plasma Hcy within hours (J. Geisler et al., unpublished).

Summary and Conclusion

It is mandatory that a technique to be established in a clinical chemistry laboratory measures all Hcy species in plasma/serum, i.e., total Hcy. This is a prerequisite for the technique to be compliant with the routines for sample collection, processing, and transport in the clinical setting.

Several new Hcy methods have been developed and old techniques have been refined. The practical aspects of these techniques are quite different, as summarized in Table 1. Choice of method depends on the personnel and instrumental resources available, the training of the technical staff, the anticipated number of samples to be analyzed, and special interests concerning the codetermination of other metabolites.

Determination of plasma/serum total Hcy together with determinations of other metabolites, such as methylmalonic acid, is of value for the diagnosis and follow-up of cobalamin and folate deficiency, especially in cases of subtle or atypical deficiency, where the classical signs are lacking. These conditions seem to be rather common. Thus, the laboratory diagnosis of cobalamin and folate deficiency will probably account for the largest number of plasma/serum Hcy determinations. In addition, assessment of risk for cardiovascular disease and clinical evaluation of premature vascular disease may become important applications in the near future. Plasma/serum Hcy also plays a pivotal role for the diagnosis of different forms of homocystinuria, but these are rare conditions.

Our original work cited in this review was supported by The Norwegian Cancer Society, the Norwegian Council on Cardiovascular Diseases, the Norwegian Research Council for Science and Humanities, and the Nordic Insulin Foundation (P.M.U., H.R.); The National Institute on Ageing (AG-09834) (S.P.S.); The National Institute of Diabetes and Digestive and Kidney Diseases (DK-21365) (R.H.A.); The National Institute of Health (RR 00163-32) (M.R.M.); and by grants from The Medical Faculty, University of Lund (A.A.).

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