CLIN. CHEM. 35/9, 1921–1927 (1989)

Fully Automated Fluorescence Assay for Determining Total Homocysteine in Plasma

Helga Refsum, Per Magne Ueland, and Asbjørn Martin Svardal

Homocysteine exists in human plasma as various (mixed) disulfides. Most plasma homocysteine (about 70%) is protein bound, probably via a disulfide bond to albumin, whereas homocysteine–cysteine mixed disulfide is the predominating form in the free fraction. We here present a method for the determination of total homocysteine, which includes both fractions. Plasma was initially treated with sodium borohydride to reduce the disulfide bonds, and the liberated thiols were derivatized with monobromobimane. The derivatized sample, still containing the plasma proteins, was injected onto a strong cation-exchange column, from which the homocysteine derivative was directed by column switching into a cyclohexyl silica (CH) column. The homocysteine derivative was top-concentrated on the CH column, then rapidly eluted with a steep gradient of methanol. Both the derivatization procedure and chromatography were performed with a combined sample processor and sample injector from Gilson (Model 232-401). Within-run and between-run precision (CV) was <4%, and the detection limit of 0.2 pmol was sufficiently low for monitoring homocysteine in plasma. We verified the assay against two established manual methods for the determination of total homocysteine in plasma. This, the first fully automated assay for total plasma homocysteine, allows the unattended analysis of 70 samples per 24 h.

Additional Keyphrases: *amino acids · chromatography, cation-exchange*

Homocysteine is a sulfur-containing amino acid, formed from methionine during *S*-adenosylmethionine-dependent transmethylation reactions. Intracellular homocysteine may be remethylated to methionine, and in most tissues, this reaction is catalyzed by methionine synthase (5-methyltetrahydrofolate–homocysteine methyltransferase; EC 2.1.1.13), which requires 5-methyltetrahydrofolate as methyldonor and vitamin B_{12} as cofactor. Catabolism via cystathionine to cysteine, an alternative route of homocysteine disposal, depends on two vitamin B_6-dependent enzymes. Thus, the metabolic fate of homocysteine is coupled to reduced folates, vitamins B_{12} and B_6, and sulfur amino acids, and may be defined as a branch-point metabolite (*1*).

When the intracellular production of homocysteine exceeds the metabolic capacity, homocysteine is efficiently exported into the extracellular space, and the intracellular content is kept low (*2*). Thus, the amount of homocysteine in extracellular fluids like plasma and urine may reflect the balance between homocysteine production and utilization. This balance may be perturbed by clinical states characterized by decreased activity of enzymes or low quantity of cofactors involved in homocysteine metabolism (*3*). This is the basis for the marked increase in homocysteine in plasma in some inborn errors of metabolism (homocystinuria) (*1*) and during vitamin B_{12} (*4, 5*) and folate deficiency (*4, 6*). In such conditions, monitoring plasma homocysteine may be of value both in diagnosis and follow-up. A more moderate increase of plasma homocysteine has been reported in renal failure (*7, 8*), some malignant states (*9*), and psoriasis (*10*), and its concentration in plasma may be affected by various drugs (*3*). Thus, plasma homocysteine may be a useful measure of the identity as well as the severity of several clinical states. Furthermore, it may itself be a pathogenic factor. Increasing evidence suggests that moderately increased homocysteine in plasma is an independent risk factor for premature cardiovascular disease (*3, 11*).

The first reports on homocysteine in plasma from healthy subjects or patients without homocystinuria were based on results obtained with amino acid analyzers (*12–16*). These assays, which measure acid-soluble homocysteine–cysteine mixed disulfide, are time consuming, and care should be taken during sample handling and storage to avoid artifically low values attributable to a progressive association of homocysteine with plasma protein(s). Recently, we developed a radioenzymic assay for total and free homocysteine (*17*). This, as well as other assays measuring total homocysteine (*18–22*), requires the liberation of protein-bound homocysteine by cleavage of the disulfide bridge in the presence of a reducing agent. Such methods have the advantage that there is no interference from redistribution between the various homocysteine species in plasma, and stored plasma samples can be used for analysis. However, most of these methods are laborious and time consuming.

The increasing demand for the determination of total homocysteine in plasma in the clinical setting prompted us to develop a fully automated and rapid chromatographic assay. The method is based on use of a combined sample processor and sample injector from Gilson, Model 232-401, for reduction of oxidized homocysteine, precolumn derivatization, and column switching. The column-switching device allows injection of plasma samples without removal of protein. The method is characterized by high precision and sample output.

Materials and Methods

Materials

Reagents. DL-Homocysteine, L-homocystine, dithioerythritol (DTE), and *N*-ethylmorpholine were obtained from Sigma Chemical Co., St. Louis, MO.[1] Sodium borohydride ($NaBH_4$) was from Fluka Chemie AG, Buchs, Switzerland. Monobromobimane (mBrB; Thiolyte®) is a product of Calbiochem–Behring Diagnostics, La Jolla, CA. "HPLC-grade" methanol was from Rathburn Chemicals, Ltd., Walkerburn, Scotland, U.K. One stainless-steel column (0.46 × 10 cm) was packed with 10-μm-diameter particles

The Clinical Pharmacology Unit, Department of Pharmacology and Toxicology, University of Bergen, N-5021 Haukeland Hospital, Norway.

Received April 24, 1989; accepted June 27, 1989.

[1] Nonstandard abbreviations: DTE, dithioerythritol; mBrB, monobromobimane; DMSO, dimethyl sulfoxide; SCX, strong cation-exchange; and CH, cyclohexyl silica.

of Partisil-10 SCX from Whatman Inc., Clifton, NJ; another (0.46 × 15 cm) was packed with 5-μm-diameter particles of Bondasil CH from Analytichem International, Harbor City, CA. Dimethyl sulfoxide (DMSO) was from Merck AG, Darmstadt, F.R.G. 2-Octanol was purchased from BDH Chemicals Ltd., Poole, U.K. Other reagents, used in the radioenzymic assay for homocysteine, have been described previously (*17*).

Standard solutions. DL-Homocysteine or L-homocystine was dissolved in water to give a concentration of 100 μmol/L. These stock solutions were further diluted with water to known concentrations. If not otherwise stated, all standards contained 100 μmol of DTE per liter.

Instrumentation

The programmable sample processor, Model 232-401, was manufactured by Gilson Medical Electronics, Inc., Middleton, WI. We used it for automated sample processing, derivatization, sample injection, and the column-switching procedure. It operates in a concurrent sequential mode with the HPLC system; one sample undergoes the chromatographic analysis while the next one is being prepared. The program for derivatization, injection, and switching was developed in our laboratory, and can be obtained upon request.

This autosampler was equipped with both a column-switching valve and an injector valve (Rheodyne Model 7010), the latter being fitted with a 20-μL sample loop. We used two ternary solvent-delivery systems (Model SP 8800), both from Spectra-Physics, San Jose, CA. Both columns were mounted in a column heater (Model SP 8792; Spectra-Physics). The effluent from the strong cation-exchange (SCX) column was either directed to the cyclohexyl silica (CH) column or to waste by use of a column-switching valve, and the tubing from the waste port was fitted with a restrictor offering a back-pressure of ~2 MPa at a flow rate of 1.5 mL/min. This minimized the pressure surge through the system during the column switching. The fluorescent material in the column effluent was detected with a Model SFM 25 fluorescence detector (Kontron, Zurich, Switzerland). We adjusted the wavelength of the primary light path to 400 nm and recorded the fluorescence emission at 475 nm, using an integrator (Model SP 4290; Spectra-Physics) that was started by a signal from the Gilson sample processor.

Procedures

Sample collection and storage. Blood was collected into evacuated tubes containing EDTA, immediately placed on ice, and plasma separated within 10 min. Plasma was then analyzed immediately or stored at −80 °C until analysis.

Solutions, and storage of samples and reagents during unattended analysis. Plasma samples were stored in narrow conical plastic vials placed in a cooled (2–3 °C) rack of the sample tray. We protected each sample (50–100 μL) from air exposure and evaporation by covering it with an octanol layer (20 μL). $NaBH_4$ (6 mol/L) was dissolved in NaOH, 0.1 mol/L, then mixed with DMSO (2/1, by vol) and stored in glass vials in a cooled rack. The glass vials (Chromacol Ltd., London, U.K.) were broad-based with a small-bore cone in the center, which trapped precipitating material. mBrB (25 mmol/L) in acetonitrile was placed in cooled, dark glass vials, to protect this reagent from light. 2-Octanol was stored in the cooled rack. The *N*-ethylmorpholine buffer (1 mol/L), hydrochloric acid (1 mol/L), and glacial acetic acid were kept at room temperature.

Automated sample preparation and derivatization. The sampler collects 30 μL of plasma from the sample vial and, together with 20 μL of octanol and 30 μL of $NaBH_4$/DMSO solution, places this in the derivatization vial. The reduction by $NaBH_4$ is then accelerated by adding 30 μL of 1 mol/L HCl. After 1.5 min, the sample is diluted with 150 μL of *N*-ethylmorpholine buffer (1 mol/L) and 350 μL of water. The sampler then adds 20 μL of the fluorescence reagent mBrB (25 mmol/L) in acetonitrile, carefully mixes and, after 3 min, stops the reaction by adding 40 μL of glacial acetic acid. After thorough mixing, 20 μL of the sample is immediately injected into the SCX column.

The timing for this procedure, including some additional details, is shown in the upper panel of Figure 1.

Chromatography. Chromatography was carried out at a constant temperature of 50 °C. Both columns were equilibrated with 20 mmol/L ammonium formate buffer, pH 3.5, and the flow rate was 1.5 mL/min. The CH column was supplied from pump A. The SCX column received its mobile phase from pump B, except during the column-switching interval, in which both columns, being in-line, were supplied by pump A. The sample (20 μL) was injected into the SCX column immediately after derivatization and was eluted isocratically from the SCX column after about 4 min. The switching interval started at about 3 min after injection and lasted for 2.5 min; the integrator was started at the end of this interval. Because the CH column was pre-equilibrated with a mobile phase containing no organic

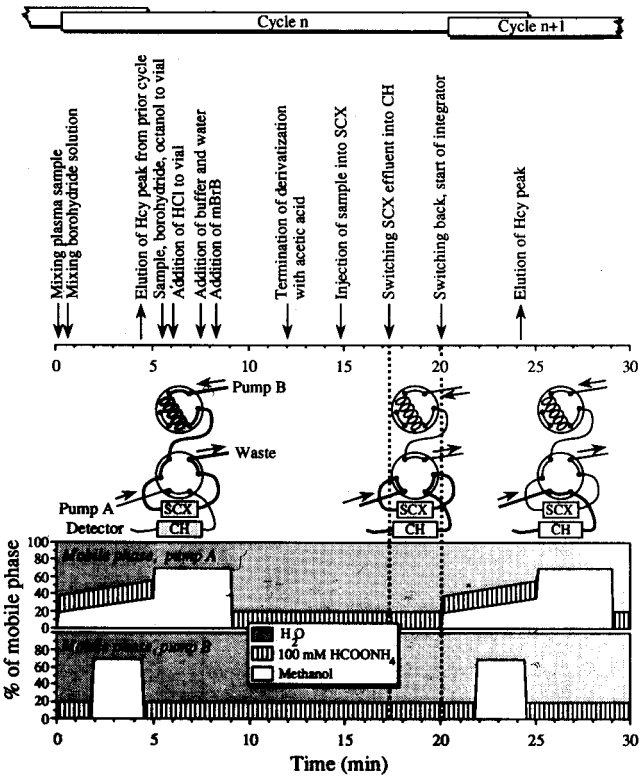

Fig. 1. Time-table for one cycle of sample preparation and chromatography carried out by the Gilson combined sample processor and sample injector

The *upper panel* shows the sample handling, including the derivatization procedure; the *middle panel* the column-switching device; and the *lower panel* the composition of the mobile phases delivered by pumps A and B. *Hcy*, homocysteine

solvent, the homocysteine derivative was concentrated at the top of this column. Immediately after termination of the switching interval, the CH column was eluted with a linear methanol gradient (from 17% to 35% in 5 min) in 20 mmol/L formate buffer. The homocysteine derivative was eluted from the CH column at a retention time of 4.5 min, corresponding to about 10 min after injection.

After the elution of the homocysteine peak, both the SCX column and the CH column were washed with 700 mL/L methanol in formate buffer.

The column switching and the composition of the mobile phases from pump A and B are summarized in the middle and lower panels of Figure 1.

Standard curve and linearity of the assay. We prepared a standard curve by adding known concentrations (0.5–100 μmol/L) of either homocysteine or homocystine to water. The assay was tested for linearity by determination of total homocysteine in plasma diluted (two- to 200-fold) with water.

Precision and comparison of methods. Plasma samples containing low normal (7.3 μmol/L), high normal (13.0 μmol/L), and above-normal (39.8 μmol/L) homocysteine, as determined by a radioenzymic assay (*17*), were selected for the evaluation of precision. The above-normal sample was obtained from a patient undergoing methionine loading. To determine the within-run precision, we assayed 10 replicates of each plasma sample in one single run. The between-run precision was determined by assaying the same plasma samples on 10 different days within four weeks. These experiments were performed by two different operators and the samples analyzed on a single pair of columns.

The present method for total homocysteine in plasma was compared with a radioenzymic assay (*17*) and a procedure for the determination of total homocysteine based on use of an amino acid analyzer (*23, 24*). The samples for the comparison studies were obtained from Dr. Lars Brattström, and the amino acid analyses were performed at his laboratory at the University of Lund, Sweden.

Results

Stability of samples and reagents. This automated assay depends on the stability of the reagents and plasma samples for the period of unattended analysis, i.e., up to 24 h. Plasma samples stored at room temperature in glass vials showed about a 30–50% decrease in assayable homocysteine within 24 h. However, the samples were stable for at least 24 h when placed in plastic instead of glass vials and protected against air with an octanol layer. The temperature was lowered to 2–3 °C by placing the vials in a cooled sample tray.

$NaBH_4$ was unstable in water, but was stable for days when dissolved in 0.1 mol/L NaOH and kept at 2–3 °C. Under these conditions there was a time-dependent formation of precipitating material, which was partly prevented by mixing the $NaBH_4$ solution with DMSO, as described in *Materials and Methods*. The small amount of precipitate that still formed was trapped in the cone at the bottom of the glass vial. The $NaBH_4$/DMSO solution was always thoroughly mixed before being added to the derivatization vial.

mBrB is subject to photolysis (*25*), and was unstable in water, and at room temperature. It was relatively stable for at least 24 h when dissolved in acetonitrile and stored at 2–3 °C in dark glass vials. Even under optimal conditions, there was a time-dependent formation of multiple fluorescent peaks, some of which co-chromatographed with the homocysteine derivative. The mBrB solution should be changed after two to three days of storage in the cooled sample tray. Routinely, we prepared the mBrB solution freshly every day before the start of unattended analysis.

Reduction and derivatization. Automated sample processing has certain limitations in comparison with manual derivatization. The sample processor cannot handle powder or remove precipitated material. At least 20 μL is needed to obtain a quantitative transfer to an empty derivatization vial or when a reagent is added above the surface of the mixture. The sample tray should accommodate up to 60 samples, with derivatization vials and the necessary amounts of reagents—which restricts the multiple dilutions and transfer steps often used in manual sample processing. In our assay, reduction and derivatization were performed in a single 700-μL vial. During optimization of the method we carried out every experiment in such a way that the steps could be performed by the Gilson sample processor. For simplicity these experiments were performed manually; only the optimal design was programmed into the processor and then verified against manual derivatization.

A reducing agent is necessary both for the reduction of the disulfide bonds and to keep the thiol in a reduced form until start of derivatization. When the reduced thiol homocysteine, 100 μmol/L, was carried through the assay procedure, only 40% was recovered in the absence of a reducing agent (Figure 2). $NaBH_4$ at 0.5 mol/L in the initial mixture of standard solution, $NaBH_4$ and HCl (reduction step), which corresponds to 40 mmol/L of $NaBH_4$ in the derivatization step, gave maximal yield of the mBrB derivative (Figure 2). When the concentration of $NaBH_4$ during de-

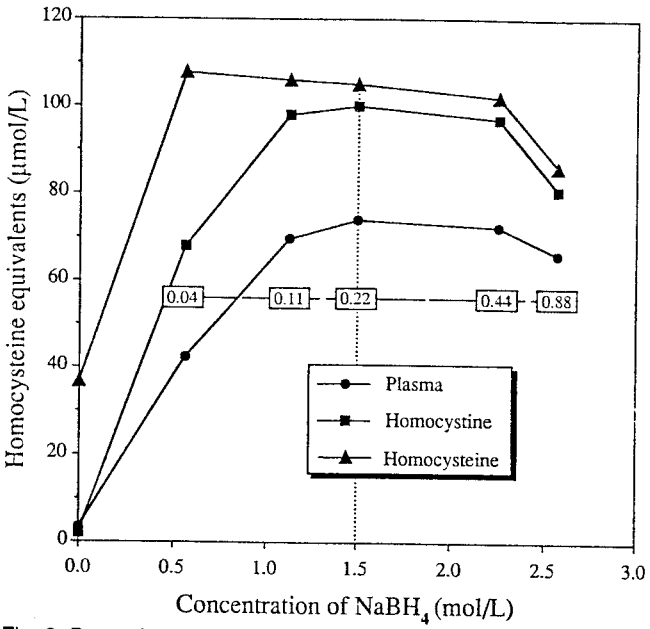

Fig. 2. Determination of homocysteine in standard solutions and in plasma in the presence of $NaBH_4$

The standard solutions were 100 μmol/L. The plasma sample was obtained from a person subjected to methionine loading, and contained 73 μmol of total homocysteine per liter, as determined with a radioenzymic assay. The concentration of $NaBH_4$ on the *x*-axis refers to the amount present after mixing sample, $NaBH_4$/DMSO, and HCl. The values in the boxes are the $NaBH_4$ concentrations (mol/L) during derivatization, i.e., after water, buffer, and mBrB were added. The concentrations of $NaBH_4$ in these two steps do not vary proportionally, owing to restrictions regarding added volumes and solubility of $NaBH_4$

rivatization exceeded 0.5 mol/L, the yield of the mBrB derivative decreased. However, this decrease could be prevented by further dilution of $NaBH_4$ reagent before addition of mBrB.

We investigated the dose–response for the reduction of the disulfide homocystine and for homocysteine in plasma in the presence of $NaBH_4$. Assayable homocysteine increased as a function of the concentration of $NaBH_4$, reaching a plateau with $NaBH_4$ between 1 and 2.3 mol/L at the reduction step. Above this concentration there was a marked decrease in yield (Figure 2) as seen for the reduced form, homocysteine. Notably, in the presence of optimal concentration of $NaBH_4$, the yield of homocysteine-mBrB derivative was the same for homocysteine and homocystine. Furthermore, the dose–response curve for homocystine in plasma and in water was almost identical, suggesting that equal concentrations of $NaBH_4$ are required for reduction of different homocysteine mixed disulfides, including the disulfide formed with plasma protein(s).

The time course for the reduction of homocystine or total homocysteine in plasma in the presence of optimal concentration of $NaBH_4$ (1.5 mol/L during reduction) reached a plateau within 2 min (data not shown).

The ability of $NaBH_4$ to reduce homocysteine disulfides was pH dependent. When we decreased the pH during reduction by adding HCl, we observed that the maximal yield of the mBrB derivative of homocysteine was observed between pH 8 and 9 (data not shown). In this experiment we kept the pH at ~9 during derivatization.

There were some problems related to the high concentration of $NaBH_4$ required for rapid reduction of the different homocysteine species. The avid formation of gas during reduction resulted in foaming, which we prevented by adding octanol. The plasma samples (30 μL) were diluted (20-fold) and then degassed by suction and purging through the injector needle. Only small, residual gas bubbles remained and did not influence the injection volume (20 μL). The extensive dilution also prevented a low yield of homocysteine-mBrB derivative and the appearance of multiple peaks observed at high (>0.5 mol/L final) concentrations of $NaBH_4$.

We investigated the dose–response curve for mBrB because fluorescent material co-chromatographing with the homocysteine derivative was formed in proportion to the amount of mBrB present. The minimal concentration of mBrB giving maximal yield of homocysteine-mBrB derivative was 0.3 mmol/L (data not shown). We routinely kept the concentration of mBrB at 0.8 mmol/L. At this concentration, the maximum amount of the homocysteine-mBrB-derivative was formed within 1–2 min (data not shown).

Chromatography. We analyzed the derivatized samples with numerous systems based on separation on reversed-phase columns (C_{18}, C_8, C_6, CH, and phenyl) or a strong cation-exchange column (Partisil-10 SCX). Using a single column system, we could not separate the homocysteine-mBrB derivative from small amounts (corresponding to 0.5–1 μmol/L of homocysteine in plasma) of interfering compound(s).

The resolution was greatly improved by directing the homocysteine peak from the SCX column into a CH column by column switching. The homocysteine derivative was eluted as a single sharp peak, and baseline separation from interfering peaks was obtained (Figure 3). Thiols such as glutathione, cysteine, or DTE did not interfere with the assay, and the blank samples contained no interfering

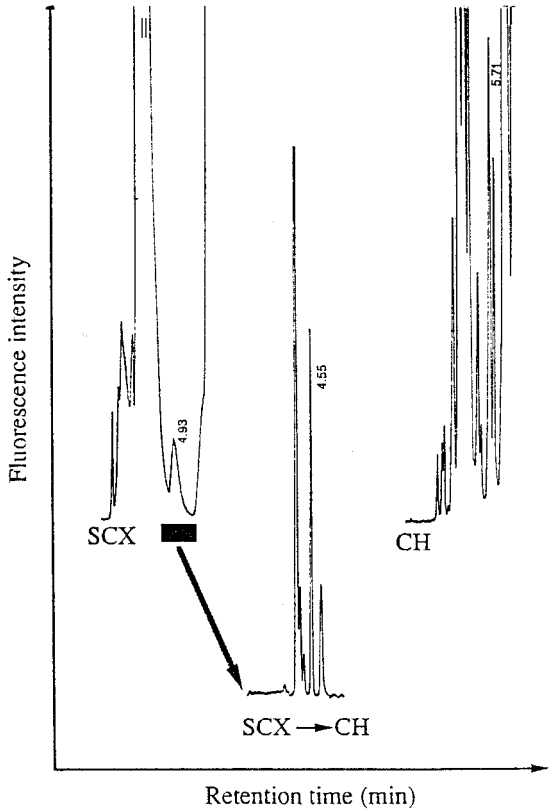

Fig. 3. Improved chromatographic resolution by column switching

A plasma sample was derivatized and stored at room temperature for 12 h. Storage increased the amount of interfering fluorescent material. The sample was chromatographed on a SCX column and on a CH column. The trace marked SCX → CH shows the chromatogram obtained when effluent from the SCX column containing the homocysteine derivative was switched (switching interval between 3.5 and 6 min) into the CH column

material (data not shown).

Upon storage of the derivatized sample, there was a time-dependent formation of fluorescent material, some of which co-chromatographed with the homocysteine peak. Therefore, we analyzed the plasma samples immediately after the derivatization was finished.

The 20-μL samples injected into the SCX column contained plasma proteins (60–70 μg) from ~1 μL of plasma. The presence of DMSO in the samples solubilized the proteins and prevented pressure build-up, even after multiple injections. The retention time of the homocysteine-mBrB derivative on the SCX column became shorter as the column aged, but the switching interval was sufficiently long to allow for some drift in retention time. The SCX column tolerated more than 1000 injections before column replacement was required.

Standard curve, linearity of the assay, sensitivity, and specificity. The standard curve for both reduced and oxidized homocysteine was linear over a wide concentration range (1–100 μmol/L), but deviated from linearity at concentrations below 1 μmol/L (Figure 4). This deviation from linearity, which was most pronounced for homocysteine, was abolished by including DTE, 100 μmol/L, in the standard solution.

We also tested the linearity of the method by assaying plasma diluted to various extents. In this case also, DTE was necessary to accomplish linearity at high dilution (Figure 4). Notably, both with plasma and with standard solutions, the deviation was more pronounced and was only

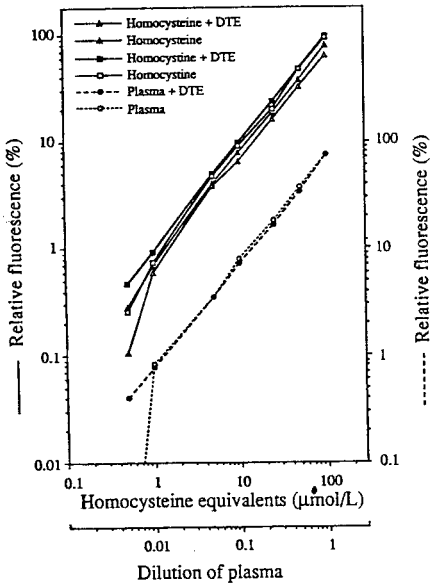

Fig. 4. Standard curves for homocysteine and homocystine, and linearity of the assay for homocysteine in plasma

The concentrations of standards corresponded to 0.5–100 μmol/L of homocysteine equivalents and the plasma (73 μmol/L, undiluted) was diluted to various extents before assay. The standard solution and the samples of diluted plasma were supplemented with 0 or 100 μmol of DTE per liter. Standards and plasma samples were analyzed together, and the results given (on identical scales) as relative fluorescence

partly prevented with DTE when we used glassware instead of plastic vials.

The sensitivity of the assay, defined as a signal-to-noise ratio ≥5, is about 0.2 μmol/L, corresponding to 0.2 pmol of homocysteine equivalents in the plasma sample.

We verified the specificity of the assay by treating the plasma sample with *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1), adenosine, and DTE. Under these conditions plasma homocysteine is quantitatively converted to *S*-adenosylhomocysteine, and the homocysteine peak is selectively removed (Figure 5). This treatment confirmed the identity of the homocysteine peak, and excluded the presence of endogenous compound(s) co-chromatographing with the homocysteine derivative.

The assay has been used for the determination of plasma homocysteine in healthy subjects, and in patients with cancer, dermatological diseases, cardiovascular conditions, and epilepsy: in all, more than 500 subjects. Although these patients were treated with several cardiovascular drugs, cancer chemotherapeutic agents, analgesics, anesthetics, anticonvulsants, or psychotropic drugs, no interference with the homocysteine peak was detected in their samples.

Precision and correlation studies. The within-run precision (CV) was 2.9% at 7.3 μmol/L, 2.8% at 13.8 μmol/L, and 1.8% at 41.8 μmol/L. The corresponding between-run CVs were 2.1%, 3.0%, and 2.0% (Table 1).

There was a good correlation ($y = -1.0547 + 1.0096x$, $r = 0.989$) between total homocysteine concentrations determined in 160 plasma samples with the present method and the values obtained for the same samples with an established radioenzymic method for total homocysteine (*17*) (Figure 6). The correlation between the fluorescence assay (x) and an assay involving reduction of samples with DTE followed by conventional amino acid analysis (y) (*23, 24*) showed the following relation between the two sets of values: $y = -1.5077 + 1.1285x$ ($r = 0.975$) (Figure 6).

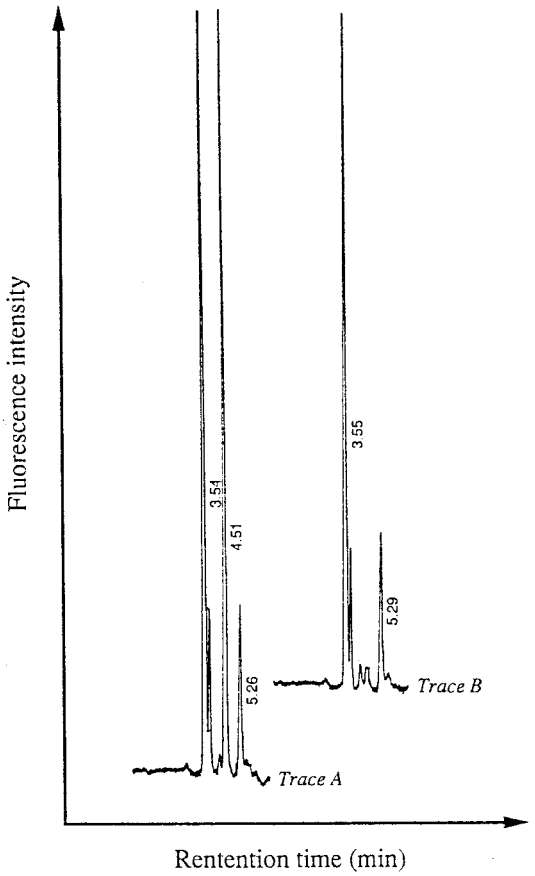

Fig. 5. Identification of the homocysteine-mBrB derivative by enzymic modification

Plasma was incubated (37 °C) with adenosine and DTE in the absence (*A*) and presence (*B*) of the enzyme *S*-adenosylhomocysteine hydrolase. In the presence of enzyme, plasma homocysteine is almost quantitatively converted to *S*-adenosylhomocysteine. The amount of plasma homocysteine in trace *A* is about 20 μmol/L; that in trace *B* is <0.5 μmol/L

Table 1. Performance Statistics of the Fluorescence Assay

Samples	Concn range, μmol/L[a]	Fluorescence assay range, μmol/L	Precision (n = 10); CV, %	
			Within-run	Between-run
Low	6.98–7.66	6.96–7.82	2.91	2.13
Medium	12.52–13.36	12.78–14.22	2.83	3.01
High	37.46–41.86	39.16–42.57	1.80	2.00

[a] Concentrations of total homocysteine in these samples were determined with the radioenzymic assay.

Discussion

Microprocessor-controlled sample processors have recently been introduced on the market, at a price comparable with that of ordinary HPLC autosamplers. Some of these instruments can be programmed to carry out column switching and complex sample-handling procedures such as precolumn derivatization. They have sample trays that can be cooled or heated, and they also can function as a sample injector for HPLC. We designed a fully automated method adjusted to the performance by the Gilson 232-401 sample processor.

Determination of total homocysteine requires the reduction of several species of homocysteine disulfides in plasma. Among the several reducing agents (*26*) that can be used

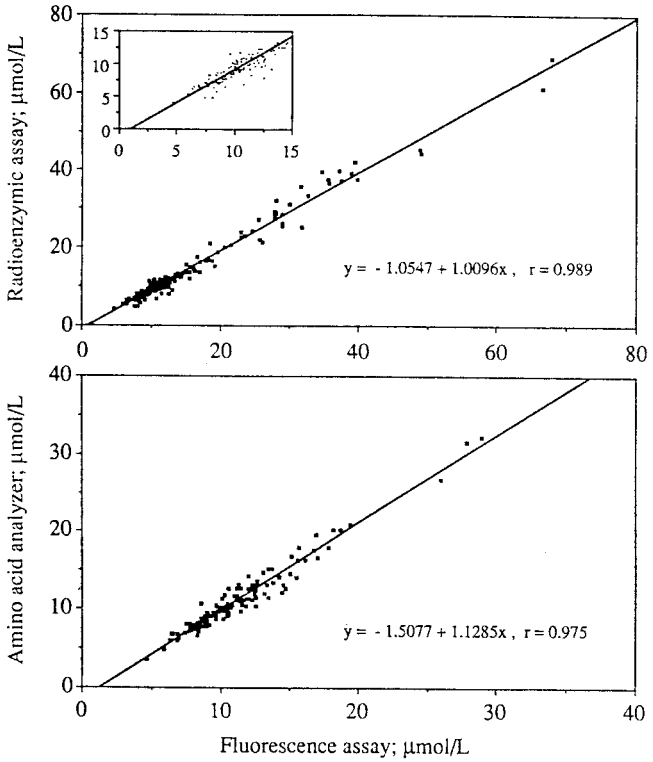

Fig. 6. Correlation between plasma homocysteine values obtained by different assays

Scatterplots for the fluorescence assay vs the radioenzymic assay (*upper panel*) and vs data obtained with an amino acid analyzer (*lower panel*)

for liberating homocysteine or other sulfur compounds from plasma proteins are DTE (*17, 23, 24*), tributylphosphine (*20*), mercaptoethanol (*19, 27*), and $NaBH_4$. Because the derivatization and chromatography are carried out in sequence in our assay, we selected a reducing agent ($NaBH_4$) and conditions suitable for fast and complete reduction and thiol liberation. Thus, the reduction step did not markedly prolong the overall analysis time. In addition, the reducing agents must be compatible with the thiol-specific derivatization agent. Thiols such as 2-mercaptoethanol and DTE are not suitable in our assay because they consume mBrB and may produce interfering fluorescent material (*28*).

In addition to the reduction of homocysteine disulfides, $NaBH_4$ serves another important function, i.e., to prevent re-oxidation of homocysteine before the derivatization of the sulfhydryl group. In the absence of a reductant, oxidation of homocysteine is pronounced (*19*), and in our assay, only 30–40% of the thiol standard could be recovered (Figure 2). Total recovery of homocysteine required less $NaBH_4$ than did total recovery of homocysteine (Figure 2), which suggests that, under the conditions of our assay, lower concentrations of reductant are necessary to prevent oxidation of the sulfhydryl group of homocysteine than to cleave the disulfide bridge by reduction.

Of the various thiol-specific derivatization reagents (*25, 28–30*), we chose mBrB for the following reasons. The fluorescence intensity of the homocysteine-mBrB derivative is sufficiently high to measure homocysteine in plasma. Furthermore, mBrB reacts rapidly with free thiols at room temperature, this step being completed within 2 min (data not shown). Other reagents, e.g., 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, require higher temperatures and long reaction times (*20*).

The column switching was an essential step in this analytical procedure and had the following impact on the analytical performance:

(*a*) We and others (*28, 31*) have observed that impurities were present in the mBrB reagent and interfering material was formed during the reaction. Such compound(s) co-chromatographed with the homocysteine-mBrB derivative in several chromatographic systems. With the present two-dimensional HPLC system, the homocysteine derivative was separated from interfering material, and procedures to remove excess reagent (*31, 32*) were not required.

(*b*) The homocysteine-derivative was loaded on the CH column with a mobile phase containing no organic solvent, and was therefore top-concentrated on the latter column. This allowed rapid elution, high chromatographic resolution, and therefore increased sensitivity.

(*c*) The sample processor did not remove plasma proteins from samples before injection into the SCX column. Moreover, the chromatographic performance of this column was not critical for the resolution of the system (Figure 3), and was only slowly deteriorated by protein loading. In addition, the SCX column served as a guard column and protected the CH column against the detrimental effect of the reagents and plasma constituents.

The identity of the homocysteine peak in the chromatogram was established by removal of homocysteine from plasma by treatment with *S*-adenosylhomocysteine hydrolase (Figure 5). The thiol groups of other sulfur compounds are not blocked by this treatment because the enzyme shows high substrate specificity towards homocysteine (*33*).

The sensitivity of our method (detection limit 0.2 pmol) compares favorably with that of other homocysteine assays (*17–22*). Major determinants of the sensitivity are the high chromatographic resolution; the horizontal, stable baseline and low noise; baseline separation from interfering compounds; high fluorescence intensity of the homocysteine derivative; and linearity of the standard curve. The linearity of the standard curve below 1 µmol/L was produced by including DTE in the standard solutions (Figure 4), which possibly prevented binding of free thiol to the surface of the reaction vials.

The chromatographic features of the assay listed above also contribute to the high analytical precision (Table 1). Notably, such precision was obtained without including an internal standard. Objections to the inclusion of internal standard in assays for sulfur compounds based on derivatization of free thiol groups include: different reactivity of homocysteine and the chosen standard with the derivatization agents (*28*) or with $NaBH_4$ as a source of erratic results; long retention time of the internal standard, for prolonged analysis time; and different chromatographic behavior of these two derivatives, which may be incompatible with column switching.

We compared the present assay with two established assays (*17, 23, 24*) for total homocysteine in human plasma. In both, homocysteine is liberated from plasma protein by using DTE as a reducing agent. The values obtained with the present fluorescence assay showed a good correlation with those obtained with both comparison methods (r = 0.989 vs the radioenzymic assay, and r = 0.975 vs the amino acid analyzer). In the lower range the amino acid analyzer gave slightly lower values than those obtained with the two other methods (Figure 6, lower panel); this may be related to the fact that such low concentrations

approach the detection limit of the ninhydrin-based method.

In conclusion, the present method is a fully automated procedure for the determination of total homocysteine in human plasma. Multiple steps in the sample-handling procedure and chromatography are carried out by an inexpensive and versatile microprocessor-controlled sample processor. The analysis shows high precision, and has been verified by two comparison methods. The present method has been found reliable as a routine assay in our laboratory, and about 70 plasma samples can be left for unattended analysis in 24 h. Obviously, the method can be easily adopted for the assay of free homocysteine, and can also be modified to measure other sulfur compounds in which a large fraction is bound to plasma proteins via a disulfide linkage.

The technical assistance of G. Kvalheim and E. Leirnes is highly appreciated. This work was supported by grants from the Norwegian Cancer Society and the Norwegian Society for Science and the Humanities.

References

1. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beadet AL, Sly WS, Valle D, eds. The metabolic basis for inherited diseases. New York: McGraw-Hill, 1989: in press.
2. Ueland PM, Refsum H, Svardal AM, Djurhuus R, Helland S. Perturbation of homocysteine metabolism by pharmacological agents in experimental and clinical use. In: Aarbakke J, Chiang PK, Koeffler HP, eds. Tumor cell differentiation. Biology and pharmacology. Clifton, NJ: Humana Press, 1987:269–78.
3. Ueland PM, Refsum H. Plasma homocysteine, a risk factor for vascular disease. Modulation by pharmacological intervention and pathological states. J Lab Clin Med 1989: in press.
4. Stabler SP, Marcell PD, Podell ER, Allen RH, Savage DG, Lindenbaum J. Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. J Clin Invest 1988;81:466–74.
5. Lindenbaum J, Healton EB, Savage DG, et al. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. N Engl J Med 1988;318:1720–8.
6. Kang SS, Wong PWK, Norusis M. Homocysteinemia due to folate deficiency. Metabolism 1987;36:458–62.
7. Wilcken DEL, Gupta VJ. Sulphur containing amino acids in chronic renal failure with particular reference to homocystine and cysteine-homocysteine mixed disulphide. Eur J Clin Invest 1979;9:301–7.
8. Wilcken DEL, Gupta VJ, Betts AK. Homocysteine in the plasma of renal transplant recipients: effects of cofactors for methionine metabolism. Clin Sci 1981;61:743–9.
9. Kredich NM, Hershfield MS, Falletta JM, Kinney TR, Mitchell B, Koller C. Effects of 2′-deoxycoformycin on homocysteine metabolism in acute lymphoblastic leukemia [Abstract]. Clin Res 1981;29:541A.
10. Refsum H, Helland S, Ueland PM. Fasting plasma homocysteine as a sensitive parameter to antifolate effect. A study on psoriasis patients receiving low-dose methotrexate treatment. Clin Pharmacol Ther 1989; in press.
11. Brattström L. Homocysteine in vascular disease. A clinical study of homocysteine metabolism in health and in vascular disease. Thesis, University of Lund, Sweden, 1989.
12. Gupta VJ, Wilcken DEL. The detection of cysteine–homocysteine mixed disulphide in plasma of normal fasting man. Eur J Clin Invest 1978;8:205–7.
13. Wilcken DEL, Gupta VJ. Cysteine–homocysteine mixed disulphide: differing plasma concentrations in normal men and women. Clin Sci 1979;57:211–5.
14. Boers GH, Smals AG, Trijbels FJ, Leermakers AI, Kloppenborg PW. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. J Clin Invest 1983;72:1971–6.
15. Brattström LE, Hardebo JE, Hultberg BL. Moderate homocysteinemia—a possible risk factor for arteriosclerotic cerebrovascular disease. Stroke 1984;15:1012–6.
16. Murphy-Chutorian DR, Wexman MP, Grieco AJ, et al. Methionine intolerance: a possible risk factor for coronary artery disease. J Am Coll Cardiol 1985;6:725–30.
17. Refsum H, Helland S, Ueland PM. Radioenzymic determination of homocysteine in plasma and urine. Clin Chem 1985;31:624–8.
18. Kang SS, Wong PWK, Cook HY, Norusis M, Messer JV. Protein-bound homocyst(e)ine. A possible risk factor for coronary artery disease. J Clin Invest 1986;77:1482–6.
19. Stabler SP, Marcell PD, Podell ER, Allen RH. Quantitation of total homocysteine, total cysteine, and methionine in normal serum and urine using capillary gas chromatography–mass spectrometry. Anal Biochem 1987;162:185–96.
20. Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. J Chromatogr 1987;422:43–52.
21. Brattström L, Israelsson B, Norrving B, et al. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease—effects of pyridoxine and folic acid treatment. Atherosclerosis 1989, submitted.
22. Chu RC, Hall CA. The total serum homocysteine as an indicator of vitamin B_{12} and folate status. Am J Clin Pathol 1988;90:446–9.
23. Brattström L, Israelsson B, Lindgärde F, Hultberg B. Higher total plasma homocysteine in vitamin B_{12} deficiency than in heterozygosity for homocystinuria due to cystathionine β-synthase deficiency. Metabolism 1988;37:175–8.
24. Brattström LE, Israelsson B, Jeppsson JO, Hultberg BL. Folic acid—an innocuous means to reduce plasma homocysteine. Scand J Clin Lab Invest 1988;48:215–21.
25. Kosower NS, Kosower EM. Thiol labeling with bromobimane. Methods Enzymol 1987;143:76–84.
26. Jocelyn PC. Chemical reduction of disulfides. Methods Enzymol 1987;143:246–56.
27. Kang SS, Wong PWK, Curley K. The effect of D-penicillamine on protein-bound homocyst(e)ine in homocystinurics. Pediatr Res 1982;16:370–2.
28. Fahey RC, Newton GL. Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. Methods Enzymol 1987;143:85–96.
29. Imai K, Toyo'oka T. Fluorometric assay of thiols with fluorobenzoxadiazoles. Methods Enzymol 1987;143:67–75.
30. Jocelyn PC. Spectrophotometric assay of thiols. Methods Enzymol 1987;143;44–67.
31. Baeyens W, van der Weken G, Lin Ling B, de Moerloose P. HPLC determination of *N*-acetylcysteine in pharmaceutical preparations after pre-column derivatization with Thiolyte® MB using fluorescence detection. Anal Lett 1988;21:741–57.
32. Fahey RC, Newton GL, Dorian R, Kosower EM. Analysis of biological thiols: quantitative determination of thiols at the picomole level based upon derivatization with monobromobimanes and separation by cation-exchange chromatography. Anal Biochem 1981;111:357–65.
33. Guranowski A, Jakubowski H. Substrate specificity of *S*-adenosylhomocysteinase. Cysteine is a substrate of the plant and mammalian enzymes. Biochim Biophys Acta 1983;742:250–6.