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Rapid HPLC Determination of Total Homocysteine and Other Thiols in Serum and Plasma: Sex Differences and Correlation with Cobalamin and Folate Concentrations in Healthy Subjects

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High-performance liquid chromatography with fluorescence detection has been utilized for the rapid determination of total homocysteine, cysteine, and cysteinylglycine in human serum and plasma. Our earlier procedure (Anal Biochem 1989;178:208), which used monobromobimane to specifically derivatize thiols, has been extensively modified to allow for rapid processing of samples. As a result, >80 samples a day can be assayed for total homocysteine, cysteine, and cysteinylglycine. The method is sensitive (lower limit of detection ≤4 pmol in the assay) and precise (intra- and interassay CV for homocysteine, 3.31% and 4.85%, respectively). Mean total homocysteine concentrations in plasma and serum were significantly different, both from healthy male donors (9.26 and 12.30 μmol/L, respectively; $P < 0.001$) and healthy female donors (7.85 and 10.34 μmol/L, respectively; $P < 0.001$). The differences in total homocysteine between sexes were also significant ($P = 0.002$ for both plasma and serum). Similar differences were found for cysteine and cysteinylglycine. We found a significant inverse correlation between serum cobalamin and total homocysteine in men ($P = 0.0102$) and women ($P = 0.0174$). Serum folate also inversely correlated with total homocysteine in both sexes.

Indexing Terms: *hyperhomocysteinemia/cysteine/cysteinylglycine/fluorometry/chromatography, reversed-phase/monobromobimane/sex-related differences*

Hereditary cystathionine β-synthase deficiency and certain inborn errors of cobalamin (B_{12}) and folic acid transport and metabolism can result in severe hyperhomocysteinemia and homocystinuria (*1–3*). An early recognizable clinical manifestation of homocystinuria is an unusually high incidence of premature cardiovascular disease (*4, 5*), often the cause of patient mortality (*1*). Recently, several clinical studies (reviewed in *6–10*) reported an association between milder degrees of hyperhomocysteinemia and coronary artery disease (*11, 12*), cerebrovascular disease (*13, 14*), and peripheral arterial occlusive disease (*14, 15*). In many of these studies, hyperhomocysteinemia was an independent risk factor for the cardiovascular disease (*11, 14, 15*). The etiology of mild to moderate hyperhomocysteinemia—in the absence of frank deficiency of cobalamin, folic acid, pyridoxine (B_6)—in patients with cardiovascular disease has not been established with certainty. Evidence suggests that some of these individuals are heterozygous for cystathionine β-synthase deficiency (*11, 16*). However, the phenotypic determinants currently used to establish heterozygosity show considerable overlap with normal individuals (*17*). Thermolabile methylenetetrahydrofolate reductase, an inherited enzyme defect that also results in hyperhomocysteinemia (*18*), has recently been reported to be an independent risk factor for coronary artery disease (*19*). The roles played by homocysteine in atherogenesis and thrombogenesis and the effect of therapeutic lowering of plasma homocysteine on cardiovascular disease are unknown. Accurate determination of serum and plasma concentrations of homocysteine is essential for understanding the role of homocysteine in the pathogenesis of vascular disease. Because plasma homocysteine concentrations can be lowered by administration of folic acid (*20–22*) or cobalamin (*23, 24*), assessment of homocysteine status in subjects involved in dietary modification or vitamin supplementation programs, as well as in cardiovascular disease patients at large, will require rapid and reproducible assays.

Early studies of plasma from patients with homocystinuria reported an assortment of abnormal sulfur-bearing amino acids, including homocystine and homocysteine-cysteine mixed disulfide, which, at the time, were undetectable in normal plasma (*25*). Only later were these metabolites also found in normal plasma at very low concentrations (*26–28*). It is now known that 75–90% of the homocysteine in normal plasma is covalently bound to plasma proteins by disulfide bonds (*29–31*). To determine total plasma homocysteine—the sum of all protein-bound forms, oxidized low-molecular-mass forms, and free reduced homocysteine—it is necessary to reduce disulfide bonds.

An early radioenzymatic method for total plasma homocysteine included dithioerythritol as the reducing agent to break disulfide bonds (*30*). More recent methods for determining total plasma homocysteine have utilized 2-mercaptoethanol as the reducing agent and

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gas chromatography–mass spectrometry (*32*); sodium borohydride as the reducing agent and monobromobimane as the thiol-specific fluorochromophore, followed by HPLC with fluorescence detection (FD) (*31, 33, 34*); tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate as the fluorochromophore, followed by HPLC-FD (*35–37*); sodium borohydride as the reducing agent and HPLC with electrochemical detection (ED) (*15*); or dithiothreitol as the reducing agent, followed by automated ion-exchange chromatography (*38*) or HPLC and thiol-specific postcolumn derivatization with ultraviolet absorbance detection (UVD) (*39*).[8] Most of these methods have been reviewed in greater detail by Ueland et al. (*40*) and Jacobsen (*41*).

Here we describe a new method for the rapid determination of total plasma (or serum) homocysteine by using simultaneous sodium borohydride reduction of disulfide bonds and derivatization of sulfhydryl groups with monobromobimane followed by HPLC-FD. Based on methodology developed earlier (*31*), the new method eliminates some of the steps required in the previous method, namely, removal of excess fluorochromophore and solid-phase extraction of samples before HPLC-FD. There being no lengthy incubations and only minimal sample processing, ~85 samples plus appropriate calibrators and quality-control serum samples can be analyzed within 24 h. The method also provides quantitative information on total plasma cysteine and cysteinylglycine (CysGly). Besides describing the method, we also report its use to determine significant differences in the concentration of total homocysteine between plasma and serum and significant differences between healthy men and women in the concentrations of total homocysteine, cysteine, and CysGly.

Materials and Methods

Reagents. Monobromobimane (Thiolyte®) and L-homocysteine thiolactone were obtained from Calbiochem-Behring Diagnostics (La Jolla, CA). Monobromobimane was also obtained from Molecular Probes (Eugene, OR). Glutathione, L-cysteine, L-cysteinylglycine (CysGly), L-homocystine, sodium borohydride, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), Trizma base (Tris), cyanogen bromide, ethylenediamine, Sepharose 4B-CL, and *N*-acetylhomocysteine thiolactone were from Sigma Chemical Co. (St. Louis, MO). Cystine-bis-diglycine was from Serva Fine Chemicals (Westbury, NY). Perchloric acid, HPLC-grade acetonitrile, and HPLC-grade methanol were from Fisher Scientific (Fair Lawn, NJ). HPLC-grade water was produced by a Milli-Q water purification system from Millipore (Bedford, MA). Amyl alcohol was from Curtin Matheson Scientific (Houston, TX). Other chemicals were of HPLC, analytical, or reagent grade and obtained from Fisher Scientific. Sepharose-SH [Sepharose(4BCL)-ethylamido(*N*-acetyl)-homocysteine] beads were prepared as described previously (*31*) by slight modifications of the methods of March et al. (*42*) and Cuatrecasas (*43*). Briefly, this solid-phase thiol is prepared by reacting Sepharose-ethylamine with *N*-acetylhomocysteine thiolactone. The immobilized primary amine opens the thiolactone to form the amide of *N*-acetylhomocysteine and a free -SH group. Sepharose-SH is used to scavenge excess monobromobimane during the preparation of the thiol-bimane standards as described below.

Blood collection and subjects. Blood was obtained by venipuncture from fasting apparently healthy laboratory personnel. The mean (± SD) age for male donors (n = 36) was 34.4 ± 9.4 years; for female donors (n = 35), it was 33.8 ± 6.5 years (ranges 22–66 and 25–48, respectively).[9] For serum, blood was collected in evacuated tubes, allowed to clot for 1 h at room temperature, and centrifuged; the serum supernate was stored at −20°C. For plasma, blood was collected in chilled evacuated tubes containing EDTA, gently mixed, placed on ice, and centrifuged within 30 min of collection; the plasma supernate was stored at −20°C. The procedures used on human subjects in this study were in accordance with ethical standards and were approved by the Institutional Review Boards of the Cleveland Clinic Foundation and the Oregon Health Sciences University.

Determination of total serum homocysteine, cysteine, and CysGly. Serum or plasma samples (100 μL) were pipetted into 1.5-mL conical snap-cap polypropylene tubes (no. 214-3418-030; Evergreen Scientific, Los Angeles, CA) along with 10 μL of water (to compensate for volume dilution during calibration curve determination as described below) and 5 μL of *n*-amyl alcohol. The samples were then gently vortex-mixed. Sodium borohydride (35 μL of 1.43 mol/L reagent in 0.10 mol/L sodium hydroxide) was added to each tube, followed by mixing. After adding hydrochloric acid (35 μL of 1.0 mol/L) and mixing, we added 50 μL of 10.0 mmol/L monobromobimane in 4.0 mmol/L sodium EDTA (pH 7.0). (The latter reagent was prepared by diluting 0.50 mL of 50 mmol/L monobromobimane in acetonitrile with 2.0 mL of 5.0 mmol/L sodium EDTA, pH 7.0.) The tubes were capped, mixed, and incubated at 42°C for 12 min. After the samples cooled to room temperature, we added 50 μL of 1.50 mol/L perchloric acid, vortex-mixed the samples, and kept them at room temperature for 10 min. Protein was removed by centrifugation (Microfuge 12; Beckman Instruments, Fullerton, CA) at 12 200*g* for 10 min. The acidic clear supernate was adjusted to pH 4 in the centrifuge tube by adding 25 μL of 2.00 mol/L Tris, gently mixing, and centrifuging again for 1 min. An aliquot (100 μL from a final volume of 310 μL) of the supernate was transferred to a glass conical insert (no. 200-238; Sun Brokers, Wilmington, NC) contained in a 12 × 32 mm glass sample vial and then sealed with a

[8] Nonstandard abbreviations: FD, fluorescence detection; ED, electrochemical detection; and UVD, ultraviolet absorbance detection.

[9] Primary data showing donors' age; plasma and serum homocysteine, cysteine, and cysteinylglycine; and serum cobalamin, folate, and methylmalonate can be provided upon request.

10-mil (0.01-in., ~0.25-mm) Teflon disc (no. 95280; Alltech, Deerfield, IL) for HPLC analysis.

HPLC-FD. A fixed-volume autosampler (SP8875; Spectra Physics, San Jose, CA) with an 80-sample capacity was used for sample injection. Samples were kept at room temperature on the autosampler for as long as 24 h without deterioration. While on the autosampler, they were shielded from room lighting with an opaque cover. The autosampler injected 20-μL aliquots onto a 4.6 × 250 mm RP8 Ultrasphere column (no. 235332; 5-μm column packing; Beckman Instruments) equipped with Brownlee RP18 New Guard column (no. 0711-0092; 7-μm column packing; Applied Biosystems, Foster City, CA). Two Kratos Spectroflow 400 HPLC pumps (Applied Biosystems) were used to develop a methanol gradient. The buffer for pump A consisted of water: methanol:acetic acid (94.75:5.00:0.25 by vol) titrated to pH 3.40 with 5.0 mol/L NaOH. Pump B contained 100% methanol.

For routine determination of cysteine-*S*-bimane, Cys(-*S*-bimane)Gly, and homocysteine-*S*-bimane, the column was developed at a flow rate of 2.0 mL/min as follows: 0–1 min, 0% B; 1–3 min, 0–10% B; 3–9 min, 10–15% B; 9–10 min, 15–100% B; 10–11 min, 100% B; 11–12 min, 100–0% B; and 12–15 min, 0% B. If glutathione-*S*-bimane was also to be determined, an extra isocratic segment was inserted from 10 to 11 min (15% B), thus extending the overall program to 16 min. Thiol-bimane adducts were detected fluorometrically with a Kratos Spectroflow 980 HPLC detector (Applied Biosystems) with the excitation wavelength set at 390 nm and the emission wavelength >418 nm with a cutoff filter. The sensitivity range and rise time of the detector were set at 0.1 and 2 s, respectively. The fluorescence detector output was recorded on an integrating recorder (Chrom-Jet; Spectra Physics).

Calibration curves for homocysteine and other thiols. The calibration curve for total serum homocysteine was established as follows: 100-μL aliquots of quality-control sera were fortified with 10 μL of solutions containing L-homocystine of 10.0 to 1000 μmol/L. The blank consisted of sera with 10 μL of water only. The fortified and blank sera were analyzed for total serum homocysteine as described above. The integrated peak areas for homocysteine-*S*-bimane in the fortified samples (minus endogenous homocysteine in the blank) were plotted against the final concentration of added homocysteine. The regression analysis equation obtained from the calibration curve was used to calculate the concentration of homocysteine in normal and patients' sera. Calibration curves for cysteine and CysGly were established similarly.

Preparation of homocysteine-S-bimane and other thiol-bimane standards. L-Homocysteine-*S*-bimane was prepared from L-homocystine as follows: 0.20 mL of 0.25 mmol/L L-homocystine in 1.00 mmol/L sodium EDTA (pH 7.0) was incubated with 0.10 mL of 2.00 mmol/L monobromobimane in 1.00 mmol/L sodium EDTA (pH 7.0), 0.70 mL of 14.3 mmol/L ammonium bicarbonate containing 1.43 mmol/L sodium EDTA (pH 8.0), and 0.05 mL of 0.26 mol/L sodium borohydride in 0.05 mol/L Tris-HCl (pH 8.5) for 10 min at room temperature. Sepharose-SH beads (0.20 mL of an equivolume suspension of beads in 5.0 mmol/L sodium EDTA, pH 6.0) were added to remove excess monobromobimane. After mixing for 20 min at room temperature, we removed the beads by centrifugation, acidifed the supernate containing L-homocysteine-bimane using 0.10 mL of 1.74 mol/L acetic acid, and stored the supernate at −20°C. L-Cysteine-bimane, L-Cys(-*S*-bimane)Gly, and glutathione-*S*-bimane were prepared from L-cysteine, L-Cys(-SH)Gly, and reduced glutathione, respectively, as described above, except that 0.20 mL of 0.50 mmol/L thiol was used without sodium borohydride reduction. The thiol concentration of freshly prepared stock solutions was determined by the method of Ellman (*44*). The four acidified thiol-bimane standards were stable for as long as 3 months when stored at 4°C in the dark.

Characterization of homocysteine-S-bimane and other thiol-bimane standards. To determine the ultraviolet–visible absorbance spectra, relative fluorescence quantum yields, and fluorescence excitation and emission maxima, we purified the thiol-bimane standards by reversed-phase HPLC-UVD as follows: 0.50 mL of the standard preparations just described was injected onto a 4.6 × 250 mm RP8 Ultrasphere column with a Brownlee RP18 New Guard column, as described above for HPLC-FD. The column was developed isocratically at 2.0 mL/min by using a Beckman HPLC system consisting of two 114M pumps, a Model 421A controller, and a Model 160 absorbance detector operating at 254 nm. The solutions for pump A and pump B were also as described above. Pump volume ratios (A/B) were 91/9 for the purification of homocysteine-*S*-bimane and glutathione-*S*-bimane and 95/5 for cysteine-*S*-bimane and Cys(-*S*-bimane)Gly. Baseline-resolved peaks, detected by their absorbance and corresponding to the individual thiol-bimane standards, were collected manually. The samples were evaporated to dryness in a Savant Speed-Vac, reconstituted in 1.0 mL of buffer A (see HPLC-FD):methanol (88:12 by vol), and stored in the dark at 4°C. The thiol-bimane standards were >98% pure as judged by reanalysis with HPLC-UVD and HPLC-FD.

Comparison of methods. Random specimens from 118 individuals undergoing total plasma homocysteine determination were analyzed by the current method and by an independent method based on HPLC-ED (*15*) at the Oregon Health Sciences University.

Statistical analysis. The Wilkes–Shapiro test and examination of quintile–quintile plots were used to evaluate distribution of data. Within-subject serum vs plasma comparisons were examined by Student's paired *t*-test. Overall group comparison (e.g., male vs female plasma homocysteine) involving continuous variables that were not in a gaussian distribution were analyzed by the nonparametric Wilcoxon rank sum test. Gaussian-distributed group data were compared by using Student's unpaired *t*-test. Pearson correlation coefficients and probabilities were calculated for the relationships between serum and plasma total homocysteine and serum cobalamin and folate levels. Comparison of

results obtained by this method and an independent method for the determination of total plasma homocysteine was done by linear regression analysis as well as by the Bland–Altman procedure (*45*).

Other methods. Donors' sera were analyzed for folic acid and cobalamin with a kit assay (no. 262226; Becton Dickinson, Orangeburg, NY). In the Cleveland Clinic laboratory the reference range for serum folic acid is >2.3 μg/L (>5.2 nmol/L) and for serum cobalamin is 170 to 700 ng/L (125 to 517 pmol/L). All of the healthy subjects in this study were within reference range values for serum folate and cobalamin. Methylmalonic acid was also determined in donors' sera by stable-isotope dilution and gas-chromatography–mass spectrometry by the method of Rasmussen (*46*) except that we used 5.0 nmol of [C^2H_3]methylmalonic acid instead of 1.0 nmol. The reference range (mean ± 3 SD) for serum methylmalonic acid in healthy males and females in this laboratory is 79–376 nmol/L. Fluorescence excitation and emission spectra were obtained with an Aminco-Bowman spectrofluorometer (Model J4-8960A; SLM Instruments, Urbana, IL). Absorbance spectra were obtained with an ultraviolet–visible recording spectrophotometer equipped with a printer/plotter (Uvikon 860; Kontron, Everett, MA). We confirmed the concentration of L-homocysteine (primary standard) by amino acid analysis with a Beckman automated amino acid analyzer (Model 6300).

Results

Assay Performance

Determination of total serum homocysteine, cysteine, and CysGly. As shown in Fig. 1A, the four thiol-bimane standards are separated with near-baseline resolution; retention times are: cysteine-*S*-bimane, 6.21 min; Cys(-*S*-bimane)Gly, 6.63 min; homocysteine-*S*-bimane, 8.69 min; and glutathione-*S*-bimane, 10.73 min. A typical HPLC-FD chromatogram of normal human serum (Fig. 1B) shows retention times for cysteine-*S*-bimane, Cys(-*S*-bimane)Gly, and homocysteine-*S*-bimane of 6.20, 6.65, and 8.70 min, respectively. The homocysteine peak at 8.70 min in Fig. 1B corresponds to a serum concentration of 10.7 μmol/L. Sera from individuals with hyperhomocysteinemia, primarily from pernicious anemia, were pooled and used as high-concentration quality-control samples. The HPLC chromatogram for serum containing above-normal homocysteine is shown in Fig. 1C. The homocysteine-*S*-bimane peak (retention time 8.70 min) corresponded to a serum concentration of 52.4 μmol/L.

Calibration curves and linearity. Calibration curves were obtained by assaying normal human serum with added known amounts of L-cysteine, L-CysGly, or L-homocystine and correcting for the endogenous thiol content in the serum. The calibration curve for homocysteine (Fig. 2) was linear over the concentration range of 0–200 μmol/L for added homocysteine ($y = -3.33 + 3.38x$; $r = 0.9995$). The calibration curves for cysteine, 0–492 μmol/L, and CysGly, 0–123 μmol/L, were also linear: $y = -0.06 + 3.61x$ ($r = 0.988$) and $y = -2.75 + 3.18x$ ($r = 0.990$), respectively (data not shown). The calibration curves were used for calculating concentrations of total homocysteine, cysteine, and CysGly in unknown serum and plasma samples from healthy donors and patients.

Detection limits. The assay easily detects serum and plasma total homocysteine concentrations <1 μmol/L. In our experience, total plasma homocysteine <3 μmol/L is exceedingly rare in either patients or healthy donors. The minimal amount of homocysteine-*S*-bimane detected in the assay was 2–4 pmol (signal-to-noise ratio of 3). Because the relative quantum yields of cysteine-*S*-bimane and Cys(-*S*-bimane)Gly are similar to that of homocysteine-*S*-bimane, we estimate the detection sensitivity for these analytes to be at least 10 pmol.

Precision. The intraassay CV for total serum homocysteine, cysteine, and CysGly was determined by including normal-concentration quality-control serum (n

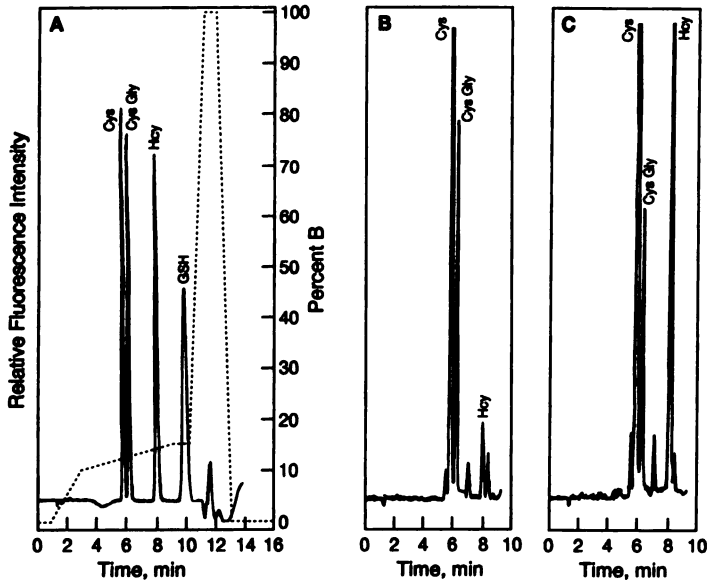

Fig. 1. HPLC-FD of thiol-bimane standards (*A*), normal human serum (*B*), and hyperhomocysteinemic serum (*C*).

(*A*) A mixture of standards, including purified cysteine-*S*-bimane (*Cys*), Cys(-*S*-bimane)Gly (*CysGly*), homocysteine-*S*-bimane (*Hcy*), and glutathione-*S*-bimane (*GSH*) was eluted from the column with a methanol gradient (••••) as described in *Materials and Methods*. Total cysteine, CysGly, and homocysteine were determined in normal human serum (*B*) or in hyperhomocysteinemic serum (*C*) and analyzed by HPLC-FD as described in the text. The gradient used was identical to that in *A* except that the isocratic segment from 9 to 10 min was omitted.

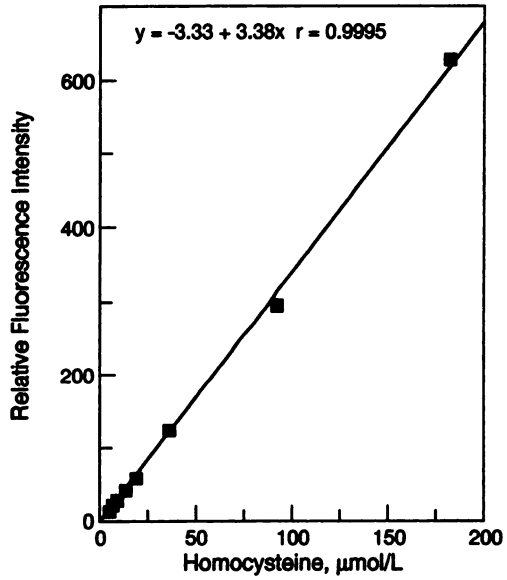

Fig. 2. Calibration curve for total serum homocysteine.

= 7) and hyperhomocysteinemic serum (n = 7) at evenly spaced intervals in a single assay of patients' samples. The interassay CV was determined by averaging the values from two normal quality-control sera and two hyperhomocysteinemic quality-control sera for seven different assays done during 1 month; the mean ± SD of the average values (n = 7) were then used to calculate the interassay CV for total serum homocysteine, cysteine, and CysGly. For total homocysteine, the intra- and interassay CVs for sera from healthy donors (means = 10.63 and 10.97 μmol/L) were 3.31% and 4.85%, respectively; for hyperhomocysteinemic sera (means = 52.8 and 56.0 μmol/L), intra- and interassay CVs were 3.78% and 3.49%, respectively. For total cysteine, the intra- and interassay CVs for normal sera (means = 208.4 and 214.5 μmol/L) were 2.35 and 3.00%, respectively; for total CysGly (means = 24.84 and 25.97 μmol/L), these were 3.96% and 7.46%, respectively.

Comparison of methods. Patients' plasma samples (n = 118) were assayed by the current method and by a previously published method based on HPLC-ED (*15*). Results from the two assays are plotted in Fig. 3A. Linear regression analysis of the data ($y = 1.19 + 0.91x$; $r = 0.974$; $S_{y|x} = 0.02$ μmol/L) showed good agreement between the two assays. Because duplicate values were available for 117 patients' samples analyzed by HPLC-FD and HPLC-ED, we analyzed the data by the Bland–Altman procedure for assessing agreement between two methods of clinical measurement. When the difference between the HPLC-ED and HPLC-FD means was plotted against the mean of the HPLC-ED and HPLC-FD means, there was good agreement and no bias between the two methods (Fig. 3B). That is, the two methods would disagree by no more than 3 μmol/L with 95% confidence.

Characterization of Thiol-Bimane Standards

The absorbance spectrum of HPLC-purified homocysteine-*S*-bimane exhibited absorbance maxima at 234 nm (relative absorbance = 1.00), 256 nm (0.603), and

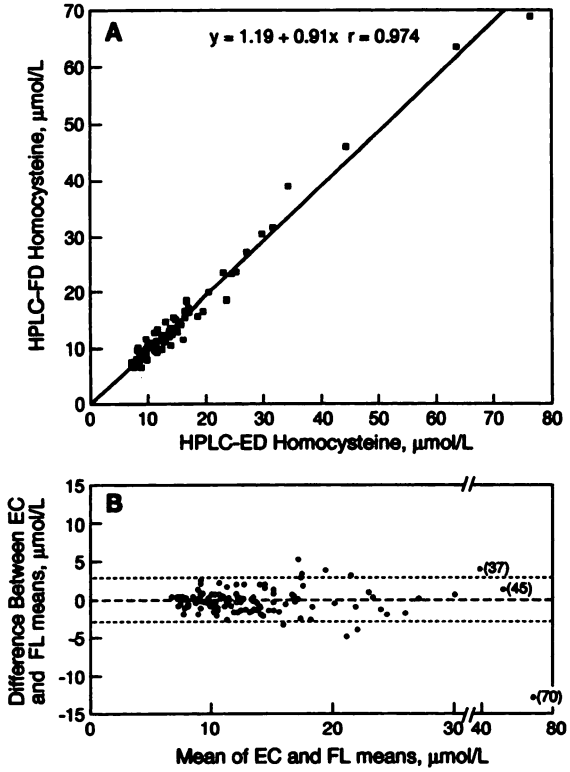

Fig. 3. (*A*) Comparison of total plasma homocysteine concentrations in patients determined by two independent methods, HPLC-FD (the current method) and HPLC-ED; (*B*) comparison of the same methods by the Bland–Altman procedure (*45*).
EC, electrochemical; FL, fluorescence.

389 nm (0.287) as shown in Fig. 4A. Similar absorbance spectra were obtained for HPLC-purified cysteine-*S*-bimane [maxima at 234 nm (1.00), 256 nm (0.593), and 389 nm (0.279)], Cys(-*S*-bimane)Gly [234 nm (1.00), 256 nm (0.582), and 389 nm (0.273)], and glutathione-*S*-bimane [234 nm (1.00), 256 nm (0.592), and 389 nm (0.279)]. The uncorrected excitation and emission spectra of homocysteine-*S*-bimane (Fig. 4B) exhibited maxima at 390 and 478 nm, respectively. Excitation and emission maxima for cysteine-*S*-bimane, Cys(-*S*-bi-

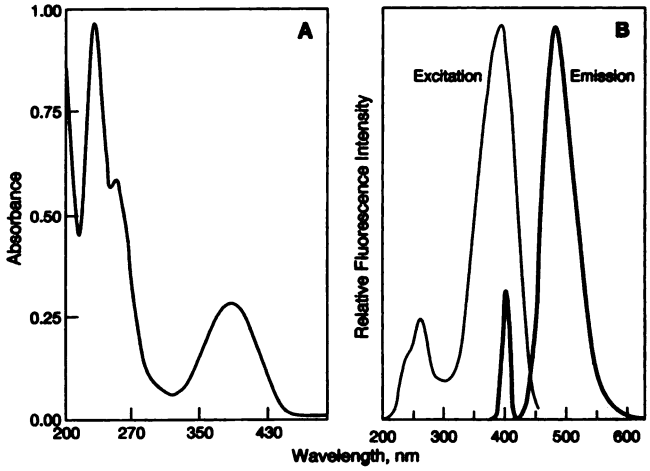

Fig. 4. Spectral characterization of homocysteine-*S*-bimane purified by reversed-phase HPLC: (*A*) ultraviolet–visible absorbance spectrum; (*B*) excitation and emission spectra.

mane)Gly, and glutathione-*S*-bimane were 392/480 nm, 392/480 nm, and 392/479 nm, respectively (data not shown). The relative fluorescence intensities of homocysteine-*S*-bimane, cysteine-*S*-bimane, Cys(-*S*-bimane)Gly, and glutathione-*S*-bimane at their emission maxima and at equivalent concentrations, based on absorbance at 389 nm, were 94.8, 97.0, 76.9, and 84.6, respectively.

Plasma and Serum Reference Values in Healthy Donors

Homocysteine. Total plasma and serum homocysteine were determined in 36 healthy men and 35 healthy women. Serum cobalamin and folate values were also determined for each donor (see footnote [9]). The plasma and serum values for total homocysteine in the men were not normally distributed, but the natural logarithm transformation of these values was. For ln-transformed values, the range (±SD) was first determined on the ln scale and then retransformed back to the original units by exponentiation. The mean (±SD) total homocysteine in plasma and serum from men (see Table 1) was 9.26 (±1.88) and 12.30 (±2.30) μmol/L, respectively, a highly significant difference (P <0.001). The ranges (mean ± 2 SD) in plasma and serum were 6.18–13.37 and 8.56–17.12 μmol/L, respectively.

In women, the plasma and serum values for total homocysteine were normally distributed, with means (±SD) of 7.85 (±2.29) and 10.34 (±3.23) μmol/L, respectively (significantly different: P <0.001). The ranges (mean ± 2 SD) for total plasma and serum homocysteine were 3.27 to 12.43 and 3.92 to 16.84 μmol/L, respectively (Table 1).

For every donor in this study, the total plasma homocysteine concentration was less than the total serum homocysteine concentration. The differences between men's and women's mean plasma and serum total homocysteine concentrations were significant: P = 0.002 for both plasma and serum (Table 1).

Men showed a significant negative correlation (Pearson) between serum cobalamin and total plasma homocysteine (r = −0.499, P = 0.0019) and between serum cobalamin and total serum homocysteine (r = −0.423, P = 0.0102). In women the negative correlation between serum cobalamin and total serum homocysteine was significant (r = −0.400, P = 0.0174), but not between total plasma homocysteine and serum cobalamin (r = −0.254, P = 0.1411). There were also significant negative correlations between serum total homocysteine and serum folate in men and women (r = −0.356, P = 0.0330; and r = −0.360, P = 0.0337, respectively) and between plasma total homocysteine and serum folate (r = −0.3059, P = 0.070; and r = −0.409, P = 0.0147, respectively). Results of linear regression analyses between serum homocysteine and serum cobalamin and folate by sex are shown in Fig. 5.

Cysteine. The plasma and serum values for total cysteine in healthy men and women were normally distributed, with means (±SD) of 209.6 (±28.42) and 266.9 (±34.23) μmol/L (P = 0.032), respectively, in men and 190.7 (±27.47) and 230.5 (±32.7) μmol/L, respectively, in women (Table 1). The plasma/serum difference was highly significant in women (P <0.001). The ranges (mean ± 2 SD) for total plasma and serum cysteine in men were 152.8 to 266.5 and 198.4 to 335.3 μmol/L, respectively, and 135.8 to 245.7 and 165.1 to 295.9 μmol/L, respectively, in women. The differences between mean plasma and serum cysteine concentrations by sex were also significant (Table 1): P = 0.006 for plasma and P <0.001 for serum.

Cysteinylglycine. The plasma and serum values for total CysGly were normally distributed in both sexes. In men the means (±SD) in plasma and serum were 30.79 (±4.48) and 40.71 (±4.99) μmol/L, respectively, significantly different (P <0.001); the mean ± 2 SD ranges were 21.83–39.74 and 30.73–50.69 μmol/L, respectively (Table 1). The means (±SD) for total plasma and serum cysteine in women were 26.53 (±3.65) and 34.10 (±4.32) μmol/L, respectively, marginally significant (P = 0.022), and the mean ± 2 SD ranges were 19.24–33.82 and 25.47–42.74 μmol/L, respectively. The sex-related differences between mean total plasma and serum CysGly were highly significant (Table 1): P <0.001 for both plasma and serum.

Table 1. Comparison of plasma and serum normal values in men and women.

			Thiol conc, μmol/L						
Variable	Group	n	Mean	SD	Min	Median	Max	Range (mean ± 2 SD)	P
Plasma homocysteine	M	36	9.26	1.88	5.94	8.71	14.98	6.18–13.37	
	F	35	7.85	2.29	4.30	7.52	14.00	3.27–12.43	0.002[a]
Serum homocysteine	M	36	12.30	2.30	8.79	11.76	20.35	8.56–17.12	
	F	35	10.34	3.23	5.89	9.97	18.89	3.92–16.84	0.002[a]
Plasma cysteine	M	36	209.60	28.42	144.00	208.30	291.40	152.80–266.50	
	F	35	190.70	27.47	117.60	191.30	258.30	135.80–245.70	0.006
Serum cysteine	M	36	266.90	34.23	163.60	267.10	353.60	198.40–335.30	
	F	35	230.50	32.70	160.90	238.40	305.10	165.10–295.90	<0.001
Plasma CysGly	M	36	30.79	4.48	22.89	30.35	43.29	21.83–39.74	
	F	35	26.53	3.65	17.24	26.08	35.19	19.24–33.82	<0.001
Serum CysGly	M	36	40.71	4.99	30.22	40.07	54.79	30.73–50.69	
	F	35	34.10	4.32	23.11	34.28	43.47	25.47–42.74	<0.001

[a] Male vs female; homocysteine values (plasma and serum) assessed by Wilcoxon's rank sum test; *t*-test was used for the others.

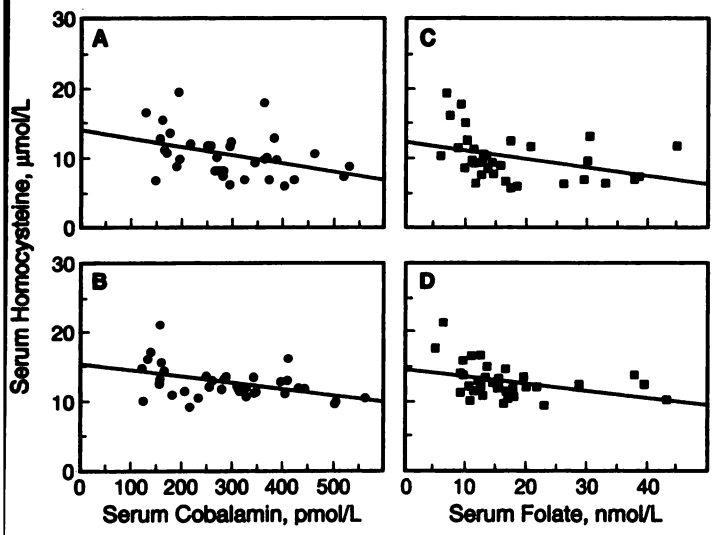

Fig. 5. Linear regression analysis of serum homocysteine vs serum cobalamin in women (*A*), serum homocysteine vs serum cobalamin in men (*B*), serum homocysteine vs serum folate in women (*C*), and serum homocysteine vs serum folate in men (*D*).

A: $y = 13.94 - 0.012x$ ($r = 0.37$; $S_{y|x} = 0.005$); *B*: $y = 15.21 - 0.009x$ ($r = 0.425$; $S_{y|x} = 0.003$); *C*: $y = 12.56 - 0.111x$ ($r = 0.344$; $S_{y|x} = 0.05$); and *D*: $y = 14.41 - 0.099x$ ($r = 0.363$; $S_{y|x} = 0.043$).

Discussion

The aims of this study were to establish a rapid and accurate assay for total homocysteine and other low-molecular-mass serum thiols in serum or plasma, to characterize the homocysteine-*S*-bimane and other thiol-bimane adducts spectrophotometrically and fluorometrically, and to establish a normal range for total homocysteine and other thiols in plasma and serum from healthy adults.

Total plasma or serum homocysteine, cysteine, and CysGly were rapidly determined by using (*a*) borohydride reduction of disulfide bonds and derivatization of thiols with monobromobimane; (*b*) perchloric acid precipitation of protein; and (*c*) HPLC-FD analysis of thiol-bimanes in the supernate. The present method, a refinement of our earlier method (*31*), has been simplified by combining the reduction/derivatization step and eliminating the reversed-phase solid-phase extraction and drying steps. Interference peaks are avoided in the new method without extensive clean-up by: (*a*) combining the disulfide reduction and thiol derivatization steps, thereby decreasing hydrolysis product formation from monobromobimane by shortening reaction and processing time; (*b*) using an excitation wavelength of 390 instead of 300 nm, thereby increasing the signal-to-noise ratio; and (*c*) using a 4.6 × 250 mm C_8 reversed-phase column instead of a 4.6 × 150 mm C_{18} reversed-phase column and a flow rate of 2.0 instead of 1.0 mL/min. Under these conditions the bimane conjugates are resolved within 8 to 9 min with little or no interference. Potentially interfering substances are retained longer than the analytes of interest but are completely eluted during column regeneration (10–13 min after sample injection; see Fig. 1A for gradient profile). All steps, including final pH adjustment of the perchloric acid supernate, are carried out in a single 1.5-mL conical snap-cap polypropylene tube. As many as 85 samples and quality-control sera can be processed in <4 h, then analyzed overnight by automated HPLC-FD. The method is sensitive, precise, and capable of detecting total plasma homocysteine over a wide concentration range.

We analyzed 118 patents' plasma samples for total homocysteine by this method and an independent method. Comparison of the two methods by linear regression analysis and the Bland–Altman procedure demonstrated very good agreement. Furthermore, the concentrations of total plasma homocysteine, cysteine, and CysGly obtained in this study are in good agreement with previously reported values obtained by others. Andersson et al. (*39*) reported total plasma homocysteine, cysteine, and CysGly in healthy men (n = 10) at 9.7 ± 2.0, 268 ± 25, and 35.8 ± 9.4 µmol/L, respectively, using postcolumn derivatization HPLC-UVD. Mansoor et al. (*47*) reported total plasma homocysteine, cysteine, and CysGly in healthy men (n = 8) at 11.85 ± 1.51, 264.3 ± 33.31, and 31.77 ± 5.36 µmol/L, respectively, using HPLC-FD.

Highly purified thiol-bimane standards were conveniently prepared from homocysteine, cysteine, CysGly, and glutathione by reacting these compounds with an excess of monobromobimane under slightly alkaline conditions, removing excess monobromobimane with Sepharose-SH, and then purifying the standards by reversed-phase HPLC-UVD. The thiol-bimane standards are useful for assessing the performance of the chromatography system and are routinely incorporated at the beginning and end of each patient run. The availability of highly purified thiol-bimane standards has made it possible to characterize the spectrophotometric properties of these compounds. The four standards had very similar absorbance spectra, with peaks at 234, 256, and 389 nm. The fluorescence excitation and emission spectra of the four compounds were also very similar, with excitation and emission maxima occurring at 390–392 and 478–480 nm, respectively. There were only minor differences in the relative quantum yields of the four thiol-bimane standards.

Total homocysteine, cysteine, and CysGly were determined in serum and plasma obtained from healthy fast-

ing donors. To standardize conditions for this study, we obtained the serum and plasma specimens from each donor at the same time, and the EDTA plasma specimen was collected in a chilled tube, placed on ice, and processed within 15 min at 4°C. The serum specimen was obtained from blood that had been allowed to clot for precisely 1 h at room temperature (22–23°C here). Significant differences were found in total homocysteine, cysteine, and CysGly concentrations between serum and plasma samples from both sexes of donors. Although the lower concentrations of the three analytes in plasma may be partially explained by fluid redistribution in plasma, this is probably not the only explanation. Erythrocytes appear to export homocysteine as a byproduct of *S*-adenosylmethionine metabolism (*48, 49*), which may account for the higher homocysteine values found in serum from both men and women. Cellular metabolism and transport may also be responsible for the higher concentrations of total cysteine and CysGly found in serum. The significantly higher concentrations of total homocysteine in serum than in plasma and the possibility of time-dependent erythrocyte export or leukocyte release of homocysteine (and for that matter cysteine and CysGly) suggest that investigative protocols used to study the role of homocysteine in atherogenesis and thrombogenesis must be rigorously standardized.

Significantly higher concentrations of total homocysteine were found in serum or plasma from men (Table 1). In this study the female donors were all premenopausal, so these findings are therefore consistent with most other studies, as reviewed by Ueland et al. (*10*). In contrast, Andersson et al. (*50*) reported no significant difference in total plasma homocysteine between premenopausal women and age-matched men but found that postmenopausal women had lower values than age-matched men. Additional studies with larger numbers of individuals in every age decade should be carried out to resolve the issues of normal range and gender differences. In this study we found significantly higher concentrations of cysteine and CysGly in men, irrespective of whether the sample was plasma or serum.

There was a significant inverse correlation between serum cobalamin and total serum homocysteine and between serum folate and total serum homocysteine in both men and women. Although the donors participating in this study were apparently healthy individuals whose combined mean age was 34 years, several individuals had serum cobalamin concentrations <300 ng/L (221 pmol/L) and four donors had <200 ng/L (148 pmol/L). Because serum methylmalonic acid is a highly sensitive and specific indicator of functional cobalamin deficiency (*51, 52*), we also determined serum methylmalonic acid in all of the donors enrolled in this study. All of the donors, including those with low normal concentrations of serum cobalamin, had serum methylmalonic acid values within the normal range. In contrast to homocysteine, serum methylmalonic acid concentrations did not negatively correlate with concentrations of serum cobalamin. Andersson et al. (*50*) also found negative correlations between concentrations of plasma homocysteine and of serum cobalamin and serum folic acid in apparently healthy individuals. Total homocysteine is clearly increased in states of frank cobalamin and folate deficiency, as reported by Stabler et al. (*53*), and in an unusually high percentage of elderly individuals with normal concentrations of serum cobalamin (*54*). Thus, total plasma homocysteine may be a highly sensitive indicator of both cobalamin and folic acid status, not only in patients with frank deficiencies of these vitamins, but in "normal" individuals as well. We are currently studying the effect of both cobalamin and folate supplementation in these donors to see whether total plasma homocysteine concentrations can be significantly lowered.

In conclusion, this rapid assay for total homocysteine, cysteine, and CysGly in plasma and serum is highly sensitive and precise and has the capability of high sample throughput. Using this assay, we have been able to accurately establish significant differences between plasma and serum samples and significant difference between the sexes.

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