CLIN. CHEM. 39/2, 263–271 (1993)

Homocysteine and Other Thiols in Plasma and Urine: Automated Determination and Sample Stability

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We have developed a modified version of our fully automated column-switching HPLC method for determining total plasma homocysteine based on single-column (reversed-phase) separation. Homocysteine, cysteine, and cysteinylglycine in plasma (total concentrations), acid-precipitated plasma (non-protein-bound concentrations), and urine can be determined. The derivatization and chromatography were performed automatically by a sample processor. The successful separation of all thiol species (within 15 min) was accomplished by accurate adjustment of the pH of the mobile phase to 3.65 (plasma) or 3.50 (acid-precipitated plasma, urine). Maximal fluorescence yield of cysteine, cysteinylglycine, and, to a lesser degree, homocysteine was dependent on optimal concentrations of EDTA and dithioerythritol during reduction (with $NaBH_4$) and derivatization (with monobromobimane). The method is sensitive (detection limit ~0.05 pmol) and has a high degree of precision (CV <5%). The sample output is ~70 samples in 24 h. Serum and heparin plasma can also be analyzed. Hemolysis up to ~2.0 g/L of hemoglobin did not interfere with the analytical recovery of homocysteine or cysteine. Collection of blood, separation of plasma from whole blood, and acid precipitation must be standardized to obtain reproducible thiol results. Our modifications and the standardization of blood-sampling procedures have substantially improved the method and broadened its applications.

Indexing Terms: *cysteine · cysteinylglycine · sample handling · fluorometry · chromatography, reversed-phase*

The introduction of methods for determination of total plasma homocysteine in the mid-1980s greatly facilitated research on the clinical role of homocysteine (*1–3*). Clinical studies of >1600 patients indicate that hyperhomocysteinemia is an independent risk factor for premature cardiovascular disease (*4*). Furthermore, determination of homocysteine in plasma and serum is useful both in the diagnosis and follow-up of folate and cobalamin deficiencies and the rare inborn errors causing homocystinuria (*5*). Thus plasma and serum homocysteine are established as indicators of several common disease states, so that homocysteine determination is attractive as a routine analysis.

In normal plasma and serum, ~70% of homocysteine (*1*) and 30% of cysteine (*6*) are protein bound. Storage of whole plasma and serum causes redistribution of plasma thiols so that the protein-bound fraction increases at the expense of the free, acid-soluble fraction. Therefore, determination of total (both free and protein-bound) homocysteine is preferable in the clinical setting (*3*).

Several procedures for determining total homocysteine have been described (*1, 2, 7–14*), but these techniques require either laborious sample processing and derivatization or operation of sophisticated chromatographic instrumentation. Four years ago we constructed the first fully automated method for determining total homocysteine in plasma (*9*). One major drawback of this method was a column-switching step, which required two solvent-delivery systems. Such switching also causes a pressure surge through the system, which may cause column deterioration and reduce column life.

Introduction of total plasma homocysteine determination in the university hospital laboratory in Bergen motivated the construction of a simple but fully automated single-column technique. Major advantages are longer column life; simultaneous determination of cysteine and cysteinylglycine; and total analytical recovery of all three thiols measured in plasma collected in EDTA or heparin, or in serum, urine, or acid-treated plasma. Procedures for sample collection and handling were also developed and evaluated.

Materials and Methods

Materials

L-Homocystine, L-cystine, cysteinylglycine (reduced form), *N*-ethylmorpholine, and dithioerythritol (DTE) were obtained from Sigma Chemical Co. (St. Louis, MO)[3]. $NaBH_4$ and EDTA tripotassium salt were from Fluka Chemie AG (Buchs, Switzerland). Dimethyl sulfoxide (DMSO), 1-octanol, 5-sulfosalicylic acid (dihydrate), hydrochloric acid, nitric acid (65%), formic acid, and acetic acid were purchased from Merck AG (Darmstadt, Germany). Monobromobimane was obtained from Molecular Probes, Inc. (Eugene, OR). Acetonitrile (HPLC-grade) was obtained from Lab-Scan Ltd. (Dublin, Ireland). The column (150 × 4.6 mm; Shandon Scientific Ltd, Cheshire, UK) for reversed-phase chromatography was slurry-packed at 13.3 kg/cm^2 with Shandon Hypersil ODS material, 3-μm particle size. The guard column (25 × 4.6 mm, from Shandon) was packed with Pelliguard LC 18 from Supelco Inc. (Bellefonte, PA).

Standard Solutions

L-Homocystine (50 μmol/L), L-cystine (600 μmol/L), and cysteinylglycine (150 μmol/L, reduced form) were dissolved in 0.1 mol/L HCl containing DTE, 100 μmol/L,

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Received June 22, 1992; accepted August 31, 1992.

[3] Nonstandard abbreviations: DTE, dithiothreitol; DMSO, dimethyl sulfoxide.

and then diluted to known concentrations with the same solution.

Instrumentation

A programmable sample processor, Model 232-401 (Gilson Medical Electronics, Inc., Middleton, WI), was used for automated sample processing, derivatization, and injection, as previously described (*9*). The ternary solvent delivery system (Model SP8800) and the integrator (SP4290) were from Spectra-Physics (San Jose, CA). The fluorescence signal was detected by means of a Shimadzu (Kyoto, Japan) RF-535 fluorometer operating at an excitation wavelength of 365 nm and emission wavelength of 475 nm.

Methods

Sample collection and storage. Blood was collected by venipuncture into a Vacutainer Tube (Becton Dickinson, Rutherford, NJ) containing EDTA. Plasma for determination of total thiol concentrations was obtained without delay by centrifuging the blood at 2000 × *g* for 5 min at 0–2 °C.

Acid-precipitated plasma was used for determining free concentrations of the thiols. Blood was collected into a cooled EDTA Vacutainer Tube, and the blood cells were removed without delay by centrifugation for ~0.5 min at 10 000 × *g*. Within 2 min after collection of the blood, plasma (0.5 mL) was added directly to a vial containing 50 μL of 500 g/L sulfosalicylic acid. The acid-treated plasma was then allowed to stand for 5–10 min on ice before removal of the acid precipitate by centrifugation for ~1 min at 10 000 × *g*. Plasma and the acid supernate were stored at −20 °C until analysis.

Derivatization procedures. The automated derivatization procedure of plasma samples was presented elsewhere (*9*). Briefly, the autosampler is filled with as many as 60 samples (~20 h analysis time) and the reagents necessary for derivatization. (All reagents were tested and found to be stable for the duration of the unattended analysis, i.e., ≥24 h.) The sample vials and most reagent reservoirs are stored in cooled racks (2–3 °C), whereas the acids, ethylmorpholine buffer, and water are kept at room temperature. The sample processor collects the sample and the reagents and dispenses them into one derivatization vial (a 1000-μL vial), where reduction and derivatization are carried out at room temperature.

We modified the composition of reagents used for reduction and derivatization. Plasma, serum, and undiluted urine were derivatized as follows: 30 μL of sample was mixed with 30 μL of 4 mol/L $NaBH_4$ in 0.066 mol/L NaOH and 333 mL/L DMSO, 10 μL of 2 mmol/L EDTA and 1.65 mmol/L DTE, 10 μL of 1-octanol, and 20 μL of 1.8 mol/L HCl. After 3 min (to allow reduction of disulfides), 100 μL of 1.5 mol/L ethylmorpholine buffer, 400 μL of H_2O, and 20 μL of 0.025 mol/L monobromobimane were added. The derivatization (with monobromobimane) was terminated after 3 min by adding 40 μL of glacial acetic acid.

The procedure was slightly different for the derivatization of acid-treated plasma: 45 μL of sample was mixed with 15 μL of 167 g/L sulfosalicylic acid with 4.5 mmol/L DTE, 30 μL of 4 mol/L $NaBH_4$ in 0.066 mol/L NaOH and 333 mL/L DMSO, and 10 μL of 1-octanol. After 3 min, 20 μL of 5 mmol/L EDTA, 100 μL of 1.5 mol/L ethylmorpholine buffer, 400 μL of H_2O, and 20 μL of 0.025 mol/L monobromobimane were added. The derivatization (with monobromobimane) was terminated after 3 min by adding 40 μL of glacial acetic acid.

Chromatography. Twenty microliters of derivatized sample was injected into a 150 × 4.6 mm ODS Hypersil column, equilibrated with 30 mmol/L ammonium nitrate and 40 mmol/L ammonium formate buffer, pH 3.65 (plasma) or pH 3.50 (acid-precipitated plasma and urine). The three thiols were eluted from the column with a linear gradient of acetonitrile (from 0% to 10.5% in 11 min) in the same buffer. Ambient temperature was used and the flow rate was 2 mL/min. The retention times of cysteine, cysteinylglycine, and homocysteine were ~8, 9.5, and 10.5 min, respectively.

Precision and Recovery

Precision. To determine within-day precision, we assayed 10 replicates of the biological sample in one run. Between-day precision was obtained by determining the same biological samples on 10 different days over 1 month.

Recovery studies. Known concentrations of homocysteine, cysteine, and cysteinylglycine equivalents were added to water containing 100 μmol/L DTE or to a biological sample (plasma or acid-precipitated plasma or urine). The concentration of thiols determined in 10 replicates of water matrix (standards, S) was used to calibrate the system. Then the concentrations in biological samples (B) and in biological samples with added standards (BS) were determined in 10 replicates and analytical recovery was calculated as follows:

$$\text{Recovery (\%)} = 100 \cdot (BS - B)/S$$

Sampling Procedures

Comparison between plasma and serum. Blood was collected into Vacutainer Tubes containing EDTA, heparin, citrate, or no addition (for serum). The tubes containing anticoagulant were placed on ice and the blood cells were removed by centrifugation within 10 min. The plasma fraction was collected without delay. Serum was obtained by leaving whole blood at room temperature and collecting the serum fraction within 60 min. In another experiment, EDTA plasma and serum were left with the blood cells for the same period of time.

Evaluation of stability of total plasma homocysteine during storage of whole blood. Changes in total plasma homocysteine in whole blood from 40 consecutive patients were determined as a function of time and temperature. We either prepared the plasma immediately (control) or left the vial containing whole blood at room temperature (22 °C) or on ice (0–2 °C) for 4 and 24 h before removing the blood cells. To test whether plasma homocysteine or plasma methionine concentrations af-

fected stability of plasma homocysteine in whole blood, we gave a healthy volunteer an oral methionine load of 100 mg/kg body weight. Six blood samples were collected (before and 1, 2.5, 4, 8, and 30 h after the methionine load), and either plasma was prepared immediately or whole blood was left at room temperature (22 °C) or on ice (0–2 °C) for 1, 4, and 24 h before preparation of plasma.

Evaluation of stability of total plasma homocysteine in plasma. Blood samples collected from 40 consecutive patients were immediately cooled and centrifuged and the plasma fraction was either removed and analyzed without delay or left at room temperature for 4 days before analysis.

Changes in free, bound, and total plasma thiols during storage of whole blood or plasma. Time- and temperature-dependent changes in free, bound, and total plasma thiols during storage of whole blood and plasma were measured in six blood samples, each divided into two portions. Plasma was prepared from one portion within 1 min and total thiol concentration was determined (control total concentration). A fraction of this plasma was immediately treated with acid to obtain the control values for free thiols. Another fraction of plasma was left either at room temperature or at 0–2 °C for 0.5, 1, 4, or 24 h before determination of total thiol concentration or acid treatment to obtain the free fraction. The other portion of whole blood was left either at room temperature or at 0–2 °C for 0.5, 1, 4, or 24 h before preparation of plasma; in plasma so obtained, total thiols and free thiols (after acid treatment) were determined.

Hemolysis. Blood from one donor was collected into three Vacutainer Tubes containing EDTA. The hemoglobin concentration of the sample was determined in one tube. Blood from another tube was subjected to rapid freezing and thawing (three times) to obtain hemolysis. Blood in the third tube was centrifuged and plasma was separated from the blood elements. The hemolyzed sample (with known concentration of hemoglobin) was then added to plasma to obtain increasing concentration of hemoglobin (and other cellular components) in the plasma sample. Maximal dilution of plasma with the hemolyzed sample was ~6%.

Statistics

Data from precision and recovery studies are presented as mean ± SD or coefficient of variation (CV). The test for stability of homocysteine in whole blood or in plasma stored under various conditions was performed by using a Friedman test followed by a Wilcoxon signed-rank test for paired samples (*15*). In addition, sample stability was evaluated by using the test statistics (T.S.) described by Thiers et al. (*16*): The sample constituent was considered stable when its mean concentration changed by <1 CV of the method, allowing a 5% risk of error in decision ($2\alpha = \beta = 0.05$). The results were presented by using the truncated normal sequential test in which

$$\text{T.S.} = \sum_{i=1}^{i=n} \ln(S_i/F_i)/\text{CV}$$

where S_i is the measured concentration of the ith sample after storage, F_i is the concentration measured in the fresh (reference or control) sample, and CV is the coefficient of variation of the method, which was set at 3%, based on our results from the precision studies.

The test for stability of homocysteine in whole blood as a function of the initial concentration was performed by using a Spearman rank correlation coefficient or a Kruskal–Wallis test followed by a Mann–Whitney U-test with a Bonferroni correction (*15*).

Results

Optimization and Performance of the Assay

Optimization of reduction and derivatization. We found that the optimal sodium borohydride concentration (1.3 mol/L) in the homocysteine assay (*9*) was also optimal for the measurement of oxidized species of cysteine and cysteinylglycine (data not shown). However, the concentration of monobromobimane required to obtain a maximal fluorescence yield of cysteine and cysteinylglycine was higher than for assay of homocysteine, and the final concentration of monobromobimane during derivatization was therefore raised to ~800 μmol/L.

EDTA was added before derivatization. This significantly increased the fluorescence yield of cysteinylglycine and cysteine (~60%) in standard solutions and biological fluids not containing EDTA. Furthermore, the inclusion of EDTA significantly reduced the size of several unidentified peaks in the chromatogram.

DTE was required in dilute samples and in standard solutions to obtain maximal yield of all thiol components, as previously reported for the determination of homocysteine (*9*). The effect from DTE was particularly pronounced when acid-treated samples were assayed (data not shown).

Chromatography. Figure 1 shows the chromatograms of the three thiol components in plasma from a healthy person. Cysteine, cysteinylglycine, and homocysteine eluted in this order, and their retention times were only moderately affected by changing the pH of the mobile phase from 3.5 to 3.8. However, the chromatographic behavior of several unidentified peaks and glutathione was highly dependent on pH, with these peaks eluting more rapidly at higher pH (Figure 1). We exploited the pH-dependent elution of this material to avoid chromatographic interference with the cysteinylglycine and homocysteine peaks. In addition, the system was further optimized by adding nitric acid to the mobile phase, which improved the separation of homocysteine from a peak that eluted at a slightly later retention time. In plasma (total concentrations), optimal separation of homocysteine from interfering peaks was obtained at pH 3.65 (Figure 1).

In urine and in acid-precipitated plasma (free concentrations), the material eluting as peak X in Figure 1 was

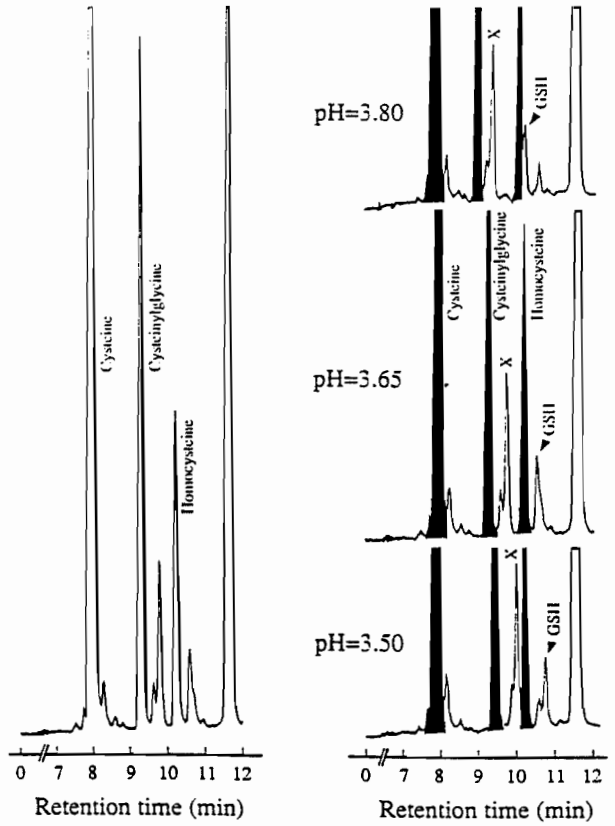

Fig. 1. Separation of cysteine, cysteinylglycine, and homocysteine from interfering material in plasma by varying the pH of the mobile phase

Left: the optimal resolution obtained at pH 3.65; *right*: pH-dependent elution of interfering compounds; X, unidentified compound; GSH, glutathione

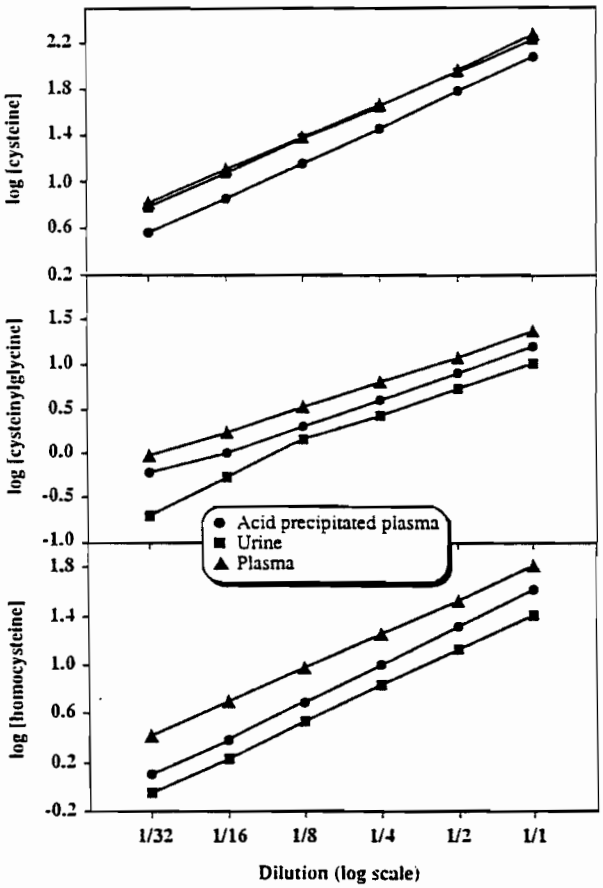

Fig. 2. Linearity of the assay results vs concentrations of cysteine, cysteinylglycine, and homocysteine in plasma, acid-precipitated plasma, and urine

Plasma, acid-precipitated plasma, and urine samples were diluted with water containing 100 μmol/L DTE; the system was calibrated with standards (from 0.5 to 1000 μmol/L) diluted in water containing 100 μmol/L DTE

not present, whereas glutathione (in acid-precipitated plasma) and material eluting close behind homocysteine (in urine) were abundant. Resolution of cysteine, cysteinylglycine, and homocysteine from this interfering material was obtained by adjusting the pH of the mobile phase to 3.5 (data not shown).

Linearity, precision, and recovery. The linearity of the assay results plotted vs concentrations of cysteine, cysteinylglycine, and homocysteine in plasma, acid-precipitated plasma, and urine is shown in Figure 2. In the absence of added DTE and EDTA, linear curves were obtained, except at high dilution or for thiol standards dissolved in buffer (data not shown).

The results from precision and recovery studies of the optimized assay are shown in Table 1. Except for homocysteine in urine, the within- and between-day CVs for cysteine, cysteinylglycine, and homocysteine were <5%. The analytical recovery was 85–100% for all thiol components.

Blood Sampling, Sample Processing, and Sample Stability

Stability of plasma homocysteine concentration in whole blood. When whole blood was stored at room temperature (22 °C) for 4 h, there was a significant increase in total plasma homocysteine [mean 20.1 (SD 11)%, P <0.01]; after 24 h, the increase was marked [mean 62.1 (SD 33)%, P <0.01]. When whole blood was left on ice, total plasma homocysteine was stable for 4 h [mean increase 1 (SD 4)%, P >0.05] and moderately increased after 24 h [mean 7 (SD 5)%, P <0.01].

Stability of total homocysteine in whole blood was further evaluated by using the test statistics of Thiers et al. (*16*), which confirmed that total homocysteine was stable for 4 h at 0–2 °C but unstable at low temperature for 24 h or at room temperature for 4 or 24 h (Figure 3).

We measured the percentage change in plasma homocysteine as a function of initial concentration (range 4.5–31.6 μmol/L) in 40 blood samples. When the data points were treated separately by using Spearman rank correlation coefficient, we found a negative correlation between percentage increase and the homocysteine concentration in blood stored for 4 h ($r = -0.31, P = 0.052$) or 24 h ($r = -0.52, P < 0.01$) on ice and for 4 and 24 h at room temperature (for both; $r = -0.78, P < 0.01$). Figure 4 shows these data grouped by low, intermediate, and high initial plasma homocysteine concentration. The absolute change in plasma homocysteine (in μmol/L) during storage of whole blood was relatively constant (upper panel), causing a marked difference in percentage change (lower panel).

We also compared stability of plasma homocysteine during storage of whole blood in six samples collected from one person immediately before and 1–30 h after an oral methionine load (Figure 5). Notably, in the samples

Table 1. Precision and Recovery of the Assay

	Within day (n = 10)				Between day (n = 10), μmol/L (CV)
	Sample, μmol/L (CV)	Added, μmol/L	Measured, μmol/L (CV)	Recovered, %	
Plasma					
Cysteine	232.2 ± 5.1 (2.2)[a]	180.0	399.0 ± 8.8 (2.2)[a]	93.1 ± 3.2[a]	234.5 ± 5.4 (2.3)[a]
Cysteinylglycine	22.4 ± 0.4 (1.9)	25.0	44.8 ± 0.8 (1.7)	89.8 ± 2.8	22.5 ± 0.4 (1.8)
Homocysteine	9.2 ± 0.2 (2.6)	15.0	23.5 ± 0.3 (1.2)	95.3 ± 3.3	9.3 ± 0.2 (2.2)
Acid-precipitated plasma					
Cysteine	151.8 ± 1.5 (1.0)	60.0	211.1 ± 1.5 (0.7)	99.1 ± 2.1	151.2 ± 2.7 (1.8)
Cysteinylglycine	12.7 ± 0.2 (1.9)	8.3	20.9 ± 0.5 (2.2)	98.7 ± 4.6	12.9 ± 0.4 (2.8)
Homocysteine	3.8 ± 0.1 (2.8)	5.0	8.6 ± 0.2 (4.0)	94.8 ± 2.9	3.7 ± 0.1 (3.7)
Urine					
Cysteine	140.4 ± 1.3 (1.0)	90.0	218.1 ± 4.0 (1.8)	84.9 ± 4.0	140.9 ± 3.1 (2.2)
Cysteinylglycine	7.4 ± 0.1 (1.2)	12.5	19.1 ± 0.3 (1.6)	93.0 ± 3.8	7.5 ± 0.2 (2.2)
Homocysteine	12.2 ± 0.2 (1.9)	7.5	19.6 ± 0.3 (5.9)	98.1 ± 6.8	12.4 ± 0.8 (6.8)

[a] Mean ± SD.

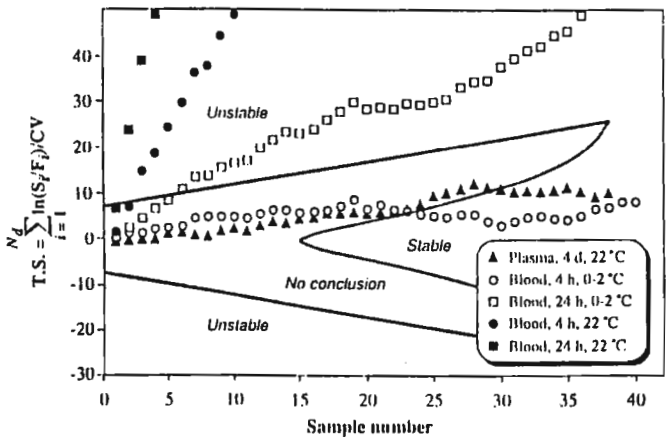

Fig. 3. Stability of homocysteine in plasma under different storage conditions

For each successive pair of homocysteine values in stored sample (S_i) and in the corresponding fresh or optimally prepared sample (F_i), a test statistic, T.S., is calculated and plotted. When T.S. crosses a boundary, a decision can be made regarding stability

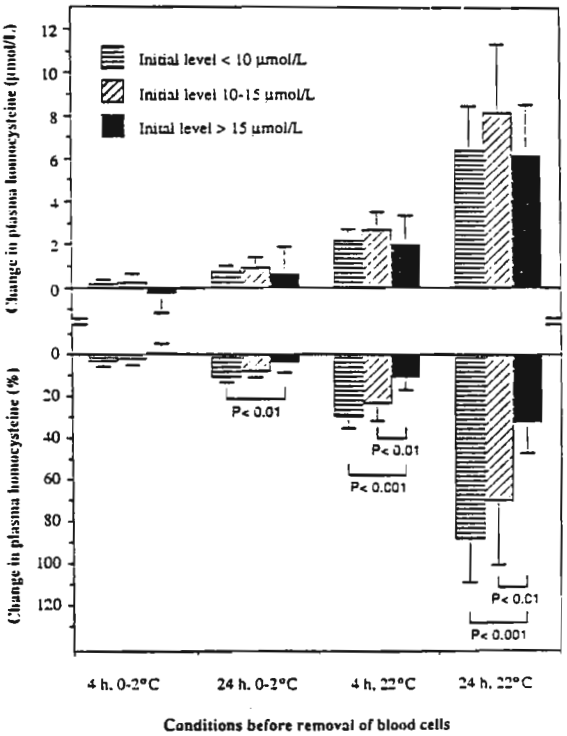

Fig. 4. Time- and temperature-dependent changes in plasma homocysteine during storage of whole blood as a function of initial homocysteine concentration

Initial homocysteine concentration <10 μmol/L [mean 7.3 (SD 1.8) μmol/L, n = 13; ▤], 10 to 15 μmol/L [mean 11.9 (SD 1.7) μmol/L, n = 13; ▨], and >15 μmol/L [mean 20.4 (SD 4.6) μmol/L, n = 14; ■]. *P* values were obtained by using the Mann–Whitney U test with a Bonferroni correction

with markedly high concentrations of plasma homocysteine, both the percentage and absolute increase in plasma homocysteine during storage of whole blood at 22 °C were lower or even negative compared with samples with low homocysteine concentration (Figure 5, lower panel). These differences between high and low plasma homocysteine concentration were also observed when whole blood was stored on ice, but were less pronounced (data not shown).

Stability of total homocysteine in plasma. Storage of plasma for 4 days at room temperature induced a small (~2%) but significant ($P < 0.05$) increase in total homocysteine. However, when the stability was tested by the method of Thiers et al. (*16*), the plasma samples were found to be stable under this condition (Figure 3).

Redistribution and changes of plasma thiol components in whole blood and plasma. The total plasma concentrations of homocysteine, cysteine, and cysteinylglycine remained constant in plasma incubated at 20 °C for 24 h (data not shown). When whole blood was kept on ice for 24 h, there were only minimal changes (<10%) in total plasma concentrations of homocysteine, cysteinylglycine (increase), and cysteine (decrease), whereas at 20 °C, total plasma homocysteine and cysteinylglycine increased markedly (by 45% and 122%, respectively) and cysteine decreased (by 17%) (data not shown).

A time-dependent redistribution between free and protein-bound thiol components in plasma occurred during storage of whole blood or plasma, both at low and high temperature (Table 2). In whole blood and plasma kept at 4 °C and in plasma kept at 4 or 20 °C, free homocysteine, and to a lesser extent free cysteinylgly-

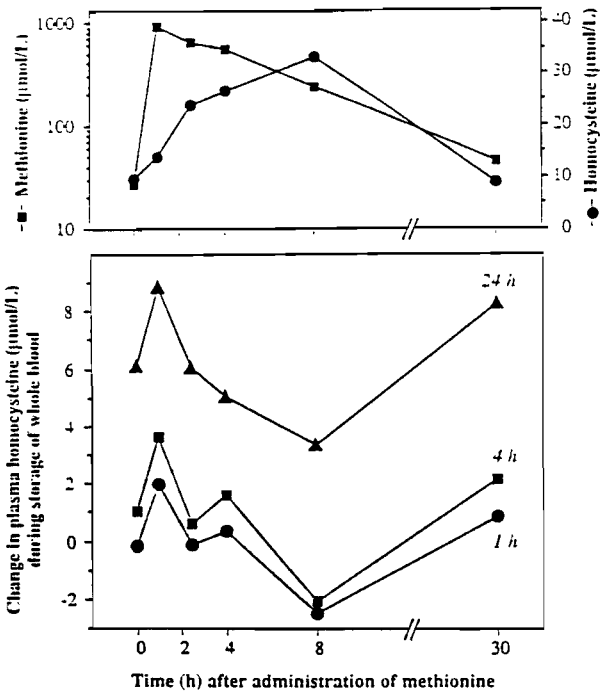

Fig. 5. The effect of an oral methionine load on changes in plasma homocysteine during storage of whole blood at room temperature

Upper panel: plasma methionine and plasma homocysteine concentrations after a methionine load in one healthy volunteer; the plasma was prepared without delay. *Lower panel:* changes in plasma homocysteine during storage of whole blood at room temperature for 1, 4, and 24 h, before plasma was separated from the blood cells

cine, usually increased temporarily within 0.5 h after blood sampling. The increase was accompanied by a transient decrease in the protein-bound fraction and may be explained by release of these thiol components from plasma proteins in fresh samples. After prolonged incubation (up to 24 h), the protein-bound fractions of all thiol components increased at the expense of the free fraction. In whole blood incubated at 20 °C, this decrease in the free fraction of homocysteine and cysteinylglycine seemed to be outbalanced by the release of these compounds (or their precursors) from the formed elements of blood (Table 2).

Anticoagulants; plasma vs serum. The modified assay is compatible with heparin and citrate anticoagulants in addition to EDTA. We compared the total homocysteine concentration in EDTA plasma and in the corresponding sera from 40 consecutive patients. When the blood cells were removed at the same time (60 min) from both the serum and plasma samples, there were no significant differences ($P > 0.05$) in the total homocysteine concentrations. However, when we compared optimally treated plasma (separated immediately) and serum prepared within 60 min, the plasma homocysteine concentrations were significantly higher [mean 7 (SD 3)%, $P < 0.01$] in serum than in plasma.

Hemolysis. When we assayed EDTA-plasma with the present method, total homocysteine and cysteine concentrations remained stable in the presence of up to ~2 g of hemoglobin per liter of plasma (the color of this plasma is deep red). Beyond this amount of hemolysis, the recovery of both thiols decreased in proportion to the plasma hemoglobin content. In contrast, plasma cysteinylglycine increased upon hemolysis (Figure 6), probably because of release of its precursor, glutathione (*17*), from the intracellular compartment.

Discussion

Practicality and low instrument cost are central features of a routine laboratory test. We have therefore substantially modified the fully automated method for the determination of total plasma homocysteine described by us 4 years ago (*9*). This modified version, which is based on a single solvent-delivery system, is easier to operate, consumes less mobile phase, is more reliable, and puts less mechanical strain on the HPLC column than did the original method. Furthermore, the new method measures cysteine and cysteinylglycine in addition to homocysteine, whereas only homocysteine was detected by the column-switching technique.

The basic principle of the present techniques was adopted from the original method (*9*). Monobromobimane is still preferred as a derivatization reagent because it is sufficiently reactive at room temperature to allow automatization. In addition, the fluorescent yield is high and the samples can be extensively diluted so that no deproteinization is required. However, monobromobimane does not specifically react with thiols, and monobromobimane and its hydrolysis products are fluorogenic (*18*). This causes chromatographic interference, which in the original method (*9*) was overcome by a chromatographic "heart-cut" performed with a column-switching device and two solvent-delivery systems; i.e., effluent containing eluting (and possibly interfering) material before and after the compound(s) of interest was switched outside of the analytical column. The modified technique exploits the dependence of the chromatographic elution of interfering material on the pH of the mobile phase; resolution of cysteine, cysteinylglycine, and homocysteine in plasma was obtained at pH 3.65 (Figure 1).

The simultaneous quantification of all three thiols is a feature shared by some other homocysteine assays (*7, 13, 19*). This may be an important asset because of the apparent relation between homocysteine and other thiols in plasma (*20–22*). Moreover, we recently showed that the ratio between homocysteine and cysteine is more strongly correlated to serum folate than is plasma homocysteine in patients with rheumatoid arthritis (*23*).

The present method required the presence of EDTA and DTE during derivatization for analytical recovery of cysteine and cysteinylglycine. Conceivably, EDTA exerts its effect by chelating transition metals that catalyze thiol oxidation (*24, 25*); DTE may prevent reoxidation of thiols, especially in dilute solutions (*19, 26*).

With the present technique, homocysteine in acid-treated plasma can be measured. In the clinical setting, however, we recommend the determination of total plasma homocysteine concentration (*3*), because free thiols are highly unstable in the presence of plasma proteins, and plasma must be prepared and acid-precip-

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Urine					
Cysteine	140.4 ± 1.3 (1.0)	90.0	218.1 ± 4.0 (1.8)	84.9 ± 4.0	140.9 ± 3.1 (2.2)
Cysteinylglycine	7.4 ± 0.1 (1.2)	12.5	19.1 ± 0.3 (1.6)	93.0 ± 3.8	7.5 ± 0.2 (2.2)
Homocysteine	12.2 ± 0.2 (1.9)	7.5	19.6 ± 0.3 (5.9)	98.1 ± 6.8	12.4 ± 0.8 (6.8)

[a] Mean ± SD.

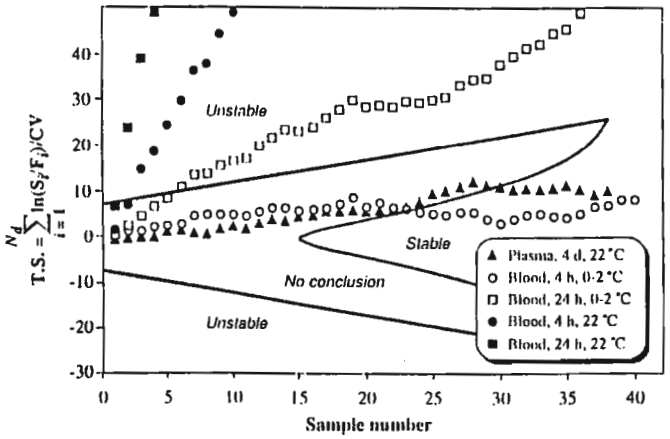

Fig. 3. Stability of homocysteine in plasma under different storage conditions

For each successive pair of homocysteine values in stored sample (S_i) and in the corresponding fresh or optimally prepared sample (F_i), a test statistic, T.S., is calculated and plotted. When T.S. crosses a boundary, a decision can be made regarding stability

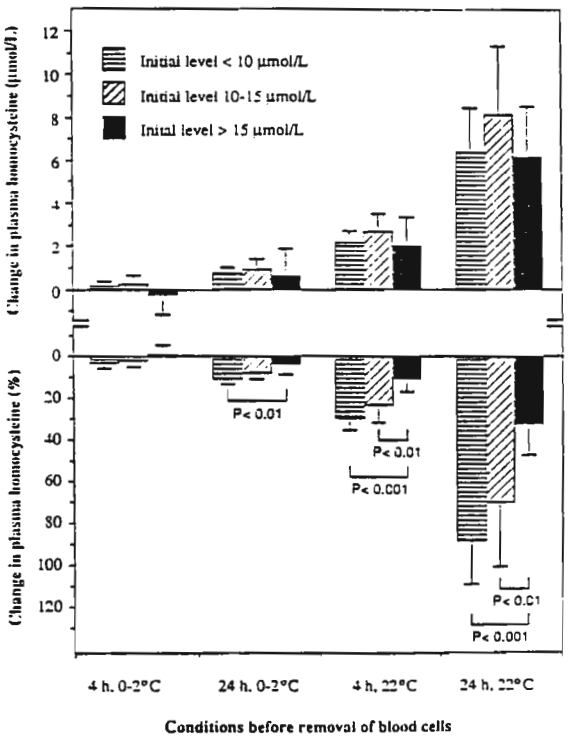

Fig. 4. Time- and temperature-dependent changes in plasma homocysteine during storage of whole blood as a function of initial homocysteine concentration

Initial homocysteine concentration <10 μmol/L [mean 7.3 (SD 1.8) μmol/L, n = 13; ▤], 10 to 15 μmol/L [mean 11.9 (SD 1.7) μmol/L, n = 13; ▨], and >15 μmol/L [mean 20.4 (SD 4.6) μmol/L, n = 14; ■]. *P* values were obtained by using the Mann–Whitney U test with a Bonferroni correction

with markedly high concentrations of plasma homocysteine, both the percentage and absolute increase in plasma homocysteine during storage of whole blood at 22 °C were lower or even negative compared with samples with low homocysteine concentration (Figure 5, lower panel). These differences between high and low plasma homocysteine concentration were also observed when whole blood was stored on ice, but were less pronounced (data not shown).

Stability of total homocysteine in plasma. Storage of plasma for 4 days at room temperature induced a small (~2%) but significant ($P < 0.05$) increase in total homocysteine. However, when the stability was tested by the method of Thiers et al. (*16*), the plasma samples were found to be stable under this condition (Figure 3).

Redistribution and changes of plasma thiol components in whole blood and plasma. The total plasma concentrations of homocysteine, cysteine, and cysteinylglycine remained constant in plasma incubated at 20 °C for 24 h (data not shown). When whole blood was kept on ice for 24 h, there were only minimal changes (<10%) in total plasma concentrations of homocysteine, cysteinylglycine (increase), and cysteine (decrease), whereas at 20 °C, total plasma homocysteine and cysteinylglycine increased markedly (by 45% and 122%, respectively) and cysteine decreased (by 17%) (data not shown).

A time-dependent redistribution between free and protein-bound thiol components in plasma occurred during storage of whole blood or plasma, both at low and high temperature (Table 2). In whole blood and plasma kept at 4 °C and in plasma kept at 4 or 20 °C, free homocysteine, and to a lesser extent free cysteinylgly-

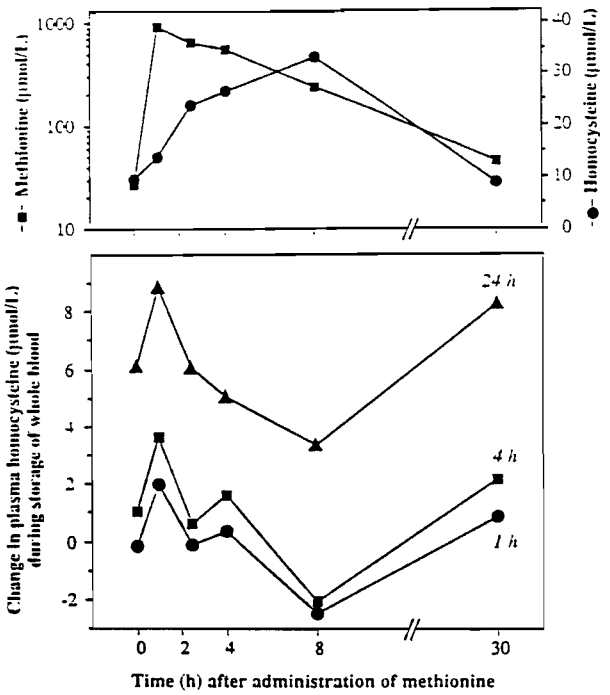

Fig. 5. The effect of an oral methionine load on changes in plasma homocysteine during storage of whole blood at room temperature

Upper panel: plasma methionine and plasma homocysteine concentrations after a methionine load in one healthy volunteer; the plasma was prepared without delay. *Lower panel:* changes in plasma homocysteine during storage of whole blood at room temperature for 1, 4, and 24 h, before plasma was separated from the blood cells

cine, usually increased temporarily within 0.5 h after blood sampling. The increase was accompanied by a transient decrease in the protein-bound fraction and may be explained by release of these thiol components from plasma proteins in fresh samples. After prolonged incubation (up to 24 h), the protein-bound fractions of all thiol components increased at the expense of the free fraction. In whole blood incubated at 20 °C, this decrease in the free fraction of homocysteine and cysteinylglycine seemed to be outbalanced by the release of these compounds (or their precursors) from the formed elements of blood (Table 2).

Anticoagulants; plasma vs serum. The modified assay is compatible with heparin and citrate anticoagulants in addition to EDTA. We compared the total homocysteine concentration in EDTA plasma and in the corresponding sera from 40 consecutive patients. When the blood cells were removed at the same time (60 min) from both the serum and plasma samples, there were no significant differences ($P > 0.05$) in the total homocysteine concentrations. However, when we compared optimally treated plasma (separated immediately) and serum prepared within 60 min, the plasma homocysteine concentrations were significantly higher [mean 7 (SD 3)%, $P < 0.01$] in serum than in plasma.

Hemolysis. When we assayed EDTA-plasma with the present method, total homocysteine and cysteine concentrations remained stable in the presence of up to ~2 g of hemoglobin per liter of plasma (the color of this plasma is deep red). Beyond this amount of hemolysis, the recovery of both thiols decreased in proportion to the plasma hemoglobin content. In contrast, plasma cysteinylglycine increased upon hemolysis (Figure 6), probably because of release of its precursor, glutathione (*17*), from the intracellular compartment.

Discussion

Practicality and low instrument cost are central features of a routine laboratory test. We have therefore substantially modified the fully automated method for the determination of total plasma homocysteine described by us 4 years ago (*9*). This modified version, which is based on a single solvent-delivery system, is easier to operate, consumes less mobile phase, is more reliable, and puts less mechanical strain on the HPLC column than did the original method. Furthermore, the new method measures cysteine and cysteinylglycine in addition to homocysteine, whereas only homocysteine was detected by the column-switching technique.

The basic principle of the present techniques was adopted from the original method (*9*). Monobromobimane is still preferred as a derivatization reagent because it is sufficiently reactive at room temperature to allow automatization. In addition, the fluorescent yield is high and the samples can be extensively diluted so that no deproteinization is required. However, monobromobimane does not specifically react with thiols, and monobromobimane and its hydrolysis products are fluorogenic (*18*). This causes chromatographic interference, which in the original method (*9*) was overcome by a chromatographic "heart-cut" performed with a column-switching device and two solvent-delivery systems; i.e., effluent containing eluting (and possibly interfering) material before and after the compound(s) of interest was switched outside of the analytical column. The modified technique exploits the dependence of the chromatographic elution of interfering material on the pH of the mobile phase; resolution of cysteine, cysteinylglycine, and homocysteine in plasma was obtained at pH 3.65 (Figure 1).

The simultaneous quantification of all three thiols is a feature shared by some other homocysteine assays (*7, 13, 19*). This may be an important asset because of the apparent relation between homocysteine and other thiols in plasma (*20–22*). Moreover, we recently showed that the ratio between homocysteine and cysteine is more strongly correlated to serum folate than is plasma homocysteine in patients with rheumatoid arthritis (*23*).

The present method required the presence of EDTA and DTE during derivatization for analytical recovery of cysteine and cysteinylglycine. Conceivably, EDTA exerts its effect by chelating transition metals that catalyze thiol oxidation (*24, 25*); DTE may prevent reoxidation of thiols, especially in dilute solutions (*19, 26*).

With the present technique, homocysteine in acid-treated plasma can be measured. In the clinical setting, however, we recommend the determination of total plasma homocysteine concentration (*3*), because free thiols are highly unstable in the presence of plasma proteins, and plasma must be prepared and acid-precip-

Table 2. Changes (%) in Free, Bound, and Total Plasma Concentrations of Thiols during Storage of Blood and Plasma

	Whole blood						Plasma			
	Bound		Free		Total		Bound		Free	
Time,[a] h	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
Cysteine										
0	100	100	100	100	100	100	100	100	100	100
0.5	95 (8)	90 (6)	101 (1)	99 (2)	98 (5)	95 (4)	99 (3)	102 (6)	101 (2)	98 (5)
1	93 (11)	91 (8)	101 (2)	96 (2)	97 (5)	94 (4)	100 (2)	107 (8)	100 (2)	94 (8)
4	105 (3)	104 (12)	99 (1)	78 (4)	102 (1)	89 (4)	105 (3)	148 (14)	96 (2)	60 (9)
24	122 (18)	147 (19)	74 (4)	30 (8)	95 (7)	83 (4)	150 (13)	208 (28)	60 (5)	12 (1)
Cysteinylglycine										
0	100	100	100	100	100	100	100	100	100	100
0.5	99 (16)	97 (16)	102 (2)	108 (3)	101 (8)	103 (8)	99 (2)	97 (5)	101 (2)	105 (6)
1	96 (13)	97 (12)	102 (3)	117 (4)	99 (7)	107 (7)	100 (1)	97 (3)	100 (2)	103 (3)
4	102 (12)	121 (23)	109 (3)	135 (5)	106 (6)	128 (12)	100 (3)	115 (9)	100 (2)	84 (7)
24	109 (13)	283 (39)	106 (7)	169 (35)	107 (6)	222 (29)	118 (9)	181 (40)	81 (7)	19 (2)
Homocysteine										
0	100	100	100	100	100	100	100	100	100	100
0.5	94 (10)	93 (7)	116 (5)	122 (11)	100 (7)	101 (6)	93 (4)	95 (4)	111 (2)	110 (9)
1	92 (10)	92 (10)	123 (6)	131 (19)	101 (7)	103 (8)	90 (6)	103 (6)	115 (3)	94 (14)
4	89 (3)	119 (22)	140 (5)	100 (27)	104 (4)	110 (13)	89 (9)	146 (19)	116 (13)	25 (5)
24	129 (17)	168 (33)	65 (14)	103 (59)	107 (6)	145 (32)	151 (23)	157 (27)	18 (3)	10 (3)

Values are mean (SD); n = 6.

[a] For whole blood, time refers to the interval before removal of the blood cells. For plasma, time refers to the interval after removal of the blood cells until acid precipitation of the sample.

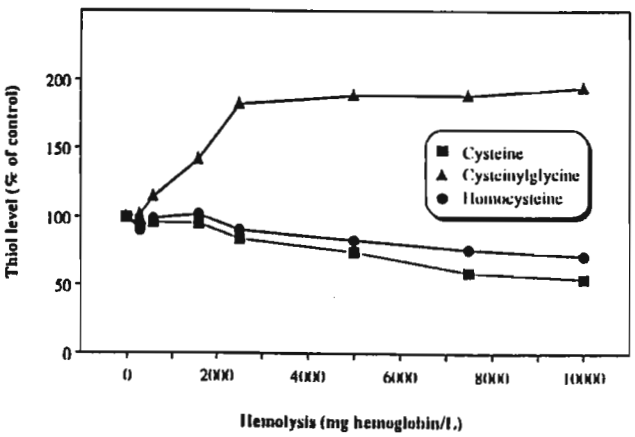

Fig. 6. Interference from hemolysis

Cysteine, cysteinylglycine, and homocysteine were assayed in plasma containing increasing concentrations of hemoglobin

itated within minutes after blood collection (Table 2) (*6*). However, determination of free homocysteine concentration may provide additional information on the regulation of plasma homocysteine and also on the relation between homocysteine and other plasma thiols (*20–22*).

This modified version of our method also allows for the determination of homocysteine in urine. Quantitative analysis of homocysteine in urine can be performed with several methods (*1, 2, 27*), and determination of homocysteine in urine is used in the diagnosis of homocystinuria (*28*). Apart from homocystinuria, only two clinical studies have included data on urinary homocysteine concentrations during disease or drug therapy (*29, 30*). Notably, in both studies, urinary homocysteine concentrations mirrored the change in plasma. Determination of urinary homocysteine may be a valuable noninvasive method, especially in children, and further investigations are warranted.

An aim here was to establish practical procedures for sample collection. Total homocysteine was stable in plasma for a sufficiently long time (4 days) (Figure 3) to allow transport of unfrozen samples to the laboratory. However, in whole blood, the time- and temperature-dependent changes in plasma homocysteine (Figure 3, Table 2) may cause serious artifacts in the determination of homocysteine and other plasma thiols.

We observed that the absolute amount of homocysteine released from the blood cells in vitro was essentially independent of the initial plasma concentration (Figure 4, upper panel). Thus, the homocysteine release from blood cells may cause particularly large errors in the determination of plasma homocysteine concentrations <15 μmol/L, whereas the percent increase is moderate at higher concentrations (Figure 4, lower panel).

A methionine loading test is currently used to reveal defects in homocysteine metabolism (*3*), and knowledge of the stability of plasma homocysteine in whole blood sampled during this procedure is therefore important. In blood collected at the maximal homocysteine response, two processes could be distinguished (Figure 5). A small transient decline was observed after blood was stored for a short time, which may reflect net homocysteine influx into blood cells. This was followed by a time-dependent increase in homocysteine at a rate independent of homocysteine or methionine concentra-

tion, probably caused by homocysteine production from the blood cells (*31*). Thus, from a practical point of view, the stability of homocysteine in whole blood obtained during methionine loading resembles that observed with high-concentration fasting samples, as presented in Figure 4. The percent increase upon storage of whole blood is small compared with the changes observed in samples containing low concentrations of homocysteine.

A possible initial influx of homocysteine (Figure 5) suggests that homocysteine in blood cells is not in equilibrium with that in plasma, and that in vivo plasma homocysteine (during methionine loading at least) stems from cells or tissues (probably liver) other than blood cells. Furthermore, in vitro, homocysteine production from blood cells is not affected by the presence of excess methionine or products of methionine metabolism. This agrees with the observation that several hematological cell lines show no homocysteine response when cultured in the presence of high methionine. In contrast, liver cells in primary culture respond markedly to methionine loading (*32*).

Our data on the kinetics of production and release of homocysteine from blood cells into plasma (Figure 3) or serum at room temperature suggest that rapidly prepared serum (within 60 min after collection) gives <10% higher values for total homocysteine than from optimally prepared plasma. Therefore, we initially recommended the use of both serum and plasma separated from cells within 1 h after collection. This routine led to an unexpected number of serum samples with increased homocysteine, which could not be confirmed by repeated blood sampling and analysis (data not shown). We now recommend that for the determination of total plasma thiols, whole blood should be placed on ice and the plasma fraction immediately prepared. If serum is used, it must be aspirated immediately after clot retraction. Our findings and these recommendations confirm and extend those recently published by Vester and Rasmussen (*14*).

We thank P. E. Lønning and S. Sandberg for helping with the statistical analysis. The technical assistance of Björn Lilletvedt is highly appreciated. This work was supported by grants from the Norwegian Cancer Society and the Norwegian Society for Science and the Humanities.

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