Clinical Chemistry 44:2
311–316 (1998)

Automation and
Analytical Techniques

Enzyme conversion immunoassay for determining total homocysteine in plasma or serum

Frank Frantzen,[1]* Arne Ludvig Faaren,[1] Ingrid Alfheim,[2] and
Arne Kristian Nordhei[1]

A rapid and precise immunoassay for quantification of total homocysteine in blood samples is presented. The method avoids the use of radioisotopes and chromatographic separations and relies on enzymatic conversion of homocysteine to *S*-adenosyl-L-homocysteine, followed by quantification of *S*-adenosyl-L-homocysteine by an enzyme-linked immunoassay in microtiter format. The within- and between-assay imprecision is <6% and 8%, respectively, and results by the method show good correlation with those by HPLC. Including controls and calibrators in duplicates, 82 samples can be analyzed within 2.5 h. The procedure can be fully automated.

The concentration of total homocysteine (tHcy)[3] in plasma or serum is an established marker of common diseases. It is a strong and independent risk factor for cardiovascular disease, a sensitive marker of cobalamin and folate deficiencies, and is used to diagnose the inborn errors in metabolism termed homocystinuria *[1–4]*. The tHcy concentrations have also been related to birth defects in pregnant women and to cognitive impairment in the elderly. Thus, the diagnostic utility of tHcy determinations is well documented in several clinical conditions, and the establishment of a tHcy assay is warranted in medium to large routine clinical chemistry laboratories. However, the current assays, based on HPLC or GC-MS, are time consuming and expensive and require highly skilled technical staff, and so are not an option for routine clinical chemistry laboratories.

We present here a new method that allows fully automated analysis of tHcy, avoiding the use of radioisotopes and tedious chromatographic separations. The method is based on enzymatic conversion of Hcy to *S*-adenosyl-L-homocysteine (SAH) by the action of SAH hydrolase (SAHase; EC 3.3.1.1), followed by quantification of SAH in a competitive immunoassay with use of a monoclonal anti-SAH antibody *[5]*. This principle has been used in an automated tHcy assay on the Abbott IMx instrument *[6]*. The method presented here, however, is not limited to specialized equipment, but allows the use of this technology in different formats and on various analytical platforms.

Materials and Methods

MATERIALS

Apparatus. A Multiscan RC microtiter reader (Labsystems OY) was used to measure absorbances on the microtiter plates. The microtiter plates ("Maxisorp" from A/S Nunc) were washed with a Well Wash 4 washing machine (Denley Instruments, Billinghurst, UK).

Chemicals. SAH, casein, bovine serum albumin (BSA), Tween 20, L-dithiothreitol (DTT), thimerosal, NaN_3, D,L-homocysteine, L-cysteine, and L-methionine were purchased from Sigma Chemical Co. L-Cystathionine and SAM were purchased from Fluka Chemie. For comparison, we obtained L-homocystine from both Sigma and Fluka; the Sigma product was the one generally used. Merck provided Na_2HPO_4, NaCl, NaOH, NaF, citric acid, and sulfuric acid. Bovine γ-globulin was provided by Miles Inc. SAHase was obtained from bovine liver by an in-house purification procedure. Adenosine (Ado) and adenosine deaminase (Adoase) were purchased from Boehringer Mannheim.

The monoclonal mouse anti-SAH antibody used was provided by Abbott Labs. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody was from Dako. Tetramethylbenzidine (TMB/E) solution from Chemicon International Inc. (Temecula, CA) was used as peroxidase substrate. The microtiter plates used in the assay were precoated with a BSA–SAH conjugate made by Axis Nord.

[1] Axis Nord, P.O. Box 6073, N-8018 Mørkved, Norway.
[2] Axis Biochemicals ASA, P.O. Box 2123, Grünerløkka, N-0505 Oslo, Norway.
*Author for correspondence.
[3] Nonstandard abbreviations: tHcy, total homocysteine; SAH, *S*-adenosyl-L-homocysteine; SAHase, SAH hydrolase; SAM, *S*-adenosyl-L-methionine; BSA, bovine serum albumin; DTT, dithiothreitol; Adoase, adenosine deaminase; HRP, horseradish peroxidase.
Received June 9, 1997; revision accepted September 8, 1997.

Reagents. The assay relies on two buffers: assay buffer 1, which contains 100 mmol/L Na_2HPO_4, 150 mmol/L NaCl, and 14 mmol/L NaN_3, adjusted to pH 8.5; and assay buffer 2, which is 100 mmol/L Na_2HPO_4, 150 mmol/L NaCl, and 0.1 g/L thimerosal, to which is added, after adjustment to pH 7.4, 0.1 g/L bovine γ-globulin, 2 g/L BSA, and 0.5 mL/L Tween 20.

Assay buffer 1 diluted 1:10 in water is used as washing solution in all washing steps.

Coating of microtiter plates. To each well of the microtiter plate is added 250 μL of coating solution containing 0.5 mg/L SAH–BSA conjugate and 2 mg/L Hcy-stripped BSA dissolved in 10 mmol/L Na_2HPO_4 and 150 mmol/L NaCl, adjusted to pH 7.4. After incubation overnight at 4 °C, the plates are emptied by inversion. Then 300 μL of blocking solution (25 g/L sodium caseinate in 10 mmol/L Na_2HPO_4 and 150 mmol/L NaCl, adjusted to pH 7.4) is added to each well, and the plates are incubated overnight at 4 °C. Finally, the plates are washed 3 times with 400 μL of washing solution and dried by inversion on absorbent paper.

ASSAY METHOD

Principle. The Hcy in serum/plasma samples is mainly in protein-bound form *[7, 8]*. Adding DTT to the sample cleaves disulfides, mixed disulfides, and protein-bound Hcy, releasing the free, reduced form of Hcy. Use of SAHase and excess Ado converts the reduced Hcy to SAH, as illustrated in Fig. 1, and tHcy in the sample is determined as SAH in a competitive immunoassay with an anti-SAH antibody. Fig. 2 depicts each step in the assay procedure. As part of sample pretreatment before the immunoassay step, any excess Ado remaining after the Hcy is converted is removed by Adoase, which avoids interference by Ado in the succeeding step. Because of the reversible nature of the SAHase reaction shown in Fig. 1, thimerosal, an inhibitor of SAHase, is included to prevent hydrolysis of SAH back to Hcy when the Ado is removed. The enzyme-treated samples are transferred to immunoplates coated with BSA–SAH and a competitive immunoassay for SAH is run.

The enzyme assay. Before start of the analysis, we made up a "sample preparation solution," sufficient for 35 samples/calibrators, by adding 1 mL of SAHase-solution (40 kU/L in assay buffer 1) and 1 mL of Ado/DTT solution (0.2 mmol/L Ado and 20 mmol/L DTT dissolved in 2.5 mmol/L citric acid, pH 3) to 18 mL of assay buffer 1.

This solution (500 μL) is added to 25 μL of serum sample or calibrator solution in a test tube. After this is incubated at 37 °C for 30 min, 500 μL of 1.4 g/L thimerosal (in 100 mmol/L Na_2HPO_4/150 mmol/L sodium chloride solution, adjusted to pH 8.5) is added, mixed, and incubated at ambient temperature (18–25 °C for 15 min). Then 500 μL of Adoase solution (0.1 kU/L in assay buffer 1) is added, mixed, and incubated for 3 min at ambient temperature.

The immunoassay. Portions (25 μL) of the SAHase-treated samples or calibrators are transferred to coated microtiter wells, 200 μL of anti-SAH antibody (67 μg/L in assay buffer 2) is added to each well, and the samples are incubated at ambient temperature (18–25 °C) for 30 min. The plate is washed 3 times, each time with 400 μL of washing solution per well, after which 200 μL of HRP-conjugated antibody (1.3 mg/L in stabilization buffer) is added to each sample. The plate is incubated at ambient temperature for 30 min, then washed 3 times with 400 μL of washing solution per well. After addition of 200 μL of the HRP substrate to each well, the plate is incubated at ambient temperature for 10 min. The HRP reaction is stopped by adding 100 μL of 0.8 mol/L sulfuric acid per well, and the yellow color produced is measured at 450 nm.

Calibration and calculation of results. Calibrators were prepared by dissolving SAH (from a stock solution of 5 mmol/L SAH) in assay buffer 1 to give concentrations of 2, 4, 8, 20, 30, and 50 μmol/L. To construct a calibration curve, we plotted the $\log_{10}$ values of the concentrations of the SAH calibrators against their absorbance readings and fitted the curve with a four-parameter logistic equation. The resulting curve was then used to calculate the tHcy values for unknown plasma samples on the basis of their absorbance readings.

For comparison, all samples were also analyzed for tHcy by HPLC at the University of Bergen *[9]*.

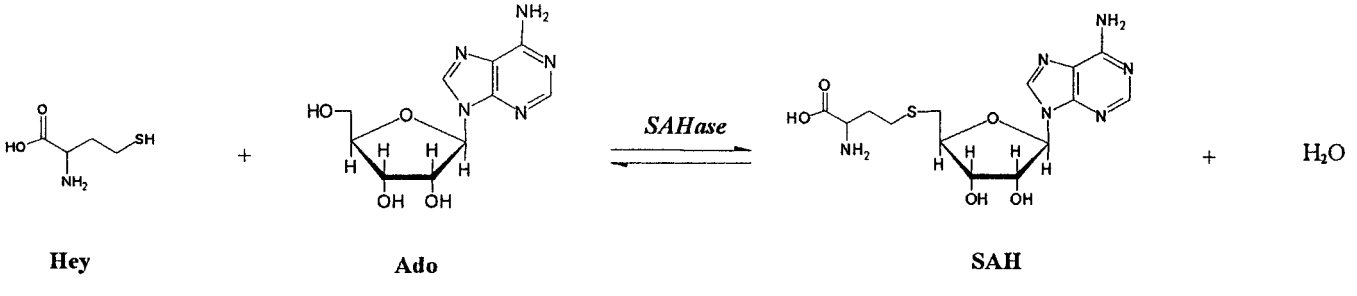

Fig. 1. Enzymatic conversion of Hcy to SAH by SAHase (EC 3.3.1.1).

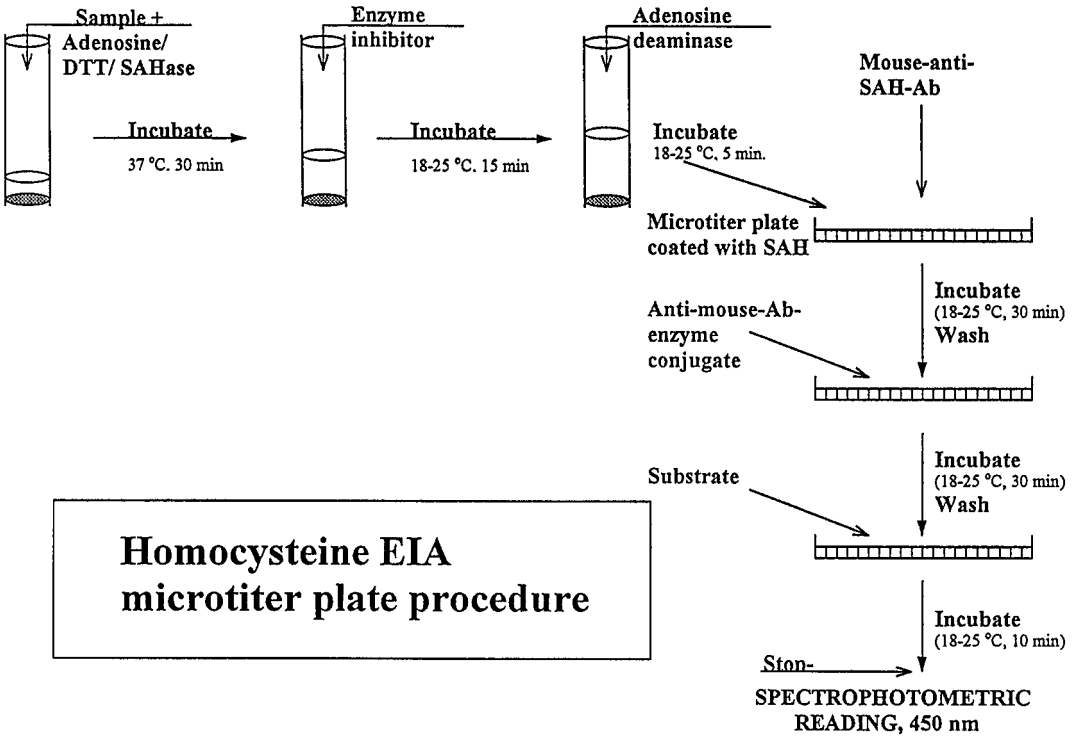

Fig. 2. Enzymatic conversion immunoassay for the determination of Hcy: sketch of the assay procedure.

BLOOD SAMPLES

Whole blood was collected into evacuated blood-collecting tubes without additives (for preparation of serum), or containing EDTA, heparin, or citrate as anticoagulants (for preparation of plasma). Blood tubes containing the stabilizer NaF with or without heparin were also evaluated. The samples were kept cold (<4 °C) after collection and centrifuged within 1 h after sampling. The serum and plasma supernatant were transferred to new vials and stored at −20 °C until analyzed.

Results

Calibration curve. A representative dose–response curve constructed with SAH calibrators is shown in Fig. 3. Calibrators were always run in duplicate. Although some variation is seen, the calibration curves made from SAH or crystalline L-homocystine are superimposed (see *Discussion*). Samples with Hcy concentrations between 2 and 50 μmol/L can be determined by the assay without additional dilution. Samples with tHcy >50 μmol/L should be diluted in assay buffer 1.

Linearity and recovery. Table 1 lists the results for analysis of dilutions of a sample with high Hcy concentration (40.7 μmol/L, as determined by HPLC *[9]*, and then diluted in assay buffer 1). Linear regression of the observed tHcy (y) vs the calculated expected tHcy (x) gave the following: $y = 1.04x - 2.25$ ($r = 0.996$, $S_{y|x} = 1.05$).

Recovery of known amounts of SAH added to samples is shown in Table 2. Because crystalline L-homocystine gave lower recovery and less consistent results than SAH, Hcy plasma samples (with tHcy concentrations >80 μmol/L) were also used. A summary of the results obtained with either SAH or L-homocystine (crystalline/blood samples) in the recovery experiments is presented in Table 3.

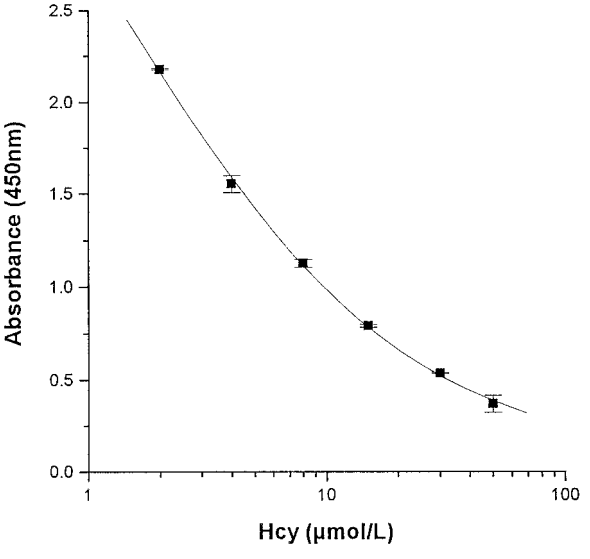

Fig. 3. Typical calibration curve prepared with SAH calibrators.

The calibrator concentrations were 2, 4, 8, 20, 30, and 50 μmol/L. The calibration curve was made by fitting the concentration/absorbance data with a four-parameter logistic function.

Table 1. Dilution linearity.

tHcy, μmol/L		
Calculated[a]	Observed	Recovery, %[b]
33.5	31.1	92.8
25.1	25.6	102.0
16.8	17.3	103.0
8.4	9.2	109.5

[a] A sample containing 40.7 μmol/L tHcy (by HPLC) was diluted in assay buffer 1 to the concentrations indicated.
[b] Observed × 100%/calculated.

Precision and comparison of methods. Table 4 lists between- and within-assay imprecision of the method. The within-assay variation was estimated after analyzing three samples containing 8.1, 13.6, and 27.3 μmol/L tHcy in 21 parallel determinations. Each of the samples were assayed in duplicate in the immunoassay step to test the imprecision of this step separately. The between-assay variation was estimated from results for 21 successive analytical set-ups.

A comparison of the results obtained with the method presented here (y) and those of an HPLC method *[9]* (x) indicated good agreement between the two methods (Fig. 4).

Specificity and interfering compounds. The cross-reactivity of the monoclonal SAH antibody against potentially interfering compounds was tested by adding those compounds to the calibrators. The compounds and concentration intervals tested (given as final concentration when added to the calibrators) were: cysteine (0–3 mmol/L), SAM (0–1 mmol/L), cystathionine (0–0.13 mmol/L), methionine (0–5 mmol/L), and Ado (0–50 μmol/L). In addition, these compounds, at concentrations of 30, 10, 1.3, 50, and 1 mmol/L, respectively, were also assayed as samples. Except for the SAM, none of the tested compounds affected the calibration curve. When assayed as samples, the concentrations recorded as tHcy were all <0.5 μmol/L. Again, SAM was the only compound found to substantially affect the performance of the assay; when present in samples at >10 μmol/L, it led to falsely increased results for Hcy concentrations (see *Discussion*).

Table 2. Recovery of SAH added to plasma.

SAH, μmol/L			
Added	Calculated	Observed[a]	Recovery, %
Plasma 1			
0	—	10.8	—
7.5	18.3	19.4	115
15.0	25.8	25.5	98
30.0	40.8	38.6	93
Plasma 2			
0	—	11.3	—
7.5	18.8	19.8	113
15	26.3	24.8	90
30	31.3	40.6	98
Average (and CV, %)			101 (9.4)

[a] Mean of four experiments.

Table 3. Summary of recovery experiments.

Concn. (μmol/L) of compounds added to plasma samples	Average recovery, %	CV, %
SAH, 7.5–30	101	9.4
Hcy,[a] 7.5–30	92	14
Hcy,[b] 10–40	100	9.5

[a] Added as crystalline L-homocystine.
[b] Added in the form of plasma samples with predetermined tHcy concentrations.

Anticoagulants and stabilizers. As long as the samples were treated as described in *Materials and Methods*, the tHcy concentrations were nearly the same (<6% difference) in serum and plasma, independent of type of anticoagulant. To test the effect of fluoride as a stabilizer *[11, 12]* in blood sample tubes, NaF was added to the sample to a concentration of 8 g/L (twice that ordinarily used in sample collection tubes) without affecting the assay results. However, plasma from vials with added fluoride/heparin showed ~10% lower tHcy concentration than the corresponding EDTA-plasma samples. No differences were found when using vials with added citrate and heparin; however, the number of samples used was limited, and more-thorough studies need to be done.

Discussion

Most methods for determining tHcy in serum or plasma are time-consuming and cumbersome *[8, 9, 13]*. With the present method, 82 samples, 2 controls, and 6 calibrators (controls and calibrators in duplicate) can be analyzed in ~2.5 h. Multiple plates can be analyzed simultaneously without much extra effort or, alternatively, the method can be partially or fully automated by using appropriate robotic equipment. The method has acceptable precision (CV <6–8%), has sufficient analysis range for most clinical applications, and shows good correlation with an established HPLC technique. Thus, this assay may be an acceptable option for routine clinical chemistry laboratories.

For tHcy concentrations within the range of the calibration curve, i.e., 2–50 μmol/L, the enzymatic conversion to SAH is complete, as illustrated by the superim-

Table 4. Within- and between-run imprecision of the assay.

Sample Hcy, μmol/L	CV, %	
	Within-assay	Between-assays
8.1	5.0	5.4
13.6	4.3	6.2
27.3	5.5	8.2

The samples were run in 21 duplicates. Between-assay variation is based on results from 21 succeeding analytical set-ups performed over 3 days.

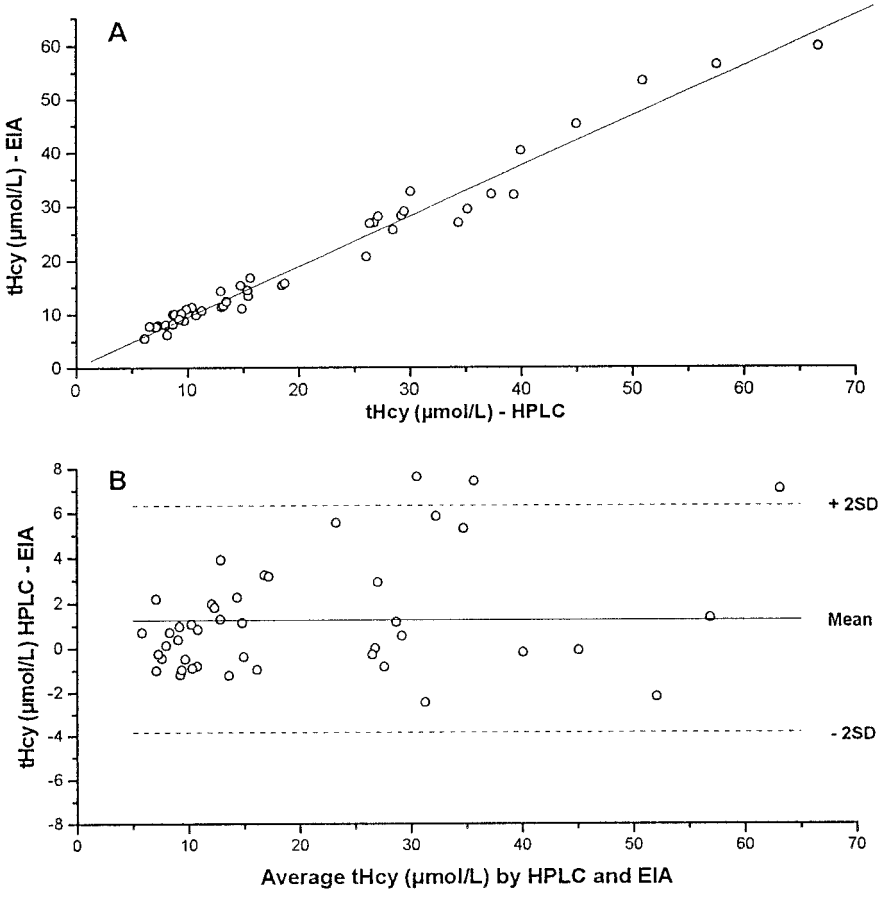

Fig. 4. Comparison of tHcy measured by the enzyme/immunoassay (*y*) vs HPLC (*x*) *[9]*: (*A*) by linear regression analysis, $y = 0.933x + 0.184$ (n = 44, $r = 0.986$, $S_{y|x} = 2.357$); (*B*) in a Bland–Altman plot *[10]*, which shows the difference in tHcy results between the two methods as a function of their mean value.

posed calibration curves derived from L-homocystine and SAH. However, similar to what was seen in the recovery experiments utilizing crystalline L-homocystine, there was also some variability of the dose–response curve prepared with crystalline L-homocystine, particularly in the lower concentration range. Because of the good recovery achieved when plasma samples with high tHcy concentrations were used for the recovery studies (Table 3), the crystalline compound may act differently from native blood samples. The reason for this discrepancy and for the greater variation when using crystalline L-homocystine is unknown; in part, however, it seems to be related to reduction of the disulfide bond of the commercial product. If the crystalline compound is a bit more difficult to reduce, slightly less L-homocysteine will be available and subsequently less SAH will be generated than when native blood samples are used. However, given differences observed between commercial L-homocystine products, the purity of the crystalline compound also seems to be of importance. Although not successful, we tried varying several assay conditions (including concentrations of Ado and DTT, amount of enzyme, and pH) to address this problem. Given these problems, as well as the lower stability of Hcy calibrators prepared from the crystalline products, we have been using SAH calibrators in the assay.

The use of SAH in the recovery experiments tests the immunoassay part of the presented assay. For evaluation of all steps in the procedure, including the enzymatic conversion, L-homocystine should be used. Because the enzyme conversion step of Hcy to SAH by SAHase is not controlled by using SAH as calibrator, plasma or serum control samples with known tHcy concentrations should be assayed together with the calibrators and samples.

Although the tHcy results obtained correlate well with HPLC results, the method presented measures slightly lower concentrations than the HPLC method—on average, 5–10% lower. This difference generally reflects the lack of standardization in tHcy assays; as long as this is the case, results from HPLC and the enzyme immunoassay presented should not be used interchangeably. The observed difference between the methods could be a result of the purity of the chemicals used and the preparation of calibrators. Given the specificity of SAHase reaction, enantiomeric purity of the Hcy calibrators is essential. However, the similarities of the calibration curves obtained with either SAH or crystalline L-homocystine suggest that this is not an important source of error. Another possibility is related to the unique chemical characteristics of Hcy in plasma/serum, of which almost all is protein-bound in thawed plasma samples. Thus, it is possible that the reduction of oxidized Hcy species, the

enzyme conversion, and the immunoassay differ between plasma/serum and water-based calibrators. However, this is not supported by the recovery experiments, which indicate equal performance with plasma samples and water-based calibrators. In any case, slope differences have also been reported between chromatographic methods *[13]*, and further studies are therefore necessary to establish good interlaboratory correlation for the Hcy analyte.

We used thimerosal to inhibit SAHase before adding Adoase to remove excess Ado. Although more specific inhibitors and inactivators of SAHase have been tested, among them 2-chloroadenosine, aristeromycin, eritadenine, adenosine dialdehyde, and adenine arabinofuranoside *[14]*, they either competed with the anti-SAH antibody or failed to inactivate the enzyme completely within acceptable incubation times or concentrations (data not shown).

Cross-reactivity studies have shown that the monoclonal anti-SAH antibody does cross-react somewhat with Ado. To eliminate this problem, we used Adoase to convert the excess Ado into inosine, which does not cross-react with the antibody. The specificity of the anti-SAH antibody was found to be directed against the Ado residue/thio-ether region of SAH. High concentrations of both methionine and cysteine, as well as Hcy alone, do not cross-react with the antibody, nor does cystathionine. The fact that only SAM was found to affect the determination of Hcy is explained by the structural resemblance of this compound to SAH and the corresponding recognition by the anti-SAH antibody. Although SAM in concentrations >10 μmol/L did show an effect, this is >50–100 times greater than the concentration usually found in human plasma *[15]*. Similarly, endogenous SAH does not interfere because of its low concentration in human plasma *[15]*.

Determination of tHcy requires special care in handling the blood samples. The concentrations of tHcy show a time- and temperature-dependent increase in whole-blood samples related to a continuous production and release of Hcy from blood cells *[9, 11]*. Artifactual increases are low when the blood samples are centrifuged and plasma separated within 1 h after collection. Because addition of anticoagulants such as EDTA or heparin allows immediate centrifugation, this is the main reason for recommending plasma rather than serum samples for the analysis of tHcy in blood.

Fluoride has been suggested as an additive to blood samples to prevent cells from producing and leaking intercellular Hcy into the plasma after collection *[11, 12]*. However, the decrease in measured tHcy in collection tubes containing added fluoride/heparin, in comparison with those containing EDTA, makes it necessary to investigate the use of this type of vial in more detail.

Unlike all chromatographic methods, the present method makes use of inexpensive apparatus available in many laboratories. The method therefore serves as an attractive alternative to HPLC analysis, both in routine and research laboratories. This new analytical method should help clinical laboratories in the diagnosis of vitamin deficiency as well as benefit clinical researchers in large population-based studies on the potential use of homocysteine as an independent risk factor for premature cardiovascular disease.

Thanks are due H. Refsum (University of Bergen) for discussions and critical reading of the manuscript.

References

1. Ueland PM, Refsum H, Brattstrøm L. Plasma homocysteine and cardiovascular disease. In: Francis RB Jr, ed. Atherosclerotic cardiovascular disease, hemostasis and endothelial function. New York: Marcel Dekker, 1992:183–236.
2. Stampfer MJ, Malinow MR. Can lowering homocysteine levels reduce cardiovascular disease. N Engl J Med 1995;332: 328–9.
3. Allen RH, Stabler SP, Savage DG. Diagnosis of cobalamin deficiency I. Usefulness of serum methylmalonic acid and total homocysteine concentrations. Am J Hematol 1990;34:90–8.
4. Nexø E, Hansen M, Rasmussen K, Lindgren A, Gräsbeck R. How to diagnose cobalamin deficiency? Scand J Clin Lab Invest 1994;54: 61–76.
5. Sundrehagen E. Homocysteine assay. Pat Appl 1993;PCT/Gb93/ 00138.
6. Shipchandler MT, Moore EG. Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx® analyzer. Clin Chem 1995;41:991–4.
7. Kang S-S, Wong PWK, Curley K. The effect of D-penicillamine on protein-bound homocyste(e)ine in homocystinurics. Pediatr Res 1982;16:370–2.
8. Refsum H, Helland S, Ueland PM. Radioenzymic determination of homocysteine in plasma and urine. Clin Chem 1985;31:624–8.
9. Refsum H, Ueland PM, Svardal AM. Fully automated fluorescence assay for determining total homocysteine in plasma. Clin Chem 1989;35:1921–7.
10. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;8476:307–10.
11. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ. The effect of blood sample aging and food consumption on plasma total homocysteine levels. Clin Chim Acta 1992;207:119–28.
12. Møller J, Rasmussen K. Homocysteine in plasma: stabilization of blood samples with fluoride [Tech Brief]. Clin Chem 1995;41: 758–9.
13. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: methods and clinical applications [Review]. Clin Chem 1993;39: 1764–79.
14. Zimmerman TP, Wolberg G, Duncan GS, Elion GB. Adenosine analogues as substrates and inhibitors of *S*-adenosylhomocysteine hydrolase in intact lymphocytes. Biochemistry 1980;19: 2252–9.
15. Loehrer FMT, Haefeli WE, Angst CP, Browne G, Frick G, Fowler B. Effect of methionine loading on 5-methyltetrahydrofolate, *S*-adenosylmethionine and *S*-adenosylhomocysteine in plasma of healthy humans. Clin Sci (Colch) 1996;91:79–86.