We have developed an HPLC assay for HCySH suitable for clinical laboratory use. The method is simple and precise, enabling comparatively easy processing of large numbers of specimens. The performance characteristics of the method presented here are comparable with those utilizing fluorescence detection. The major advantage of this method is the avoidance of derivatization. The use of penicillamine as an internal standard for HCySH analysis had not been reported previously. It is an excellent internal standard because it has a similar chemical structure to HCySH and is electrochemically active and readily available.

References

Method Comparison for Total Plasma Homocysteine between the Abbott IMx Analyzer and an HPLC Assay with Internal Standardization, Christine M. Pfeiffer,1* Della Twite,1 Jessie Shih,2 Shelley R. Holets-McCormack,2 and Elaine W. Gunter1 1National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341, and 2Abbott Laboratories, Abbott Park, IL 60064; *author for correspondence: fax 770-488-4609, e-mail cfp8@cdc.gov

The increasing interest in measuring total homocysteine in plasma has led to the development of several different methods (1). Although most publications describe HPLC methods with manual sample preparation, the first fully or partially automated kits were introduced recently: a fluorescence polarization immunoassay on the IMx® analyzer (Abbott Laboratories, Abbott Park, IL), a microtiter plate enzyme immunoassay (Bio-Rad Laboratories), and an HPLC kit with electrochemical detection (BAS). We evaluated the Abbott IMx analyzer automated method for total homocysteine and compared it with our HPLC assay with internal standardization.

The “Abbott Homocysteine (HCY) assay” is a fluorescence polarization immunoassay based on the highly selective enzymatic conversion of homocysteine to S-adenosyl-l-homocysteine, which is then recognized by a monoclonal antibody (2). The assay requires 50 μL of sample, with no sample pretreatment. A batch of 20 samples can be processed within 1 h. We assessed the intra- and interassay variabilities, the recovery of added homocysteine, the analytical sensitivity, the stability of the calibration curve, the specificity, and the cross-reactivity towards other thiols. EDTA plasma samples were obtained within a maximum of 30 min after blood collection. Serum samples were obtained from whole blood allowed to clot at room temperature for 30–60 min. Blood specimens were collected by the Emory University Hospital Blood Collection Service under an agreement with the Centers for Disease Control and Prevention (including an omnibus informed consent and Human Subjects Review protocol). All serum and plasma samples were stored at −70 °C.

We sequentially analyzed 20 replicates (one carousel) of each of the three serum-based quality-control (QC) pools supplied by Abbott to assess intraassay imprecision (CV). The within-run CV was 1.0% for the low-concentration QC pool (7.0 μmol/L), 1.5% for the mid-concentration QC pool (12.8 μmol/L), and 1.4% for the high-concentration QC pool (26.0 μmol/L). The intraassay CV was also determined by measuring five replicates each (in one analytical run) of 20 plasma samples from healthy volunteers. The mean CV was 2.1%, with a range of 0.7–8.3%.

Interassay imprecision was determined over the course of 20 days with the use of three in-house plasma-based QC pools (single determination). The between-run CV was 3.2% for the low-concentration QC pool (6.0 μmol/L), 4.9% for the mid-concentration QC pool (11.4 μmol/L), and 2.5% for the high-concentration QC pool (28.8 μmol/L). The 20 plasma samples mentioned above were also analyzed in single determinations on each of 5 days. The average CV was 3.2%, with a range of 1.0–8.3%.

We studied the recovery of l-homocystine added to a base plasma pool at three concentrations in duplicate: 5, 10, and 20 μmol/L free thiol. The mean recovery (± SD) was 101.9% ± 1.9%. To test the influence of the matrix on recovery, we added l-homocystine corresponding to 20 μmol/L free thiol to three serum and six plasma samples from apparently healthy volunteers. The mean recovery (± SD) was 99.6% ± 1.2%.

To test the stability of the calibration curve (range, 0–50 μmol/L), we analyzed the six calibrators in duplicate on 6 days over the course of 1 month with a machine-stored calibration curve. The measured values corresponded well with the expected values (y = 0.1058 + 0.9838x; R² = 0.9999), and the CV was 1.0–7.2% (increasing CV with decreasing concentration of calibrator).

We measured the zero calibrator (calibrator A, no homocysteine added) in two or more replicates during 10 days. The limit of detection, 0.35 μmol/L total homocysteine, was calculated as 3 SD above the mean response of the zero calibrator (0.08 ± 0.117 μmol/L).

To test for specificity and cross-reactivity, we analyzed six thiol calibrator solutions in duplicate at four different concentrations (20, 40, 200, and 400 μmol/L): cysteine, cysteinylglycine, glutathione, cysteamine, N-acetylcy-
teinie, and mercaptopropionylglycine. The observed values were between 0.06 and 0.14 μmol/L, and thus were very close to the observed value of the zero calibrator.

We prepared solutions of t-homocysteine at 20, 40, 100, 200, and 300 μmol/L free thiol. Each concentration was analyzed in triplicate, the three highest concentrations with the use of the dilution assay provided with the software for total homocysteine concentrations exceeding 50 μmol/L (which performs a 1:10 dilution of the sample). The measured values agreed well with the expected values: $y = 0.3628 + 0.9348x$; $r^2 = 0.9995$.

An extensive method comparison was performed between the Abbott Homocysteine (HCY) assay and an HPLC assay with internal standardization. If samples were not analyzed simultaneously with both methods, they were stored at −70 °C for not more than 1 month between the assays. The performance of the HPLC assay and the validation results will be described in detail in a separate paper. Briefly, the assay, which is a modification of the method of Vester and Rasmussen (3) and Gilfix et al. (4), uses tris(2-carboxyl-ethyl)phosphine, a novel water-soluble derivative of tri-butyolphosphine, to reduce protein-bound and oxidized thiols; trichloroacetic acid to precipitate proteins; and ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) to derivatize reduced thiols for fluorescent detection. Cystamine was used as internal standard. The separation of the obtained thiol derivatives was performed isocratically within 7 min, with the use of an acetate mobile phase with mild pH. Recoveries of thiols added to plasma at different concentrations were nearly complete (98.7% ± 2.5%). The intraassay imprecision was between 1.1% and 1.8%; the interassay imprecision was between 2.4% and 6.7% (5).

Total homocysteine concentrations determined for ~800 different serum and plasma samples from healthy subjects (n = 713) and subjects with low or low-normal serum folate and/or serum vitamin B₁₂ (n = 87) gave good agreement between the two methods: $y = (0.16 ± 0.044) + (0.96 ± 0.004)x$; $r^2 = 0.9866$; $S_{y|x} = 0.642$; Fig. 1. An even better agreement was obtained for samples in the range up to 50 μmol/L total homocysteine, thus excluding three samples that had to be diluted for the Abbott assay: $y = (-0.1098 ± 0.043) + (0.9923 ± 0.004)x$; $r^2 = 0.9855$; $S_{y|x} = 0.568$. The correlations were very similar between serum [$y = (-0.2361 ± 0.086) + (1.0046 ± 0.007)x$; $r^2 = 0.9882$; $S_{y|x} = 0.571$; n = 275] and plasma [$y = (-0.0271 ± 0.052) + (0.9808 ± 0.006)x$; $r^2 = 0.9832$; $S_{y|x} = 0.564$; n = 540]. Subjects with low or low-normal serum folate and/or serum vitamin B₁₂ had significantly increased total homocysteine (14.8 ± 7.0 μmol/L) compared with apparently healthy subjects (8.3 ± 3.9 μmol/L).

During the course of the method comparison, we found one very lipemic sample that showed some discrepancy between the total plasma homocysteine determined by HPLC (10.7 μmol/L) and by the Abbott assay (13.1 μmol/L). However, after the sample was diluted 1:2 with saline, the Abbott result (11.0 μmol/L) was nearly identical to the HPLC result.

At present, we are performing an interlaboratory com-

parison study with 14 laboratories that use different methods for the determination of total plasma homocysteine to study the interlaboratory variation within one method type and the intermethod variation under field conditions. The fully automated Abbott IMx method is included in this project.

In conclusion, we found that the Abbott Homocysteine (HCY) assay had within-day and day-to-day imprecision (CV) <5%, 99.6% recovery of added homocysteine, and no significant cross-reactivity with the tested thiols. The comparability between this assay and an HPLC assay with internal standardization was excellent for the range up to 50 μmol/L of total homocysteine. The throughput of the fully automated Abbott Homocysteine (HCY) assay is ~100–120 patient samples/8 h. Although a similar throughput can be obtained with the HPLC assay, the effective time necessary to perform the Abbott assay is less than the time needed to perform the sample preparation for the HPLC assay.

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References