lates mostly as disulfides complexed with albumin, measured by laboratory tests. This study was carried out with the cooperation of Becton Dickinson Vacutainer Tube Systems, Franklin, NJ, who provided the evaluation tubes and covered the costs of laboratory tests.

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References


Measurement of Plasma Total Homocysteine by HPLC with Coulometric Detection, Steven C. Martin,* Ioannis Tsakas-Ampatzis, William A. Bartlett, and Alan F. Jones (Department of Clinical Chemistry, Heartlands Hospital, Birmingham, West Midlands, B9 5SS, United Kingdom; *author for correspondence: fax 44 121 766 8693, e-mail s.c.martin@bham.ac.uk)

Homocysteine (HcySH) is an intermediate in the metabolism of methionine. High plasma HcySH concentrations are an independent risk factor for stroke, peripheral vascular disease, deep venous thrombosis, coronary disease, and neural tube defects (1). Because HcySH circulates mostly as disulfides complexed with albumin, measurement of plasma total HcySH involves reduction of these disulfide bonds and detection of the generated HcySH. Once reduced, HcySH can be separated chromatographically using ion-paired reversed-phase HPLC and is amenable to oxidative mode coulometric electrochemical detection, which avoids time-consuming pre- or postcolumn derivatization. The use of electrochemical detection with an appropriate internal standard, penicillamine, has led to substantial improvements over our previously reported method (2).

The assay required 400 μL of EDTA plasma, which was reduced with 100 μL of 50 mmol/L dithiothreitol [DTT; modified from Ref. (3)] containing the internal standard (200 μmol/L penicillamine) at 37 °C for 15 min. After incubation, protein precipitation was achieved by the addition of 100 μL of 150 g/L sulfosalicylic acid. We injected 20 μL of the supernatant onto a 250 × 4.6 mm octadecyl silica column (Spherisorb ODS-2; particle size, 5 μm; Jones Chromatography Ltd.), with a 10 × 4.6 mm guard column packed with the same material. The mobile phase (flow rate, 1 mL/min) consisted of 10 mmol/L diammonium hydrogen phosphate adjusted to pH 2.58 (2.63 mmol/L H⁺ ion) with orthophosphoric acid, 137.5 mL/L methanol, and 12 mmol/L octane sulfonate (OSA). The mobile phase was filtered through a 0.45 μm cellulose membrane before use and was not recycled during chromatography.

We detected HcySH with an ESA Coulotech 5100A electrochemical detector (ESA Analytical Ltd), using an ESA 5010 coulometric cell and with electrode 1 (E1) set at a screening potential of +0.4 V and electrode 2 (E2) set at +1.0 V. An ESA 5020 guard cell was set to +1.1 V and installed between the pump and the autosampler (ISS200; Perkin-Elmer). The guard cell cleans the mobile phase electrochemically before addition of sample and reduces the background current at detector E2. The signal (peak height) resulting from oxidation of HcySH was measured at E2 and processed using a computer integrator (Gilion Unipoint Software).

HcySH calibrators (0.625–40 μmol/L) were prepared by dissolving l-homocystine (Sigma Chemical Co.) in 2 mmol/L disodium hydrogen phosphate. l-Homocystine has been reported to be purer than commercially available d,l-homocystine (4). The internal quality control was citrated plasma (time-expired, fresh-frozen plasma; West Midlands Blood Transfusion Service) with HcySH added at 8.5 and 26 μmol/L.

Each component of the mobile phase was adjusted independently to achieve optimal separation of HcySH from other electrochemically active components. When an initial mobile phase of 10 mmol/L diammonium hydrogen phosphate buffer, pH 2.75, containing 6 mmol/L OSA and 100 mL/L methanol was used, sulfosalicylic acid was not retained by the column. Reduced DTT eluted early (at ~5.7 min), and oxidized DTT eluted at ~9.6 min. When the phosphate concentration of the mobile phase was increased, the retention times for all peaks were decreased without alteration of their elution sequence. Previous methods used sodium dihydrogen phosphate as the
buffer salt (2, 5). We used diammonium hydrogen phosphate because ammonium ions tend to block residual uncapped silanol groups on the column packing. These groups may complicate the chromatography by facilitating normal rather than reversed-phase mechanisms. The effects are usually apparent in the form of poor peak symmetry and tailing (6), and the use of the ammonium rather than the sodium salt improved peak characteristics (data not shown).

An increase in the OSA concentration had a greater effect on the retention of HCySH and penicillamine than on oxidized or reduced DTT. At concentrations >6 mmol/L, the increase in the retention time of HCySH caused it to elute after the oxidized DTT peak. The retention time of HCySH increased at OSA concentrations up to 12 mmol/L, the concentration chosen for additional experiments.

We chose a final mobile phase pH of 2.58 after investigating the effect of increasing the hydrogen ion concentration in ~0.4 mmol/L steps (Table 1), which gave a pH range of 3.50 to 2.50. Lower pH values were not investigated because of the risk of column damage. pH values <2.75 allowed resolution of all the components of interest.

Increasing the methanol concentration of the mobile phase reduced the retention times of all peaks without changing the order in which they eluted. The use of 138 mL/L methanol allowed separation of HCySH from oxidized DTT in an acceptable elution time.

Penicillamine, cysteamine, and 3,4-dihydroxybenzylamine were tested as candidate internal standards. 3,4-Dihydroxybenzylamine did not produce a signal under the conditions used, and cysteamine eluted too early. Penicillamine (a close analog of HCySH with an extra methyl group) eluted late, in a position free of other peaks. A final concentration of 34 μmol/L was adopted in subsequent investigations (Fig. 1).

The mobile phase was not recycled because the baseline drifted and the peak shape deteriorated when recycled mobile phase was used. These problems may be caused by the accumulation of oxidized products in the mobile phase.

The purity of the HCySH peak was established electrochemically by plotting the peak areas of the putative HCySH peak in plasma samples against those of a pure calibrator at various E2 applied voltages. The resulting plot was a straight line through the origin with a correlation coefficient of 1.000.

The intraassay CVs were 7.5% at 0.625 μmol/L (aqueous, n = 20), 2.2% at 7 μmol/L (plasma, n = 20), and 2.4% at 20 μmol/L (plasma, n = 20). The interassay CVs were 8.6% at 8.5 μmol/L (plasma, n = 8) and 6.8% at 25 μmol/L (plasma, n = 8). The recovery of HCySH was 93.4% at 6.5 μmol/L and 95% at 20 μmol/L. Under the conditions chosen, the detection limit for HCySH (95% confidence), defined (7) as 1.65 SD below the lowest measured concentration (0.625 μmol/L), was 0.56 μmol/L.

The geometric mean for total HCySH in plasma samples from 33 fasting laboratory volunteers (obtained after approval from the hospital ethics committee) was 8.2 ± 1.3 μmol/L, similar to values in the literature (4).

## Table 1. The effect of varying pH on peak retention times.

<table>
<thead>
<tr>
<th>pH</th>
<th>Reduced</th>
<th>Oxidized</th>
<th>HCySH</th>
<th>Penicillamine</th>
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<tr>
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<td>5.7</td>
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<td>9.5</td>
<td>—</td>
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</tr>
<tr>
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<td>2.50</td>
<td>6.6</td>
<td>11.2</td>
<td>15.6</td>
<td>26.9</td>
</tr>
</tbody>
</table>

* The HCySH peak coeluted with reduced DTT.

* The HCySH peak coeluted with oxidized DTT.

![Fig. 1. Typical chromatograms achieved with a mobile phase consisting of 10 mmol/L diammonium hydrogen phosphate (pH 2.58) containing 137.5 mL/L methanol and 12 mmol/L OSA (flow rate, 1 mL/min).](image)

(A), 1.25 μmol/L calibrator; (B), 40 μmol/L calibrator; (C), plasma containing 20 μmol/L HCySH. Peak 1, reduced DTT; peak 2, oxidized DTT; peak 3, HCySH; peak 4, penicillamine (internal standard).
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

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.

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 methods with manual sample preparation, the first fully automated 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 variability, 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 samples 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%.

Intraassay 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-homocysteine 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-homocysteine 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-acetylcyso-