Rapid, Fully Automated Measurement of Plasma Homocyst(e)ine with the Abbott IMx® Analyzer

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We have developed a totally automated fluorescence polarization immunoassay for homocyst(e)ine with no pretreatment or chromatographic steps. Comparison with four well-established chromatographic methods yielded r values ranging from 0.892 to 0.997 and slopes from 1.030 to 1.493. Inter- and intraassay CVs ranged from 0.0% to 8.0% and from 0.0% to 6.4%, respectively. Imprecision (CV) of measuring six plasma samples on three instruments ranged from 6.3% to 10.2%. The assay was linear for plasma samples diluted with buffer from 0 to 8-fold. Mean recovery of homocysteine added to two plasma samples was 97.1% and 99.9%. The assay exhibited almost no cross-reactivity towards cysteine and methionine, and a batch of 20 samples can be processed in 60 min.

Indexing Terms: fluorescence polarization immunoassay/cardiovascular disease/risk factors

The acceptance of an assay for total plasma or serum concentrations of homocyst(e)ine (HCY) as a marker of cardiovascular risk, nutritional assessment, or neonatal homocystinurea, depends on the test’s availability for the routine laboratory. The test should be rapid, minimize operator exposure to biohazards, and meet the stringent requirements of a clinical assay. Current methods of measuring plasma or serum HCY, comprehensively reviewed in a recent article (1), use some form of chromatography; only a few are totally automated, and the throughput is relatively low. For example, in the totally automated C18-based HPLC method of Fiskerstrand et al. (2) and Jacobson et al. (3), throughput is 60–80 samples per day.

HCY exists in numerous forms in plasma—as a small percentage in the free form, as disulfides with itself and cysteine, and as disulfides with albumin (~70%) (4). Reducing agents, such as sodium borohydride, phosphines, and dithiothreitol (DTT), are used to reduce disulfide bonds and to yield free HCY. The next step in quantification is chromatography, either as a derivatized or an undervatized form (1). Total HCY is the clinically relevant measure, with reference values in fasting subjects of 5 to 15 μmol/L (1). Even a moderate increase in plasma HCY, referred to as hyperhomocyst(e)inemia, is considered a common, independent, and reversible risk factor for premature cardiovascular diseases in the general population (5–9).

Antibody-based assays are popular formats amenable to automation with high throughputs. In the present study, we chose the fluorescence polarization immunoassay (FPIA) methodology, a commonly used sensitive and precise method for measuring small molecules (10). For direct measurement of HCY by FPIA, we would have had to procure or produce an antibody that could differentiate HCY from similar amino acids, e.g., L-cysteine and L-methionine, which are present in ~25- and ~3-fold molar excess concentrations, respectively (11). An indirect approach, based on the highly selective enzyme conversion of HCY to S-adenosyl-L-homocysteine (SAH), provided an attractive alternative (12). We raised a monoclonal antibody that recognized SAH and a fluoresceinated analog of SAH. Using these reagents, we developed a new automated assay method on an Abbott (Abbott Park, IL) IMx® analyzer (13), in which no manual pretreatment steps are required and the throughput is 20 samples per hour (14).

Materials and Methods

Materials

**Chemicals, instrument, and supplies.** SAH, DTT, L-cysteine hydrochloride, L-methionine, and adenosine were purchased from Sigma Chemical Co. (St. Louis, MO). L-Homocysteine was purchased from Fluka Chemika-BioChemika (Ronkonkoma, NY). Clinical serum samples were provided by R. H. Allen of the University of Colorado (Denver, CO). Clinical plasma samples were provided by M. R. Malinow, Oregon Regional Primate Research Center (Beaverton, OR). Plasma containing 504 μmol/L HCY was obtained from Helga Refsum, University of Bergen (Bergen, Norway). Plasma samples, obtained from healthy donors at Abbott Labs. (Abbott Park, IL), were analyzed by Refsum and Anders Andersson, University of Lund (Lund, Sweden).

The IMx analyzer, disposable cuvettes and cartridges, and the FPIA line buffer (0.1 mol/L phosphate buffer, pH 7.4, containing 0.1 g/L bovine gamma globulin and 0.5 g/L sodium azide) were obtained from Abbott Labs. A monoclonal antibody against SAH (4-481-244) and an SAH analog fluoresceinated tracer (S-adenosyl-L-cysteine-6-carboxyfluorescein amide) were also produced at Abbott Labs. Bovine liver SAH hydrolase enzyme was prepared as described in the literature (15).

**HCY calibrators and other reagents.** These were made from a stock solution of L-homocystine corre-
sponding to 274.14 μmol/L HCY equivalent in the line buffer (9.196 mg in 250 mL, dissolved by warming) and diluted further in the same buffer to give calibrators of 0, 3.75, 7.50, 15, 30, and 60 μmol/L HCY equivalent.

**IMx Assay**

To establish a calibration curve, the six calibrators are loaded (~70 μL each), in duplicate, into the sample cups (13). The automated assay sequence begins with the pipetting and mixing, by a robotic arm (probe), of 25 μL of the calibrator (or, later, sample), 25 μL of combined adenosine (200 μmol/L) and DTT (10 mmol/L) solution, 60 μL of SAH hydrolase, and 70 μL of the line buffer in the reaction well (see Fig. 1). Mixing is enhanced by the aspiration and dispense cycles of the probe. After 30 min of incubation at 34 °C, 23 μL of the reaction mixture and 65 μL of the mouse monoclonal antibody solution are transferred to the reaction cuvette and diluted to 1 mL with the line buffer. After 10 min of incubation, a background fluorescence reading is taken. The fluoresceinated tracer (85 μL) and a second aliquot of the reaction mixture (23 μL) are then added and diluted to 2 mL with the line buffer. After 10 min, the second reading is obtained. A microprocessor calculates the polarization in mP units, corrects for the background, and constructs a calibration curve from the six points by using a four-parameter logistic curve formula. The microprocessor-stored curve is then used to calculate the values for unknown plasma samples run in the same or later cycles. Results for as many as 20 samples are available for printout, typically in ~60 min (13).

**Results**

**Analytical Performance**

**Inter- and intraassay CVs.** Intraassay CVs were obtained by assaying the calibrators in triplicate on the same carousel. This process was repeated three times to determine the interassay CV (Table 1).

**Precision.** Six plasma samples were measured on three different IMx instruments on each of 5 days. The combined CVs (n = 15) for plasma samples containing 5.0, 8.6, 9.6, 11.9, 20.4, and 33.2 μmol/L HCY equivalents were 10.2%, 6.3%, 5.9%, 5.8%, 5.7%, and 8.6%, respectively.

**Stability of the calibration curve.** A typical calibration curve is shown in Fig. 2. The six calibrators were analyzed on days 0, 4, 9, 13, and 16 in duplicate with use of a machine-stored calibration curve. The CVs ranged from 0.4% to 1.7%. A plasma sample measured at 10.80 μmol/L HCY equivalent in the same run gave a CV of 2.3% (Malinor MR, Oregon Regional Primate Research Center, Beaverton, OR; unpublished data).

**Comparison with other methods.** A regression equation for results for 42 plasma clinical samples analyzed by this method (γ), as single determinations, and by an established HPLC method (κ) with thiol-specific electrochemical detection (16, 17) was: \( y = 1.030x + 0.184 \) (\( r = 0.980, S_{yx} = 1.183, n = 42 \)). Comparisons (also as single determinations) with other methods yielded: (α) with a thiol-specific HPLC monobromobimane derivatization method in plasma, \( y = 1.212x - 0.319 \) (\( r = 0.995, S_{yx} = 0.457, n = 10 \) normal plasma samples) (2); (β) with a 4,4′-dithiopyridine postcolumn derivatization method, \( y = 1.119x + 0.167 \) (\( r = 0.996, S_{yx} = 0.557, n = 8 \) normal plasma samples) (18); and (c) with gas chromatography–mass spectrometry, \( y = 1.493x - 1.145 \) (\( r = 0.997, S_{yx} = 0.877, n = 21 \) clinical serum samples) (19).

**Dilution parallelism.** Two plasma samples containing moderately high HCY values were diluted with the line FPIA buffer from 0 to 8-fold. Table 2 summarizes the results. In addition, a patient’s plasma containing 408 μmol/L HCY (University of Bergen) was similarly diluted 16-, 32-, and 64-fold. The ratios of observed/calculated values were 1.17, 1.16, and 1.20, respectively. As shown above, for the FPIA method correlated with the University of Bergen method (2), the slope is 1.212.

**Mixing of plasmas.** One plasma with a high concentration (50.31 μmol/L) and one with low (14.41 μmol/L)
were mixed in equal proportions. The measured value of 32.23 μmol/L was 99.6% of the expected value.

Analytical recovery. Mean recoveries of l-homocysteine (introduced as l-homocystine dissolved in FPIA line buffer) added to two plasma samples were 97.1% and 99.9% (Table 3).

Cross-reactivity. l-Cysteine (hydrochloride) and l-methionine at 5 and 4.5 mmol/L, respectively, in the line buffer were assayed with the HCY FPIA. The observed values were 0 and 0.1 μmol/L, respectively.

Analysis of Plasma Samples for Endogenous SAH

In this experiment, the enzyme reagent bottle was replaced with one filled with the FPIA line buffer (Fig. 1). The assay was run in the same protocol with 18 normal plasma samples and 2 containing added SAH (16.2 μmol/L) as positive controls. In the absence of the enzyme, no conversion of HCY to SAH took place; thus, we could measure the endogenous SAH. The average polarization value for the normal plasma samples was 161.58 mP (±1.57 mP, CV <1), which corresponds to SAH concentrations close to zero read from the calibra-

tion curve (Fig. 2). The two positive controls had mP values of 94.96 and 94.10, corresponding to concentrations of 19.00 and 19.20 μmol/L, respectively, which indicated that all the reagents were functional (Malinow MR, unpublished data).

Discussion

The biochemical principles used in our method relate to those of Kredich et al. (20) and Refsum et al. (21). In brief, DTT reduces HCY bound to albumin and to other small molecules through the disulfide bond. SAH hydrolase catalyzes conversion of HCY to SAH in the presence of added adenosine. The reduction and the enzymatic steps have been combined to shorten the assay time substantially. In the subsequent steps, the specific monoclonal antibody and the fluoresceinated SAH analog tracer constitute the FPIA detection system (10). The correlations are excellent (r = 0.980–0.997), but the slopes range from 1.030 to 1.493. These slope variations could possibly result from the use of different calibrators (commercial origin) with different degrees of chemical purity in the different laboratories. The assay steps involving the enzyme conversion of HCY to SAH will be sensitive to enantiomeric purity as well. Slope differences ranging from 0.850 to 1.009 have also been reported among established chromatographic methods (1).

The high precision and very low inter- and intraassay CVs may eliminate the need to make duplicate or more determinations. As mentioned above, excellent correlations were also obtained for single determinations in all cases. The high selectivity of the enzyme for HCY as substrate is reflected by the absence of measurable cross-reactivity with the closely related amino acids cysteine and methionine, which are present in

Table 2. Linearity of plasma dilution with buffer.

<table>
<thead>
<tr>
<th>Buffer: Plasma vol. ratio</th>
<th>HCY, μmol/L</th>
<th>Obs. × 100%</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Calculated</td>
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<tr>
<td><strong>Plasma 1</strong></td>
<td></td>
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</tr>
<tr>
<td>0:1</td>
<td>47.51</td>
<td>47.51</td>
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<tr>
<td>1:3</td>
<td>33.78</td>
<td>35.63</td>
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<tr>
<td>1:2</td>
<td>32.74</td>
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<tr>
<td>1:1</td>
<td>25.29</td>
<td>23.76</td>
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<tr>
<td>3:1</td>
<td>12.79</td>
<td>11.88</td>
</tr>
<tr>
<td>7:1</td>
<td>6.81</td>
<td>5.94</td>
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<tr>
<td><strong>Mean (SD)</strong></td>
<td></td>
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<td></td>
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<td>104.5 (6.9)</td>
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| **Plasma 2**             |             |             |
| 0:1                      | 40.40       | 40.40       | 100.0 |
| 1:3                      | 29.14       | 30.30       | 96.2  |
| 1:2                      | —           | —           | —     |
| 1:1                      | 19.48       | 20.20       | 96.4  |
| 3:1                      | 10.09       | 10.10       | 99.9  |
| 7:1                      | 5.13        | 5.00        | 102.6 |
| **Mean (SD)**            |             |             |
|                          |             | 99.0 (2.7)  |
plasma in much higher concentrations. The stability of
the stored calibration curve for 2 weeks eliminates
frequent standardization of the equipment. Dilution
parallelism and analytical recovery of added analyte
are within acceptable limits. The nonmeasurable con-
centrations of endogenous SAH in plasma permit one
to measure in vitro-generated SAH (from HCY), result-
ing in extremely good correlations to the HPLC and gas
chromatography—mass spectrometric methods, which
are free from such an interference.

Hyperhomocyst(e)inemia has been described as "a
common and easily reversible risk factor for occulsive
atherosclerosis" (22). In a recent study of 1100 elderly
patients, Selhub et al. concluded that 29.3% of the
cohort exhibited hyperhomocyst(e)inemia, with 67% of
this being associated with one or more manifestations
of vitamin B deficiency (23). This study has led
Stamper and Willett (24) and Stamper and Malinow
(25) to emphasize adequate dietary intake of folate and
vitamin supplements.

We conclude that this rapid, fully automated
method, with no manual deproteinization or cumber-
some chromatography step, will potentially benefit
clinical researchers and nutritionists alike in large
population-based studies such as those carried out in
Norway (1) and the US (26). The lowering of plasma
HCY concentrations in hyperhomocyst(e)inemia by
simple and inexpensive vitamin therapy may lead to
intervention not only in elderly patients but also in
the general population, with potentially concomitant
reductions in mortality and morbidity from cardiovascu-
lar diseases. An accurate, precise, and automated
method for quantifying total HCY will help identify
patients who might benefit from the intervention.

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