Performance Characteristics of Six Homocysteine Assays

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Key Words: Homocysteine; Method comparison; Imprecision

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Abstract

Elevated concentrations of homocysteine (Hcy) are associated with a range of disorders. Linearity, imprecision, interference, method comparison, and accuracy were evaluated on the ADVIA Centaur (Siemens Healthcare Diagnostics, Deerfield, IL), ARCHITECT i2000SR (Abbott Diagnostics, Abbott Park, IL), AxSYM (Abbott Diagnostics), and IMMULITE 2000 (Siemens Healthcare Diagnostics) methods and analyzers and the Catch (Equal Diagnostics, Exton, PA) and Diazyme (Diazyme Laboratories, San Diego, CA) methods, both on the Modular P analyzer (Roche Diagnostics, Indianapolis, IN). All methods were linear with maximum deviations from target recoveries of less than 10%. Total coefficients of variation ranged from 1.7% to 9.4%. The effects of hemolysis, icterus, and lipemia were assessed. Method comparisons were performed using high-performance liquid chromatography as the comparison method. Correlation coefficients were 0.95 to 0.99. Bland-Altman plots demonstrated percentage bias of −29.3% (IMMULITE) to 7.2% (Centaur). Accuracy using the National Institute of Standards and Technology Standard Reference Material 1955 showed varying results with only 1 method within the certified range for all 3 levels. All methods demonstrated acceptable performance except the IMMULITE, which is less precise and accurate. Standardization of most methods seems acceptable, although continuing efforts are warranted.

Homocysteine (Hcy) is a sulfur-containing amino acid formed as an intermediate product of methionine metabolism. Clinically, various disorders are associated with elevated concentrations of Hcy.1 Measurement of Hcy is most commonly used as an independent risk factor for cardiovascular disease.2,3 However, it is not recommended to use total Hcy to screen the general population.1 Owing to the cofactors involved in Hcy metabolism, it is also a sensitive marker for folate and cobalamin deficiency.4,5 Measurement of Hcy, along with methionine, is useful in diagnosing homocystinuria, a rare autosomal recessive disorder involving deficiencies of cystathionine β-synthase.6

Clinical testing for Hcy has improved during the last 20 years, particularly with the introduction of automated platforms. In practice, 3 assay methods are used: chromatography, enzymatic methods, and immunoassay. In the present study, we evaluated the correlation of all 3 methods, along with the performance characteristics of 2 enzymatic assays and 4 immunoassays, including a newly developed immunoassay. Previously, numerous studies evaluated the comparability of various methods.7-30 Standardization was a topic of concern for most, if not all, studies. Consequently, Standard Reference Material (SRM) 1955, homocysteine and folate in human serum, was formulated by the National Institute of Standards and Technology (NIST) to assess the standardization of Hcy assays.31 We obtained SRM 1955 and tested it on all methods as an assessment of trueness.

Materials and Methods

Linearity, imprecision, interference, and method comparison were evaluated on the ADVIA Centaur (Siemens Healthcare Diagnostics, Deerfield, IL).
Healthcare Diagnostics, Deerfield, IL), ARCHITECT \(i2000_{SR}\) (Abbott Diagnostics, Abbott Park, IL), AxSYM (Abbott Diagnostics), Catch homogeneous enzymatic assay (Equal Diagnostics, Exton, PA) on a Modular P analyzer (Roche Diagnostics, Indianapolis, IN), Diazyme enzymatic cycling assay (Diazyme Laboratories, San Diego, CA) on a Modular P analyzer, and IMMULITE 2000 (Siemens Healthcare Diagnostics). All assays were performed according to manufacturers’ instructions. The nominal throughput estimates for homocysteine testing were 240, 200, 60, 800, and 100 samples per hour for the ADVIA Centaur, ARCHITECT \(i2000_{SR}\), AxSYM, Modular P, and IMMULITE 2000 analyzers, respectively. We did not independently verify the nominal throughput estimates provided by the manufacturers of the analyzers. All testing, except for the high-performance liquid chromatography (HPLC) comparison method, was performed at ARUP Laboratories, Salt Lake City, UT.

Linearity was assessed by diluting a high patient serum pool, with an analyte concentration near the upper reportable limit, with a low patient serum pool to yield concentrations of 100%, 75%, 50%, 25%, 10%, 5%, 2.5%, 1%, and 0% of the original high pool. All dilutions were tested in duplicate. The same pools were used for linearity assessment of all methods. The University of Utah Institutional Review Board (Salt Lake City) approved all studies using human samples.

Imprecision was determined by using commercially available quality control material. Three concentration levels of Bio-Rad Liquichek Cardiac Markers Plus Control LT (Bio-Rad Laboratories, Irvine, CA) were used according to the manufacturer’s instructions. Multiple bottles for each level were thawed, pooled, divided into aliquots, and stored at 4°C until use, with a fresh aliquot used for each run. Controls were run twice a day, for 5 days, in replicates of 2, with a minimum of 2 hours separating each run.

Interference studies were performed as indicated previously. A serum pool was supplemented with various concentrations of hemoglobin, conjugated bilirubin, and Intralipid (Fresenius Kabi, Uppsala, Sweden) triglycerides and tested in concentrations of hemoglobin, conjugated bilirubin, and Intralipid. The maximum deviation from target recovery was not known. NIST SRM 1955 was tested on all methods done and the estimated glomerular filtration rate (eGFR) was calculated for each sample using the following equation:

\[
\text{GFR} (\text{mL/min/1.73 m}^2) = 175 \times (\text{S_e})^{-1.154} \times (\text{Age})^{0.203} \times (0.742 \text{ if female}) \text{ (conventional units)}
\]

No correction was made for ethnicity because the ethnicity of the subjects from whom these samples were collected was not known. NIST SRM 1955 was tested on all methods using the same calibration as patient samples.

EP Evaluator Release 8 software (David G. Rhoads Associates, Kennett Square, PA) was used to calculate linearity and imprecision. Deming and linear regression and Bland-Altman plots were performed using Analyse-It, version 2.04 (Analyse-It Software, Leeds, England).

## Results

The target value for each linearity sample was calculated based on the samples with the lowest and highest concentrations within the analytic measurement range for each method. The maximum deviation from target recovery ranged from 4.3% for the ARCHITECT to 10.0% for the Centaur. The AxSYM, Diazyme, and Catch methods were the most precise for levels 1, 2, and 3, respectively. The method comparison was evaluated using 101 specimens with Hcy results ranging from 4.4 to 52.7 µmol/L measured by the HPLC method. Only serum and heparin plasma samples were used because EDTA is not an approved specimen type for the Diazyme assay. Before testing, specimens were thawed, mixed thoroughly, centrifuged for 5 minutes at 3,000 rpm, and checked for clots. Samples were analyzed on the same day for all enzymatic and immunoassay determinations.

### Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Maximum Deviation From Target Recovery (%)</th>
<th>Concentration at Which Maximum Deviation From Target Recovery Occurred (µmol/L)</th>
<th>Measured Range (µmol/L)</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADVIA Centaur</td>
<td>10.0</td>
<td>7.2</td>
<td>4.9-38.0</td>
<td>0.999</td>
</tr>
<tr>
<td>ARCHITECT (i2000_{SR})</td>
<td>4.3</td>
<td>15.4</td>
<td>4.8-47.7</td>
<td>0.998</td>
</tr>
<tr>
<td>AxSYM</td>
<td>9.3</td>
<td>14.6</td>
<td>5.1-38.0</td>
<td>0.995</td>
</tr>
<tr>
<td>Catch</td>
<td>7.0</td>
<td>5.0</td>
<td>5.0-42.5</td>
<td>0.999</td>
</tr>
<tr>
<td>Diazyme</td>
<td>9.2</td>
<td>31.1</td>
<td>7.9-60.6</td>
<td>0.997</td>
</tr>
<tr>
<td>IMMULITE 2000</td>
<td>7.5</td>
<td>3.0</td>
<td>2.0-34.4</td>
<td>0.999</td>
</tr>
</tbody>
</table>

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Centaur was the least precise for level 1, and the IMMULITE was the least precise for levels 2 and 3.

Potential interference from hemolysis, icterus, and lipemia was evaluated. Interference causing 10% or less deviation was assessed for all methods and compared with the manufacturers’ claims Table 3. All methods met the manufacturers’ claims except the Catch (manufacturer’s claim <10% effect at 10 g/L of hemoglobin), which showed a 10% effect at 7 g/L of hemoglobin, Diazyme (manufacturer’s claim <10% effect at 25 g/L of triglycerides), which showed a 10% effect observed at 17 g/L of triglycerides, and IMMULITE (manufacturer’s claim no effect at 30 g/L of triglycerides), which showed a 10% effect at 22 g/L of triglycerides.

Table 3

<table>
<thead>
<tr>
<th>Concentrations in Which 10% Interference Was Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>ADVIA Centaur</td>
</tr>
<tr>
<td>ARCHITECT i2000sr</td>
</tr>
<tr>
<td>AxSYM</td>
</tr>
<tr>
<td>Catch</td>
</tr>
<tr>
<td>Diazyme</td>
</tr>
<tr>
<td>IMMULITE 2000</td>
</tr>
</tbody>
</table>

* Did not meet manufacturers’ claims.

All methods compared well with HPLC by Deming regression with slopes ranging from 0.90 to 0.93, correlation coefficients of 0.95 to 0.99, and y-intercept from –2.86 to 3.05 µmol/L Figure 1. Method comparison was also evaluated by Bland-Altman plots Figure 2. The percentage of bias ranged from ~29.3% (IMMULITE) to 7.2% (Centaur). Statistical outliers were repeated and did not change significantly from the original result. An average of the original and repeated result was used in the final method comparison analysis. The eGFR was examined as a possible explanation for outliers with an absolute bias greater than 2 SD. All outliers had an eGFR less than 67 mL/min/1.73 m², with all but one less than 56 mL/min/1.73 m².

Testing of SRM 1955 showed varying results between methods Table 4. The Centaur was the only method for which all 3 levels fell within the certified concentration range. The ARCHITECT, AxSYM, and Catch had only level 1 (3.98 µmol/L) outside the certified concentration range, whereas Diazyme and IMMULITE had all 3 levels out of range.

Discussion

All methods were linear with maximum deviations not exceeding 10% for any method. Similar linearity results were observed on the Centaur, AxSYM, and IMMULITE previously.9,10,12,23,26 Imprecision demonstrated total CVs less than 10% for all methods. Nevertheless, Refsum et al1 recommended that imprecision for Hcy assays should preferably be less than 5%. When using these guidelines, 3 methods did not fulfill recommended criteria: Centaur for level 1, Catch for level 1, and IMMULITE for levels 1, 2, and 3. There is a prominent matrix effect noted for the ADVIA Centaur method with these quality control materials. Imprecision results from other studies have also shown the Centaur and IMMULITE not meeting this guideline.10,19,20,23,26

Interference from hemolysis, icterus, and lipemia was assessed. Three methods did not meet the manufacturers’ claims: Catch for hemolysis and Diazyme and IMMULITE previously.
for lipemia. Repeated analysis using different analyzers, a new lot of reagents, and different sample sets for each method did not improve results. Two other studies evaluated Catch reagents: one found more than 20% change at 7 g/L of hemoglobin, whereas the other saw no interference at 16 g/L. Caution should be used with these 3 methods when testing samples with interfering substances exceeding the thresholds indicated in Table 3.

Deming regression demonstrated good correlation with HPLC for all methods with correlation coefficients of 0.95 to 0.99. Slopes were 0.90 to 0.93 and y-intercepts were –2.86 to 3.05 µmol/L. Only the ARCHITECT and AxSYM had intercepts with absolute values less than 1.0 µmol/L. In the past, Hcy methods generally compared well with regards to regression analysis. However, differences are seen even with a strong correlation. This is evident in our study when looking at the percentage bias as calculated by Bland-Altman plots. For the IMMULITE, there is excellent correlation with HPLC (r = 0.98) but clearly a substantial calibration bias (–29.3%). Several efforts were made to address this observed bias. These included performing new calibrations, testing on a different analyzer, and use of a new reagent lot. In all instances, the manufacturer’s controls were within acceptable ranges, but results for the SRM were consistently outside the certified concentration range. Owing to the known variation among methods, Refsum et al recommend bias being less than 10%. Excluding the IMMULITE, the percentage bias ranged from –5.2% to 7.2% for all other methods and was within this guideline. It is noteworthy that results outside the limits of agreement on the Bland-Altman plots were exclusively from subjects with diminished renal function. An explanation for this observation is not evident because this phenomenon is not unique to one particular method.

Numerous method comparison studies comment on concerns regarding standardization of Hcy assays. In response, SRM 1955 was developed and characterized. All levels of the material were prepared from a human serum master pool. Level 2 is unfortified and equivalent to the master pool. Level 1 was prepared by diluting level 2 with phosphate-buffered saline. Level 3 was prepared by spiking level 2 with the appropriate quantity of Hcy. Values were assigned by gas chromatography–mass spectrometry and

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**Figure 1** Deming regression of homocysteine (Hcy) methods using high-performance liquid chromatography (HPLC) with fluorescence detection as the designated comparison method. A, y = 0.91x + 3.05; r = 0.95. B, y = 0.93x + 0.64; r = 0.99. C, y = 0.92x + 0.46; r = 0.98. D, y = 0.93x + 1.85; r = 0.98. E, y = 0.91x + 3.03; r = 0.97. F, y = 0.90x + 2.86; r = 0.98.
Figure 2: Bland-Altman plots showing the percentage difference between each of the 6 homocysteine (Hcy) assays and high-performance liquid chromatography (HPLC) on the y-axis. An ideal mean percentage difference of 0 is indicated by a dotted line. The mean percentage difference is indicated by a dark, solid line. The limits of agreement for the mean percentage difference, as defined by 95% confidence limits, are indicated by dashed lines. A, The Centaur gave a mean percentage difference of 7.2% with 95% confidence limits of –18.4% and 32.7%. B, The ARCHITECT gave a mean percentage difference of –3.4% with 95% confidence limits of –16.9% and 10.2%. C, The AxSYM gave a mean percentage difference of –5.3% with 95% confidence limits of –18.3% and 7.8%. D, The Catch Hcy assay on the Roche Modular P gave a mean percentage difference of 2.1% with 95% confidence limits of –13.4% and 17.66%. E, The Diazyme Hcy assay on the Roche Modular P gave a mean percentage difference of 6.7% with 95% confidence limits of –11.1% and 24.4%. F, The IMMULITE 2000 gave a mean percentage difference of –29.3% with 95% confidence limits of –53.0% and –5.6%.

Table 4: NIST SRM 1955 Testing

<table>
<thead>
<tr>
<th>Method</th>
<th>Level 1 (µmol/L)</th>
<th>Level 2 (µmol/L)</th>
<th>Level 3 (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADVIA Centaur</td>
<td>3.96</td>
<td>9.00</td>
<td>17.6</td>
</tr>
<tr>
<td>ARCHITECT 2000&lt;sub&gt;SR&lt;/sub&gt;</td>
<td>4.57&lt;sup&gt;†&lt;/sup&gt;</td>
<td>9.12</td>
<td>17.8</td>
</tr>
<tr>
<td>AxSYM</td>
<td>4.41&lt;sup&gt;†&lt;/sup&gt;</td>
<td>9.12</td>
<td>17.1</td>
</tr>
<tr>
<td>Catch</td>
<td>3.00&lt;sup&gt;†&lt;/sup&gt;</td>
<td>9.00</td>
<td>18.0</td>
</tr>
<tr>
<td>Diazyme</td>
<td>2.50&lt;sup&gt;†&lt;/sup&gt;</td>
<td>10.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td>20.0&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMMULITE 2000</td>
<td>2.79&lt;sup&gt;†&lt;/sup&gt;</td>
<td>6.47&lt;sup&gt;†&lt;/sup&gt;</td>
<td>14.3&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NIST, National Institute of Standards and Technology; SRM, Standard Reference Material.

<sup>†</sup>SRM 1955 is homocysteine and folate in human serum formulated by the NIST.
<sup>‡</sup>Outside the NIST-certified range: level 1, 3.80-4.16 µmol/L; level 2, 8.25-9.45 µmol/L; level 3, 16.6-18.8 µmol/L.
liquid chromatography combined with mass spectrometry and tandem mass spectrometry. All 3 levels of SRM 1955 were tested on each method during the same calibration interval as patient samples. The Centaur was the only method with all 3 levels within the NIST-certified concentration range, which is interesting considering it was the method with the highest positive bias (7.2%) relative to the HPLC comparison method.

It is also worth considering the comparison of the linearity pools with the SRM. The low end of the linearity pools, where the Centaur, ARCHITECT, AxSYM, and Catch assays gave comparable results (Table 1), is not reflected in level 1 of the SRM for the same methods. The ARCHITECT, AxSYM, and Catch methods were outside the certified range only for level 1. For the ARCHITECT and AxSYM, there was a slight negative bias with regard to patient samples (−3.4% and −5.3%, respectively), yet for level 1, both reported values higher than the certified range. It is interesting that the opposite effect was observed with the Catch method in which patient samples had a positive bias (2.1%), but level 1 was lower than the certified range. For Diazyme, the bias plot shows a roughly opposite outcome of what was expected based on the SRM results. Despite these results, trueness for level 1 may not be critical because the upper reference limit of Hcy is closer to the concentrations of level 2 and 3, depending on the population.1 Also, another study found a substantial bias for level 1 of SRM 1955.18 These findings indicate a possible matrix effect for this material and also suggest the need for improved reference materials.

All Hcy methods show acceptable performance and compare well with HPLC, although not all assays met precision criteria of less than 5% or manufacturers’ package insert claims for interferences. Bias relative to the comparison method was within acceptable limits with the exception of the IMMULITE. This method requires improvements in trueness and precision. The newly developed ARCHITECT i Hcy assay demonstrated excellent performance characteristics with low CVs, good comparison with HPLC, and low percentage bias. Previous reports have recommended that serial Hcy testing be done using the same method and sample type as the initial testing event.1,36 Given the results from the present study, it appears that, with the exception of the IMMULITE 2000, the Hcy methods we evaluated give comparable results and could probably be used interchangeably for serial testing.

References


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