Candidate Reference Method for Determining Creatinine in Serum: Method Development and Interlaboratory Validation

Thomas G. Rosano,¹ Robert T. Ambrose,² Alan H.B. Wu,³ Thomas A. Swift,¹ and Priscilla Yadegari¹

We describe a “high-performance” liquid chromatographic (HPLC) method for accurately determining creatinine in serum. After prechromatographic precipitation of protein, we performed isocratic ion-exchange chromatography with ultraviolet detection (234 nm). Analytical results showed linearity up to 1770 μmol/L, a detection limit of 22 μmol/L, an average analytical recovery of 101%, and a CV ranging from 3% to 11%. We used certified human serum (National Institute of Standards and Technology), and additional lyophilized serum pools also assayed by definitive isotope-dilution mass spectrometry, to validate the accuracy of the HPLC method. In addition, the isocratic HPLC results showed close agreement with those obtained with a step-gradient HPLC method. We also compared the isocratic HPLC method with alkaline picrate and enzymatic methods. Our findings with samples from nonuremic, uremic, and diabetic ketoacidotic patients confirmed the positive bias previously reported with the alkaline picrate method. Interlaboratory transferability of the method was demonstrated with various commercial instruments and analytical columns. We evaluated column stability and possible interference from endogenous or exogenous compounds. On the basis of our analytical findings, we recommend the isocratic HPLC method as a candidate Reference Method for determining creatinine in serum.

Additional Keyphrases: chromatography; ion-exchange variation, source of

Creatinine in the clinical laboratory is routinely measured primarily by colorimetric and enzymatic methods. Colorimetric methods, based on the Jaffé reaction, are nonspecific (1–4), and numerous method modifications such as continuous-flow dialysis (5) and monitoring reaction rate (6) have had only limited success in improving specificity. Newer enzymatic methods may provide more specific routine methods, but their analytical performance is often evaluated against the Jaffé-based methods (7, 8). Therefore, a reference system is needed to ascertain the accuracy of routine creatinine methods, to assign target values for reference serum pools, and to guide the development of new methods. Figure 1 outlines the relationship of methods and reference material for the National Reference System (9–11).

The first steps in developing a reference system for creatinine are complete. Certified Reference Material is available from the National Institute of Standards and Technology (NIST, Gaithersburg, MD) and a Definitive Method of creatinine quantification by isotope-dilution mass spectrometry has been developed (12). A Reference Method, however, is needed to provide a link between the Definitive and routine methods. Based on established criteria, a Reference Method must be precise, have minimal and well-defined inaccuracy as compared with the Definitive Method, and be within the technical reach of a number of laboratories (10). HPLC has been proposed as a Reference Method for serum creatinine, and a number of reversed-phase (13–17), ion-exchange (18, 19), and normal-phase (20) HPLC applications have been reported. These methods, however, either require nonisocratic conditions or have not been evaluated against all Reference Method criteria. The purpose of this study, therefore, is to develop an isocratic HPLC method for serum creatinine and to evaluate its precision, accuracy, and interlaboratory transferability.

Materials and Methods

Apparatus. HPLC systems in three laboratories were used to develop and evaluate the method: laboratory A (Albany Medical Center, Albany, NY), an HPLC pump

¹ Department of Pathology and Laboratory Medicine, Albany Medical Center, Albany, NY 12208.
² Eastman Kodak Co., Rochester, NY 14650.
³ Department of Pathology and Laboratory Medicine, University of Texas Medical School, Houston, TX 77025.

Received January 23, 1990; accepted September 12, 1990.

4 Nonstandard abbreviations: SRM, Standard Reference Material; NIST, National Institute of Standards and Technology; and CDC, Centers for Disease Control.
formed 1952 interlaboratory calibrated been also centrifugal vortex-mix trichloroacetic acid mobile a used also.


We prepared a stock solution of creatinine (88.4 mmol/L) by dissolving 200.0 ± 0.05 mg of SRM 914 creatinine in distilled water (20 °C) to a total volume of 200 mL, intermediate standards (concentrations 44–3540 μmol/L) by dilution in distilled water, and final working creatinine standards (concentration 22–1770 μmol/L) by diluting the intermediate standards with an equal volume of a 140 g/L aqueous solution of bovine serum albumin (Fraction V; Kodak Chemicals, Rochester, NY). Final working standards with creatinine concentrations >1770 μmol/L were also prepared to test recovery and linearity, but were not used for routine analysis.

Chromatographic conditions included a flow rate of mobile phase at 0.8 mL/min and ultraviolet detection at 234 nm. Mobile phase (20 mmol/L succinic acid, pH 5.8) used in the chromatographic procedure was prepared weekly from a stock succinic acid solution (200 mmol/L, pH 5.8). During the method development, the pH and ionic strength of the mobile phase were varied over a range of 5.0–7.0 and 2–50 mmol/L, respectively.

Procedure. For each sample of working standard or serum, pipet 200 μL of sample and 200 μL of 100 g/L trichloroacetic acid solution into a centrifuge tube and vortex-mix for 15 s. Centrifuge the samples at a relative centrifugal force >1000 × g for 5 min and collect the supernate by aspiration. A 10-μL injection volume is recommended, although injection volumes 10–50 μL have been used without affecting analytical performance. Both manual and automated injectors have shown acceptable analytical performance. Because we found no internal standard with acceptable chromatographic retention, we calibrated with an external standardization method. A calibration curve of peak height vs creatinine concentration was used to quantify unknown samples.

Evaluation protocol. To evaluate precision, accuracy, and interlaboratory correlation, laboratories A, H, and R performed replicate analyses of six serum pools that had been prepared and distributed by laboratory A. Pool A was NIST-Certified Human Serum SRM 909; pools B–D were CDC serum pools also analyzed for creatinine concentra-

tion by isotope-dilution mass spectrometry (12) by the NIST; serum pools E and F were pooled aliquots of patients sera prepared and distributed by laboratory A. Additional individual serum samples (n = 198) from patients with a wide range of renal dysfunction were analyzed in both laboratories A and H to further assess interlaboratory correlation with clinical samples.

To determine optimum chromatographic conditions, we analyzed the effect of mobile-phase pH and ionic strength on creatinine peak efficiency, retention time, and resolution from potential interference. For recovery studies, we analyzed pooled serum supplemented with pure creatinine (SRM 914) over a concentration range of 138–2210 μmol/L; for interference studies, we analyzed pooled serum supplemented with purified compounds ranging in concentration from 0.1 to 50 mg/L. Intermethod comparisons included step-gradient HPLC (18), linked-enzyme reactions involving creatinine iminohydrolase and glucose dehydrogenase (21), and a colorimetric alkaline picrate technique. The enzymatic technique was automated and performed with a Cobas-Bio centrifugal analyzer (Roche Diagnostics, Montclair, NJ) and analytical-grade reagents. For the alkaline picrate method, we used an RA-1000 random-access analyzer (Technicon Instruments, Tarrytown, NY) with the manufacturer’s reagents and protocol. To eliminate standardization bias, each laboratory included in the method analysis a creatinine standard solution prepared in laboratory A. Intermethod comparison was extended in laboratory A to include serial serum samples from ketoacidotic patients. Fresh nonfrozen serum obtained from venous blood was analyzed by both the isocratic HPLC and colorimetric alkaline picrate methods. Serum was also qualitatively tested for ketones (Chemstrip 7L; Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN), and arterial blood pH was determined by blood-gas analysis (ABL-3 Blood Gas Analyzer; Radiometer, Inc., Westlake, OH).

Methods were compared statistically by a biometric evaluation that considers error components for both the x and y variables. We used a principal component standardization procedure with a least-squares estimation to calculate the regression line and the 95% confidence interval for the slope and y-intercept (22).

Results and Discussion

Analysis of creatinine by ion-exchange HPLC has required either step-gradient chromatography (18) or a post-column reaction detector (19). To simplify the procedure, we evaluated isocratic conditions with a micropellicular column and determined optimum chromatographic conditions for retention and efficient elution of creatinine. Experiments to determine optimum mobile-phase pH and molar strength for creatinine retention and peak efficiency are summarized in Figure 2. We used succinic acid to provide adequate buffering capacity around pH 6.0. A final pH of 5.8 and molar strength of 20 mmol/L were selected to optimize creatinine peak efficiency and analysis time. Only minor variations in creatinine retention were observed with two commercial analytical columns. A typical series of chromatograms for working standards and serum preparations is shown in Figure 3. In each chromatogram, the creatinine peak shows complete resolution from earlier eluting compounds, without significant peak trailing. Analytical columns were stable for approximately 300 injections under the chromatographic conditions selected for routine analysis.
Analytical variables of the method were studied in each laboratory. The results of the method varied linearly with creatinine concentration up to at least 1770 μmol/L, based on calibration curves and serum dilution studies. The detection limit, defined as the minimum creatinine concentrations distinguishable from baseline with 95% confidence, was 22 μmol/L. Data from replicate analyses of serum pools are summarized in Table 1 and show comparable interlaboratory precision, with CVs ranging from 3% to 11%. To evaluate the accuracy of the HPLC method, we analyzed sera with creatinine values assigned by the Definitive Method. For all pools, the mean creatinine concentration determined by HPLC was not significantly different (P > 0.05) between laboratories A, H, and R. For pools A–D, analyzed by the Definitive Method, the HPLC-determined concentration agreed closely with the value assigned by the Definitive Method. For all laboratories the mean concentration of creatinine measured by HPLC was within 97% to 102% of that by the Definitive Method. Analytical recovery (Table 2) varied from 99% to 102% (average 100.8%, SD 1.5%).

Transfersability of the method was validated by the interlaboratory precision and accuracy data presented in Table 1. In addition, in laboratories A and R, HPLC results showed close agreement with sera (n = 198) from patients with or without uremia: slope = 1.002, y-intercept = 4 μmol/L, and r = 0.987. The slope and y-intercept were indistinguishable from 1 and 0, respectively, indicating no significant difference in results between laboratories.

Our isocratic HPLC measurement of creatinine in patients' sera was also compared with those by step-gradient HPLC, enzymatic, and alkaline picrate methods (Figure 4).

Table 1. Accuracy and Interassay Precision of Serum Creatinine Measurement by HPLC

<table>
<thead>
<tr>
<th>Pool</th>
<th>Lab. A</th>
<th>Lab. R</th>
<th>Lab. H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
<td>Def.</td>
</tr>
<tr>
<td>A</td>
<td>149</td>
<td>10</td>
<td>6.6</td>
</tr>
<tr>
<td>B</td>
<td>56</td>
<td>5</td>
<td>9.0</td>
</tr>
<tr>
<td>C</td>
<td>411</td>
<td>12</td>
<td>2.9</td>
</tr>
<tr>
<td>D</td>
<td>231</td>
<td>9</td>
<td>3.7</td>
</tr>
<tr>
<td>E</td>
<td>69</td>
<td>6</td>
<td>8.6</td>
</tr>
<tr>
<td>F</td>
<td>393</td>
<td>15</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* See text for description of pools analyzed. Data for each pool represent statistical analysis of 10 interassay determinations by each laboratory.

** Percent of the mean concentration determined by the Definitive (Def.) Method.

Table 2. Analytical Recovery of Creatinine by HPLC

<table>
<thead>
<tr>
<th>Added Conc., (SD) pmol/L</th>
<th>Measured Conc., (SD) pmol/L</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (85 (9))</td>
<td>85 (9)</td>
<td>—</td>
</tr>
<tr>
<td>138 (217 (12))</td>
<td>217 (12)</td>
<td>98.6</td>
</tr>
<tr>
<td>276 (364 (12))</td>
<td>364 (12)</td>
<td>102.5</td>
</tr>
<tr>
<td>553 (634 (31))</td>
<td>634 (31)</td>
<td>100.0</td>
</tr>
<tr>
<td>1105 (1214 (31))</td>
<td>1214 (31)</td>
<td>102.5</td>
</tr>
<tr>
<td>2210 (2301 (32))</td>
<td>2301 (32)</td>
<td>100.5</td>
</tr>
</tbody>
</table>

*All pools were extracted in triplicate with duplicate analyses of each extract. For each creatinine concentration tested, 50 μL of creatinine solution was added to 1 mL of a serum pool.

Fig. 2. Effect of mobile-phase pH and succinic acid concentration on chromatographic retention of creatinine

Fig. 3. Representative chromatograms of calibration standards and serum samples

Working standards, S1 (89 μmol) and S5 (442 μmol/L); patients' sera, PT88 and PT78
Concentrations determined by isocratic HPLC and step-gradient HPLC could not be distinguished, based upon statistical parameters of the biometric analysis. The enzymatic method correlation produced a slope significantly greater than 1 (P < 0.05), but without a significant y-intercept bias. The alkaline picrate method gave the greatest discrepancy in results; concentrations determined by the alkaline picrate method were consistently higher, resulting in a statistically significant difference in slope and y-intercept. Within the study, bias was observed in sera from patients with or without uremia. The positive bias exhibited by the alkaline picrate method may be attributed to the nonspecificity of the Jaffé reaction (1). Other investigators (3, 4, 23) have shown clinically significant bias, especially in the diabetic patient during ketoacidosis. To assess the degree of bias in ketotic serum, we compared HPLC and alkaline picrate measurements in serial samples of fresh sera from diabetic patients during ketoacidotic episodes. Positive bias in the alkaline picrate method was greatest when acidosis existed and serum ketones were qualitatively positive; sample freezing or prolonged storage decreased but did not fully eliminate the bias observed (data not shown). This discrepancy between the HPLC and the alkaline picrate methods was even greater than that observed in the correlation study and was consistent with alkaline picrate interference studies reported by others (23).

We also assessed interference by exogenous or other endogenous compounds. Compounds eluting near or after creatinine were not observed in the numerous serum samples analyzed throughout the method evaluation. In addition, we tested 37 pure compounds for potential interference (Table 3) and observed responses from several of the drugs in the chromatographic procedure, but only isoniazid and phenobarbital were eluted near creatinine. To further test the relative chromatographic behavior of these two drugs, we determined retention with various concentrations of succinic acid in the mobile phase (Table 4). Under all mobile-phase conditions tested, creatinine was eluted later than phenobarbital and these two peaks were completely resolved. Isoniazid was not fully resolved from creatinine with succinic acid ≥20 mmol/L, but could be completely resolved at lower concentrations of succinic acid, owing to the selective retention of creatinine. The potential interference of isoniazid, however, is minimal in the clinical setting. Therapeutic concentrations of isoniazid are below the detection limit of the creatinine method; therefore, a change in mobile-phase conditions is not necessary, given the minimal interference in clinical samples. The potential for bias by phenobarbital and isoniazid has been well characterized and has not been found to significantly affect the accuracy of the creatinine measurement.
In summary, the candidate Reference Method is easy to perform and demonstrates acceptable interlaboratory transferability. The precision of the proposed Reference Method is comparable with that of routine methods used in the clinical laboratory and exceeds the criteria for medically useful precision as determined by Skendzel et al. (24). Accuracy has been demonstrated by both recovery studies and direct comparison with the Definitive Method. Correlations with routine methods further reinforce the need for a Reference Method that is traceable in accuracy to the Definitive Method. The HPLC method reported here meets the criteria for such a Reference Method.

References