Automated Determination of Urinary Creatinine without Sample Dilution: Theory and Practice

Martin H. Kroll, Ruth Chesler, Claire Hagengruber, David W. Blank, Jane Kestner, and Michael Rawe

The rate of the Jaffé reaction depends on the concentration of sodium hydroxide; the pseudo-first-order rate constant of the reaction, at 37°C in 10 mmol/L picrate solution, is 0.004 mmol/L. We formulated an automated method to determine urinary creatinine directly without manual sample dilution. The conditions are as follows: 10 mmol/L picrate and 60 mmol/L sodium hydroxide (final concentrations); ratio of sample to final volume, 1:41; temperature, 37°C; wavelengths of measurement, 500 or 510 nm; interval of measurement, 30 to 90 s; and mode of measurement, kinetic. Determinations of creatinine in patients' samples by the new method compared favorably with those obtained with the AutoAnalyzer and aca. The run-to-run CVs were 3.6% or less, and the method was accurate for concentrations of creatinine up to 3000 mg/L. We recommend this method as a good replacement for the AutoAnalyzer or aca methods.

Additional Keyphrases: kinetic analysis - variation, source of - random-access analysis - centrifugal analyzer - continuous-flow and discrete analysis compared

Because the concentration of creatinine in urine is at least 10-fold that in serum, for many automated methods urine samples may need to be diluted before creatinine is determined. Modern automated instruments allow one to manipulate the volumes of sample and reagent and to decrease the ratio of sample to total volume, which thus may eliminate the necessity of manual dilution. Manipulation of sample volume alone, however, may be insufficient to obtain satisfactory results with the Jaffé reaction because, at high concentrations of creatinine, the reaction rate is too fast. Previously we demonstrated (1) the dependence of the rate of the Jaffé reaction on the picrate concentration, but this dependence did not show promise for developing a nondilution method for urinary creatinine.

Here we examine the dependence of the reaction rate on the concentration of sodium hydroxide and express the rate of the Jaffé reaction as a function of the concentrations of creatinine, picrate, and hydroxide. Application of the rate expression led us to formulate new reaction conditions in developing a method involving low concentrations of hydroxide. We compared results obtained by this new method with those obtained by more conventional ones, and with a method based on decreased sample volume—this last method being included to demonstrate the superiority of modifying the rate instead of the sample size.

Materials and Methods

Instruments

Sodium hydroxide study. All absorbance measurements were made with a Model 25 spectrophotometer (Beckman Instruments, Inc., Brea, CA 92621).

Automated method for urinary creatinine. The automated "low-hydroxide" method was tailored to the RA-1000 random-access analyzer (Technicon Instruments Corp., Tarrytown, NY 10591) and Cobas Bio centrifugal analyzer (Roche Analytical Instruments, Inc., Nutley, NJ 07110). We compared the results obtained with the low-hydroxide method determined in the RA-1000 with results obtained with the AutoAnalyzer II continuous-flow analyzer (Technicon), the ASTRA discrete analyzer (Beckman), the aca discrete analyzer (Du Pont Instruments, Wilmington, DE 19896), and a method based on decreased sample volume performed with the RA-1000 (a low-sample-volume method).

Reagents

Sodium hydroxide study. Creatinine [Standard Reference Material (SRM) no. 914, National Bureau of Standards, Gaithersburg, MD 20234]; cefoxitin sodium, sterile (lot no. 0673H; Merck Sharp & Dohme, West Point, PA 19486); picric acid (99+%; Gold Label; Aldrich Chemical Co., Milwaukee, WI 53201); and reagent-grade NaOH, NaH2PO4, and Na2HPO4.

We dissolved all reagents in double distilled, de-ionized water, except for cefoxitin, which we dissolved in phosphate buffer. The molarity of our stock picric acid solution was determined by titration with NaOH. The creatinine and cefoxitin solutions were freshly prepared, daily.

Automated method for urinary creatinine. For the low-hydroxide method, performed with the RA-1000 (user-1 method) and the Cobas Bio, we mixed equal volumes of picric acid, 42 mmol/L (Technicon), and sodium hydroxide, 240 mmol/L (creatinine diluent, Technicon), then diluted this alkaline picrate mixture with an equal volume of double distilled, de-ionized water, adding one drop of "wetting agent W" (Technicon) for every 6.5 mL of working creatinine reagent. This reagent is stable for two weeks. For the low-sample-volume method (user-2 method), we mixed equal volumes of picric acid, 42 mmol/L, and sodium hydroxide, 240 mmol/L (both from Sigma Diagnostics, St. Louis, MO 63178), but did not dilute the final mixture.

For the AutoAnalyzer method, we used picric acid, 30% Brij-35 solution (both from Technicon), and reagent-grade NaOH and NaCl. Reagents and calibrators for the aca and ASTRA were from the manufacturer. We used creatinine (SRM no. 914) as the calibrator for the user-1, Cobas Bio, and AutoAnalyzer methods, and a serum calibrator (Sigma Diagnostics) for the user-2 method.

For interference studies we used cefoxitin (Merck Sharp & Dohme), cephalothin sodium for injection (Eli Lilly & Co.,

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Indianapolis, IN 46285), protein (Lancer Microprotein Rapid STAT Standard, Sherwin Medical, St. Louis, MO 63105), bilirubin reference material (Sigma), glucose, urea (J. T. Baker Chemical Co., Phillipsburg, NJ 08865), and acetone (Aldrich).

Experimental Design

**Sodium hydroxide study.** We added 0.9 mL of alkaline picrate to 0.1 mL of creatinine in a cuvette and recorded absorbance as a function of time at 500 nm and 37 °C (final concentrations were 0.05 mmol of creatinine per liter (5.95 mg/L) and 10 mmol of picric acid per liter). We varied the final concentration of sodium hydroxide from 25 to 200 mmol/L in 25-mmol/L intervals. We also performed identical studies, replacing creatinine with cefoxitin (0.1 mmol/L, final concentration).

We calculated the pseudo-first-order rate constants from the slope of plots of \(-\ln A_{\text{max}} - A_t\) vs time, \(A_{\text{max}}\) being the maximum absorbance and \(A_t\) the absorbance at time \(t\) (1), and plotted the pseudo-first-order rate constants vs the concentration of sodium hydroxide. We analyzed the results by linear regression, with the concentration of sodium hydroxide as the independent variable. We calculated the equilibrium constants and molar absorptivities by means of the Benesi–Hildebrand equation (1).

**Automated methods for urinary creatinine.** The **user-1** method is based on a slower rate of reaction than is found with most serum methods; we achieved the slower rate by decreasing the concentration of sodium hydroxide. The **user-2** method is based on a decreased concentration of creatinine in the final reaction mixture, achieved by decreasing the sample volume while increasing the reagent volume. The instrument settings for the RA-1000 user-1 and user-2 methods, and those for the Cobas Bio method, are listed in Table 1; otherwise, the settings are the same as in the Technicon RA-1000 method for serum creatinine (2).

The **user-1** method is calibrated with a 1000 mg/L solution of the SRM creatinine standard; this standard is usually stable for one month. We calibrated the **user-2** method the same way as for the RA-1000 serum method, with a 30 mg/L serum-based standard (Sigma). After calibration for the **user-2** method we changed the RA-1000 settings for sample volume, reagent volume, linearity factor, and first limit to the **user-2** settings listed in Table 1, and changed the multiplication factor to 10. We calibrated the Cobas Bio method with SRM creatinine in aqueous solutions at concentrations of 500, 1000, and 2000 mg/L. We operated the AutoAnalyzer according to a previously described method (3) and the **ASTRA** and **aca** according to the manufacturers' instructions. For the **aca** method, we diluted urine samples 10-fold with enzyme diluent (4). For the **ASTRA** method, we used the urinary mode of the instrument so that the sampler picked up only one-third the volume of sample that it does for serum.

We determined urinary creatinine in samples from 104 patients, using the RA-1000 (user-1 and user-2 methods), AutoAnalyzer, **ASTRA**, and **aca**. We analyzed the results by analysis of variance, Tukey's test, standard linear regression (arbitrarily choosing to use the AutoAnalyzer results as the independent variable), and Deming debiased linear regression (5, 6). In a separate study, we determined urinary creatinine in 100 samples with the low-hydroxide method (user-1 and Cobas Bio versions). We analyzed the results by linear regression, using the results from the RA-1000 as the independent variable.

For the new methods, we determined precision by assaying triplicate and low-concentration urinary pools (prepared in our laboratory) and assessed accuracy by linear regression analysis of observed creatinine concentration vs the concentration of SRM creatinine standards.

We recorded absorbance at 510 nm as a function of time for creatinine reacted in two different reagent configurations: the low-hydroxide method (user-1 and Cobas Bio methods), and the low-sample-volume method (user-2 method). We mixed the creatinine samples and picrate reagents directly in the cuvettes, maintaining the temperature at 37 °C.

To test for interference from compounds thought to have the potential to interfere, we dissolved various concentrations of the compounds with pooled urine, then determined creatinine by all the analyzers and analyzed the results by linear regression. The interference, which is equal to the slope of the regression line, is expressed as milligrams of "pseudo-creatinine" per gram of compound. The highest final concentrations of the compounds tested were protein 10 g/L, bilirubin 9.5 g/L, acetone 800 mg/L, ascorbic acid 2 g/L, glucose 5 g/L, cephalothin 4 g/L, cefoxitin 4 g/L, and urea 5 g/L.

**Results**

**Sodium Hydroxide Study**

The rate for the Jaffé reaction, whether reacting with creatinine or with cefoxitin, is directly proportional to the concentration of hydroxide (Figure 1) and is reducible to pseudo-first-order. The relationship between the pseudo-first-order rate constant, and the concentration of hydroxide (at 10 mmol/L picrate concentration) can be expressed as \(k = 0.004 [\text{OH}^-] - 0.06 (S_p = 0.038, r = 0.989, \text{standard error of the slope} = 2.4 \times 10^{-4}, \text{standard error of the y-intercept} = 0.03)\) for creatinine, and \(k = (3.2 \times 10^{-4}) [\text{OH}^-] - 0.006 (S_p = 2.91 \times 10^{-3}, r = 0.991, \text{standard error of the slope} = 1.8 \times 10^{-4}, \text{standard error of the y-intercept} = 0.002)\) for cefoxitin. At equilibrium, the molar absorptivities of the Jaffé reaction for creatinine and cefoxitin did not change significantly as the concentration of hydroxide was varied. The equilibrium constant for the reaction with creatinine did change, however, the constant being 1.5 and 0.75 L/mmol at 125 and 50 mmol of sodium hydroxide per liter, respectively. The equilibrium constant for the reaction with cefoxitin did not change significantly.

**Table 1. Settings for Automated Methods for the Determination of Urinary Creatinine**

<table>
<thead>
<tr>
<th>RA-1000</th>
<th>user-1</th>
<th>user-2</th>
<th>Cobas Bio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample vol, µL</td>
<td>9</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Reagent vol, µL</td>
<td>360</td>
<td>400</td>
<td>240</td>
</tr>
<tr>
<td>Diluent vol, µL</td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Time of first reading, s</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Time between readings, s</td>
<td>30</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>No. of readings</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Wavelength, nm</td>
<td>500</td>
<td>500</td>
<td>510</td>
</tr>
<tr>
<td>Temp, °C</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Maximum concn, mg/L</td>
<td>3000</td>
<td>2500</td>
<td>3000</td>
</tr>
<tr>
<td>Final [picrate], mmol/L</td>
<td>10.2</td>
<td>21</td>
<td>9.5</td>
</tr>
<tr>
<td>Final [NaOH], mmol/L</td>
<td>59</td>
<td>120</td>
<td>55</td>
</tr>
<tr>
<td>Linearity factor</td>
<td>2.6</td>
<td>2.6</td>
<td>—</td>
</tr>
<tr>
<td>First limit</td>
<td>1.00</td>
<td>1.00</td>
<td>—</td>
</tr>
</tbody>
</table>

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The identical slope intercepts, and significantly worse than those for the user-2 method (Figure 2). The absorbance as a function of time is linear during the time interval of measurement for the Jaffé reaction configured as in the user-1 and Cobas Bio methods (low-hydroxide method), but is curved exponentially for the user-2 method (low-sample-volume method) (Figure 2).

Analysis of variance showed differences among the methods. The mean for the Astra method differed significantly from the means for the user-1, user-2, acc., and AutoAnalyzer methods (Tukey's test), but the means for the user-1, user-2, acc., and AutoAnalyzer methods did not differ significantly among themselves (Table 2). The user-1, user-2, and the acc. methods all had nearly identical slopes and y-intercepts, but the Astra method had a significantly lower slope (Table 2, Figure 3). Included in Figure 3 are the results of Deming debiased regressions; the slopes, y-intercepts, and $S_{xy}$ values for the Deming debiased regressions are nearly identical to their counterparts calculated for standard linear regressions.

The user-1 method is accurate to 3000 mg of creatinine per liter, while the user-2 method is accurate only to 2500 mg/L (Figure 4). The CVs are <3.6% for both RA-1000 methods for run-to-run and <1.4% for within-run determinations (Table 3).

The two antibiotics, cephalothin and cefoxitin, interfered with all the methods (Table 4). On the average, the observed values of creatinine determined in urine containing ascorbic acid, glucose, urea, protein, and acetone differed from unadulterated urine by <1% for the user-1 and Cobas Bio methods, and 2% for the user-2 method; in urine containing bilirubin, interference was <3% for all three methods. Similarly, the observed values of creatinine determined by the AutoAnalyzer, acc., and Astra for urine containing these compounds, excluding cefoxitin and cephalothin, differed from those for unadulterated urine by <4%. Further, we observed no trend as the concentrations of these compounds were increased; therefore, we conclude that ascorbic acid, glucose, urea, protein, acetone, and bilirubin do not interfere with any of the methods tested for urinary creatinine.

In a separate study we compared the results for the low-hydroxide method as performed by the Cobas Bio with those for the user-1 method. The mean creatinine concentrations were in close agreement for both methods—839 mg/L for the Cobas Bio method ($y$) and 822 mg/L for the RA-1000 method ($x$)—and results of linear regression analysis were excellent: $y = 0.997x + 19$ mg/L ($r = 0.988$, $S_{xy} = 18$, standard error of the slope = 0.006, and standard error of the y-intercept = 6). Results of a Deming debiased regression analysis were nearly identical to the results of the standard regression: $y = 0.998x + 18$ mg/L, $S_{xy} = 22$. The run-to-run CV for the Cobas Bio was 1.1% (mean creatinine, 1160 mg/L). The Cobas Bio method was accurate to 3000 mg/L and showed excellent agreement between the values of standards ($x'$) and the observed creatinine concentrations ($y'$); $y' = 0.993x' + 20$ mg/L ($S_{xy'} = 47$ mg/L, $r = 0.999$, standard error slope = 0.019, and standard error y'-intercept = 24 mg/L). Only cefoxitin and cephalothin interfered with the Cobas Bio method, the degree of interference being similar to that of the user-1 method (Table 4).

**Discussion**

**Dependence of the Jaffé Reaction Rate on the Concentration of Hydroxide**

Modern methods for the determination of creatinine with the Jaffé reaction are kinetic, being either one-point, two-point, or multi-point, according to the classification scheme developed by Purdue (7). The theory of reaction rates should apply to the Jaffé reaction and describe the formation of product in solution—the product being the chromophore, and picrate the chromogen. To be of value, a theory explaining the Jaffé reaction should be able to predict the absorbance of the reaction, especially as the concentrations of the reactants are changed. The absorbance of the Jaffé reaction has been mathematically expressed as

$$A_t = eBC_0(1 - e^{kt}) + A_0$$

where $e$ is the molar absorptivity of the product, $b$ is the pathlength of the cuvette, $C_0$ is the initial concentration of creatinine, $A_0$ is the absorbance of the blank at $t = 0$, $k$ is a first-order rate constant, and $t$ represents time (8). This mathematical expression, though simple, is infelicitous, because it neglects the importance of the concentrations of picrate and hydroxide and predicts the true absorbance...
Table 2. Comparison of Results for Urinary Creatinine by Various Methods

<table>
<thead>
<tr>
<th></th>
<th>USER-1</th>
<th>USER-2</th>
<th>AutoAnalyzer</th>
<th>ACS</th>
<th>ASTRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, mg/L</td>
<td>1044</td>
<td>1029</td>
<td>1046</td>
<td>1035</td>
<td>916*</td>
</tr>
<tr>
<td>Standard linear regressionb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.95 (0.01)</td>
<td>0.96 (0.01)</td>
<td>0.96 (0.02)</td>
<td>0.95 (0.02)</td>
<td>0.86 (0.02)</td>
</tr>
<tr>
<td>y-Intercept, mg/L</td>
<td>45 (28)</td>
<td>22 (26)</td>
<td>26 (29)</td>
<td>19 (35)</td>
<td></td>
</tr>
<tr>
<td>$s_{yy}$, mg/L</td>
<td>27</td>
<td>26</td>
<td>27</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>.992</td>
<td>.993</td>
<td>.992</td>
<td>.985</td>
<td></td>
</tr>
<tr>
<td>CV&lt;sub&gt;y&lt;/sub&gt;, %</td>
<td>2.6</td>
<td>2.5</td>
<td>2.6</td>
<td>3.3</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from other means (Tukey's test, p<.05, minimum significant difference = 22). bMean (and 2 SD) vs AutoAnalyzer results. n = 104.

![Graphs A, B, C, D](image)

Fig. 3. Comparison of four methods for the determination of urinary creatinine with results by AutoAnalyzer method

- Solid line, results of Deming debiased regressions assuming equal errors in both y- and x-axis; dashed line, the line of identity. n = 104

In reality, the Jaffe reaction is third-order, being dependent on the concentrations of creatinine, picrate, and hydroxide, but can be reduced to a pseudo-first-order reaction by adjusting the concentrations of picrate and hydroxide so that they change less than 5% as the product is formed. Previously, we discussed the reaction's dependence on the concentration of picrate (1). Now we turn our attention to the dependence on hydroxide. The number of published studies concerning this dependence on the concentration of hydroxide are few and have many deficiencies. The authors failed to express the results as a rate constant, used concentrations of hydroxide exceeding 200 mmol/L, or measured absorbance only at 25 °C (9-13). Measurements at 25 °C are unacceptable for instruments operated at 37 °C, e.g., the RA-1000, because the Jaffe reaction rate is sensitive to temperature (12, 14). Moreover, most modern methods
are performed with solutions containing less than 200 mmol of hydroxide per liter, because high alkalinity suppresses color formation [15]. Thus, we had to determine the rate constant ourselves. [Although Butler showed that the rate constant can be reduced to pseudo-first-order at 25 °C, he did not calculate its value (10).] The pseudo-first-order rate constant for the Jaffé reaction, in 10 mmol/L picoate solution at 37 °C, is $4.02 \times 10^{-3}$ min$^{-1}$ for creatinine and $3.2 \times 10^{-4}$ min$^{-1}$ for cefoxitin.

The rate of the Jaffé reaction is strongly dependent on the concentration of hydroxide. The linear relationship between the rate constants and the concentration of hydroxide contradicts a previously accepted model for the reaction (9):

$$\text{POH}^* + C \xrightarrow{k_1} \text{PC} + x\text{OH}^-$$  \hspace{1cm} (2)

where \(\text{POH}^*\) is activated picoate, \(C\) is creatinine, \(\text{PC}\) is the picoate-creatinine product, \(x\) is the number of moles of hydroxide released at the end of the reaction, \(k_1\) is the forward rate constant, and \(k_2\) the reverse rate constant. One must infer from equation 2 that the reaction rate becomes slower as the hydroxide concentration increases, which may be true for concentrations of hydroxide $>500$ mmol/L, but not for those $<200$ mmol/L. At concentrations of hydroxide exceeding 500 mmol/L, picoate forms a second complex with hydroxide, the formation of which allows the rate of the Jaffé reaction (15). We therefore propose the following model:

$$\text{HP} + \text{H}_2\text{O} \xrightarrow{k_4} \text{P}^- + \text{H}_2\text{O}^+ + \text{OH}^- \rightarrow \text{P}^- + 2\text{H}_2\text{O}$$  \hspace{1cm} (3)

$$\text{P}^- + \text{OH}^- \rightarrow (\text{P}^- + \text{OH}^-)^*$$  \hspace{1cm} (4)

$$\text{(P}^--\text{OH}^-)^* + C \rightarrow (\text{P}^- - C - \text{OH}^-)$$  \hspace{1cm} (5)

where \(\text{HP}\) is picric acid, \(K_4\) is the dissociation constant for acid, \(\text{P}^-\) is picoate, \((\text{P}^- - \text{OH}^-)^*\) is activated picoate, \(C\) is creatinine, and \((\text{P}^- - C - \text{OH}^-)\) is the creatinine-picoate complex. To obtain an analogous model for cefoxitin, one need only replace creatinine by cefoxitin in equations 3 through 5. The effect of varying the concentration of sodium hydroxide on the cefoxitin-picoate reaction parallels that involving creatinine. Cefoxitin interferes no more with the low-hydroxide method than with conventional kinetic methods, because its dependence on the concentration of hydroxide parallels that of creatinine.

Absorbance in the Jaffé reaction depends on the molar absorptivity, pathlength, equilibrium constant, temperature, and concentrations of creatinine and picoate, as well as the concentration of hydroxide. We can express this dependency for the rate of change in absorbance as

$$\frac{dA}{dt} = \epsilon_b \left( \frac{K([\text{picoate}]_0)}{1 + K([\text{picoate}]_0)} \right)$$

$$K([\text{creatinine}][\text{picoate}]_0) [\text{OH}^-]^{n_1 - [\text{picoate}]_0[\text{OH}^-]^{n_2}}$$  \hspace{1cm} (6)

where \(K\) is the equilibrium constant, \([\text{picoate}]_0\) the initial concentration of picoate, \([\text{OH}^-]_0\) the initial concentration of hydroxide, \(k\) the pseudo-first-order rate constant (independent of [picoate] and [OH$^-$]), \(t\) is time, and \(\epsilon\) is the exponential function. This expression differs from the one derived by Bowers and Wong (17) in that we do not use the model

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**Table 3. Precision (CV, %) of RA-1000 Methods**

<table>
<thead>
<tr>
<th>Creatinine concn</th>
<th>user-1</th>
<th>user-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run (n = 20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.63 (455)*</td>
<td>1.22 (460)</td>
</tr>
<tr>
<td>Middle</td>
<td>1.32 (1380)</td>
<td>0.86 (1120)</td>
</tr>
<tr>
<td>High</td>
<td>0.86 (2170)</td>
<td>1.24 (2130)</td>
</tr>
<tr>
<td>Run-to-run (n = 34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3.6 (477)</td>
<td>3.1 (456)</td>
</tr>
<tr>
<td>High</td>
<td>2.6 (1330)</td>
<td>3.0 (1310)</td>
</tr>
</tbody>
</table>

*Mean creatinine concentrations (mg/L) in pooled urines are given in parentheses.

**Table 4. Interferences by Cefoxitin and Cephalothin with Different Methods for the Determination of Urinary Creatinine**

<table>
<thead>
<tr>
<th></th>
<th>Cefoxitin</th>
<th>Cephalothin</th>
</tr>
</thead>
<tbody>
<tr>
<td>USER-1</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>USER-2</td>
<td>49</td>
<td>-7</td>
</tr>
<tr>
<td>AutoAnalyzer</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>aca</td>
<td>45</td>
<td>-12</td>
</tr>
<tr>
<td>ASTRA</td>
<td>37</td>
<td>27</td>
</tr>
<tr>
<td>Cobas Bio</td>
<td>38</td>
<td>32</td>
</tr>
</tbody>
</table>

*From the slope of the linear regression, expressed as milligrams of "pseudocreatinine" per gram of compound tested."
proposed by Vasiliaides (9). With equation 6, anyone can
determine how fast the absorbance will change and what
the total absorbance (minus the blank) will be at the end of
the reaction or measurement interval. One simply uses the
pseudo-first-order rate constant and inserts the desired
concentrations of reactants. Our value for the pseudo-first-
order rate constant assumes a picrate concentration of 10
mmol/L.

For a sample of urine containing 1000 mg of creatinine
per liter (an average concentration), the absorbance would
be 2.6 at 1.5 min, as determined with the RA-1000 method
for serum. Most photometers cannot measure the absorb-
bance precisely when the percent transmittance is less than
1.0% (i.e., A > 2.0), because of the relative concentration
error (18). Values for dA/dt will be imprecise for most
urinary samples measured this way, and accuracy lost. Thus
the method for creatinine in serum cannot be used to
measure creatinine directly in urine. Either the sample
must be diluted or the characteristics of the Jaffé reaction
changed. Because use of a fully automated method for
urinary creatinine precludes manual dilution of urine, the
reaction's characteristics must be changed.

The equation for dA/dt as a function of the reactant
concentrations and time (equation 6) divides the character-
stics of the Jaffé reaction into its elemental components.
From this equation, one can calculate dA/dt and the total
absorbance for any given moment. The maximum absorb-
ance of the reaction is given by εcreatinine, ε = 12.0
L/mmol at 500 nm and 37 °C (1). A urine sample containing
3000 mg of creatinine per liter and diluted 20-fold (by the
instrument) would have a concentration in the measure-
ment cuvette of 1.32 mmol/L and a final absorbance in the
Jaffé reaction of 15.8. This absorbance is much too high for
photometers to measure, so the sample must be diluted
further or the absorbance measured long before the reaction
reaches equilibrium. The most obvious approach would be to
dilute the sample further, which is the rationale of the user-
2 method. Here, a urine sample containing 3000 mg of
creatinine per liter would have a final concentration of 0.13
mmol/L and a maximum absorbance of 1.56.

Thus a method based on a very-low-sample volume would
seem to be the solution, but is invalid only if the rate of
the reaction is ignored. The reaction as configured for the user-
2 method is too fast (Figure 2). The half-life of the reaction is
1.2 min and the reaction is halfway over in the middle of the
measurement interval. The velocity of the reaction is given
by K[creatinine][picrate]0[OH–]0, and the shape of the rela-
tionship between absorbance and time is given by exp(–k ·
[picrate]0[OH–]0t) of equation 6. The absorbance–time rela-
tionship is an inverse exponential curve for both the low-
hydroxide (user-1) and the low-sample-volume (user-2)
methods, but where on the exponential curve the measure-
ment interval falls is determined by the speed of the
reaction. In the early part of an exponential curve, which
approximates a straight line, is where we want the meas-
urement interval to be (Figure 2A), not in the middle or
later part of the exponential curve (Figure 2B), because
accuracy and precision are maximized in the linear region.

It is imperative, therefore, to slow the rate. The most
obvious way is to decrease the concentration of picrate, but a
decreased concentration of picrate leads to two problems. In
the Jaffé reaction, not all of the creatinine is converted to
product at equilibrium. The fraction of creatinine converted
to product (I),

\[
\frac{K[\text{picrate}]_0}{1 + K[\text{picrate}]_0}
\]
is determined by the law of mass action and decreases
considerably with the decrease in the concentration of
picrate. But random errors in measurement, which are
inversely related to the fraction of creatinine converted
to product, can be minimized if the fraction is 90% or greater;
therefore, the ratio of picrate to creatinine must be kept
>10:1. Moreover, the direct, simple, pseudo-first-order rate
relationship in equation 6 depends on the maintenance of
pseudo-first-order conditions; in other words, the concentra-
tion of creatinine should be the only variable changing with
time. The ratio of [picrate] to [creatinine] must in fact be
20:1 or greater to maintain pseudo-first-order conditions;
otherwise, the rate depends on the changing concentration
of both creatinine and picrate. Therefore, we have little
leeway to decrease the concentration of picrate.

The concentration of hydroxide is at an even higher
concentration in relation to creatinine. Therefore, we can
decrease the concentration of hydroxide and slow the rate of
reaction without compromising the fraction of creatinine
converted to product or the pseudo-first-order nature of the
reaction. The half-life of the reaction for the low-hydroxide
method is 4.6 min, and the measurement interval falls on the
linear part of the absorbance-time curve (Figure 2A). The
slower reaction rate makes the user-1 method better than the
user-2 method. The user-2 method could be optimized if measure-
ments were taken between 7 and 15 s after mixing, but such a timed interval is beyond the ability of
many commercial instruments.

The Comparison Studies

In spite of the differences in the rates of reaction, both the
user-1 and user-2 methods gave results that compared well
with those of the other methods for the determination of
urinary creatinine (Table 2, Figure 3). The astra was the
only method that compared poorly with the others. The
lower values for the mean and slope determined with the
astra method were probably caused by the presence of
protein in the calibrators vs none in urine. In contrast, we
calibrated the RA-1000 and AutoAnalyzer with nonprotein-
containing calibrators; although the aao is calibrated with
protein-based material, one dilutes the urinary samples
with a protein-based diluent (4). The slopes for the user-1
and user-2 method are close to that of the AutoAnalyzer
method, and the precisions for the user-1 and user-2
methods are almost equal. The major difference between the
two methods is that the user-1 method is more nearly
accurate and more robust than the user-2 (Figure 4). Thus
the user-1 method is a good replacement for the AutoAna-
lyzer or oo methods.

Subtler differences make the user-1 method even more
attractive. One calibrates the user-2 method as for use with
serum samples, then changes the multiplication factor and
the sample and reagent volumes. Such indirect calibration
increases the possibility of operator errors, e.g., failure to
change the multiplication factor or the volumes used, and
also is slower than the direct approach. The user-1 method
offers flexibility in calibration material, so that one could
use a standard reference material, such as NBS no. 914
creatinine, in aqueous solution (dilute HCl, 0.2 mmol/L)
rather than in a serum matrix, the former better resembling
urine. The major additional difference between the user-1

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method for urinary creatinine and the RA-1000 method for serum creatinine is the one-to-one (by volume) dilution of the alkaline picrate reagent with water in the former, a task requiring some skill but little time. Otherwise, the ussa-1 method is as simple to use as the RA-1000 serum method, whereas the ussa-2 method is not. Therefore, we chose the low-hydroxide, ussa-1 method with the RA-1000 to replace our AutoAnalyzer method for the determination of creatinine in urine.

In a separate study, we compared the low-hydroxide method for urinary creatinine as implemented in the Cobas Bio and in the RA-1000. Statistically, the two methods are identical. The method with the Cobas Bio is accurate to 3000 mg of creatinine per liter and is precise. Interference was the same as with the ussa-1 method. The low-hydroxide method for urinary creatinine is not instrument dependent, and can be implemented with any instrument capable of adequately mixing the reagents and sample and measuring absorbance over the required time interval.

Advantages of a Direct Method for Urinary Creatinine

Besides its good accuracy and precision, the low hydroxide method for the determination of creatinine is fast, principally because the reagent is diluted instead of the sample. Determining urinary creatinine with the RA-1000 for our typical daily workload of 30 samples takes about 8 min, in contrast to the 45 min our AutoAnalyzer method used to take. The css takes considerably longer, because all the samples must be diluted. Even though the determination of urinary creatinine by the ASTRA appears to be quick, it may be necessary to dilute the samples with a protein diluent to obtain accurate results, which would add considerable time and cost to the whole procedure. The reagents for the low-hydroxide method are inexpensive, current prices being $1.28 for 1 L of picric acid (Aldrich), $0.72 for 1000 mg of creatinine per liter (NBS no. 914), which is stable for about one month, and $0.08 for 1 L of 120 mmol/L NaOH solution (Aldrich)—or less than U.S. $0.01 per sample (assuming 30 samples/day for one month). The method is as safe as any creatinine method based on the Jaffé reaction (sodium hydroxide and picric acid are corrosive). The standards used for calibration must be inspected visually, because creatinine precipitates out of solution in high concentrations, especially when refrigerated. Interferences with the low-hydroxide method are caused mainly by cepha antibiotics, and samples containing high concentrations of them should be avoided. The other potential interferents—protein, glucose, acetone, bilirubin, urea, ascorbic acid—did not affect results.

A negative interference may occur if the sample collectors are too zealous in adding sulfamic acid as a preservative. Too much sulfamic acid (e.g., 3 g of sulfamic acid in 10 mL of urine) interferes negatively with the low-hydroxide method by neutralizing the hydroxide. This situation is most likely to occur for urine collected from children, because their urinary volumes are smaller than adults. The proper concentration of sulfamic acid has no effect on the low-hydroxide method.

In conclusion, the low-hydroxide method for the determination of urinary creatinine, performed with either the RA-1000 or the Cobas Bio, is rapid, cost-effective, easy to perform, and a good replacement for the AutoAnalyzer or css methods. Because the low-hydroxide method is easy to run and automate, the difficulties of determining patients' creatinine clearance—time, cost, inconvenience—are primarily those of collecting the 24-h urinary sample. We hope that with the adoption of simple, automated methods for urinary creatinine, more creatinine clearances will be performed, determinations of creatinine clearance being superior to serum creatinine in evaluating renal function (19). In addition, by applying the kinetic theory of the Jaffé reaction, we formulated new reaction conditions and evaluated the weaknesses of different methods for the determination of urinary creatinine.

References