Determination of Creatinine in Serum and Urine by a Rapid Liquid-Chromatographic Method

R. Paroni, C. Arcelloni, I. Fermo, and P. A. Bonini

We describe an HPLC ion-pair procedure for rapid and specific evaluation of creatinine in serum and urine. We used a 15 cm × 4.6 mm ODS column with a 50/50 (by vol) mixture of sodium decanesulfonic acid (10 mmol/L, pH 3.2) and methanol and measured absorbance at 236 nm. Serum (100 μL) or 30-fold-diluted urine (100 μL) was added to 400 μL of acetone. After centrifugation, the supernates (300 μL) were dried, reconstituted with the mobile phase, and injected into the HPLC. Assay precision was tested for concentrations of 10, 29, and 130 mg/L and yielded, respectively, 3.1%, 2.1%, and 1.1% for within-day CV and 2.8%, 2.1%, and 2.2% for total CV. Analytical recovery was 102 (±6.7)%. Linearity was demonstrated in the 0–200 mg/L range for serum and 0–3.5 g/L range for urine (r = 0.999). The detection limit for creatinine (signal-to-noise ratio = 3) was 0.5 mg/L. We used cimetidine for internal standardization. Correlation was good between this procedure and the Jaffé kinetic, the enzymatic (creatinine amidohydrolase), and the Fuller’s earth alkaline picrate methods.

Additional Keyphrases: chromatography, reversed-phase cimetidine as internal standard

Creatinine concentrations in serum and urine are very useful indexes for evaluating glomerular filtration rate and, in general, for indicating renal function. Clinically, the automated Jaffé procedure (1), based on the alkaline picrate reaction, is the method most widely used to determine the creatinine concentration; however, the low specificity of this assay is well known. Positive interactions between picric acid and many endogenous chromogens or interfering substances (glucose, acetacetic acid, acetone, pyruvic acid, etc.) (2) may give an overestimated value of the creatinine concentration. High concentrations of bilirubin can also mask the reaction between creatinine and picric acid (3), yielding negative values for the creatinine determination.

Various modifications of the original Jaffé reaction have been adopted to improve the specificity of the assay (4), e.g., utilizing cation-exchange resins (5), adsorption on Lloyd’s reagent (6), or dialysis (7) to separate creatinine from the interfering compounds, or performing a differential measurement of the Jaffé reaction (before and after enzymatic degradation of creatinine) (8). Larsen (9) observed that the initial formation rate of the colored compound produced by the reaction with picric acid in alkaline solution is proportional to the true concentration of creatinine (and is different from the reaction rate of the pseudo-creatinine chromogens present in the system). In fact, use of kinetic methods, which result in substantially improved specificity, is widespread; however, results obtained with these methods are critically time-dependent. A series of enzymatic methods utilizing one (10) or several enzymes (11) are also available for creatinine determination, and have been successfully applied to kinetic analyzers. These methods are generally considered more specific than the preceding ones; but, again, bilirubin apparently interferes in the peroxidase-mediated reaction (12) and the antibiotic 5-fluorocytosine interferes in the popular iminohydrolase-coupled enzymatic procedure (13), the latter being used in the solid-phase chemistry of multilayer film systems.

In the last 10 years many procedures for creatinine determination have been developed based on "high-performance" liquid chromatography (HPLC). The first ones were based on ion-exchange chromatography followed by post-column alkaline picrate reaction (14, 15) or by direct ultraviolet detection of creatinine (16–19); normal-phase (20, 21), paired-ion (22), and reversed-phase (23–31) chromatographic techniques were developed subsequently. Some of these methods are characterized by tedious, time-consuming, or expensive sample pretreatments, i.e., solid-phase extraction (30), ultrafiltration (26, 28), or deproteinization with special pre-columns (29). Moreover, some of these HPLC separations need particular temperature conditions (19, 26), complex elution gradients (24), or a long column conditioning time (22). In addition, some of the proposed HPLC methods showed poor specificity (a retention time not long enough to separate the creatinine peak from other peaks) (24, 26) or required a chromatographic apparatus not easily reproducible in clinical laboratories (14, 25, 30).

Because of the various drawbacks presented by the chromatographic methods so far proposed, we decided to develop an alternative liquid-chromatographic method for creatinine determination in biological fluids. This method should be characterized by high precision, accuracy, and specificity and, at the same time, should be simple, sufficiently rapid, and easily practicable in a clinical chemistry laboratory as a comparison method.

Materials and Methods

Reagents

We used HPLC-grade solvents and doubly distilled water throughout. Decane-, octane-, and hexanesulfonic acid (C10, C8, C6), sodium salts, were supplied by Fluka Chemie (Buchs, Switzerland). Creatinine standard was obtained from BDH (Milano, Italy). All reagents for the picrate kinetic method and for the amidohydrolase enzymatic method (test combination creatinine-PAP) were supplied by Boehringer Mannheim (Mannheim, F.R.G.). The Fuller’s earth (BDH) was kindly supplied by Prof. C. Franzini, Lab. Clin. Invest., Hospital of Rho (Milan, Italy).

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Lyophilized sera of different pools (n = 4) previously analyzed by isotope dilution mass spectrometry (IDMS) (32) were a gift of Prof. L. Siekmann, Institut für Klinische Biochemie, Universität Bonn, Bonn, F.R.G.

Creatinine standard solutions. Creatinine stock solution (1 g/L), prepared by dissolving pure creatinine in 10 mmol/L HCl solution, was stored at 4 °C for a month. Weekly, we prepared working standard solution (20 mg/L) by diluting the stock solution with water; a 20-μL aliquot of this solution was used daily as a control to check retention time, etc. of the HPLC procedure.

Internal standard solution. Cimetidine solution (100 g/L) was prepared by dissolving the drug in 10 mmol/L HCl solution. We added 5 μL of this solution to each serum sample before extraction.

Calibration curves. Calibration curves, both with and without internal standard, were prepared by adding to serum (or water) and urine increasing amounts of creatinine (10–200 mg/L for serum or water and 0.3–3.5 g/L for urine). For daily calibration we repeatedly injected aliquots of the 20 mg/L standard in water.

Apparatus

The HPLC system we used was a "System Gold" (Beckman Instruments, San Ramon, CA) with two dual-piston pumps (Model 126) and a variable double-beam ultraviolet detector (Model 167) with a fast wavelength drive allowing the automatic recording of peaks' spectra during the analysis. The whole apparatus was computer controlled (IBM, Model PS/50) with the "System Gold" software, which permits the handling, storage, and reprocessing of the chromatographic data. Injections were automatically done with a autosampler (Model 460, Kontron, Zurich, Switzerland) connected to the HPLC apparatus.

A Kontron spectrophotometer (UVikon 930) was used for colorimetric and enzymatic determinations.

Procedures

HPLC proposed procedure. We used a Beckman ODS Ultrasphere C18 reversed-phase column [15 cm × 4.6 mm (i.d.), 5-μm particles]. The mobile phase consisted of equal volumes of decaconuclidean acid (10 mmol/L, pH 3.2) and methanol. Before use, the mobile phase was always filtered through 0.45-μm pore-size filters (Millepore, Bedford, MA) and further degassed by sonication. The flow rate was 0.7 mL/min and the retention time for creatinine was 8.1 min. The column was equilibrated with the mobile phase for 2 h at the beginning of each working week and washed with water every night. Each daily reconditioning required 30 min. The absorbance detector was set at 266 nm. The analysis was run at room temperature. Peaks area was stored and quantified by the on-line data processor.

Sample preparation procedure. For protein precipitation, we added 100 μL of serum samples, 30-fold-diluted urines (diluted with water), or working standard solution (20 mg/L) to 400 μL of acetone in plastic Eppendorf centrifuge tubes, vortex-mixed for 10 s, and centrifuged at 8000 × g for 3 min in a Microfuge 11 (Beckman). The supernates (300 μL) were dried in a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, NY). After reconstitution with 100 μL of the mobile phase, 20 μL was automatically injected into the HPLC. When the procedure with internal standard was used, we mixed 100 μL of sample, 5 μL of cimetidine, and 400 μL of acetone in an Eppendorf tube, then proceeded as above.

Comparison Methods

Alkaline picrate–kinetic bichromatic method. We used the Hitachi 737 discrete automated analyzer (Hitachi Ltd., Tokyo, Japan). Ten microliters of non-deproteinized serum or of 10-fold-diluted urine was mixed with 250 μL of sodium hydroxide, 0.2 mol/L; after 3 min at 37 °C, the picric acid solution (25 mmol/L, 50 μL) was added. After a 1-min incubation, the rate of color formation was monitored for 72 s at 505 and 570 nm. The daily calibration was performed with the "Calibrator for Automated System" (Boehringer), a lyophilized serum with known creatinine concentration (about 20 mg/L).

Enzymatic method. Creatininase (creatinine amidohydrolase, EC 3.5.2.10) was used to convert creatinine to creatine, and creatinase (EC 3.5.3.3) to convert creatine in urine and sarcosine. Sarcosine oxidase catalyzed the conversion of sarcosine to glycine, formaldehyde, and hydrogen peroxide, which is detected colorimetrically. We mixed 100-μL samples in disposable test tubes with 2 mL of either blank or test reagent. After 20 min at room temperature their absorbances at 510 nm were measured against the reagent blank.

Fuller's earth method. We followed the procedure reported by Haeckel et al. (33). After deproteinization of serum (100 μL) with tungstic acid, creatinine in the supernatant fluid was absorbed onto purified Fuller's earth and then determined with an alkaline picrate technique.

Results

Optimization of Chromatographic Conditions

Creatinine is a weak base (Kb = 3.57 × 10^-11 at 40 °C), very soluble in water. A selective control of its retention time on a reversed-phase column may be obtained by using an acidic buffer eluent, with a paired-ion technique; in fact, alkylsulfonates have often been used as pairing-ions to resolve cationic species (34). We tested several variables to optimize the separation of the creatinine peak from that of other endogenous compounds.

Figure 1A illustrates the relationship between the creatinine retention time (RT) and the alkyl chain length of the pairing-ion agent (C6, C8, C10), with a fixed concentration (10 mmol/L) and pH (3.2) of pairing-ion. Using the same protocol, we also studied the effects of the percentage of the organic solvent (Figure 1A). A longer RT was obtained for creatinine by increasing the chain length of the pairing-ion molecules from C6 to C10. Increasing the percentage of methanol in the mobile phase allowed a more rapid elution of creatinine.

Figure 1B shows the variation of creatinine RT as the C10 concentration was varied from 3 to 20 mmol/L, with a fixed proportion of methanol (50/50, by vol). Unlike published data (22) for paired-ion reversed-phase techniques, we found that the RT decreased as the C10 concentration increased; however, Tomlinson et al. (34) have described a nonlinear relationship between RT and paired-ion concentration, which they attributed to different aggregations between the pairing-ions (C10) and the sample cation (creatine), or to the pairing-ions themselves, which tend to form dimers and trimers in the aqueous phase when their concentration increases.

Figure 1C shows the modifications of creatinine RT as the pH of the mobile phase ranges from 3.0 to 6.0. As expected, in the 5.0–6.0 pH range, the RT did not show appreciable variations because this pH range exceeded the...
creatinine $K_0$ (5.02) so that the molecule remained in neutral form. The RT greatly increased when the pH was lowered from 4.5 to 3.0, the creatinine being sufficiently cationic there to form a "paired-ion" with the counter-ion.

We studied the spectral behavior of creatinine in the HPLC mobile phase over the wavelength range of 180–300 nm (Figure 2). Although the maximum absorbance was 200 nm, we preferred to perform the analyses at 236 nm, where the baseline was more stable.

On the basis of these results we decided to adopt the chromatographic conditions reported in HPLC proposed procedure, which represent a good compromise between the need to obtain a good separation of the creatinine peak from the solvent peak or from the other interfering peaks, and to keep the total analysis time reasonably short. Figure 3 shows chromatograms from the analyses of pure creatinine standard and of serum with high or low creatinine concentration under the chromatographic conditions finally adopted: the retention time was 8.1 min, and the creatinine peak was well resolved from other interferences. Figure 3D shows the chromatogram of a serum sample with the internal standard cimetidine (5 µg) added. Because the retention time of cimetidine is 14 min, the total analysis time was increased to 17 min.

**Analytical Variables**

*Linearity.* The absolute creatinine peak area, or the ratio for creatinine/internal standard peak areas, was plotted against the concentration values ($x$) in serum or water, and least-squares regression analysis was done. The equations calculated without and with cimetidine addition $y = 2.22(±0.44)x + 3.28(±0.70)$ and $y = 0.15(±0.003)x + 0.25(±0.049)$, respectively; values in parentheses indicate SD for slope and intercept] show a good linearity in the concentration range tested both for serum and urine specimens ($r > 0.999$).

*Analytical recovery.* Twenty-two routine sera obtained from the clinical laboratory and selected without conscious bias were analyzed before and after addition of pure creatinine, 10 mg/L. Analytical recovery was 102 ± 6.7% (CV = 6.5%).

*Precision.* The within-day and the total (between-day) imprecision was assessed by analyzing, four times per day for 10 days, three sera pools with different creatinine concentrations: 10, 29, and 130 mg/L. The within-day CV ranged from 3.1% to 1.1% for the 10 and 130 mg/L pools, whereas the between-day CV was 2.8%, 2.1%, and 2.2% for the 10, 29, and 130 mg/L pools, respectively. The overall imprecision was 4.2%, 3.0%, and 2.4%, respectively.

*Detection limit.* At a signal-to-noise ratio of 3, the detection limit of the method for creatinine in biological fluids was 0.5 mg/L, i.e., 2.5 ng per injection.

*Specificity of the method.* The specificity was evaluated by analyzing solutions of various compounds that interfere with the Jaffé reaction and drugs frequently administered to patients. None of the compounds listed in Table 1 showed a retention time interfering with the creatinine peak. We further confirmed the specificity of the HPLC method by using the Model 167 Beckman detector, which permits automatic recording of the spectra of peaks during the chromatographic analysis and prints simultaneously two chromatograms at different selected wavelengths. Figure 4 shows the spectra of the peak for creatinine eluted in a serum specimen (A) and for an aqueous standard (A*). The
spectra were recorded at the up slope and at the downslope creatinine peak. The perfect overlapping, in the wavelength range tested (200–350 nm), of the two superimposed spectra (Figure 4B) confirmed the absence in biological specimens of compounds interfering with the creatinine peak.

As a further confirmatory test, we superimposed the first (Figure 4C) and the second (Figure 4D) derivatives of the absorbance spectra obtained during analysis.

### Table 1. Interfering Compounds Tested

<table>
<thead>
<tr>
<th>Compound</th>
<th>Interfering Compounds Tested</th>
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<tbody>
<tr>
<td>Acetone</td>
<td>Furosemide</td>
</tr>
<tr>
<td>Glucose</td>
<td>Polythiazide</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Clofibrate</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>Methylclozide</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Methyldopetrazide</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>2-Methyl-4-phenylacetamide</td>
</tr>
<tr>
<td>Warfarin</td>
<td>5,10-Dimethylaminobenzamide</td>
</tr>
<tr>
<td>Methylimidazole</td>
<td>Leucine</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>Carboxymethylcysteine</td>
</tr>
<tr>
<td>Barbitol</td>
<td>Tropone</td>
</tr>
<tr>
<td>Histamine</td>
<td>Fenquizone</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>Triasenic acid</td>
<td>Quinidine sulfate</td>
</tr>
</tbody>
</table>

two subgroups of 19 icteric samples (bilirubin = 12–412 mg/L) and 30 hypertriglyceridemic samples (triglycerides = 2200–8430 mg/L) yielded respectively

\[
y = 0.91x - 0.05\]

\[r = 0.911\] and

\[
y = 0.910 - 0.02\]

\[r = 0.988\].

For comparison of the liquid-chromatographic method with the enzymatic assay and with the Fuller's earth procedure, we analyzed 52 serum samples with creatinine concentrations of 2–120 mg/L. As shown in Figures 5B and C, results of the HPLC method closely agreed with those of the enzymatic and of the colorimetric methods (intercepts not significantly different from zero, \[r = 0.986\] and \[r = 0.990\], respectively). HPLC of 42 urines from the routine laboratory of our Institute also showed good agreement (\[r = 0.974\]) with the Jaffe's method (Figure 5D) for these specimens, which are known to contain less creatinine-like Jaffe-positive chromogens than serum.

Comparison with IDMS. Four sera for which the creatinine content had been determined by IDMS (32) were measured with our HPLC procedure and with the enzymatic method, the Fuller's earth procedure, and the Hitachi 737. Table 2 summarizes the concentrations measured by IDMS and the percentage differences (means of three determinations) found with the test methods. The regression analysis of the values obtained with HPLC and IDMS gave an intercept of 0.03, a slope of 0.984, and \(r^2 = 0.999\).

### Discussion

The aim of this work was to develop a specific, simple, and inexpensive HPLC method for the determination of creatinine in serum and urine, to be eventually proposed as a reference method.

The reversed-phase technique, even though it may appear less specific, is generally considered more versatile than other methods. Our method uses acetone as deproteinizing agent, with a 1:4 volume ratio of sample:organic solvent. Deproteinization is complete and the supernate...
may be quickly evaporated in only a few minutes under a nitrogen stream and easily injected after reconstitution with the mobile phase.

The sensitivity of our method was always satisfactory, even for pediatric specimens, despite the reading of absorbance at a wavelength (236 nm) that is not the maximum absorbance.

The specificity of the method has been tested on most of the substances known to give Jaffé reaction interference. Conceivably other "weak bases," not tested here, could interfere with creatinine peak—a possibility that is, in our opinion, the most important limit of any HPLC assay. The purity of the endogenous creatinine peak, however, may be confirmed by the calculation of chromatographic signal-ratios at two different wavelengths, and by superimposition of recorded spectra.

The final chromatographic conditions adopted (pairing-ion type and concentration, methanol percentage, pH, etc.) were a compromise between analysis time, peak shape and symmetry, and resolution from any other interfering substances. Analysis length could obviously have been shortened by using a higher methanol concentration, or a higher flow-rate, but these modifications gave a loss of resolution and specificity. Because chromatographic methods serve potentially as "reference methods," and are not commonly used in large routine laboratories, we preferred to adopt longer analysis time rather than losing resolution and specificity.

The choice of cimetidine as internal standard obviously means the subject must not be receiving treatment with this drug. On the basis of the RT, none of the compounds studied for creatinine interference would interfere with the internal standard peak. From our data the use of an internal standard, which means a longer analysis time (17 min instead of 10), is not strictly necessary; in fact, the use of an automatic injector and a refrigerated sample tray results in very good precision with reasonably high throughput.

The shorter analysis time obtained by Soldin and Hill (22), who used an ion-paired chromatographic method with sodium lauryl sulfate, is compensated by the very long conditioning time (4 h) of the column, which is not necessary with our conditions. Other disadvantages of their method are the higher flow rate (2.3 mL/min instead of our 0.7 mL/min), greater column length (30 cm instead of 15), and analysis at 30 °C (we work at room temperature).

"Novel" analytical methods should be compared with a reference method; unfortunately, we could verify our HPLC procedure only with a limited number of samples previously assessed with IDMS, obtaining an acceptable concordance, the r-value being apparently higher than with the other techniques. Analysis of a larger number of samples with the picrate alkaline method usually used in our laboratory yielded, in agreement with the literature (18, 22, 27, 28), creatinine values a bit higher than by the HPLC method. Only the icteric sera showed statistically lower concordance between the two methods (r = 0.911), nevertheless confirming the same trend as above, i.e., lower HPLC values.

We were able to analyze 52 sera samples by HPLC, by an enzymatic method recognized to have very good accuracy (35), and by the reliable Fuller's earth procedure proposed.

Fig. 4. (A) Spectrum recorded at the upslope of creatinine peak in serum extract; (A') spectrum recorded at the upslope of creatinine peak in a standard solution; (B) superimposition of the two spectra reported in A and A'; (C) superimposition of the first derivatives of the two spectra reported in A and A'; (D) superimposition of the second derivatives of the two spectra A and A'.
Differences, 1.32 ± 0.008

Table 2. Creatinine Determinations in Four Sera
Previously Analyzed by IDMS

<table>
<thead>
<tr>
<th>Serum</th>
<th>Creatinine concn, mg/L</th>
<th>HPLC</th>
<th>PAP</th>
<th>Fuller</th>
<th>Hitachi</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.50 ± 0.030</td>
<td>-1.4</td>
<td>-2.3</td>
<td>-6.6</td>
<td>+4.8</td>
</tr>
<tr>
<td>2</td>
<td>1.50 ± 0.010</td>
<td>+3.3</td>
<td>+1.9</td>
<td>+3.3</td>
<td>+13.3</td>
</tr>
<tr>
<td>3</td>
<td>4.09 ± 0.050</td>
<td>-0.5</td>
<td>-7.8</td>
<td>-14.6</td>
<td>+7.6</td>
</tr>
<tr>
<td>4</td>
<td>1.32 ± 0.008</td>
<td>-2.3</td>
<td>-3.0</td>
<td>+6.0</td>
<td>+12.9</td>
</tr>
</tbody>
</table>

* IDMS values kindly provided by Prof. L. Siekmann, Bonn.

& Differences calculated from means of three determinations performed in the same day.

Fig. 5. Comparison between creatinine determinations with the HPLC and (A) Jaffé kinetic method (Hitachi 737) for 235 unselected sera from hospitalized patients; (B) enzymatic assay (creatinine-PAP) of 52 patients' sera with creatinine concentration 2–120 mg/dL; (C) Fuller's earth procedure with the same sera as in (B); (D) Jaffé kinetic method (Hitachi 737) for 43 unselected urines from our routine laboratory.

Cost per assay, currently about US $1.50 in our country (including labor but not the instrument amortization), even if obviously higher than for a routine technology, is quite reasonable.

We consider this method suitable for proposal as a possible reference method.

We thank Prof. C. Franzini and Drs. L. Prencipe and F. Ceriotti for their criticism. This work was partly supported by grants from the Consiglio Nazionale delle Ricerche, Roma, Italy.

References

by Haeckel et al. (33); for all comparisons, we obtained a good correlation.

For analysis of urine, we compared results only with the Jaffé kinetic method because of the lower concentration of "pseudocreatinine" interferences.

In our experience, when an HPLC technique is used as a reference method, good performance of the whole chromatographic apparatus is essential. This means always using an appropriate pre-column and replacing it whenever the peaks start to deteriorate. Moreover, overnight washing with water keeps the column from being clogged with salts and thereby increasing the backpressure.

Finally, because our method requires little sample handling for extract preparation and the chromatographic procedure, it can be also performed by not particularly well-trained personnel.