A simplified method for HPLC determination of creatinine in mouse serum

Yuen, Peter S. T., Stephen R. Dunn, Takehiko Miyaji, Hideo Yasuda, Kumar Sharma, and Robert A. Star. A simplified method for HPLC determination of creatinine in mouse serum. Am J Physiol Renal Physiol 286: F1116–F1119, 2004.—Mouse models are frequently used to study renal function. However, mouse serum contains chromagens that interfere with standard picric acid-based assays for serum creatinine. Several alternative methods exist for serum creatinine measurements, including assay by high-performance liquid chromatography (HPLC), but only one has been adapted to mouse serum. Creatinine was measured in serum by acetonitrile deproteinization, followed by isocratic, cation-exchange HPLC. The HPLC method was compared with a standard alkaline picrate colorimetric assay, using serum from animals with low-to-moderate renal injury. Acidification of acetonitrile with HCl in the deproteinization step produced variable results, including an extra peak that interfered with integration of the creatinine peak or loss of the creatinine peak. Deproteinizing with acetonitrile alone resulted in a more reliable measurement of serum creatinine, which was validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variation from 1.6 to 5.1%. The picric acid assay overestimated serum creatinine, which was validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variation from 1.6 to 5.1%. The picric acid assay overestimated serum creatinine, which was validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variation from 1.6 to 5.1%. The picric acid assay overestimated serum creatinine, which was validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variation from 1.6 to 5.1%. The picric acid assay overestimated serum creatinine, which was validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variation from 1.6 to 5.1%. The picric acid assay overestimated serum creatinine, which was validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variation from 1.6 to 5.1%. The picric acid assay overestimated serum creatinine, which was validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variation from 1.6 to 5.1%.

METHODS

All animal studies were performed in compliance with National Institutes of Health criteria for the care and use of laboratory animals in research. Mice were subjected to a cecal ligation puncture model of acute renal failure (7) and various treatments. At various times after surgery (or sham surgery), blood was collected from anesthetized animals via the abdominal aorta, and serum was stored at −80°C until assayed.

We initially used a HPLC creatinine assay based on the method of Johns et al. (4) with some modifications by Dunn et al. (1). HPLC mobile phase (5 mM sodium acetate, pH 5.1) was freshly made and filtered (0.2 μm) before use; premade creatinine standards (in 20 mM HCl) and creatinine powder were obtained from Sigma (St. Louis, MO). Creatinine powder was dissolved in HPLC mobile phase and used as the creatinine standard after confirmation of its concentration with premade creatinine standards, by HPLC assay. HPLC grade acetonitrile alone (0.5 ml) or acidified acetonitrile (0.5 ml + 20 μl of 20 mM HCl) was added to serum (5 μl), vortexed, and centrifuged for 15 min at 13,000 g in a microfuge at 4°C. In some cases, known amounts (10, 20, 30, 40, or 60 ng) of creatinine standard were added to the serum before precipitation. The supernatant fraction was completely transferred to a 2-ml HPLC autosampler vial, evaporated to dryness by SpeedVac (Thermo Savant, Holbrook, NY), and resuspended immediately in 100 μl of HPLC mobile phase (overnight storage of the dried samples resulted in variable results). Duplicate injections (25 μl each) were performed for each sample using an autosampler on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA), with a 100 × 4.1-mm PRP-X200 cation exchange column (Hamilton, Reno, NV). Isocratic HPLC was performed at a flow rate of 1 ml/min, and UV absorbance at 234 nm was monitored for 10 min after each injection. The creatinine peak eluted at ~4 to 4.5 min, and integration was performed with ChemStation software (Agilent). For some samples, the chromatogram contained a trough immediately preceding the creatinine peak, but the baseline was consistently flat between 6 and 10 min. Therefore, this baseline was used for integration, to define the creatinine peak and distinguish it from the trough. A standard curve of 10, 20, 30, 40, and 50 ng of pure creatinine was performed with each experiment, and the creatinine concentrations of the unknown samples were determined by linear regression analysis. Creatinine was also measured with a standard picric acid-based colorimetric assay (Astra 8 autoanalyzer; Beckman Instruments, Fullerton, CA). In experiments where known amounts of creatinine were added to mouse serum, the expected value for creatinine concentration was the sum of the known added creatinine concentration and the measured value of creatinine concentration without any additions.

The concentration of creatinine in serum is routinely used as a simple surrogate indicator for the glomerular filtration rate in patients and in animal models (5). Creatinine in serum is most commonly measured via a colorimetric reaction (Jaffé method) with picric acid that produces a derivative absorbing at 485–520 nm (3). In 1985, Meyer et al. (6) reported that chromagens in mouse serum result in a fivefold overestimation of serum creatinine in the picric acid assay and that creatinine could be more accurately measured by high-performance liquid chromatography (HPLC). Despite the popularity of mouse models to study renal function, the HPLC assay is not commonly used. Dunn et al. (1) recently developed an improved HPLC assay that is capable of reliably measuring low levels of creatinine in 25 μl of mouse plasma and correlated their HPLC creatinine clearances with inulin-based clearances. We describe a simplified HPLC method, demonstrate its accuracy, and illustrate why it should be widely adopted for mouse serum creatinine measurements.
RESULTS

We attempted to measure creatinine in mouse serum by a HPLC procedure, essentially using the method developed by Johns et al. (4) with some modifications from Dunn et al. (1). However, under those conditions, we had difficulty identifying creatinine in normal mouse serum (Fig. 1B) because of an extra peak that preceded the peak corresponding to pure creatinine (Fig. 1A). When we eliminated the hydrochloric acid from the acetonitrile precipitation step, the extra peak disappeared (Fig. 1D). The identity of the correct peak was validated by adding creatinine standard to the serum before precipitation (Fig. 1, C and E). In some studies, we found that the inclusion of HCl in the deproteinization step could result in a complete loss of the creatinine peak. Therefore, all subsequent HPLC assays were performed with only acetonitrile in the deproteinization step.

We tested the reproducibility of our assay in serum from six mice (n = 5 for each mouse) with average serum creatinine values of 0.164 to 0.942 mg/dl, and the coefficient of variation ranged from 1.64 to 5.06% (Table 1). To validate the accuracy of the HPLC assay, we added known amounts of creatinine (2, 4, 6, 8, or 12 ng/μl serum) to serum from three mice with normal, moderate, or high serum creatinine values. Over the entire range, there was a linear relationship between measured values and expected values (r = 0.997), with an average recovery of 101.1% (Fig. 2).

Finally, we compared serum creatinine values from our HPLC assay with values from the standard picric acid assay. To examine a broad range of serum creatinine values, sera were collected from normal mice and mice subjected to cecal ligation puncture (7). The picric acid assay overestimated...
serum creatinine in almost all cases (Fig. 3A). The banding pattern seen in the plot reflects the lower number of significant digits in the autoanalyzer readout. The degree and range of overestimation by the picric acid assay were more pronounced at lower HPLC creatinine values (<0.5 mg/dl; Fig. 3B), but the picric acid assay significantly overestimated creatinine values by an average of 23%, compared with the HPLC assay, even at creatinine values over 0.5 mg/dl ($n = 57$, $P < 0.001$). In our determinations, sham-treated, 10-mo-old C57BL6 mice have an average serum creatinine value of 0.207 ± 0.012 (SE) mg/dl ($n = 13$).

**DISCUSSION**

The long-standing problem of noncreatine chromagen interference in the picric acid-based serum/plasma creatinine assays has been addressed over the decades with a number of alternative approaches, including HPLC- and enzymatic-based assays. Despite the scores of publications describing methods to measure creatinine more accurately in human samples (2), only one has specifically addressed the inadequacy of the picric acid assay in mouse serum. In this paper, Meyer et al. (6) report a 5- to 8.5-fold overestimation of mouse serum creatinine by the picric acid assay, in contrast to the 20–50% overestimation of human serum creatinine by the picric acid assay.

A number of methods have been developed to measure creatinine in serum by HPLC that use either a cation exchange or a reverse-phase column for separation and UV for detection (2). When one works with serum, a prechromatographic cleanup step is necessary to prolong the life of the HPLC column. Our method, and that of Dunn et al. (1), is based on the method of Johns et al. (4). The three methods are compared in detail in Table 2. Most of the differences between our method and the Dunn et al. method are minor, and we believe that the underlying principles of these methods are robust enough to allow for modifications to customize and optimize the analysis for any HPLC system. The major differences between our method and the Dunn et al. method (1) are a smaller sample volume, fewer pipetting steps, and the omission of acid in the acetonitrile precipitation step. Based on our data that HCl could cause an artifact peak, Dunn et al. successfully substituted acetic acid for HCl, whereas we found that the acidification of the acetonitrile was not necessary to deproteinize the serum sample (data not shown). We speculate that after precipitation, the initially low concentration of HCl becomes high enough during evaporation to alter an acetonitrile-soluble component of mouse serum, so that it absorbs at 234 nm. Precipitation without HCl allowed collection of minimal volumes of blood to monitor multiple time points in a single mouse, without complications of significant blood loss.

We demonstrated a HPLC method that is simple, reproducible, accurate, and sensitive. We also clearly showed that the picric acid assay can also overestimate serum creatinine up to sixfold over the HPLC assay. The degree of overestimation, as well as the variability in the overestimation, is especially high when HPLC-derived serum creatinine values are low (<0.5 mg/dl). This finding is significant, not only for determining normal or baseline values, but also for determining the effectiveness of treatments for impaired renal function. Because the picric acid assay overestimates serum creatinine, it can result in underestimation of drug/treatment effectiveness. Therefore, HPLC determination of serum creatinine provides a more accurate assessment of whether a treatment can restore renal function.

![Fig. 3. Overestimation of serum creatinine values by the picric acid-based Jaffe assay. Serum samples ($n = 256$) were collected from mice with different stages of cecal ligation puncture-mediated kidney injury and/or drug treatment to compare the assay methods over a broad range of creatinine values. A: direct comparison between picric acid values and HPLC values demonstrates overestimation of serum creatinine by picric acid assay; line of equivalency is shown. B: degree of overestimation, represented by the ratio of picric acid serum [creatinine]/HPLC serum [creatinine], is shown as a function of HPLC serum [creatinine]. Brackets indicate concentration.](https://ajprenal.physiology.org/10.1152/ajprenal.00352.2003)
### Table 2. Differences between HPLC creatinine methods

<table>
<thead>
<tr>
<th>Present Study</th>
<th>Ref. 1</th>
<th>Ref. 4</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume of sample</strong></td>
<td>5 μl Mouse serum</td>
<td>25 μl Mouse plasma</td>
<td>50 μl Monkey serum</td>
</tr>
<tr>
<td><strong>Precipitant</strong></td>
<td>500 μl Acetonitrile</td>
<td>100 μl Acetonitrile</td>
<td>500 μl Acetonitrile</td>
</tr>
<tr>
<td><strong>Transfer of supernatant fluid</strong></td>
<td>Directly to autosampler vial</td>
<td>To microfuge tube</td>
<td>10 μl 20 mM HCl HPLC</td>
</tr>
<tr>
<td><strong>Evaporation</strong></td>
<td>Speedvac</td>
<td>Speedvac</td>
<td>Stream of N₂ gas</td>
</tr>
<tr>
<td><strong>Resuspension</strong></td>
<td>120 μl Mobile phase</td>
<td>25 μl Mobile phase</td>
<td>300 μl mobile phase</td>
</tr>
<tr>
<td><strong>Autosampler vial</strong></td>
<td>Standard, flat-bottom</td>
<td>Low dead volume</td>
<td>Standard, flat-bottom</td>
</tr>
<tr>
<td><strong>Volume injected, corresponding plasma/serum volume</strong></td>
<td>25 μl (1.04 μl)</td>
<td>3.0 μl (3.0 μl)</td>
<td>50 μl (8.33 μl)</td>
</tr>
<tr>
<td><strong>HPLC system</strong></td>
<td>Agilent 1100</td>
<td>PerkinElmer Auto</td>
<td>Waters 2690</td>
</tr>
<tr>
<td><strong>HPLC SCX column length × width</strong></td>
<td>100 × 4.1 mm</td>
<td>Agilent Zorbax SCX</td>
<td>SynchroPak CS103-10</td>
</tr>
<tr>
<td><strong>(chemistry)</strong></td>
<td>(polymer-based)</td>
<td>50 × 2.1 mm</td>
<td>150 × 4.6 mm (silica-based)</td>
</tr>
<tr>
<td><strong>HPLC column oven temperature</strong></td>
<td>Room temperature</td>
<td>Room temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td><strong>Mobile phase</strong></td>
<td>5 mM Sodium acetate</td>
<td>4.76 mM Sodium acetate</td>
<td>5 mM Lithium acetate</td>
</tr>
<tr>
<td><strong>(pH 5.1)</strong></td>
<td>pH 4.2</td>
<td>pH 4.9</td>
<td>pH 4.9</td>
</tr>
<tr>
<td><strong>UV detection</strong></td>
<td>234 nm</td>
<td>234 nm</td>
<td>234 nm</td>
</tr>
</tbody>
</table>

HPLC, high-performance liquid chromatography; GFR, glomerular filtration rate.

**ACKNOWLEDGMENTS**

Present address of T. Miyaji: First Department of Medicine, Hamamatsu University, 1-20-1 Handayama, Hamamatsu 431-3192, Japan.

**GRANTS**

Funding for this work was partially provided by Grant DK-60995 from the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases “Mouse Models for Human Diabetic Nephropathy.”

**REFERENCES**