A Simplified Method for HPLC Determination of Creatinine in Mouse Serum

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ABSTRACT

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 to 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 an extra peak that interfered with integration of the creatinine peak. Deproteinizing with acetonitrile alone resulted in a more accurate measurement of serum creatinine, as validated by a series of known additions of creatinine standard. The HPLC assay was reproducible with coefficients of variations from 1.6 to 5.1%. The picric acid assay overestimated serum creatinine, when directly compared with the HPLC assay. The extent of overestimation, up to 6-fold, was greatest at normal (0.1 to 0.2 mg/dl) to moderately elevated (0.5 mg/dl) serum creatinine levels. Mouse serum contains substances that interfere with standard picric acid assays for creatinine. Our new HPLC assay can accurately detect creatinine from 5 microliters of mouse serum. These results support the widespread adoption of HPLC to accurately measure serum creatinine in mouse models of renal injury.
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 [1]. 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[2]. In 1985, Meyer et al. [3] reported that chromagens in mouse serum result in a 5-fold 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. [4] have recently developed an improved HPLC assay that is capable of reliably measuring low levels of creatinine in mouse plasma and correlated their HPLC creatinine clearance 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.
METHODS

All animal studies were performed in compliance with NIH criteria for the care and use of laboratory animals in research. Mice were subjected to a cecal ligature puncture model of acute renal failure [5] and various treatments. At various times after surgery (or sham surgery) blood was collected from anesthetized animals via the abdominal aorta.

The HPLC creatinine assay we initially used was based on the method of Johns et al. [6] and Dunn et al. [4]. 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 x g in a microfuge. In some cases, known amounts of creatinine were added to the serum before precipitation. The supernatant fraction was completely transferred to a 2 ml HPLC autosampler vial, dried either by SpeedVac (Thermo Savant, Holbrook, NY) or a stream of nitrogen and resuspended in 120 µl of the filtered (0.2µm) HPLC mobile phase (5 mM sodium acetate pH 5.1), which was filtered (0.2µ). 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 x 4.1 mm PRP-X200 cation exchange column (Hamilton, Reno NV). Isocratic HPLC was performed at a flow rate of 1 ml/min, and the creatinine peak was detected by UV absorbance at 234 nm. Creatinine was also measured with a standard picric acid-based colorimetric assay (Astra 8 autoanalyzer; Beckman Instruments, Fullerton, CA). Creatinine standards and creatinine powder were obtained from Sigma (St. Louis, MO).
RESULTS

We attempted to measure creatinine in mouse serum by a HPLC procedure, essentially using the method developed by Johns et al. [6] with some suggestions from Dunn et al. [4]. 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 1C, E).

We tested the reproducibility of our assay in serum from 6 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 (2-6 ng/µl serum) to serum. We found that the relationship between added creatinine and total creatinine was linear ($r^2 = 0.996$, Fig 2). Indirect measurement of serum creatinine by extrapolation (0.126 mg/dl) corresponded to the directly measured value (0.122 ± 0.002 mg/dl). In contrast, precipitation with acetonitrile/HCl resulted in a substantial discrepancy between the indirect/extrapolated value (0.140 mg/dl) and the directly measured value (0.197 ± 0.005 mg/dl; 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 [5]. 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 was more pronounced at lower HPLC creatinine values (<0.5 mg/dl, Fig. 3B), but the picric acid assay agreed with the HPLC assay at higher creatinine values (n = 8, p = 0.87). In our determinations, sham-treated, 10 month 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 non-creatinine 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 [7], only one has specifically addressed the inadequacy of the picric acid assay in mouse serum. In this paper, Meyer et al. 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 [3].

A number of methods have been developed to measure creatinine in serum by HPLC. Typically they used either a cation exchange or a reverse phase column for separation and UV for detection[7]. When working with serum, a prechromatographic cleanup step is necessary to prolong the life of the HPLC column. We used the method of Johns et al [6] as a starting point and 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. The acidified acetonitrile also caused a discrepancy between the directly measured value for serum creatinine and the indirect, extrapolated value from a series of added creatinine standard (Fig. 2). This discrepancy was virtually eliminated if HCl was omitted (Fig 2). Precipitation without HCl allowed us to routine detection of creatinine from 5 µl of mouse serum, with excellent reproducibility (Table 1) and accuracy (Fig. 2). The small sample volume allows collection of minimal volumes of
blood to monitor multiple time points in a single mouse, without complications of significant blood loss.

We have demonstrated a HPLC method that is simple, reproducible, accurate and sensitive. We have also clearly shown that the picric acid assay can also overestimate serum creatinine up to six-fold 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.

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REFERENCES


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n=5 for each mouse
FIGURE LEGENDS

Fig. 1. Serum creatinine detection is affected by precipitation with acidified acetonitrile. Representative chromatograms illustrating isocratic HPLC of creatinine standard (A), or normal mouse serum after precipitation with acidified acetonitrile (B, C) or acetonitrile (D, E). Creatinine standard was added to serum before precipitation (C, E) to validate creatinine peaks. Absorbance was measured at 234 nm. Arrows mark the location of the creatinine peak.

Fig 2. Validation of HPLC assay for serum creatinine by addition of creatinine standard. Increasing amounts of creatinine standard were added to normal mouse serum and precipitated by acetonitrile (circles, ACN) or acidified acetonitrile (squares, ACN/HCl). Duplicate precipitations and duplicate injections per precipitation were performed for each condition. Extrapolated values were calculated by linear regression analysis; $r^2 = 0.999$ (acetonitrile) and 0.996 (acidified acetonitrile).

Fig 3. Overestimation of serum creatinine values by the picric acid-based Jaffé assay. Serum samples ($n = 141$) were collected from mice with different stages of CLP-mediated kidney injury and/or drug treatment, in order to compare the assay methods over a broad range of creatinine values. Direct comparison (A) between picric acid values and HPLC values demonstrates overestimation of serum creatinine by picric acid assay; line of equivalency is shown. (B) The degree of overestimation, represented by
the ratio of picric acid serum [creatinine]/HPLC serum [creatinine], is shown as a function of HPLC serum [creatinine].
Fig 1

A standard

B serum (HCl/ACN ppt.)

C serum + standard (HCl/ACN ppt.)

D serum (ACN ppt.)

E serum + standard (ACN ppt.)

absorbance units (arbitrary scales)

1 min
Fig 2

\[ y = 0.0352x + 0.1222 \]
\[ R^2 = 0.999 \]

\[ y = 0.0192x + 0.1404 \]
\[ R^2 = 0.9956 \]

[creatinine] (mg/dl) vs added creatinine (ng/5 µl serum)
Fig 3

A

HPLC serum creatinine (mg/dl)

picric acid serum creatinine (mg/dl)

B

HPLC serum creatinine (mg/dl)

picric acid/HPLC ratio