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Short communication

Simple high-performance liquid chromatographic method to analyze serum creatinine has several advantages over the Jaffé picric acid reaction as demonstrated with a cimetidine dose response in rhesus monkeys

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Abstract

A simple method for creatinine determination was developed using high-performance liquid chromatography (HPLC) to more accurately monitor serum creatinine levels in experimental animal models when compared to the Jaffé method. The new HPLC procedure will replace the traditional Jaffé method for rhesus monkey kidney function studies. We developed an isocratic method using a polymeric, hydrophilic, silica-based strong cation-exchange bed with a 5.0 mmol/l lithium acetate matrix, pH 4.9, which isolates creatinine with no detectable impurities as determined by three-dimensional ultraviolet–visible spectral analysis. Sample preparation includes deproteination with acetonitrile, evaporation, and resolubilization in mobile phase followed by quantitation with UV detection at 234 nm. Extraction efficiency across the measured range was 96±2%. From numerous extracted rhesus monkey creatinine curves (n=38) a slope of 251 100±756 (95% CI) and an intercept of 675.6±712.7 (95% CI) was calculated. Extraction efficiency and peak purity tests with human plasma were cross-compared with rhesus monkey serum producing equivalent results. An average of 120 samples can be run daily.

Keywords: Creatinine; Jaffé picric acid; Cimetidine

1. Introduction

The most widely recognized clinical method for creatinine determination is the Jaffé picric acid reaction. Use of the Jaffé method [1] is an issue of continued debate. Alkaline picrate reacts with creatinine to form a chromogen which absorbs light between 490 and 520 nm. Alkaline picrate also reacts with other substances including glucose, proteins, and acetoacetate to form chromophores with similar absorbance profiles to the picrate–creatinine reaction. This lack of specificity [2,3] has been repeatedly addressed by concerned clinicians and scientists without indisputable resolution for the known inaccuracies of the picric acid reaction. The picric acid reaction is also limited by its sensitivity to variations in conditions; alterations in temperature,

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pH, hardware, and concentrations of sodium hydroxide and picric acid including differences between lots, preparations and suppliers, all of which have the potential to influence results. Variation among picric acid methods has been evaluated for accuracy [4], demonstrating a need for standardization and proficiency testing for clinical laboratories. In recent years viable alternatives to the picric acid reaction have been developed [5] to measure creatinine but none have gained the same widespread acceptance of the Jaffé method.

In parallel with chemical determinations of serum creatinine concentration, more specific methods using high-performance liquid chromatography (HPLC) have been developed to isolate and quantitate creatinine [6–9]. Included among the HPLC methods are methods that have been developed for species specific models such as rat and human. Still, others look to simultaneously distinguish and quantitate multiple analytes along with creatinine.

Our objective was to develop a fast, simple method to more accurately measure creatinine concentration in rhesus monkey serum to replace the Jaffé method presently used. We also required enhanced sensitivity to measure subtle changes in serum creatinine ($\pm$0.01 mg/l) to study effects on renal tubular secretion of creatinine. The isocratic ion-exchange method we developed was an adaptation of a step gradient first described by Ambrose et al. [10]. Our method removes protein from the serum or plasma sample using acetonitrile. At a pH of 4.9 (near the pK_a, 5.02, of creatinine) sufficient isolation of creatinine was achieved to eliminate all interfering molecules while achieving our sensitivity goals at 234 nm.

2. Experimental

2.1. Materials and reagents

2.1.1. Extraction reagents for HPLC creatinine

The extraction required HPLC-grade acetonitrile (ACN) and 20 mmol/l hydrochloric acid (HCl) prepared from 1 *M* HCl certified stock, both from Fisher Scientific (Fair Lawn, NJ, USA).

2.1.2. Creatinine standards

Creatinine standards purchased from Sigma (St. Louis, Mo, USA), 925-11, were serially diluted using 0.025 mmol/l HCl.

2.1.3. Ion-exchange chromatography for creatinine

The column properties included a silica-based strong cation-exchange resin, 300 Å, 5 μm, 150×4.6 mm, CS103-10 (SynChropak; Eichrom Industries, Darien, IL, USA).

2.1.4. Chromatography buffer

The buffer contained 5.0 mmol/l lithium acetate (LiAc) (Sigma–Aldrich, Milwaukee, WI, USA), pH 4.9, vacuum degassed and was adjusted using 10% glacial acetic acid (Fisher).

2.2. Instruments

A Waters 2690 separations module with an integrated auto-injector and a Waters 996 photodiode array detector (Waters, Milford, MA, USA); a Multivap Analytical evaporator (Organomation, South Berlin, MA, USA); and a Jouan GR-422 centrifuge (Winchester, VA USA) were used. HPLC methods were managed using Waters Millenium32 software, vs. 3.05.

2.3. Precipitation of protein from serum and isolation of creatinine by cation-exchange chromatography

Samples were stored at −70°C, processed at 4°C and centrifuged at 2500 *g* for 10 min in all procedures unless otherwise stated. Thawed samples were centrifuged to remove any solids before beginning assay. Serum (50 μl) was injected into 500 μl ACN containing 10 μl of 20 mmol/l HCl. Control (untreated) serum spiked with creatinine standard was injected into acetonitrile. After vigorous mixing denatured samples were centrifuged. The supernatant (450 μl) was transferred to a fresh tube and evaporated under a steady stream of nitrogen at 40–45°C. Samples and standards were resolubilized in 300 μl of mobile phase. The column was preequilibrated

with degassed mobile phase at 0.6 ml/min, 25°C. Injections (50 μl) were analyzed for 7 min at a scan rate of 1.0 scan/s between 210 and 300 nm with a 3.6 nm resolution. A spectrum was extracted at 234 nm for peak area analysis.

2.4. Measurement of creatinine concentration using the Jaffé method

Samples were analyzed using a modification of the Jaffé method described by Schiapparelli Biosystems [11]. The assay was automated using a blood chemistry analyzer (ACE) which performs a colorimetric determination on creatinine using the following calculation: creatinine (mg/l)= {[(AU505nmT2 − AU573nmT2) − (AU505nmT1 − AU573nmT1)]−[(AERB505nmT2−AERB573nmT2) −(AERB505nmT1−AERB573nmT1)} × calibration factor. Where: AU=absorbance of unknown at specified wavelength; AERB=absorbance of external reagent blank at specified wavelength; T1=first reading taken at specified time; T2=second reading at specified time. For this method the within-run precision relative standard deviations (RSDs) at 1.3 and 1.9 mg/l were 2.7 and 2.9%, respectively. The total assay precisions were found to be 4.4 and 3.6% RSD at concentrations of 1.3 and 1.9 mg/l, respectively.

2.5. Cimetidine dosing of rhesus monkeys and serum collection

Male and female rhesus monkeys, weighing 4.3 to 10.4 kg were fasted overnight and placed in a primate restraint chair for the duration of the experiment. Cimetidine was administered through a peripherial vein with a bolus injection (5 ml) followed by an infusion (0.1 ml/min) for 240 min. Saline served as the vehicle for cimetidine administration except where polyethylene glycol (PEG)–saline (20:80) was used for solubility reasons. Blood samples for serum were collected from peripherial veins at predetermined times then transferred to a Vacutainer Gel tube for a 60 min incubation at room temperature before separation and storage.

3. Results and discussion

3.1. Deproteination and extraction efficiency for creatinine

Other extraction methods have been used to compliment creatinine isolation by HPLC including ultra-filtration [7], treatment with trichloroacetic acid [10] and various organic solvents [8,12]. We used acetonitrile for its efficiency in denaturing protein, high solubility characteristics for creatinine and volatility for rapid vaporization.

Across our measured range the average creatinine yield after deproteination for each of five standards was 96±2% (n=38). The extraction efficiency was determined by the following equation: % yield= {[(TPA−CSA)/USA]×100}. Where TPA=total peak area; CSA=control serum area; and USA= unextracted standard area. The consistency and high yield is in line with the fact that creatinine does not bind to serum albumin.

3.2. Specificity for creatinine

Because of endogenous creatinine in control serum the determination of spectral homogeneity was necessary. Spectral homogeneity for creatinine was determined by three-dimensional spectral analysis of the column eluate. Potential peak contamination was detected by analysis of solvent angles and noise angles between spectra generated from each data point in the integrated peak and the peak apex spectrum. Subsets of untreated rhesus sera from the colony used for kidney function studies were tested individually to determine variation between animals. We found no variation in the spectral purity of creatinine isolates among all animals tested (n=7). Stocks of three different male human plasma preparations were also evaluated for interference using our protocol. All three human stocks produced results equivalent to rhesus serum. Beyond potential endogenous coeluents, the potential for infused compounds to coelute was addressed first by comparing retention times of cimetidine or other compounds with creatinine followed by peak purity analysis to double check for potential metabolite coelution.

Resolution after repeat injections was also ad-

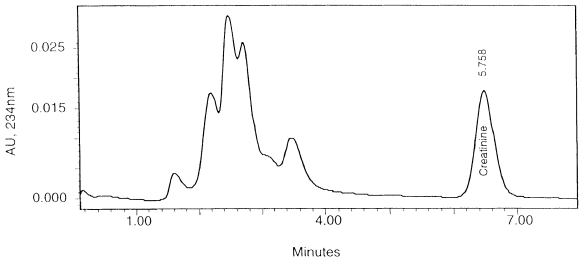


Fig. 1. Chromatogram after deproteination of 0.05 ml rhesus monkey serum containing 0.5 mg/l creatinine.

dressed to determine the efficiency and robustness of the column. Two columns (serial Nos. 277230 and 11616411) were compared. Each column maintained resolution after 1000 injections (Fig. 1). Periodic washing with 1 *M* NaCl in mobile phase helped maintain column pressure.

3.3. Calibration and sensitivity for creatinine

One advantage to the step gradient isolation of creatinine by Ambrose et al. [10] is that creatinine elutes at pH 7.1 predominately in its uncharged form. Creatinine has the greatest molar absorptivity in its uncharged form with a molar absorptivity of 6471 l cm^{-1} mol^{-1} at pH 7.1 and $\lambda_{max}=234$ nm. While the step gradient offers the greatest absolute absorptivity for creatinine, this level of sensitivity is not necessary for physiological measurements. Importantly, the step gradient method is time consuming compared to our method. Linearity has been demonstrated between 0.13 and 4.00 mg/l with routine curves generated between 0.13 and 2.00 mg/l with the following linear relationship $y=251,100x+676$, correlation coefficient=0.9990.

3.4. Precision for creatinine

Creatinine standards prepared from a 10.00 mg/l stock solution, serially diluted to 5.00, 2.50, 1.25 and 0.63 mg/l were evaluated over a 120-day period for precision. Precision was monitored using the data from experimental standard curves. The total assay precision RSD was calculated from peak areas after subtracting the background concentration of the serum blank (Table 1). Within-day variation was calculated from the differences in areas between duplicate concentrations (Table 1) using independently processed samples without control subtraction so that basal creatinine variability could be included with the spiked standards.

We did however observe a slight trend toward higher variation in RSD with decreasing creatinine concentration for total assay precision below 0.5 mg/l. The variability in total assay precision at concentrations below 0.5 mg/l does not correlate with extraction efficiency variability. Variation in basal creatinine levels between control lots of serum has been seen to have the greatest effect on the variability of total assay precision. Use of control serum with a fixed creatinine concentration would reduce variability across all concentrations therefore, an internal standard was considered. Since our method had comparable total assay precision to the Jaffé method in the normal range of rhesus serum creatinine (0.6 to 1.2 mg/l) and the extraction efficiency across the measured range was consistent we elected not to incorporate an internal standard.

3.5. Comparison with Jaffé method

To assess the value of this HPLC method of

Table 1
Between-day and within-day variations in determination of creatinine in rhesus monkey serum

SD (mg/l)	Mean peak area[a]	Total assay precision RSD (%) $n=38$	Within-day[b] precision RSD (%), $n=15$
0 (control)	–	–	1.6
0.13	34,117	11.9	0.74
0.25	64,401	7.4	0.88
0.50	123,722	5.9	1.1
1.00	251,440	3.8	1.2
2.00	503,501	2.7	1.7

[a] Mean peak area calculated using the equation: average (spiked peak area−control peak area).
[b] Within-day 95% RSD calculated from spiked peak areas and control serum areas directly.

creatinine determination, a study in rhesus monkeys was conducted to evaluate the effect of blocking tubular secretion of creatinine on serum creatinine levels with increasing doses of cimetidine. In this study, five groups of monkeys ($n=2$) were given either a bolus of cimetidine, 10 mg/kg, or vehicle (matching volume) followed by an intravenous (i.v.) infusion (vehicle or 0.1, 0.2, 0.3, 0.4, 0.5 mg/kg/min cimetidine) for the subsequent 240 min. Base–acid extractions of serum cimetidine samples using ethyl acetate were performed using a laboratory method. Chromatographic analysis of cimetidine was performed using C_{18} hydrophobic interaction chromatography. Our analysis of cimetidine levels show that further serum elevation beyond the spike of the initial bolus occurs with infusions $\geq$0.4 mg/kg/min with steady state levels being initially achieved at 0.3 mg/kg/min (Fig. 2). The relationship between serum cimetidine and serum creatinine does not directly correlate but rather creatinine lags cimetidine in both accumulation and elimination. Time is required for creatinine to accumulate after cimetidine blockade of the renal creatinine transporter.

Also important to us is the ability to discern between similar infusion rates so that we can accurately titrate monkeys for creatinine transport blocking. Even with low numbers of monkeys, differential effects of cimetidine on serum creatinine begin to be revealed among infusions (Fig. 3a). The results of the same samples analyzed by the Jaffé method

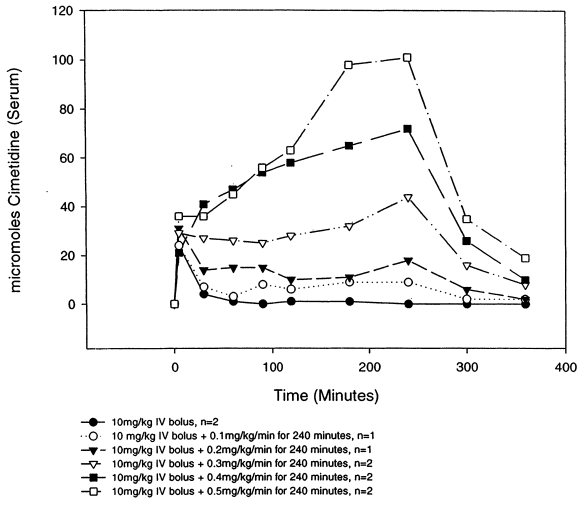
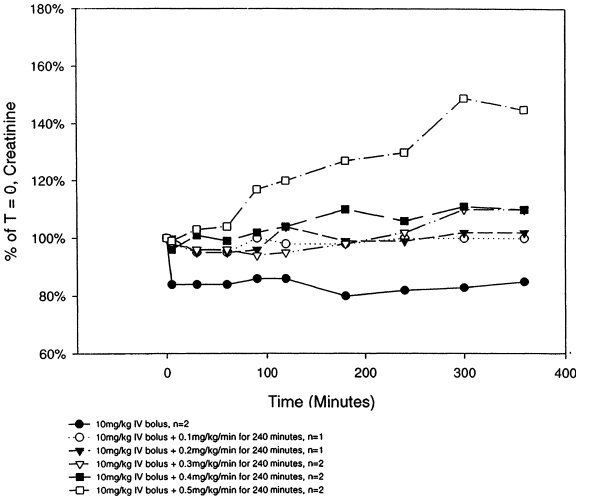


Fig. 2. Serum cimetidine levels with increasing cimetidine infusion rates.



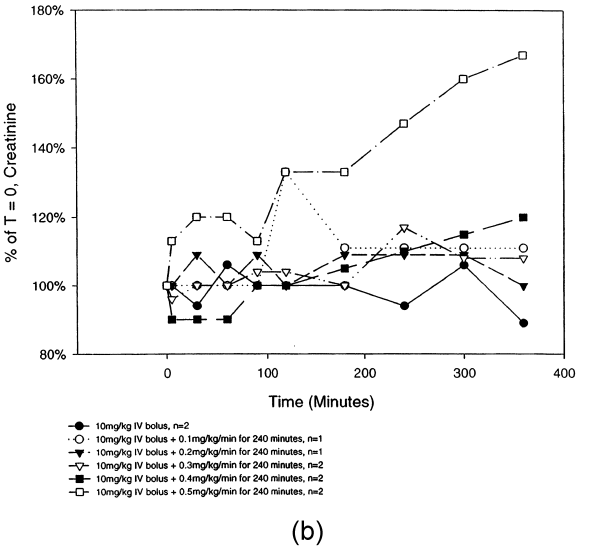


Fig. 3. (a) Serum creatinine shown as a % of predose creatinine levels ($T=0$) as analyzed by HPLC. (b) Serum creatinine shown as a % of predose creatinine levels ($T=0$) as analyzed by the Jaffé method.

shown in Fig. 3b are not discernible except at the highest infusion rate.

To examine the variations between the two methods that cannot be explained by the differences in precision, serum samples from control animals dosed with an i.v. bolus infusion of vehicle were also evaluated (Fig. 4). The Jaffé method regularly generated values 30 to 50% greater than the corre-

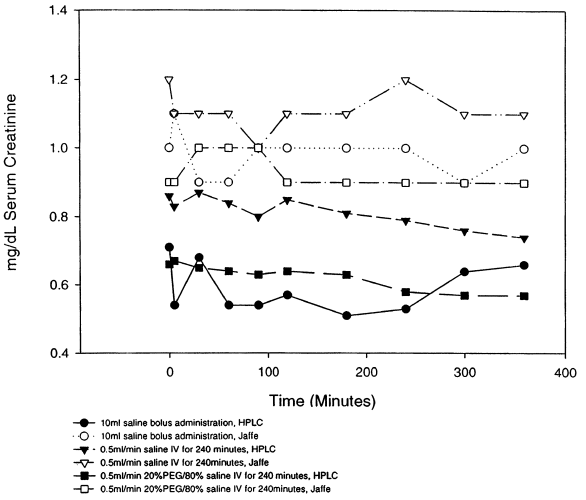


Fig. 4. Creatinine levels following vehicle infusion and/or bolus administration using the described HPLC and Jaffé methods.

sponding HPLC result. Interfering chromophores have been commonly blamed for the consistently high absolute creatinine values of the Jaffé method [3,4]. We believe that the difference between the absolute values of the HPLC and Jaffé can be attributed mainly to interfering chromophores with the Jaffé picric acid reaction (Fig. 4).

4. Conclusion

We have met our objective to develop a simple method of creatinine determination which eliminates the interference associated with colorimetric or enzymatic methods. We conclude that the Jaffé method for creatinine determination is not suitable for our renal studies and a proposed method has been offered which improves the accuracy and offers similar precision. This HPLC method is easily scalable to the simplest form of isocratic HPLC with a single channel UV detector. The excellent reproducibility of the assay results over time and between columns offers the potential for good laboratory practice validation with single- or dual-point calibration.

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