Evaluating an alternative method for rapid urinary creatinine determination

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Abbreviations CR, creatinine; CRc, creatinine concentration; CV, Coefficient of Variation; DI, Deionized; KJ, Kinetic Jaffé; LC-TOF/MS, Liquid Chromatography Time-of-Flight Mass Spectrometry; ln, Natural Log; OD, Optical Density; PJ, Plateau Jaffé; MS, Mass Spectrometry; WHO, World Health Organization

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Abstract

Creatinine (CR) is an endogenously-produced chemical that is routinely assayed in urine specimens to assess kidney function and sample dilution. The industry-standard method for CR determination, known as the Kinetic Jaffé (KJ) method, relies on an exponential rate of a colorimetric change, and can therefore require automated processing equipment for moderate- to high-throughput analysis (hundreds to thousands of samples per day). This study evaluates an alternative colorimetric method, the "Plateau Jaffé" (PJ) method, which utilizes the chemistry of the KJ method, a commercially-available kit, and a multi-point calibration curve. This method is amenable to moderate-throughput sample analysis and does not require automated processing equipment. Thirty-two spot urine samples from healthy adult volunteers were analyzed for creatinine concentration (CRc) using the KJ and PJ methods. Samples were also analyzed using a liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) method, which acted as an analytical control. Replicate measurements of spot samples (natural log-transformed values) were used to estimate method precision, and linear regression models were used to evaluate method accuracy (LC-TOF/MS measurements were considered the analytical benchmark). Measurement precision was comparable across all three methods, with coefficent of variation estimates ranging from 3 to 6%. Regression models generally showed good agreement across methods with R^2 estimates ranging from 0.996 to 0.998, slope estimates ranging from 0.944 to 0.986, and y-intercept estimates ranging from 0.111 to 0.303. Minor bias (between 2 and 16%) was observed across methods at the tails of the measurement distributions. The provided regression equations can be used to adjust for this bias and improve CR measurement comparisons across studies employing different methods. Considering these results, the PJ method is a suitable alternative to the industry standard KJ method for urinary

CRc determination. It can be implemented for moderate-throughput sample analysis using modest and commonly-available lab instrumentation, and manual sample preparation techniques.

1.0 Introduction

Creatinine (CR) is an endogenous compound that is released from skeletal muscle, filtered in the kidney through the glomerulus, and eliminated in urine. For a healthy individual, the excretion of CR from the body is thought to occur at a fairly constant rate (mass/time) (Narayanan and Appleton 1980; Boeniger et al. 1993; Gaines et al. 2010). Creatinine is generally measured in spot or pooled urine samples and reported in units of concentration (mass/volume). These creatinine concentration (CRc) levels are a function of both CR excretion rate and urine output (volume/time). Since urine output differs between individuals, and within individuals over time (Fortin et al. 2008), urinary CRc levels are variable. According to the World Health Organization (WHO), valid urine specimens from adults are expected to have CRc levels in the range of 30 to 300 mg/dl (WHO 1996).

Urinary CRc measurements are widely used in drug testing, medicine, and environmental exposure and health research. In drug testing, CRc levels are evaluated to help determine urine sample reliability. Here, an exceeding low CRc level may suggest that a sample has been intentionally diluted (Lafolie et al. 1991; Cook et al. 2000; Barbanel et al. 2002; Levy et al. 2007). In medicine, urinary CRc is assessed during routine evaluations to evaluate kidney function (Narayanan and Appleton 1980; Jafar et al. 2005). For this application, urinary CRc levels are used with blood-based CR measurements to estimate glomerular filtration rate (GFR). In environmental exposure and health research, CRc is used to "normalize" urinary levels of other analytes – this adjustment is performed most frequently on urinary metabolites of exogenous chemicals. Here, concentrations of chemical biomarkers (reflecting either exposures (Koch et al. 2005; Baker et al. 2005; Naeher et al. 2010) or biological responses (Bales et al. 1984; Price et al. 2005; Emond et al. 2013)) vary with changing urine output. Dividing chemical

biomarker concentration by CRc (yielding a creatinine-adjusted concentration) might remove some variation produced by increased or decreased urine output (Barber and Wallis 1986; Boeniger et al. 1993).

Robust analytical methods exist for measuring CRc with accuracy and precision (Pino et al. 1965; Jaynes et al. 1982; Toffaletti et al. 1983; Kochansky and Strein 2000). However, *specific* methods often utilize mass spectrometry (MS) (Patel and George 1981; Stokes and O'Connor 2003; Park et al. 2008), which can be cost prohibitive for small labs, and not conducive to high-throughput analysis. As such, for decades most scientific and medical labs have utilized the Kinetic Jaffé (KJ) method (Jaffe 1886; Narayanan and Appleton 1980; Spencer 1986; Boeniger et al. 1993)), a low-cost and non-specific colorimetric assay, for estimating urinary CRc. This method involves measuring the absorbance or optical density (OD) of a prepared standard and urine sample at two time points within the first 10 min following a reaction with two working reagents (sodium hydroxide [NaOH] and a picrate solution) (Vasillades 1976). The differences in the OD of the unknown from time 2 (unk_2) to time 1 (unk_1) (i.e., $OD_{unk_2} - OD_{unk_1}$), and of the single standard from time 2 (std_2) to time 1 (std_1) (i.e., $OD_{std_2} - OD_{std_1}$), are used to calculate CRc, according to equation (1).

$$CRc = \left(\frac{OD_{unk_2} - OD_{unk_1}}{OD_{std_2} - OD_{std_1}} \right) \times n \times \text{std conc} \quad (1)$$

Here, "std conc" is the concentration of a prepared CR standard (mg/dl) and *n* is a dilution factor.

The KJ method can be utilized for low-throughput sample analysis (tens of samples per day) via a single cuvette spectrophotometer, or for increased-throughput analysis using multi-

well plate readers. Most moderate- (hundreds of samples per day) and high-throughput (thousands of samples per day) CRc measurement platforms rely on costly (upwards of $100,000) automated processing and analysis equipment to maintain accuracy and precision (for examples see Roche/Hitachi MODULAR® and cobas® analytical systems [Roche Diagnostics, 9115 Hague Road, Indianapolis, IN, USA; http://www.roche.com/products-us.htm]). Given the time sensitivity of the KJ method – that is, the need for absorbance measurements at two specific time points within 10 min following the reaction – it can be difficult to perform even moderate-throughput CRc analysis using manual preparation techniques (e.g., pipetting). Thus, a need exists for a low cost and less time-sensitive method that still allows moderate-throughput CRc measurement. This study evaluates an alternative method for rapid CRc determination, referred to as the "Plateau Jaffé" (PJ) method, which is based on the proven chemistry of the KJ method, a commercially-available kit, and a multi-point calibration curve.

Table 1 shows specific criteria that need to be considered when selecting a methodology for urinary CR determination. Labs with the need to analyze thousands of samples per day, on a continuous basis, are well-suited for automated analytical systems utilizing the traditional KJ method. Here the need for consistent high-throughput measurement justifies the increased cost of the analytical platform. For labs that require CR determination in only tens to hundreds of samples, on an infrequent basis, the most practical option may be the use of a contract lab, where the cost is about $10/sample. As shown in Table 1, the PJ method is well-suited for labs that require CR determination in hundreds to thousands of urine samples on an intermittent basis (e.g., for environmental or molecular epidemiology studies that occur months to years apart). Here, the number of samples and frequency of analysis may not justify the expense of an automated platform, but may prove too costly for contract lab analysis.

As part of this investigation, common sample aliquots were analyzed by a contract lab using the standard KJ method, and in our lab using the PJ method. All samples were also analyzed in our lab using a liquid chromatography (LC) time-of-flight (TOF)/MS methodology for overall quality assurance. The purpose of this investigation was to thoroughly evaluate the performance of the PJ method, when employed in a multi well-plate format, against both the industry standard KJ method, and a mass-specific analytical benchmark.

2.0 Materials and Methods

2.1 Urine samples for evaluating method performance

Individual urine samples used for method comparison were from nominally healthy adult donors collected as part of a longitudinal study in North Carolina. The longitudinal study was performed with approval from the University of North Carolina Biomedical Institutional Review Board (IRB 09-0741) and with subjects' informed consent. Measurements of 32 urine samples from study subjects are considered in this investigation; two samples were provided from each of 8 woman (ages 21-42) and 8 men (ages 22-48). Study samples were analyzed in duplicate using the PJ method, the KJ method (via a contract lab), and the LC-TOF/MS method. Pooled control urines were also prepared from study samples and used for methods development.

2.2 Plateau Jaffé method

2.2.1 Development of a standard curve

The PJ method uses the reagents (Picrate solution and NaOH) in the Quantichrom creatinine assay kit (DICT-500) from BioAssay Systems (Hayward, CA, USA). Stock solution concentrations were first prepared to achieve (after dilution) 6 working standards in the range of

1 to 40 mg/dl using creatinine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. C6257-100G) in deionized (DI) water. Blanks were prepared and evaluated alongside CR standards to allow compensation for DI water absorbance levels. Thirty µl aliquots of blanks and standards were added to individual wells of a 96-well plate (purchased from Eppendorf International, Hamburg, Germany; Cat. No. 951040081). Next, 200-µl of a reagent solution - consisting of two parts DI water, one part picrate reagent, and one part NaOH reagent - was added using an 8-channel autopipette (Eppendorf Cat. No. 2246143-5). The plate was tapped gently to promote mixing and placed onto a 96-well microplate reader (BioTek ELx808 absorbance microplate reader, BioTek Instruments Inc., Winooski, VT, USA). The entire microplate reader was covered with aluminum foil due to light sensitivity of the reaction. The microplate reader, equipped with BioTek Gen5 software, was then used to measure absorbance at 490 nm. Optical density readings of each standard and blank were taken at 5 min intervals, beginning at one min and ending at 90 min. After background subtraction, sequential OD readings for standards were plotted versus time (Figure 1). A non-linear (saturable) increase in OD occurred for each standard, such that a period of rapid rise was observed between one and ~15 min, followed by a period of slower elevation up ~40 min, and then a plateau period out to 90 min. This observation is consistent with a first-order reaction, and published reports that suggest urine contains only small amounts of non-creatinine chromogens (Husdan and Rapoport 1968; Narayanan and Appleton 1980; Boeniger et al. 1993). Optical density readings of each standard at 60 min were used to create a second-order polynomial calibration curve (R^2 = 0.9999). Sixty min was selected as the measurement time point based on visual inspection of the OD vs. time plots (see Figure 1). This was considered a conservative time point in that small variations in timing (seconds to minutes) were unlikely to appreciably affect OD measurements.

2.2.2 Analysis of study urine samples

The 32 study urine samples were evaluated (in duplicate) after a 10-fold dilution in DI water (since urine samples are generally expected to be in the range of 30 - 300 mg/dl (WHO 1996)). Specifically, 100 µl of each urine sample was added to 900 µl of DI water in a 1.5-ml microcentrifuge tube (Sigma-Aldrich, Plastibrand® Microcentrifuge Tubes, Cat. No. Z336769). Diluted urine samples were vortexed to ensure proper mixing and then centrifuged (500 × g) for 5 min. Thirty µl of each sample supernatant layer was transferred into a well of a 96-well plate. Two-hundred µl of reagent solution was then added to each well. The plate was tapped and placed onto the microplate reader, which was then covered with aluminum foil. Optical density readings (490 nm) at 60 min were recorded. The calibration curve was used to calculate sample CR concentrations after adjusting for blank values and a 1:10 dilution factor.

2.3 Mass spectrometry method

Stock solutions of CR in DI water were prepared using solid creatinine hydrochloride (Sigma-Aldrich, Cat. No. C6257-100G). Stock solution concentrations were prepared to achieve (after dilution) 9 working standards in the range of 1 to 500 mg/dl. Blanks were prepared and evaluated alongside CR standards to verify the absence of matrix contaminates. Working standards/samples were prepared by transferring a 100-ul aliquot of each stock solution or sample into a clean 15 ml polypropylene vial (BD Biosciences, San Jose, CA) containing 10 ml of DI water and 10-ng of ^{13}C-methyl-creatinine (Cambridge Isotope Labs, Andover, MA). These standards/samples were further diluted by transferring 100 ul of the previous dilution after vortexing to a clean 15 ml polypropylene vial containing 10 ml of DI water to achieve a final

10,000-fold dilution. A 400 ul aliquot of each diluted standard/sample was transferred in a 500 ul vial (Lab Supply Distributors Corporation) and used for LC-TOF/MS analysis.

Analysis of prepared standards and study urine samples was performed using an Agilent 1100 series HPLC interfaced with a 6200 series Accurate-Mass LC-TOF system (Agilent Technologies, Palo Alto, CA). The MS was operated in electrospray positive ion mode (ESI) and any drift in the mass accuracy of the LC-TOF was continuously corrected by infusion of two reference compounds (purine [m/z = 121.0509] and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazene [m/z = 922.0098]) via dual-ESI sprayer. The LC-TOF/MS method consisted of a 4 min gradient run and an initial mobile phase of 90 % solvent A (2 mM ammonium acetate in 5:95 methanol: H_2O) and 10 % solvent B (2-mM ammonium acetate in 5:95 DI: methanol). The flow rate was 200 µl/min with CR separated from matrix compounds using a Waters Sunfire C8 column (3 mm × 50 mm, 5.0 µm) with an injection volume of 5 µl. The LC-TOF/MS was operated in 4 GHz mode from 50-1700 m/z. Creatinine concentrations in unknown urine samples were calculated based on a 1/x weighted quadratic fit (R^2 = 0.9988) of the standard curve concentration versus area ratio (analyte/internal standard). As shown in Figure 2, the extracted ion chromatograms (EIC) monitored were the [M+H]+ ion, which was 114.0662 for CR and 115.0695 for ^{13}C-methyl CR, respectively.

2.4 Kinetic Jaffé analysis

Study samples and prepared CR standards were sent in duplicate to a contract lab (Laboratory Corporation of America [LabCorp], Burlington, NC 27215; test number: 013672) for analysis using the KJ method on a Roche/Hitachi MODULAR® P analyzer (Roche

Diagnostics, 9115 Hague Road, Indianapolis, IN, USA). All samples and standards were randomized and given unique IDs to prevent measurement bias.

2.5 Statistical analysis

All data were evaluated using Microsoft Excel (Office 2007, Microsoft Corporation, Redmond, WA, USA), GraphPad Prism (v. 4.03, GraphPad Software, San Diego, CA, USA), and SAS statistical software (v. 9.3, SAS Institute, Cary, NC, USA). The analytical limit of quantitation (LOQ) for the PJ method was based on 10 times the standard deviation (SD) of 4 replicate measurements of the lowest calibration standard (1 mg/dl). Urine sample measurement distributions were evaluated using the Shapiro-Wilk test and by visual inspection of histograms and quantile-quantile plots (SAS PROC UNIVARIATE). All measurements were (natural) log-transformed to satisfy assumptions of normality. Method precision was determined using ln-transformed measurements of the 32 replicate study samples. Specifically, within-sample variance was estimated ($\hat{\sigma}_w^2$) (SAS PROC NESTED) and used to calculate the coefficient of variation (CV) for each method according to equation 2 (Rappaport 1991; Serdar et al. 2003).

$$\text{estimated CV} = \sqrt{e^{\hat{\sigma}_w^2} - 1} \qquad (2)$$

Method accuracy was based on results of regression models (SAS PROC REG), with LC-TOF/MS measurements serving as the analytical gold standard (note that this is the only tested method that is specific for CR). For all regression models, replicate measurements of urine samples were first averaged and then ln-transformed, giving 32 unique measurements associated with each method. Significant differences in regression intercept and slope estimates were determined at $\alpha = 0.05$.

3.0 Results

Method performance statistics and descriptive statistics for 32 urine samples are shown in Table 2. The LOQ for the LC-TOF/MS method was set as the lowest working standard concentration (i.e., 1 mg/dl); this level was sufficiently low to measure all study samples. The LOQ for the KJ method was set as 15 mg/dl; the contract lab that implemented the KJ method would not support measurements below this value. The LOQ for the PJ method was calculated as 2.29 mg/dl using replicate measurements (n = 4) of the lowest working standard.

Measurement precision was based on repeated measurements of the 32 urine samples. Precision estimates were similar across the three methods, with %CV levels of 2.59, 4.19, and 5.56 for the PJ, LC-TOF/MS, and KJ method, respectively. LC-TOF/MS measurements of CRc ranged from 13.9 to 326 mg/dl with a geometric mean (GM) of 94.7 mg/dl. Geometric mean estimates of CRc based on the KJ method (99.3 mg/dl) and the PJ method (99.2 mg/dl) were slightly larger than the LC-TOF/MS estimate. All three methods yielded measurements that were outside of the WHO-defined guidance range of 30 – 300 mg/dl (WHO 1996). Specifically, for each method, three measurements were below 30 mg/dl and three measurements were above 300 mg/dl (see Table 2 for min and max values). Thus, each method identified 6 out of 32 samples (19%) that were outside of the guidance range.

Results from linear regression analyses are shown in Figure 3; here all data points represent ln-transformed measurements of CRc based on 32 urine samples. Figure 3a shows the regression of KJ measurements on LC-TOF/MS measurements, Figure 3b shows the regression of PJ measurements on LC-TOF/MS measurements, and Figure 3c shows the regression of PJ measurements on KJ measurements. Strong agreement is shown across all methods with R^2

estimates ranging from 0.996 to 0.998. The slope estimates were close to one in each model, and ranged from 0.944 (Figure 3c) to 0.986 (Figure 3a). However, only the slope estimate for KJ vs. LC-TOF/MS (Figure 3a) was statistically indistinguishable from one (95% CI: 0.963 to 1.009). Y-intercept estimates were close to zero in each model, and ranged from 0.111 (Figure 3a) to 0.303 (Figure 3b). While the y-intercept for KJ vs. LC-TOF/MS was closest to zero (0.111), estimates were significantly different than zero in each model ($p \leq 0.04$).

4.0 Discussion

This study evaluates an alternative colorimetric method for CR determination that can be used to support moderate-throughput assessments of urine samples without the need for expensive automated sampling equipment or reliance upon a time-crucial technique. A modified version of the well-known industry standard KJ method is examined with adjustments for measurement timing and the use of a standard curve. With the PJ method, absorbance measurements are made during a plateau phase (~60 min) following reaction with two reagents where small changes in measurement timing are unlikely to impact final quantitative results. Since the PJ method relies less heavily on the timing of sampling events (compared to the KJ method), it can be performed using manual preparation techniques with multi-well plates. Furthermore, it is noteworthy that this method may be further optimized for shorter run times given the stabilization of absorbance measurements starting at ~40 min (see Figure 1).

Thirty-two urine samples from anonymous healthy donors were analyzed for CR using the PJ method, the industry standard KJ method, and a LC-TOF/MS method. Method precision and sensitivity were comparable across all three methods, with estimated CV values less than 6% in each case, and LOQ that were below all measured sample concentrations. Interestingly, each

method identified nearly 20% of study urine samples that were outside of the WHO guidance range of 30 – 300 mg/dl. This result is consistent with a report from Barr et al. (2005) based on CRc data from the Centers for Disease Control and Prevention's (CDC) Third National Health and Nutrition Examination Survey (NHANES). In their study, Barr et al. reported ~13% of CRc measurements being outside the WHO guidance range when looking across males and females, ages 20-49, from the U.S. population (Barr et al. 2005).

Results of the regression models showed similar results across the three methods. R-squared estimates were greater than 0.995 in each model, indicating that over 99.5% of the measurement variance from any one method might be explained using measurements from any other method. In each model, slope estimates were near one and y-intercept estimates were near zero. To evaluate method accuracy, LC-TOF/MS measurements were considered the "analytical gold standard" in the regression models. Figures 3a and 3b show that KJ measurements were slightly more accurate than PJ measurements when compared with LC-TOF/MS data. According to the regression equations, the KJ and PJ methods would be expected to measure (on average) CRc levels of 16.1 and 17.4 mg/dl, respectively, given an LC-TOF/MS measurement of 15 mg/dl. These levels represent a respective 8 and 16% overestimation of CRc by the KJ and PJ methods at the minimum of the measurement distribution. At the maximum of the measurement distribution – that is, 330 mg/dl – the KJ and PJ methods would be expected to measure (on average) CRc levels of 340 and 322 mg/dl, respectively. Here the KJ concentration would overestimate the LC-TOF/MS level by 3%, whereas the PJ concentration would underestimate the LC-TOF/MS level by 2%.

While the LC-TOF/MS is the only "specific" method evaluated in this comparison (and therefore considered our "analytical gold standard"), the KJ method is widely considered the

industry standard for CRc analysis. As such, it is prudent to evaluate PJ method performance with respect to KJ method performance. Figure 3c shows that PJ measurements were slightly higher than KJ measurements on the low end of the measurement distribution, and slightly lower than KJ measurements on the high end. Given KJ estimates of 15 and 330 mg/dl, the PJ method would be expected to give average estimates of 16.3 and 312.9 mg/dl, respectively. These values represent an overestimation of KJ by 9% on the low end, and an underestimation by 5% on the high end.

While only minor differences across methods were observed here, it is important to remember that the provided regression equations can be used to make measurement adjustments. That is, KJ and PJ measurements can be adjusted to better reflect LC-TOF/MS data, and PJ measurements can be adjusted to better reflect KJ data. This allows complete flexibility when comparing CRc levels across studies that employed different measurement methodologies. Of course, discrepancies between methods were shown here to be minor, and mostly occurring at the low end of the measurement distributions. Thus the use of correction factors need to be considered an optional means to maximize comparability across CR measurement studies.

5.0 Conclusion

The KJ method continues to be the industry standard for urinary CRc analysis but generally utilizes costly sampling equipment for moderate- to high-throughput sample analysis. Lower-cost kit-based methods exist, but are subject to timing imprecision when using manual preparation techniques and multi-well plates. The PJ method builds on the widely-used KJ method and allows moderate-throughput sample analysis, based on manual preparation techniques, at a modest cost. Indeed, the cost of the measurement platform described here was

less than $10,000 and is capable of producing hundreds of sample measurements per day. Using this platform, smaller labs can analyze urine samples for CRc more efficiently and with no loss in measurement sensitivity or precision. Bias across colorimetric (KJ and PJ) and mass-specific (LC-TOF/MS) methods were shown here to be minimal, and can now be easily corrected using the simple linear models.

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LEGEND TO FIGURES

Figure 1. Time-course absorbance measurements (at 490 nm) of 6 working standards in the range of 1 – 40 mg/dl. Rapid concentration-dependent increases are shown for each standard between 0 and ~15 min, followed by a period of slower increase, and ultimately a plateau. A grey box drawn vertically at 60 min denotes the time at which absorbance measurements were made to generate standard curves and quantify study samples.

Figure 2. Extracted Ion Chromatograph (EIC) of creatinine and ^{13}C-creatinine from a 500 mg/dl standard total ion chromatograph (TIC). Inset (a) shows a background subtracted full spectrum from the TIC. Region (1) shows the compounds of interest and the reference mass for purine. Region (2) shows the HP0921 reference mass.

Figure 3a. Linear regression of CRc measurements [ln(mg/dl)] from the KJ method on CRc measurements [ln(mg/dl)] from the LC-TOF/MS method. The regression parameters are as follows: slope = 0.986 (95% CI: 0.963 to 1.009); y-intercept = 0.111 (95% CI: 0.005 to 0.216); $R^2 = 0.996$.

Figure 3b. Linear regression of CRc measurements [ln(mg/dl)] from the PJ method on CRc measurements [ln(mg/dl)] from the LC-TOF/MS method. The regression parameters are as follows: slope = 0.944 (95% CI: 0.923 to 0.965); y-intercept = 0.303 (95% CI: 0.206 to 0.400); $R^2 = 0.997$.

Figure 3c. Linear regression of CRc measurements [ln(mg/dl)] from the PJ method on CRc measurements [ln(mg/dl)] from the KJ method. The regression parameters are as follows: slope = 0.956 (95% CI: 0.940 to 0.972); y-intercept = 0.202 (95% CI: 0.127 to 0.278); $R^2 = 0.998$.

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