HHS Public Access

Author manuscript

Clin Mass Spectrom. Author manuscript; available in PMC 2021 August 18.

Published in final edited form as:

Clin Mass Spectrom. 2019 January ; 11: 1–7. doi:10.1016/j.clinms.2018.11.002.

A fully automated high-throughput liquid chromatography tandem mass spectrometry method for measuring creatinine in urine

Neelam Zahoor, **Uliana Danilenko***, **Hubert W. Vesper**

Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Hwy NE, MS F25, Atlanta, GA 30341, USA

Abstract

Reliable creatinine measurements are important to evaluate kidney function and for creatinine correction to reduce biological variability of other urinary analytes. A high-throughput, accurate liquid chromatography tandem mass spectrometry method for quantitation of human urinary creatinine has been developed and validated.

Sample preparation was fully automated including cryovial decapping, sample ID scanning and two serial dilution steps. Quantitation was performed using a stable isotope-labeled internal standard. Multiplexed chromatographic separation of creatinine was achieved within a one-minute analysis and followed by tandem mass spectrometry in positive electrospray ionization mode. The precursor and product ions of creatinine and D_3-creatinine were monitored in selected reaction monitoring mode.

Method validation results showed reproducibility with within-run precision of 3.59, 3.49 and 2.84% and between-run precision of 4.01, 3.28 and 3.57% for low, medium and high quality control materials prepared from pooled donor urine, respectively. The method showed excellent accuracy with a bias of −1.94%, −0.78% and −1.07% for three levels of certified reference material. The calibration curve was linear throughout a 7.50–300 mg/dL (0.663–26.5 mmol/L) measurement range (R^2 = 0.999), with the mean slope of 0.0115 (95%CI, 0.0108–0.0122) and intercept of 0.0027 (95%CI, 0.0003–0.0051). The limit of detection (LOD) of the method was 3.17 mg/dL (0.280 mmol/L). Analytical specificity was achieved by chromatographically separating creatinine from potentially interfering creatine within a one-minute run and monitoring the Quantitation Ion/Confirmation Ion (QI/CI) ratios in samples.

A simple, accurate, high-throughput method was successfully developed for measuring creatinine in human urine samples.

*Corresponding author. UDanilenko@cdc.gov (U. Danilenko).

5. **Publisher's Disclaimer:** Disclaimer

Publisher's Disclaimer: The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service and the US Department of Health and Human Services.

Conflict of Interest

None of the authors has any conflicts of interest to disclose.

Keywords

Mass spectrometry; Urine creatinine; Automation; High throughput

1. Introduction

Creatinine is a breakdown product of muscle creatine that is excreted in urine at a constant rate. Thus, in normal individuals, creatinine concentration in urine is proportional to muscle mass [1,2]. Urinary creatinine together with other analytes, such as albumin, has been used for years as a marker of renal health and to diagnose kidney disease [1,3]. Creatinine is also used in biological monitoring and toxicological studies to compensate for variability in fluid balance and related variation in analyte concentration of other urinary analytes [4,5] such as urinary proteins [6], exposure biomarkers [7,8,9], heavy metals [10,11], as well as in regulatory settings for drug and doping testing [12]. Creatinine adjusted concentrations are expressed as amount of analyte per gram of creatinine.

Several analytical methods are available for the determination of creatinine in urine. Commonly used commercial clinical analyzer-based methods apply Jaffe or enzymatic reactions [13,14,15,16,17]. Jaffe reaction-based methods are known to overestimate creatinine concentrations due to the lack of specificity [18,19]. Enzymatic assays are usually limited by low throughput caused by long analysis time. Both types of methods can have notable reagent and calibrator lot-to-lot variability, and limited shelf-life of reagents. Mass spectrometry can provide highly specific creatinine measurements and methods have been described for measuring creatinine in combination with flow injection [20,21], gas chromatography [22] or high performance liquid chromatography [23,24]. Flow injection approaches may lead to unwanted detection of interfering compounds, variability in sensitivity due to ion suppression, and may require frequent instrument maintenance due to salt and other compounds accumulating on the instrument. The use of chromatography can minimize these limitations. However, the current length of chromatographic separation (i.e., at least 4 min) of previously published methods [23,24,25] may impede high throughput analysis. Additional information comparing run times and sample preparations strategies of the existing mass spectrometry-based and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods is provided in Table 1.

The goal of this study was to develop a mass spectrometry-based method for urinary creatinine that utilized chromatography without compromising sample throughput. The presented method achieves excellent chromatographic separation of creatinine within one minute, uses complete automation optimized for stored study samples with a liquid handling system, and implements multiplexing of ultra-high performance liquid chromatography (UHPLC) in combination with tandem mass spectrometry.

2. Materials and methods

2.1. Chemicals and material

Creatinine standard reference material (SRM) 914a with a purity of 99.7 ± 0.3% for preparation of calibrators was obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD). Creatinine-methyl-D_3 (D_3-creatinine) with an isotopic purity of 98 atom % D from Sigma Aldrich (Bellefonte, PA) was used as an internal standard. HPLC-MS grade acetonitrile, water and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (Fluka, LC-MS grade) was ordered from Sigma Aldrich (St. Louis, MO).

2.2. Calibration standards and quality control (QC) materials

An initial calibrator stock solution at a concentration of 5000 mg/dL (442 mmol/L) was prepared from NIST SRM 914a using an acetonitrile:water mixture (40:60, v/v) with addition of 0.1 mol/L hydro-chloric acid, as recommended by the manufacturer (Sigma Aldrich, St. Louis, MO). The subsequent 500 mg/dL (44.2 mmol/L) stock solution, as well as final calibrator working solutions at concentrations of 7.50,10.0, 20.0, 30.0, 100, 150 and 300 mg/dL (0.663, 0.884, 1.76, 2.65,8.84, 13.3, 26.5 mmol/L), were prepared from the initial stock solution using an acetonitrile:water mixture (40:60, v/v). An internal standard working solution (50.0 mg/dL, 4.42 mmol/L) was prepared by dissolving D_3-creatinine in an acetonitrile:water mixture (40:60, v/v).

Three levels of bench quality control (QC) materials at 41.7 mg/dL (3.68 mmol/L) (low), 135 mg/dL (12.0 mmol/L) (medium) and 266 mg/dL (23.5 mmol/L) (high) were prepared from pooled human urine samples collected according to the CDC-approved IRB protocol for anonymous urine collection. The QC limits for each level were established, as described elsewhere, using the multi-rule quality control system developed for three QC pools per run scenario [26]. The QC materials were analyzed with each sample batch in duplicate. Calibrator solutions and QC materials were processed in the same manner as study samples.

2.3. Sample preparation

Frozen urine samples, QC materials, internal standard working solutions and calibrators were allowed to reach room temperature by rotating for 30 min. Typically, one batch of samples consisted of a set of seven calibrators, 82 donor samples, three levels of QC materials, and one solvent blank (acetonitrile:water, 40:60, v/v). An automated decapper (FluidX, San Bruno, CA) was used to simultaneously open 48 cryovials (FluidX 2 ml 2D coded external thread jacket cryo tubes, FluidX, San Bruno, CA) with urine samples in their storage boxes. Samples in their storage box were transferred to an automated liquid handling system for further processing.

Samples, calibrator solutions and QC materials were processed using a TECAN Freedom EVO 200 liquid handler (TECAN, Morrisville NC) equipped with a 2D barcode reader (XTR-96 Tetra Flex Cryo, 4 rack reader, FluidX, San Bruno, CA) to simultaneously scan 4 storage boxes containing the cryovials with urine samples. 100 μL of each calibrator, urine sample, QC and solvent blank were transferred into a 2-mL 96 deep-well plate (Arctic White

LLC, Bethlehem, PA). The internal standard working solution (100 μL) was added to each well, and the mixture was diluted with 1300 μL of dilution solvent (acetonitrile:water, 40:60, v/v). After mixing with a 96-chanel system, 100 μL from each well were transferred to a second 2-mL 96 deep-well plate and further diluted with 1400 μL of dilution solvent. After mixing the sample solutions, the well plates were sealed and loaded on the auto sampler for UHPLC-MS/MS analysis.

2.4. Liquid chromatography tandem mass spectrometry conditions

All samples were analyzed using a UHPLC system (Dionex UltiMate 3000, Thermo Electron, San Jose, CA) equipped with two binary pumps and coupled with a triple quadrupole mass spectrometer (Quantum, Thermo Electron, San Jose, CA) with heated electrospray ionization (HESI).

Chromatographic separation was performed on a reversed phase column (Aquity UHPLC HSS C18 SB 1.8 μm, 2.1 × 50 mm, Waters Corporation, Milford, MA) with a guard column (VanGuard™ Pre-Column HSS C18 SB, 1.7 μm, 2.1 mm × 5 mm, Waters, Milford, MA). The flow rate, oven temperature, and injection volume were 700 μL/min, 55 (± 2) °C, and 5 μL, respectively. Isocratic separation was carried out with an acetonitrile:water mixture (40/60 v/v, with 0.01% formic acid). Multiplexing was achieved by concurrent chromatographic separation of samples on two columns using a switching valve. Using the instrumentation described with 2 columns, a total cycle time of 1.7 min (injection to injection) was achieved. Faster cycle times and the use of more columns could be possible with different instrumentation.

The mass spectrometer was operated in positive ion mode with the ion spray voltage set at 4000 V; capillary temperature at 350 °C; and collision (argon), sheath (nitrogen) and auxiliary (nitrogen) gases set at 20 psi, 40 psi and 10 psi, respectively. The collision energy was set at 20 V.

Selected reaction monitoring mode was carried out for the following ion transitions: m/z 114 → 44 (quantitation ion (QI), creatinine), m/z 117 → 47 (QI, D_3-creatinine), m/z 114 → 72 (confirmation ion (CI), creatinine) and m/z 117 → 75 (CI, D_3-creatinine) (Fig. 1).

2.5. Data analysis

Dionex Chromatography MS Link 2.14 (Thermo Fisher Scientific, San Jose, CA) and Xcalibur 2.2 (Thermo Scientific, San Jose, CA) were used to operate the UHPLC-MS/MS system and acquire data. Chromatographic peaks were integrated using automated Indigo Ascent (Indigo BioAutomation, Indianapolis, IN) software. The ratio of the creatinine QI area count to the D_3-creatinine QI area count was used for concentration calculations. Possible shifts in measurement accuracy were assessed by comparing bench QC material data against predefined acceptance limits and trend rules using an in-house-developed SAS-based program (SAS Version 9.4, SAS Institute Inc., NC, USA) as outlined in section 1.2, 1.6.1, and described previously [26].

2.6. Method validation

2.6.1. Accuracy and precision—Accuracy of the method was evaluated by analyzing three levels of urine-based NIST certified reference materials (NIST SRM 3667, NIST SRM 3672, NIST SRM 3673) in duplicate over 5 days. The measurement results were compared to the certified values and evaluated for agreement using NIST Special Publication 829 [27]. The difference between the certified value of the material and measured value was determined. Taking into account the provided expanded uncertainty and variability of the measurements, the significance of this difference was evaluated.

Within-run and between-run imprecision were assessed by measuring urine pools with low, medium and high creatinine concentrations in duplicate over 22 days. Imprecision was calculated using Analysis of Variance (ANOVA). ANOVA produces mean square estimates for the total variation, the variation between runs, and the variation within runs [28]. Using these estimates, the maximum allowable deviations from the target (i.e., 2× SD and 3× SD) were calculated. Calculated imprecision was expressed as a percentage of the coefficient of variation (% CV). In addition, the long-term stability of the method was evaluated by calculating the between-day % CV observed with QC materials over an extended period of time and results were compared to the initial between-day % CV calculations.

2.6.2. Linearity and analytical measurement range—Linearity of the measurement range was evaluated following principles described in the CLSI document, EP6-A [29]. In brief, seven levels of calibrator working solutions were measured over the course of 20 days and the ratios of creatinine QI to D_3-creatinine QI area counts were plotted against creatinine concentration. Linearity of the measurement range (i.e., the range between lowest and highest calibrator concentrations: 7.50–300 mg/dL [0.663–26.5 mmol/L]) was assessed using residuals and linear and polynomial fitting models. The mean sum of squared residuals (ASSR) and the mean relative sum of squared residuals (RASSR) from 20 sets of calibration curves analyzed over 20 days were used to choose the best fitting regression model from among linear and polynomial models. We further assessed the effect of different weights, such as no weighting, weights of $1/X$, $1/X^2$, or 1/(Variance of Y), where X represents the calibrator concentration and Y represents the instrument response (ratio of area count versus internal standard). The ASSR was calculated from the sum of the squared differences between the model-predicted and the observed Y values. The RASSR was calculated by dividing the ASSR by the average of the Y values. Mean slope and mean intercept were calculated using results obtained from 20 independent calibration curves. All calculations were performed using SAS (SAS Version 9.4, SAS Institute Inc., NC, USA).

2.6.3. Sensitivity and specificity—The limit of detection (LOD) was determined in urine matrix according to Taylor's method [30], by calculating the standard deviation (SD) of repeat measurements at different creatinine concentrations. Medium concentration in-house QC material was diluted with dilution solvent (acetonitrile:water, 40:60, v/v) to yield five different creatinine concentrations, including LOD. Seven replicates of each level were prepared using the regular sample preparation procedure and analyzed on five different days. The absolute values of the SDs were then plotted against concentration and extrapolated to a zero concentration SD (SD_0). The LOD was calculated as SD_0 multiplied by 3.

The lower limit of quantitation (LLOQ) was defined as the concentration of the lowest calibrator. Imprecision and accuracy (expressed as percent recovery) were evaluated at the concentration level corresponding to the lowest calibrator. Synthetic urine (Dyna-Tek, Lenexa, KS), not containing creatinine, was spiked with 7.50 mg/dl (0.663 mmol/L) creatinine and analyzed in 5 replicates over 2 days. Imprecision of the measurement and percent recovery compared to expected values were calculated. Calculated imprecision and accuracy (i.e., recovery) were compared to the values recommended for bioanalytical methods [31,32] to evaluate assay performance as discussed in section 2.3.

Creatinine was identified when the chromatographic peak had the same retention time as the IS (± 2.5%) and the QI/CI ratio was the same as that determined in neat calibrator solutions (+/−20%) [33].

Matrix effects (ME) were assessed in four matrices (0.9% saline solution, synthetic urine and individual donor human urine samples with low and high creatinine concentrations) and compared to the neat dilution solvent (acetonitrile: water, 40:60, v/v, matrix-free). Synthetic urine was obtained from Dyna-Tek (Lenexa, KS), and saline solution was purchased from Fisher Scientific (Hanover Park, IL). Seven-point calibration curves ranging from 7.50 to 300 mg/dL (0.663 to 26.5 mmol/L) were prepared in these matrices. The calibrators in these matrices underwent the sample preparation and analysis described in the previous section. The area count ratios of analyte to IS were compared in all four matrices, after blank subtraction, to those analyzed in neat samples. Sample ME was calculated with the following equation: ME % = B/A × 100, where B is the area count ratio of analyte to IS obtained from samples in matrix, and A is the area count ratio in matrix-free samples based on principles described elsewhere [34,35,36]. In addition, R^2 and slopes were evaluated and compared in all matrices.

2.7. Method comparison

Our method was compared to results obtained with an enzymatic creatinine assay operated on a Roche Cobas c311 clinical analyzer (Roche, Indianapolis, IN). 160 individual donor adult urine samples covering the clinically relevant concentration range were collected according to the CDC-approved IRB protocol for anonymous urine collection or obtained from Bioreclamation IVT (Westbury, NY). The company has IRB approval to collect human specimens and obtained informed consent from donors. CDC's use of urine is consistent with the IRB approval and donor consent. No personal identifiers were provided to CDC. Donor samples were analyzed using both methods, and the results were compared using Deming regression plots and the Bland-Altman bias analysis (Analyze-It Software Ltd., 4.65.2, Leeds, UK).

3. Results and discussion

3.1. Sample preparation and automation

This method was developed for analyzing stored samples from large epidemiological studies. Therefore, automation components included automated decapping of cryovials and barcode scanning without removing the vials from the storage box, transferring the

specimens from the cryovial into 96-well plates and further processing using 96-channel pipetting systems. This enabled fully automated processing of eight 96-well plates (656 urine samples) within 8 h. The chromatographic conditions allowed for elution of creatinine in less than 30 s (retention time: 0.48 min) while ensuring separation from creatine (retention time: 0.38 min). The excellent peak symmetry (Fig. 1) facilitated automated integration with less than 1% of integrations requiring correction.

3.2. Method specificity

The method is highly specific, which was achieved by chromatographically separating creatinine from creatine. Even though creatine has both a different mass/charge (*m*/*z*: 132) and ion transitions than creatinine, we chose to separate this compound to avoid an unlikely, but potential, interference caused by water loss due to the high temperature of the ion source. In addition to using transitions for QI, we also monitored transitions for CI. Both QI and CI transitions were detectable in all urine samples. The mean ratio of QI/CI ratio was 38.1 (95% CI, 37.6 to 38.6, N = 21) calculated using calibrators in neat solution processed in 3 separate preparations. The QI/CI ratios determined in 160 urine samples ranged between 34.3 and 42.4 and were all well within the suggested limit of ± 20% from the ratios obtained in neat calibrators [33]. Comparable QI/CI ratios in donor and neat samples suggest that there were no interferences present in these 160 urine samples.

3.3. Analytical performance

The proposed method is highly accurate and sensitive. When assessing NIST certified standard reference materials the measurement results were not significantly different from the target values with mean biases of −1.94%, −0.78% and −1.07% for NIST SRM materials 3667, 3673 and 3672, respectively (Table 2).

The accuracy bias of the method was well below the maximum suggested of 12.2%, as calculated from intra-and inter-individual biological variation [37,38]. The accuracy was verified every six months using NIST certified standard reference material and monitored in every run using QC materials. The LOD of the method was found to be3.17 mg/dL (0.280 mmol/L), which is profoundly lower than the lowest concentration typically observed in human urine [1]. The LLOQ of the method was set at the concentration of the lowest calibrator (7.50 mg/dL, 0.663 mmol/L). At this concentration, the imprecision was 5.71% and inaccuracy determined using creatinine spiking experiments was11.86% (95% CI: 7.90% to 15.83%). Both imprecision and accuracy estimations at the LLOQ level were well within those suggested for bioanalytical methods, which should not exceed 20% at the LLOQ level [31,32].

The method was found to have excellent precision. Measuring three QC levels (low, medium and high) over 22 days, the precision expressed as coefficient of variation in percent (% CVs) was 3.59%, 3.49% and 2.84% for within-run precision and 4.01%, 3.28% and 3.57% for between-run precision, respectively (Table 3); this is well below the maximum suggested imprecision for bioanalytical methods of 15% [31,32] and the suggested desirable imprecision of 18.2% obtained from intra-and inter-individual biological variation [37,38]. In addition, the long-term between-day precision for the method based on QC results from

112 independent runs analyzed over 9 months period using multiple operators, UHPLC columns, calibrator and reagent batches was 4.57%, 4.33% and 4.26% for low, medium and high QCs. The long-term between-day % CVs were consistent with between-day % CVs calculated during method evaluation phase suggesting long-term reliability of the method when used for routine analysis.

The measurement range of this method spans from 7.50 to 300 mg/dL (0.663 to 26.5 mmol/L) and covers the biological ranges typically observed in humans.

The calibration curve for the method was linear throughout its measurement range with no significant polynomial relationship observed. Analysis of sets of calibration curves from 20 independent runs indicated non-uniformity of measurement error variance across the range of sample concentrations, suggesting that a weighted model should be considered. Further analyses indicated that a 1/X weighted model provides a better fit (smaller ASSR) than other weighted models ($1/\sqrt{X}$, $1/X^2$) and better fit than a non-weighted model (ASSR: unweighted = 0.076; 1/X weighted = 0.031; RASSR: unweighted = 0.031; 1/X weighted = 0.026). Therefore, a 1/X weighted calibration curve was used for quantitation. Using this model, the calibration curves were consistent with a mean slope of 0.0115 (95%CI,0.0108 to0.0122) and a mean intercept of 0.0027 (95% CI, 0.0003 to0.0051). The mean coefficient of correlation (R^2) was 0.999.

Matrix effects were found to have minimal impact with ME% ranging between 94.14% and 95.73% (Table 4) indicating minimal ion suppression. The slopes in 0.9% saline, synthetic urine and donor urine, with low and high creatinine concentrations, were identical to the slope in neat solution (i.e., 0.011) suggesting that any matrix effect is effectively compensated for by the internal standard, resulting in no impact on quantitation. Due to the high natural concentration of creatinine in urine, two consecutive sample dilution steps on the liquid handler introduced a 225-fold dilution that minimized the amount of protein and other non-relevant compounds injected into the UHPLC-MS/MS and effectively eliminated any significant matrix effects.

3.4. Method comparison

This method was compared to a commercially available enzymatic method performed on a clinical analyzer by measuring 160 donor urine samples with both methods. The results determined with our UHPLC-MS/MS method ranged between 7.69 and 284 mg/dL (0.680 to25.1 mmol/L). Using Deming regression analysis, no significant difference between methods was observed with a slope of 0.9937 (95% CI, 0.9789 to 1.0090; P = 0.41) and intercept of 0.3261 (95%CI: −0.4591 to 1.1110; P = 0.41) (Fig. 2A). The correlation coefficient was 0.998 suggesting that the methods are comparable. Bland-Altman bias analysis (Fig. 2B) showed a non-significant mean bias of 0.54%, (95%CI: −0.15% to 1.24%). The suggested total error (TE) limit for urine creatinine is 42.1% [38]; none of the individual urine samples used in the study comparison exceeded this limit.

When compared with previously published LC-MS/MS-based methods, the presented method has the shortest run time and utilizes a high level of automation throughout all steps of the sample preparation and data anlysis process to achieve maximum throughput [23,24].

The newly developed method combines advantages of chromatographic separation that are superior to direct flow, while maintaining a very short run time (Table 1). A higher level of measurement accuracy and reproducibility was achieved by using a sample volume of 100 μL. Chromatographic separation and use of QI/CI ratios in neat and human urine samples ensure that the analysis is free from other potentially interfering compounds that may be present when direct flow injection is used [20,21]. Multiplexing allowed for an increase in sample throughput and, hence, a more efficient use of the mass spectrometer.

4. Conclusion

An accurate and reliable liquid chromatography tandem mass spectrometry method for the quantification of creatinine in human urine was developed. Automation of the method minimizes errors caused by manual handling and data entry. This high-throughput automated one-minute method is suitable for sample analysis for routine clinical measurements, large biomonitoring studies and other activities in research and public health settings.

Acknowledgments

We thank Sam Caudill, PhD for his support with statistical analysis. We thank Sarah Warren for her support with laboratory measurements, review and suggestions; Enada Archibold, PhD for her help with experimental design; Douglas Wirtz for his help with method automation; Neha Ahuja for creatinine measurements using commercial enzymatic assay; Dr. Julianne Botelho, Dr. Candice Ulmer and Cindy Tse for review of the manuscript (all with the National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia).

Funding

This work was supported by the Division of Laboratory Sciences from the Centers for Disease Control and Prevention.

Abbreviations:

CI	Confirmation ion
CV	coefficient of variation
HESI	heated electrospray ionization
IRB	institutional review board
LC-MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
LLOQ	lower limit of quantitation
ME	matrix effects
NIST	National Institute of Standards and Technology
QI	quantitation ion
QC	quality control

SD	standard deviation
SRM	standard reference material
UHPLC	ultra-high performance liquid chromatography

References

[1]. Burtis C, Ashwood E, Bruns D, Tietz textbook of clinical chemistry and molecular diagnostics, 5 ed., W.B. Saunders, 2012.

[2]. Henry JB, Ed., Clinical diagnosis and management by laboratory methods. 20th ed., 2001, p. 182.

[3]. KDIGO2012 clinical practice guideline for the evaluation and management of chronic kidney disease. Kidney International Supplements. 2013, 3(1).

[4]. Cocker J, Mason HJ, Warren ND, Cotton RJ, Creatinine adjustment of biological monitoring results, Occup. Med61 (5) (2011) 349–353.

[5]. Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, Pirkle JL, Urinary Creatinine Concentrations in the U. S. Population: Implications for Urinary Biologic Monitoring Measurements, Environ. Health Perspect113 (2) (2005) 192–200. [PubMed: 15687057]

[6]. Newman DJ, Pugia MJ, Lott JA, Wallace JF, Hiar AM, Urinary protein and albumin excretion corrected by creatinine and specific gravity, Clin. Chim. Acta294 (1–2) (2000) 139–155. [PubMed: 10727680]

[7]. Ye X, Wong LY, Bishop AM, Calafat AM, Variability of urinary concentrations of bisphenol A in spot samples, first morning voids, and 24-hour collections, Environ. Health Perspect119 (7) (2011) 983–988. [PubMed: 21406337]

[8]. Christensen K, Sobus J, Phillips M, Blessinger T, Lorber M, Tan YM, Changes in epidemiologic associations with different exposure metrics: a case study of phthalate exposure associations with body mass index and waist circumference, Environ Int. 73 (2014) 66–76. [PubMed: 25090576]

[9]. Berg CJ, Schauer GL, Ahluwalia JS, Benowitz NL, Correlates of NNAL levels among nondaily and daily smokers in the college student population, Curr. Biomarker Find2 (2012) 87–94.

[10]. Adams SV, Newcomb PA, Cadmium blood and urine concentrations as measures of exposure: NHANES 1999–2010, J. Expo Sci. Environ. Epidemiol24 (2) (2014) 163–170. [PubMed: 24002489]

[11]. Park H, Lee K, Moon CS, Woo K, Kang TS, Chung EK, Son BS, Simultaneous Exposure to Heavy Metals among Residents in the Industrial Complex: Korean National Cohort Study, Int. J. Environ. Res. Public Health12 (2015) 5905–5917. [PubMed: 26024361]

[12]. Lafolie P, Beck O, Blennow G, Boreus L, Borg S, Elwin CE, Karlsson L, Odelius G, Hjemdahl P, Importance of creatinine analyses of urine when screening for abused drugs, Clin. Chem37 (11) (1991) 1927–1931. [PubMed: 1934467]

[13]. Jaffé M, Ueber den Niederschlag welchen Pikrinsaure in normalen Harn erzeugt und uber eine neue Reaction des Kreatinins, Zeitschrift für physiologische Chemie10 (5) (1886) 299–308.

[14]. Bonsnes RW, Taussky HH, On the colorimetric determination of creatinine by the Jaffe reaction, J. Biol. Chem158 (3) (1945) 581–591.

[15]. Horio M, Orita Y, Comparison of Jaffé rate assay and enzymatic method for the measurement of creatinine clearance, Nippon Jinzo Gakkai Shi. (Jpn. J. Nephrol.)38 (7) (1996) 296–299.

[16]. Lindbäck B, Bergman A, A new commercial method for the enzymatic determination of creatinine in serum and urine evaluated: comparison with a kinetic Jaffé method and isotope dilution-mass spectrometry, Clin Chem. 35 (5) (1989) 835–837. [PubMed: 2541950]

[17]. Junge W, Wilke B, Halabi A, Klein G, Determination reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaffé method, Clin. Chim. Acta344 (2004) 137–148. [PubMed: 15149882]

[18]. Leung EM, Chan W, A novel reversed-phase HPLC method for the determination of urinary creatinine by pre-column derivatization with ethyl chloroformate: comparative studies with the standard Jaffé and isotope-dilution mass spectrometric assays, Anal. Bioanal. Chem406 (2014) 1807–1812. [PubMed: 24408302]

[19]. Chen C, Lin MS, A novel structural specific creatinine sensing scheme for the determination of the urine creatinine, Biosens. Bioelectron31 (1) (2012) 90–94, 10.1016/j.bios.2011.09. [PubMed: 22047975]

[20]. Niesser M, Koletzko B, Peissner W, Determination of Creatinine in Human Urine with Flow Injection Tandem Mass Spectrometry, Ann. Nutr. Metab61 (4) (2012) 314–321. [PubMed: 23208161]

[21]. Hušková R, Chrastina P, Adam T, Schneiderka P, Determination of creatinine in urine by tandem mass spectrometry, Clin. Chim. Acta350 (1–2) (2004) 99–106. [PubMed: 15530465]

[22]. Dimitrios Tsikas AWolf, Mitschke A, Gutzki FM, Will W, Bader M, GC–MS determination of creatinine in human biological fluids as pentafluorobenzyl derivative in clinical studies and biomonitoring: inter-laboratory comparison in urine with Jaffé, HPLC and enzymatic assays, J. Chromatogr. B878 (27) (2010) 2582–2592.

[23]. West CE, Rhodes BN, Determination of urinary creatinine in Washington state residents via liquid chromatography/tandem mass spectrometry, Int. J. Anal. Chem (2014) 1–6.

[24]. Park EK, Watanabe T, Gee S, Schenker M, Hammock B, Creatinine Measurements in 24 h Urine by Liquid Chromatography-Tandem Mass Spectrometry, J. Agric. Food Chem56 (2) (2008) 333–336. [PubMed: 18092755]

[25]. Hou H, Xiong W, Song D, Tang G, Hu Q, LC-MS-MS measurements of urinary creatinine and the application of creatinine normalization technique on cotinine in smokers' 24 hour urine, J. Anal. Meth. Chem2012; article ID 245415.

[26]. Caudill SP, Schleicher RL, Pirkle JL, Multi-rule quality control for the age-related eye disease study, Stat. Med27 (2008) 4094–4106. [PubMed: 18344178]

[27]. Becker RC, Currie L, Diamondstone B, Eberhardt K, Gills T, Hertz H, Klouda G, Moody J, Parris R, Schaffer R, Steel E, Taylor J, Watters R, Zeisler R, Use of NIST standard reference materials for decisions on performance of analytical chemical methods and laboratories, Gaithersburg, MD: NIST Special Publication829; 437, 1992.

[28]. Statistical Methods, Snedecor George W., Cochran William G., 6th Edition, 1967, The Iowa State University Press, Ames, Iowa, U.S.A., pp. 258–298.

[29]. CLSI, EP6-A. Evaluation of the linearity of 1uantitative measurement procedures: a statistical approach; Approved Guideline-first edition, Clinical and Laboratory Standards Institute, Wayne, PA, 2003.

[30]. Taylor JK, Quality assurance of chemical measurements.1st, ed., CRC, 1987.

[31]. FDA, Guidance for industry, Bioanalytical method validation. DOI.2001.

[32]. European Medicines Agency, Guideline on bioanalytical method validation. 2011.

[33]. CLSI. C62-A, Liquid chromatography–mass spectrometry methods, Clinical and Laboratory Standards Institute, Wayne, PA, 2014.

[34]. Matuszewski BK, Constanzer ML, Chavez-Eng CM, Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC–MS/MS, Anal. Chem75 (13) (2003) 3019–3030, 10.1021/ac020361s. [PubMed: 12964746]

[35]. Matuszewski B, Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis, J. Chrom. B830 (2) (2006) 293–300, 10.1016/j.jchromb.2005.11.009.

[36]. Botelho JC, Shacklady C, Cooper HC, Tai SS, Uytfanghe KV, Thienpont LM, Vesper HW, Isotope-dilution liquid chromatography-tandem mass spectrometry candidate reference method for total testosterone in human serum, Clin. Chem59(2) (2012) 372–380, 10.1373/clinchem.2012.190934C. [PubMed: 23213081]

[37]. Ricós V, Alvarez F, Cava JV, Current databases on biological variation: pros, cons and progress, Scan. J. Clin. Lab. Inv59 (7) (1999) 491–500.

[38]. Desirable Biological Variation Database specifications – Westgard, Retrieved from https://www.westgard.com/biodatabase1.htm.

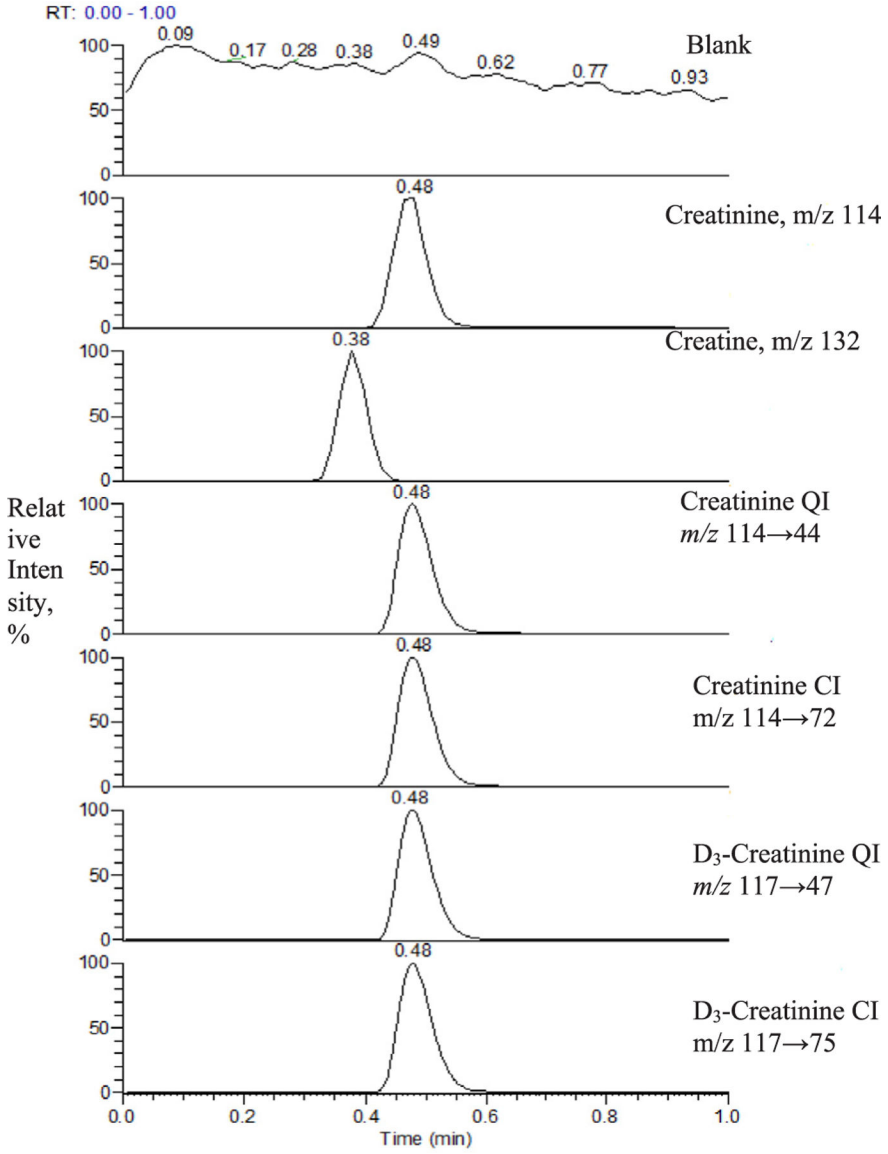

Fig. 1.

Selected ion chromatograms of creatinine in single donor urine sample (46.3 mg/dL). Creatinine quantitation ion (QI) and confirmation ion (CI) transitions are *m*/*z* 114 → 44 and *m*/*z* 114 → 72, respectively. The D_3-creatinine quantitation and confirmation ion transitions are *m*/*z* 117 → 47 and *m*/*z* 117 → 75), respectively. Chromatogram of the blank, and chromatographic separation of creatinine from potentially interfering creatine are also demonstrated. Blank sample was acetonitrile:water mixture (40:60, v/v).

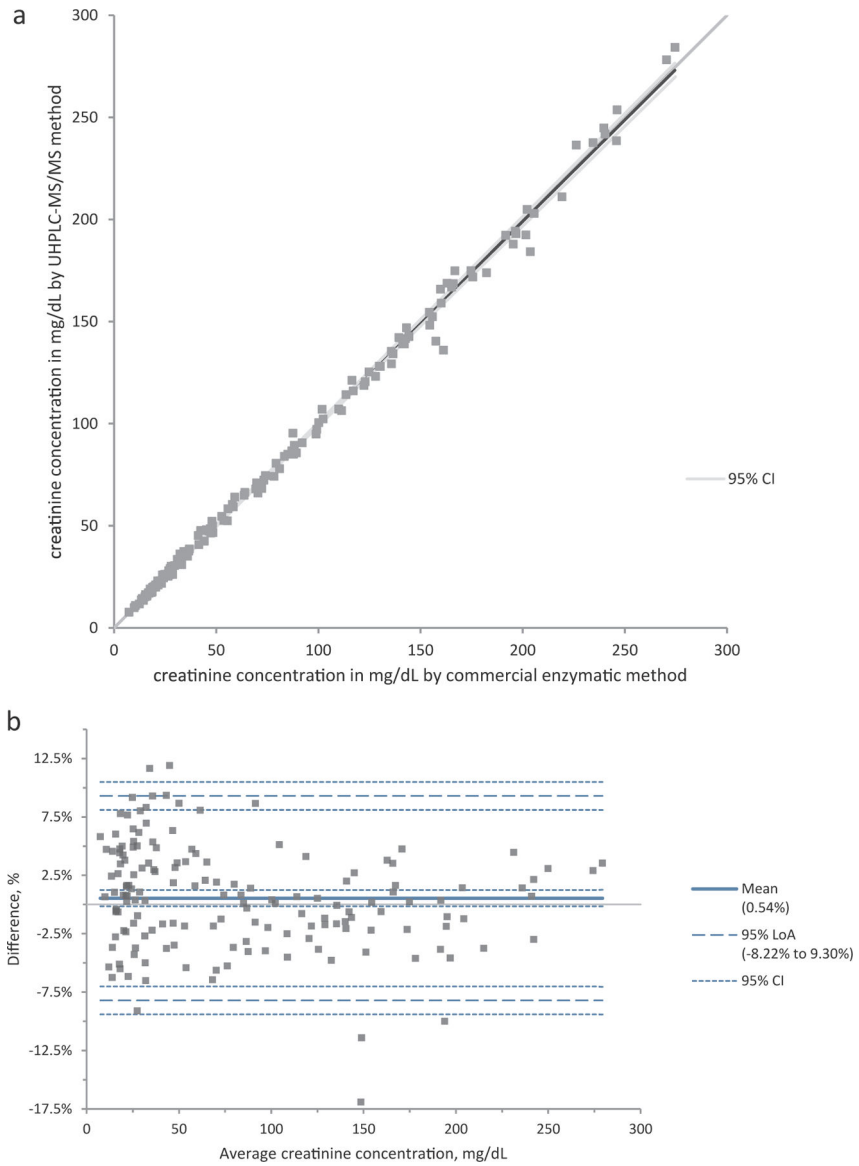

Fig. 2.

Comparison of UHPLC-MS/MS method with a commercial clinical analyzer-based method using 160 human urine samples. 2A. Deming regression analysis 2B. Bland- Altman analysis. Mean creatinine concentrations calculated based on results obtained using both methods. Dashed lines indicate 95% CI and 95% limits of agreement.

Table 1

Comparison of the LC-MS/MS method reported here with previously published mass spectrometry-based and LC-MS/MS methods.

Method	Length of chromatographic run (min)	Multiplexing on LC	Sample volume	Sample preparation strategy
LC-MS/MS method described in this manuscript	1 min	Yes	100 μL	Sample is diluted 225 times (with acetonitrile:water, 40:60, v/v) before analysis
[20]	Flow injection without chromatographic separation	Not available	40 μL	Sample is diluted 250 times (with acetonitrile) and centrifuged before analysis
[21]	Direct flew injection	Not available	50 μL	Sample preparation 1: Sample is diluted 10 times (with 0.01 mol/L HCl) before analysis Sample preparation 2: Sample is diluted 10 times (with acetonitrile:water, 1:1; 0.02% formic acid) and applied to ion-exchange SPE before analysis
[23]	4 min	no	50 μL	Sample is diluted 10 times (with acetonitrile:water, 1:1) before analysis
[24]	4 min	no	Not available	Sample is diluted 100 or 1000 times (with methanol) before analysis
[25]	4 min	no	1 ml	Sample is centrifuged, filtered and diluted 2000 times (with water) before analysis

Table 2

Accuracy compared to Reference Material Accuracy determined by measuring NIST certified reference materials (NIST 3667, 3673 and 3672) in duplicate over 5 days and comparing the measured value against the nominal value.

			Measured concentration, mg/dL								
Reference material	**Replicate**	**Nominal value**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**CV (%)**	**Difference from nominal value (%)**
SRM 3667	1	61.8	59.5	61.1	61.2	63.3	62.7	60.6	2.29	3.78	−1.94
	2		63.1	58.1	58.3	56.8	61.8				
SRM 3673	1	51.5	52.1	53.5	53.6	52.1	51.0	51.1	1.96	3.84	−0.78
	2		49.4	51.3	48.5	47.8	51.4				
SRM 3672	1	74.8	74.9	77.0	75.3	82.7	75.0	74.0	4.14	5.60	−1.07
	2		69.8	68.7	74.0	69.7	72.6				

Table 3

Precision of the method. Within-run and between-run method precision in quality control (QC) samples determined measuring human urine at 3 concentration levels in duplicates over 22 days.

Sample Description	Mean , mg/dL	Within-run precision, %CV	Between-run precision, %CV
Low QC	41.7	3.59	4.01
Medium QC	135	3.49	3.28
High QC	266	2.84	3.57

Table 4

Matrix effects assessment in neat (acetonitrile:water, 40:60, v/v), saline, synthetic urine, low concertation urine and high concentration urine. Matrix Effects (ME) were calculated at seven calibration points.

	Acetonitrile:Water (40:60 v/v)	0.9% Saline	Synthetic urine	Low creatinine human urine (from individual donor)	High creatinine human urine (from individual donor)
Slope	0.011	0.011	0.011	0.011	0.011
R^2	0.9995	0.9994	0.9988	0.9992	0.9982
ME, %		94.14	95.36	95.45	95.73
ME Mean. %	n/a	95.15 (95% CI 91.74–98.55)			