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Creatinine Measurements in 24 h Urine by Liquid Chromatography−Tandem Mass Spectrometry

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A simple, sensitive, and specific liquid chromatography−tandem mass spectrometry (LC−MS/MS) method for determining urinary creatinine was developed and used to evaluate 24 h urine samples collected during an exposure study. Urine (1 μL) was diluted with methanol and then directly applied to LC−MS/MS. Under electrospray ionization (ESI) conditions, the transition molecules of creatinine and creatinine-d_3 were observed at m/z 114 > 44 and m/z 117 > 47, respectively. The retention time of creatinine was 0.59 min. The linear range was 1−2000 ng/mL, with a detection limit in urine of 1 ng/mL. LC−MS/MS and colorimetric end-point methods were significantly associated ($R^2 = 0.8785$, $p < 0.0001$). The LC−MS/MS method to determine creatinine in 24 h urine samples had shorter retention times, was more sensitive, reliable, reproducible, simple, selective, and used a smaller sample size than other LC−MS/MS or commercial methods.

KEYWORDS: Creatinine; urine; LC−MS/MS; validation

INTRODUCTION

Urine is a widely used matrix in biological monitoring because nonpersistent chemicals (i.e., chemicals that have short biologic half-lives, such as pesticides) are excreted through the kidney and the collection is noninvasive and easy (*1–4*). Continuous and complete 24 h urine collection yields more accurate results, because spot urine sampling may not provide a valid overview of the entire pesticide exposure profile (*5–7*). However, 24 h urine collection is not a simple task when compared to spot urine sampling (*6*). Incomplete and improper collection of 24 h urine could result in an underestimation of the exposure profiles of agricultural chemicals, decreasing the potential advantages of the 24 h collection. Because creatinine excretion from the body is relatively constant (*8*), its measurement is an assessment of the completeness of the 24 h urine collection (*6, 9*). The concentrations of absorbed compounds are then corrected to obtain a more accurate picture of exposure (*10–12*). In the case of spot urine collection, urinary creatinine is commonly used to "normalize" the concentrations of absorbed chemicals (i.e., concentrations are reported as the weight of analyte per gram of creatinine). Although there is some disagreement over the value of creatinine measurements for this purpose, these measurements nevertheless continue to be considered integral values in exposure studies. Thus, there is need to accurately measure urinary creatinine.

Several analytical methods to measure urinary creatinine have been reported: the Jaffe reaction method (*13, 14*), an enzymatic method (*15, 16*), a high-pressure liquid chromatography (HPLC) (*17–19*), and liquid chromatography−tandem mass spectrometry (LC−MS/MS) (*20, 21*) method. LC−MS/MS is becoming widely used for a broad range of research and in laboratories performing routine screening (*22, 23*). It is the reference method of choice for creatinine (*24*). The Jaffe reaction or enzymatic methods for determining creatinine have been widely used and modified; however, these methods are adversely affected by endogenous interfering substances (*13, 25*). An interlaboratory comparison of several test methods has demonstrated as much as a 30% difference in measurements, and none of these methods compared acceptably to the reference mass spectrometry method (*26*).

This study was designed to develop a simple, sensitive, and specific method for determining urinary creatinine by the LC−MS/MS. The method was applied to samples collected in an exposure study and compared to the laboratory standard, a colorimetric end-point assay.

MATERIALS AND METHODS

Chemical and Urine Sample Preparation. All chemicals were of analytical reagent grade. Creatinine, creatinine-d_3, and the creatinine colorimetric test reagents were from Sigma (St. Louis, MO). Water (>18.0 MΩ) was purified by a NANO pure II system (Barnstead, Dubuque, IA). A total of 99 24 h urine samples were collected during a exposure study for the herbicide paraquat (*27*) and were stored at −20 °C until analysis. Frozen urine samples were thawed at room temperature. After vortex mixing, an aliquot of urine was spiked with

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the internal standard (creatinine-d_3) and directly diluted 100- or 1000-fold with methanol and analyzed by LC−MS/MS or diluted 10-fold in water for colorimetric analysis.

LC−MS/MS. The LC−MS/MS analysis was carried out using a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, U.K.) equipped with an atmospheric pressure ionization source [atmospheric z-spray pressure chemical ionization (APcI) or electrospray ionization (ESI) interface]. The mass spectrometer was coupled to the outlet of the HPLC system that consisted of a Waters model 2790 separation module (Waters Corporation, Milford, MA), including an autosampler with refrigerated sample compartment and inline vacuum degasser, and a Waters model 2487 dual wavelength absorbance detector. MassLynx software (version 4.0) was used for data processing. The ESI mass spectrometric detection was performed in the positive-ion mode, with a capillary voltage at 1.0 kV. Cone gas (N_2) and desolvation gas (N_2) were maintained at flow rates of 130 and 630 L/h, respectively. The source and the desolvation temperatures were set at 100 and 300 °C, respectively. The optimum cone and collision voltages for creatinine and creatinine-d_3 were 40 and 15 V, respectively. The collision energy of 15 V was employed. Mass spectra of the precursor ions were obtained by syringe pump infusions at the flow rate of 10 μL/min, while scanning over the range of m/z 20−200 at 2 s/scan. Data were acquired in the multichannel analysis (MCA) and continuum modes. Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode, with a dwell time of 600 ms. Ultra pure argon (99.9999%) was used as a collision gas at a pressure of 2.5 millitorr for collision-induced dissociation (CID). An XTerra MS C_{18} column (30 × 2.1 mm i.d., 3.5 μm; Waters Corporation, Milford, MA) was used with a flow rate of 0.3 mL/min at ambient temperature. Isocratic separation was performed with 50% acetonitrile containing 0.1% formic acid. Solvents were filtered through a 0.45 μm membrane and degassed by a vacuum before use. Aliquots (5 μL) of the standard or diluted urine samples containing internal standard were injected onto the LC−MS/MS system. Results from samples were calculated from the calibration curve and corrected for recovery using an internal standardization method.

Quality Assurance. The creatinine standard dissolved in methanol was measured on 3 different days to assess reproducibility. Urine samples (n = 15) were divided in half, and each half (n = 15) was analyzed as a separate sample with the LC−MS/MS on different analysis days.

Creatinine Measurement by a Colorimetric End-Point Assay. The urinary creatinine measurement was also carried out using a colorimetric end-point assay with a commercial kit (Sigma Diagnostics, St. Louis, MO). In principle, creatinine reacts with picric acid under alkaline conditions to form a characteristic yellow−orange complex. Creatinine concentrations were measured in triplicate using a 96-well plate format kinetic assay. Frozen urine samples were thawed and vortexed, and a 100 μL urine aliquot was diluted 10-fold with MilliQ water. Each sample was run against an independent 10 000−150 000 ng/mL calibration curve. Absorbance at 500 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA). Further dilutions were prepared as necessary to bring samples within the calibration range.

Comparison of Colorimetric and LC−MS/MS Methods. The urinary creatinine values (n = 84) determined by LC−MS/MS were compared to those by a colorimetric end-point assay. Results of the method comparisons are presented using Bland−Altman plots (*28*). The plot consisted of the average of the differences and the 95% limits of agreement, which was performed with GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA).

Statistical Analysis. Correlations between the assays and duplicate urine samples were calculated using Pearson's correlation. $p < 0.05$ was selected for statistical significance. Results are reported as mean ± standard deviation. All analysis was performed using GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

The standard curve created using creatinine dissolved in methanol was linear (R^2 = 0.9995) in the analytical range from 1 to 2000 ng/mL. The limit of detection for creatinine dissolved in methanol was 1 ng/mL that was estimated from a signal-to-noise (S/N) ratio of 3. The interday precision of the LC−MS/MS expressed as the coefficient of variation (% CV) measured for the same standard (1 ng/mL) during 3 different days did not exceed 6.0%. Intraday precision of creatinine assayed with standard concentrations of 31, 250, and 1000 ng/mL were 31.6 (±2.8), 243 (±2.1), and 1001.6 (±17.8) ng/mL, respectively. Intra- and interday results showed the method to be reliable.

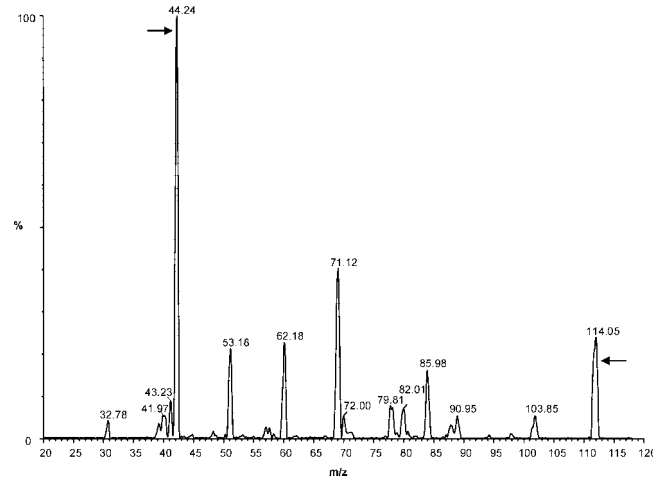

Figure 1. Mass ESI tandem mass spectra of creatinine with parent ion scan (m/z 114) and the product ion (m/z 44).

For the colorimetric assay, the % CV on standards did not exceed 20% and most typically ranged from 5 to 7%. The % CV on five random samples analyzed on 3 or 4 different days did not exceed 20%.

Under the conditions of ESI, the protonated molecules ([M + H]$^+$) of creatinine and creatinine-d_3 were observed at m/z 114 and 117, respectively. The most abundant product ions were m/z 44 for creatinine and m/z 47 for creatinine-d_3, respectively. A tandem mass spectrum of creatinine is shown in **Figure 1**.

The retention time of creatinine was 0.59 min. Chromatograms of creatinine-d_3 and creatinine dissolved in methanol are presented in **Figure 2A**. Both compounds were detected in a diluted urine sample (**Figure 2B**). The chromatograms are nearly identical, indicating little interference from the urine sample. The creatinine elution time in our study was faster than previous reports of 3.015 min (*21*) and 12.4 min (*20*). The rapid elution could result in a peak containing a mixture of compounds including creatinine (m/z 114) and creatine (m/z 132). Selective mass detection should serve to distinguish these because an interference would need to have not only the same chromatography but also the same parent and daughter ions.

The performance of the LC−MS/MS was verified using duplicate blind urine samples. A subset of urine samples (n = 15) was divided in half, and each half was determined independently on different days. The results from blind duplicate samples measured by the LC−MS/MS indicated a good correspondence with a Pearson r = 0.9738 and R^2 = 0.9483 ($p < 0.0001$) (**Figure 3**), which demonstrated that the method is constant, reproducible, and stable for the determination of urinary creatinine.

The use of creatinine has been accepted for determining the completeness of 24 h urine collection and adjustment of absorbed compound concentrations, especially in occupational exposure studies. However, different measurement methods have resulted in major differences in the creatinine concentration (*26*). To check completeness of the urine collection, we measured

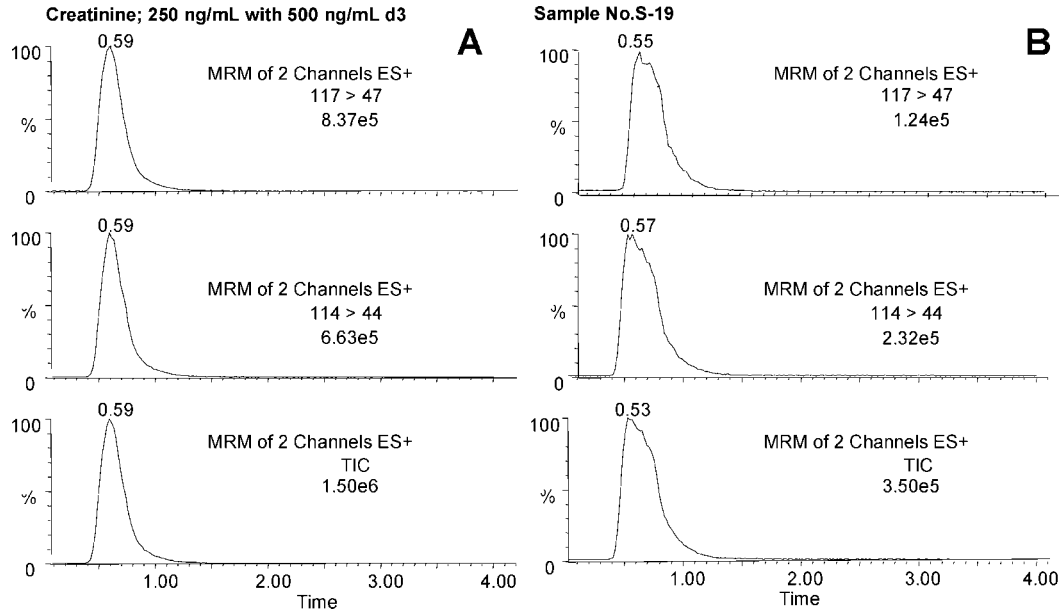

Figure 2. (**A**) Chromatograms of creatinine-d_3 (top), creatinine standard (middle), and total ion (bottom), both eluting from the column at 0.59 min. (**B**) Chromatograms of creatinine-d_3 (top), creatinine (middle), and total ion (bottom) in diluted urine samples, both eluting from the column at 0.57 min.

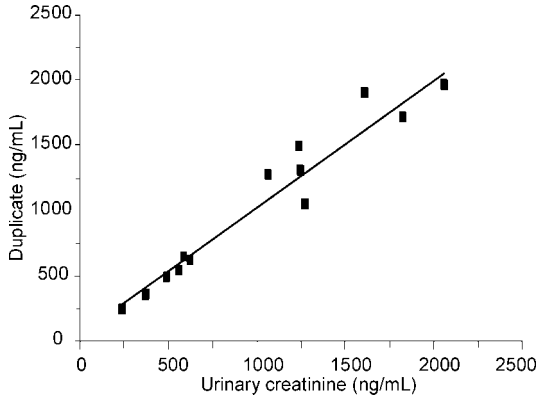

Figure 3. Correlation between duplicate urine samples analyzed by the LC−MS/MS. A set of urine samples ($n = 15$) was divided in half, and each half was analyzed as a separate sample with the LC−MS/MS at different days.

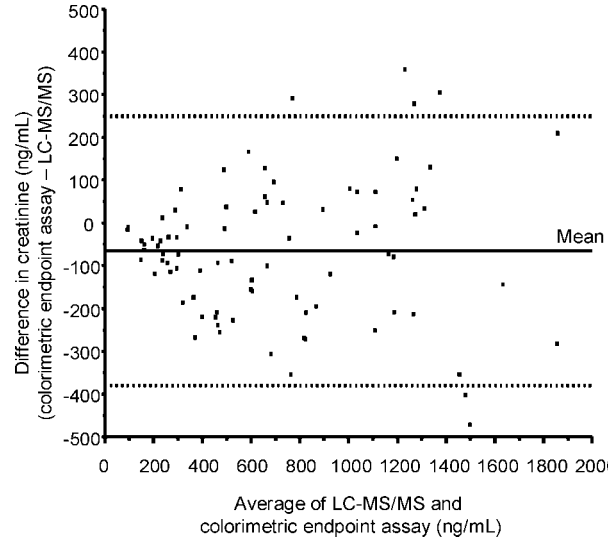

Figure 4. Bland−Altman plot comparing the LC−MS/MS method and the colorimetric end-point method for 84 urine samples. The dotted line indicates the ±1.96 standard deviation.

urinary creatinine in 24 h urine samples using the LC−MS/MS with a simple one-step dilution and the colorimetric methods for the same set of urine samples run ($n = 84$) in a double-blind study. The creatinine values measured by the colorimetric end-point and LC−MS/MS methods were positively associated (Pearson $r = 0.9373$ and $R^2 = 0.8785$, $p < 0.0001$). The Bland−Altman analysis also showed that no systematic difference occurred between the LC−MS/MS and the colorimetric end-point assay (**Figure 4**). Only 7.1% ($n = 6$) of the points were outside of ±1.96 standard deviation. However, there was a slight bias toward lower values for the colorimetric method. Test kits that are applied to serum analysis of creatinine may be compensated to account for the effect of protein, which is known to cause overestimation of creatinine. Because there is little protein in urine samples, urinary creatinine levels measured by the colorimetric method may be underestimated compared to those measured by the LC−MS/MS method in this study.

To determine urinary creatinine, a simple dilution with methanol was used in this study. It was reported that pretreatment of urine with solid-phase extraction was not a necessary step for urinary creatinine measurement and that simple dilution of the urine sample without pretreatment provided high selectivity for creatinine (*21*). The chromatography step and the selectivity of the detector in the LC−MS method obviate the need for a cleanup step. In comparison to previous reports with the LC−MS/MS, our method required less sample (1 μL compared to 50 μL urine) (*20, 21*) because of the 23-fold greater limit of detection. Because urine is generally available in large amounts, a reduction in the sample volume from 50 to 1 μL is not important from a sample standpoint. However, applying a smaller volume to the LC−MS/MS should reduce the impact of the matrix and thus allow more samples to be processed before cleaning is necessary.

The method to determine urinary creatinine using the LC−MS/MS developed in this study had a lower limit of detection than both the previous LC−MS/MS method (*21*) and the colorimetric method (ng/mL versus μg/mL) and thus used a smaller sample volume (1 versus 50 μL). The reliability as assessed by inter- and intraday precision for the LC−MS/MS for standards was <8.0% compared to <20% for the colorimetric assay. Sample preparation (dilution with methanol) was

simpler than methods using solid-phase extraction prior to analysis (*21*). Rapid chromatography and mass selective detection imparted a greater selectivity than the colorimetric assay. The LC−MS/MS method used in this study is clearly more expensive than the colorimetric assay. Nevertheless, the LC−MS/MS could become a more effective tool for measuring pesticide exposures, if the pesticides of interest and creatinine in urine could be measured in the same run.

ABBREVIATIONS USED

APcI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; % CV, percent coefficient of variation; HPLC, high-performance liquid chromatography; ESI, electrospray ionization; LC−MS/MS, liquid chromatography−tandem mass spectrometry; MCA, multichannel analysis; MRM, multiple reaction monitoring; S/N, signal-to-noise ratio.

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