Assessment of $^1$H NMR-based metabolomics analysis for normalization of urinary metals against creatinine

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A B S T R A C T
Background: Proton nuclear magnetic resonance ($^1$H NMR, or NMR) spectroscopy and inductively coupled plasma-mass spectrometry (ICP-MS) are commonly used for metabolomics and metal analysis in urine samples. However, creatinine quantification by NMR for the purpose of normalization of urinary metals has not been validated. We assessed the validity of using NMR analysis for creatinine quantification in human urine samples in order to allow normalization of urinary metal concentrations.

Methods: NMR and ICP-MS techniques were used to measure metabolite and metal concentrations in urine samples from 10 healthy subjects. For metabolite analysis, two magnetic field strengths (600 and 700 MHz) were utilized. In addition, creatinine concentrations were determined by using the Jaffe method.

Results: Creatinine levels were strongly correlated ($R^2 = 0.99$) between NMR and Jaffe methods. The NMR spectra were deconvoluted with a target database containing 151 metabolites that are present in urine. A total of 50 metabolites showed good correlation ($R^2 = 0.7–1.0$) at 600 and 700 MHz. Metal concentrations determined after NMR-measured creatinine normalization were comparable to previous reports.

Conclusions: NMR analysis provided robust urinary creatinine quantification, and was sufficient for normalization of urinary metal concentrations. We found that NMR-measured creatinine-normalized urinary metal concentrations in our control subjects were similar to general population levels in Canada and the United Kingdom.

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1. Introduction

Metal concentrations in urine samples of workers are commonly used in occupational health as markers of exposure to environmental insults such as welding fumes [1–5] or residual oil fly ash [6], since collection of air samples for exposure measurement is not always practical. Urinary metal concentrations are routinely normalized to creatinine levels to account for variability and the hydration status of subjects [7–10]. In addition, measurement of urinary creatinine levels allows for selection or rejection of samples for monitoring purposes. The World Health Organization (WHO) has adopted guidelines to accept urine samples that have creatinine levels of 0.3–3.0 g/l, and any sample outside these limits must be rejected for normalization [11]. The American Conference of Governmental Industrial Hygienists (ACGIH) publishes guidance values for urine samples based on normalized metal concentrations [12]. Although these values are not always linearly correlated to exposure levels, they provide guidance for occupational health specialists to verify if workers may have been overexposed to toxic environmental threats at their workplace. It is therefore important to assess creatinine concentrations accurately and precisely in urine samples.

Generally, clinical quantification of urinary creatinine is done by various spectrophotometric methods [13–15]. The standard analytical technique used for creatinine quantification is the Jaffe reaction method [13,16,17]. Other methods may be used to assess creatinine levels, including proton nuclear magnetic resonance ($^1$H NMR, or NMR) analysis, which has become increasingly important for metabolomic studies of human biological samples [18–22]. Several features make NMR a versatile method for metabolite analysis in biological fluids including urine samples. Samples are typically analyzed by NMR without the need for derivatization and/or column separation, and in particular, this technique affords the ability to noninvasively and simultaneously analyze numerous metabolites in an individual sample [23]. However, many experimental and instrumental variables, especially dilution factors of urine based on the hydration status of subjects, can affect the observed
intensities of metabolites in NMR analysis of urine samples. In addition, the type of spectrometer used as well as the particular NMR parameters can also influence the results [24]. Nicholson et al. [25] pioneered the use of high-field NMR spectroscopy to analyze low molecular weight compounds in human urine. In another study focused on creatinine levels determined by NMR, Bales et al. [26] used 400 and 500 MHz spectrometers to determine creatinine concentrations in five replicate urine samples. These estimates agreed closely with corresponding results obtained by the Jaffe method; however, these did not show correlation values between NMR and the colorimetric Jaffe method for measurement of creatinine. While NMR spectroscopy has been shown in recent studies to be useful for determining urinary creatinine levels for the purpose of medical diagnosis [27–29], few attempts have been made to validate NMR-based creatinine measurements in comparison with standard methods of creatinine quantification. Moreover, to our knowledge, validation of NMR measurements of urinary creatinine for the purpose of normalization of urinary metal concentrations has not been reported.

With successive improvements to NMR software and hardware over the past 30 y, we have seen a dramatic improvement in spectral detail and metabolite recognition. Consequently, to consider urinary creatinine values obtained from NMR analysis for normalization of urinary metals, it is important to optimize and validate the quantification of creatinine with NMR by using higher magnetic fields, and compare these values with those obtained with the gold standard Jaffe reaction method.

2. Materials and methods

Urine samples were collected from 10 healthy individuals (2 males and 8 females, aged 20–53 y) at the Pulmonary Research Group (Department of Medicine, University of Alberta). All samples were collected in the early morning following 12 h of fasting using standard collection protocol. Samples were collected in pre-cleaned polystyrene urine collection bottles and placed at 4 °C until processed for ICP-MS and NMR studies.

2.1. Sample processing for metal analysis

Within 3 h of collection, 500 μl of each sample was pipetted into 15 ml pre-cleaned polyethylene tubes. The samples were made up to 10 ml by adding 9.4 ml of distilled water and 100 μl of nitric acid. Samples were kept at 4 °C before analysis by ICP-MS. On the day of analysis, samples were warmed to room temperature under a laminar flow hood and vortexed for 1 min to obtain a homogeneous solution. Reference standard solutions were prepared by pipetting 500 μl of both low- and high-level certified reference materials (CRM), Clinchem I and II (Recipe) into 15 ml pre-cleaned tubes along with 9.4 ml distilled water and 100 μl of nitric acid. CRM samples were analyzed at the start and end of each analytical run.

All sample analyses were conducted using ICP-MS at the “SWAMP Laboratory”, Department of Renewable Resources, Faculty of Agricultural, Life & Environmental Sciences (University of Alberta). All elements were determined on an iCAP-Q ICP-MS (Thermo Scientific). The typical ICP-MS conditions were as follows: PFA-400 Nebulizer (Elemental Scientific) was used to nebulize the solution, with a sample introduction speed of 400 μl/min. The spray chamber was cooled to 2.7 °C to prevent larger particles from entering the torch. Four replicates of blank samples were analyzed and the average value of the blanks was subtracted from each sample.

2.2. Sample processing for NMR

Within 3 h of collection, 10 ml of each urine sample was transferred into a separate sterile 15 ml conical centrifuge tube and centrifuged at 2000 rpm, 4 °C for 10 min. Supernatants were transferred into a new 15 ml sterile conical tube without disturbing the bottom of the tube. Samples were then frozen at −80 °C until NMR analysis. On the day of NMR analysis, the samples were thawed at room temperature, vortexed for a minimum of 5 min to ensure uniformity, and then placed on ice. A test sample of 2.0 ml from each participant was prepared using 1.8 ml urine added to 200 μl of internal standard (IS) solution (IS-1, Chenomx IS: DSS with added imidazole), followed by adjustment to pH 7.0 ± 0.25 using minimal appropriate volumes of NaOH. A portion of each sample was transferred into a 3 mm NMR tube (250 μl) and a 5 mm NMR tube (750 μl) (Wilmad Labglass).

Using 5 mm NMR tubes, NMR spectra were acquired at the NANUC facility on an Oxford 14.09 Tesla (600 MHz) VNMRS spectrometer equipped with a 5-mm inverse-proton (HX) probe with z-axis gradient coil and Varian 768AS robotic system. One-dimensional NMR spectra were collected at 25 °C by using the first increment of a 2-dimensional-1H,1H-NOESY with a transmitter presaturation delay of 900 ms for water suppression and 100 ms mixing time and a spectral width of 7200 Hz. All spectra were collected with 8 steady-state scans, an acquisition time of 4 s, a 90° proton pulse of ~10.7 μs, and the number of acquired scans was 256 per free induction decay. The data were apodized with an exponential window function corresponding to a line broadening of 0.1 Hz, zero-filled to 64 complex points, Fourier-transformed, phased, and baseline-corrected for further analysis. Reference deconvolution using the methyl peak of DSS as peak of reference was used to correct for line shapes.

1H NMR spectra were also obtained at the Chemistry NMR Facility, Department of Chemistry, University of Alberta on a 16.44 Tesla (700 MHz) Agilent VNMR 4-channel spectrometer utilizing a 5 mm inverse-detection, cryo-cooled HCN triple resonance probe with Z-gradients. Novel 5 mm to 3 mm adaptors were used for the ceramic spinners allowing use of 3 mm NMR tubes. The spectrometer was equipped with an Agilent 7620 automatic sample handling system and operated by VNMRJ 4.2 software. Each sample was individually tuned and matched, with shimming comprising first z-axis gradients and then a software version of VNMRJ pre-released human simulated iterative shimming containing extensive in-house modifications. The temperature of the probe head was calibrated for samples to be exactly 25 °C.

2.3. Sample preparation for creatinine assay

The most common clinical method used for creatinine estimation in urine samples is the standardized Jaffe reaction method [16]. Urine samples were sent to the Department of Laboratory Medicine and Pathology, University of Alberta for Jaffe method analysis for validation.

2.4. Statistical analysis

Metabolite identification and quantitation for all NMR spectra was done using Chenomx NMR Suite 8.0 software. Baseline correction was done by adjusting the peak to an internal reference standard (DSS). After quantification was established, NMR data sets were analyzed by subtracting the mean value of each metabolite concentration from the individual concentration value of respective metabolite and then dividing the product by the standard error of the mean (SEM) for each metabolite [24]. Urinary metabolite concentrations determined at 600 and 700 MHz were compared by linear regression (Pearson’s correlation) using Prism 6 (GraphPad Software, Inc.). Bland-Altman (BA) plots for urinary creatinine obtained for NMR (600 and 700 MHz) and the Jaffe method were also constructed using Prism 6. Further, urinary metal concentrations were normalized to creatinine concentrations to adjust for variability in urine dilution due to hydration states of subjects at the time of sample collection. Uncorrected values were expressed in μg/l and creatinine-corrected values in μg/g creatinine or μmol/mmol creatinine.
3. Results

3.1. Correlation between NMR analysis and the Jaffe reaction method for quantification of creatinine in human urine

Creatinine concentrations of 10 urine samples in our study, measured using the standard Jaffe reaction method, were distributed within a normal range (i.e. 0.8–3.0 g/l) and therefore all samples were considered valid for further analysis and normalization as specified by WHO guidelines [11]. Creatinine concentrations in urine samples obtained by the Jaffe reaction method were compared to creatinine levels determined by NMR in Fig. 1A (600 MHz) and Fig. 1B (700 MHz). A strong correlation between these 2 methods was observed with R-squared values of 0.988 and 0.984, respectively.

Further, we determined values for bias from comparison of clinical measurement of creatinine values obtained with the Jaffe reaction method and NMR measurements, using BA plots. BA plots are used to test the efficacy of two different methods for measurement of a given clinical marker. These plots quantify bias and provide a 95% confidence limit for the bias (represented by dotted lines) [30]. If there is no difference between two methods for measuring creatinine, then the bias should be close to zero.

Fig. 2A and B shows the BA plots constructed from comparing the Jaffe method with NMR measurements for creatinine assessment at 600 MHz and 700 MHz, respectively. Negative values of 1.300 and 1.480 for bias were found, which implies that, on average, the NMR method measures ~1 mM (~0.1 g/l) less creatinine than the Jaffe reaction method across the 10 samples. However, the variability between the two methods is well within the range of the estimated 95% confidence limit, and further, the % bias represents less than 20% of the total urinary creatinine concentrations detected in our samples (range 0.8–3.0 g/l).

Similarly, Fig. 2C illustrates the BA plot obtained for the NMR technique performed with two different magnets (600 and 700 MHz) based at our campus. A bias of 0.18 showed that there were negligible differences between creatinine values obtained in the same urine samples by 600 and 700 MHz.

3.2. NMR identification and quantification of metabolite concentrations in human urine samples

A typical NMR spectrum of urine obtained from a healthy subject is shown in Fig. 3, with an abundance of peaks that indicate a plethora of metabolites. The urine spectra in this study were deconvoluted with a database containing 151 metabolites that were chosen based on known urinary metabolites, using a computer-based algorithm. This number of metabolites is similar to those reported in previous studies estimating that ~200 compounds are potentially detectable in human urine by NMR analysis [31,32]. Metabolites obtained at 600 and 700 MHz were compared by assessing Pearson linear regression curves for all metabolites in our study.
Fig. 3. Typical 600 MHz NMR spectrum of control human urine sample. An example NMR spectrum is shown that was collected on a Varian VNMRS 600 MHz spectrometer. Several of the abundant organic metabolites are indicated. DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) represents the internal standard peak calibrated at 0 ppm.

A

Glycine

\[ y = 1.0258x + 0.0325 \]

\[ R^2 = 0.9997 \]

B

Creatinine

\[ y = 1.0139x + 0.5509 \]

\[ R^2 = 0.9973 \]

C

Methionine

\[ y = 0.4173x + 0.0004 \]

\[ R^2 = 0.5995 \]

Galactose

\[ y = 0.4359x + 0.0471 \]

\[ R^2 = 0.5105 \]

Sarcosine

\[ y = 0.8310x + 0.0201 \]

\[ R^2 = 0.1245 \]

Lysine

\[ y = 0.1526x + 0.1186 \]

\[ R^2 = 0.0713 \]

Fig. 4. Pearson’s correlation plots for selected metabolites. Representative metabolites showing a (A) high degree of correlation \((R^2 = 0.7–1.0)\); (B) moderate degree of correlation \((R^2 = 0.5–0.7)\); and (C) low degree of correlation \((R^2 < 0.5)\). All data indicate correlations for metabolite concentrations \((\text{mM})\) acquired using 600 and 700 MHz magnets.
Pearson linear regression plots of selected urinary metabolites with high \((R^2 = 0.7\text{--}1.0)\), moderate \((R^2 = 0.5\text{--}0.7)\), and low \((R^2 < 0.5)\) degrees of correlation between 600 and 700 MHz magnets are shown in Fig. 4. In addition to their respective \(R^2\) values, bias may be ascertained from the slope linearity of each metabolite. Based on \(R^2 \) (0.7\text{--}1.0) values and bias \((<0.50)\), 50 metabolites exhibited a strong correlation between the two magnetic fields used for NMR analysis. This represents 33\% of the total urinary metabolites used for deconvolution in our study. The list of metabolites with their \(R^2\) values is shown in Supplementary Data Table S1.

3.3. Identification and quantification of metals present in human urine samples by ICP-MS

To assess the validity and reproducibility of urinary metal analysis in our samples, we determined metal levels in quality control CRM samples by ICP-MS. Table S1 (Supplementary Data) shows the quality control data for CRM levels 1 and 2 with their respective percentage recoveries. Except for Pb, metals were within an acceptable range of percentage recovery (80\%--120\%).

Normalized metal concentrations (in \(\mu\)mol/mol creatinine, determined by 600 MHz NMR in this study) are shown in Table 1 along with their respective limit of quantification (LOQ) values. All urinary metals were found to have concentrations well above their respective LOQ values. NMR-measured creatinine was used to normalize trace metals that could result in elevated blood, glucose, ketones, or protein levels in urine. However, we speculate that several biomolecules and proteins present at abnormally high levels in urine from diseased or otherwise unhealthy subjects could interfere with the measurement of metabolites by NMR or the Jaffe method. For example, proteins and biomolecules that bind to metal ions exhibit altered NMR spectra [35, 36], and in occupational hazards or diseases that result in proteinuria or hematuria [37,38], there may be abnormal constituents or drugs that may interfere with the Jaffe method [39]. Thus, a limitation of our study is that we measured urinary metabolites and metals only in normal, healthy individuals, with no apparent disease or associated conditions that could alter the measurement of these components in their urine samples. Therefore, a re-validation of these techniques is required in disease states to confirm the observed high correlation of creatinine levels measured by NMR and the Jaffe method in normal urine samples [39,40].

We found that 33\% of urinary metabolites showed a strong correlation (0.7\text{--}1.0) between two different magnets. The diminished correlation values for the remainder of urinary metabolites were not due to differences in peak-fitting methods, since their spectra were identically analyzed using Chenomx NMR Suite software. Several possible reasons exist for the observed reduction in correlation values for a large number of urinary metabolites measured on two different magnets. Firstly, a proportion of the error in metabolite measurements may be caused by a number of unknown compounds in the Chenomx software used for the 700 MHz results, which has not yet had a dedicated metabolite library established at this field strength. Second, discrepancies in metabolite measurements may be due to spectral interference that preclude accurate quantification, particularly in spectral regions that have significant and overlapping clusters of peaks. Finally, many metabolites are present at insufficiently low levels in urine samples to allow detection by NMR. Thus, these findings indicate that specific criteria must be established for the selection of metabolites using appropriate quality control samples in NMR metabolomics analysis.

Our study demonstrates that NMR may be used to evaluate creatinine concentrations for the purpose of urinary metal normalization. Two NMR spectrometers with different field strengths and probes were reproducibly shown to quantify creatinine levels in urine samples. Creatinine concentrations measured by NMR exhibited strong correlations, along with low bias values, when compared with the standard Jaffe reaction method. This suggests that the use of NMR-measured creatinine levels may be applied to normalization of urinary metals, as well as other urinary metabolites within the same sample.

The subjects in this study were free of any metabolic or other disorders that could result in elevated blood, glucose, ketones, or protein levels in urine. However, we speculate that several biomolecules and proteins present at abnormally high levels in urine from diseased or otherwise unhealthy subjects could interfere with the measurement of metabolites by NMR or the Jaffe method. For example, proteins and biomolecules that bind to metal ions exhibit altered NMR spectra [35, 36], and in occupational hazards or diseases that result in proteinuria or hematuria [37,38], there may be abnormal constituents or drugs that may interfere with the Jaffe method [39]. Thus, a limitation of our study is that we measured urinary metabolites and metals only in normal, healthy individuals, with no apparent disease or associated conditions that could alter the measurement of these components in their urine samples. Therefore, a re-validation of these techniques is required in disease states to confirm the observed high correlation of creatinine values measured by NMR and the Jaffe method in normal urine samples [39,40].

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Our previous study on the effects of NMR calibration stated that special attention to appropriate calibration settings in cross-center studies is critical for obtaining reproducible results [24]. Small changes in specific parameters such as saturation power levels may significantly influence NMR resonance signal intensities, and thus alter metabolite quantification. We ensured that these parameters were appropriately calibrated in our present study for comparison of identical urine samples between 600 and 700 MHz magnets.

**Table 1**

Trace element levels in urine of healthy human control subjects \((n = 10)\) and comparison with UK and Health Canada reports.

<table>
<thead>
<tr>
<th>Element</th>
<th>LOQ (μg/l) in urine samples</th>
<th>Current study creatinine ((\mu)mol/mol) ((n = 10))</th>
<th>Median</th>
<th>95th percentile</th>
<th>UK study creatinine ((\mu)mol/mol) ((n = 132))</th>
<th>Median</th>
<th>95th percentile</th>
<th>Health Canada Survey creatinine ((\mu)mol/mol) ((n &gt; 5400))</th>
<th>Median</th>
<th>95th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>2.74</td>
<td>50.11</td>
<td>104.90</td>
<td></td>
<td>18.48</td>
<td>215.19</td>
<td></td>
<td>19.73</td>
<td>102.31</td>
<td></td>
</tr>
<tr>
<td>As</td>
<td>0.01</td>
<td>10.46</td>
<td>30.23</td>
<td></td>
<td>19.07</td>
<td>254.43</td>
<td></td>
<td>1.07</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>Be</td>
<td>0.21</td>
<td>3.53</td>
<td>6.44</td>
<td></td>
<td>0.077</td>
<td>0.221</td>
<td></td>
<td>0.39</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>0.004</td>
<td>0.11</td>
<td>0.79</td>
<td></td>
<td>0.17</td>
<td>0.57</td>
<td></td>
<td>0.39</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>0.003</td>
<td>0.72</td>
<td>1.94</td>
<td></td>
<td>0.50</td>
<td>2.47</td>
<td></td>
<td>0.39</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>0.02</td>
<td>0.97</td>
<td>1.41</td>
<td></td>
<td>0.92</td>
<td>2.85</td>
<td></td>
<td>0.39</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0.13</td>
<td>18.20</td>
<td>26.74</td>
<td></td>
<td>18.66</td>
<td>35.41</td>
<td></td>
<td>18.91</td>
<td>36.35</td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>0.10</td>
<td>0.54</td>
<td>1.01</td>
<td></td>
<td>&lt;LOQ</td>
<td>1.31</td>
<td></td>
<td>0.19</td>
<td>1.44</td>
<td></td>
</tr>
<tr>
<td>Mo</td>
<td>0.14</td>
<td>49.42</td>
<td>139.65</td>
<td></td>
<td>39.91</td>
<td>106.48</td>
<td></td>
<td>50.94</td>
<td>143.35</td>
<td></td>
</tr>
<tr>
<td>Ni</td>
<td>0.08</td>
<td>3.44</td>
<td>5.32</td>
<td></td>
<td>5.01</td>
<td>10.66</td>
<td></td>
<td>2.54</td>
<td>8.69</td>
<td></td>
</tr>
<tr>
<td>Pb</td>
<td>0.004</td>
<td>0.06</td>
<td>0.17</td>
<td></td>
<td>0.31</td>
<td>4.07</td>
<td></td>
<td>0.32</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.03</td>
<td>0.44</td>
<td>0.92</td>
<td></td>
<td>4.59</td>
<td>10.69</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
In the last part of the study, metal quantification in human urine of the 10 subjects was performed by ICP-MS. All metal concentrations were normalized to creatinine as measured by NMR on our 600 MHz magnet. While most median metal concentrations presented similar values compared to those in the UK and Health Canada reports, median concentrations appeared to differ for Be, Pb and V. In particular, the median concentration of Pb was five times lower in our study than in UK and Canada studies. However, Pb measurement is problematic in urine samples. Guolson et al. [41] showed that urinary Pb concentrations in a 50-year-old male subject could vary by a factor of 5 over a 24-month period. Moreover, we determined that Pb demonstrated a lower than acceptable percentage of recovery in our CRM samples, suggesting that Pb levels were underestimated in our urine samples. Thus, urinary Pb measurements are unlikely to be accurate or precise using our analysis.

We also found that the median concentration of urinary Be was higher, while V was lower, in our study compared with the UK study. These discrepancies may be explained by possible contamination during sampling or error in analysis due to extremely low concentrations of these metals in urine [41–43]. Moreover, ICP-MS may not be sensitive enough to accurately determine very low levels of urinary Be and V.

The 95th percentiles for some metals, such as Al and As, were considerably increased in the UK study compared to their respective 95th percentiles in our group of 10 subjects. These discrepancies may be explained by large deviations in concentrations for several elements, as the UK study was carried out on a larger group of people (n = 132). Similarly, the larger 95th percentile value for several analyte concentrations observed in Canadian population may be attributed to a very large sample size (n = 5,400).

5. Conclusions

In this study, we report the validity of using NMR-based metabolomics as a robust and reliable tool for measurement of urinary creatinine for the purpose of normalization of urinary metal concentrations. It was found that this method allows for both the precise and accurate quantification of creatinine concentrations, without the need for complex sample treatment and preparation, in order to normalize urinary metal concentrations. In addition, comparison of 600 and 700 MHz magnet measurements demonstrated that special attention must be given to quantification of urinary metabolites with a computer-based algorithm. Finally, metal concentrations observed in our urine samples were similar to values obtained from the general population in both Canada and the United Kingdom.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2016.10.037.

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