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A ^{1}H-NMR based Method for Quantifying Creatinine in Urine Samples from Human Intervention Studies

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ABSTRACT

To overcome problems arising from the coloration of urine samples and the limitations of the picric acid method when determining creatinine in urine, we have established an alternative method using ^{1}H-NMR. The method is practically straight forward, provides good recoveries of 105 ± 2%, and a linear regression yielded an R^2 value of 1. The method revealed a similar precision to the standard procedure based on the Jaffé reaction for urine samples without interfering components.

Keywords: anthocyanins, creatinine, ^{1}H-NMR, human interventions, urine

INTRODUCTION

Many intervention studies involve collecting urine samples and analyzing their contents of selected biomarkers. However, the measured concentrations of excreted pharmaceuticals and other analytes can vary due to variation in urinary volumes. This problem can be overcome by simultaneously determining the samples' contents of creatinine and normalizing the levels of the other analytes against that of creatinine [1]. The level of creatinine in the urine is an indicator of the glomerular filtration rate and kidney function [2], and its excretion correlates with the volunteers' muscle mass, which is usually constant. If the amount of creatinine released per day is known, urine samples collected at different intervals can be normalized based on their creatinine contents, concentrations, and volumes [3]. A first method for quantifying creatinine was reported by Jaffé in 1886 [4]. In 1914, Otto Folin reported a colorimetric method for quantifying the creatinine content of a urine sample by treating it with alkaline picrate to form an orange-colored Meisenheimer complex [5]. Over the last 130 years, this method has been widely used to determine creatinine in urine, plasma, and serum samples [6-9]. More recently, kits using 96-well plates have been developed, making it possible to quickly and easily determine creatinine levels in multiple samples simultaneously. Unfortunately, because this method relies on photometric determination of the Meisenheimer complex at 490-530 nm, it is subject to interference caused by various compounds including urinary metabolites [2, 10-12]. For example, certain antibiotics and biomolecules such as bilirubin, glucose and acetone reportedly interfere with the Jaffé method [13], leading to false positive results. In addition, the method is not applicable to some colored urine samples. Such samples may be produced by individuals who have recently ingested and excreted colored food components that absorb in the same range as the Meisenheimer complex, such as anthocyanins from red-colored fruits and vegetables [14]. An alternative way of determining the creatinine content of such samples is to use HPLC-MS. We have developed a reliable HPLC-MS/MS method using an isotopically labeled standard for this purpose [15]. Another method reported to quantify creatinine in urine is the use of capillary zone electrophoresis [16], but these analyses are costly to perform. Therefore, we aimed to develop a fast and less expensive ^{1}H-NMR method for quantitative determination of creatinine in urine samples.

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MATERIALS & METHODS

Chemicals

Deuterium oxide (99.96 atom % deuterium), 3-trimethylsilyl propionic acid-2,2,3,3-d_4 (TSP, deuteration degree ≥ 98 %), reference creatinine (anhydrous, purity ≥ 98%), picric acid (purity ≥ 98 %), and sodium chloride were purchased from Sigma-Aldrich (Taufkirchen, Germany).

^{1}H-NMR Measurements of Creatinine

Stored urine samples (-80 °C) were thawed in a water bath at 37 °C (Köttermann, Uetze, Germany). The reference 3-trimethylsilyl propionic acid (TSP) was weighed on a high precision scale (XP6U Mettler Toledo, Germany) and then dissolved in deuterium oxide to produce a standard solution (c = 0.5 mg/ml TSP; 75.3 mg TSP in 150.6 ml D_2O). A 1.5 ml vial (Eppendorf, Hamburg, Germany) was charged with 400 µl urine and 400 µl of the TSP standard solution, which were mixed thoroughly and stored at 4 °C. The mixed solution was then transferred to an NMR tube and stored at 4 °C prior to analysis.

The samples were analyzed in duplicate by ^{1}H-NMR at 600 MHz (Avance, Bruker, Karlsruhe, Germany). A water suppression technique was used due to the samples' with high water content.

The acquired spectra were evaluated using the Bruker Topspin software package. First, the chemical shift of TSP was set to 0 ppm. Then, the creatinine signal at 3 ppm was used for quantification by comparing the integral of the 3 ppm creatinine peak to that of the TSP peak. Specifically, the concentration of TSP (M = 172.27 g/mol) was known to be 0.5 mg/ml, which corresponds to a molar concentration of 2.9024 µmol/ml. The equivalent mass concentration of creatinine (M = 113.2 g/mol) is 0.3284 mg/ml. However, TSP contains 9 hydrogen atoms whereas creatinine has 3, so the mass concentration of creatinine in the sample is equal to the three times the product of 0.3284 mg/ml and the ratio of the creatinine signal integral to the TSP integral.

Photometric Quantification

The spectrophotometric determination of creatinine is based on the formation of a colored complex via the Jaffé reaction [4, 5]. Urine samples (without any of the previously mentioned interfering substances) were diluted 1:4 with bidistilled water. Then, 1000 µl of picric acid solution (9.0 g NaCl, 1.31 g picric acid in 1 liter water) was added to 400 µl of diluted urine and mixed in a cuvette. To start the reaction, 400 µl of sodium hydroxide solution (1 molar) was added and incubated for 10 min. The absorption of the Meisenheimer complex was measured at 510 nm using a UV-VIS spectrophotometer (Shimadzu, Duisburg, Germany). In a second step 5 µl acid was added to destroy the complex. After 20 min incubation the sample's extinction was measured, and the difference between the two values was calculated and used for creatinine determination. Urine samples were analyzed in duplicate, and the range of the calibration curve was 30 to 1100 µM creatinine (R^2=0.99).

Validation of the ^{1}H-NMR Method

To check the linearity of the quantification method, creatinine solutions in deuterium oxide were prepared at three concentrations (0.5, 1.0 and 1.5 mg/ml), mixed with TSP (0.5 mg/ml), and analyzed by ^{1}H-NMR. The recovery of the measurements was 105 ± 2%, and a linear regression yielded an R^2 value of 1. To demonstrate the precision of the method a standard solution of creatinine (1 mg/ml) was measured in triplicate. The results after ^{1}H-NMR measurements ranged from 0.96 to 1.01 mg/ml with an average deviation of 0.05 mg/ml and a standard deviation of 0.03 mg/ml and shows a deviation of 3% of the concentration used. The standard deviation from run to run with 10 different samples ranged from 0.6 to 3%. Furthermore, the recovery of creatinine in the urine samples was determined by adding a creatinine standard. First, the urine's endogenous creatinine content was measured, and found to be 0.14 mg/ml. Exogenous creatinine was then added to the urine sample to increase its creatinine concentration by 0.5 mg/ml and the modified sample's creatinine concentration was measured again, yielding a value of 0.65 mg/ml, which was 102% of the expected concentration. Finally, the precision of the quantitative ^{1}H-NMR method was compared to that of the Jaffé reaction by using both methods to determine the creatinine contents of 10 different urine samples in duplicate. The average deviation between the two methods was 4 ± 2% (**Table 1**).

Table 1. Determination of creatinine levels in urine samples by quantitative ^{1}H-NMR and the Jaffé reaction

Sample	^{1}H-NMR creatinine [mg/ml]	Jaffé creatinine [mg/ml]	SD
1	0.15	0.15	/
2	0.30	0.31	0.01
3	0.32	0.30	0.02
4	0.33	0.32	0.01
5	0.35	0.37	0.02
6	0.44	0.44	/
7	0.87	0.92	0.05
8	1.31	1.35	0.04
9	1.38	1.44	0.06
10	1.69	1.78	0.09

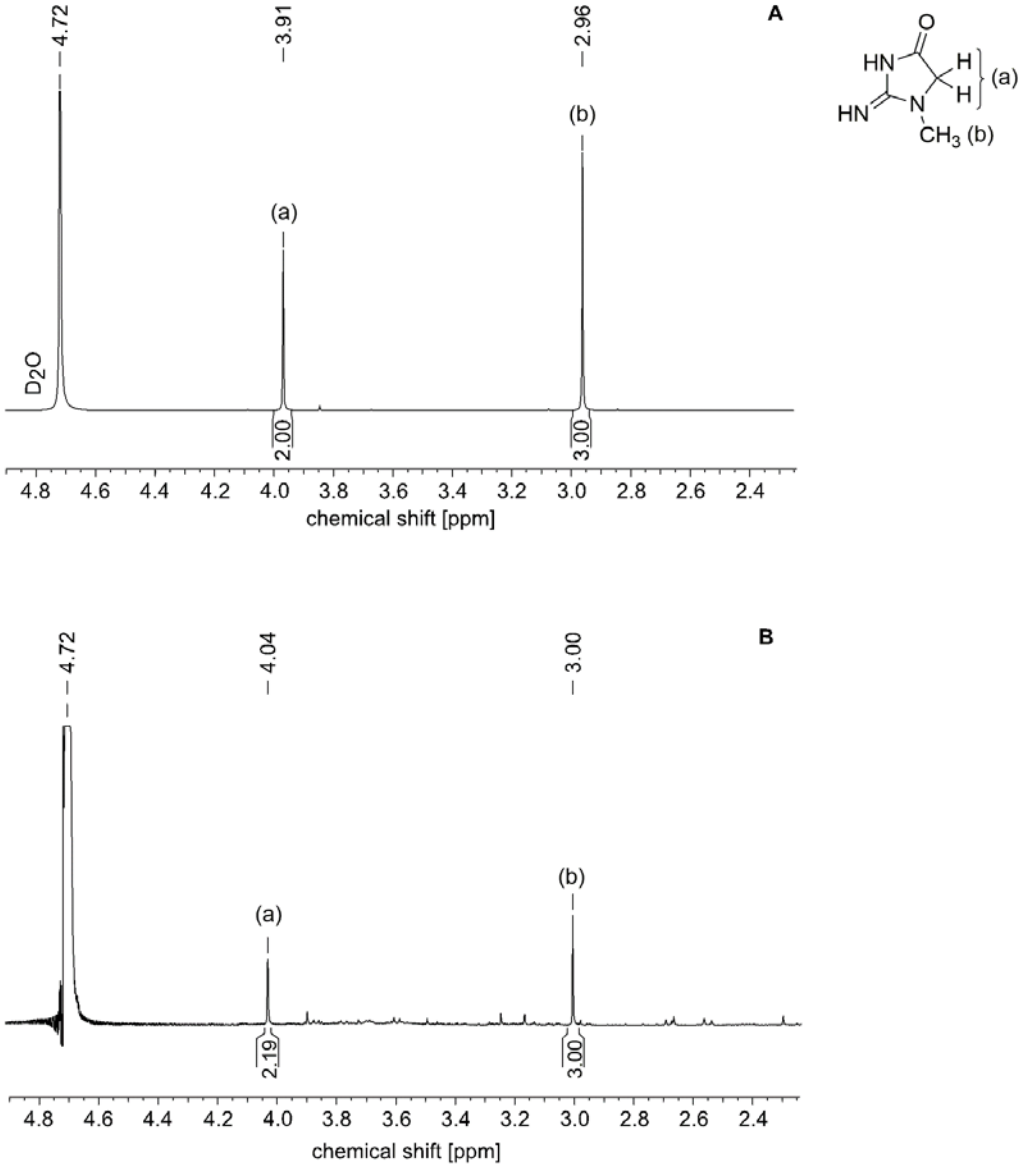

Figure 1. A: ^{1}H-NMR spectrum of creatinine (1 mg/ml) in deuterium oxide and B: ^{1}H-NMR spectrum of a typical urine sample at 600 MHz with water suppression

RESULTS

Because creatinine is a major constituent of urine and produces two characteristic NMR signals, the method presented here is a good alternative to existing methods for quantitating creatinine in urine samples. **Figure 1** shows the ^{1}H-NMR spectrum of the creatinine standard in deuterium oxide (A) and the spectrum of a urine sample (B).

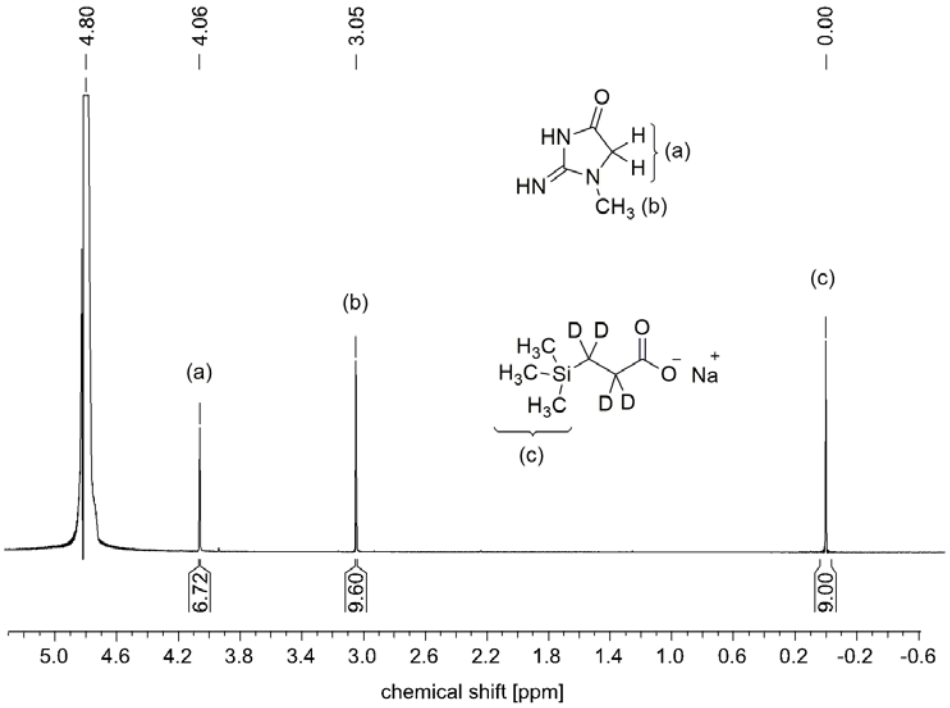

Figure 2. ^{1}H-NMR spectrum of urine (600 MHz, water suppression) with added standard TSP (0 ppm) for quantification of creatinine concentration. The signals a (4.06 ppm) and b (3.05 ppm) represented the marked protons of creatinine and the signal c (0.00 ppm) represented the marked protons of TSP

The creatinine peaks in the 600 MHz ^{1}H-NMR spectra of the urine samples were assigned unambiguously by comparison to the spectra of the standard. The quantification of creatinine in urine samples by ^{1}H-NMR requires the addition of an internal standard to the studied sample. This standard should be chemically stable under the assay conditions, not present in urine naturally, and have a chemical shift that is not disturbed by other compounds present in urine. The standard used in this work, i.e. the sodium salt of d_4-trimethylsilyl propionic acid (3-trimethylsilyl propionic acid-2,2,3,3-d_4, TSP), satisfies all these criteria. The chemical shift of the trimethylsilyl group protons of TSP was arbitrarily set to 0 ppm (**Figure 2**), and defined quantities of the standard were added to each urine sample to be analyzed. Creatinine gave rise to two NMR signals, one at 3 ppm (-CH_3) and another at 4 ppm (**Figure 2**).

By comparing the integral of the 3 ppm signal to that of the TSP trimethylsilyl group and accounting for the expected differences in signal intensity, it was possible to calculate the creatinine content of the samples. Recovery experiments using defined amounts of creatinine (0.5 to 1.5 mg/ml) and 0.5 mg/ml TSP gave recovery rates of 105 ± 2% based on the signal at 3 ppm and 110 ± 1% based on the signal at 4 ppm (**Figure 3**). The higher divergent recovery based on the 4 ppm signal may be due to complications caused by the water signal at 5-4.6 ppm. Consequently, the 3 ppm signal was used preferentially for quantification. The method was validated by analyzing one solution containing only urine and TSP, and another containing TSP and creatinine. A satisfactory recovery of 102 % was achieved in these experiments. The precision of the ^{1}H-NMR method was comparable to that of the standard Jaffé method – the deviation between the two methods was only 4 ± 2%.

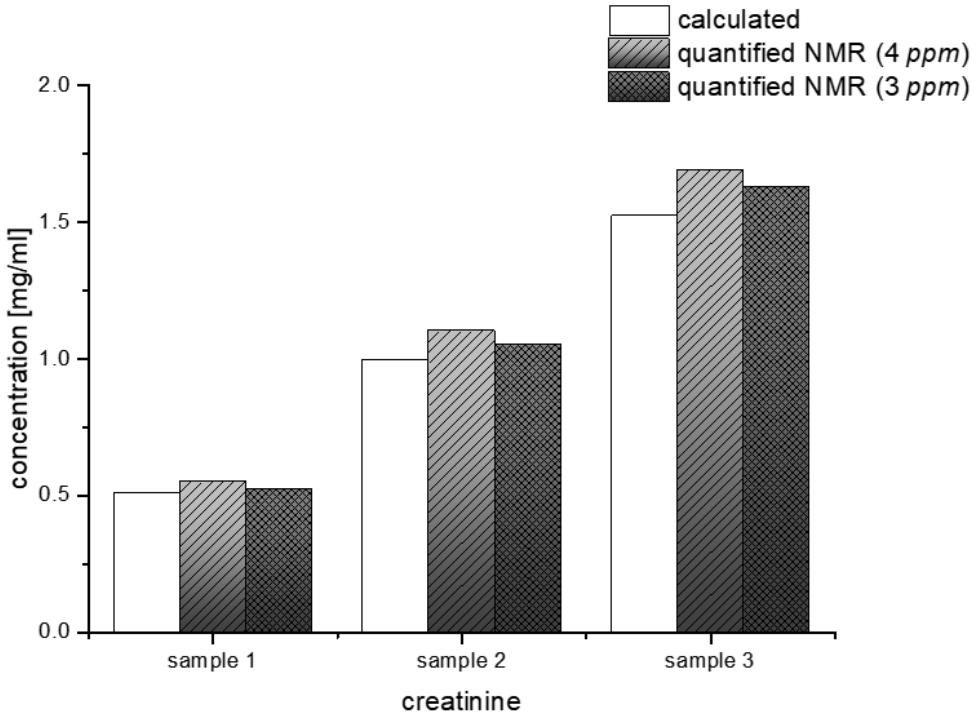

Figure 3. Quantification of creatinine in urine samples (0.5, 1.0, 1.5 mg/ml) based on the proton signals at 3 ppm and 4 ppm

DISCUSSION

To date, the picric acid assay has been the preferred method for quantifying creatinine in urine samples from human intervention studies because it is a convenient and easily performed colorimetric method. Unfortunately, it is not applicable to samples that are colored red because of compounds such as anthocyanins and their metabolites, which absorb in the same wavelength as the Meisenheimer complex formed in the assay. The use of HPLC-MS/MS or capillary zone electrophoresis as reported in literature is costly and time consuming. Therefore, the ^{1}H-NMR method presented in this work was developed to quantify creatinine in urine samples from a human intervention study involving the ingestion of an anthocyanin-rich bilberry extract [14] or any other urine samples from human intervention studies when the photometrical method using picric acid is not applicable due to red colored samples by e.g. anthocyanins and their metabolites. It could be a simple alternative to avoid false results of creatinine quantification, when urine samples contain biomolecules such as bilirubin, glucose and acetone reportedly to interfere with the Jaffé method [13]. The recovery rates, precision and slight standard deviation of the quantitative ^{1}H-NMR method determined here, demonstrate a usable alternative to the standard methods. The quantitative ^{1}H-NMR method has the advantage of being easy to perform and requiring almost no sample preparation.

CONCLUSION

A simple ^{1}H-NMR method has been established to determine creatinine in urine samples whenever the Jaffé method is not applicable e.g. when urine contains compounds absorbing at the same wavelength.

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