Talanta 83 (2011) 1707–1710

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of uric acid and creatinine in human urine using hydrophilic interaction chromatography

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ARTICLE INFO

Article history:
Received 2 November 2010
Accepted 28 November 2010
Available online 4 December 2010

Keywords:
Uric acid
Creatinine
Hydrophilic interaction chromatography
Hyperuricemia
Antioxidant
Urine

ABSTRACT

Uric acid is the end-product of purine metabolism and a major antioxidant in humans. The concentrations of uric acid in plasma and urine are associated with various diseases and routinely measured in clinical and biomedical laboratories using enzymatic conversion and colorimetric measurement. In this study a hydrophilic interaction chromatographic (HILIC) method was developed for simultaneous determination of uric acid and creatinine, a biomarker of urine dilution and renal function, in human urine. Urine samples were pretreated by dilution, protein precipitation, centrifugation and filtration. Uric acid and creatinine were separated from other components in urine samples and quantified using HILIC chromatography. A linear relationship between the ratio of the peak area of the standards to that of the internal standard and the concentration of the standards was obtained for both uric acid and creatinine with the square of correlation coefficients >0.999 for both analytes. The detection limits were 0.04 μg/mL for creatinine and 0.06 μg/mL for uric acid. The described HILIC method has proved to be simple, accurate, robust and reliable.

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1. Introduction

In humans, uric acid (Fig. 1) is a main antioxidant as well as the final product of catabolization of the purine nucleosides, adenosine and guanosine [1,2]. Abnormal concentrations of uric acid in blood and urine are related to various medical conditions, such as gouty arthritis, hyperuricemia, hypertension, pneumonia, and kidney damage [3–6]. Recent studies have also indicated that serum uric acid is a strong and independent risk factor for type 2 diabetes [7,8]. Increased serum levels of uric acid have been implicated in cardiovascular diseases [9]. However, the contribution of uric acid to atherosclerotic vascular disease is still somewhat controversial. Several mechanisms have been suggested through which uric acid may be involved in the atherosclerotic process and its clinical complications. Uric acid can act as a prooxidant, particularly at increased concentrations, and may therefore be an indicator of oxidative stress, but it may also have a therapeutic role as an antioxidant [10,11]. Plasma uric acid concentrations correlated with longevity in primates and other mammals, a characteristic that is presumably a function of uric acid's antioxidant properties [12]. A more recent study suggested that low serum/cerebrospinal fluid levels of uric acid are a biomarker of development of Parkinson's disease and that high urate levels could slow this disease [13]. Thus, rapid, reliable and accurate analytical methods for the determination of uric acid in human fluids are needed for both early diagnoses of these diseases and for studying the effects of uric acid in the disease development and on human health.

Conventional methods for the determination of uric acid are based on the enzymatic conversion of urate to allantoin using uricase followed by colorimetric measurement. However, these methods require expensive and labile reagents and suffer from interferences from other compounds such as ascorbic acid and dopamine, which are present in biological fluids. In the past decade, ion-exchange, paired-ion, reversed-phase, and size-exclusion liquid chromatographic (LC) methods have been used to determine uric acid and creatinine; capillary electrophoresis (CE) has also been employed [14–24]. Among all chromatographic techniques used, reversed-phase HPLC is one of the most commonly used approaches for the simultaneous determination of uric acid and creatinine. Creatinine is the most widely used marker of urine dilution and renal function [25]. It results from the irreversible, non-enzymatic dehydration and loss of phosphate from phosphocreatine. However, separation of strongly polar solutes like uric acid and creatinine on a reversed-phase column requires the use of a highly aqueous mobile phase, which may cause the retention loss of the solutes on continuous run [18–20]. Recently, we have developed an environmentally friendly reversed-phase HPLC method for simultaneous determination of creatinine and uric acid in human urine sam-

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doi:10.1016/j.talanta.2010.11.073

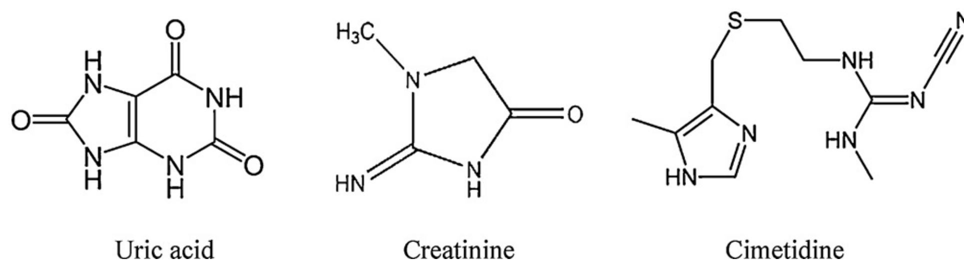

Fig. 1. Chemical structures of uric acid, creatinine and cimetidine.

ples [18], in which a small volume of acetonitrile was employed after the analyte separation to prevent the retention loss. In this paper, we report a simple, rapid and accurate hydrophilic interaction chromatographic method for the determination of uric acid and creatinine in human urine samples. This reported method has been successfully applied to study the effects of consumption of cranberry juice on the urinary concentrations of uric acid.

2. Materials and methods

2.1. Chemicals and materials

Creatinine and uric acid were purchased from Acros Organics (Geel, Belglum, NJ, USA). Cimetidine was obtained from Spectrum (Gardena, CA, USA). Sodium dihydrogen phosphate obtained from Fisher Scientific (Fair Lawn, NJ, USA) was used in preparing buffer solutions. Other solvents used in the experiments, water and acetonitrile, supplied by Pharmco Products (Brookfield, CT, USA), were HPLC grade. All other chemicals were of reagent grade and used without further purification. All standards were dissolved with distilled-deionized water. The mobile phase solvents were vacuum-filtered and degassed before use. Ocean Spray cranberry fruit juice was purchased from a local supermarket and stored at 4 °C until used in this study.

2.2. Standard solutions

Stock standard solutions (1.00 mg/mL) of creatinine and cimetidine were freshly prepared in water. Uric acid standard was dissolved in water (1.00 mg/mL) by adding 0.1 M sodium hydroxide solution to adjust the pH to 10.35. The working solution contained 50.0 μg/mL of creatinine and uric acid was prepared from the stock standard solution. The internal standard working solution contained 100 μg/mL of cimetidine. Calibration curves were prepared over the concentration of 0.0–25 μg/mL for both creatinine and uric acid.

2.3. Preparation of urine samples

Urine samples were collected from healthy volunteers in plastic containers. Urine samples were diluted 100-fold with mobile phase, in which acetonitrile could precipitate the proteins, followed by centrifugation at 5000 × *g* for 15 min. The supernatants were filtered through 0.45 μm membrane filters (Fisher Scientific brand) after adjusting the pH to 6.85 using 0.1 M sodium hydroxide. The 1 mL solutions containing pretreated samples and an internal standard (cimetidine, 15 μg/mL) were then prepared for HPLC determination. The injection volume was 20 μL.

2.4. Chromatographic conditions

A Dionex high performance liquid chromatograph (Dionex Corporation, Sunnyvale, CA, USA) equipped with a P680 pump, a UVD-170U spectrophotometer detector and Chromeleon 6.60 software was used for analysis. The analytical column used in the experiments was a Waters Spherisoro S_5NH_2 column (250 mm × 4.6 mm, 5 μm; Waters) fitted with an Adsorbosil NH_2 guard column (7.5 mm × 4.6 mm, 5 μm). An isocratic elution was employed for separation. The mobile phase consisted of 50% acetonitrile and 50% sodium phosphate buffer solution (10 mM) of pH 4.75 and the flow rate was 1.2 mL/min. The detection of creatinine and uric acid was carried out by UV absorbance at 205 nm.

2.5. Identification and quantification

The identification of creatinine and uric acid in the samples was achieved by comparing the retention times with those of authentic standards, and by spiking the urine samples with the standard of each analyte. In each sample, the quantification of creatinine and uric acid was carried out by calculating the ratio of peak areas of the identified compounds to that of the internal standard, cimetidine (15 μg/mL).

3. Result and discussion

Reversed-phase (RP) HPLC is the most commonly employed separation technique for the determination of creatinine, uric acid and other metabolites and was used in our previous work [18,26]. However, creatinine and uric acid are very polar small molecules (see Fig. 1) showing only poor retention on reversed-phase columns. Their separation requires a highly aqueous mobile phase, which causes the retention loss of the analytes on the reversed-phase column, and reequilibration of the column with an organic solvent after each run. An alternative for analyzing these polar metabolites is hydrophilic interaction chromatography (HILIC) [27–30]. In contrast to reversed-phase HPLC, a polar stationary phase is used in HILIC chromatography. But like in reversed-phase HPLC, aqueous mobile phases, commonly consisting of acetonitrile and water or an aqueous buffer, are used in HILIC chromatography. The presence of water in the mobile phase is critical for the establishment of a stagnant enriched aqueous layer on the surface of the stationary phase into which analytes may selectively partition, as described by Alpert [27]. The elution order in HILIC is usually the reverse of that in RP-HPLC.

In order to verify the suitability of HILIC chromatography for the simultaneous determination of uric acid and creatinine, after multiple preliminary assays with several commercially available HILIC columns using different mobile phase combinations, a Waters Spherisoro S_5NH_2 column (250 mm × 4.6 mm, 5 μm; Waters) fitted with an Adsorbosil NH_2 guard column (7.5 mm × 4.6 mm, 5 μm) and an isocratic elution program using acetonitrile–aqueous phosphate buffer (pH 4.75) were chosen. Fig. 2 shows the separation of a standard mixture of uric acid, creatinine and internal standard, cimetidine. A baseline separation was achieved in a short separation time of less than 5.5 min under the HPLC conditions described above. Fig. 3 presents a typical HPLC chromatogram for

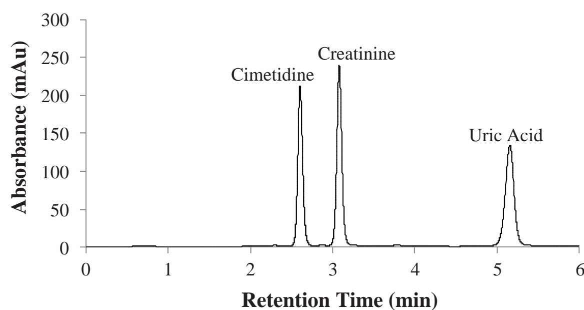

Fig. 2. HPLC chromatogram of a uric acid and creatinine standards mixture with internal standard cimetidine (15.0 μg/mL for each compound). Injection volume, 20 μL.

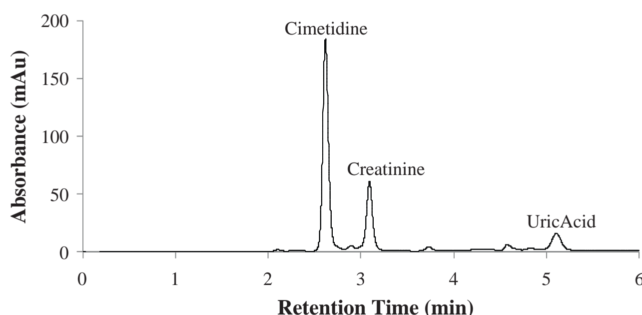

Fig. 3. A typical HPLC chromatogram of a urine sample with internal standard (15 μg/mL). Injection volume, 20 μL.

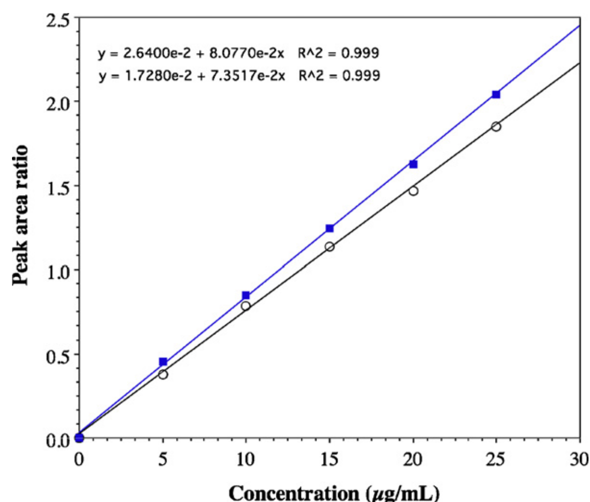

Fig. 4. Calibration curves for uric acid (open circles) and creatinine (squares).

the separation of a freshly collected urine sample. It is not surprising that the elution order for the two analytes is the same as in the RP-HPLC, since the amino functional groups on the surface of the HILIC packing is positively charged at pH 4.75. Besides the common partition mechanism in HILIC chromatography, the electrostatic interactions between charged analytes and the stationary phase played a major part in the separation of uric acid, creatinine and the internal standard. The retention time of the internal standard, creatinine and uric acid were: 2.61 ± 0.01 min, 3.08 ± 0.01 min, and 5.10 ± 0.01 min, respectively. The relative standard deviation (RSD) values of the retention times were smaller than 0.4%, indicating that the developed separation method was very stable and had high reproducibility.

Hypoxanthine has been used as the internal standard for the determination of creatinine and uric acid in our previous study [18]. Hypoxanthine may present at trace concentrations in biological fluids although at which it does not have significant effect on the quantification of creatinine and uric acid in biological fluids. In this study, cimetidine, a medical drug for the treatment of heartburn, was selected as the internal standard. Cimetidine was used as the internal standard for analyzing creatinine and pseudouridine in an earlier study [31]. The UV light absorbance of cimetidine was determined at a wavelength of 220 nm, differing from that used for measurement of analytes at 262 nm, which may increase the uncertainty of the quantification of analytes. In this developed method, both internal standard, cimetidine, and analytes, uric acid and creatinine, were detected at 205 nm. As previously reported, greater sensitivity and fewer matrix interferences were observed at 205 nm than at 220 nm or 235 nm [18]. Chromatograms of typical urine samples without the addition of internal standard revealed that there was no possible interferent that co-eluted with cimetidine. The constant ratios of absorbance at different wavelength across the peak profile of cimetidine, creatinine and uric acid confirmed that the internal standard and analyte peaks were free from co-eluting substances.

Calibration graphs for uric acid and creatinine were obtained using a series of standard solutions over the concentration range from the limits of quantification of each analytes to 25 μg/mL. A linear relationship between the ratio of the peak area of the standards to that of the internal standard and the concentration of the standards was obtained for both uric acid and creatinine, as shown in Fig. 4. The square of correlation coefficients > 0.999 for both uric acid and creatinine. The detection limits (S/N = 3) were 0.04 μg/mL for creatinine and 0.06 μg/mL for uric acid.

Accuracy of the method was estimated by adding three levels of standard uric acid and creatinine to a urine sample and determining the amounts of standard recovered. The average percentage recoveries were found to be 94.0–102% for uric acid and 90.0–94.2 for creatinine, as presented in Table 1.

Urine samples from eight volunteers (five males and three females) were analyzed using the developed hydrophilic interaction chromatographic method under selected conditions. As shown in Fig. 3, the peaks of uric acid, creatinine and the internal standard were well separated from other peaks, and no interference occurred. Results are presented in Table 2. The concentration of uric acid and creatinine are in the range of 419–848 μg/mL and 438–1480 μg/mL, respectively. The precision of the chromatographic determination for the proposed method, expressed as a relative standard deviation (RSD), was calculated by three replicate injections of urine samples in each of four days. The RSDs for the analysis of uric acid and creatinine were 2.08 and 1.03, respectively. This developed method was also applied to a preliminary study on the effect of consumption of a cranberry fruit juice on the concentration of urinary uric acid. Fig. 5 presented a time course

Table 1
The recoveries of creatinine and uric acid in human urine samples.

Compound	Standard added (μg/mL)[a]	Mean recovery[b] (%) (± SD)
Uric acid	3.00	102.2 (±1.0)
	6.00	98.2 (±1.8)
	12.0	94.0 (±2.0)
Creatinine	3.00	90.0 (±1.8)
	6.00	94.2 (±2.0)
	12.0	92.0 (±1.8)

[a] Concentrations of standards are expressed as the equivalent concentrations added in the final injected solutions. Each level of standard added was replicated six times.
[b] Calculated as % recovery = [(amount observed − original amount)/amount added] × 100.

Table 2
Analytical results of uric acid and creatinine in human urine sample[a]

Urine sample	Uric acid		Creatinine	
	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)
No. 1	534	1.7	728	0.65
No. 2	848	1.9	456	0.55
No. 3	688	1.7	915	0.70
No. 4	438	1.3	846	0.62
No. 5	419	2.6	1152	2.3
No. 6	611	3.6	916	2.1
No. 7	656	3.7	1480	1.2
No. 8	447	0.13	1382	0.15

[a] The urine samples were measured at 100-fold dilution.

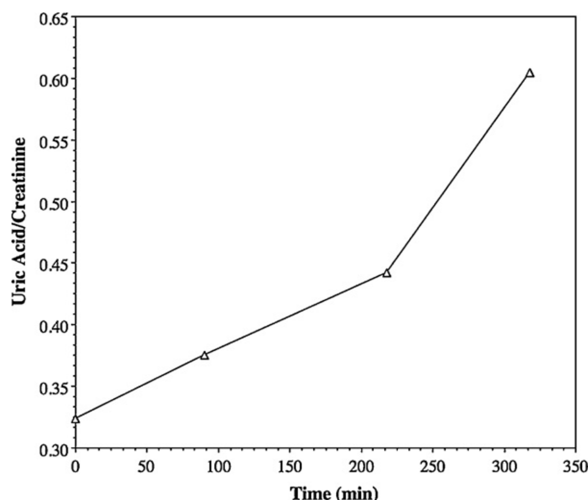

Fig. 5. A time course of the concentration ratios of uric acid to creatinine after consumption of a cranberry fruit juice (250 mL).

of the concentration ratios of uric acid to creatinine after a volunteer consumed 250 mL of a cranberry fruit juice. Further studies are needed to understand the mechanisms how the consumption of cranberry, and other fruits and vegetables affects the concentrations of uric acid and other biomarkers in biological fluids, and human health.

4. Conclusions

A fast, accurate and reliable hydrophilic interaction chromatographic (HILIC) method has been developed for the simultaneous determination of uric acid and creatinine in human urine. Compared with reversed-phase HPLC methods, the developed HILIC method has proved to be simple and robust. It eliminated the re-equilibrium procedure required in the reversed-phase HPLC for the determination of uric acid, creatinine and other highly polar small metabolites. The developed HILIC method has been successfully applied to separate and quantify uric acid and creatinine in urine samples from eight volunteers and to a study of effect of consumption of cranberry fruit juice on the levels of urinary uric acid. The method developed in this study could also be used in the measurement of uric acid and creatinine in other biofluids for disease diagnoses.

Acknowledgements

This work was partly supported by the Massachusetts Agriculture Research Fund and the University of Massachusetts Dartmouth.

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