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Reduction of urinary uric acid excretion in patients with proteinuria

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ABSTRACT

Serum uric acid (UA) concentration is positively associated with proteinuria. However, the relationship between proteinuria and urinary metabolites of purine metabolism remains unknown. This study developed a hydrophilic interaction chromatography (HILIC)-based HPLC method with ultraviolet detection (UV) to quantify creatinine (Cr), UA, xanthine, and hypoxanthine in human urine simultaneously. The urinary concentrations of UA and Cr obtained by our method are consistent with those measured by an autoanalyzer. The HPLC-HILIC-UV method was validated as selective and robust with simple sample preparation for measuring UA, xanthine, hypoxanthine and Cr, which is suitable for large clinical studies. The UA/Cr ratios in random urine samples were 5.5 times lower in proteinuria patients (0.077 ± 0.008) than in healthy individuals (0.424 ± 0.037). Moreover, the UA/hypoxanthine ratio in proteinuria patients was approximately 10 times lower than that in healthy individuals. Our findings revealed a reduced urinary UA excretion, which is one of the factors leading to increased serum UA in proteinuria patients.
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1. Introduction

Proteinuria is a condition characterized by the presence of a greater than normal amount of protein in the urine. Moreover, proteinuria is a sign of a chronic disease of or damage to the kidneys given that microalbuminuria results from endothelial dysfunction [1]. Indeed, urinary albumin is a sensitive marker for early changes in glomerular permeability [2]. Uric acid (UA) is the terminal oxidation product of purine metabolism in humans and in higher primates. UA is synthesized from xanthine, which is derived from hypoxanthine; both reactions are catalyzed by xanthine oxidase. Therefore, UA, hypoxanthine, and xanthine are important intermediates or end products of nucleic acid metabolism. The formation, distribution, and excretion of these compounds in the body are in a state of balance under normal conditions. Epidemiologic studies have shown that high serum UA level is a marker of oxidative stress, tissue injury, and renal dysfunction and is an independent risk factor for cardiovascular disease [3–5]. Multiple studies have shown that serum UA concentration is positively associated with proteinuria in patients with type 2 diabetes mellitus [2,6,7] and heart failure [8]. Compared with blood collection, urine sample collection is more convenient and encourages better patient compliance. However, to the best of our knowledge, the relationship between proteinuria and urinary UA (and its precursors hypoxanthine and xanthine) has not yet been reported.

Creatinine (Cr) is the breakdown product derived from creatine in muscles, and urinary Cr concentrations are generally inversely proportional to urine flow [9]. Therefore, metabolite concentrations are widely normalized to Cr concentrations to rectify the variation in urine volume during biomonitoring [10–12]. For this reason, we developed a high-performance liquid chromatography (HPLC) method for the simultaneous quantification of Cr, UA, hypoxanthine, and xanthine in human urine samples. Fig. 1 shows the chemical structures of these compounds.

The Cr and UA in biofluids are traditionally measured using the Jaffé alkaline picrate reaction [13,14] and the phosphotungstate reduction [15,16], respectively. However, these photometrical methods lack specificity because various endogenous and exogenous substances can react with alkaline picrate or can reduce phosphotungstate. Various HPLC methods based on C18 columns, such as HPLC-coupled ultraviolet (UV) detection [17–19] and mass spectrometry (MS) [20–22] have been developed and validated for the isolation and quantitation of Cr, UA, and other metabolites in

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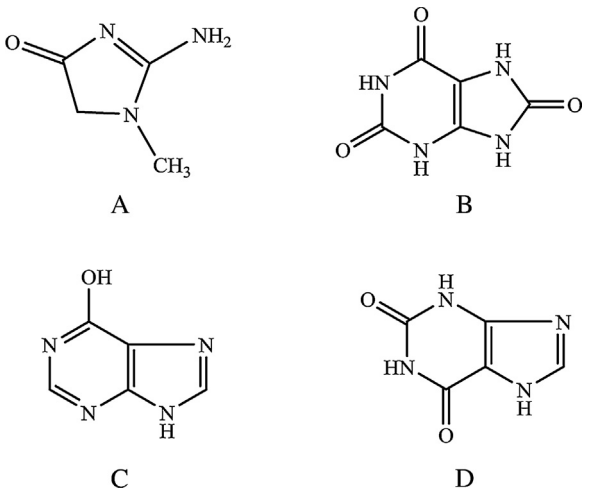

Fig. 1. Chemical structures of creatinine (A), uric acid (B), hypoxanthine (C) and xanthine (D).

biological fluids to avoid interference in sample matrices. However, given the high polarity of Cr, a poor resolution of Cr from other metabolites in human urine is generally obtained on the reverse-phase HPLC system [23].

Hydrophilic interaction chromatography (HILIC), which is similar to normal-phase chromatography, was developed in the 1990s, with water used as a strong eluting solvent [24]. The mechanism of HILIC separation is somewhat complicated, as it involves partitioning, hydrogen bonding and electrostatic interactions [25]. HILIC is suitable for hydrophilic and polar compounds, such as sugars, oligosaccharides, and phosphorylated amino acids. The technology has been widely applied to the separation of various highly polar compounds [26–29].

The present study aimed to explore the relationship between proteinuria and the urinary metabolites of purine metabolism in humans. A reproducible and sensitive HILIC-based HPLC-UV method was developed for the simultaneous measurement of UA, Cr, xanthine, and hypoxanthine in urine. Basing from the quantitative results for the metabolites in random human urine samples, we evaluated the ratios of UA, xanthine, and hypoxanthine to Cr both in healthy individuals and in proteinuria patients.

2. Experimental

2.1. Subjects

We randomly collected urine samples from 31 healthy volunteers (normal group; 20–40 years old; 14 females and 17 males) and 55 outpatients who were positive for proteinuria in accordance with the dipstick screening test (proteinuria group; 25–50 years old; 34 females and 21 males) conducted in the Hospital of Nanchang University (China). The ratio of urinary protein to Cr in all patients was 0.1–3.0. Our study protocol was approved by the institutional review board of Nanchang University Hospital, and all subjects provided a written informed consent. The urine samples were stored at −20 °C after collection.

The total protein in the urine samples was quantified using the latex turbidimetric method, whereas urinary Cr and UA were measured using a fully automated analyzer (BS-380, Mindray, China).

2.2. Materials and reagents

Xanthine and hypoxanthine were purchased from Aladdin Reagent Co., Ltd. (China). UA and Cr were obtained from Apotheker Reagent Company (China) and Sigma Chemical Co., Ltd. (USA), respectively. HPLC-grade acetonitrile, phosphoric acid, and ammonium acetate were obtained from Aladdin Reagent Co., Ltd. China. All other chemicals were of analytical grade and used without further purification.

2.3. Sample preparations

Urine samples (200 μL) were thawed at room temperature and were diluted fivefold with acetonitrile for protein precipitation prior to centrifugation at 12,000 × *g* for 10 min. A 20 mL aliquot of the supernatant was then injected into the HPLC system.

Individual stock solutions of each standard (400 μg/mL) were prepared in 80% (v/v) acetonitrile–water solution. Appropriate dilutions of these solutions were prepared to produce mixed solutions and quality control samples (QCs) at concentrations of 2.80, 14.0, and 70.0 μg/mL for Cr, and 1.6, 6.2, and 31.0 μg/mL for UA, hypoxanthine, and xanthine, respectively.

2.4. HPLC-HILIC-UV analysis

Chromatographic separation of the samples was achieved on an Agilent series 1260HPLC system equipped with Agilent series 1260 DAD detector (Agilent, USA).

The chromatographic separation of the samples was achieved on SeQuant ZIC-HILIC (250 mm × 4.6 mm, 5 μm, Merck KGaA, Germany) protected by a HILIC pre-column. Solvent A consisted of 5 mm ammonium dihydrogen phosphate and 0.1% phosphoric acid (v/v), whereas Solvent B consisted of acetonitrile. The mobile phase flow rate was 0.8 mL/min, and the gradient elution conditions were as follows: 0–3 min, isocratic 83% B; 3–15 min, linear gradient 83–65% B; 15–18 min, isocratic 65% B; 18–20 min, linear gradient 65–83% B; and 20–30 min, isocratic 83% B. The column was maintained at 38 °C and the injection volume of samples was 20 μL. The detection wavelength was set at 215 nm for Cr, 270 nm for UA and hypoxanthine, and 250 nm for xanthine.

2.5. Method validation

The specificity, precision, method recovery, linearity, limit of quantification (LOQ), and stability of the metabolites were validated in the current study. The specificity of the method was determined by analyzing three human urine samples, and the urine samples were spiked with each analyte to determine if any compound was eluting at the same retention time as the target metabolites. The method recovery was established by separately spiking 8.0, 10.0, and 12.0 μg/mL Cr and 3.0, 3.75, and 4.5 μg/mL UA, hypoxanthine, and xanthine, respectively, with three aliquots of urine, whose amounts of analytes were predetermined via HPLC analysis. At each level, analysis was performed in duplicate, and the method recovery was calculated using the amount of analytes, which was expressed as a percentage of the theoretical amount presented in the medium.

The intra- and inter-day precisions were defined as the relative standard deviation (R.S.D.) in the analysis of five replicates of QCs within 1 day or in 3 separate days, whereas the accuracy was determined by calculating the relative error (R.E.). This study also evaluated the stability following the time of storage. For storage stability, the QCs were analyzed in five replicates before and after 1, 3, and 5 days of storage under laboratory bench conditions (25 ± 2 °C).

The final solutions (seven standard solutions for each calibration curve) containing the four metabolites were prepared from the mixed stock solutions in 80% acetonitrile–water (v/v) to generate calibration curves from 0.78125 μg/mL to 100 μg/mL (UA, xanthine, and hypoxanthine) and 1.5625 μg/mL to 400 μg/mL (Cr). Each final solution was injected in duplicate, and the peak area was

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