Simultaneous Determination of Creatinine and Uric Acid in Human Urine by High-Performance Liquid Chromatography

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An environmentally friendly reversed-phase HPLC method for simultaneous determination of creatinine and uric acid in human urine samples has been developed. Human urine samples were pretreated by dilution, protein precipitation, centrifugation and filtration, followed by HPLC separations using a reversed-phase C18 column with an aqueous mobile phase of phosphate buffer. The retention loss of a C18 column when using the highly aqueous mobile phases was avoided by employing a gradient elution using a small volume (<0.23 mL) of acetonitrile and phosphate buffer at pH 4.75. This developed method provides a simple, rapid separation and sensitive detection for the species of interest in 10 min with UV detection at 205 nm. Quantitation was carried out by relating the peak areas of the identified compounds to that of hypoxanthine as an internal standard. The detection limits for creatinine and uric acid were 0.045 and 0.062 μg mL⁻¹, respectively. The recoveries of the standards added to urine samples were 87.3 - 102.2% for creatinine and 97.3 - 108.6% for uric acid, and the relative standard deviation for both analytes was less than 1.0%. This method has been successfully applied to estimating of creatinine and uric acid in human urine.

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Introduction

Uric acid (Fig. 1) is the final oxidation product of purine metabolism in humans and other higher primates. The concentration levels of uric acid in serum and urine are associated with various diseases, such as cardiovascular disease, renal disease, diabetes, and hypertension,10,11 and are routinely determined especially in clinical and biomedical laboratories. Creatinine (Fig. 1) is a breakdown product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body depending on muscle mass. Therefore, the concentration of uric acid and other diagnostic markers in urine is commonly corrected by the urinary creatinine concentration, and the “creatinine ratio” is often used as diagnostic markers.

The measurement of creatinine and uric acid in plasma and urine is traditionally performed by colorimetical methods, such as those based on the Jaffé alkaline picrate reaction for creatinine10,11 and the reduction of phosphotungstate for uric acid.12,13 However, these photometrical methods lack specificity and suffer from interferences from various endogenous and exogenous substances that react with alkaline picrate or reduce phosphotungstate. A number of enzymatic methods have also been adopted for routine clinical measurements of creatinine and uric acid.11,14 Although the enzymatic methods generally have less interference than colorimetical methods, there have been reports of various substances that still interfere.11,13,15 To avoid interferences in the sample matrices, many HPLC methods for the isolation and quantitation of creatinine, uric acid, and other metabolites in biological fluids have been described, including ion-exchange, normal-phase, reversed-phase, and reversed-phase ion-pair chromatography.16-21 Reversed-phase HPLC methods with a highly aqueous mobile phase have been increasingly attractive in light of the recent movement of green analytical chemistry, which encourages the development of analytical methods without or with reduced consumption of organic solvent, energy and time.17,19,22-24 However, many studies have reported that the use of a highly aqueous mobile phase (>90% water) with C18 and C8 columns causes an anomalous chromatographic behavior, and a retention loss of analytes.19,25 These anomalies have been attributed to (i) aggregation of the bonded hydrocarbon chains in the presence of a highly aqueous mobile phase, rendering them inaccessible to analytes, and (ii) extrusion of the highly aqueous mobile phase from the pores of the particles of the stationary phase.25-27 Our preliminary experiments in this study showed a rapid loss in the retention time of creatinine and uric acid on the C18 column with highly aqueous mobile phases reported in some recent publications.19,21

In this paper, a simple, fast, accurate and environmentally friendly reversed-phase HPLC method is described for the

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![Fig. 1 Chemical structure of uric acid, creatinine and hypoxanthine.](image-url)
simultaneous determination of creatinine and uric acid in human urine. The method was optimized through gradient elution to remove hydrophilic and hydrophobic residues, and to maintain the C18 column resolution following separation of the analytes using an aqueous mobile phase of phosphate buffer.

**Experimental**

**Chemicals**

Standards of creatinine, uric acid and hypoxanthine were purchased from Acros Organics (Geel, Belgium, NJ). Sodium phosphate and phosphorous acid were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide was obtained from CMS Inc. (Houston, TX). Acetonitrile was supplied by Pharmco Products (Brookfield, CT). Except where noted, all reagents were of analytical grade, and all solutions were prepared using distilled-deionized water, and were filtered through 0.45-µm membranes (Fisher Scientific) before HPLC analysis. The mobile-phase solvents were degassed before use.

**Standard solutions**

Stock standard solutions (200 µg mL⁻¹) of creatinine and uric acid (adjusted to pH 10.35) were freshly prepared in water. Stock internal standard solutions (160 µg mL⁻¹ hypoxanthine) were freshly prepared by dissolving 8.0 mg in 50 mL of water. All solutions were stored in brown glass bottles and kept at 4°C. A series of 1.6 mL mixed working standard solutions containing a 10.0 µg mL⁻¹ internal standard were prepared by adding a 100-µL stock internal standard solution and appropriately diluting the individual stock standard solutions to obtain concentrations in the range of 0.0 – 25 µg mL⁻¹. Calibration curves were constructed by linear regression of the peak area ratio of individual standards to the internal standard versus the concentration.

**Sample preparation**

Urine samples were collected from four healthy volunteers in plastic containers. Urine samples were diluted 100-fold with distilled water and acidified to pH 2.35 with phosphorous acid to precipitate protein before centrifugation at 5000g for 15 min. The supernatants were filtered through 0.45 µm membrane filters (Fisher Scientific brand) after adjusting the pH to 6.85 using 0.010 N sodium hydroxide and 10% phosphorous acid. The 1.6 mL solutions containing pretreated samples and an internal standard (10.0 µg mL⁻¹) were prepared in duplicate for HPLC analysis. Twenty microliters of the pretreated urine samples or standard solutions were directly injected into the HPLC system. Precautions have always been taken to minimize sample contamination. All sample containers, glassware and filtration devices were thoroughly cleaned with a 0.10 M HCl solution, and then finally with doubly distilled-deionized water.

**HPLC analysis**

A Dionex high-performance liquid chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with a P680 HPLC pump, a UVD-170U spectrophotometer detector, a Gina 50 autosampler, and Chromeleon 6.60 software was used for all experiments. The analytical column used was a symmetry C18 reversed-phase column (150 × 3.00 mm i.d., 5 µm; Waters) fitted with a 10-mm C18 guard column. The detection of creatinine and uric acid was carried out by direct UV absorbance at 205 nm. Solvent gradients were formed by a dual pumping system by varying the proportion of solvent A (sodium phosphate buffer; pH 4.75) to solvent B (acetonitrile; HPLC grade). The solvent gradient elution program is presented in Table 1.

**Identification and quantification**

The identification of creatinine and uric acid in each urine sample was achieved by comparing the HPLC chromatographic retention times. In each sample, the quantification of both creatinine and uric acid was carried out by relating the peak areas of the identified compounds to that of the internal standard, hypoxanthine, at a concentration of 10.0 µg mL⁻¹. A trace amount of hypoxanthine may be present in urine, but its concentration has always been found to be below the limit of quantification, and thus does not significantly affect the quantification of analytes (Fig. 3A). All of the calibration standards and urine samples were measured in triplicate.

**Results and Discussion**

**Chromatographic separation**

It has been reported that the HPLC retention time gradually decreases and even becomes almost no retention when reversed-phase C18 columns are used for long-time continuous operation with a highly aqueous mobile phase. A similar retention loss of uric acid and creatinine was also observed on a C18 column with previously reported aqueous mobile phases after two runs, or less than 20 min. The retention losses are attributed to (1) a folding of the stationary phase alkyl chains in the presence of a highly aqueous mobile phase, and (2) the highly aqueous mobile phase being forced out of the pores when the flow is stopped and the pressure released. Retention is lost because the mobile phase is no longer in contact with the interior surface of the particles, where most of the surface area is located. To maintain good performance of the column, after multiple
preliminary assays, gradient elution with a small volume of acetonitrile (less than 0.23 mL) was employed, following an isocratic elution of analytes using an aqueous phosphate buffer mobile phase in this study.

Figure 2 shows a chromatogram of a standard mixture of creatinine and uric acid. A base-line separation can be achieved in a short elution time of less than 10 min. Figures 3A and 3B show a typical HPLC separation of a freshly collected urine sample without and with the addition of internal standard, respectively. Chromatogram B was obtained through about 20 chromatographic runs after chromatogram A. No detectable change in the retention time of the analytes has been observed. The mean values of the retention time for creatinine, uric acid and internal standard hypoxanthine were: creatinine, 2.970 ± 0.031 min; uric acid, 3.920 ± 0.038 min; and hypoxanthine, 5.540 ± 0.054 min, respectively. The relative standard deviation (RSD) values of the retention times and the peak areas were generally smaller than 1%, indicating that the developed separation method was very stable and had high reproducibility. The chosen wavelength of 205 nm provides a higher sensitivity with a clean chromatogram than the wavelength of 220 or 235 nm employed in previous studies.\textsuperscript{19-21} The peak purity was confirmed by constant signal ratios (relative absorbances at different wavelength) across the peak profile of creatinine, uric acid and the internal standard.

Quantitative analysis

The calibration curves for both creatinine and uric acid were linear over the concentration ranges tested: 0.00 - 25.0 \( \mu \text{g mL}^{-1} \).
uric acid in human urine. This method has successfully prevented a retention loss of reversed-phase C18 column encountered in previous studies using a highly aqueous mobile phase, by introducing a brief gradient elution with a small volume of acetonitrile (<0.25 mL), following analyte separation. The method developed in this study could also be used in the separation and quantitative measurement of creatinine and uric acid in other biological fluids.

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