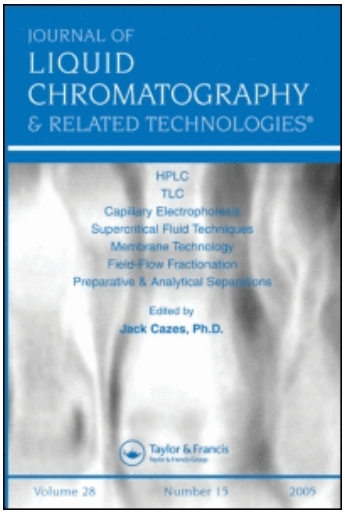

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous Determination of Creatinine and Uric Acid in Serum by High Performance Ion Exchange Chromatography with Direct Injection

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To cite this Article Etoh, T. , Iwatake, M. , Miyazaki, M. , Harada, K. , Nakayama, M. , Sugii, A. , Uji, Y. and Okabe, H.(1992) 'Simultaneous Determination of Creatinine and Uric Acid in Serum by High Performance Ion Exchange Chromatography with Direct Injection', Journal of Liquid Chromatography & Related Technologies, 15: 9, 1565 — 1575
To link to this Article: DOI: 10.1080/10826079208018309
URL: http://dx.doi.org/10.1080/10826079208018309

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(9), 1565-1575 (1992)

SIMULTANEOUS DETERMINATION OF CREATININE AND URIC ACID IN SERUM BY HIGH PERFORMANCE ION EXCHANGE CHROMATOGRAPHY WITH DIRECT INJECTION

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ABSTRACT

An anion-exchange chromatographic method for simultaneous determination of creatinine and uric acid in human serum is described. Serum samples were analyzed by direct injection high performance liquid chromatography which a column of quaternized resin cross-linked with ethylene glycol dimethacrylate (PCMS-Q). This allowed all proteins to elute at void volume. Thus, small analytes were readily separated from macromolecules. The method was found to be precise and accurate. There was a good correlation between the results of the new method and those of spectrophotometric methods for measuring creatinine and uric acid in human serum.

INTRODUCTION

Creatinine and uric acid levels in human serum are important parameters of renal function. Recently, several high performance liquid chromatography (HPLC) methods have been used to determine creatinine or uric acid, or both in human plasma, serum and urine. These include reversed phase (1,2,3), cation-exchange (4), normal phase (5) and multi- (6) modes of liquid chromatography. In most cases, it is necessary to precipitate the proteins and then extract the analytes. Disadvantages of these methods include the loss of analytes during extraction and deproteinizaton, difficulties with reproducibility, and increased analysis time.

Direct serum injection HPLC which does not require tedious sample preparation, can be used for routine analysis, some approaches, such as using column switching (3) and multi-mode column packing (6), have been used to determine creatinine or uric acid, or both. Although the column switching method is promising, for clinical applications it is also important to develop a new column packing suitable for direct injection HPLC.

PCMS-Q, an anion-exchanger prepared from in our laboratory, only interacts with small molecules in the presence of biological macromolecules (7). All macromolecules were eluted in one peak at the void volume because of this size-exclusion effect. However, small molecules do permeate into the pores and interact with internal functional groups without being affected by the macromolecules. The characteristics of the PCMS-Q column may be

comparable to those of the "restricted access" columns reported by Pinkerton, Regnier and others (8,9).

In this study, we evaluated this method for simultaneous determination of creatinine and uric acid in human serum with the PCMS-Q column in direct injection ion-exchange HPLC.

MATERIALS AND METHODS

Chloromethylstyrene (CMS, a mixture of meta and para isomers) and ethylene glycol dimethacrylate (EGDMA) were purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. Creatinine and uric acid were obtained from Wako Pure Chemical Ind. Ltd., Osaka, Japan. Serum samples stored at -80 ℃ and were injected directly onto the HPLC column without prior treatments. All other chemicals were of reagent grade.

Appropriate amounts of creatinine and uric acid were dissolved in distilled water and lithium carbonate solution, respectively, to make 1 mg/ml stock standards. These solutions were further diluted with distilled water to produce spiking standards. The mixed solution contained 0.04 ml of creatinine stock standard and 0.1 ml of uric acid stock standard, made up to 1 ml with distilled water.

Polymerization of PCMS-Q was performed in a nitrogen atmosphere. A toluene solution containing CMS, EGDMA and benzoyl peroxide (1% of monomers) was suspended in a 1% hydroxyethylcellulose aqueous solution containing 20% sodium

chloride. After stirring for 7 hours at 70 ℃, the resulting resin, PCMS, was washed with hot water until it was free of adhering stabilizer. Finally, PCMS was classified in methanol, and the fraction with particle diameters of 10-15 μm was allowed to react with trimethylamine in a pressure bottle at 80 ℃ for 20 hours. The product, PCMS-Q, was filtered off, then washed repeatedly with methanol and acetone. The ion-exchange capacity and specific surface area of PCMS-Q were 1.48 mmol/g and 0.37 m^2/g, respectively.

Chromatographic measurements were carried out with a Hitachi L-6200 intelligent pump equipped with a Hitachi L-4000 UV detector or a Hitachi L-3300 refractive index detector and a Hitachi D-2000 chromatointegrater (Hitachi Co., Ltd., Tokyo, Japan). The mobile phase was 0.05 M tris(hydroxymethyl)aminomethane-hydrochloric acid (Tris-HCl) buffer (pH 7.0) containing 0.5 M sodium chloride. It was filtered through a 0.45-μm cellulose nitrate membrane filter (Advantec Toyo, Tokyo, Japan) and was degassed before use. PCMS-Q was packed into stain-less steel guard and analytical columns (10 mm x 4 mm I.D. and 250 mm x 4 mm I.D., respectively) and was conditioned with the mobile phase. Chromatography was performed at ambient temperature with detection at 230 nm. The flow rate was 0.5 ml/min.

The pore size of PCMS-Q in the wet state was evaluated by size-exclusion chromatography (SEC) with standard dextrans. The hold-up volume of the column was measured with heavy water. The retention of carbohydrates was measured with a refractive index detector.

RESULTS AND DISCUSSION

When the pore size and the number of ion-exchange groups on the outer surface of the column packing are insufficient for interaction with large molecules, these components are eluted sharply at void volume. The improvement of the peak sharpness was achieved by addition of neutral salt in mobile phase. In contrast, small molecules can penetrate the pores and interact with ion-exchange groups on the internal surfaces. The exclusion limit of the PCMS-Q column, as determined with carbohydrates, was about 5000 daltons. Therefore, creatinine and uric acid (molecular weights 113.12 and 168.11, respectively) can permeate the pores of the PCMS-Q but macromolecular proteins cannot.

The advantage of the PCMS-Q column for the assay of creatinine and uric acid is that it can be used to separate these small analytes from macromolecular species in serum. Detection at 230 nm was found to give clear peaks for both analytes with minimal background interference. FIGURE 1 shows the chromatograms of a mixed sample of creatinine and uric acid and a normal sample of blank serum. Creatinine and uric acid peaks are clear. Creatinine has no negative charge at pH 7.0, so it may be separated from serum proteins by a molecular sieve effect. Uric acid that has a negative charge is retained by partition and ion-exchange interactions.

The calibration curves obtained with duplicate injections of the spiked serum were linear from 0 to 20 mg/dl. The creatinine peak overlapped with the serum protein peak, so the results based on peak heights gave better precision than those based on peak areas.

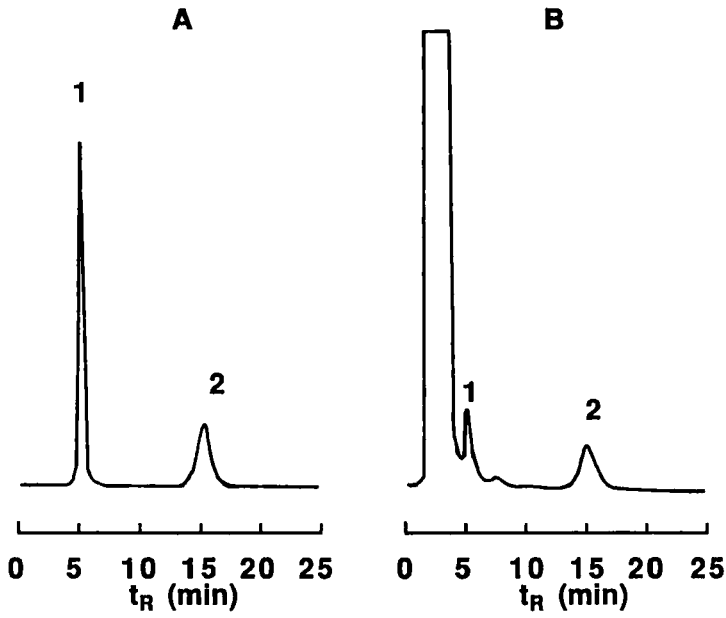

FIGURE 1. Separation of creatinine and uric acid from the components of serum with PCMS-Q column. (A) standard solution; (B) serum sample, Peaks: 1, creatinine; 2, uric acid, Eluent: 0.05 M Tris-HCl buffer + 0.5 M NaCl (pH 7.0), Flow rate: 0.5 ml/min, Detection: UV 230 nm.

The peak heights of the spiked sample were compared with the heights of the creatinine and uric acid solution to determine the absolute recovery. TABLE 1 shows that the recoveries of creatinine and uric acid were 98-103 % and 95-98 %, respectively. The accuracy increased with the spiked level.

Within-day and between-days reproducibility were assessed by analyzing a serum sample containing a known amount of creatinine and uric acid. Within-day and between-days analyses were repeated 8 times on 1 day and 4 times on 4 days, respectively. As

TABLE 1
Recovery of Spiked Creatinine and Uric Acid from Human Serum.

	spiked level mg/dl	average content mg/dl	recovery %	C.V.* %
creatinine	0.00	7.31	-	-
	3.66	10.72	97.8	1.58
	7.31	15.14	102.9	1.34
uric acid	0.00	5.84	-	-
	2.92	8.41	95.8	1.96
	5.84	11.43	97.3	1.75

* coefficient of variation

TABLE 2
Precision of Within-day and Between-days Assays.

	within-day assay (n=8)		between-days assay (n=4)	
	average content mg/dl	C.V. %	average content mg/dl	C.V. %
creatinine	11.71	1.14	11.46	1.27
uric acid	6.79	1.86	6.61	2.09

shown in TABLE 2, the coefficients of variation (C.V.) were less than 3%.

It is well documented that creatinine and uric acid levels in blood after haemodialysis (HD) are markedly lower than those before HD (10). Chromatograms of HD patient serum (before and after HD) are shown in FIGURE 2. The uric acid peak was easily

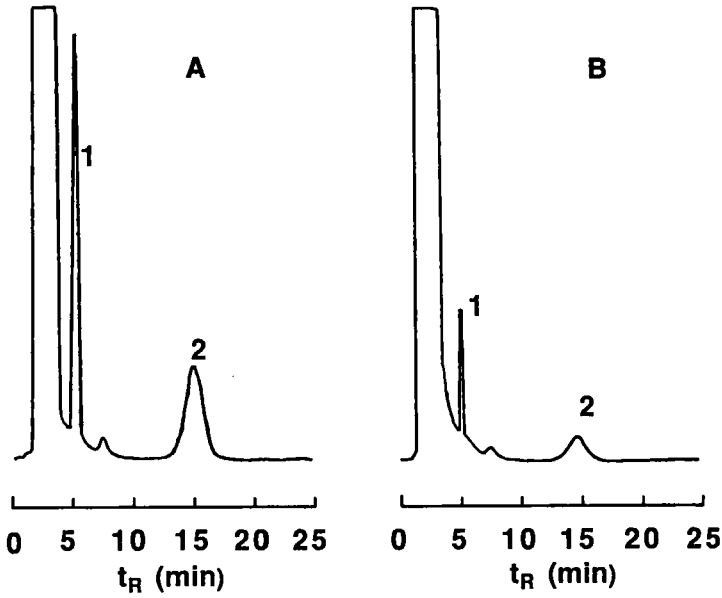

FIGURE 2. Chromatograms of haemodialysis (HD) patient serum with PCMS-Q column. (A) before HD; (B) after HD, Peaks: 1, creatinine; 2, uric acid, Chromatographic conditions are the same as FIGURE 1.

distinguished from other peaks, but the creatinine peak overlapped to some extent with the that of endogenous components, involving mainly serum proteins. There was an obvious change in the heights of the creatinine and uric acid peaks after HD. The change in mobile phase pH and the addition of organic solvents did not affect the resolution of creatinine and uric acid. There were no irreversible increases in column back-pressure or changes in retention times of creatinine and uric acid with more than 200 serum injections.

The specificity of this direct injection HPLC method for creatinine and uric acid was tested by comparing it with the

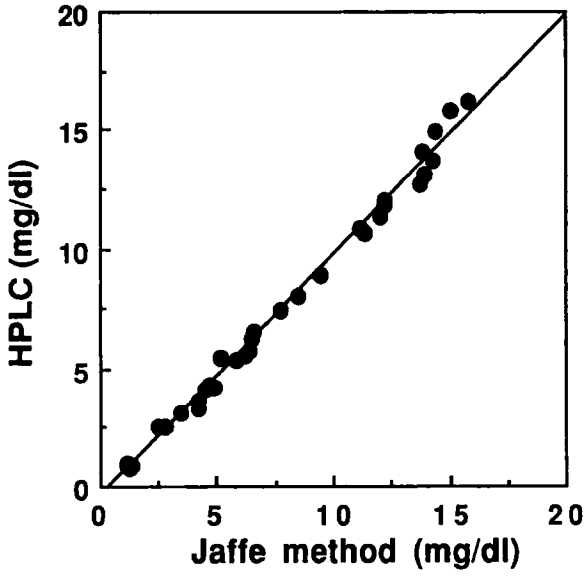

FIGURE 3. Correlation between uric acid contents measured by uricase-peroxidase method and by direct injection HPLC.

spectrophotometric methods used in commercial kits, i.e., the alkali-picrate method (Jaffe method) (11) and the uricase-peroxidase method (12) for measuring creatinine and uric acid, respectively. The results are shown in FIGUREs 3 and 4. Thirty-six serum samples were analyzed by both methods. Good correlations were obtained for both creatinine and uric acid. The regression equations were $Y=0.955\ X + 0.606$ for creatinine, with a correlation coefficient (r) of 0.991, and $Y=0.928\ X + 0.658$ for uric acid, with $r=0.967$.

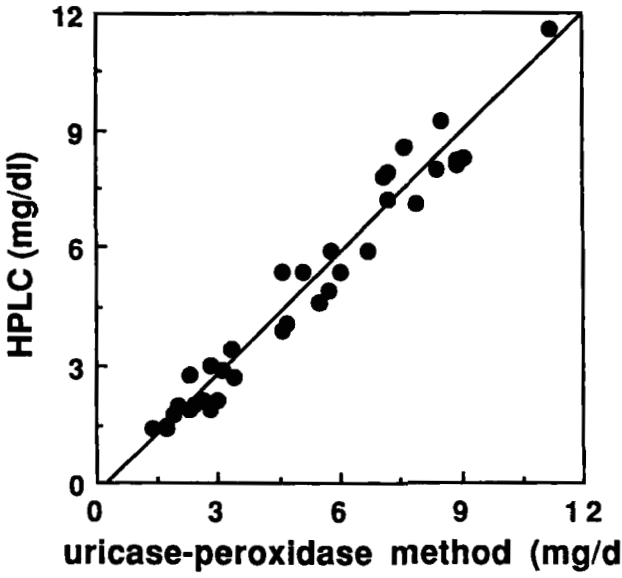

FIGURE 4. Correlation between creatinine contents measured by Jaffe method and by direct injection HPLC.

CONCLUSION

A PCMS-Q column, that restricts access of proteins to internal surfaces, was applied to the direct injection HPLC methods for simultaneous determination of creatinine and uric acid in serum. This method does not require troublesome sample pretreatment, and analytical errors are minimized. The accuracy and precision of the proposed assay system are adequate for clinical use. This direct injection HPLC method with the PCMS-Q column may be useful for measuring small ionic analytes in various biological fluids.

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