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Simultaneous determination of uric acid and creatinine in biological fluids by column-switching liquid chromatography with ultraviolet detection

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Abstract

A column-switching liquid chromatographic method for the simultaneous determination of uric acid and creatinine in human serum and urine was developed. Creatinine and uric acid were separated by size-exclusion chromatography on a hydrophilic gel column (C1) and creatinine eluted from C1 was separated from proteins by filtration through a longer hydrophilic gel column (C2). The creatinine fraction eluted from C2 was transferred to a weakly acidic cation-exchange column (C3) and then to a strongly acidic cation-exchange column (C4). Uric acid eluted from C1 after creatinine was transferred to an anion-exchange column (C5) and then to a hydrophilic gel column (C6). The mobile phase was a mixed buffer of pH 5.1 (propionic acid–succinic acid–NaOH, 60:15:60 mmol/l in water). Diluted serum and urine could be injected onto C1, and C1 was backflushed after the transfer of uric acid from C1 to C5.

Creatinine and uric acid in the eluate were determined by measuring their ultraviolet absorption at 234 and 290 nm, respectively. The recovery of uric acid and creatinine added to diluted serum (20-fold dilution, concentration 20 and 5 μmol/l, respectively) was 98.9±0.56% and 100.9±1.29%, respectively. The recovery of uric acid and creatinine added to diluted urine (100-fold dilution, concentration 50 and 100 μmol/l, respectively) was 99.4±0.72% and 98.7±1.45%, respectively (mean±R.S.D., $n=6$).

Keywords: Column switching; Uric acid; Creatinine

1. Introduction

Measurement of creatinine and uric acid in serum and urine is routinely performed by photometric methods such as the alkaline picrate reaction according to Jaffé [1] or enzymatic method for creatinine, and reduction of phosphotungstate or enzymatic method for uric acid. The chemical methods give higher values due to the presence of endogenous and exogenous substances that react with alkaline picrate (creatinine) or reduce phosphotungstate (uric acid). Enzymatic assays have higher specificity, but still suffer from interference by various exogenous and endogenous substances. To avoid these interferences, high-performance liquid chromatographic methods were developed. Ion-exchange [1–8], paired-ion [9–12] and reversed-phase [13–15] chromatography were used for the analysis of creatinine, and ion-exchange [16–18], paired-ion [12,19], reversed-phase [20,21] chromatography and size-exclusion

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chromatography [22] were used for uric acid. Most of these chromatographic methods require deproteination before analysis, but direct analysis of serum uric acid and creatinine was also successful [13,17,18,22], especially by column-switching liquid chromatography [15,23,24].

We could separate creatinine and uric acid by size-exclusion chromatography on a hydrophilic gel column (C1); size-exclusion chromatography on a longer hydrophilic gel column (C2) of the creatinine fraction transferred from C1 separated creatinine from serum protein. Therefore, we have tried to develop a column-switching liquid chromatographic method to determine creatinine and uric acid simultaneously with injection of diluted samples. The creatinine fraction eluted from C2 was transferred to a weakly acidic cation-exchange column and then to a strongly acidic cation-exchange column. Uric acid eluted from C1 after creatinine was purified further by anion-exchange and size-exclusion chromatography by column switching. As the same mobile phase is used for filtration of a sample through C1 and for backflushing of C1 after the transfer of uric acid from C1 to an anion-exchange column, C1 could be used repeatedly and the whole process could be automated.

2. Experimental

2.1. Materials

Creatinine and uric acid of special grade were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were of analytical grade from Yashima Pharmaceutical (Osaka, Japan). Stock solution of creatinine (20 m*M*) was prepared in 0.01 *M* hydrochloric acid, and that of uric acid (5 m*M*) was prepared in 0.01 *M* aqueous lithium carbonate solution. They were diluted with the mobile phase described below to give standard solutions of various concentrations.

2.2. Apparatus

The liquid chromatographic system equipped with automatic column-switching valves consisted of five Model 880-PU constant-flow pumps (Jasco, Tokyo, Japan), a Model SP-024-2 dual-head pump (Jasco), a Model KSST-601 automatic injector (Kyowa Seimitsu, Tokyo, Japan) and six columns: C1, a hydrophilic gel column, 10×0.76 cm I.D. (Shodex Asahipak GS-320 7C); C2, a hydrophilic gel column, 30×0.76 cm I.D. (Shodex Asahipak GS-320 HQ); C3, a weakly acidic cation-exchange column, 10×0.76 cm I.D. (Shodex Asahipak ES-502C 7C); C4, a strongly acidic cation-exchange column, 7.5×0.8 cm I.D. (Shodex IEC SP-825); C5, a weakly basic anion-exchange column, 10×0.76 cm I.D. (Shodex Asahipak ES-502N 7C); C6, a hydrophilic gel column, 25×0.76 cm I.D. (Shodex Asahipak GS-320 7E) and a hydrophilic gel column, 10×0.76 cm I.D. (Shodex Asahipak GS-320 7C) connected in series. The particle size is 9±0.5 μm for the materials packed in C1 and C3–C6, and gel of a smaller diameter is packed into C2 (Showa Denko K.K., Tokyo, Japan). Other equipment included a Model 821-09 automatic 6-port valve (Jasco), five Model MVA-4U7H automatic 4-port valves (Sanuki Kohgyo, Tokyo, Japan), two Model 870 UV spectrophotometers (Jasco) and a Model RC-125 recorder (Jasco). These components were assembled as shown in Fig. 1. The sample injection and switching events were controlled by using eleven timer units (T1–T11) [24]. The first timer unit (T1), controlling the automatic injector and a 4-port valve (V5), is equipped with a Model KS-1500 programmable timer (Koizumi Computer, Kobe, Japan), which repeats on and off modes of electric supply (100 V ac), at preset time intervals, to the relay circuit of T1 and those of T2–T11. Each timer unit T2–T11 is equipped with a motor timer with maximum graded time of 6, 12 or 30 min (Models SYS-6M, -12M, and -30M; Omron Electronics, Kyoto, Japan), and also with a relay circuit. When the preset time of the motor timer has elapsed, the 100 V a.c. supply to the motor timer is shut off and the 100 V a.c. supply is switched to an other timer unit. Timer units T2–T6, T8 and T10 are connected to T1. T7, T9 and T11 are connected to T6, T8 and T10, respectively. The circuits of timer units T2–T11 are reset when the supply of 100 V a.c. from T1 is off. Timer units T2 and T3 control valve V2, T4 controls V5, T5 controls V1, T6 and T7 control V3, T8 and T9 control V4 and T10 and T11 control V6.

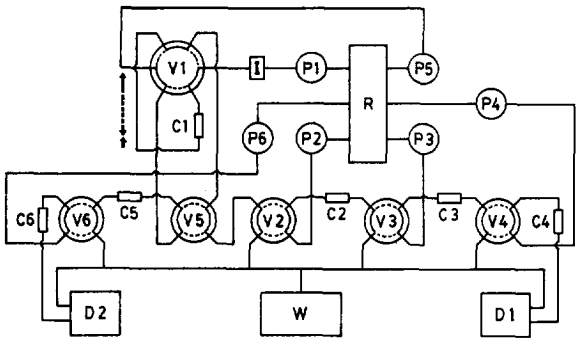

Fig. 1. Diagram of the column-switching equipment. R=mobile-phase reservoir; I=automatic injector; P1–P4 and P6=880-PU; P5=dual-head pump; C1=Shodex Asahipak GS-320 7C column (30°C); C2=Shodex Asahipak GS-320 HQ column (40°C); C3=Shodex Asahipak ES-502C 7C column (40°C); C4=Shodex IEC SP-825 column (40°C); C5=Shodex Asahipak ES-502N 7C column (40°C); C6=Shodex Asahipak GS-320 7E column+Shodex Asahipak GS-320 7C column (40°C); V1=6-port automatic valve (full line and broken line represent rotor positions B and A of the valve, respectively); V2–V6; 4-port automatic valve (full line and broken line represent rotor positions L and R, respectively); D1 and D2=spectrophotometers measuring absorbance at 234 and 290 nm, respectively; W=waste. When the rotor position of V1 is B and that of V2–V6 is L, mobile phase flows through the channels drawn with full lines, and when the rotor position of V1 is A and that of V2–V6 is R, the mobile phase flows through the channels drawn with broken lines. The arrows drawn with full line indicate the direction of flow of mobile phase pumped by P1 when the rotor position of V1 is B (for 10.6 min from the time of sample injection and for 5 min after backflushing of C1). When the rotor position of V1 is A, mobile phase pumped by P5 flows in the direction indicated by the arrow drawn with broken line and back-flushes C1 (after 10.6 min to 37 min from the time of sample injection, Table 1).

2.3. Mobile phase

A mixed buffer of pH 5.1 (propionic acid–succinic acid–NaOH, 60:15:60 mmol/l H_2O) was used as the mobile phase. Water of ultra-pure grade, purified by reverse osmosis (ROpure 40, Barnstead, Boston, MA, USA), ion exchange and charcoal adsorption (NANOpure II, Barnstead) in series, was used to prepare the mobile phase. It was filtered through a membrane filter (Type HV, pore size, 0.45 μm; Millipore, Bedford, MA, USA) and degassed before use. The flow-rate of the mobile phase was 1.0 ml/min.

2.4. Sample preparation

Fresh sample or samples in plastic vials frozen at −70°C were used. The latter were thawed at 37°C before use. They were diluted 15- to 500-fold with the mobile phase. The diluted samples were filtered through a disposable membrane filter (Shodex DT MX-13K, pore size 0.5 μm; Showa Denko K.K.) and poured into a vial. For the determination of recovery, 100 μl of serum was mixed with 1.0 ml of the mobile phase containing 40 μmol/l of uric acid and 10 μmol/l of creatinine and 0.9 ml of the mobile phase to give a diluted serum sample with a uric acid and creatinine concentration of 20 μmol/l and 5 μmol/l, respectively; 500 μl of urine was mixed with 10 ml of the mobile phase containing 250 μmol/l of uric acid and 500 μmol/l of creatinine and the mixture was diluted with the mobile phase to give a diluted urine sample with a uric acid and creatinine concentration of 50 μmol/l and 100 μmol/l, respectively.

2.5. Column switching and detection

The rotor of V1 was set at position B and the rotors of V2–V6 were set at position L. Under these settings of the rotor valves, the mobile phase flowed through the channels of the rotors of the valves drawn with full lines in Fig. 1. Mobile phase, pumped by P1, flowed through the hydrophilic gel column (C1) to waste, and the mobile phase pumped by P5 flowed through the anion-exchange column (C5). When the switch of the programmable timer of T1 was turned on, T2–T11 were switched on, and a sample (300 μl) was injected onto C1. Creatinine eluted from C1 was transferred to C2 via V2 by column switching, and then uric acid was transferred from C1 to C5 by rotating the rotor of V5 to position R. After the transfer of uric acid fraction to C5 was completed, the rotor of V1 rotated to position A to disconnect C1 and C5, and at the same time to backflush C1 by the mobile phase pumped by P5. The creatinine fraction eluted from C2 was purified further by ion-exchange chromatography on the weakly acidic cation-exchange column (C3) and on the strongly acidic cation-exchange column (C4). The uric acid fraction was transferred from C5 to hydrophilic gel columns (Shodex Asahipak GS-320

7E+Shodex Asahipak GS-320 7C, C6). Thirty-seven minutes after sample injection, the programmable timer of T1 was off, T2–T11 were reset, the rotor of V1 rotated to position B and backflushing of C1 ended, and at the same time the rotor of V5 rotated to position L. Five minutes later, the timer of T1 was on, and the next sample was injected (Table 1).

Creatinine and uric acid were determined from their peak heights on the chromatogram, obtained by ultraviolet detection at 234 nm and 290 nm, respectively. The output lines of the two detectors (D1 and D2) were connected to the relay of a timer unit (T12), which was controlled by the timer unit T1. The input line of the recorder was also connected to the relay of T12. From 0 to 26 min, D2 was connected to the recorder and from 26 to 37 min D1 was connected to the recorder.

3. Results

As shown in Fig. 2, creatinine and uric acid were separately eluted from C1. The creatinine fraction from C1 was transferred to C2 to separate creatinine from proteins and other high-molecular-mass components. Cation-exchange chromatography on a weakly acidic ion-exchange column (C3) and strongly acidic ion-exchange column (C4) gave the

Table 1
Timing and sequence of events of automated column switching for the determination of creatine and uric acid

Time (min)	Rotor position of valves						Events
	V1	V2	V3	V4	V5	V6	
0	B	L	L	L	L	L	Sample injection on to C1
4.9	B	R	L	L	L	L	Connection of C1 and C2. Transfer of creatinine fraction from C1 to C2
7.3	B	L	L	L	L	L	Disconnection of C1 and C2. End of transfer of creatinine fraction from C1 to C2
7.6	B	L	L	L	R	L	Connection of C1 and C5. Transfer of uric acid fraction from C1 to C5
10.6	A	L	L	L	R	L	Backflushing of C1 wit P5. End of transfer of uric acid fraction
15.5	A	L	R	L	R	L	Connection of C2 and C3. Transfer of creatinine fraction from C2 to C3
18.4	A	L	L	L	R	L	Disconnection of C2 and C3. End of transfer of creatine fraction from C2 to C3
22.3	A	L	L	R	R	L	Connection of C3 and C4. Transfer of creatine fraction from C3 to C4
24.5	A	L	L	R	R	R	Connection of C5 and C6. Transfer of uric acid fraction from C5 to C6
26.0	A	L	L	L	R	R	Disconnection of C3 and C4. End of transfer of creatine fraction from C3 to C4
29.2	A	L	L	L	R	L	Disconnection of C5 and C6. End of transfer of uric acid fraction from C5 to C6
37.0	B	L	L	L	L	L	End of backflushing of C1
42.0	B	L	L	L	L	L	Injection of next sample

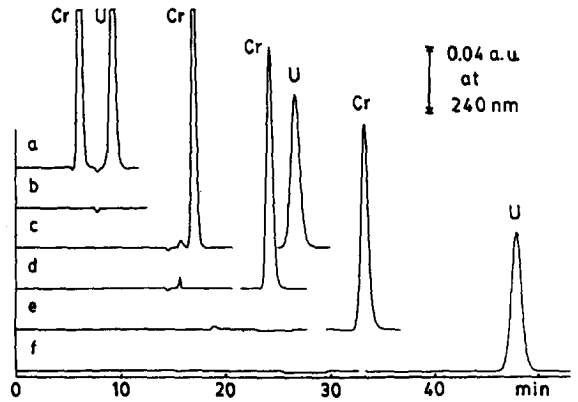

Fig. 2. Elution patterns showing steps of column-switching. (a) Elution of creatinine (Cr) and uric acid (U) from C1, (b) transfer of Cr and U from C1 to C2 and C5, (c) elution of Cr from C2 and U from C5, (d) transfer of Cr from C2 to C3 and elution of Cr from C3, (e) transfer of Cr from C3 to C4 and elution of Cr from C4, (f) transfer of U from C5 to C6 and elution of U from C6.

creatinine fraction free from UV-absorbing impurities (Fig. 3). Anion-exchange chromatography of the uric acid fraction from C1 on a weakly basic ion-exchange column (C5) removed most of the impurities. As is shown in Fig. 2, the uric acid fraction from C5 was further purified on hydrophilic gel columns. The height of the peak of UV-absorbing compound remaining after uricase treatment of a urine sample was less than 0.2% of that of uric acid peak of the

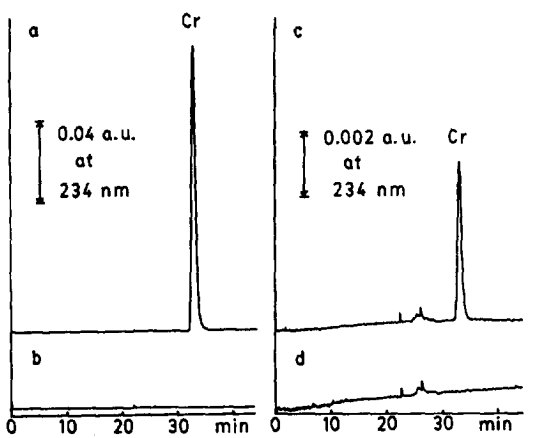

Fig. 3. Elution of creatinine from C4. (a) Urine sample diluted 50-fold with mobile phase, (b) the urine sample treated with creatinine deiminase, (c) serum sample diluted 20-fold with mobile phase, (d) the serum sample treated with creatinine deiminase.

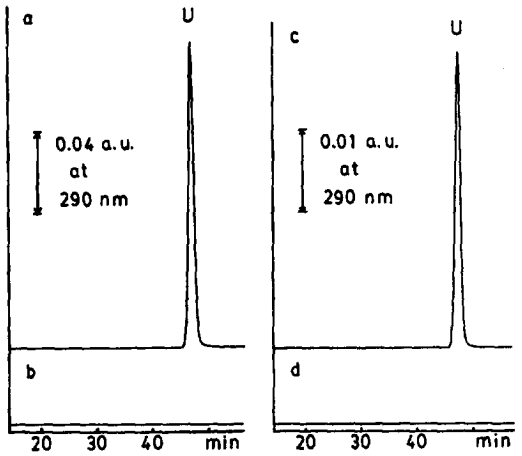

Fig. 4. Elution of uric acid from C6. (a) Urine sample diluted 50-fold with the mobile phase, (b) the urine sample treated with uricase, (c) serum sample diluted 20-fold with the mobile phase, (d) the serum sample treated with uricase.

urine sample (Fig. 4b). Shodex Asahipak GS-320 7E and Shodex Asahipak GS-320 7C were connected to get a retention time for uric acid longer than that of creatinine from C4 so that the outputs of the two detectors could be recorded alternately on a recorder chart (Fig. 5).

The limit of detection was 0.2 μmol/l for creatinine and 0.1 μmol/l for uric acid at a signal-to-noise ratio of 3. The relationship between the peak

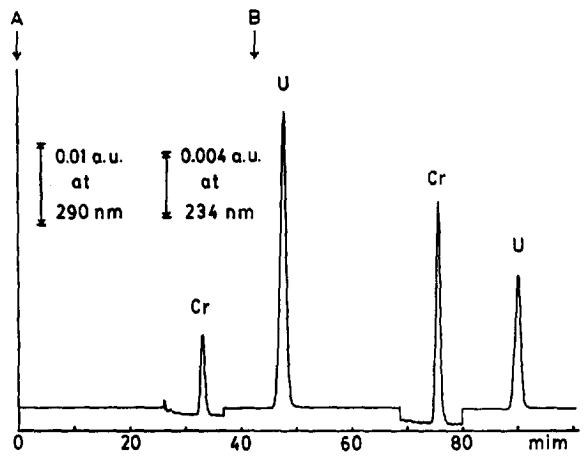

Fig. 5. Elution of creatinine and uric acid from C4 and C6, respectively. Arrows indicate the time of injection of a diluted serum sample (A, 20-fold dilution) and a diluted urine sample (B, 500-fold dilution). Concentrations of analytes calculated are, from the left, Cr=3.48, U=14.4, Cr=10.5, U=6.42 (mol/l).

Table 2
Recovery of creatinine and uric acid added to diluted serum and urine

Analyte	Concentration of analyte added (μmol/l)	Recovery[a] (%)
Serum (20-fold dilution)		
Creatine	5	100.9±1.29
Uric acid	20	98.9±0.56
Urine (100-fold dilution)		
Creatine	100	98.7±1.45
Uric acid	50	99.4±0.72

[a] Mean±R.S.D. (n=6).

height (x) and the concentration of the analyte (y) was linear: in the ranges 1.0–200 μmol/l for creatinine and 0.5–100 μmol/l for uric acid in the mobile phase, the equations were $y=0.604x-0.054$ ($r=0.99997$) and $y=0.332x-0.014$ ($r=1$), respectively. The recovery of creatinine and uric acid added to serum and urine was satisfactory as is shown in Table 2.

4. Discussion

The column-switching system described is fairly complex, but the system was automated and a mixed buffer of pH 5.1 was used as the mobile phase. Since regeneration of C1 was performed by back-flushing with the same mobile phase, no other mobile phase was needed. Propionic acid was incorporated in the mixed buffer because propionate prevents the growth of microorganisms. Addition of succinic acid to the buffer reduced the retention time of uric acid from C5 relative to those of impurities from the column. Creatine and creatinine were eluted together from C1 and creatine eluted from C2 faster than creatinine with partial overlapping but the former was completely separated from the latter by ion-exchange chromatography on a weakly acidic cation-exchange column (C3). Conversion of creatine to creatinine was 0.63% when a 2 mM solution of creatine in mobile phase was incubated at 40°C for 3 h. Since the time needed to elute creatine through C1 and C2 was only 18 min, conversion of creatine to creatinine during chromatography will be less than 0.1%.

Pretreatments of the sample to be injected onto C1 were dilution of the sample with mobile phase and filtration of the diluted sample through a disposable membrane filter. The sensitivity of the method was high enough to determine the concentration of creatinine and uric acid in diluted serum and urine samples. Every 42 min a sample could be analyzed. This time is longer than that obtained with other HPLC methods, but the use of a mobile phase that is stable for several weeks makes this method suitable for routine analysis. Furthermore, simultaneous determination of uric acid and creatinine is useful for diagnosis and treatment of hyperuricemia.

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