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Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1621–1626

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Short communication

Simultaneous determination of creatinine, hypoxanthine and uric acid in biological samples by column-switching liquid chromatography with ultraviolet detection[1]

Tokuichiro Seki [a,*], Yoshimasa Orita [a], Kenji Yamaji [a], Akiko Shinoda [b]

[a] *School of Allied Health Sciences, Faculty of Medicine, Osaka University, Suita-Shi, 1-7, Yamadaoka, Osaka 565, Japan*
[b] *Specialty Chemicals Division, Showa Denko K.K., 13-9, Shiba Daimon 1 Chome, Minato-ku, Tokyo 105, Japan*

Received 25 September 1996; accepted 19 December 1996

Keywords: Creatinine; Hypoxanthine; Uric acid; Liquid chromatography; Column-switching

1. Introduction

The determination of hypoxanthine and xanthine, which are precursors of uric acid and also important intermediates in purine metabolism, were performed by liquid chromatography with ultraviolet detection [1–3], column-switching liquid chromatography with ultraviolet detection [4,5], fluorescence detection using enzyme reactors [6] and gas chromatography-mass spectrometry [7]. These methods required deproteinization of plasma and blood samples for sample preparation.

In a previous paper we have shown that creatinine and uric acid in human serum and urine could be determined by column-switching liquid chromatography by using a mixed buffer of pH 5.1 without any sample pretreatment other than dilution and filtration [8]. Hypoxanthine could be separated from uric acid by chromatography on a weakly basic ion-exchange column, and use of a mixed buffer of pH 5.75 and additional purification by reversed-phase chromatography of the hypoxanthine fraction permitted determination of hypoxanthine in diluted human urine, serum and plasma filtered through a membrane filter.

2. Experimental

2.1. Materials

Creatinine, hypoxanthine and uric acid of special grade were purchased from Wako (Osaka, Japan). Other chemicals were of analytical grade from Yashima Pharmaceutical (Osaka, Japan). Stock solution of creatinine (10 mM) was pre-

* Corresponding author.
[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August, 1996, Osaka, Japan.

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PII S0731-7085(97)00011-3

pared in 0.01 M hydrochloric acid, and those of uric acid (0.4 mM) and hypoxanthine (0.4 mM) were prepared in a mixed buffer (propionic acid–succinic acid–NaOH–Na_2EDTA, 60:15:80:0.5 mmol kg^{-1} H_2O; Na_2EDTA, disodium ethylenediaminetetraacetate). They were diluted with the mixed buffer to give standard solutions of various concentrations. The mixed buffer was also used as the diluent to prepare diluted urine, serum and plasma samples.

2.2. Apparatus

The liquid chromatographic system equipped with automatic column-switching valves consisted of three model 880-PU and model PU-980 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 five columns: (C1, a hydrophilic gel column, 10 × 0.76 cm i.d. (Shodex Asahipak GS-320 7C); C2, a hydrophilic gel column, 25 × 0.76 cm i.d. (Shodex Asahipak GS-320 7E); C3, a strongly acidic cation-exchange column, 7.5 × 0.8 cm i.d. (Shodex IEC SP-825) and a weakly acidic cation-exchange column, 10 × 0.76 cm i.d. (Shodex Asahipak ES-502C 7C) connected in series; C4, a weakly basic anion-exchange column, 10 × 0.76 cm i.d. (Shodex Asahipak ES-502N 7C) and a hydrophilic gel column, 25 × 0.76 cm i.d. (Shodex Asahipak GS-320 7E) connected in series; C5, a polystyrene-divinylbenzene gel column, 15 × 0.6 cm i.d. (Shodex RSpak RP18-613) and a hydrophilic gel column, 5 × 0.76 cm i.d. (Shodex Asahipak GS-2G 7B) connected in series. The particle size is 9 ± 0.5 μm for the materials packed in C1–C4 and the GS-2G7B column and gel of a smaller diameter is packed into the RP18-613 column (Showa Denko, Tokyo, Japan)). Other equipment included a model 821-09 automatic 6-port valve (Jasco), four model MVA-4U7H automatic 4-port valves (Sanuki Kohgyo, Tokyo, Japan), three UV-spectrophotometers (D1, a model UVIDEC-100-III (Jasco); D2, a model 870-UV (Jasco); D3, a model UV-7500 (Advantec, Osaka, Japan)), 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 eight timer units (T1–T8) [8]. The first timer unit (T1), controlling the automatic injector and a 4-port valve (V4), is equipped with a model KS-1500 programmable timer (Koizumi Computer, Kobe, Japan), which repeats on and off modes of electric supply (100 V a.c.), at preset time intervals, to the relay circuit of T1 and those of T2–T8. Each timer unit of T2–T8 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 is over, the 100 V a.c. supply to the motor timer is shut off and the 100 V a.c. supply from the timer unit

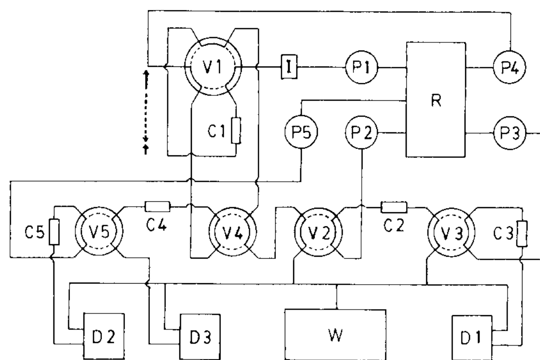

Fig. 1. Diagram of the column-switching equipment. R, mobile-phase reservoir; I, automatic injector; P1–P3, 880-PU; P4, dual-head pump; P5, PU-980; C1, Shodex Asahipak GS-320 7C column (25°C); C2, Shodex Asahipak GS-320 7E column (25°C); C3, Shodex IEC SP-825 + Shodex Asahipak ES-502C 7C column (20°C); C4, Shodex Asahipak ES-502N 7C column + Shodex Asahipak GS-320 7E column (25°C); C5, Shodex RSpak RP18-613 column + Shodex Asahipak GS-2G 7B column (40°C); V1, 6-port automatic valve; V2–V5, 4-port automatic valves; D1, D2 and D3, spectrophotometers measuring absorbance at 235 nm, 250 nm and 290 nm, respectively; W, waste. The arrows drawn with full line indicate the direction of flow of the mobile phase pumped by P1. It flows through the channel of V1 drawn with a full line (for 9.5 min from the time of sample injection and for 4 min after backflushing of C1). The arrow drawn with a broken line indicates the direction of flow of the mobile phase pumped by P4. It flows through the channel of V1 drawn with a broken line, backflushes C1, flows through the channels of V1 and V4 drawn with a broken line and that of V2 drawn with a full line and flows into the waste (9.5 min to 41 min from the time of sample injection).

to the other timer unit is on. Timer units T2–T4, T5 and T7 are connected to T1, and T6 and T8 are connected to T5 and T7, respectively. The circuits of timer units T2–T8 are reset when the supply of 100 V a.c. from T1 is off. Timer unit T2 controls valve V2, T3 controls V2 and V4, T4 controls V1, T5 and T6 control V3, T7 and T8 control V5.

2.3. Mobile phase

A mixed buffer of pH 5.75 (propionic acid–succinic acid–NaOH, 60:15:80 mmol kg^{-1} H_2O) was used as the mobile phase. Water of ultrapure grade, purified by reverse osmosis (ROpure 40, Barnstead, Boston, MA), ion-exchange and charcoal adsorption (NANOpure II, Barnstead) in series, was used to prepare the mobile phase and the diluent described above. The mobile phase was filtered through a membrane filter (Type HV, pore size, 0.45 μm; Millipore, Bedford, MA) and degassed before use. The flow-rate of the mobile phase was 1.1 ml min^{-1}.

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 5- to 100-fold with the mobile phase. The diluted samples were filtered through a disposable membrane filter (Shodex DT MK-13X, pore size 0.5 μm; Showa Denko) and poured into a vial. For the determination of recovery, 200 μl plasma was mixed with 800 μl the diluent containing 20 μmol l^{-1} creatinine, 1.0 μmol l^{-1} hypoxanthine and 100 μmol l^{-1} uric acid to give a diluted plasma sample with concentration of creatinine, hypoxanthine and uric acid increased by 16, 0.8 and 80 μmol l^{-1}, respectively.

2.5. Column-switching and detection

The position of the rotors of V1–V5 were set so that the mobile phase flows through the channels of the rotors of the valves drawn with full lines in Fig. 1. Mobile phase pumped by P1 flowed through C1 to waste, and the mobile phase pumped by P4 flowed through C4. When the switch of the programmable timer T1 was turned on (0 min), T2–T8 came on, and a sample (300 μl) was injected onto C1. Creatinine eluted from C1 was transferred to C2 via V2 by column switching (4–6.4 min), and then uric acid + hypoxanthine was transferred from C1 to C4 by rotating the rotor of V4 (6.4 min). When the transfer of uric acid + hypoxanthine fraction to C4 was over, the rotor of V1 rotated to disconnect C1 and C4, and at the same time to back flush C1 with the mobile phase pumped by P4 that flows through the channels of V1 drawn with broken line (9.5 min). The creatinine fraction eluted from C2 was purified further by ion-exchange chromatography on C3 (time of transfer 12.5–15.5 min). The hypoxanthine fraction eluted from C4 was transferred to C5 (26.4–30.6 min) and purified by reversed-phase mode. 41 Min after sample injection, the programmable timer of T1 was off, T2–T8 were reset, the rotor of V1 and V4 rotated to the original position and backflushing of C1 ended. 4 Min later the timer T1 came on and the next sample was injected. Creatinine, hypoxanthine and uric acid were determined from their peak heights on the chromatogram, obtained by ultraviolet detection at 235, 250 and 290 nm, respectively. The output lines of two detectors, D3 and D1, were connected to the relay of a timer unit (T9) and the output line of D2 was connected to the relay of another timer unit (T10). They were equipped with a motor timer and received 100 V a.c. supply from timer T1. The relay of T9 was connected to the relay of T10 and the input line of the recorder was also connected to the relay of T10. When T1 was on (0 min), D3 was connected to the recorder via T9 and T10. After 18 min, D1 was connected to the recorder via T9 and T10, and from 29 min, D2 was connected to the recorder via T10. After 41 min from the time of sample injection, T1 was off, T9 and T10 were reset, and D3 was connected to the recorder. Therefore, the output of three detectors could be alternatively recorded on a recorder chart.

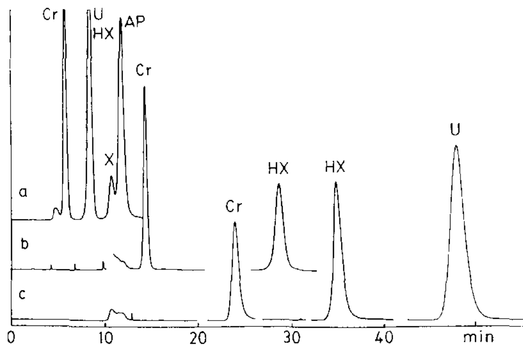

Fig. 2. Elution patterns showing steps of column-switching. (a) Elution of creatinine (Cr) uric acid (U), hypoxanthine (HX), xanthine (X) and allopurinol (AP) from C1, (b) transfer of Cr from C1 to C2, and U and HX from C1 to C4, elution of Cr from C2 and HX from C4 (elution of U from C4 was not recorded), (c) transfer of Cr from C2 to C3, elution of Cr from C3, transfer of HX from C4 to C5, elution of HX from C5 and elution of U from C4.

3. Results

As shown in Fig. 2, creatinine was separated from uric acid and hypoxanthine by elution from a hydrophilic gel column (C1). The creatinine fraction from C1 was transferred to C2 to separate creatinine from proteins and other high-molecular-weight components. Cation-exchange chromatography on a weakly acidic ion-exchange column and strongly acidic ion-exchange column (C3) removed UV-absorbing impurities from the creatinine fraction (Fig. 3b, Fig. 4b). The height of the UV-absorbing peak remaining after creatinine deiminase treatment of a urine sample was less than 0.2% of that of creatinine peak of the urine sample (Fig. 3b). Anion-exchange chromatography of the uric acid + hypoxanthine fraction from C1 on a weakly basic ion-exchange column and filtration through a hydrophilic gel column removed impurities having absorption at 290 nm. In some urine samples the UV-absorbing peak, about 0.3% as high as the original uric acid peak, remained after uricase treatment (Fig. 3b). The peak of hypoxanthine disappeared after treatment of diluted serum and urine with xanthine oxidase (Fig. 3b, Fig. 4b). C1 and C2, packed with a hydrophilic gel, were very stable when used with the mobile phase and the elution pattern of the analyses did not change after repeated injection of more concentrated plasma samples (Fig. 5a).

The limit of detection was 0.2 μmol l^{-1} for creatinine and uric acid, and 0.1 μmol l^{-1} for hypoxanthine at a signal-to-noise ratio of 4 (Fig. 5b). The relationship between the peak height (x) and the concentration of the analyte (y) was linear in the ranges 1.0–100 μmol l^{-1} for creatinine and uric acid and 0.2–20 μmol l^{-1} for hypoxanthine in the diluent. The equations were $y = 0.466x - 0.115$ ($r = 1$), $y = 0.646x - 0.052$ ($r = 1$) and $y = 0.182x + 0.133$ ($r = 0.9999$), respectively. The recovery of creatinine (16 μmol l^{-1}), uric acid (80 μmol l^{-1}) and hypoxanthine (0.8 μmol l^{-1}) added to a plasma sample diluted 5-fold was 101 ± 0.3, 99.4 ± 0.46 and 103 ± 1% (mean ± R.S.D., $n = 5$), respectively.

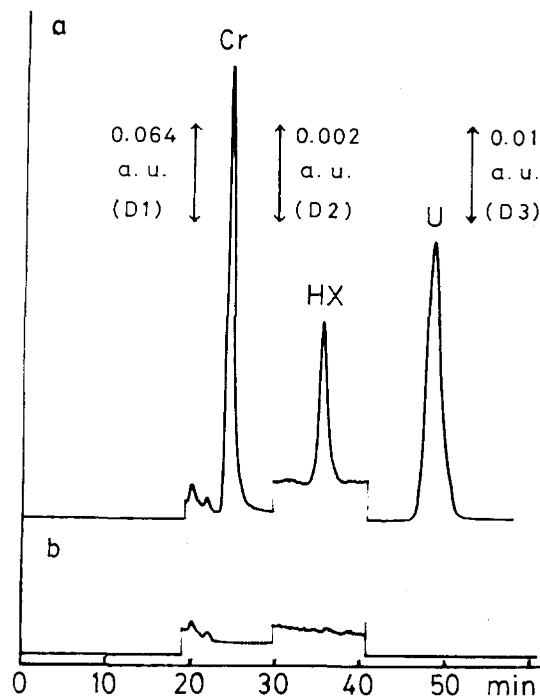

Fig. 3. Elution of creatinine, hypoxanthine and uric acid from C3, C5 and C4, respectively. (a) A urine sample diluted 50-fold, (b) the urine sample treated with creatinie deiminase, xanthine oxidase and uricase. D1, D2 and D3 are detector numbers. Concentration of the analyses calculated are Cr = 169, HX = 1.63 and U = 91.6 μmol l^{-1}.

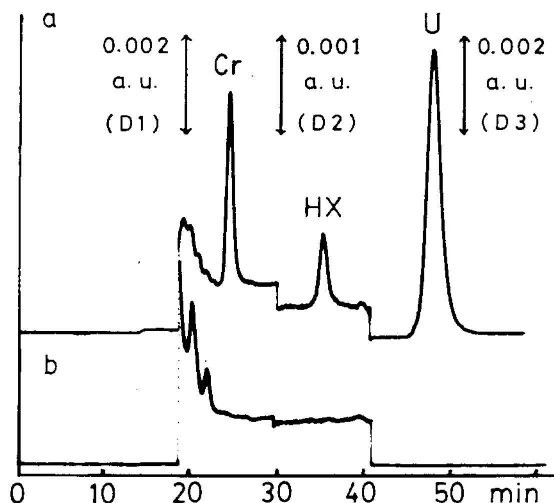

Fig. 4. Elution of creatinine, hypoxanthine and uric acid from C3, C5 and C4, respectively. (a) A serum sample diluted 20-fold, (b) the serum sample treated with creatinine deiminase, xanthine oxidase and uricase. Concentration of the analyses calculated are Cr = 2.24, HX = 0.35 and U = 18.7 μmol l^{-1}.

4. Discussion

The column-switching system described is fairly complex, but the system was automated and a mixed buffer of pH 5.75 that is stable for several weeks was used as the mobile phase. Propionic acid was incorporated in the mixed buffer, because propionates do not allow microorganism to grow. Since regeneration of C1 was performed by backflushing with the same mobile phase, no other mobile phase was needed. 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 cation-exchange chromatography on C3. Conversion of creatine to creatinine was 0.078% after a solution of creatine in the diluent at concentration of 10 mM was incubated in C2 for 150 min at 25°C. Since the time needed to elute creatine through C1 and C2 was 14 min, conversion of creatine to creatinine during chromatography will be 0.0073%, and the value is smaller than the R.S.D. of the recovery of 16 μmol l^{-1} of creatinine added to a diluted plasma sample (0.3%).

The sensitivity of this method was high enough to determine the concentration of creatinine and uric acid in diluted serum (20- to 50-fold dilution) and urine (50- to 500-fold dilution) samples. But, for the determination of hypoxanthine in human plasma, samples were diluted 5-fold (Fig. 5a) because concentration of the analyse is low [7]. Samples were analyzed every 45 min. This time is longer than the other HPLC method, but use of a mobile phase that is stable for several weeks makes this method suitable for routine analysis. Furthermore, simultaneous determination of uric acid, hypoxanthine and creatinine is useful for diagnosis and treatment of hyperuricemia.

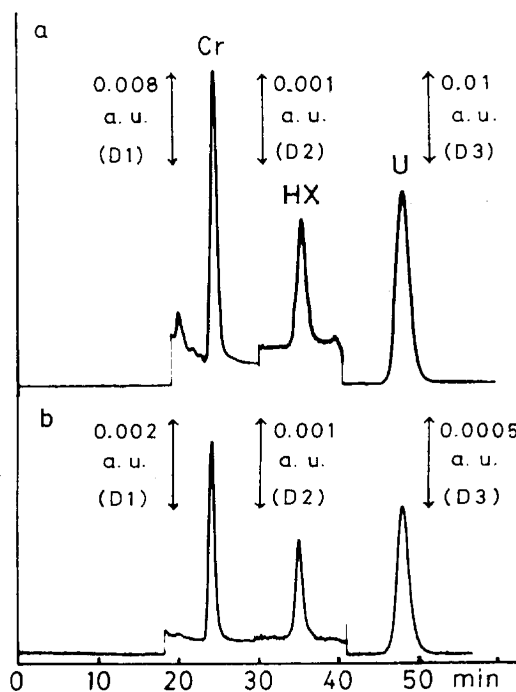

Fig. 5. Elution of creatinine, hypoxanthine and uric acid from C3, C5 and C4, respectively. (a) A plasma sample diluted 5-fold, (b) a standard sample solution (Cr = 2.5, HX = 0.5 and U = 2.5 μmol l^{-1}). Concentration of the analyses calculated are Cr = 14.7, HX = 0.65 and U = 66.7 μmol l^{-1}.

Acknowledgements

This work was supported by a grant for protection against the intractable disease 'progressive renal disease' by the Ministry of Health and Welfare of Japan.

References

[1] R.J. Simmonds and R.A. Harkness, J. Chromatogr., 226 (1981) 369–381.
[2] T. Yamamoto, Y. Moriwaki, S. Takahashi, T. Hada and K. Higashino, J. Chromatogr., 382 (1986) 270–274.
[3] T. Kojima, T. Nishina, M. Kitamura, N. Kamatani and K. Nishioka, Clin. Chem., 32 (1986) 287–290.
[4] K. Nagao, Jpn. J. Clin. Chem., 23 (1994) 182–187.
[5] S. Sumi, K. Kidouchi, S. Ohba and Y. Wada, J. Chromatogr. B, 670 (1995) 376–378.
[6] M. Kito, R. Tawa, S. Takeshima and S. Hirose, J. Chromatogr., 278 (1983) 35–42.
[7] C. Lartigue-Mattei, J.L. Chabard, H. Bargnoux et al., J. Chromatogr., 529 (1990) 93–101.
[8] T. Seki, K. Yamaji, Y. Orita, S. Moriguchi and A. Shinoda, J. Chromatogr. A, 730 (1996) 139–145.