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Simultaneous determination of urinary creatinine and aromatic amino acids by cation-exchange chromatography with ultraviolet detection

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ABSTRACT

A cation-exchange chromatographic procedure for the simultaneous determination of urinary creatinine and aromatic amino acids is described. Creatinine and amino acids were separated from organic acids and/or neutral species in urine by using a preparative cation-exchange resin column. A column packed with a cation-exchange resin of low capacity was used for the analytical separations. The elution of creatinine and aromatic amino acids was monitored at 210 nm by means of an ultraviolet detector. The relationships between concentration and peak heights were reproducible with a coefficient of variation of less than 2%, and were linear from 5 to 200 μM for each compound. Overall recoveries of the analytes were more than 95%. The method was applied to the analysis of urine of patients with disorders of amino acid metabolism, such as phenylketonuria. The concentration ratios of phenylalanine to creatinine in the patients' urine were accurately and easily determined, and were quite different from those in the urine of healthy newborns.

INTRODUCTION

Determination of creatinine in human serum and urine is important and routinely performed in many clinical laboratories, because the concentration ratio of a specific analyte to creatinine is considered to be a more useful indicator than the analyte concentration itself. In gereral, creatinine is determined separately from other compounds in the samples. The classical Jaffe alkaline picrate method [1] is the most widely used. However, it is non-specific and subject to interference from constituents of serum and urine [2]. Recently, several specific methods for creatinine determination have been developed, using gas chromatography–mass spectrometry (GC–MS) [3] and high-performance liquid chromatography (HPLC) [4–9]. The sample preparation and derivatization necessary for the GC–

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MS method is quite complicated. For the HPLC methods, the sample preparation is simple. A deproteinized and/or filtered serum or urine sample is directly analysed. It is considered, however, that sample preparation prior to determination is useful for reliable and reproducible analyses of such complicated mixtures as urine. It is desirable to separate the sample into acidic, neutral and basic fractions beforehand.

This paper describes a newly developed method for the specific determination of urinary creatinine and of several aromatic amino acids. Prepurification of urine samples by preparative cation-exchange chromatography, prior to HPLC, was useful. High-performance cation-exchange chromatography using a low-capacity cation exchanger [10] and UV detection facilitated the simultaneous determination of creatinine and aromatic amino acids.

EXPERIMENTAL

Analytical HPLC

The analytical chromatography system consisted of a Tosoh (Tokyo, Japan) Model CCPE pump, a Shodex (Tokyo, Japan) Model DP-1 pulse dampener, a Rheodyne (Cotati, CA, U.S.A.) 7125 sample injector with a 50-μl loop, a Jasco (Tokyo, Japan) Model 875-UV UV–VIS detector and a Hitachi (Tokyo, Japan) Model D-2000 integrator. The column was a stainless-steel column (100 mm × 4.6 mm I.D.) packed with a cation-exchange resin (sulphonated styrene–divinylbenzene; 4% DVB; average diameter 11 μm; ion-exchange capacity 24 μequiv./ml) manufactured by Tosoh. The theoretical plate number was 1200 for a peak of phenylalanine. A 30% (v/v) aqueous solution of methanol, containing 7 m*M* perchloric acid, was used as the mobile phase at a flow-rate of 1.0 ml/min. All separations were carried out at ambient temperature.

Preparative column chromatography

The preparative column chromatography system consisted of a Rheodyne 5012 six-position rotary valve for changing the mobile phase (0.1 *M* HCl, water, and 0.1 *M* NH_3), a Gasukuro Kogyo (Tokyo, Japan) Model MPD-3MG single-plunger middle-pressure pump, a Rheodyne 5020 sample injection valve, a Shimadzu (Kyoto, Japan) Model UVD-4 fixed-wavelength (254 nm) UV detector, and an Ohkura (Tokyo, Japan) Model DR-1111 chart recorder. The preparative column was a glass column (100 mm × 6 mm I.D., 65 mm bed length, 0.27 mequiv. total capacity) packed with TSKgel SP-Toyopearl 650 (Tosoh, cation exchanger; 0.15 mequiv./ml).

Reagents

Creatinine, L-phenylalanine, L-tyrosine, L-tryptophan, hydrochloric acid, perchloric acid, methanol, and ammonia water were purchased from Wako (Osaka, Japan). Other amino acids and bases were from Wako or Sigma (St. Louis, MO,

U.S.A.). All reagents were of guaranteed grade and used without further purification. Methanol and deionized water were distilled before use.

Urine samples

The urine samples, collected from four patients with phenylketonuria (PKU) and one patient with hypertyrosinemia, were furnished by Shimoshizu National Hospital and Sanatorium. The urine samples of normal controls were collected from ten healthy newborns at Yokohama City University Hospital. All urines were stored at −20°C.

Sample treatment procedure

The preparative cation-exchange resin (SP-Toyopearl 650) was conditioned to the H^+ form with 0.1 *M* HCl and washed with water until neutral. The urine was filtered through a 0.2-μm cellulose acetate filter (Advantec, Tokyo, Japan), then a 100-μl aliquot was introduced into the column from the sample injection valve using a Hamilton (Reno, NV, U.S.A.) 1725 gas-tight syringe. After organic acids and/or neutral compounds had been eluted with water (Fig. 1), the mobile phase was changed to 0.1 *M* NH_3. The adsorbate eluted with a large peak was collected in a 20-ml Erlenmeyer flask, and is referred to as the 'amino acid fraction'. The column was again conditioned and used repeatedly. The fraction was freeze-dried in order to remove ammonia. The residue was redissolved in 1.00 ml of water, and a 20-μl aliquot was injected into the analytical column using a Hamilton 1710 gas-tight syringe.

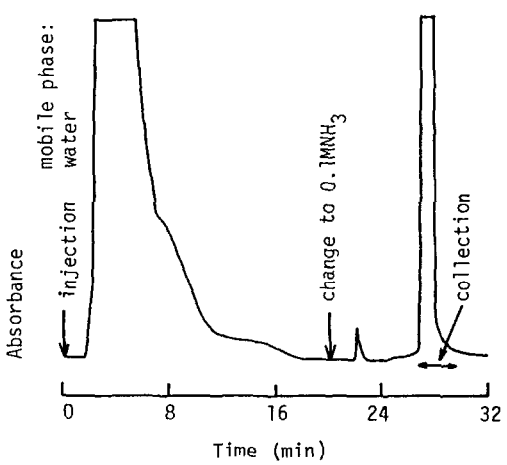

Fig. 1. Preparative separation of a normal urine sample: sample size, 100 μl; UV monitor, 254 nm, 0.64 a.u.f.s.

RESULTS AND DISCUSSION

Pretreatment

The Toyopearl gels, which are hydrophilic vinyl polymers, used for sample preparation do not change in volume when the eluent is changed. Because the ion-exchange capacity of the resin is relatively small, the concentration of the eluting ion can be reduced.

The preparative separation of a urine sample from a normal newborn is shown in Fig. 1, in which two large peaks appear. The first peak contains organic acids and neutral compounds, and the latter contains amino acids, among others. The volume of water required to wash out the acid and neutral compounds was dependent on the urine concentration, and varied from 6 to 20 ml. The effluent could be used for further analysis of organic acids. About 2 ml of 0.1 *M* NH_3 were needed to elute the analytes, and a rather large portion (*ca.* 3 ml) of the eluate was collected considering the dead volume behind the UV monitor. Since the amount of protein in urine is generally small, deproteinization was not necessary. The pH of the urine samples, ranging 4.5 to 8.5, was not adjusted.

Composition of mobile phase for the analytical separation

In the present cation-exchange chromatography the eluting ion was H^+. Perchloric acid was chosen because of its weak corrosive effects and UV transparency. When 7 m*M* perchloric acid was used as the mobile phase, creatinine and tyrosine were eluted close to the retention times of 15 and 16 min, respectively. However, phenylalanine was hydrophobically retained in the column, and appeared only at 54 min as a peak with a width of 15 min. Therefore methanol was added to the mobile phase in order to reduce the retention of the hydrophobic amino acids, such as tyrosine and phenylalanine.

The effect of the perchloric acid concentration in the mobile phase containing 30% (v/v) methanol, and of the concentration of methanol in the mobile phase of 7 m*M* perchloric acid on the retention times of creatinine, tyrosine, and phenylalanine are shown in Figs. 2 and 3, respectively. The retention time of each analyte was dependent on the concentration of perchloric acid. The retention time of creatinine was independent of the methanol concentration in the mobile phase. The mixture of 7 m*M* perchloric acid containing 30% (v/v) methanol (pH 2.2) was used as the optimum mobile phase for the separation and the quantification of creatinine and aromatic amino acids. A typical chromatogram of a standard mixture is shown in Fig. 4.

Detection wavelength

Fig. 5 shows the relationship between the detection wavelength and the chromatographic responses (peak height) for creatinine, tyrosine, and phenylalanine. Many workers have used a wavelength of 235 nm for the detection of creatinine. In this chromatographic system, however, the sensitivity of creatinine was very

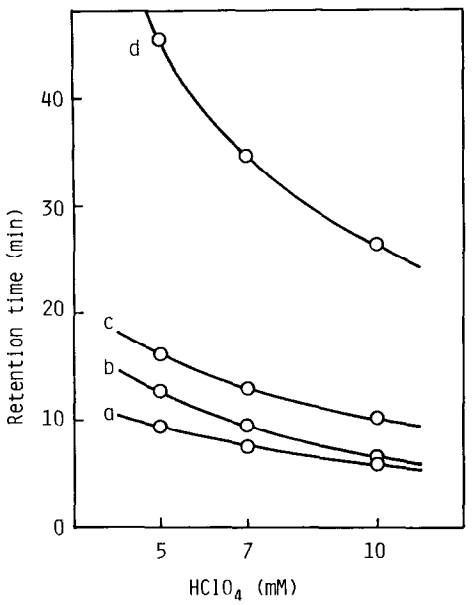

Fig. 2. Effect of perchloric acid concentration in the mobile phase on the retention times of the analytes; methanol concentration, 30% (v/v): (a) tyrosine; (b) creatinine; (c) phenylalanine; (d) tryptophan.

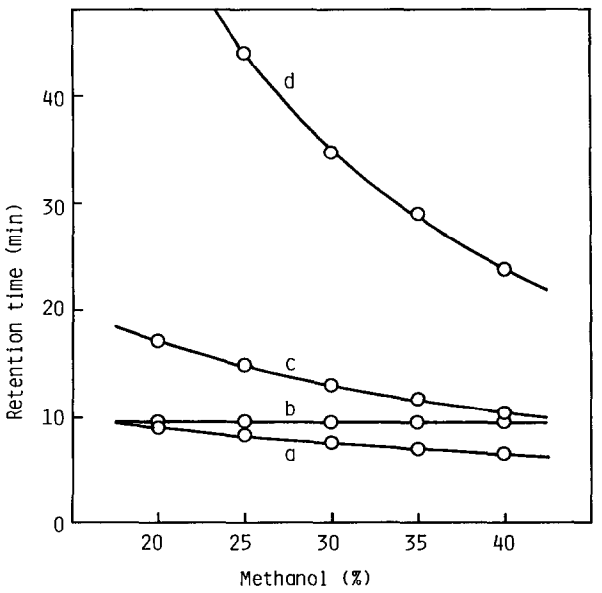

Fig. 3. Effect of methanol concentration in the mobile phase on the retention times of the analytes; perchloric acid concentration, 7 m*M*: (a) tyrosine; (b) creatinine; (c) phenylalanine; (d) tryptophan.

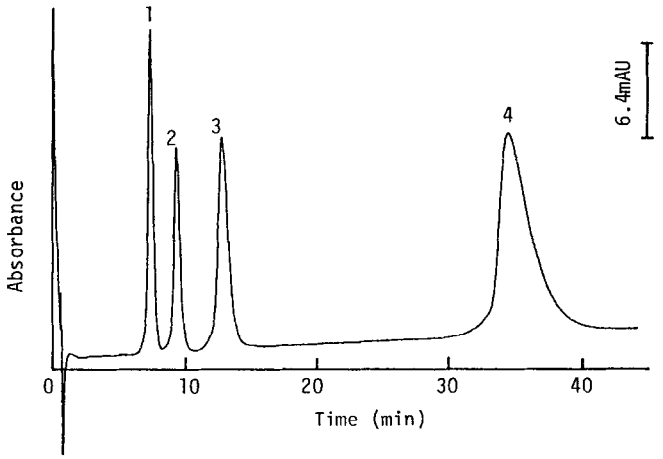

Fig. 4. Chromatogram of a standard mixture of creatinine and aromatic amino acids. Mobile phase, 7 m*M* perchloric acid–30% (v/v) methanol; detection wavelength, 210 nm. Peaks: 1 = tyrosine; 2 = creatinine; 3 = phenylalanine; 4 = tryptophan (100 μM each).

low at 235 nm. It was advantageous to determine the analytes, especially phenylalanine, at a wavelength below 220 nm. Therefore 210 nm was used for the analysis, with regard to sensitivity and baseline noise, which increased as the wavelength decreased.

Quantification

Analyses at five different concentrations of aqueous standard mixtures of creatinine, tyrosine, and phenylalanine were performed in triplicate, and the peak

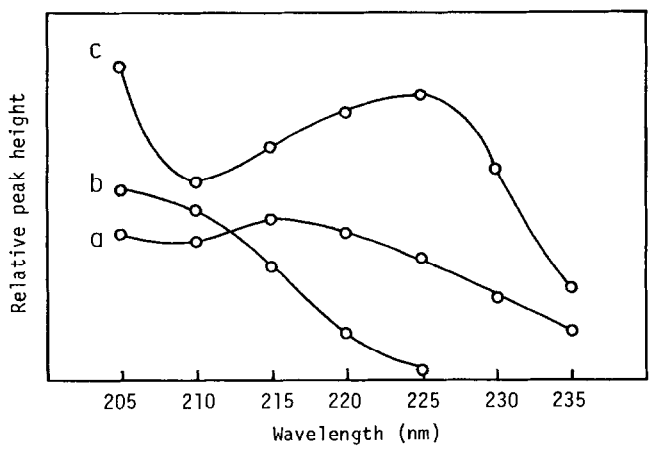

Fig. 5. Relationship between detection wavelength and peak height: (a) creatinine; (b) phenylalanine; (c) tyrosine.

height of each analyte was determined. Coefficients of variation (C.V.) of the peak height were lower than 2.0%. The peak height was linearly related to the concentration of each analyte between 5 and 200 μM using 20-μl samples. The following regression lines were obtained for tyrosine, creatinine, and phenylalanine, respectively: $y = 1.98 \cdot 10^2 x - 2.7 \cdot 10^2$ ($r = 0.9999$), $y = 1.29 \cdot 10^2 x - 2.1 \cdot 10^2$ ($r = 0.9999$), and $y = 1.25 \cdot 10^2 x - 0.3 \cdot 10^2$ ($r = 0.9999$), where y is the peak height and x is the concentration (μM) of the analytes.

The detection limit for creatinine was 1 μM when a 20-μl sample was injected into the column. The sensitivity of the method was higher than necessary for the analysis of urinary creatinine. The aromatic amino acids were also detectable, even in normal urine.

The overall recoveries of creatinine and phenylalanine, in triplicate experiments using a 100 μl aliquot of a standard mixture containing 1 mM creatinine and 300 μM phenylalanine (which was prepared to simulate PKU urine) ranged from 98.3 to 101.1% (mean 99.5%) and from 96.2 to 98.7% (mean 97.8%), respectively.

Tryptophan

Tryptophan was strongly retained on the column because of its hydrophobicity, and was eluted as a broad peak in the isocratic system described. Since the amount of urinary tryptophan is small, tryptophan did not affect the separation, and was not considered in the present analyses. If the methanol and perchloric acid concentrations in the mobile phase were increased to more than 40% and 10 mM, respectively, tryptophan could be determined.

Interferences

Although methionine gave an observable response in the chromatographic procedure, it did not affect the determination of creatinine or that of the aromatic amino acids, because it was eluted before tyrosine. The sensitivity of methionine determination was about one fifth of that of tyrosine.

Most of the neutral and acidic amino acids with weak UV absorbance were eluted near or at the solvent front.

Since the divalent cations, such as calcium, magnesium, and basic amino acids, gradually accumulated on the column under the elution conditions described, they reduced the number of available cation-exchange sites of the resin. Consequently, the retention times of the analytes gradually became shorter. Therefore, the analytical column was periodically washed with 100 ml of 0.1 M NaCl. Otherwise, a short guard column would be effective.

Several UV-absorbing bases were also retained by the column and separated as shown in Fig. 6, where the retention time of the peak of cytosine was close to that of creatinine shown in Fig. 4. In practice, however, cytosine will not affect the determination of creatinine because its concentration in urine is too low.

The amounts of UV-absorbing urinary amines are generally small compared

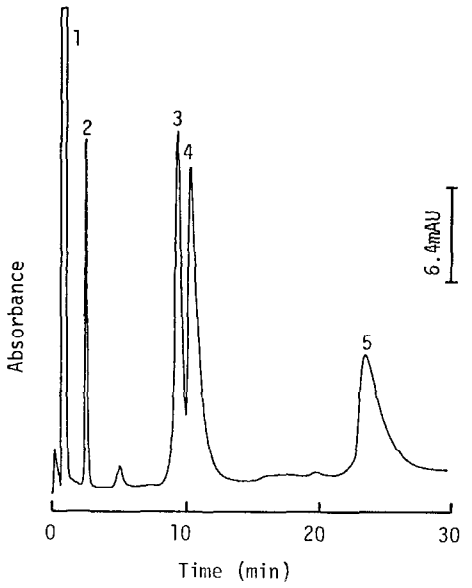

Fig. 6. Chromatogram of a standard mixture of bases: mobile phase, 7 m*M* perchloric acid–30% (v/v) methanol; detection wavelength, 210 nm. Peaks: 1 = uracil, thymine, xanthine; 2 = hypoxanthine; 3 = cytosine; 4 = guanine; 5 = adenine (50 μM each).

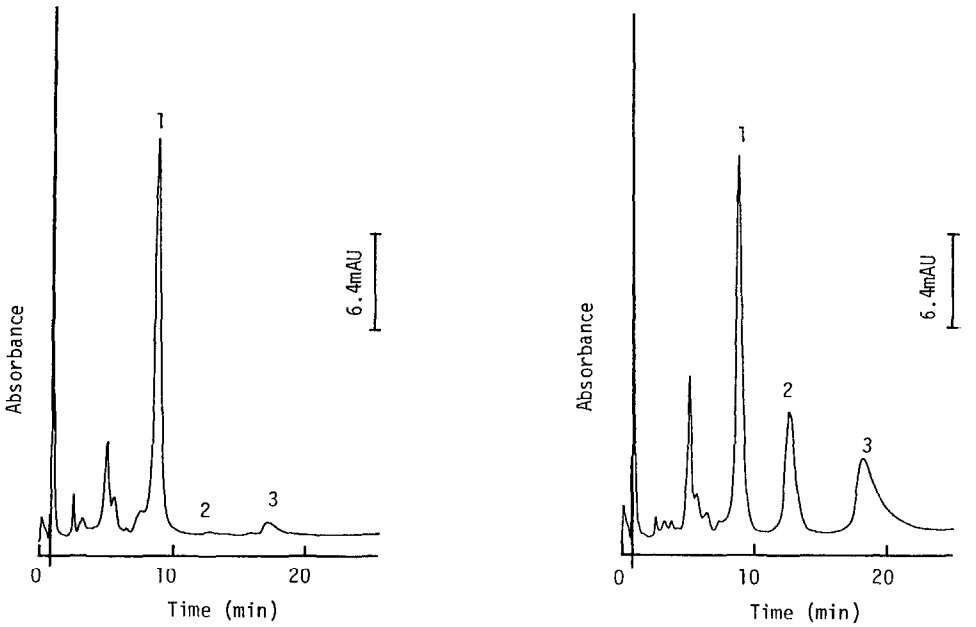

Fig. 7. Typical chromatogram of a normal urine: mobile phase, 7 m*M* perchloric acid–30% (v/v) methanol; detection wavelength, 210 nm. Peaks: 1 = creatinine; 2 = phenylalanine; 3 = unknown.

Fig. 8. Typical chromatogram of a PKU urine: mobile phase, 7 m*M* perchloric acid–30% (v/v) methanol; detection wavelength, 210 nm. Peaks: 1 = creatinine; 2 = phenylalanine; 3 = unknown.

with those of creatinine and amino acids, and are below the detection limit of the method.

Application to urine of patients with inherited metabolic disorders

The method was applied to the analysis of urine from four patients with PKU, one patient with hypertyrosinemia, and ten healthy newborns.

Figs. 7 and 8 show typical chromatograms obtained from a healthy newborn and a patient with PKU, respectively. Creatinine was clearly separated as a dominant peak in both samples, and was determined to be 1.94 and 1.71 m*M*. Phenylalanine was observed in the PKU urine as shown in Fig. 8, at a concentration of 0.568 m*M*. The unknown peak eluting after phenylalanine is probably an amine. The concentration ratio of phenylalanine to creatinine was 0.331. This value is extremely high compared with that of normal urine (average 0.008).

The results of the urine analyses are summarized in Table I. The concentration of creatinine ranged from 0.222 to 8.96 m*M*. The concentration of phenylalanine in the urine of patients with PKU ranged from 0.169 to 1.07 m*M*. The ratios of phenylalanine to creatinine for the patients with PKU were above 0.3, and were significantly high compared with those for the healthy newborns. The ratio of tyrosine to creatinine for hypertyrosinemia was 0.107, which was also high compared with that of controls.

TABLE I

ANALYTICAL RESULTS FROM THE URINE SAMPLES

Sample		Creatinine (C) (μM)	Phenylalanine (F) (μM)	F/C ratio	Tyrosine (Y) (μM)	Y/C ratio
Normal	1	6050	28	0.005	51	0.008
	2	1951	22	0.010	29	0.015
	3	1939	19	0.010	16	0.008
	4	1279	15	0.012	11	0.009
	5	787	12	0.015	N.D.[a]	–
	6	3375	19	0.006	11	0.003
	7	7233	46	0.006	85	0.012
	8	8957	65	0.007	101	0.011
	9	1953	12	0.006	21	0.011
	10	2835	18	0.006	21	0.007
PKU	1	1712	568	0.331	15	0.009
	2	3385	1071	0.316	17	0.005
	3	222	169	0.761	12	0.054
	4	1005	304	0.302	15	0.015
Hyperty-rosinemia		977	20	0.020	105	0.107

[a] N.D. = not detected.

In order to save analysis time, the amino acid fraction can be analysed without freeze-drying if the dilution ratio of urine is measured by weighing the fraction.

The present method is useful for determining urinary creatinine and for the characterization of inborn disorders of amino acid metabolism, such as PKU and hypertyrosinemia.

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