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Short communication

Simultaneous determination of urinary creatinine and UV-absorbing amino acids using a novel low-capacity cation-exchange chromatography for the screening of inborn errors of metabolism

Yukio Yokoyama*, Keiko Yamasaki, Hisakuni Sato

Department of Analytical Chemistry, Faculty of Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan

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Abstract

A simple and versatile low-capacity cation-exchange chromatography system for the simultaneous determination of creatinine and UV-absorbing amino acids was developed. The separation column was packed with a newly developed low-capacity sulfoacylated macro-porous polystyrene-divinylbenzene resin selective for amino-acid cations. Urinary creatinine, creatine, tyrosine, histidine, phenylalanine, and tryptophan were simultaneously separated and determined by an isocratic elution with phosphate/acetonitrile eluent in 25 min. Relative standard deviations (R.S.D.) of the retention times for the analytes were between 0.28 and 1.06%. R.S.D. of peak area responses for the analytes were between 0.75 and 3.51%. The r^2 values for the calibration lines were between 0.9994 and 0.9999. The method could provide the creatinine ratios for the analytes, and was applicable to the screening and/or chemical diagnosis of several inherited disorders of amino-acid metabolism such as phenylketonuria (PKU).
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1. Introduction

The determination of urinary metabolites such as creatinine and amino acids is important in studying human diseases, and is routinely performed especially in clinical and biomedical laboratories. In recent years, a number of clinical inspecting institutes that have introduced HPLC methods for the screening of inborn errors of metabolism (IEM) have increased. The HPLC method replaces the use of classical bacterial inhibition assays [1]. Several specific instruments, commercially available amino acid analyzers, are often used for this purpose. In general, however, the cost of such specialized equipment, unsuitable for general use, is very expensive, and often requires higher running costs.

On the other hand, it is not always necessary to know all amino acids in biological fluids of interest such as urine, as required in the screening of IEM [2]; and the analytes can be limited to several kinds of amino acids. For example, in the screening of IEM, phenylalanine is the diagnostic marker for phenylketonuria (PKU), and tyrosine for tyrosinemia [3–7].

To evaluate the concentration of such diagnostic markers in urine, it is required that the analyte concentration should be corrected by the urinary creatinine concentration, to give the "creatinine ratio". In general, however, creatinine is determined separately by means of the colorimetric method based on the classical alkaline picrate reaction [8]. As alternatives to such non-specific methods, several specific methods for the simultaneous determination of creatinine and target metabolites have been proposed using cation-exchange [9], ion-pair [10–12], and reversed-phase [13–20] HPLC techniques. In addition, the simultaneous determination of urinary creatine and creatinine is very important from the biological

* Corresponding author. Tel.: +81 45 339 3939; fax: +81 45 339 3939.
E-mail address: yokyuk@ynu.ac.jp (Y. Yokoyama).

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or biomedical aspects, which has been reviewed recently [21,22].

For separating specific or diagnostic amino acids with low analytical costs, it is favorable from the analytical and economical viewpoints to use a general purpose HPLC as an alternative to the specialized amino acid analyzer. Recently we have presented a low-capacity cation-exchange separation of creatinine and aromatic amino acids using a permanent coating ion-pair chromatography [23], but the system is unsuitable for gradient elution in principle, otherwise the hydrophobically adsorbed anionic surfactant (hexadecylsulfonate, acting as cation-exchanger) will be desorbed from octadecyl-silica stationary phase. Alternatively we have developed a low-capacity sulfoacylated macroporous polystyrene-divinylbenzene cation-exchange column selective for amino acids [24,25], based on the works by Seubert and Klingenberg [26] and Klingenberg and Seubert [27].

This paper presents a simultaneous separation of creatinine and several diagnostic markers for IEM using the low-capacity cation-exchange column with an isocratic elution system. The method can facilitate the determination of creatinine ratios of diagnostic markers. Application to the screening of inherited amino acid metabolism such as phenylketonuria is also presented.

2. Experimental

2.1. Chemicals and samples

L-Histidine (His), L-tyrosine (Tyr), L-phenylalanine (Phe), and L-tryptophan (Trp) (all >98%) were purchased from Sigma (St. Louis, MO). Creatinine (Cre), creatine (Crn), phosphoric acid, and disodium hydrogenphosphate all of guaranteed grade were purchased from Wako (Osaka, Japan). Acetonitrile of HPLC grade was from Kanto Chemical (Tokyo, Japan). All reagents were used without further purification. Ultrapure water was obtained through a Nihon Millipore (Tokyo, Japan) Direct-Q water purification system just before use.

Standard mixtures containing 100, 50, 20, 10, 5, 2, and 1 μM were prepared for each amino acid except Trp, the concentration of which was decreased to 1/5 of the others, because of its higher UV absorptivity.

Urine samples from patients with phenylketonuria, tyrosinemia, and Lowe syndrome were furnished by Shimoshizu National Hospital and Sanatorium, and those from healthy newborns were from Yokohama City University Hospital. All samples were stored at −30 °C until use.

2.2. Sample pretreatment

A 100 μL aliquot of urine preliminarily filtered through a 0.2 μm filter was loaded onto the preparative H^+-formed cation-exchange resin (SP-Toyopearl 650 M, Tosoh) column (65 mm × 6 mm i.d., glass made) [9] and passed through the column with water while monitoring the UV (254 nm) baseline. After washing acidic and/or neutral species away from the column, the accumulated cationic species such as amino acids and creatinine could be eluted by passing 1–2 mL of 0.1 M NH_3. The fractionation step should take about 30 min as described previously [9]. The collected ammonia fraction was acidified by adding phosphoric acid and the resulting solution was ready for injection to the HPLC system because of UV detection. The dilution ratio of the fraction to neat urine was collected by weighing. The ammonia fractions were stored at −30 °C until use.

2.3. Instrumentation

The HPLC system consisted of a ERC (Tokyo, Japan) 3510 solvent degasser, two Shimadzu (Kyoto, Japan) LC-10AD$_{VP}$ pumps with a high-pressure solvent mixer for two liquids, a Rheodyne (Cotati, CA) Model 7725i syringe-loading sample injector with a 100 μL sample loop, a Sugai (Tokyo, Japan) U-620 column oven, a Shimadzu SPD-10A UV spectrophotometric detector, and a self-made data processing system. The analog outputs from the detector were converted to digital outputs via an Advantest (Tokyo, Japan) R6441A digital multimeter, which were acquired through an RS232C interface by a Fujitsu (Tokyo, Japan) 5166D8 personal computer with a self-made program written with Visual BASIC working under MS-DOS environments. A set of chromatographic data (text file) acquired was then processed and visualized with a self-made program working under Microsoft-Windows environments, or was transferred to Microsoft-Excel to draw a chromatogram. A Hitachi (Tokyo, Japan) D-2000 integrator was also used as an auxiliary data processor.

2.4. Analytical column

The analytical column (4.6 mm i.d. × 150 mm stainless-steel) was prepared by self-packing with a newly developed low-capacity cation exchange resin (5 μm in diameter), sulfoacylated macro-porous polystyrene-divinylbenzene (TMR-A/75) [25] with 75 μeq./column of ion exchange capacity. The base polymers used were TSKgel G1000HHR series GPC packing materials furnished by courtesy of Tosoh (Tokyo, Japan). The detailed functionalization procedure has been presented in our previous papers [24,25].

2.5. Chromatographic conditions

Two delivery solvents (A) 20 mM H_3PO_4—30 % (v/v) CH_3CN and (B) 20 mM Na_2HPO_4—30 % (v/v) CH_3CN were prepared to optimize the isocratic elution. The solvent delivery ratio A/B was set to 58/42, resulting in an eluent with pH 3.30. Other conditions set up were as follows: flow rate, 0.6 mL/min; column temperature, 40 °C; UV detection wavelength, 210 nm; and injection volume: 50 μL.

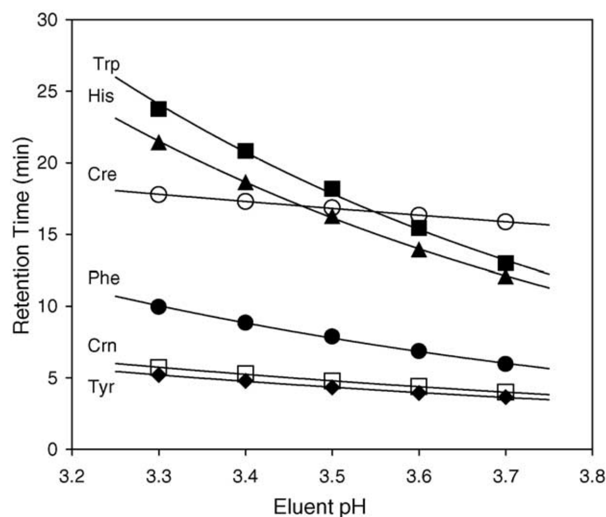

Fig. 1. Change in retention of the analytes by eluent pH.

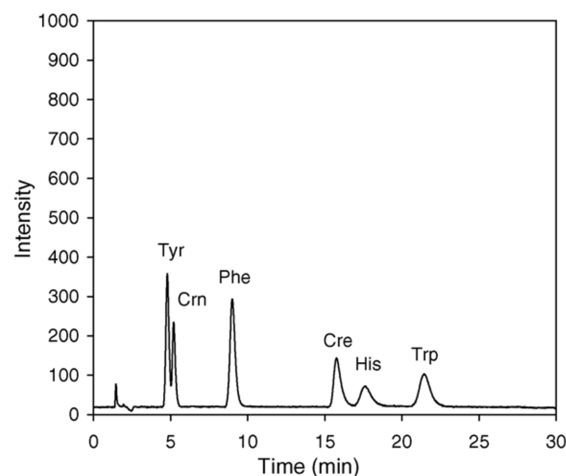

Fig. 2. Standard chromatogram of the analytes. Column: TMR-A/75 self-packed low-capacity cation-exchange column; eluent: (20 mM H_3PO_4—30 % (v/v) CH_3CN)/(20 mM Na_2HPO_4—30 % (v/v) CH_3CN) = 58/42 (pH 3.30); flow rate: 0.6 mL/min; temperature: 40 °C; detection: UV 210 nm; sample size: 50 μL × 50 μM for Tyr, Crn, Phe, Cre, and His, and 10 μM for Trp.

3. Results and discussion

3.1. Optimization study

Since a very highly cross-linked PS-DVB base polymer, 5 μm in diameter, was used for the functionalization, the chromatographic efficiency of the newly developed low-capacity cation-exchange column was higher than that of the previously described low capacity column [9] for the separation of aromatic amino acids. A phosphoric acid/sodium phosphate buffer system of 20 mM was chosen as the mobile phase considering the results from the preceding studies [24]. The addition of acetonitrile to the mobile phase was needed for the separation of the hydrophobic amino acids within a practical analysis time. From fundamental studies [24,25], 30% (v/v) acetonitrile was selected and examined as organic content for the isocratic elution.

Fig. 1 shows the change in retention time of the analytes as the eluent pH was adjusted by changing the *A*/*B* ratios. Since creatinine has a guanidino functional group, the retention time is rather independent of the acidic pH. The small change in eluent pH should affect the retention of other amino acids. From the result, the optimum pH was estimated as 3.3, which could be reached by setting the *A*/*B* delivery ratio to 58/42.

Fig. 2 shows a standard chromatogram of Tyr, Crn, Phe, Cre, His, and Trp. Although the resolution between Tyr and Crn was estimated to be near 1.0, which is capable of improvement, all analytes could be separated within 25 min. It was considered important that the hydrophobic Trp could be chromatographed within a practical operation time. In our previous work [9], Trp has been excluded from the analytes. The present chromatography system was applicable to the separation of urinary metabolites with a minimum pretreatment.

3.2. Quantification study

Table 1 lists the quantification data for the chromatography, acquired by injecting standard samples. The retention times for the analytes were very reproducible, and the relative standard deviations (R.S.D.) were between 0.28 and 1.06%.

Relationships between analyte concentration and peak area response were linear from 2 to 200 μM (by 50 μL injection) for Tyr, Crn, Phe, Cre, and His, and from 0.4 to 50 μM for Trp. R.S.D. of peak area responses for the analytes were

Table 1
Reproducibility and quantification data for analytes

Analyte	R.S.D. (%) $n = 5$				Linear range (μM × 50 μL)	r^2
	Retention time		Area intensity[a]			
	Intra-day	Inter-day	Intra-day	Inter-day		
Tyr	0.01	0.17	1.83	2.29	2–200	0.9997
Crn	0.09	0.26	1.71	1.33	2–200	0.9998
Phe	0.09	0.21	1.78	2.27	2–200	0.9999
Cre	0.24	0.21	2.17	1.85	2–200	0.9999
His	0.33	0.26	1.72	2.43	2–200	0.9994
Trp	0.20	0.36	2.03	3.74	0.4–50	0.9999

[a] Sample size, 50 μM × 50 μL.

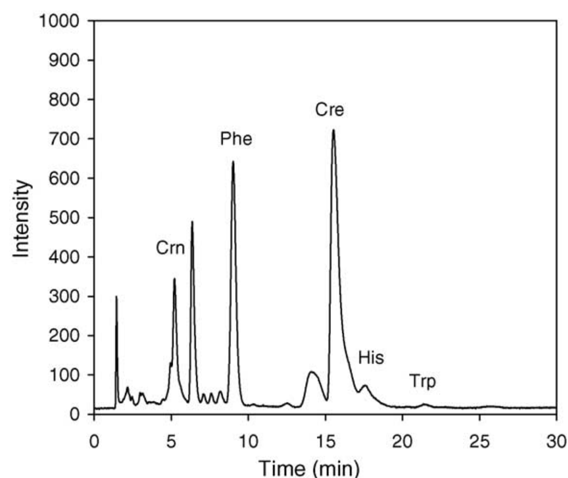

Fig. 3. Chromatogram for urine of patient with PKU. Chromatographic condition as in Fig. 2.

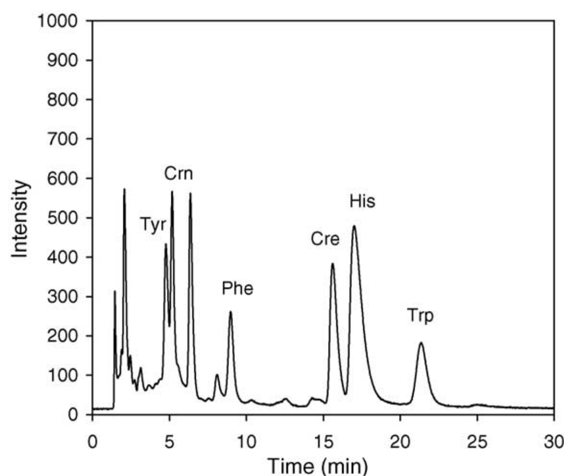

Fig. 4. Chromatogram for urine of patient with Lowe syndrome. Chromatographic condition as in Fig. 2.

good from 0.75% (Tyr) to 3.51% (His), where the sample size was 50 μmol/L × 50 μL each, except for Trp for which 10 μmol/L was used. Since histidine is a dibasic hydrophilic amino acid, the precision data for His were somewhat lower than other analytes, probably due to the peak broadening effect occurring under the eluting condition. Calibration lines with the area data were also linear for all analytes with r^2 (correlation coefficient) values between 0.9994 and 0.9999.

3.3. Application study

The chromatographic system was applied to the analyses of urine from patients with IEM such as PKU, tyrosinemia, and Lowe syndrome, and urine from healthy newborns as controls. The results are summarized in Table 2.

Fig. 3 shows a typical chromatogram of the ammonia fraction from urine of a patient with PKU. The peaks of Cre and Phe (the marker of PKU) observed were very intense, which is clearly indicative of the disease. The creatinine ratios to phenylalanine, i.e., μM Phe/μM Cre, for five PKU urines examined in this study were from 0.165 to 0.680. The values were significantly large compared with those for control urines (<0.04).

Fig. 4 shows a chromatogram for urine of a patient with Lowe syndrome, typically containing large amounts of UV-absorbing amino acids. Similarly, in this case, the creatinine ratios for diagnostic amino acids could be calculated easily as given in Table 2. In the case of tyrosinemia, the creatinine ratio of diagnostic tyrosine was 0.256, which was much higher than those for normal control urines.

In contrast, the chromatograms of control urines should provide less significant peaks of amino acids other than creatinine, as shown in Fig. 5. Creatine and histidine are relatively abundant metabolites in the urine of newborns for the control urine samples. The creatinine ratios of diagnostic amino acids for control urines were significantly lower than those for diseased urines.

Table 2
Analytical results for urines of diseased and healthy newborns

Samples\Analytes	Concentration (μM)						Creatinine ratio (μM/μM creatinine)				
	Tyr	Crn	Phe	Cre	His	Trp	Tyr/Cre	Crn/Cre	Phe/Cre	His/Cre	Trp/Cre
PKU1	<10	951	563	1856	97	9	–	0.512	0.304	0.052	0.005
PKU2	<10	958	963	3418	69	6	–	0.280	0.282	0.020	0.002
PKU3	<10	258	194	286	74	5	–	0.903	0.680	0.259	0.017
PKU4	<10	433	251	932	194	5	–	0.465	0.269	0.208	0.006
PKU5	<10	720	279	1694	153	4	–	0.425	0.165	0.090	0.003
Lowe syndrome	585	1435	381	1562	2742	167	0.374	0.919	0.244	1.756	0.107
Tyrosinemia	187	430	16	733	143	<2	0.256	0.587	0.022	0.196	–
Control-1	<10	1042	52	7384	554	27	–	0.141	0.007	0.075	0.004
Control-2	<10	849	<10	1316	234	8	–	0.645	–	0.178	0.006
Control-3	<10	37	12	270	41	<2	–	0.137	0.044	0.150	–
Control-4	<10	393	51	1978	127	3	–	0.199	0.026	0.064	0.001
Control-5	<10	78	22	752	<10	<2	–	0.103	0.030	–	–
Control-6	<10	579	95	3584	46	17	–	0.161	0.027	0.013	0.005
Control-7	<10	312	115	10924	372	29	–	0.029	0.011	0.034	0.003
Control-8	<10	399	48	2263	102	8	–	0.176	0.021	0.045	0.004

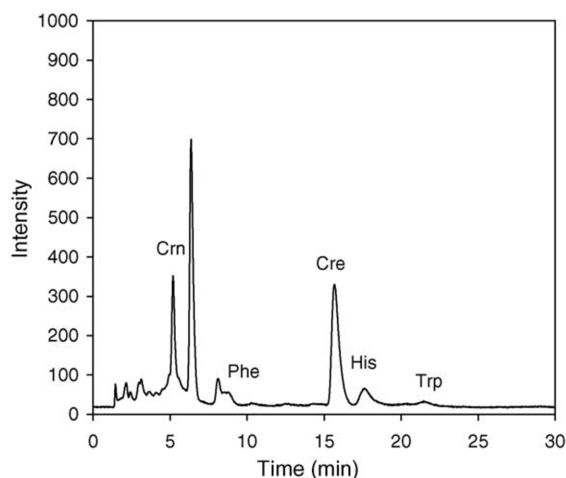

Fig. 5. Chromatogram for urine of healthy newborn. Chromatographic condition as in Fig. 2.

Since the creatinine concentrations can range from 270 to 11000 μM as found in this example study, it is naturally understandable that the absolute concentrations of the diagnostic makers should be inadequate for judging IEM risks. Therefore, it is a big advantage of the method that diagnostic amino acids and creatinine can be determined simultaneously.

All analyte peaks observed in the urine samples were identified by co-eluting. The ammonia fractions chromatographed in this study were the same samples as used in the previous work, where all analytes had been identified spectrophotometrically by means of a photodiode-array UV detection [23]. The use of such multi-wavelength detector is of course favorable to the developed chromatography system.

Some peak tailings are observed in the His bands rather than Trp. This is due to the fact that the eluting power is low for the doubly charged His at the eluent pH. This problem may be solved by using a doubly charged eluent as in previous work [23].

3.4. Column durability

The self-packed column could provide acceptable column efficiency for a long period at least during the present work. In the case of decrease in the efficiency, from our experimental experience, the column could be recovered by passing through an Na^+ eluent of high ionic strength such as 0.1 M $NaNO_3$ of 100 mL; otherwise the re-packing with the used materials would be also effective. To extend the life of a column, in general, preliminary treatment suitable for the analysis is necessary as described above.

Since urinary protein levels seemed to be significantly low, a general deproteinization process was omitted from the pretreatment. On the one hand, the resultant ammonia fraction through the preparative cation exchange column can only contain relatively hydrophilic cationic species, and thus may little affect the separation column.

In HPLC analyses of biological samples, appropriate pretreatments should be carried out to extend the column life. In general, it often takes longer analytical time for sample purification process rather than for final HPLC run time. The sample purification, adequate for the UV detection, carried out by means of the preparative chromatography required about 30 min or a little more. However, if requiring a fluorescent detection in another application, ammonia in the fraction should be removed by freeze-drying for example. In such a case, the analysis time should be greatly increased. Alternative to this, a solid-phase extraction may be useful to decrease the fractionation time, and the detailed discussion will be presented in due course.

4. Conclusion

The developed low-capacity cation-exchange column was durable enough at least during the research work, and the column efficiency was unchanged. In the case of deterioration, the column performance could be recovered by rinsing with a high concentration inorganic salt solution such as 0.1 M Na_2SO_4 or by repacking with the cation-exchanger used.

Since the column performance is still capable of improvement, further study concerning this is now under way. The chromatographic system required is simple and less expensive, so that the method can provide clinically important information and cost-effective results for the high-risk screening or chemical diagnosis of several inherited metabolic disorders such as PKU.

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