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Simultaneous determination of creatinine, creatine, and UV-absorbing amino acids using dual-mode gradient low-capacity cation-exchange chromatography

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Abstract

A simple and versatile cation-exchange chromatography technique for the simultaneous determination of urinary creatinine (Cre), creatine (Crn), methionine (Met), tyrosine (Tyr), phenylalanine (Phe), histidine (His), and tryptophan (Trp) was developed. A novel low-capacity cation-exchange column packed with a newly developed sulfoacylated hypercross-linked macroreticular polystyrene-divinylbenzene resin, referred to as TMR-A/75 (capacity: 75 μequiv/column), was successfully used with a binary dual-mode gradient eluting system. Two solvents, (A) 25 mM phosphoric acid–methanol (30:70, v/v) and (B) 25 mM disodium hydrogenphosphate–methanol (30:70, v/v) were pumped through the column by programming solvent delivery ratios as 0 to 5 min: A–B (55:45, pH 3.6); 5–21 min: A–B (49:51, pH 5.3); and 21–35 min: A–B (55:45, pH 3.6). The flow rate was simultaneously time-programmed to be 0.6 mL/min from 0 to 19 min and to be 1.0 mL/min from 19 to 35 min. This eluting system could permit the use of the UV detection at 210 nm. The analytes, Crn, Met, Tyr, His, Cre, Phe, and Trp, were well separated in this order in 27 min with minimum resolution of approximately 2, and the cycle time was about 35 min. Retention time of each analyte was very reproducible with relative standard deviations (RSDs) between 0.05 and 0.38% ($n = 5$). The peak area responses were also reproducible with RSDs between 0.74 and 2.24% ($n = 5$). Calibration lines based on area data were linear from 1 to 1000 μM with r^2 values of 0.9998 (Crn), 0.9998 (Met), 0.9999 (Tyr), 0.9999 (His), 1.0000 (Cre), 1.0000 (Phe), and 0.9999 (Trp). The method was applicable to the screening and/or chemical diagnosis of inherited metabolic disorders such as phenylketonuria (PKU), tyrosinemia, and Lowe syndrome. The creatinine ratios of diagnostic markers (μM/μM Cre) were easily determined. The Phe/Cre ratios for five urines from patients with PKU ranged from 0.162 to 0.521, and the Tyr/Cre ratio for tyrosinemia was 0.147. The ratios of Tyr/Cre, Phe/Cre, and Trp/Cre for Lowe syndrome were 0.497, 0.321, and 0.495, respectively. In contrast, the creatinine ratios for healthy newborns showed one digit lower than those for patients did. The developed method is very practical and can provide useful information and results for the clinical or biomedical researches with low analytical run costs.
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1. Introduction

The determination of urinary metabolites such as creatinine and amino acids is important in chemical diagnoses of human diseases, and is routinely carried out especially in clinical and biomedical laboratories. In recent years, the clinical testing institutes that have introduced HPLC methods for the screening of inborn errors of metabolism (IEM) are in course of increasing. This seems to be due to their specificity and reliability in comparison with classical BIA (bacterial inhibition assay) method [1]. Several specific instruments, so-called amino-acid analyzer commercially available, are often used for the purpose. In general, however, prices of such specialized equipment are very expensive, and analytical costs are also high.

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On the other hand, the determination of all amino acids in biological fluids of interest such as urine is not always required for the screening and/or chemical diagnosis of IEMs [2]; and the target amino acids are usually limited to several kinds. In the screening and/or chemical diagnosis of several IEMs, for example, L-phenylalanine is diagnostic marker for phenylketonuria (PKU); L-tyrosine is for tyrosinemia; L-methionine is for homocystinuria; and branched chain L-amino acids are for maple syrup urine disease (MSUD) [3–7].

In the case of analyzing urinary metabolites, it is required that the analyte concentration should be corrected by urinary creatinine concentration; that is, the creatinine ratio is significant value. In general, however, creatinine must separately be determined by means of the colorimetric method still based on the classical Jaffé alkaline picrate method [8]. Alternative to such non-specific method, specific HPLC methods for the simultaneous determination of creatinine and target urinary metabolites have been proposed [9–20]. In addition, latest review papers concerning urinary creatine and creatinine have been published [21,22].

It is significant and cost-effective if a conventional HPLC system can be used for the simultaneous determination of urinary creatinine, natural internal standard, and diagnostic amino acids in place of the use of expensive amino-acid analyzers. Recently we have proposed a low-capacity cation-exchange HPLC of creatinine and aromatic amino acids using a permanent coating ion-pair chromatography [23], but the system can accept only isocratic elution mode due to its fatal limitations for use. In other words, any pH or solvent gradient elution can strip the coated materials (hydrophobic anionic surfactant) from the stationary phases (ODS silica column). To solve this problem, alternatively, we have developed a low-capacity sulfoacylated macroreticular polystyrene-divinylbenzene cation-exchange column selective for amino acids [24,25], which has been based on the works by Seubert and Klingenberg [26,27]. The developed low-capacity column enabled the separation of proteinic amino acids using a conventional HPLC with low-concentration binary gradient eluent system [25]. Since such low-capacity columns can permit the use of low-concentration eluents like UV-transparent phosphate buffer and have facilitated the simultaneous determination of urinary creatinine and aromatic amino acids by an isocratic elution [23,28], practicable for the screening and/or chemical diagnosis of several IEMs such as phenylketonuria (PKU).

This paper presents a further improved methodology for the simultaneous determination of UV-detectable urinary creatinine and several amino acids using a sulfoacylated low-capacity cation-exchange column with a dual-mode [11] pH gradient elution system. The method can provide the rapid determination of creatinine ratios to the target amino acids. Some application data to the screening of IEMs such as PKU, tyrosinemia, Lowe syndrome, and homocystinuria are also presented.

2. Experimental

2.1. Materials

L-Methionine (Met), L-histidine (His), L-tyrosine (Tyr), L-phenylalanine (Phe), and L-tryptophan (Trp) were purchased from Sigma (St. Louis, MO) as a proteinic standard kit. Creatinine (Cre) and creatine (Crn) were purchased from Wako (Osaka, Japan). Phosphoric acid and disodium hydrogenphosphate both of analytically guaranteed grade were purchased from Wako. Methanol of HPLC grade was from Kanto Chemical (Tokyo, Japan). Ultra-pure water (18.2 MΩ) was obtained through a Nihon Millipore (Tokyo, Japan) Direct-Q water purification system just before use.

Standard mixtures containing 1000 μM each, 500 μM each, 200 μM each, 100 μM each, 50 μM each, 20 μM each, 10 μM each, 5 μM each, 2 μM each, and 1 μM each were prepared except Trp, the concentration of which was decreased to 1/5 of those of others because of its high UV absorbability.

Realistic urine samples from patients with PKU, tyrosinemia, and Lowe syndrome were furnished by Shimoshizu National Hospital and Sanatorium, and those from healthy newborns were from Yokohama City University Hospital. Simulated homocystinuria urine was prepared by spiking methionine (300 μM) into normal urine. All urine 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 650M, Tosoh) glass column (65 mm × 6 mm I.D. in bed length) [9], and was passed through the column with water while monitoring the UV (254 nm) baseline. After eluting acidic and/or neutral metabolites away from the column, the adsorbed cationic metabolites such as amino acids and creatinine could be eluted by passing 1.5–3 mL of 0.1 M NH_3. The fractionation step should take about 30 min as described previously [9]. The collected ammonia fraction could be directly injected to the HPLC system because of UV detection. The dilution ratio of the fraction to the intact urine was collected by weighing. If necessary, the ammonia fraction should be neutralized or acidified by adding phosphoric acid. If using a fluorescent detection, ammonia should be removed by freeze-drying for example, which should take longer analytical run time. The ammonia fractions ready for injection were also stored at −30 °C until use.

2.3. Apparatus

The binary high-pressure gradient HPLC system used consisted of an ERC (Tokyo, Japan) 3510 solvent degasser, two Shimadzu (Kyoto, Japan) LC-10AD$_{VP}$ solvent delivery pumps with a static 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 Hitachi (Tokyo, Japan) D-2000 chromatographic integrator. In addition, the chromatographic data were simultaneously acquired and processed, via an Advantest (Tokyo, Japan) R6441A digital multimeter through an RS232C interface, by using a Toshiba (Tokyo, Japan) S7/290LNKW notebook computer installed with a laboratory-written data acquisition program with Visual BASIC working under Microsoft-Windows environments. A set of chromatographic data acquired as "dat" file can be transferred to Microsoft-Excel to draw a chromatogram.

2.4. Analytical column

The analytical column (150 mm × 4.6 mm I.D., 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, which was referred to as TMR-A/75 [25] with 75 μequiv/column in ion-exchange capacity. The base polymer used was 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) 25 mM phosphoric acid–methanol (30:70, v/v) and (B) 25 mM disodium hydrogenphosphate–methanol (30:70, v/v) were prepared for the binary gradient elution. The A–B mixing ratio was stepwise changed by time-programming B% from the delivery control pump for solvent (A). An optimized dual-mode gradient program is given in Table 1. Other chromatographic conditions were: column temperature, 40 °C; detection, UV at 210 nm; and sample size, 50 μL.

3. Results and discussion

3.1. Optimization

The resolution between creatine and tyrosine by an acetonitrile eluent system has been unsatisfactory as described in the latest paper [28]. Since urinary creatine is relatively abundant in newborns and the separation of methionine has

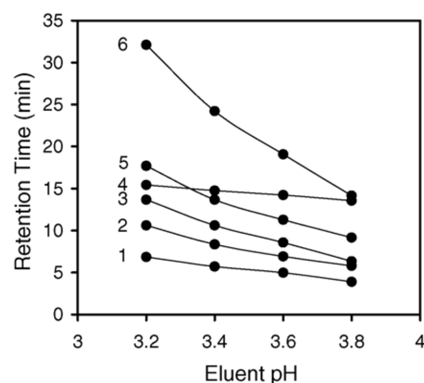

Fig. 1. Changes in retention times of Crn (1), Met (2), Tyr (3), Cre (4), His (5), and Phe (6) by eluent pH. The eluent pH was changed by changing the delivery ratio of the two mobile phases: (A) 25 mM H_3PO_4–CH_3OH (30:70, v/v) and (B) 25 mM Na_2HPO_4–CH_3OH (30:70, v/v).

been failed in the previous study [28], in this work, an alternative eluent system was studied to establish a practically specialized amino acid analysis system.

It was found that the phosphate mobile phase containing methanol in place of acetonitrile could provide considerable changes in chromatographic behaviors of amino acids of interest. In other words, significant difference in selectivity for amino acids was found between methanol and acetonitrile eluting systems. On the bases of several preliminary experiments, 25 mM phosphate–methanol (30:70, v/v) system was selected and examined.

Fig. 1 shows the changes in retention times of Crn, Met, Tyr, His, Cre, and Phe by the change in eluent pH under isocratic eluting condition, established by mixing (A) 25 mM phosphoric acid–methanol (30:70, v/v) and (B) 25 mM disodium hydrogenphosphate–methanol (30:70, v/v) in suitable pump delivery ratios. This indicated that the elution at pH 3.6 should be adequate for the simultaneous separation. Since Cre is a sort of amines, its retention time is relatively independent of the eluent pH under such acidic condition. The separations among the analytes were considerably improved by using the methanol system, comparing to those obtained by the acetonitrile system [28]. Especially, the band spacing between Crn and Tyr was dramatically improved, that is, the resolution was increased from 1.2 to 4.3. Thus, methionine was clearly separated between Crn and Tyr. In addition, the eluting order was also changed from Tyr, Crn, Phe, His, Cre, Trp (by acetonitrile system) to Crn, Tyr, His, Cre, Phe, Trp (by

Table 1
Optimized time-program for binary dual-mode gradient chromatography

Time (min)	A (%)	B (%)	pH	Time (min)	Flow rate (mL/min)
0 → 5	55	45	3.6	0 → 19	0.6
5 → 21	49	51	5.3	19 → 35	1
21 → 35	55	45	3.6	35	0.6
Stop					

(A) 25 mM H_3PO_4–30 % (v/v) CH_3OH; (B) 25 mM Na_2HPO_4–30 % (v/v) CH_3OH.

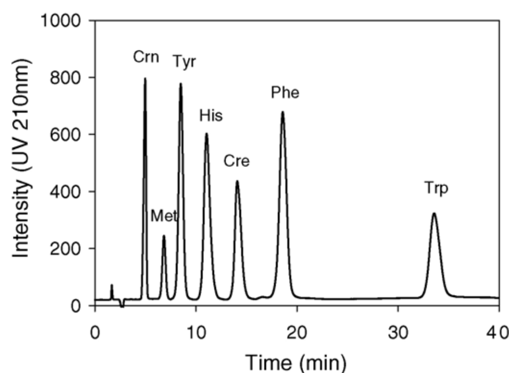

Fig. 2. pH-Gradient chromatogram for standard Crn, Met, Tyr, His, Cre, Phe, and Trp. Column: TMR-A/75 low-capacity cation-exchange column; mobile phase: (A) 25 mM H_3PO_4–CH_3OH (30:70, v/v) and (B) 25 mM Na_2HPO_4–CH_3OH (30:70, v/v) with a gradient program as in text; flow rate: 0.6 mL/min; temperature 40 °C; detection: UV 210 nm; sample size: 50 μL × 100 μM each, except Trp 20 μM.

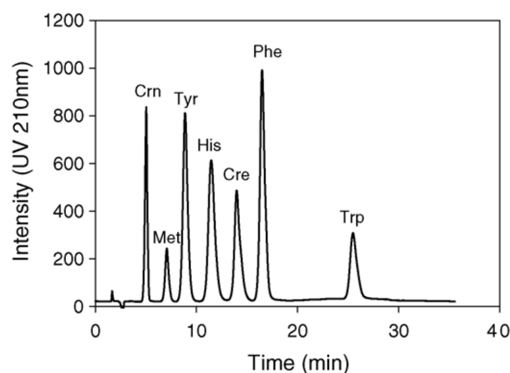

Fig. 3. Dual-mode gradient chromatogram for standard Crn, Met, Tyr, His, Cre, Phe, and Trp. Column: TMR-A/75 low-capacity cation-exchange column; mobile phase: (A) 25 mM H_3PO_4–CH_3OH (30:70, v/v) and (B) 25 mM Na_2HPO_4–CH_3OH (30:70, v/v) with dual-mode gradient program as in Table 1; temperature 40 °C; detection: UV 210 nm; sample size: 50 μL × 100 μM each, except Trp 20 μM.

methanol system). This implied that the selectivity of the low-capacity cation exchanger for hydrophobic amino acids was considerably affected by the organic constituent of the eluent, which led to the consideration that the hydrophobic interaction would be dominated for the retention by low-capacity cation exchanger.

Since the retention time of Trp was fairly longer (over 60 min) than those of others under this isocratic condition, a step gradient elution was necessary to elute Trp faster by increasing the eluent pH after separating Crn, Met, Tyr, His, Cre, and Phe at pH 3.6. Considering the isocratic results, an optimum gradient program was found to be 0–10 min, A–B (55:45, pH 3.6); 10–30 min, A–B (50:50, pH 4.0); 30–45 min, A–B (55:45, pH 3.6). The gradient elution could provide excellent band spacing for the analytes with acceptable retention times, as shown in Fig. 2. Trp was eluted at 33 min and the cycle time was about 43 min.

Although the individual band spacing was very good, however, the retention time of Trp and the cycle time were still longer than those obtained by the isocratic acetonitrile system (cycle time: 35 min) [28]. To elute Trp faster, the methanol contents of 32:68, 35:65, and 40:60 (v/v) both in (A) and (B) mobile phases were examined. However, contrary to expectation the increase in methanol contents was not so effective in decreasing retention times significantly, and besides, the band spacing between Cre and Phe became worse. This suggested that the retention of hydrophobic amino acids to the low-capacity cation exchanger could depend on the ionic strength and/or pH rather than the organic contents of the eluent.

As an alternative choice to decrease the cycle time, the flow-rate gradient elution in addition to the pH gradient, i.e. "dual-mode" gradient [11], was introduced to the chromatography. The initial flow rate at 0.6 mL/min was kept to 19 min and stepwise increased to 1.0 mL/min at 19 min through 35 min. The eluent delivery program was also reconstructed in order to elute all analytes faster than in Fig. 2. Table 1 lists a finally optimized time-program for the binary dual-mode gradient chromatography. Fig. 3 shows the optimized chromatogram for the analytes, which can provide the cycle time of about 35 min. The dual-mode gradient elution was considered useful to obtain well-balanced and -resolved chromatograms.

3.2. Quantification

The dual-mode gradient chromatography was very reproducible for each analyte as listed in Table 2. The analytical data for relative standard deviation (RSD) were sufficient for both retention times and area integrations, comparing with those observed in general HPLC methods. The linear regres-

Table 2
Reproducibility and quantification data for analytes

Analyte	RSD (%) ($n = 5$)		Linear range[a] (μM)	r^2	Detection limit[a] (μM)
	Retention time	Area intensity			
Crn	0.38	1.57	1–1000	0.9998	0.02
Met	0.30	1.10	1–1000	0.9998	0.1
Tyr	0.17	2.24	1–1000	0.9999	0.02
His	0.16	2.04	1–1000	0.9999	0.02
Cre	0.09	0.74	1–1000	1.0000	0.02
Phe	0.08	1.71	1–1000	1.0000	0.02
Trp	0.05	1.18	0.4–1000	0.9999	0.01

[a] Sample size injection was 50 μL.

sion lines between analyte concentration and peak area intensity were obtained, giving regression coefficients (r^2) of 0.9998 or over. The peak height integration was also linear up to 500 μM for each with $r^2 \geq 0.9995$, and the detection limits were estimated from the height data.

3.3. Recovery

The preliminary preparative chromatography is quantitative as mentioned previously [9,23], giving almost 100% recovery. Since the pretreated urine samples measured in the following application study were the same samples as used in previous studies [10,11,23], the recovery was thought to be almost the same. The overall recovery of Met for the urine of simulated homocystinuria was led to ca. 110%, which seemed to be reasonable with considering endogenous Met. At all events, the method has technical merits that the creatinine ratio is independent of the analyte recovery because creatinine and aromatic amino acids can act in similar manner in the preparative and analytical cation-exchange columns.

3.4. Applicability

The method was applicable to the screening and/or chemical diagnosis of IEMs, providing urinary creatinine ratios to diagnostic markers. Since endogenous creatinine is often used as internal standard to collect inconstant 'urine concentration', the exact 'creatinine ratio' is essential to evaluate the significant levels of urinary metabolites as diagnostic markers.

Fig. 4 shows a typical chromatogram of the ammonia fraction from urine of patient with PKU. The intense peaks corresponding to Cre and Phe were significantly observed and simultaneously determined. The creatinine ratio was simply calculated as μM Phe divided by μM Cre. Similarly, characteristic chromatograms indicative of tyrosinemia and of Lowe syndrome were obtained. The diagnostic amino acids were significantly observed, and their creatinine ratios were directly determined. Fig. 5 shows a chromatogram of the ammonia fraction from the urine for simulated homocystinuria, also providing the intense peak of methionine without any interference.

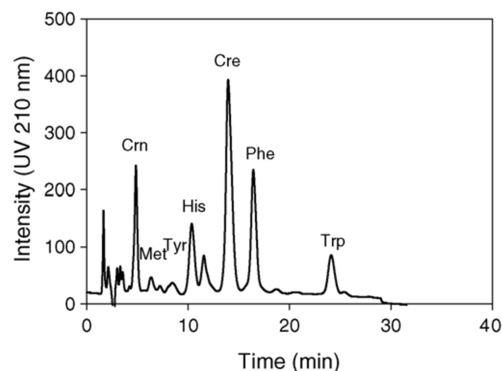

Fig. 4. Chromatogram for urine of patient with phenylketonuria. Conditions as in Fig. 3. The sample injected (50 μL) was corresponding to 22.9 times dilution of the intact urine.

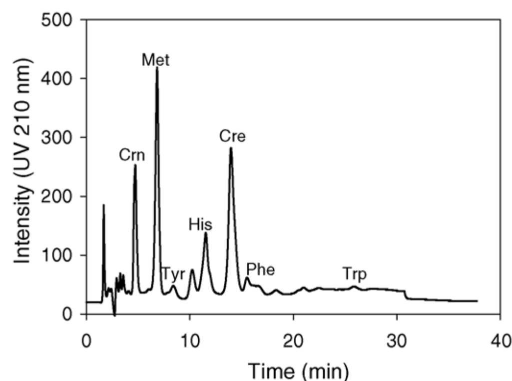

Fig. 5. Chromatogram for urine of simulated homocystinuria. Conditions as in Fig. 3. The sample injected (50 μL) was corresponding to 23.8 times dilution of the intact urine.

On the other hand, some preliminary separations are considered useful and necessary when analyzing biological fluids, although it takes longer analytical run time. In addition, the peak height ratio is directly calculable from the chromatogram, which may be also significant for judging "cut-off point" of the corresponding diagnostic metabolites.

In contrast to such diseased urines, the creatinine ratios in healthy urines were significantly lower than those found

Table 3
Diagnostic amino acid concentration and creatinine ratio for urine of patient with IEM

IEM	Cre (μM)	Met (μM)	Tyr (μM)	Phe (μM)	Trp (μM)	μM/μM Cre
PKU 1	2014	110	80	532	27	0.264 (Phe)
PKU 2	3669	113	207	1077	41	0.294 (Phe)
PKU 3	339	21	45	177	93	0.521 (Phe)
PKU 4	1139	48	71	400	43	0.350 (Phe)
PKU 5	2097	54	103	340	25	0.162 (Phe)
Tyrosinemia	2983	152	439	23	66	0.147 (Tyr)
Lowe syndrome		78	1168	–	–	0.497 (Tyr)
	2350	–	–	755	–	0.321 (Phe)
		–	–	–	1162	0.495 (Trp)
Homocystinuria[a]	1305	330	75	49	48	0.253 Met

[a] Simulated IEM.

Table 4
Diagnostic amino acid concentration and creatinine ratio for urine of healthy newborns

Control	Cre (μM)	Met (μM)	Tyr (μM)	Phe (μM)	Trp (μM)
Normal 1	8651	143	190	65	184
μM/μM Cre		0.017	0.022	0.008	0.021
Normal 2	2300	148	46	76	33
μM/μM Cre		0.065	0.02	0.033	0.014
Normal 3	2081	ND[a]	143	31	126
μM/μM Cre			0.069	0.015	0.06
Normal 4	4441	136	82	73	78
μM/μM Cre		0.031	0.018	0.016	0.017
Normal 5	1346	87	43	25	66
μM/μM Cre		0.065	0.032	0.019	0.049
Normal 6	1576	94	39	18	111
μM/μM Cre		0.06	0.025	0.011	0.07
Normal 7	2045	86	27	47	39
μM/μM Cre		0.042	0.013	0.023	0.019
Normal 8	1131	85	27	56	50
μM/μM Cre		0.076	0.024	0.05	0.044

[a] Not determined.

in IEMs, although the chromatograms for normal urines also provided peaks of amino acids in some degree in addition to the intense Cre peaks as shown in Fig. 6. The results are summarized in Tables 3 and 4. Since creatine and histidine are relatively abundant metabolites in urine of newborns, their creatinine ratios are omitted. In addition, His is not separable from other histidine derivatives such as 1-methyl- and 3-methyl- His, normal metabolites, under the present chromatographic condition. If necessary to separate them, another chromatographic system is available [23].

Since the actual values of the diagnostic markers are considerably changed from sample to sample, it seems to be difficult to judge IEM risks in terms of such absolute values. However, the individual creatinine concentrations are greatly different from sample to sample, so that, the relative concentration normalized by creatinine concentration is very indicative for judging whether normal or abnormal. The developed method can provide such critical and significant creatinine ratios for the diagnostic markers of individual IEMs.

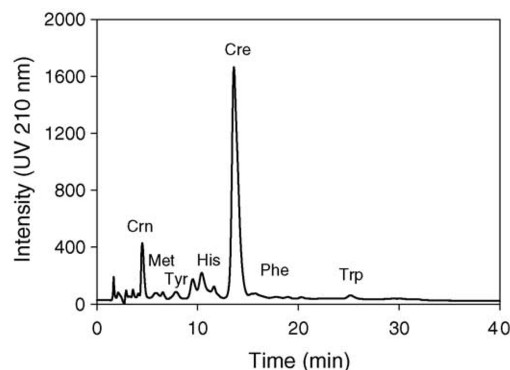

Fig. 6. Chromatogram for urine of healthy newborn. Conditions as in Fig. 3. The sample injected (50 μL) was corresponding to 22.1 times dilution of the intact urine.

4. Conclusions

The developed amino-acid analyzing system comprised of a conventional HPLC system with the novel low-capacity cation-exchange column is versatile, flexible, practical, and cost-effective for biological or clinical assays such as screening of IEMs. Alternative to the UV detection effectively used in the present work, intelligent diode-array UV detection may be powerful for yielding more qualitative information for analytes as used previously [23].

The developed low-capacity cation-exchange column is extremely durable and can keep the initial column performance for a long period exceeding one year, nevertheless depending on analytical skills. In the case of decrease in column efficiency, rinsing and/or repacking are useful to recover column performance.

Although the low-capacity cation-exchange columns newly developed for the amino-acid analyses are commercially unavailable at present, further application studies will be expected in near future.

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