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Separation and determination of amino acids, creatinine, bioactive amines and nucleic acid bases by dual-mode gradient ion-pair chromatography

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Abstract

A simple and fast high-performance liquid chromatography method for the analysis of amino acids and biological bases such as creatinine was developed using a technique termed "dual-mode gradient ion-pair chromatography". A butyl-silica reversed-phase column and water–acetonitrile eluent containing sodium dodecyl sulfate (SDS) and perchloric acid were used for the separation. A concentration gradient of acetonitrile from 15 to 35% provided a good separation of such organic cations. Since change in concentration of acetonitrile causes change in distribution equilibrium of SDS between mobile and stationary phases, a complete regeneration of the column to the initial state is required for the reproducible separations. Completion of the reequilibrium was indicated by a system peak appearing in the UV chromatogram and by conductivity measurements. The formation mechanism of the system peak was revealed. A flow-rate gradient from 1 to 2 ml/min was introduced in addition to the concentration gradient to shorten the cycle time of the chromatography. More than twenty kinds of amino acids, creatine and creatinine were simultaneously separated within 50 min and the cycle time was 80 min including the reequilibration time. A post-column derivatization fluorescence detection system was usable as well as UV detection. This elution system was also useful for the separation of bioactive amines and nucleic acid bases. The developed method was applied to the simultaneous determination of urinary creatinine and diagnostic amino acids due to inherited metabolic disorders.

Keywords: Ion-pairing reagents; Mobile phase composition; Food analysis; Derivatization, LC; Amino acids; Amines; Creatinine; Sodium dodecyl sulfate; Nucleic acid bases; Perchloric acid

1. Introduction

The analysis of amino acids is very important in biological and biomedical research, having progressed in the methodology based on the classical ion-exchange chromatography [1,2]. High-performance liquid chromatography (HPLC) techniques are now essential for the separation and determination of free amino acids. These techniques are mainly based on two different procedures. One is cation-exchange chromatographic separation followed by post-column derivatization using ninhydrine [3], *o*-phthalaldehyde (OPA) [4–6] and 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD) [7], with fluorescence detection. The other is reversed-phase chromatographic separation with fluorescence detection after pre-column derivatization using OPA [8–10], 1-N,N′-dimethylaminonaphthalene-5-sulfonyl [11,12], phenylthiohy-

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dantoin [13] and NBD [14]. Two types of amino acid analyzers based on these procedures are now commercially available and are widely accepted in scientific laboratories [15,16]. Such instruments, requiring complex eluent programming and/or specialized equipment composition, being therefore expensive, seem to be suitable for routine use but unsuitable for general purpose.

Ion-pair chromatography has also been proposed [17–19] as a relatively fast and simple procedure for amino acid analysis. This technique, however, has been unpopular because of poor reproducibility in chromatography. In general, gradient elution is required for the simultaneous separation of various kinds of amino acids by reversed-phase ion-pair HPLC. The gradient ion-pair method requires lengthy column regeneration [19]. However, the change in equilibrium of ion-pair reagent between stationary and mobile phases due to the change in concentration of organic solvent has not been realized in practice. Therefore, unfavorable reproducibility in retention of the analyte arises when samples are injected before the column is equilibrated.

On the other hand, the determination of creatinine in biological fluids is one of the most important clinical analyses. The most widely accepted method is the non-specific colorimetric procedure based on the Jaffé reaction [20]. In recent years, a variety of specific methods for creatinine determination have been reported by many researchers, using cation-exchange [21–27], normal-phase [28,29], reversed-phase [30–38] and ion-pair [30,39–43] HPLC. Further specific studies, the simultaneous determination methods of creatinine and several target compounds such as purine metabolites [44–50] and catecholamine metabolites [51–53] in biological fluids, have also been reported. In many works referred to as above, however, the urine or serum sample treated by only filtration or deproteinization has been directly injected into the analytical column. In our opinion, appropriate preliminary separation besides deproteinization and/or filtration should be made prior to analytical chromatography of such complicated mixtures. In our previous work [26,38], a urine sample has been separated into at least two fractions, acidic/neutral and basic ones, using cation-exchange preparative chromatography, while further separation of the fraction of basic species seems difficult. In other words, amino acids and biological bases such as creatinine coexist in the basic fraction. We considered, therefore, such organic cations should be separated simultaneously by an established HPLC technique [26,38].

This paper describes the optimization of acetonitrile gradient elution for the simultaneous separation of amino acids using ion-pair chromatography with a butyl-silica (C4) column and sodium dodecyl sulfate (SDS) as ion-pair reagent. The column reequilibration mechanism in gradient ion-pair chromatography is discussed, and the effect of the additional flow-rate gradient elution is also described. Applications of the new HPLC technique to clinical and food analyses are presented.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of an Erma (Tokyo, Japan) ERC-3510 solvent degasser; two Shimadzu (Kyoto, Japan) LC-9A pumps with a high-pressure solvent mixer for two liquids; a modified Toyo (Tokyo, Japan) FI-45 incubator as column oven equipped with a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading sample injector with a 100-μl sample loop; a Shimadzu SPD-10A UV spectrophotometric detector and a Hitachi (Tokyo, Japan) D-2500 integrator. A GL Sciences (Tokyo, Japan) Inertsil C4 (150 mm×4.6 mm I.D.; particle size 5 μm; carbon loading 7.5%; nominal theoretical plate number 13 000) reversed-phase column was used throughout the experiments. A Rheodyne 7335 column inlet filter was placed between the injector and the separation column. The detection wavelength was 210 nm; the temperature of the column oven was 30°C and the sample injection volume was 20 μl throughout the experiment.

A Tosoh (Tokyo, Japan) FS-8010 fluorescent detector was used for the post-column fluorescence detection of amino acids. A reaction coil (5 m×0.5 mm I.D. stainless-steel tubing) was connected to the outlet tubing of the UV detector and placed inside a Tosoh (Tokyo, Japan) CO-8000 column oven. The OPA fluorescent derivatization mixture was pumped through a Lab-Quatec (Tokyo, Japan) Model MP-

311 pump at 0.5 ml/min and was mixed with the chromatographic effluent at a mixing tee. The reaction temperature was kept at 70°C. The excitation and emission wavelengths of the detector were 340 and 430 nm, respectively.

A Tosoh CM-8000 conductivity detector was used to measure the change in background conductivity of the effluent during the gradient elution.

2.2. Preparation of mobile phase

Perchloric acid of super special grade and acetonitrile of HPLC grade were purchased from Wako (Osaka, Japan) and SDS of 98% purity was from Aldrich (Milwaukee, WI, USA). These reagents were used without further purification. Deionized water, provided by an ORGANO (Tokyo, Japan) model G-10 mixed-bed ion-exchange cartridge system, was purified by a Milli-Q Labo (Nihon Millipore, Tokyo, Japan) water purification system just before use.

In gradient elution, it is very important to use pure chemicals and solvents as far as possible because impurities in mobile phase are accumulated in the analytical column, often giving ghost peaks and causing baseline drift and noise. In this study, common reagents from several suppliers were examined for ultraviolet transparency. The reagents listed above and the Milli-Q water were the most adequate combination for the mobile phase constituents.

Mobile phase A (5 m*M* SDS/10 m*M* $HClO_4$) was prepared by diluting a 50-ml aliquot of 0.1 *M* SDS stock solution and a 10-ml aliquot of 1 *M* $HClO_4$ stock solution to 1 l. Mobile phase B was 100% acetonitrile.

2.3. Gradient program

A high-pressure binary gradient elution was performed by programming both concentration and flow-rate of mobile phase. The optimum final gradient program is shown in Fig. 1. The mobile phase composition was varied linearly from 85% A–15% B at 0 min to 80% A–20% B at 20 min, and consecutively to 65% A–35% B at 50 min, while the flow-rate was varied linearly from 1 to 2 ml/min. After returning to 85% A–15% B at 50.01 min, the initial mobile phase was kept flowing from 50.01 to 80 min at the flow-rate of 2 ml/min. The program end at 80.01 min returned the flow-rate to 1 ml/min, ready for injection.

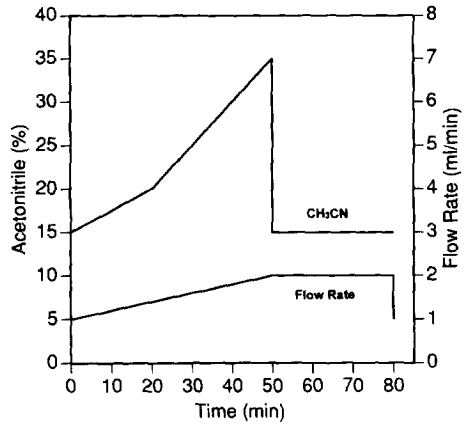

Fig. 1. A dual-mode gradient program.

2.4. Analytical standard

Creatinine (Cre), creatine (Crn), amino acids, bioactive amines and nucleic acid bases were purchased from Wako or Sigma (St. Louis, MO, USA) and were used as received. Several kinds of standard mixtures were prepared in appropriate concentrations according to the detection system used.

One-letter or three-letter symbols are used in the text and figures to represent amino acids and nucleic bases.

2.5. Preparation of post-column OPA derivatization mixture

Sodium hydroxide and boric acid of amino-acid analysis grade, OPA and 2-mercaptoethanol of biochemistry reagent grade and ethanol of HPLC grade were purchased from Wako.

A 200 mg weight of OPA was dissolved in 10 ml of ethanol. This ethanolic solution and 1 ml of 2-mercaptoethanol were added to approx. 900 ml of 0.1 *M* borate buffer (4 g NaOH and 6.2 g H_3BO_3 in 1 l) and the total volume was then adjusted to 1 l. The solution was protected from the light and was used up within a week.

In general, addition of polyoxyethylene lauryl ether (corresponding to Brij 35, ICI, UK) to the

solution (0.1%, w/v) has been recommended [6]. However, the surfactant was not used in this derivatization to decrease the background noise level, because the reagent is not so pure. The concentrations of OPA and borate buffer were both decreased to one-fourth the manufacturer's recommendation [6] for the same purpose. Consequently, the signal-to-noise ratio on fluorescence detection was improved three times. On this condition, the post-column reaction was quantitative as described in Section 3.

2.6. Application examples

The urines of patients with several inherited metabolic disorders, five with phenylketonuria (PKU), two with citrullinemia, one with tyrosinemia and one with Lowe syndrome, were furnished by Shimoshizu National Hospital and Sanatorium. Control urines from healthy newborns were obtained from Yokohama City University Hospital. All urine samples were stored at −30°C. The thawed urine was filtered and then separated into two fractions, referred to as acidic and basic fractions, by using a preparative cation-exchange chromatography with Tosoh SP-Toyopearl 650M resin. The basic fraction obtained by the elution with ammonia–water was freeze-dried to remove ammonia and the residue was then redissolved in water. The detailed procedure has been given in our previous paper [26]. The resultant ammonia-free basic fraction, corresponding to 50 or 500 times dilution of intact urine, was chromatographed.

The fresh juices, obtained by squeezing the flesh of a watermelon, a grape and an orange, were filtered through a 0.2-μm cellulose acetate filter (DIS-MIC-13 CP, Advantec, Tokyo, Japan) and frozen at −30°C. The fruit juices were also pretreated by the same procedure described above.

3. Results and discussion

3.1. Optimization of separation

A gradient elution with organic solvent is essential to separate amino acids using ion-pair HPLC [17–19]. In this gradient elution, a 100% acetonitrile of HPLC grade was chosen as the mobile phase B for the following reasons: it shows excellent UV-transparency at 210 nm compared with some other commercially available solvents for reversed-phase HPLC; the pure acetonitrile is efficient and convenient for washing out impurities in the analytical column; the mixture with water provides relatively low back pressure to the analytical column. The back pressure changed between ca. 70 and 140 kg/cm^2 during the gradient HPLC.

The use of C4 column led to the small change in UV baselines due to the small change in acetonitrile concentration required in the gradient elution.

The basic acetonitrile gradient program was constructed by considering retention behavior of amino acids on the C4 column by several isocratic elutions as shown in Fig. 2. The subsequent program optimization was made by repeated trial and error. Perchloric acid was used to lower the pH of the mobile phase because it is UV-transparent, less corrosive to stainless-steel tubing and an ultrapure reagent is

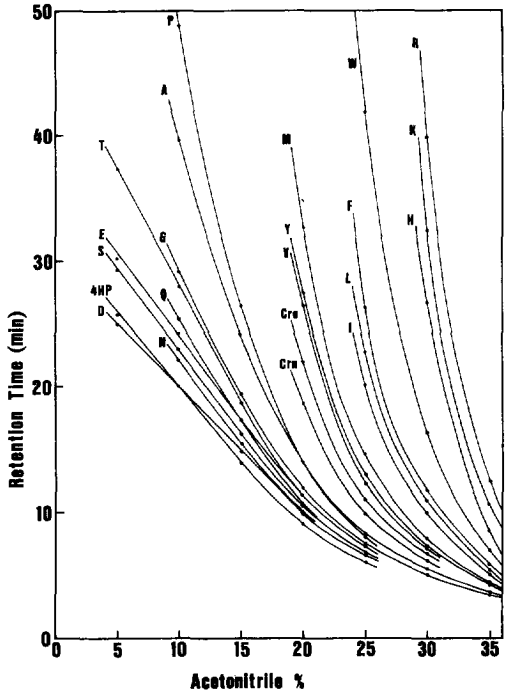

Fig. 2. Effect of acetonitrile concentration in mobile phase on retention of amino acids in ion-pair chromatography. Each eluent contains 5 m*M* SDS and 5 m*M* $HClO_4$. Flow-rate: 1 ml/min constant. 4HP=4-hydroxyproline.

available. The effect of the acid concentration (5, 7.5 and 10 m*M*) in the mobile phase A on the separation of amino acids was examined. The small change in mobile phase pH due to the acid concentration gave a subtle change in chromatogram. The separation among Pro, Cys and Ala in 10 m*M* $HClO_4$ was better than those in 5 and 7.5 m*M* $HClO_4$. Crn and Cre were eluted between Ala and Val in all $HClO_4$ concentrations. The best resolution between these four bands was obtained in 10 m*M* $HClO_4$ which led to well-balanced chromatograms. The concentration of SDS in the mobile phase A was also examined for 5, 7.5 and 10 m*M*. The separation between Val and Tyr was completed by the use of 5 m*M* SDS, although the two peaks were overlapped in the case using 10 m*M* SDS. The 5 m*M* SDS was effective in decreasing retention times of hydrophobic and basic amino acids while keeping good separations among them. Consequently, 5 m*M* SDS with 10 m*M* $HClO_4$ solution was fixed for the mobile phase A. Fig. 3 shows a chromatogram of amino acids, Crn and Cre obtained by using the concentration gradient program shown in Fig. 1. A good separation from 4-hydroxyproline (4HP) to Arg was achieved within 55 min. Although Asn and Gln are not seen, they are eluted between Asp and Ser and between Glu and Thr, respectively. The dominant peak around 18 min, a so-called ghost peak, appearing at indefinite position, was independent of impurities from samples, mobile phases and chromatographic tubing. This was indeed a system peak indicating the completion of reequilibration of the reversed-phase column.

3.2. Column reequilibration

The background conductivity of the effluent was measured to understand the change in SDS concentration during the gradient elution using conductivity detection connected with UV detection in series. Fig. 4 shows the changes in conductivity and UV absorbance of the effluent. The conductivity on the initial equilibrium was ca. 2700 μS/cm owing to SDS and $HClO_4$ in the mobile phase. Although the concentrations of SDS and $HClO_4$ in the mobile phase were decreased according to the increase in acetonitrile concentration, the conductivity was gradually increased in the early part of the gradient because of the increase in total SDS caused by SDS from stationary phase. The amount of SDS in the stationary phase depends on the acetonitrile concentration. After a maximum of ca. 3500 μS/cm, the conductivity gradually decreased to a minimum of ca. 1750 μS/cm. This decrease is due to the further decrease in both SDS and $HClO_4$ concentrations in

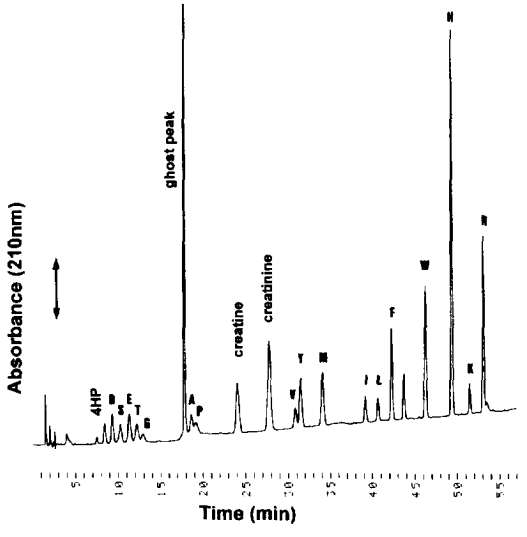

Fig. 3. Separation of amino acids, creatine and creatinine by acetonitrile gradient from 15 to 35%, as shown in Fig. 1, at a constant flow-rate of 1 ml/min. Concentrations: Crn, Cre, Met, His=100 μM; Tyr, Phe=25 μM; Trp=10 μM; others=1 m*M*. Sample size: 20 μl. Detection: UV (210 nm).

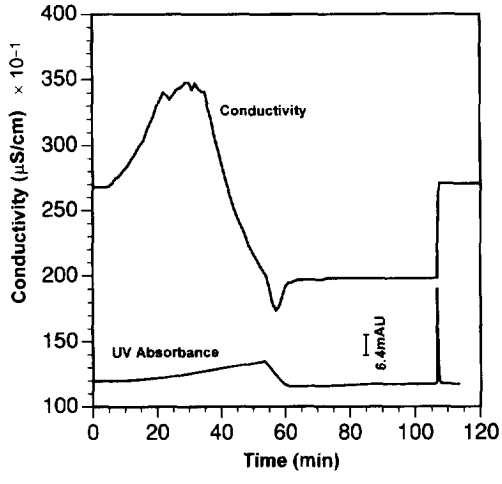

Fig. 4. Changes in conductivity and UV absorbance of the effluent due to acetonitrile gradient elution.

the mobile phase. Since the column reequilibration to the 15% acetonitrile reversed-phase condition was relatively quick, the conductivity showed a little increase from the bottom to ca. 2000 μS/cm. However, this pseudo equilibrium, as observed in the UV baseline after 60 min, is greatly different from the initial condition because SDS in the stationary phase is still left in the 35% acetonitrile equilibrium. The subsequent constant conductivity indicates that the initial SDS equilibrium is recovered from the inlet of the column. Since SDS concentration of the effluent is equalled to that of the starting eluent when reaching the initial equilibrium, a dramatic change in conductivity was observed at 107 min and the column was brought to the true reequilibrium. It can be estimated that the sharp peak observed on the UV background is due to a lens effect caused by the passing of the dense SDS front of different refractive indices through the UV cell. Since SDS is optically transparent, the absorbance is unchanged before and after the peak. This is namely the real identity of the ghost peak appearing on the consecutive chromatogram as shown in Fig. 3.

Consequently, reproducibility in retention times for amino acids was considerably improved. The relative standard deviations (R.S.D.s) before and after revealing the ghost peak were 0.4–5.5% (relatively worse for hydrophilic amino acids) and 0.3–0.7%, respectively. The peak area responses for UV-sensitive analytes were also reproducible with R.S.D. of 0.9–1.5%.

3.3. Dual-mode gradient elution

The flow-rate programming was introduced to reduce the time needed for the reequilibration. Based on several runs, the linear gradient from 1 to 2 ml/min in 50 min and the subsequent 2 ml/min was adequate for good separation and reequilibration. Although the peak distances from 4HP to Gly were slightly reduced, the separation between Val and Tyr was successfully improved. The elution system newly developed by programming both organic concentration and flow-rate, shown in Fig. 1, is termed "dual-mode gradient" elution. The dual-mode gradient program provided the system peak around 77 min and then led the cycle time to 80 min. Fig. 5 shows the improved separation of amino acids,

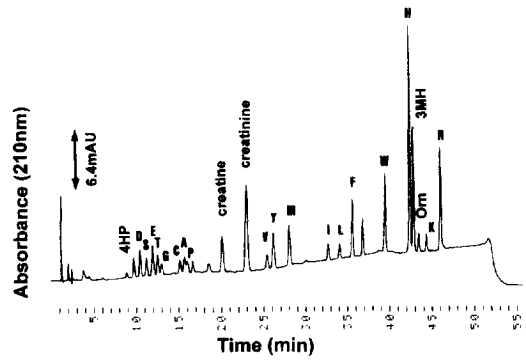

Fig. 5. Separation of amino acids, creatine and creatinine by dual-mode gradient elution shown in Fig. 1. Concentrations: the same as in Fig. 3. Symbols: 3MH=3-methylhistidine; Orn=ornithine. Detection: UV (210 nm).

Crn and Cre. The cycle time of this system is thought to be superior to those of conventional ion-exchange amino-acid analyzers. A well-balanced chromatogram of sufficient resolution was obtained.

3.4. Post-column fluorescence detection

A post-column OPA derivatization fluorescence detection system was conveniently used on this dual-mode gradient elution condition. The concentration of reaction mixture was reduced to one-fourth of the usual specification, the reaction was complete and reproducible. Since the high flow-rate was used, the reaction coil was set longer and the reaction temperature was set higher than those written in the usual specification. A standard chromatogram of amino acids with fluorescence detection is shown in Fig. 6. Crn and Cre as well as secondary amino acids such as proline were not detected in the present derivatization procedure. Since the reaction coil has a fairly large volume of ca.1 ml, the peaks were somewhat widened due to diffusion. This problem will be solved by improving reaction procedure.

3.5. Quantitation

Quantitation data by the dual-mode gradient ion-pair HPLC with UV and fluorescence detections are listed in Table 1. Relationships between concentration and peak area response were linear from 1 to 500 μM (20-μl injection) for the UV-absorbing

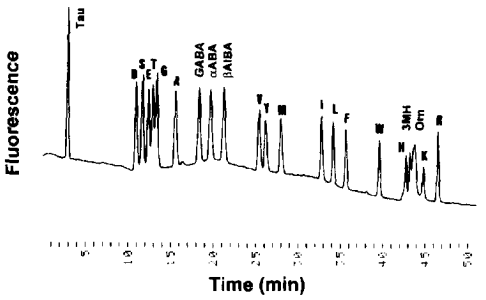

Fig. 6. A standard chromatogram of amino acids with OPA post-column fluorescence detection by dual-mode gradient ion-pair HPLC. Sample size: 20 μl×1 μM each. Symbols: Tau= taurine; GABA=γ-aminobutyric acid; αABA=α-aminobutyric acid; βAIBA=β-aminoisobutyric acid.

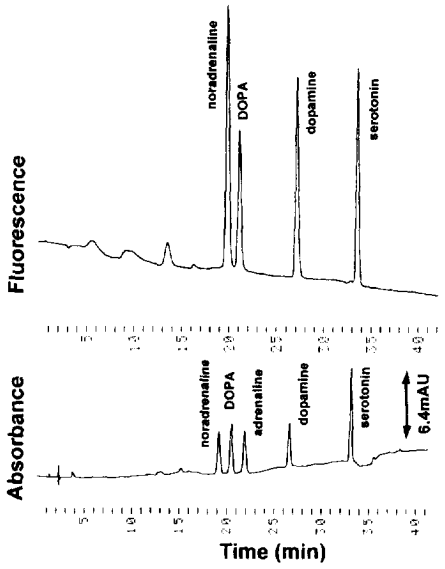

Fig. 7. Standard chromatograms of bioactive amines. Sample size: 20 μl×10 μM each.

analytes with good precision. In the fluorescence detection, relationships between concentration and peak height response were linear from 0.05 to 15 μM (20-μl injection) for primary amino acids.

3.6. Application to bioactive amines and nucleic acid bases

Figs. 7 and 8 show separations of bioactive amines and nucleic acid bases, respectively. Fortunately, the peaks of these analytes were not overlapped with those of amino acids shown in Figs. 5 and 6. The developed method is probably applicable to the determination of catecholamines in biological fluids [54,55]. Although the amines separated here are detectable both by spectrophotometric and fluorometric [54] detections except adrenaline (not an amine), electrochemical [55] detection is thought to be useful for the specificity. In Fig. 8, seven bases were well separated, although Ura, Thy and Xan, showing less basicity, were weakly retained on this condition.

3.7. Application to clinical analysis

The developed method was applied to the simultaneous determination of urinary Cre and amino acids [26,38] due to several inherited metabolic disorders. In the previous method [38], the precision in quantitation of His, Cre and Crn was somewhat inferior to that of aromatic amino acids because of their rela-

Table 1
Quantitation data for creatine, creatinine and amino acids by dual-mode gradient ion-pair HPLC with UV and fluorescence detections

Analyte	Detection	Range (sample size: 20 μl) (μM)	R.S.D. (Area) (%)	r
Crn	UV (210 nm)	1–500	1.0	1.0000
Cre		1–500	1.5	0.9999
Tyr		1–500	1.0	0.9999
Phe		1–500	1.1	0.9999
Trp		0.4–100	0.9	0.9999
His		1–500	1.5	0.9997
All amino acids	Fluorescence	0.05–15	<5 (Height)[a]	

[a] Obtained by measuring Asp, Glu and Gly because of almost the same sensitivity for amino acids as shown in Fig. 6. Peak height responses were better than areas for the determination.

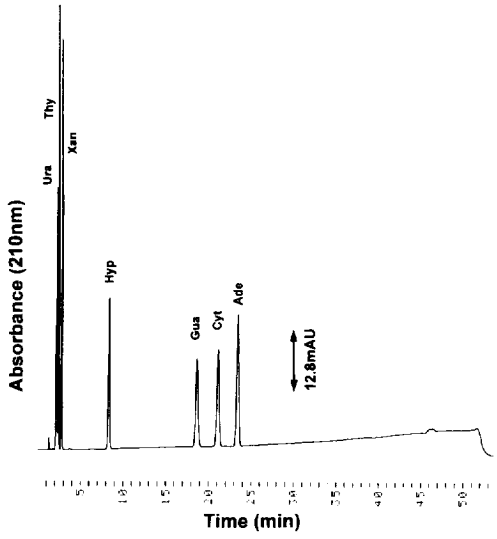

Fig. 8. A standard chromatogram of nucleic acid bases. Sample size: 20 μl×50 μM each.

tively short retention times. Such problem was solved in this work. Fig. 9 shows chromatograms of the basic fraction from urine of a patient with PKU, corresponding to 50 times dilution of the intact urine, with UV and fluorescence detections. The UV detection is sensitive enough to determine urinary Cre and Phe [26,38], one of diagnostic markers of the disease [56,57]. However, the 10 to 20 times dilution is adequate for the UV determination with 20-μl injections [26,38]. The fluorescence detection, of course, is more sensitive, giving many peaks of amino acids. Since Gly is a relatively abundant urinary metabolite [58], its large peaks were observed in chromatograms for healthy newborns. Taurine is also abundant in urine, but it is not included in the basic fraction because of its small pK_a. Asp, likewise passing through the cation-exchanger, was also not included in the fraction, although Glu was detected. This delicate difference in the cation-exchange affinity probably comes from the difference in the pH of the isoelectric point, 2.8 for Asp and 3.2 for Glu.

Table 2 lists the analytical results for several metabolic diseases with UV detection. Cre and diagnostically useful amino acids due to the diseases were determined simultaneously and the analyte concentration was easily corrected by Cre concentration (mM analyte/mM Cre). The Cre ratios for five PKU urines agreed well with those of previous results [38]. Since the concentration of citrulline (Cit) in the citrullinemia urine was outstandingly high, simultaneous UV detection of Cre and Cit was possible.

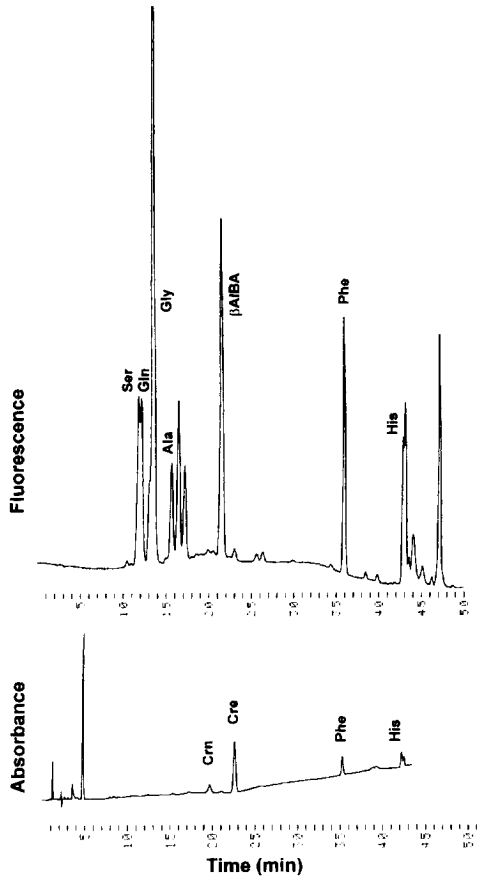

Fig. 9. Chromatograms of a basic fraction from urine of a patient with PKU.

3.8. Application to food analysis

Fig. 10 shows a chromatogram of watermelon juice obtained by fluorescence detection. The basic fraction analyzed corresponded to 304 times dilution of the fresh juice. Various kinds of amino acids were determined. The large peak out of scale is probably due to ammonia left in the sample by incomplete freeze-drying. Phe, Trp and His were also determined with UV detection by a 20-μl injection of the

Table 2
Analytical results of urinary creatinine and diagnostic amino acids for inherited metabolic disorders

Disease	No.	Concentration for intact urine (m*M*)				m*M*/m*M* Cre
		Cre	Tyr	Phe	Trp	
PKU	1	1.652	0.026	0.539	0.017	0.326 (Phe)
PKU	2	3.460	0.029	1.095	0.013	0.316 (Phe)
PKU	3	0.224	<0.02	0.168	<0.005	0.750 (Phe)
PKU	4	1.052	<0.02	0.297	0.007	0.282 (Phe)
PKU	5	1.615	<0.02	0.280	0.007	0.173 (Phe)
Tyrosinemia		1.502	0.162	0.018	0.010	0.108 (Tyr)
Lowe's syndrome		1.480	0.475	0.320	0.175	
Control (normal)		3.380	<0.02	<0.01	0.014	
		Cre	Cit			
Citrullinemia	1	10.75	201			18.7 (Cit)
	2	1.490	23.5			15.8 (Cit)

30-times diluted fraction. Orange and grape juices were also analyzed and the chromatographic patterns were dependent on the fruits.

4. Conclusion

The dual-mode gradient ion-pair HPLC method developed for amino-acid analysis has several advantages as follows: the standard instrumentation; the simple mobile phase; the simple gradient program; the sufficient separation given in reasonable analysis time and the low running cost. The analytical results are reproducible and quantitative. The use of dual detection, UV and fluorescence, is thought to be effective in practical analyses, especially in clinical laboratories. It is considered that the developed HPLC system has enough performance as a new analytical technique for amino acids and organic cations. Further applications in various research fields are expected.

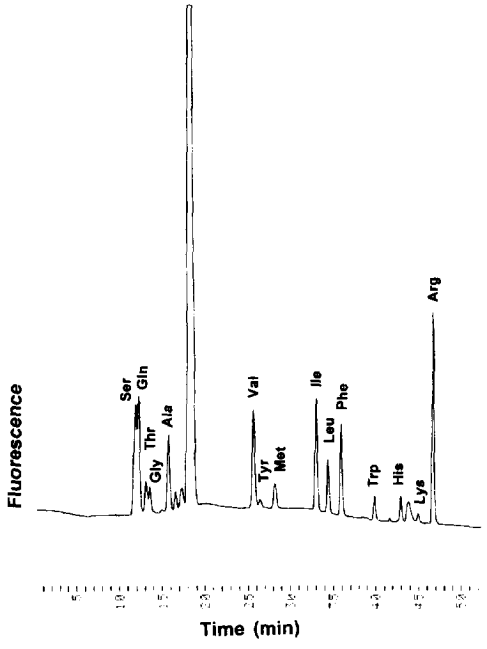

Fig. 10. Chromatogram of a basic fraction from watermelon juice.

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