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Determination of creatinine and ultraviolet-absorbing amino acids and organic acids in urine by reversed-phase high-performance liquid chromatography

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ABSTRACT

A simple and reliable method for the determination of urinary creatinine, amino acids and organic acids was developed. A urine sample was preliminarily separated into an organic acid fraction (including neutral species) and an amino acid fraction by cation-exchange chromatography. Both fractions were analysed by reversed-phase high-performance liquid chromatography, with a phosphoric acid–methanol gradient elution system and ultraviolet detection at 210 nm. Relationships between concentrations and peak heights were linear from 2 to 500 μM for the analytes. Overall recoveries were *ca.* 100%. The concentrations of creatinine for 37 urine samples, from 20 healthy newborns and from 17 patients with several inherited metabolic disorders, were 2.35 ± 2.29 mM (ranging between 0.27 and 10.15 mM). The method was applied to the determination of several diagnostically useful metabolites in urine. The concentrations of phenylalanine and phenylacetic acid for five urine samples from patients with phenylketonuria were 347 ± 177 and 282 ± 224 μM/mM creatinine, respectively. The concentrations of tyrosine and 4-hydroxyphenyllactic acid in the urine of a patient with tyrosinemia were 112 and 1871 μM/mM creatinine, respectively.

INTRODUCTION

The determination of urinary metabolites is important in the study of human diseases. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) have been widely used for the analysis of organic acids in urine [1–9] and are recognized to be essential techniques for the screening and/or the diagnosis of patients with organic acidurias [3–9]. In general, however, the sample preparation for GC and GC–MS is complicated and time-consuming, because solvent extraction and derivatization procedures are required. Tandem mass spectrometry (MS–MS) using stable isotope dilution has also been proposed [10]. However, the MS instrumentations are very expensive.

On the other hand, sample preparation for high-performance liquid chromatography (HPLC) is generally simple. Therefore, several HPLC techniques have also been proposed for the analysis of organic acids in urine [11–13] and in plasma [14], and of amino acids in serum [15–17].

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The determination of creatinine, a clinically important metabolite in serum and urine, is still based on the classical Jaffé picrate method [18]. However, the method is non-specific for creatinine. Recently, several HPLC techniques have been proposed as specific methods for the determination of creatinine [19–24]. The simultaneous determination of creatinine and several target compounds, such as uric acid [25,26], pseudouridine [27,28] and vanillylmandelic acid (VMA) and homovanillic acid (HVA) [29], have also been proposed. In many cases, however, urine or serum has been injected into the HPLC column only after filtration or deproteinization.

Recently, we have developed a cation-exchange HPLC method for the simultaneous determination of urinary creatinine and aromatic amino acids with a preliminary separation procedure using cation-exchange chromatography [30]. In the previous work, analytical cation-exchange HPLC caused difficulties in the determination of tryptophan, because of its hydrophobicity, and it was not applicable to the analysis of organic acids.

This paper describes a reversed-phase HPLC method for the determination of creatinine and UV-absorbing amino acids and organic acids in urine. The method is useful for the analysis of aromatic metabolites that result from metabolic disorders. HPLC profiling of the urine of patients with several inherited metabolic disorders is described.

EXPERIMENTAL

Reagents

Creatinine, creatine and amino acids were purchased from Wako (Osaka, Japan), and the organic acids listed in Table I were from Tokyo Kasei (Tokyo, Japan) or Sigma (St.Louis, MO, USA). Catecholamines and nucleic acid bases were from Wako or Sigma. Phosphoric acid of biochemical grade and methanol of HPLC grade were from Wako. Distilled water provided by an Advantec (Tokyo, Japan) Aquarius GSR-27 was passed through a Millipore (Bedford, MA, USA) Milli-Q Labo water purification system before use.

Urine samples

Seventeen abnormal urine samples, collected from five patients with phenylketonuria (PKU), five with methylmalonic acidemia, five with lactic acidemia, one with tyrosinemia and one with Lowe syndrome, were furnished by Shimoshizu National Hospital and Sanatorium. Twenty normal urine samples were collected from healthy newborns at Yokohama City University Hospital. All urine samples were stored at −20°C until analysis.

Reversed-phase HPLC

The HPLC system consisted of two Shimadzu (Kyoto, Japan) LC-9A pumps with a solvent mixer for a high-pressure gradient elution, a Rheodyne (Cotati, CA, USA) 7125 injector with a 100-μl sample loop, a Jasco (Tokyo, Japan) 875-UV variable-wavelength UV–VIS spectrophotometric absorbance detector and a Hitachi (Tokyo, Japan) D-2500 integrator. An Erma (Tokyo, Japan) ERC-3510 degasser was placed between the solvent reservoirs and pumps. A modified Toyo (Tokyo, Japan) FI-45 incubator was used as a column oven. A Rheodyne 7335 column inlet filter was placed between the injector and separation column. A GL Sciences (Tokyo, Japan) Inertsil ODS-2 (stainless-steel 250 mm × 4.6 mm I.D. column, particle size 5 μm, nominal theoretical plate number 19 000 plates per column) reversed-phase column was used for all analytical separations. The mobile phase consisted of 5 m*M* phosphoric acid (A) and methanol (B). The gradient elution was performed by programming the composition of the mobile phase from 99.9% A and 0.1% B to 45% A and 55% B in 60 min (see Fig. 1). The flow-rate was 1.0 ml/min. The column temperature was kept constant at 30°C. Under these conditions, the pumping pressure varied from 125 to 220 kg/cm^2, according to the increase of methanol concentration. The detection wavelength was 210 nm.

Preliminary separation

Urine was treated by the same procedure as described in the previous paper [30]. The glass column (100 mm × 6 mm I.D., 65 mm in bed

TABLE I

RELATIVE RETENTION TIMES (RRT) OF ORGANIC ACIDS

Retention times relative to that of uric acid under the conditions described in the text.

Acid	RRT	Acid	RRT
Imidazolcacetic	0.28	4-hydroxyphenyllactic (4HPLA)	4.21
Imidazolelactic	0.30	Quinaldic	4.39
Urocanic	0.43	4-Hydroxybenzoic	4.45
Malic	0.56	Hippuric	4.61
3-Hydroxypropionic	0.59	5-Hydroxyindole-3-acetic	4.65
Picolinic	0.59	Gentistic	4.86
Nicotinic	0.64	4-Hydroxyphenylacetic	4.86
Lactic	0.66	3-Hydroxyphenylacetic	5.16
Oxalic	0.67	2-Methylhippuric	5.18
Hydantoin-5-acetic	0.69	Vanillic	5.20
Malonic	0.74	2-Hydroxyphenylacetic (2HPAA)	5.28
Pseudouridine[a]	0.75	Homovanillic (HVA)	5.38
3,4-Dihydroxymandelic	0.91	4-Hydroxyphenylpropionic	5.75
Uric (UA)	1.00	Xanthurenic	5.87
Adenylic	1.10	Kynurenic	5.87
Succinic	1.18	Phenyllactic (PLA)	5.98
3-Hydroxybutanoic	1.22	4-Methylhippuric	6.10
Fumaric	1.26	3-Methylhippuric	6.14
Quinolinic	1.29	4-Hydroxycinnamic	6.17
Orotic	1.39	Phenylacetic (PAA)	6.31
2-Hydroxybutanoic	1.47	Suberic	6.43
Methylmalonic	1.64	Benzoic	6.56
Maleic	2.17	Indole-3-acetic	6.65
Vanillylmandelic (VMA)	2.19	Indole-3-propionic	7.27
Glutaric	2.26		
Homogentistic	2.79		
Ethylmalonic	3.29		
Adipic	3.78		
3,4-Dihydroxyphenylacetic	3.89		
Mandelic	3.98		

[a] Pseudouridine was not adsorbed by the cation-exchange resin.

length) was packed with TSKgel SP-Toyopearl 650M (cation exchanger, 0.15 mequiv./ml, Tosoh, Tokyo, Japan). A 100-μl aliquot of urine filtered through a 0.2-μm cellulose acetate filter (DIS-MIC-13 CP, Advantec, Tokyo, Japan) was introduced into the column, and the constituents were separated into two fractions referred to as the organic acid fraction (not adsorbed by the resin) and the amino acid fraction (eluted by 0.1 *M* ammonia water). The weight of each fraction was measured to determine the dilution ratio. A 20-μl aliquot of each fraction was injected into the analytical HPLC column.

RESULTS AND DISCUSSION

Sample preparation

Acidic and basic constituents in urine were quantitatively separated into the organic and the amino acid fractions, respectively. Although the amino acid fraction can be freeze-dried to remove ammonia, as described previously [30], freeze-

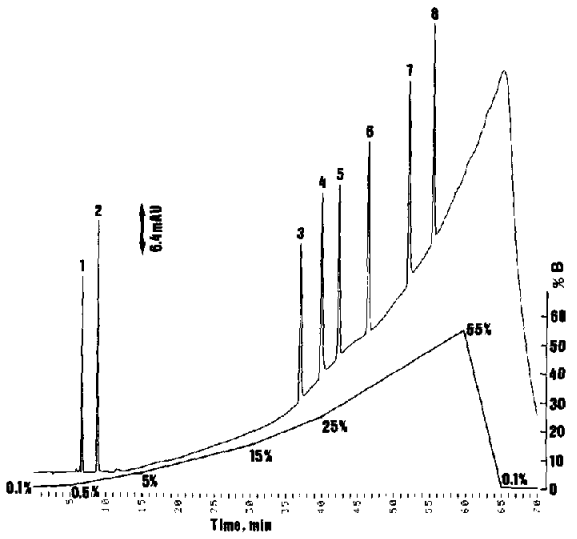

Fig. 1. Standard chromatogram of a mixture of organic acids. Peaks: 1 = pseudouridine; 2 = uric acid; 3 = 4-hydroxyphenyllactic acid; 4 = hippuric acid; 5 = 4-hydroxyphenylacetic acid; 6 = 2-hydroxyphenylacetic acid; 7 = phenyllactic acid; 8 = phenylacetic acid (20 μM pseudouridine; 50 μM each acid).

drying of the organic acid fraction is not permitted because volatile organic acids, such as lactic and phenylacetic acids, are lost partly or completely. Therefore, aliquots of both fractions were directly injected into the reversed-phase analytical column. The presence of ammonia in the amino acid fraction did not affect the determination of the analytes.

Analytical HPLC separation

The best separation was obtained by a concave gradient elution (Fig. 1) rather than by linear gradient elution. Table I lists the relative retention times of organic acids, both those that absorb in the UV region and those that are significant in metabolic diseases or in medicine.

Fig. 1 shows a standard chromatogram of the organic acid fraction. The increase in background absorbance due to the increase of methanol concentration in the mobile phase did not affect the determinations.

Fig. 2 shows a standard chromatogram of the amino acid fraction. Several hydrophobic amino acids, *i.e.* valine, methionine, leucine and isoleucine, are eluted between creatine and tyrosine, at retention times between 3 and 7 min. Although most of hydrophilic amino acids are eluted between 2 and 3 min, their UV transparency does not affect the determination of creatinine and creatine. Histidine and 3-methylhistidine, which are relatively abundant in urine [9,31], are eluted near the solvent front. Their separation was impossible under our chromatographic conditions. The concentration of histidine given in this paper includes 3-methylhistidine.

Catecholamines, *i.e.* norepinephrine, epinephrine and dopamine, and nucleic acid bases, *i.e.* purines and pyrimidines, were eluted by the proposed separation system. Their concentrations in urine are generally three orders of magnitude lower than those of the amino acids [31] and are below the detection limit of our method.

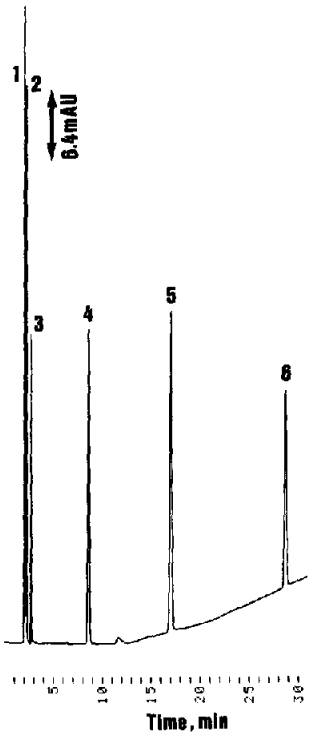

Fig. 2. Standard chromatogram of creatinine, creatine and amino acids. Peaks: 1 = histidine; 2 = creatinine; 3 = creatine; 4 = tyrosine; 5 = phenylalanine; 6 = tryptophan (10 μM tryptophan; 50 μM each).

TABLE II

REPRODUCIBILITIES OF RETENTION TIMES AND PEAK HEIGHTS OF ORGANIC ACIDS, CREATININE (Cre), CREATINE (Crn) AND AMINO ACIDS

Injection volume, 20 μl of 50 μM standard solutions (n = 3).

Compound	Retention time (min)			Peak height[a]		
	Mean	S.D.	C.V. (%)	Mean	S.D. ($\times 10^{-3}$)	C.V. (%)
UA	8.80	0.08	0.9	30.58	0.82	2.7
4HPLA	37.07	0.20	0.5	21.43	0.30	1.4
2HPAA	46.49	0.18	0.4	24.72	0.16	0.6
PLA	52.07	0.12	0.2	27.12	0.55	2.0
PAA	55.50	0.10	0.2	31.00	0.52	1.7
His	2.05	0.01	0.5	67.48	0.37	0.6
Cre	2.23	0.01	0.4	57.10	0.35	0.6
Crn	2.71	0.01	0.4	31.54	0.36	1.1
Tyr	8.26	0.09	1.1	33.50	0.12	0.3
Phe	16.30	0.18	1.1	33.25	0.25	0.8
Trp	27.76	0.21	0.8	20.42	0.10	0.5

[a] Integration units for peak height.

Quantification

Quantitative data were obtained by using uric (UA), 4-hydroxyphenyllactic (4HPLA), 2-hydroxyphenylacetic (2HPAA), phenyllactic (PLA) and phenylacetic (PAA) acids as standards for the organic acid fraction, and histidine (His), creatinine (Cre), creatine (Crn), tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) for the amino acid fraction.

Reproducibilities of the retention times and of the peak heights are given in Table II. The data were obtained by triplicate injections of 20 μl of the two 50 μM standard mixtures (Trp 10 μM). The coefficients of variation (C.V.) were below 1.1 and 2.7%, respectively.

The relationship between concentration and peak height was linear in the ranges 2–50 μM for uric acid, 2–500 μM for the other acids, 2–500 μM for histidine, creatinine, creatine, tyrosine and phenylalanine, and 0.4–100 μM for tryptophan. Although the retention time of creatinine was rather short and close to that of histidine, the separation between the two peaks was sufficient for quantitative evaluation. Only a few substances interfered with the determination of creatinine in urine. The detection limit, at a signal-to-noise ratio of 20, for the analytes was *ca.* 0.5 μM, using a 20-μl injection (0.1 μM for tryptophan).

The standard solution of uric acid was unstable. In contrast, uric acid in urine gave a reproducible peak and seemed stable.

Recoveries, using 1 mM standard solutions for the assay, were nearly quantitative (Table III).

TABLE III

OVERALL RECOVERIES OF ORGANIC ACIDS, CREATININE, CREATINE AND AMINO ACIDS

Compound	Recovery (%)	
	Mean	S.D.
UA	97.8	2.9
4HPLA	98.8	1.7
2HPAA	99.2	1.6
PLA	94.7	2.1
PAA	98.0	1.3
His	98.3	0.2
Cre	100.6	1.2
Crn	100.1	1.1
Tyr	99.4	0.6
Phe	98.3	0.8
Trp	98.2	0.8

Influence of ammonia

The presence of ammonia in the amino acid fraction increased the retention times of histidine, creatinine and creatine, but the separations were the same. The detection sensitivity of histidine was reduced under these conditions by *ca.* 50%, and that of creatinine and creatine was enhanced. Aromatic amino acids were not affected by ammonia. Since the retention times and the sensitivity depend on the pH, the standard mixture for the determination was prepared by dissolving in 0.05 *M* ammonia water.

Application of the method to urines of patients with inherited metabolic disorders

Normal urine. Fig. 3 shows typical chromatograms for amino acid (solid line) and organic acid (broken line) fractions of the urine of a healthy newborn. Only a few peaks were observed in addition to those of histidine, creatinine, creatine, uric acid and pseudouridine, which are known to be abundant constituents of urine [9,31].

The results for the amino acid fractions obtained from twenty normal urines are summarized in Table IV. The concentrations of creatinine were 2.70 ± 2.62 m*M* (2.35 ± 2.29 m*M* for all samples, *i.e.* twenty normal and seventeen abnormal urines). The concentrations of creatine were 567 ± 499 μM. These values were at the normal excretion levels for infancy [31]. In the organic acid fractions of normal urines, hippuric and 4-hydroxyphenylacetic acids were detected in several samples, but their concentrations were at normal levels [9,31].

Phenylketonuria. Fig. 4 shows typical chromatograms of the urine of a patient with PKU. Phenylalanine and 2-hydroxyphenylacetic, phenyllactic and phenylacetic acids, diagnostically useful metabolites for PKU [6], were commonly observed. An unknown peak observed in the organic acid fraction was supposed to be due to the presence of phenylpyruvic acid [6], but was not identified.

The concentrations of phenylalanine for five PKU urines were 347 ± 177 μM/m*M* creatinine as shown in Table V, and were significantly high-

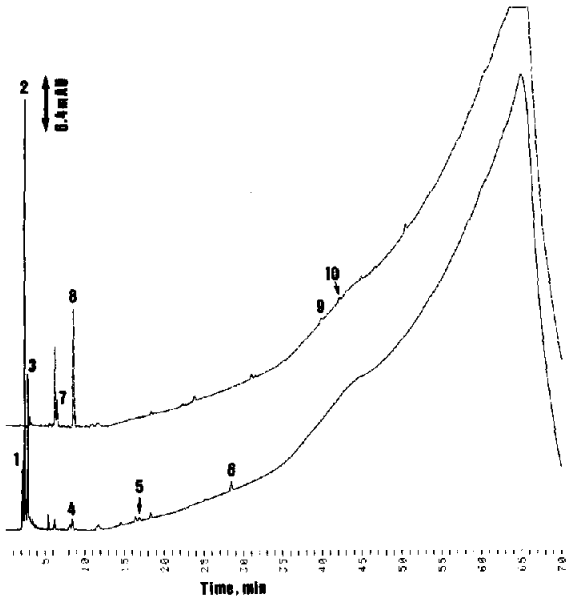

Fig. 3. Typical chromatograms of a urine of a healthy newborn (solid line, amino acid fraction; broken line, organic acid fraction). Peaks: 1 = histidine; 2 = creatinine; 3 = creatine; 4 = tyrosine; 5 = phenylalanine; 6 = tryptophan; 7 = pseudouridine; 8 = uric acid; 9 = hippuric acid; 10 = 4-hydroxyphenylacetic acid.

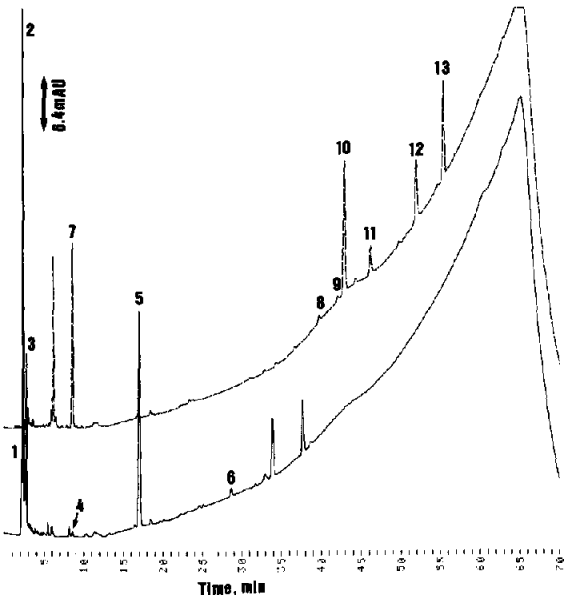

Fig. 4. Typical chromatograms of a urine of a patient with phenylketonuria (solid line, amino acid fraction; broken line, organic acid fraction). Peaks: 1 = histidine; 2 = creatinine; 3 = creatine; 4 = tyrosine; 5 = phenylalanine; 6 = tryptophan; 7 = uric acid; 8 = hippuric acid; 9 = 4-hydroxyphenylacetic acid; 10 = unknown; 11 = 2-hydroxyphenylacetic acid; 12 = phenyllactic acid; 13 = phenylacetic acid.

TABLE IV

ANALYTICAL RESULTS FROM THE AMINO ACID FRACTIONS OF URINES OF HEALTHY NEWBORNS

Sample No.	Concentration									
	μM						*μM*/m*M* creatinine			
	Cre	Crn	His	Tyr	Phe	Trp	His/Cre	Tyr/Cre	Phe/Cre	Trp/Cre
1	6824	910	1273	65	41	38	187	10	6	6
2	266	71	63	11	7	3	237	41	26	11
3	1944	245	181	21	13	6	93	11	7	3
4	1279	817	298	24	13	10	233	18	10	8
5	773	26	74	6	9	4	96	8	12	5
6	4127	320	155	31	21	13	38	8	5	3
7	7490	192	478	69	55	27	64	9	7	4
8	10145	425	1157	57	73	30	114	6	7	3
9	2106	297	227	23	18	7	108	11	9	3
10	1184	60	100	10	9	4	84	8	8	3
11	2744	166	162	21	23	11	59	8	8	4
12	1390	1213	116	12	8	4	83	9	6	3
13	707	571	91	12	5	3	129	17	7	4
14	1356	1335	319	22	13	13	235	16	10	10
15	3784	621	317	32	20	11	84	8	5	3
16	1520	416	316	29	15	9	208	19	10	6
17	1922	1812	298	29	19	17	155	15	10	9
18	827	612	309	36	12	11	354	43	14	13
19	681	58	172	19	7	7	253	28	10	10
20	2907	1167	142	28	14	7	49	10	5	2
Mean	2699	567	312	28	20	12	143	15	9	6
S.D.	2622	499	327	18	18	10	86	11	5	3

TABLE V

ANALYTICAL RESULTS FROM THE AMINO ACID FRACTIONS OF URINES OF PATIENTS WITH PHENYLKETONURIA, TYROSINEMIA AND LOWE SYNDROME

Sample No.	Concentration									
	μM						*μM*/m*M* creatinine			
	Cre	Crn	His	Tyr	Phe	Trp	His/Cre	Tyr/Cre	Phe/Cre	Trp/Cre
Phenylketonuria										
1	1819	769	708	21	563	17	389	12	310	9
2	3198	807	452	20	1029	12	141	6	322	4
3	280	311	126	11	181	5	450	39	646	18
4	920	301	194	9	260	8	211	10	283	9
5	1868	697	729	10	323	9	390	5	173	5
Mean ± S.D.							316 ± 113	14 ± 14	347 ± 177	9 ± 6
Tyrosinemia										
	1591	733	487	178	16	12	306	112	10	8
Lowe syndrome										
	1739	1423	2865	535	359	220	1647	308	206	127

TABLE VI

ANALYTICAL RESULTS FROM THE ORGANIC ACID FRACTION OF URINES OF PATIENTS WITH PHENYLKETONURIA, TYROSINEMIA AND LOWE SYNDROME

Sample No.	Concentration (μM)						
	UA	4HPLA	Hip[a]	4HPAA[b]	2HPAA	PLA	PAA
Phenylketonuria							
1	950	43	423	N.D.[c]	165	1129	676
2	2221	43	102	91	566	1218	1638
3	266	23	<20	<20	<20	173	123
4	1317	N.D.	38	36	36	<20	53
5	1020	22	97	N.D.	44	537	52
Tyrosinemia							
	429	2977	<20	453	87	N.D.	N.D.
Lowe syndrome							
	1376	42	153	256	79	N.D.	N.D.

[a] Hippuric acid.
[b] 4-Hydroxyphenylacetic acid.
[c] N.D. = not detected.

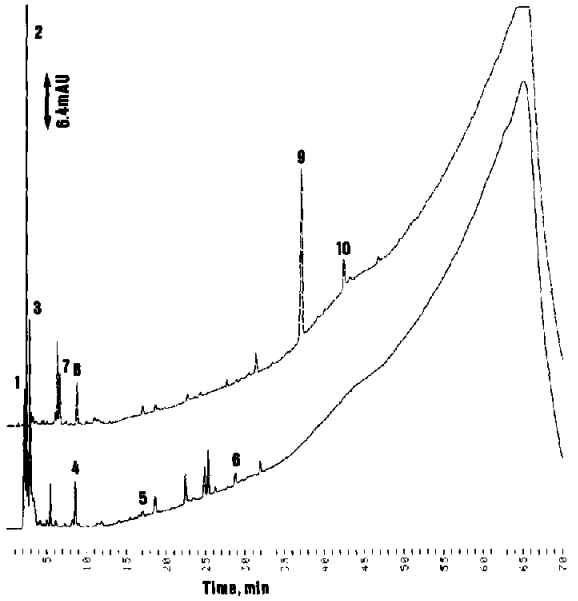

Fig. 5. Chromatograms of a urine of a patient with tyrosinemia (solid line, amino acid fraction; broken line, organic acid fraction). Peaks: 1 = histidine; 2 = creatinine; 3 = creatine; 4 = tyrosine; 5 = phenylalanine; 6 = tryptophan; 7 = pseudouridine; 8 = uric acid; 9 = 4-hydroxyphenyllactic acid; 10 = 4-hydroxyphenylacetic acid.

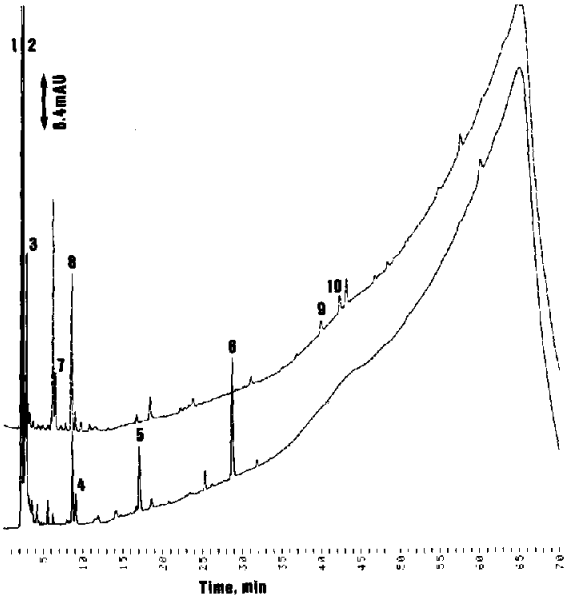

Fig. 6. Chromatograms of a urine of a patient with Lowe syndrome (solid line, amino acid fraction; broken line, organic acid fraction). Peaks: 1 = histidine; 2 = creatinine; 3 = creatine; 4 = tyrosine; 5 = phenylalanine; 6 = tryptophan; 7 = pseudouridine; 8 = uric acid; 9 = hippuric acid; 10 = 4-hydroxyphenylacetic acid.

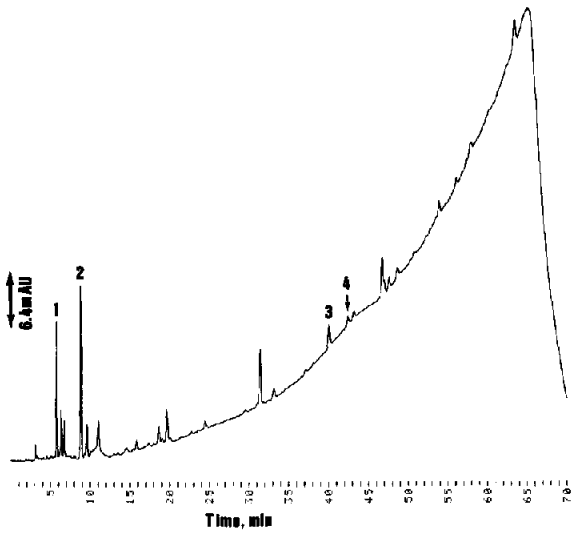

Fig. 7. Chromatogram of the organic acid fraction of a urine of a patient with lactic acidemia. Peaks: 1 = lactic acid; 2 = uric acid; 3 = hippuric acid; 4 = 4-hydroxyphenylacetic acid.

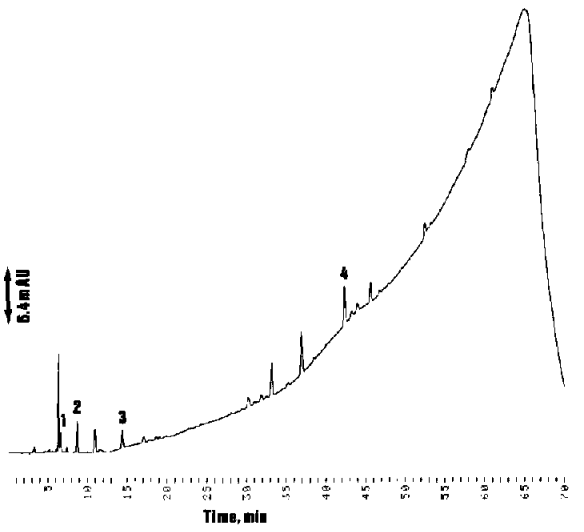

Fig. 8. Chromatogram of the organic acid fraction of a urine of a patient with methylmalonic acidemia. Peaks: 1 = pseudouridine; 2 = uric acid; 3 = methylmalonic acid; 4 = 4-hydroxyphenylacetic acid.

er than those for control urines (Table IV). The concentrations of histidine, 316 ± 113 μM/mM creatinine, were thought to be high, but those of tyrosine and tryptophan were at normal excretion levels (Table IV).

2-Hydroxyphenylacetic, phenyllactic and phenylacetic acids are not detected in normal urines but were in the urine of PKU patients (Table VI).

Tyrosinemia. Tyrosine, 4-hydroxyphenylacetic acid and 4-hydroxyphenyllactic acid, diagnostically useful metabolites for tyrosinemia [6], were observed on the chromatograms shown in Fig. 5. Their concentrations were greatly elevated (Tables V and VI).

TABLE VII

ANALYTICAL RESULTS FROM URINES OF PATIENTS WITH LACTIC AND METHYLMALONIC ACIDEMIAS

Sample No.	Concentration (mM)			Concentration (mM/mM creatinine)
	Lactic acid	Methylmalonic acid	Creatinine	
Lactic acidemia				
1	113.4	N.D.[a]	0.752	151.8
2	98.8	N.D.	0.488	202.5
3	152.8	N.D.	4.113	37.2
4	12.3	N.D.	2.363	5.2
5	12.4	N.D.	3.239	3.8
Methylmalonic acidemia				
1	N.D.	2.6	0.642	4.0
2	N.D.	7.2	0.491	14.7
3	N.D.	19.4	1.318	14.7
4	N.D.	13.3	0.636	20.9
5	N.D.	9.2	7.464	1.2

[a] N.D. = not detected.

Lowe syndrome (oculocerebrorenal syndrome). Histidine and aromatic amino acids were observed on the chromatograms (Fig. 6) and were thought to be characteristic of the disease. Their concentrations were high (Table V).

Lactic and methylmalonic acidemias. Figs. 7 and 8 show chromatograms of organic acid fractions of urines of patients with lactic and methylmalonic acidemias, respectively. Although the sensitivity of UV detection of lactic and methylmalonic acids is low, the peaks corresponding to those acids were clearly observed in the respective chromatograms, because their concentrations (Table VII) were more than two to three orders of magnitude higher than in normal urines [6,9,31].

CONCLUSION

The chromatographic profiling of patients with inherited metabolic disorders was indicative of the individual diseases, and the abnormally excreted metabolites due to the diseases could be determined precisely. Our method will facilitate the chemical diagnosis of several amino acidemias and organic acidurias. The proposed method provides more information on several inherited metabolic disorders, because urinary creatinine, amino acids and organic acids were analysed in the same urine sample. We believe that our method has a wide application to the analysis of urinary metabolites, *i.e.* uric acid, pseudouridine, VMA and HVA for neuroblastoma and for detecting organic solvent poisoning. It may also be applicable in doping tests.

ACKNOWLEDGEMENT

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