Journal of Chromatography, 239 (1982) 301–322
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,532

COMPILATION OF GAS CHROMATOGRAPHIC RETENTION INDICES OF 163 METABOLICALLY IMPORTANT ORGANIC ACIDS, AND THEIR USE IN DETECTION OF PATIENTS WITH ORGANIC ACIDURIAS

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SUMMARY

Gas chromatographic retention indices have been compiled for 163 metabolically important compounds (mostly organic acids) in the form of methylene units, as trimethylsilyl derivatives, on 10% OV-1 and 10% OV-17 columns. Comprehensive references on metabolic diseases that can be diagnosed by detection of these metabolites are cross-indexed to facilitate the use of the methylene-unit list.

The gas chromatographic method, which utilizes extraction of urine with ethyl acetate and trimethylsilylations, is described. Modified methods, one for neutral compounds and one for highly polar organic acids, both of which utilize appropriate ion exchange and lyophilization, are also described. Practical applications of these methods and the use of the methylene-unit list in the diagnosis of eleven patients with various metabolic disorders are also shown.

INTRODUCTION

Since isovaleric acidemia was identified in 1966 by use of gas chromatography (GC) and mass spectrometry (MS) coupled with GC, more than 15 additional organic acidurias (acidemias) have been identified by the use of these advanced analytical techniques[1,2]. These disorders are commonly characterized either by (a) the urinary excretion of extremely large amounts of normal metabolic intermediates that are not usually detectable, or are excreted only in trace amounts in normal urine; or by (b) the excretion of unusual metabolites that are secondarily produced from the accumulated normal intermediates by alternative pathways, owing to a block in the main degradative pathway. These organic acidurias are usually the result of a genetic aberration that results in an enzyme deficiency, but some are due to an inhibition of enzymes by an environmental toxin[3,4] or by a nutritional deficiency[5].

The use of GC and GC–MS has been essential in this development in clinical chemistry during the past 15 years. GC has provided high-efficiency resolution of numerous organic acids that are present in human urines, but unknown compounds, detected by non-specific GC detectors such as the flame-ionization detector, could be identified only by the use of GC–MS, because many of the abnormal metabolites detected in urine from patients with these organic acidurias had never been identified

0021-9673/82/0000–0000/$02.75

previously from natural sources. These include several aliphatic acylglycines such as isovalerylglycine[6], 3-methylcrotonylglycine[7], tiglylglycine[8], and *n*-hexanoylglycine[9], as well as polyfunctional compounds such as methylcitric[10], ethylmalonic[11], 3-hydroxypropionic[12], 3-hydroxyisovaleric[13], and 3-hydroxy-*n*-valeric acids[14].

GC–MS requires expensive instrumentation, and maintenance, operation and data interpretation require highly specialized training and technical expertise. In addition, a computer is almost indispensible for data processing. Thus, screening for organic aciduria has been done in only a few major medical centers, where such instruments and expertise are available.

During the past 15 years most of the metabolic diseases that can be detected by these techniques appear to have been found. The unusual urinary metabolites specific to these diseases have now been well characterized. Therefore, if the retention indices for these organic acids on more than one GC column are well defined, these organic acids may be readily identified by GC alone, eliminating the need for GC–MS and for the technical expertise.

In this paper we report retention indices, in terms of methylene units (MU), on 10% OV-1 and 10% OV-17 columns of trimethylsilyl (TMS) derivatives for 163 compounds of clinical importance. The compounds listed include normal metabolic intermediates and various unusual metabolites known to accumulate in the urine of patients with organic acidurias. Because many of the diagnostic metabolites were not available from commercial sources, they were synthesized, for the most part in our laboratory. With this list of MU values, it is now possible to make diagnoses of the well-defined organic acidurias, and practical organic aciduria screening programs may be implemented in hospitals that are not equipped with GC–MS and a mass spectrometrist.

In this report, we also describe in detail a practical GC method of urinary organic acid analysis, which was designed to be used in such organic aciduria screening programs. This method involves extraction of urine with ethyl acetate, dehydration of the extract residues, trimethylsilylation, and use of the list of retention indices to identify the organic acids. We present some typical chromatograms of urines from patients with organic acidurias who have been diagnosed in our laboratory since we completed this study. Details of this work have recently been published[16,17]. Since then, we have added seven compounds to the list. Procedures for the analysis of very polar compounds and their use for detection of patients with glycoluria and glyceric aciduria are also included.

MATERIALS AND METHODS

Organic acid standards and other chemicals

The following organic acids were synthesized by H. Ramsdell, B. Baretz and K.T. in our laboratory: 17 acylglycines[15] and ethylhydracrylic, 3-hydroxyisovaleric, 3-hydroxyvaleric, 2-hydroxyisovaleric, 2-hydroxyhexanoic, 2-hydroxyglutaric, 2-hydroxyadipic and methylcitric acids. The following compounds were the gift of other investigators: 3-hydroxyisobutyric acid (Dr. J. Craig, University of California, San Francisco, CA, U.S.A.) and 2-methyl-3-hydroxybutyric acid (Dr. O. Mamer, McGill University, Montreal, Canada). All other organic acids and hydrocarbon standards were procured from appropriate commercial sources. "TriSil-BSA formula P" was purchased from Pierce (Rockford, IL, U.S.A.).

Regular method

Urinary creatinine concentrations were first determined. Specimens were also tested for ketones and ketoacids by the dinitrophenylhydrazine–HCl (DNPH) method. When DNPH was negative, a volume of urine corresponding to 250 μg of creatinine was placed in a 110 × 13 mm screw-cap culture tube, diluted with de-ionized water to 2 ml, and the pH adjusted to 1 by dropwise addition of 6 *N* HCl. The acidified sample was extracted successively with four 2-ml aliquots of ethyl acetate, with vigorous shaking. The organic layers were combined into a second tube and 250 μg pentadecanoic acid (PDA) was added. The combined ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under a nitrogen stream. The evaporated residue was trimethylsilated with 100 μl TriSil-BSA Formula P (Pierce) at 60°C for 30 min.

When a specimen was positive for DNPH, an amount of the urine corresponding to 250 μg of creatinine was diluted with de-ionized water to 1 ml. After adjusting the pH to 14 with NaOH solution (30%), 1 ml of aqueous hydroxylamine–HCl (2.5%) was added and the sample was heated at 60°C for 30 min to form oximes of α-keto acids. After the sample had cooled to room temperature, the pH was adjusted to 1 by dropwise addition of 6 *N* HCl and the sample was extracted with ethyl acetate as described above.

Methods for very polar compounds

Some compounds with high polarity are of diagnostic significance. These include glycerol in glycerol kinase deficiency and L- and D-glyceric acids in L- and D-glyceric acidurias, respectively. Although these very polar compounds are not well extracted by extraction with ethyl acetate with yields ranging 3 to 8%, the amounts of these compounds which accumulate are extremely large. Therefore, small but significantly increased amounts of the polar compounds can be detected by the ethyl acetate-extraction method, indicating the underlying abnormality. When the increase of glycerol or glyceric acid is indicated, we process the sample by ion-exchange–lyophilization procedures. When a neutral compound such as glycerol is to be quantitated, urine which is equivalant to 250 μg creatinine is passed through a small column of a mixed bed resin (Bio-Rad AG 501) packed in a Pasteur pipette (2 cm height) and eluted with 5 ml water. The eluate is lyophilized and then derivatized with 0.5 ml TriSil-BSA-Formula P. When a polar acidic compound is to be quantitated, the urine specimen containing 250 μg creatinine is passed through a Dowex 50 column and eluted with 5 ml water.

RESULTS

List of methylene units of 163 compounds and its use for identification of urinary metabolites

Table I lists names of compounds and their MU values on 10% OV-1 and on 10% OV-17 columns. In the fourth column of Table I, abbreviated names of diseases in which the particular compound accumulates are listed, with pertinent references. A disease that is underlined indicates that the accompanying compound accumulates greatly and is diagnostic for the particular disease. Diseases not underlined are not specifically linked with that compound. Table II lists the abbreviated names of the diseases.

TABLE I

METHYLENE UNITS OF VARIOUS ORGANIC ACIDS

(I), (II) and (III) after acylglycines indicate mono-, di-, and tri-TMS, respectively.

Compound	*Columns used*		*Diseases to be considered*[§]
	10% OV-1	*10% OV-17*	
Propylene glycol	10.06	10.00	
Phenol	10.39	11.35	Normal, malabsorption[20]
Lactic acid	10.58	10.95	LA[2,21]
Hexanoic acid	10.63	11.24	GA II[22], IVS[3,4]
2-Hydroxyisobutyric acid	10.66	10.74	
Glycolic acid	10.70	11.27	
Oxalic acid	11.16	12.29	
o-Cresol	11.19	12.13	
Glyoxylic acid (oxime)	11.21	11.96	
2-Hydroxybutyric acid	11.30	11.61	LA[23]
p-Cresol	11.38	12.38	Normal, malabsorption[20]
3-Hydroxypropionic acid	11.40	11.94	PA[12,24]
Dipropylacetic acid (valproic acid)	11.47	11.87	Valp[25,26]
Pyruvic acid (oxime)	11.47	12.11	LA[21]
3-Hydroxybutyric acid	11.60	11.94	Ketosis
Heptanoic acid	11.62	12.19	
3-Hydroxyisobutyric acid	11.63	11.96	Ketosis[27,28]
2-Hydroxyisovaleric acid	11.70	11.88	MSUD[17,19,29]
2-Ketobutyric acid (oxime)	11.88	12.46	
Malonic acid	11.97	12.90	
Acetoacetic acid*	12.04	12.82	
2-Methyl-3-hydroxybutyric acid	12.09	12.25	KTD[30,31], PA[24]
Methylmalonic acid	12.12	12.86	MMA[32–34]
2-Ketoisovaleric acid (oxime)	12.13	12.69	MSUD[19,29,35]
3-Hydroxyisovaleric acid	12.14	12.35	MCC[7,36], MCD[17,24], IVA[13], Ketosis[27]
Urea	12.25	13.50	
Benzoic acid	12.28	13.73	Benzoic acid treatment, bacterial growth
2-Ethylhydracrylic acid	12.33	12.63	Normal***[37–39]
3-Hydroxyvaleric acid	12.39	12.70	PA[14,17,24]
2-Hydroxyisocaproic acid	12.41	12.61	
Acetoacetic acid*	12.43	13.18	Ketosis
Acetoacetic acid (oxime)	12.52	13.27	
Acetylglycine (I)[=]	12.53	14.86	
2-Ketovaleric acid (oxime)	12.55	13.05	
Octanoic acid	12.58	13.14	
4-Hydroxyisovaleric acid**	12.60	n.d.	
3-Ketovaleric acid**	12.72	13.44	PA[17,24]
2-Keto-3-methylvaleric acid (L-oxime)	12.73	13.24	MSUD[19,29,35]
Phosphoric acid	12.76	13.46	Normal***
Phenylacetic acid	12.77	14.37	PKU[17,40]
Ethylmalonic acid	12.78	13.48	GA II[22,41], EMA[42], IVS[3,11,39]
2-Hydroxyhexanoic acid	12.84	13.10	

TABLE I *(continued)*

Compound	*Columns used*		*Diseases to be considered*[§]
	10% OV-1	*10% OV-17*	
3-Ketovaleric acid	12.86	13.57	PA[17,24]
2-Keto-3-methylvaleric acid (D-oxime)	12.88	13.33	MSUD[19,29,35]
2-Ketoisocaproic acid (oxime)	12.89	13.34	MSUD[17,19,29,35]
Glycerol	12.92	12.63	GKD[43], HG[44]
Maleic acid	12.93	14.21	
trans-2-Octenoic acid	13.05	13.96	
Succinic acid	13.08	14.02	
Thymol	13.10	13.88	Sample preservative
2-Methylacetoacetic acid**	13.20	—	PA[45]
2-Methyl-3-ketovaleric acid*,**	13.20	13.76	PA[45]
Methylsuccinic acid	13.25	13.98	EMA[42]
2-Ketocaproic acid (oxime)	13.32	13.85	
Acrylylglycine (I)[π]	13.33	15.55	
Propionylglycine (I)[π]	13.34	15.37	PA[17,24,46]
Glyceric acid	13.43	13.60	Gly-L[47], Gly-D[48]
Fumaric acid	13.49	14.03	
2-Methyl-3-ketovaleric acid*,**	13.54	14.03	PA[35]
Nonanoic acid	13.55	14.16	
Acetylglycine (II)[π]	13.57	14.86	
Isobutyrylglycine (I)[π]	13.72	15.60	
Methacrylylglycine (I)[π]	13.88	15.92	
2-Propyl-3-hydroxypentanoic acid**	13.92	14.03	Valp[25,26]
Glutaric acid	13.96	14.87	GA[49,50], GA II[22,4]
Vinylacetylglycine (I)[π]	14.02	16.23	
Acrylylglycine (II)[π]	14.04	15.35	
Isobutyrylglycine (II)[π]	14.08	15.16	
n-Butyrylglycine (I)[π]	14.16	16.24	Hypoglycin intoxication in animals[39,51]
Propionylglycine (II)[π]	14.17	15.37	PA[17,24,46]
2-Propyl-3-oxopentanoic acid**	14.19	14.71	Valp[25,26]
3-Methylglutaric acid	14.19	15.01	Normal[53]
2-Propyl-3-oxopentanoic acid**	14.37	14.71	Valp[25,26]
Methacrylylglycine (II)[π]	14.42	15.52	
2-Methylbutylglycine (I)[π]	14.51	16.41	Hypoglycin intoxication in animals[39,51], PA[24]
Decanoic acid	14.53	15.06	
Isovalerylglycine (I)[π]	14.64	16.56	IVA[2,6]
Vinylacetylglycine (II)[π]	14.71	15.96	
n-Butyrylglycine (II)[π]	14.79	15.88	Hypoglycin intoxication in animals[39,51]
Crotonylglycine (I)[π]	14.82	17.18	
2-Propyl-5-hydroxypentanoic acid**	14.84	15.34	Valp[25,26]
2-Methylbutyrylglycine (II)	14.91	15.80	Hypoglycin intoxication in animals[39,51], PA[24]
Adipic acid	14.99	15.97	GA II[22,41], EMA[42], JVS[3,11,39], CD[52], Ketosis[53], food additive
Malic acid	15.01	15.39	

(Continued on p. 306)

TABLE I *(continued)*

Compound	*Columns used*		*Diseases to be considered*[§]
	10% OV-1	*10% OV-17*	
Salicylic (*o*-hydroxybenzoic) acid	15.05	16.32	SAL
trans-3-Hydromuconic acid	15.06	16.22	
Pyroglutamic acid	15.07	16.80	PGA[54,55], artificial diet[56]
Isovalerylglycine (II)[=]	15.10	16.02	IVA[2,6], GAD[22]
Crotonylglycine (II)[=]	15.13	16.52	
n-Valerylglycine (I)[=]	15.14	17.23	
2-Keto-4-methiol-butyric acid (oxime)	15.14	16.37	
trans-Cinnamic acid	15.21	17.17	
3-Methyladipic acid	15.28	16.20	
Oxalacetic acid (oxime)	15.28	16.14	
3-Methylcrotonylglycine (I)[=]	15.39	17.60	MCC[17], MCD[17,24]
2-Propylglutaric acid**	15.43	16.18	Valp[25,26]
Tiglylglycine (I)[x]	15.49	17.69	MCD[8,24], PA[57]
Tiglylglycine (II)[x]	15.49	16.76	KTD[30]
Undecanoic acid	15.49	16.04	
o-Hydroxyphenylacetic acid	15.59	16.90	
n-Valerylglycine (II)[=]	15.59	16.67	
m-Hydroxybenzoic acid	15.60	16.76	
3-Methylcrotonylglycine (II)[=]	15.63	16.97	MCC[7], MCD[17,24]
2-Hydroxyglutaric acid	15.75	16.32	
Phenyllactic acid	15.80	16.88	PKU[17,40], SBS[17]
Pimelic acid	15.91	16.93	
m-Hydroxyphenylacetic acid	15.98	17.33	
n-Hexanoylglycine (I)[=]	16.11	18.17	EMA[42], GA II[22], JVS[3,4,9]
3-Hydroxy-3-methylglutaric acid	16.12	16.48	HMG[58–60]
2-Furoylglycine (I)[=]	16.17	18.97	Dietary origin[61]
p-Hydroxybenzoic acid	16.22	17.30	
p-Hydroxyphenylacetic acid	16.25	17.66	Tyr[34], SBS[17], normal[62]
3,5-Furandicarboxylic acid	16.26	17.96	Dietary origin[61]
Phenylpyruvic acid (oxime)	16.32	17.81	PKU[17,40]
2-Ketoglutaric acid (oxime)	16.33	17.06	
2-Furoylglycine (II)[=]	16.47	18.33	Dietary origin[61]
n-Hexanoylglycine (II)[=]	16.48	17.48	EMA[42], GA II[22], JVS[3,4,9]
Dodecanoic acid	16.51	16.97	
2-Hydroxyadipic acid	16.83	17.39	KAA[62]
Octanedioic acid	16.91	17.94	EMA[42], GA II[22,41], JVS[3,4,9]
2-Ketoadipic acid (oxime)	17.21	17.98	KAA[63,64]
Orotic acid	17.49	18.59	Orotic aciduria[65]
Tridecanoic acid	17.49	17.99	
trans-Aconitic acid	17.52	18.40	
cis-Aconitic acid	17.54	18.42	
4-Hydroxy-3-methoxybenzoic acid	17.54	19.08	

TABLE I *(continued)*

Compound	*Columns used*		*Diseases to be considered*[§]
	10% OV-1	*10% OV-17*	
Homovanillic acid	17.58	19.32	
Hippuric acid (benzoylglycine) (II)*	17.85	20.04	Normal[17]
p-Hydroxymandelic acid	17.85	18.78	
Nonanedioic acid	17.89	18.88	
Hippuric acid (benzoylglycine) (I)*	17.96	21.10	Normal[17]
2,4-Dihydroxybenzoic acid	18.22	19.12	
Protocutechuic acid	18.26	19.11	
Citric acid	18.41	18.69	Normal[17]
Isocitric acid	18.41	18.86	
Phenylacetylglycine (II)*	18.42	21.74	
Myristic acid	18.45	18.96	
4-Decenedioic acid	18.65	19.96	Hypoglycin intoxication in animals[5,66]
Methylcitric acid	18.66	18.92	PA[10,24]
Phenylacetylglycine (I)*	18.66	20.67	
Decanedioic acid	18.90	19.95	GA II[22,41], EMA[42], JVS[3,11,39]
p-Hydroxyphenyllactic acid	19.09	19.93	Tyr[34], Liver failure,
p-Hydroxyphenylpyruvic acid (oxime)	19.43	20.58	SBS[17]
Pentadecanoic acid	19.44	19.95	
o-Hydroxyhippuric acid (III)*	19.54	21.01	SAL
Palmitic acid	20.43	20.90	JVS[3,4]
o-Hydroxyhippuric acid (II)*	20.47	23.03	SAL
p-Hydroxyphenylpyruvic acid	20.59	21.49	
p-Hydroxyhippuric acid (III)*	21.25	22.78	
Traumatic acid	21.31	22.63	
3-Indoleacetic acid	21.74	23.61	
p-Hydroxyphenylacetylglycine (II)*	21.82	24.72	
p-Hydroxyphenylacetylglycine (III)*	21.82	23.48	
5-Hydroxyindole-3-acetic acid	22.00	25.14	
Linoleic acid	22.00	23.00	
p-Hydroxyhippuric acid (II)*	22.02	24.56	
Oleic acid	22.08	22.87	
Stearic acid	22.39	22.90	
Tetradecanedioic acid	22.77	23.76	
Trichloroethanol glucuronide**	22.96	23.61	
Valproic acid glucuronide**	23.86	24.15	Valp[25,26]
Hexadecanedioic acid	>24.00	25.70	
Octadecanedioic acid	>24.00	27.59	

* Two isomers of 3-keto acid–TMS. These are due to *cis* and *trans* forms of enol–TMS.
** Identified in patient's urine using GC–MS.
*** Present in small amount in normal.
§ See Table II for abbreviations.
* (I), (II) and (III) after acylglycines indicate mono-, di-, and tri-TMS, respectively.

As can be seen in Table I, two or more compounds may have an identical or nearly identical MU value on one column, but their MU values differ considerably on the other column, permitting identification of an organic acid with satisfactorily high probability. To identify a peak positively, the MU of the peak should match within

TABLE II

ORGANIC ACIDURIAS WHICH CAN BE DIAGNOSED BY THE USE OF TABLE I

Abbreviation	*Name of disease*	*Abbreviation*	*Name of disease*
CD	Carnitine deficiency	MSUD	Maple syrup urine disease
EMA	Ethylmalonic-adipic aciduria	MCC	3-Methylcrotonyl CoA carboxylase deficiency
GA	Glutaric aciduria	MMA	Methylmalonic acidemia
GA II	Glutaric aciduria type II	MCD	Multiple carboxylase deficiency
GKD	Glycerol kinase deficiency	N	Normal
Gly-D	D-Glyceric aciduria	PA	Propionic acidemia
Gly-L	L-Glyceric aciduria	PKU	Phenylketonuria
HG	Hyperglycerolemia	PGA	Pyroglutamic aciduria (glutathione synthetase deficiency)
HMG	3-Hydroxy-3-methylglutaric aciduria		
IVA	Isovaleric aciduria	SAL	Salicylate treatment
JVS	Jamaican vomiting sickness	SBS	Short bowel syndrome (organic aciduria due to)
KAA	2-Ketoadipic aciduria		
KTD	β-Ketothiolase deficiency	Tyr	Tyrosinemia
LA	Lactic acidosis*	Valp	Valproic acid treatment

* Several diseases due to deficiency of pyruvate carboxylase and those of E_1, E_2 and E_3 of pyruvate dehydrogenase complex are included under lactic acidosis. For details, see ref. 21.

0.03 MU on both columns unless it is overlapped with other compounds. In the event a urinary metabolite is greatly increased, the sample must be diluted with either hexane or pyridine so that the amount of metabolite in 0.5–1.0 μl would be less than 2.5 μg. If the amount of compound injected is greater than 3 μg, the columns may be overloaded and the retention time of the same compound may become larger than that listed in Table I. We noted that even with this precaution, MU of urea (di-TMS) shifted considerably, particularly on the OV-1 column, for unknown reasons. Because some important metabolites such as methylmalonic acid and 3-hydroxyisovaleric acid appear in this region, peaks in this region must be identified carefully. These metabolites can be readily distinguished from urea on the OV-17 column.

Peaks for fatty acids shorter than six carbons are eluted with or before the last solvent peak on the OV-1 column, making determination of MU values of these compounds not possible. However, in diseases such as isovaleric and propionic acidemias, in which short-chain fatty acids accumulate, these acids are converted to unusual secondary metabolites and excreted in the urine. These secondary metabolites are isovalerylglycine[6] and 3-hydroxyisovaleric acid[13] in the former and 3-hydroxypropionic[12], 3-hydroxyvaleric[14], methylcitric acids[12] and tiglylglycine[8] in the latter. These two diseases can be readily diagnosed by identifying these secondary metabolites[2].

The ethyl acetate-extraction method vs. the ion-exchange–lyophilization method

In our routine analysis, we analyze urine samples by the ethyl acetate-extraction method. Although the recovery of very polar compounds such as glyceric acid and glycerol is low, it is adequate to detect them when they are increased. When unusual compounds with high polarity are detected by this method, the urine specimen is then followed by using an appropriate ion exchange (cation exchange or mixed bed) and lyophilization which gives quantitative recovery of very polar compounds.

However, the use of the ion-exchange–lyophilization method is not utilized for routine screening for several reasons. In the cation-exchange method, huge peaks of inorganic acids such as phosphoric acid elute in the region where many important diagnostic metabolites elute (Table I). Also, this procedure is time-consuming.

Analysis of normal urines

We analyzed 50 normal urines on OV-1 and OV-17 columns according to the present method (Table III). In all of the normal chromatograms we found a urea (di-TMS) peak, usually 5–20% of that of PDA. In two samples in group I, there were numerous other small peaks, all of them less than 2% of the size of the PDA peak (Fig. 1A), and including peaks that matched exactly to succinic, adipic, *p*-hydroxyphenylacetic, hippuric, and citric acids. In the chromatograms of the six cases in group II, several peaks were somewhat more prominent than others, including succinic, adipic, *p*-hydroxyphenylacetic, hippuric and citric acids and *p*-cresol (Fig. 1B). These peaks were also detected in small amounts in normal urines from other age groups. In all age groups, compounds detectable in essentially all urines are: succinic, adipic, *p*-hydroxyphenylacetic, hippuric, and citric acids concentrations in each age group of these five compounds are listed in Table III. Other compounds were detected only in some normal urines, and the range of concentration of these compounds and the number of urines in which these compounds were detected are in Table III.

Hippuric acid was detected in virtually all urines tested but the range of its concentration varies greatly, from almost 0 to 1093 μg per mg of creatinine, which is consistent with its presumed exogenous origin (as benzoic acid). Its main source has not been fully elucidated at present.

Many other smaller peaks were detected in normal urines. However, we believe that precise quantitation and unequivocal identification of these very small peaks must be done extensively with a GC–MS–computer system and are beyond the scope of this paper, which is to present a routine screening procedure.

Typical chromatograms of urines from patients with organic acidurias

Typical chromatograms of urines from several patients with different metabolic diseases are shown in this section. Peaks that are diagnostic for these diseases are very large compared with those of other organic acids. Our routine is to analyze the samples first on the OV-1 column. When very large peaks are observed, we then analyze the sample on the OV-17 column. Because the peak size of a compound does not vary significantly on either column, peaks of the same metabolites can be readily recognized on the OV-17 column. When accurate MU values of the abnormal metabolites are necessary for identification, the sample must be appropriately diluted with pyridine so that the amount of the particular metabolite to be injected will be less than 2.5 μg per injection.

Maple syrup urine disease. When a urine from an acute ketoacidotic episode of maple syrup urine disease was analyzed without oxime-formation, branched 2-keto acids were not detectable, but two major peaks (lactic and 2-hydroxyisovaleric acids) were. 2-Hydroxyisovaleric acid, presumably formed from the reduction of 2-ketoisovaleric acid, was the most important diagnostic peak in the non-oximized sample. When the oxime–TMS derivatives were made, several additional peaks were detected, as shown in Fig. 2: 2-ketoisocaproic, 2-keto-3-methylvaleric, 2-ketoisov-

TABLE III

MAJOR ORGANIC ACIDS IN NORMAL URINE

Acid	*Excretion (μg per mg creatinine)*				
	Group I (6-24 months) n = 2 mean ± SD	*Group II (2-5 years) n = 7 mean ± SD*	*Group III (6-10 years) n = 8 mean ± SD*	*Group IV (10-15 years) n = 22 mean ± SD*	*Group V (16-20 years) n = 11 mean ± SD*
Always present					
3-Hydroxybutyric + 3-hydroxyisobutyric*	0	16 ± 3	25 ± 28	12 ± 8	12 ± 6
Succinic	10	60 ± 31	32 ± 13	19 ± 10	13 ± 8
Adipic	18	50 ± 24	43 ± 20	69 ± 50	34 ± 15
p-Hydroxyphenylacetic	21 ± 6	52 ± 17	47 ± 29	35 ± 23	23 ± 11
Hippuric	18 ± 4	284 ± 146	336 ± 189	192 ± 162	293 ± 186
Citric	14 ± 3	182 ± 152	102 ± 50	88 ± 57	78 ± 32
		Range (number of samples which contain the acid)			
Present in some samples					
Glycolic			0-59 (31/50)		
Oxalic			0-63 (13/50)		
p-Cresol			0-126 (12/50)		
Octanedioic (suberic)			0-104 (21/50)		

* These two compounds are eluted very closely from each other on both OV-1 and OV-17 columns and their separate identification was difficult.

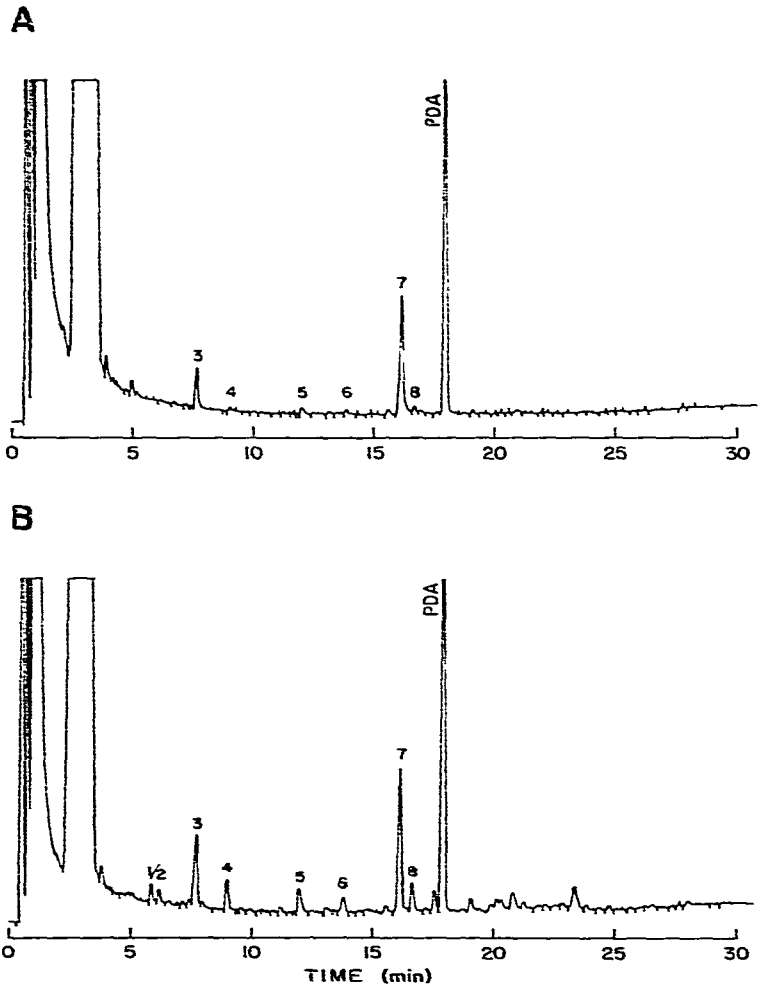

Fig. 1. Chromatograms of urinary organic acids from two normal children (A: 16 months and B: 3.5 years). A 10% OV-1 column was used for analyses. The compounds noted in the figures are TMS derivatives of the following compounds: 1 = oxalic; 2 = *p*-cresol; 3 = urea; 4 = succinic; 5 = adipic; 6 = *p*-hydroxyphenylacetic; 7 = hippuric; and 8 = citric acids. *n*-Pentadecanoic acid (PDA, 1 mg/mg creatinine) was added as an internal standard.

aleric, 2-ketoglutaric and pyruvic acids[19,29,35]. In particular, the peak sizes of the first two compounds were very large and diagnostic. In the patient we studied, these unusual 2-ketoacids all disappeared after five days of treatment with a diet low in branched-chain amino acids.

Isovaleric acidemia: When a urine was collected while the patient with isovaleric acidemia was in remission, isovalerylglycine was the only abnormal metabolite to be detected[2,6]. The amount of this metabolite was very large, far exceeding the size of the internal standard peak (PDA: 1 mg/mg creatinine; Fig. 3, top). Two peaks of isovalerylglycine were observed (the mono-TMS and di-TMS derivatives of isovaler-

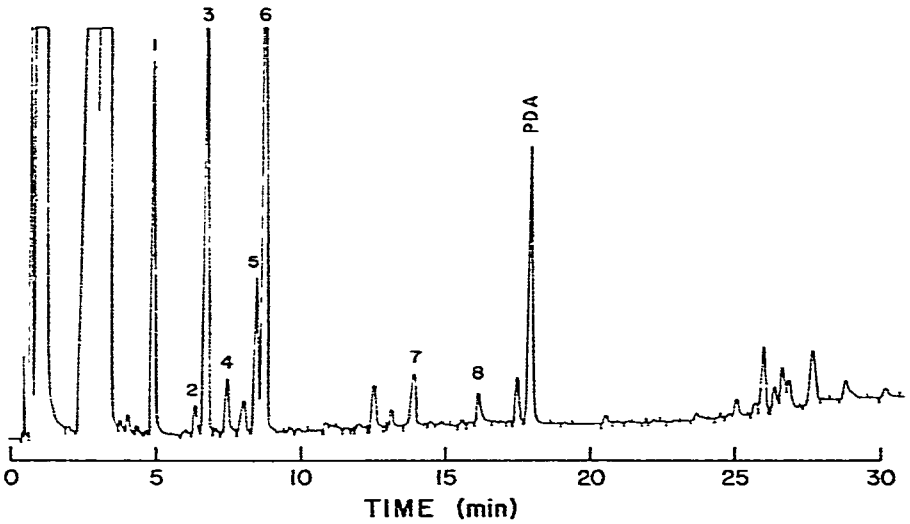

Fig. 2. Urinary organic acids from a patient with maple syrup urine disease. The column was a 10% OV-1 column. Peaks: 1 = lactic; 2 = pyruvic-oxime; 3 = 2-hydroxyisovaleric; 4 = 2-ketoisovaleric-oxime; 5 = 2-keto-3-methylvaleric-oxime; 6 = 2-ketoisocaproic-oxime. 7 = 2-ketoglutaric-oxime; 8 = hippuric acids. *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard.

ylglycine). When urine from a ketoacidotic episode of isovaleric acidemia was analyzed, several additional large peaks were detected: lactic, 3-hydroxybutyric, acetoacetic, and 3-hydroxyisovaleric acids (Fig. 3, bottom)[2,13].

Propionic acidemia. Sometimes the only diagnostic change detectable in urinary metabolite analysis of a patient with propionic acidemia who is in stable condition is a moderate accumulation of methylcitric (homocitric) acid[10], as shown in Fig. 4 (top). This change is much less dramatic than those seen in other organic acidurias and could be easily missed unless analyses of such urines and inspection of chromatograms are carefully done. In contrast, many unusual organic acids are detectable in urines collected when the propionic acidemia patient is in an acute acidotic episode (Fig. 4, bottom). These diagnostic metabolites specific for propionic acidemia are 3-hydroxypropionic[12], 2-methyl-3-hydroxybutyric[24], 3-ketovaleric[12], 3-hydroxyvaleric[14,17,24], 3-methylacetoacetic[24], 2-methyl-3-ketovaleric[24], 2-methyl-3-hydroxyvaleric and methylcitric acids and two unusual acylglycines, propionylglycine[17,24,46] and tiglylglycine[59] (Fig. 4, bottom). However, the amounts of these diagnostic metabolites are not extremely large. In addition, large peaks of nonspecific metabolites such as 3-hydroxybutyric, acetoacetic, and *p*-hydroxphenylacetic acids are detected.

Methylmalonic acidemia. Diagnosis of methylmalonic acidemia is usually very easy; a very large amount (up to 20 mg per mg of creatinine) of methylmalonic acid is readily detectable and usually is the only abnormal peak (Fig. 5). In addition, much smaller peaks of metabolites from isoleucine, such as 2-methyl-3-hydroxyburytic acid and tiglylglycine, and the secondary metabolites of propionate (3-hydroxypropionic, 3-hydroxyvaleric, methylcitric acids) may be detected. In case a large methylmalonic acid peak is not detected in a suspected urine, one must learn whether vitamin B_{12}

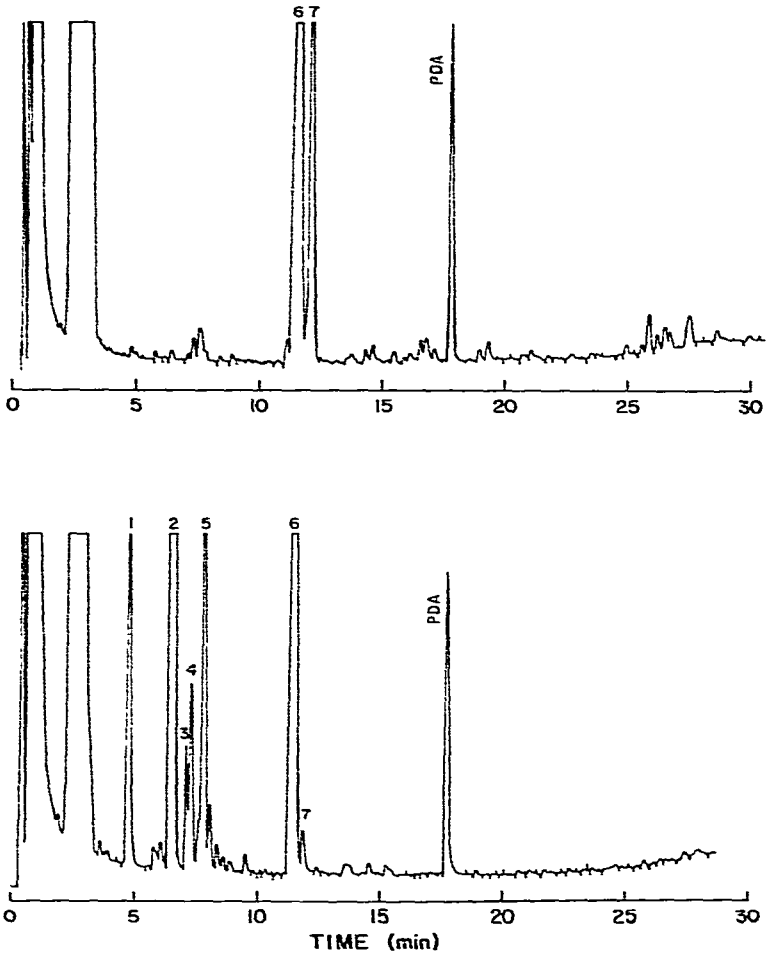

Fig. 3. Urinary organic acids from a patient with isovaleric acidemia. Top: a urine from remission. Bottom: a urine from a ketoacidotic episode. A 10% OV-1 column was used for analysis of both samples. Peaks: 1 = lactic; 2 = 3-hydroxybutyric; 3 = acetoacetic (first peak); 4 = 3-hydroxyisovaleric; 5 = acetoacetic (second (peak); 6 = isovalerylglycine (mono-TMS); 7 = isovalerylglycine (di-TMS). *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard. 3-Ketoacids such as acetoacetic acid is detected as two peaks of di-TMS of the enol forms (*cis* and *trans*).

(cobalamin) was administered to the patient; in B_{12}-responsive methylmalonic acidemia, urinary methylmalonic acid may be decreased to a very low concentration. Several different forms of methylmalonic acidemia exist[34].

Glutaric aciduria type II (GA II) and ethylmalonic-adipic aciduria (EMA). Large amounts of ethylmalonic, glutaric, adipic, octanedioic (suberic), and decanedioic (sebacic) acids are detectable in urines from patients with GA II (Fig. 6, top)[22,41]. Although in most of the urines from patients with GA II the amount of ethylmalonic acid is larger than that shown in Fig. 6, peaks of glutaric or adipic acid are the largest. In contrast, in urines from patients with EMA, ethylmalonic or adipic acids have the largest peaks, and glutaric acid usually is detectable only in small

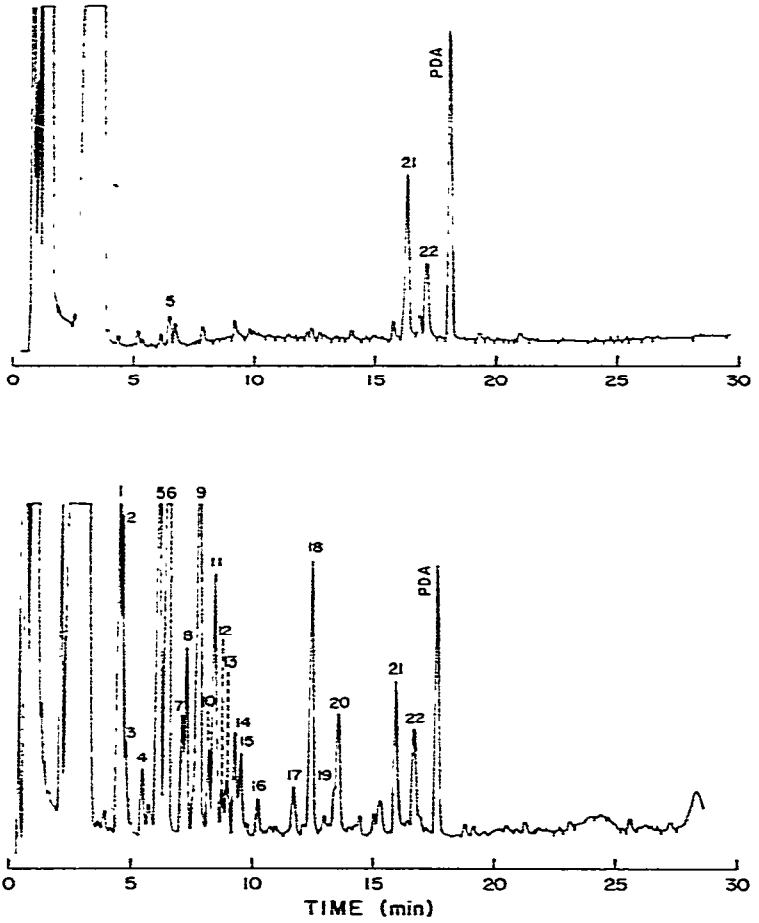

Fig. 4. Urinary organic acids from patients with propionic acidemia. Top: a urine from remission. Bottom: a urine from a ketoacidotic episode. A 10% OV-1 column was used for analyses of both samples. Peaks: 1 = oxalic; 2 = unknown; 3 = lactic; 4 = unknown; 5 = 3-hydroxypropionic; 6 = 3-hydroxybutyric; 7 = acetoacetic (first peak); 8 = 2-methyl-3-hydroxybutyric; 9 = 3-hydroxyvaleric + acetoacetic (second peak); 10 = 3-ketovaleric (first peak); 11 = 3-ketovaleric (second peak); 12 = succinic; 13 = 2-methylacetoacetic; 14 = propionylglycine; 15 = 2-methyl-3-ketovaleric; 16 = glutaric; 17 = adipic; 18 = tiglylglycine; 19 = 3-hydroxy-3-methylglutaric; 20 = *p*-hydroxyphenylacetic; 21 = hippuric; 22 = methylcitric acids. *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard. 3-Ketoacids are detected as two peaks of di-TMS derivatives of enolic forms (*cis* and *trans*).

amounts or is undectectable (Fig. 6, bottom); large accumulations of glutaric acid may occur on rare occasions when the patient is severely acidotic[42]. GA II and EMA are both caused by a similar cellular mechanism, namely, a deficiency of multiple acyl CoA dehydrogenase activities, but the biochemical causes underlying this deficiency are still unknown. The distinct difference in urinary metabolites suggests heterogeneities of these two similar diseases. This phenotypical difference is not due to difference in severity of deficiencies of acyl CoA dehydrogenase activities, because the glutaric acid peak is as prominent in a very mild case of GA II[22] in which the

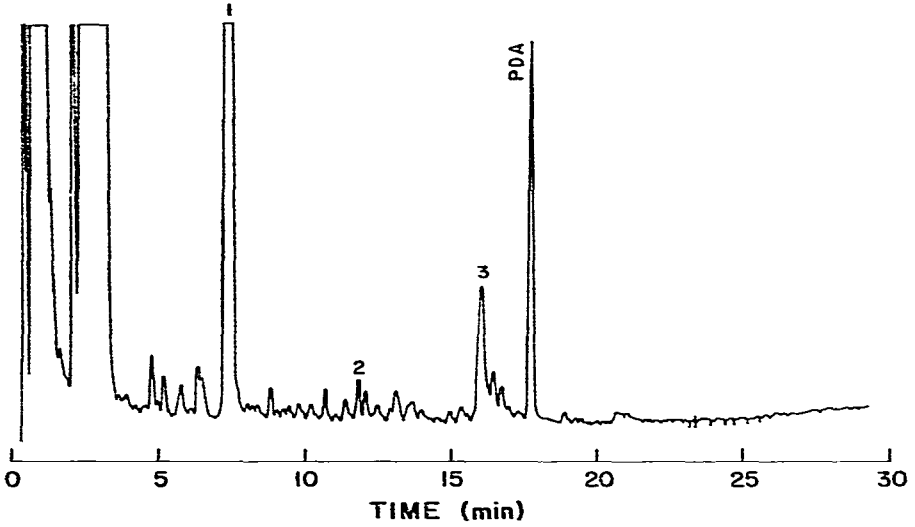

Fig. 5. Urinary organic acids from a patient with methylmalonic acidemia. A 10% OV-1 column was used for analysis. Peaks: 1 = methylmalonic; 2 = adipic; 3 = hippuric acids. *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard.

deficiency was milder than that of the EMA patient[41]. The GA II urine in Fig. 6 (top) was from a patient with mild clinical manifestations.

Multiple carboxylase deficiency. Activities of three biotin-dependent carboxylases (propionyl CoA-, 3-methylcrotonyl CoA- and pyruvate carboxylases) are low in this disease, presumably because of a deficiency of holocarboxylase synthetase or defective biotin absorption[36]. A chromatogram of urinary organic acids from a patient with this disease, recently diagnosed in our laboratory, is shown in Fig. 7. This patient, a 28-month-old boy, had a severe episode of hypoglycemia and acidosis. Large peaks of lactic, 3-hydroxypropionic, 3-hydroxybutyric, 3-hydroxyisovaleric, and acetoacetic acids and 3-methylcrotonylglycine were detected. After two weeks of treatment with biotin (10 mg/day), these unusual urinary metabolites almost entirely disappeared except for a small amount of 3-hydroxyisovaleric acid.

Phenylketonuria. Patients with phenylketonuria are usually detected by newborn screening programs with the Guthrie test or with amino acid chromatography. However, we occasionally receive urines from undiagnosed phenylketonuria patients for analysis of urinary organic acids from such a previously undiagnosed case (four-year-old boy) is shown in Fig. 8. Large peaks of phenylacetic, phenylpyruvic, and phenyllactic acids are characteristic features of these urines.

Short bowel syndrome. Obese patients who have undergone drastic small-bowel resection may have severe acidosis. We have encountered two such cases in the past two years. In both, there was a huge lactic acid peak and moderately increased amounts of phenyllactic, *p*-hydroxyphenylacetic, and *p*-hydroxyphenyllactic acids (Fig. 9). These organic acids are presumably produced by bacteria in the colon from unabsorbed amino acids. The D-configuration of urinary lactic acid in short bowel syndrome was initially suggested by us on the basis of a discrepancy between a low serum lactate concentration, as measured enzymically with L-lactic dehydrogenase,

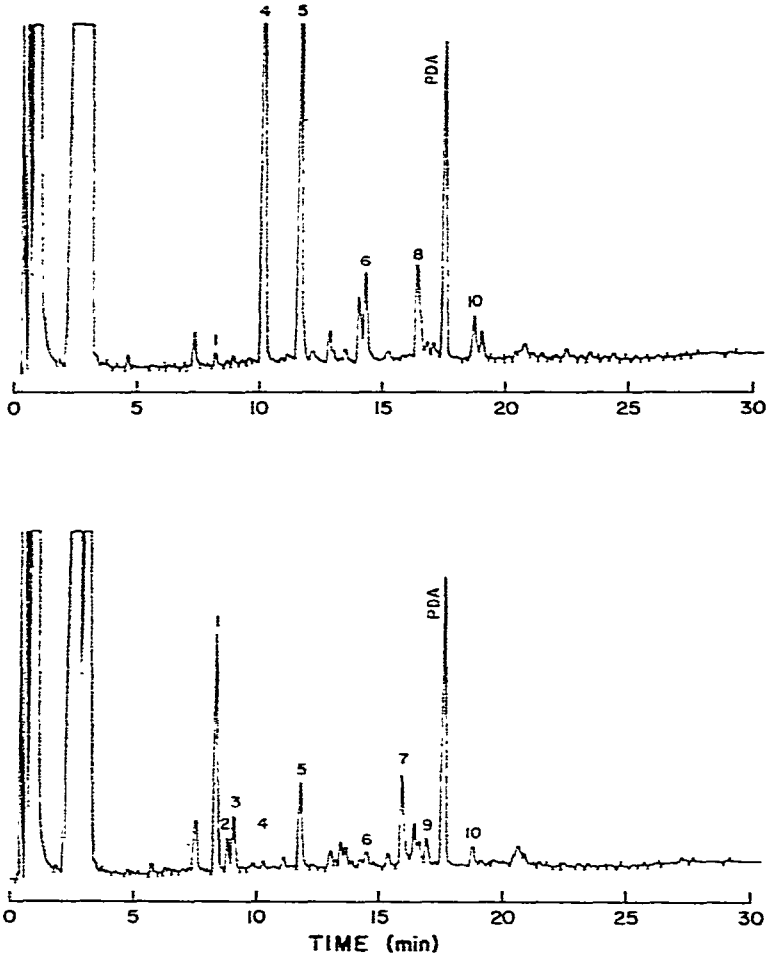

Fig. 6. Urinary organic acids from a patient with glutaric aciduria type II (acute stage) (top) and a patient with ethylmalonic aciduria (remission) (bottom). A 10% OV-1 column was used for analyses of both samples. Peaks: 1 = ethylmalonic; 2 = succinic; 3 = methylsuccinic; 4 = glutaric; 5 = adipic; 6 = octanedioic (suberic); 7 = hippuric; 8 = decenedioic; 9 = decanedioic (sebacic); 10 = palmitic acids. *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard.

and a very large amount of urinary lactic acid, as detected by GC. It has since been confirmed.

Glyceric aciduria. In the initial sample, moderately increased amounts of lactic acid and that identified as glyceric acid were found by the ethyl acetate-extraction method (Fig. 10A). When the same sample was prepared by the Dowex-50 column method, a huge amount of glyceric acid was detected. In addition, a huge peak of phosphoric acid and those of citric acid and a few unknown compounds were detected (Fig. 10B). The stereoconfiguration of glyceric acid and the nature of the enzyme deficiency are now under investigation.

Glyceroluria. This disease is due to a deficiency of glycerol kinase activity.

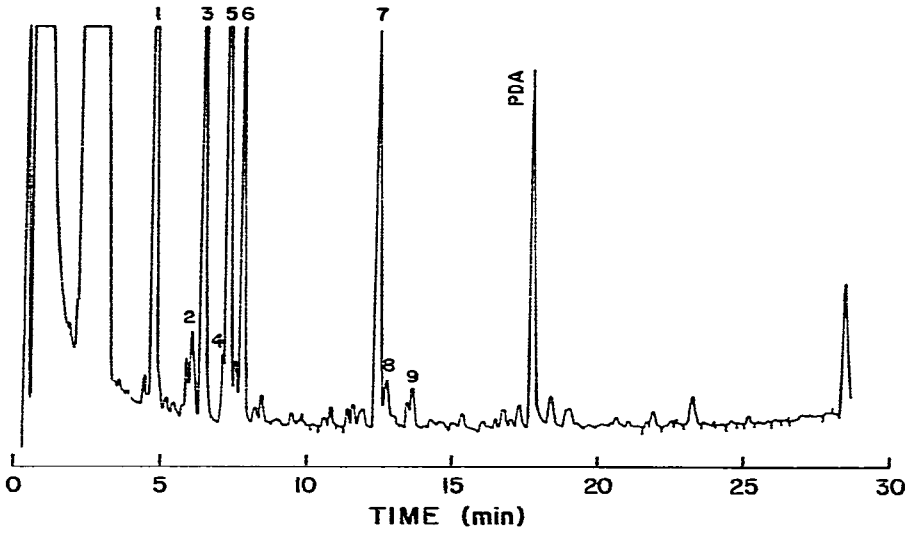

Fig. 7. Urinary organic acids from a patient with multiple carboxylase deficiency. A 10% OV-1 column was used for analysis. Peaks: 1 = lactic; 2 = 3-hydroxypropionic; 3 = 3-hydroxybutyric; 4 = 2-ethylhydracrylic; 5 = 3-hydroxyisovaleric; 6 = acetoacetic; 7 = 3-methylcrotonylglycine (mono-TMS); 8 = 3-methylcrotonylglycine (di-TMS); 9 = *p*-hydroxyphenylacetic acids. *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard.

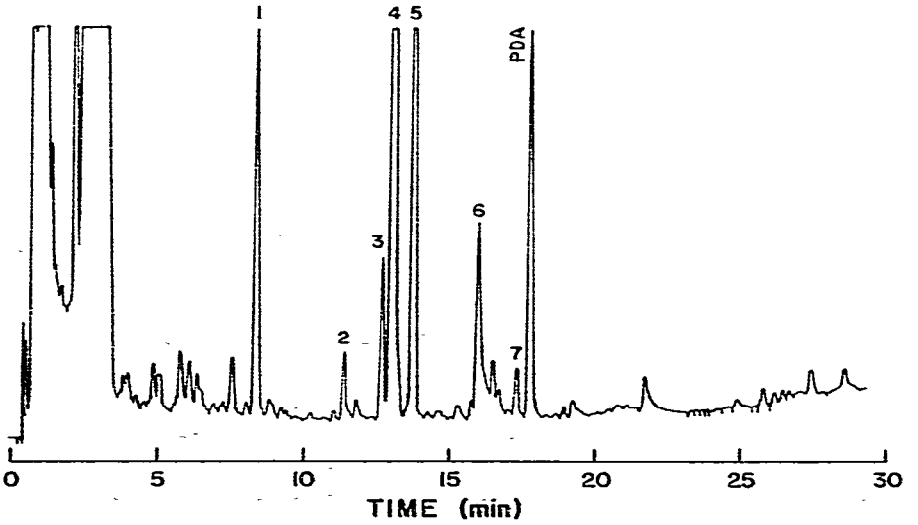

Fig. 8. Urinary organic acids from a patient with phenylketonuria. A 10% OV-1 column was used for analysis. Peaks: 1 = phenylacetate; 2 = unknown; 3 = *o*-hydroxyphenylacetic; 4 = phenyllactic; 5 = phenylpyruvic-oxime; 6 = hippuric; 7 = *p*-hydroxyphenyllactic acids. *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard.

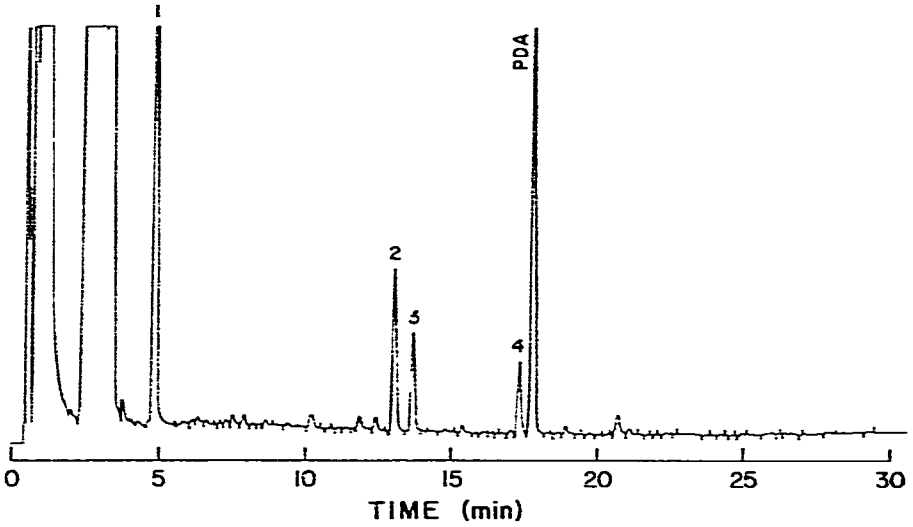

Fig. 9. Urinary organic acids from a patient with short bowel syndrome. A 10% OV-1 column was used for analysis. Peaks: 1 = lactic (D-form); 2 = phenylacetic; 3 = *p*-hydroxyphenylacetic; 4 = *p*-hydroxyphenyllactic acids. *n*-Pentadecanoic acid (PDA: 1 mg/mg creatinine) was added as an internal standard.

Although the main clinical features of the previously reported case was psychomotor retardation, spastivity, a non-specific myopathy and adrenal insufficiency[43], our patient presented severe ketoacidosis. When the first urine was analyzed using the ethyl acetate extraction, there was a large glycerol peak in addition to a huge 3-hydroxybutyric acid peak (Fig. 11A). The same urine was then fractionated by a AG 501 mixed-bed resin and the neutral fraction was trimethylsilated. A huge glycerol peak and a large urea peak were observed (Fig. 11B).

DISCUSSION

In 1966, Dalgliesh *et al.*[67] compiled an extensive list of MU values for many organic acids, pioneering in the GC analysis of urinary organic acids, but their list is now of very limited practical application in the detection of organic aciduria because of certain drawbacks. First, the stationary phase used was F-50 siloxane polymer (Dow Corning) alone, which is no longer in wide use. A more serious drawback is the fact that all compounds tested were from the shelf; thus, many diagnostic metabolites are missing from the list because this study was completed before the identification of isovaleric acidemia, the first organic aciduria to be discovered[1]. Gates *et al.*[68] recently published an extensive list of retention indices for urinary metabolites from normal adults, patients with neuroblastoma and pediatric patients with unspecified diseases, as determined by GC–MS–computer. This study also suffers from similar shortcomings in its application to the detection of patients with organic aciduria by GC alone: the use of a single stationary phase and the lack of pathological metabolites in their list. Thus, the present study represents the first comprehensive list of retention indices of organic acids that can be utilized for the diagnosis of organic acidurias.

Since completing this compilation two years ago, we have analyzed over 500

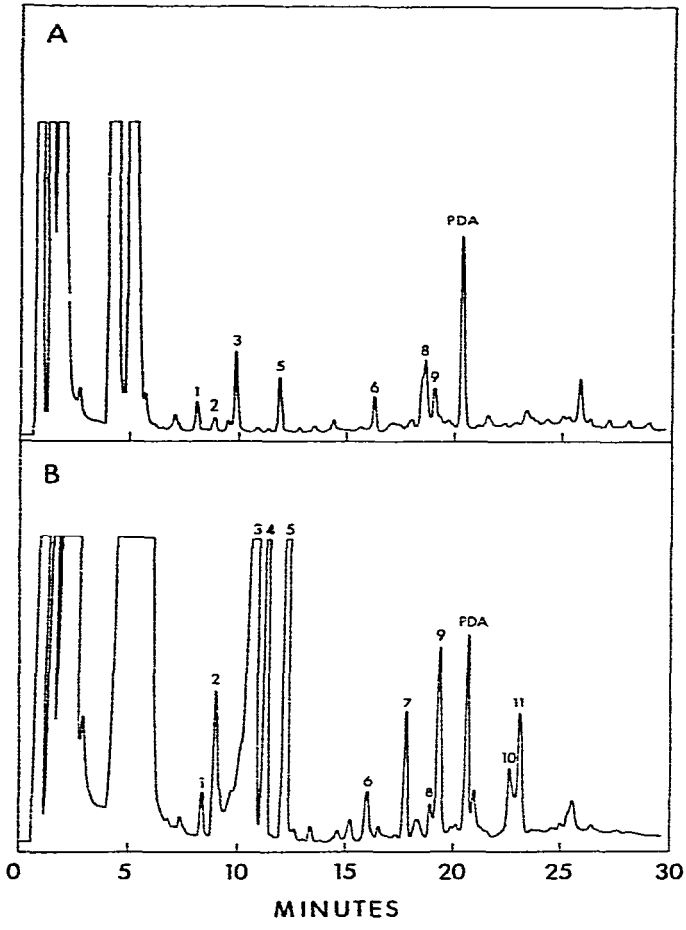

Fig. 10. Urinary organic acids from a patient with glyceric aciduria. A, ethyl acetate-extraction method; B, cation-exchange–lyophilization method. A 10% OV-1 column was used for analysis. Peaks: 1 = oxalic; 2 = 3-hydroxyisobutyric; 3 = urea; 4 = phosphoric; 5 = glyceric; 6 = *p*-hydroxyphenylacetic; 7 = unknown; 8 = hippuric; 9 = citric; 10 = unknown; 11 = unknown. PDA (1 mg/mg of creatinine) added as an internal standard.

urines by GC alone and readily identified 21 patients with well-defined organic acidurias. These include methylmalonic acidemia, isovaleric acidemia, multiple carboxylase deficiency[7,36], propionic acidemia, maple syrup urine disease, tyrosinemia[34], glyceroluria[43] and glyceric aciduria[47,48]. All of the identifications were subsequently confirmed by MS. In our experience, the diagnosis by GC alone was much easier and sometimes more convincing than with GC–MS alone, especially in a disease such as propionic acidemia, in which numerous abnormal metabolites are excreted. In these cases, interpretation of mass spectra is often obscured by overlapping peaks. For laboratories not equipped with a GC–MS, this list provides sufficient information for diagnosis of the welldefined organic acidurias listed in Table II, because we have included most of the abnormal metabolites characteristic of these diseases. For labo-

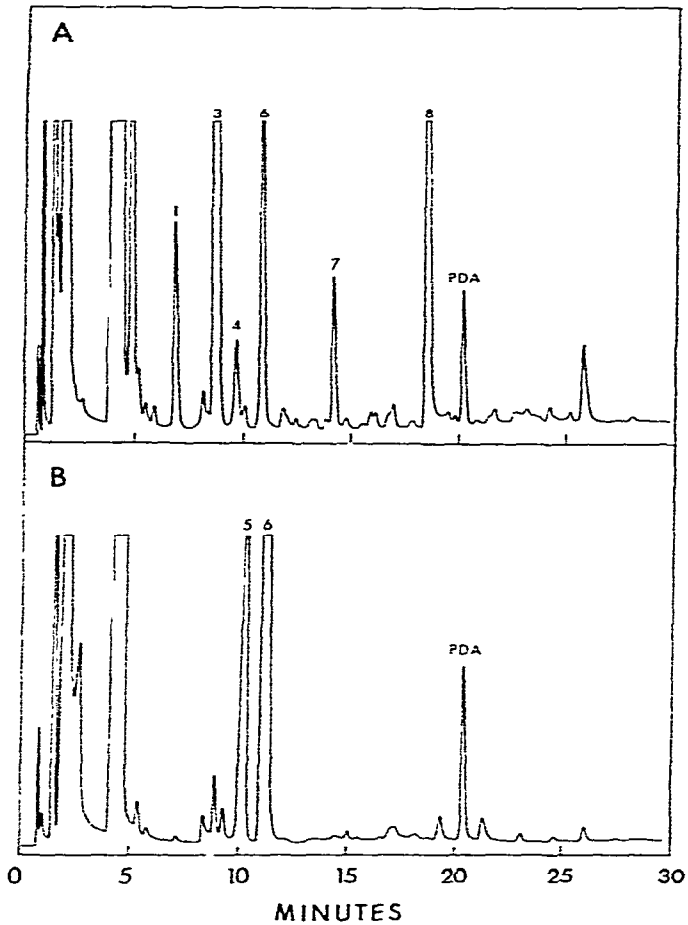

Fig. 11. Urinary organic acids from a patient with glyceroluria. A, ethyl acetate-extraction method; B, mixed-bed ion-exchange–lyophilization method. A 10% OV-1 column was used for analysis. Peaks: 1 = lactic; 2 = 2-hydroxybutyric; 3 = 3-hydroxybutyric; 4 = 3-hydroxyisovaleric; 5 = urea; 6 = glycerol; 7 = adipic; 8 = hippuric. PDA (1 mg/mg of creatinine) added as an internal standard.

ratories with a GC–MS system, use of the present MU tabulation makes it much easier to interpret mass spectra of complex urinary metabolites, by indicating possible overlapping peaks.

ACKNOWLEDGEMENTS

We thank Ms. Agnes West-Dull, Ms. Alda Saunders and Mr. Theodore Lynn for technical assistance. This study was supported in part by a NIH grant (AM 17453) and a grant from the March of Dimes-Birth Defect Foundation (1978).

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