Journal of Chromatography, 341 (1985) 1—10
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2529

IDENTIFICATION OF METABOLITES DIAGNOSTIC FOR ORGANIC ACIDURIAS BY SIMULTANEOUS DUAL-COLUMN CAPILLARY GAS CHROMATOGRAPHY

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(First received August 29th, 1984; revised manuscript received January 7th, 1985)

SUMMARY

To offer a procedure with increased resolution compared to packed-column gas chromatography, we developed a dual-capillary method of gas chromatography for diagnosing organic acidurias. We derivatized and injected organic acids repeatedly on two different bonded phase columns (DB-1, DB-1701) to establish a table of methylene units. Compounds in urine specimens were identified by their characteristic pair of methylene units. With this method, we are able to identify 120 metabolites in urine. Accordingly, the procedure provides a cost-effective alternative to routine gas chromatography—mass spectrometry.

INTRODUCTION

Organic acidurias are genetic disorders generally caused by the diminished activity or complete absence of specific enzymes involved in the metabolism of amino acids. These inborn errors of metabolism result in the excretion of specific organic acids in the urine at abnormal concentrations. Some examples of common organic acidurias are isovaleric acidemia, methylmalonic aciduria, maple syrup urine disease, and propionic acidemia [1]. Although clinical symptoms may be present, a definitive diagnosis of an organic aciduria has usually required identification of specific metabolites by gas chromatography—mass spectrometry (GC—MS). In recent years, however, gains in diagnosing organic acidurias have been made through advances in GC alone [2—5].

In the late 1960s, Horning et al. [2] investigated the separation of urinary acids and other compounds of biological interest on OV-1 and OV-17. They found that the thermostability of these packing materials increased the range of

0378-4347/85/$03.30

compounds that could be studied by GC. Methylene unit (MU) values for trimethylsilyl (TMS) derivatives of many biochemically important organic acids were later reported for OV-1 and OV-17 by Butts [3]. The two columns gave a characteristic pair of MU values for a particular compound. Tanaka and coworkers [4, 5] employed the difference in the polarity of these columns to diagnose organic acidurias without the use of GC—MS. The identity of an organic acid was determined by comparing its MU value on OV-1 and OV-17 with the MU values of known organic acids.

Since urine contains as many as several hundred compounds, it is not always possible to adequately resolve and identify the important constituents without GC—MS. Capillary column GC offers an advantage over packed-column GC. The increased number of theoretical plates available with capillary GC greatly increases the resolution of complex matrices, thus increasing the reliability of identification based on MU values. Recently, capillary columns have been employed to separate organic acids. In a limited study, De Jong [6] compared the MU values of 30 normally occurring organic acids separated on a capillary column of OV-1701 to the MU values reported earlier for a packed column of OV-17 [4].

We report here a dual-column GC method employing bonded-phase capillary columns of different polarities to screen for organic acidurias. From repeated injections of TMS derivatives, we prepared a table of reference MU values to identify 120 organic acids of biological interest.

MATERIALS AND METHODS

Reagents

All organic acids and hydrocarbon standards were purchased as free acids or stable salts from commercial vendors. Organic acids, not available commercially, were detected in selected urine samples and identified by GC—MS. Hydroxylamine hydrochloride was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). TriSil BSA Formula P was obtained from Pierce (Rockford, IL, U.S.A.). All gases were commercial grade and passed through moisture and oxygen traps (for helium carrier gas only), while all solvents were pesticide grade.

Sample preparation

Samples were prepared by a procedure essentially the same as the procedure of Tanaka et al. [5]. TMS derivatives were formed by the addition of 100 μl of TriSil BSA Formula P to the dried sample and heating at 60°C for 30 min.

Instrumentation

Fused-silica capillary columns, Durabond-1 (DB-1) and Durabond-1701 (DB-1701) (J & W Scientific, Rancho Cordova, CA, U.S.A.), were of 0.25 μm film thickness and 30 m × 0.25 mm I.D. A Hewlett-Packard 5880A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) was equipped with dual flame ionization detectors, electrometers, and data terminals. An HP 7671A autosampler injected 1 μl of sample. The inlet liner was packed with 10% OV-1 on Chromosorb W HP 80—100 mesh (Alltech Assoc., Deerfield, IL, U.S.A.) and

split the injected sample 1:50 (column flow/split inlet flow). Carrier gas flow-rate (helium) at 50°C was set at 1 ml/min. Make-up gas (helium) and hydrogen flow-rates were each set at 30 ml/min. Air flow-rate at the flame-ionization detector was set at 300 ml/min. After a 1-min delay, the oven temperature was programmed from 50°C to 270°C at 8°C/min. The final temperature was held for 5 min followed by a post-run oven temperature of 280°C for 10 min. The injector and detectors were maintained at 250°C and 330°C, respectively. This configuration permitted automated simultaneous analysis of a series of samples on two capillary columns.

The packed columns were 10% OV-1 on 100—120 mesh Gas-Chrom Q (Alltech Assoc.) and 10% OV-17 (10% SP-2250 on 100—120 mesh Supelcoport, Supelco, Bellefonte, PA, U.S.A.). Both silane-treated glass columns (1.88 m × 2 mm I.D.) were installed in a Model 3700 gas chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with dual flame ionization detectors and a single electrometer. After a 1-min delay, the oven temperature was programmed at 8°C/min from 70°C to 290°C, while the injector and detector were maintained at 250°C and 300°C, respectively. The carrier gas (helium) was set at a flow-rate of 30 ml/min. A 1-μl aliquot of the derivatized sample was injected manually.

Formulation of the methylene unit table

Oximes of the keto acid standards were formed as described [5]. The internal standard solution (pentadecanoic and capric acid 1 mg/ml in ethyl acetate) was added to each organic acid standard prior to derivatization. A sufficient amount of each organic acid was used to enable 1/4 to full scale deflection upon derivatization and injection. An on-line computer calculated methylene units by linear interpolation based on the retention times of even and odd hydrocarbon standards (C_{10} to C_{25}) for the DB-1 and DB-1701 columns, respectively.

Gas chromatography—mass spectrometry

Unknown peaks present in urine samples were identified with an HP 5985B gas chromatograph—mass spectrometer. The system consisted of an HP 5840 GC and HP 1000 minicomputer and disk drives containing a user-built library of organic acids. Methylene units were computed by a user-written program.

RESULTS

Methylene unit table

The MU values for 120 compounds chromatographed on DB-1 and DB-1701 are listed in Table I. These values represent the mean of three to ten injections on two columns of each bonded phase. There was no significant difference in MU values between the two different columns of the same bonded phase. The small standard deviation of the multiple injections permitted MU windows for identification of ± 0.03. In contrast, the packed-column method in our laboratory required MU windows of ± 0.06. For compounds not commercially available, patient urine specimens were prepared and the MU values of metabolites were computed after identification by GC—MS. These compounds are denoted by an asterisk.

TABLE I

METHYLENE UNITS OF VARIOUS COMPOUNDS ON DB-1 AND DB-1701 CAPILLARY COLUMNS

Compound	DB-1	DB-1701	Compound	DB-1	DB-1701
Lactic acid	10.61	11.16	2-Keto-3-methylvaleric acid (D-oxime)	12.88	13.41
Hexanoic acid	10.69	11.24	Glycerol	12.92	12.61
Glycolic acid	10.73	11.45	Maleic acid	12.96	14.16
Oxalic acid	11.19	12.38	Succinic acid	13.08	14.10
Glyoxylic acid (oxime)	11.20	11.91	Thymol	13.12	13.73
o-Cresol	11.27	11.97	Methylsuccinic acid	13.23	14.18
2-Hydroxybutyric acid	11.29	11.80	Propionylglycine* (I)	13.30	15.83
p-Cresol	11.42	12.16	2-Ketocaproic acid (oxime)	13.31	13.80
3-Hydroxypropionic acid	11.42	12.07	Glyceric acid	13.41	13.89
Pyruvic acid (oxime)	11.48	12.04	Fumaric acid*	13.44	14.38
Dipropylacetic acid	11.56	12.02	Acetylglycine (II)	13.59	15.17
3-Hydroxybutyric acid	11.64	12.15	Glutaric acid	13.91	15.02
3-Hydroxyisobutyric acid*	11.70	12.16	3-Methylglutaric acid	14.16	15.17
2-Hydroxyisovaleric acid	11.71	12.13	Capric acid	14.51	15.09
2-Ketobutyric acid (oxime)	11.88	12.40	Isovalerylglycine (II) (oxime)	14.58	16.26
2-Hydroxyvaleric acid	11.99	12.49	Mandelic acid	14.73	15.77
2-Methyl-3-hydroxy-butyric acid*	12.00	12.48	Adipic acid	14.97	16.12
Acetoacetic acid	12.00	13.10	Malic acid	14.98	15.79
2-Ketoisovaleric acid (oxime)	12.09	12.58	*trans*-3-Dihydro-muconic acid	15.03	16.31
Methylmalonic acid	12.09	13.02	Isovalerylglycine (I) oxime	15.05	17.03
3-Hydroxyisovaleric acid*	12.16	12.58	Salicylic acid	15.07	16.17
Urea	12.30	14.65	4-Phenylbutyric acid	15.10	16.40
2-Ethylhydracrylic acid	12.31	12.81	Pyroglutamic acid	15.10	17.05
Benzoic acid	12.33	13.45	2-Keto-4-methiolbutyric acid (oxime)	15.12	16.19
3-Hydroxyvaleric acid*	12.38	12.86	2-Phenylglycine	15.20	16.11
2-Hydroxyisocaproic acid	12.38	12.84	*trans*-Cinnamic acid	15.26	16.78
Acetoacetic acid	12.40	13.10	3-Methyladipic acid	15.28	16.40
2-Hydroxy-3-methyl-valeric acid	12.47	12.88	Tiglylglycine* (I)	15.41	17.93
2-Ketovaleric acid (oxime)	12.52	13.05	Undecanoic acid	15.49	16.10
Acetylglycine (II)	12.53	15.06	*cis*-Oxalacetic acid	15.53	16.21
Glyoxylic acid	12.59	13.09	*o*-Hydroxyphenyl-acetic acid	15.60	16.81
Octanoic acid	12.63	13.19	*m*-Hydroxybenzoic acid	15.60	16.70
2-Keto-3-methylvaleric acid (L-oxime)	12.70	13.13	2-Hydroxyglutaric acid	15.76	16.66
Phosphoric acid*	12.73	13.90	Phenyllactic acid	15.83	16.89
Ethylmalonic acid	12.77	13.66	Pimelic acid	15.92	17.12
2-Hydroxycaproic acid	12.82	13.30	*m*-Hydroxyphenyl-acetic acid	15.96	17.22
Phenylacetic acid	12.82	14.06	3-Hydroxy-3-methyl-glutaric acid	16.06	16.85
2-Ketoisocaproic acid (oxime)	12.86	13.31	*p*-Hydroxybenzoic acid	16.22	17.43
			2-Ketoglutaric acid (oxime)	16.27	17.20
			p-Hydroxyphenylacetic acid	16.27	17.57

TABLE I (continued)

Compound	DB-1	DB-1701	Compound	DB-1	DB-1701
Phenylpyruvic acid (oxime)	16.30	17.43	*p*-Hydroxyphenylglycine	18.75	19.73
Dodecanoic acid	16.47	17.06	3-Indoleacetic acid	18.80	22.42
Tartaric acid	16.55	17.21	Decanedioic acid	18.84	20.09
Octanedioic acid	16.87	18.10	*p*-Hydroxyphenyllactic acid	19.06	20.10
2-Ketoadipic acid (oxime)	17.07	18.08	*p*-Hydroxyphenylpyruvic acid (oxime)	19.34	20.44
trans-Aconitic acid	17.44	18.72	3,4-Dihydroxymandelic acid	19.36	20.19
cis-Aconitic acid	17.44	18.70	Pentadecanoic acid	19.43	20.04
Orotic acid	17.44	18.47	*o*-Hydroxyhuppuric acid (III)	19.52	21.03
Tridecanoic acid	17.48	18.09	Palmitic acid	20.40	21.02
4-Hydroxy-3-methoxybenzoic acid	17.54	18.94	*o*-Hydroxyhippuric acid (II)	20.50	23.26
4-Hydroxy-3-methoxyphenylacetic acid	17.59	19.12	*trans*-2-Dodecenedioic acid	21.23	22.79
Gentisic acid	17.80	18.82	5-Hydroxyindole-3-acetic acid	21.99	24.01
Nonanedioic acid	17.82	19.08	Linoleic acid	22.01	22.95
Hippuric acid (II)	17.92	19.80	Linolenic acid	22.05	23.09
Hippuric acid (I)	18.05	21.34	Palmitoleic acid	20.16	20.92
2,4-Dihydroxybenzoic acid	18.18	19.21	Oleic acid	22.08	22.85
Homogentisic acid	18.34	19.47	Stearic acid	22.39	23.07
Citric acid	18.35	19.17	Tetradecanedioic acid	22.69	23.99
Isocitric acid*	18.35	19.27	Arachidonic acid	23.59	24.70
Tetradecanoic acid	18.40	19.03	Hexadecanedioic acid	24.77	26.09
Methylcitric acid* (2*R*,3*S*)	18.58	19.25			
Methylcitric acid* (2*S*,3*S*)	18.66	19.34			

*Compounds identified by GC—MS in patient specimens of known organic acidurias.

Column efficiency

To determine if capillary columns provided greater resolution, we calculated several chromatographic parameters for the capillary and packed columns (Table II). The separation number (*TZ*, Trennzahl value) is an approximation of the number of compounds that may be resolved between two consecutive *n*-alkanes of known peak width at one-half peak height. Four to five compounds can be resolved on DB-1 or DB-1701 for each compound resolved on OV-1 or OV-17. Capillary columns had significantly more theoretical plates per unit length and total theoretical plates than the packed columns.

Analysis of selected urine specimens from patients with organic acidurias

We further demonstrated the increased resolution of capillary GC by analyzing some representative patient samples. To verify the value of the MU method for identification, we compared the identities of compounds provided by this procedure with those provided by GC—MS. In the organic acidurias studied, the GC—MS confirmed the identities provided by the MU method.

Propionic acidemia

In propionic acidemia, the absence of propionyl coenzyme A (CoA)

TABLE II

COMPARISON OF COLUMN EFFICIENCY

$$TZ = \frac{t_{R(x+1)} - t_{R(x)}}{W_{h(x+1)} + W_{h(x)}} - 1 \qquad N = 5.54(t_R/W_h)^2$$

$t_{R(x+1)}$ = Retention time of the hydrocarbon peak (C_{13} or C_{18}); $t_{R(x)}$ = retention time of hydrocarbon peak (C_{12} or C_{17}); $W_{h(x+1)}$ = width of hydrocarbon peak at half height (C_{13} or C_{18}); $W_{h(x)}$ = width of hydrocarbon peak at half height (C_{12} or C_{17}); N = the number of plates.

	OV-1	OV-17	DB-1	DB-1701
TZ (C_{12}—C_{13})	6.4	5.9	31.6	30.3
TZ (C_{17}—C_{18})	4.5	4.5	20.5	20.8
Total plates (C_{18})	4330	3670	109,600	110,700
Plates per m (C_{18})	2405	2040	3650	3690

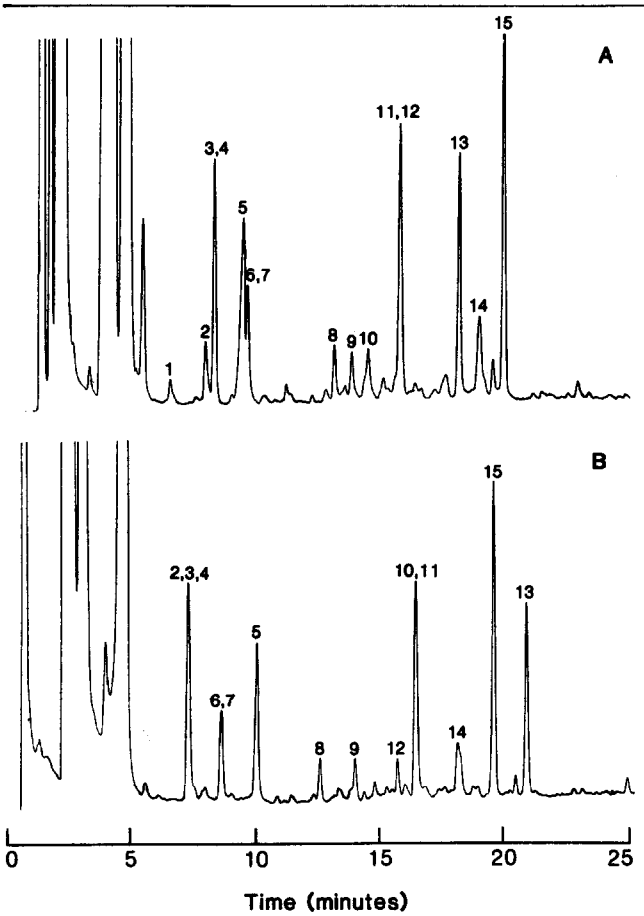

Fig. 1. OV-1 (A) and OV-17 (B) urinary organic acid profiles of a patient with propionic acidemia. Compounds identified are: 1 = lactic acid, 2 = 3-hydroxypropionic acid, 3 = 3-hydroxybutyric acid, 4 = 3-hydroxyisobutyric acid, 5 = urea, 6 = 3-hydroxyvaleric acid, 7 = 2-ethylhydracrylic acid, 8 = capric acid, 9 = adipic acid, 10 = 3-methylcrotonylglycine (I), 11 = *p*-hydroxyphenylacetic acid, 12 = 2-ketoglutaric acid (oxime), 13 = hippuric acid, 14 = methylcitric acid, 15 = pentadecanoic acid. The MU table from Tanaka et al. [4] was used for compound identification.

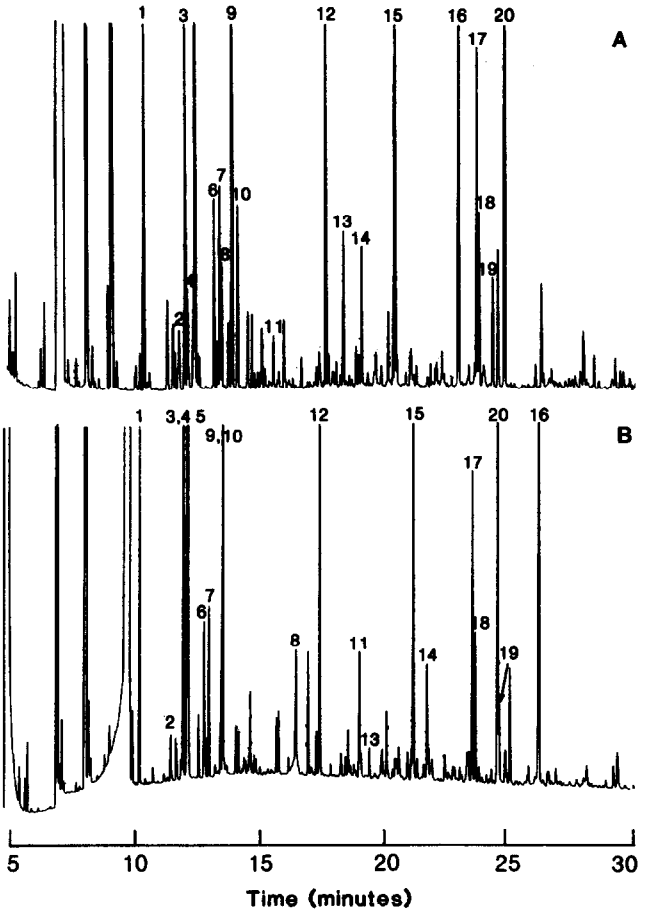

Fig. 2. DB-1 (A) and DB -1701 (B) urinary organic acid profiles of a patient with propionic acidemia. Compounds identified are: 1 = lactic acid, 2 = 2-hydroxybutyric acid, 3 = 3-hydroxypropionic acid, 4 = pyruvic acid (oxime), 5 = 3-hydroxybutyric acid, 6 = 2-hydroxyvaleric acid, 7 = 2-ketoisovaleric acid (oxime), 8 = urea, 9 = 2-hydroxyisocaproic acid, 10 = 2-hydroxy-3-methylvaleric acid, 11 = propionylglycine (I), 12 = capric acid, 13 = adipic acid, 14 = tiglylglycine (I), 15 = *p*-hydroxyphenylacetic acid, 16 = hippuric acid (I), 17 = methylcitric acid (2*R*,3*S*), 18 = methylcitric acid (2*S*,3*S*), 19 = *p*-hydroxyphenyllactic acid, 20 = pentadecanoic acid.

carboxylase prevents the conversion of propionyl CoA to methylmalonyl CoA in leucine and isoleucine metabolism. As a result, several metabolites accumulate in the urine which are useful for diagnosing the disorder. A patient with propionic acidemia was analyzed by the packed-column (Fig. 1) and capillary column (Fig. 2) procedures. As seen in the packed-column chromatograms, several peaks had shoulders indicating the presence of additional compounds. These compounds could not be identified by GC alone because of the peak overlap. Using capillary columns, however, these peaks were well resolved, enabling compound identification.

For example, methylcitrate (the most important metabolite diagnostic for propionic acidemia), was not readily identified by packed column GC and con-

firmation by GC—MS was necessary. The poorly resolved peak on OV-17 did not permit identification of methylcitrate. Identification was not a problem with capillary GC. In fact, the diastereomers of methylcitrate were resolved. In addition, secondary metabolites [propionylglycine (I), tiglyglycine] were identified by capillary GC but not by packed GC.

Maple syrup urine disease

Maple syrup urine disease, also known as branched-chain keto aciduria, results from an enzymatic defect in the degradation of the branched-chain amino acids: leucine, isoleucine, and valine. Fig. 3 shows the capillary chromatograms of a patient with maple syrup urine disease; the presence of lactic, pyruvic, and

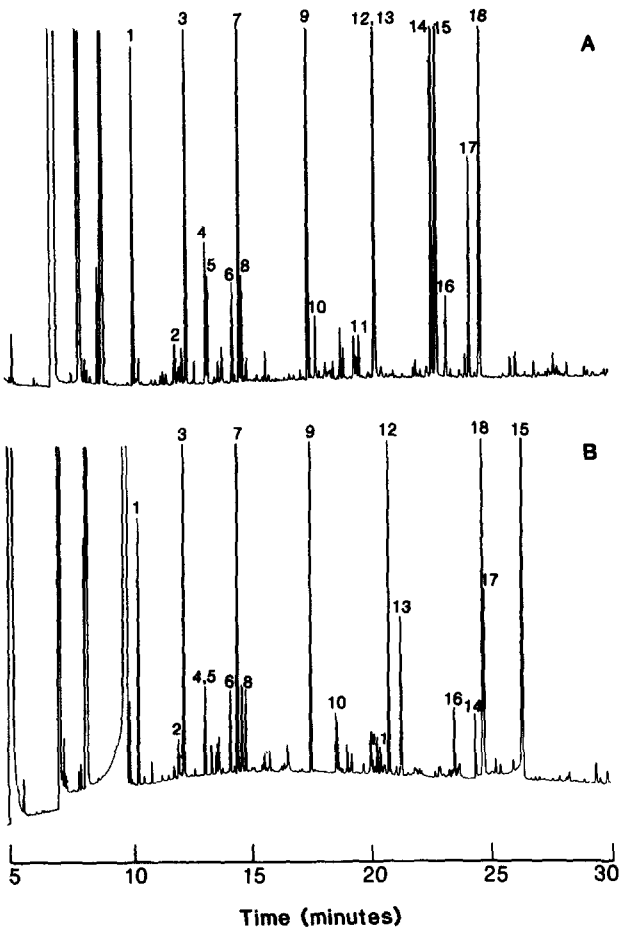

Fig. 3. DB-1 (A) and DB-1701 (B) urinary organic acid profiles of a patient with maple syrup urine disease. Compounds identified are: 1 = lactic acid, 2 = pyruvic acid (oxime), 3 = 2-hydroxyisovaleric acid, 3-hydroxybutyric acid, 4 = 2-ketoisovaleric acid (oxime), 5 = 3-hydroxyisovaleric acid, 6 = 2-keto-3-methylvaleric acid (L-oxime), 7 = 2-ketoisocaproic acid (oxime), 8 = 2-keto-3-methylvaleric acid (D-oxime), 9 = capric acid, 10 = mandelic acid, 11 = phenyllactic acid, 12 = 2-ketoglutaric acid (oxime), 13 = *p*-hydroxyphenylacetic acid, 14 = hippuric acid (II), 15 = hippuric acid (I), 16 = citric acid, 17 = *p*-hydroxyphenyllactic acid, 18 = pentadecanoic acid.

3-hydroxyisovaleric acids also indicates ketoacidosis. Of particular interest is the resolution of 2-ketoisovaleric acid (oxime) and 3-hydroxyisovaleric acid on the DB-1 and the resolution of 2-ketoisocaproic acid (oxime) and 2-keto-3-methylvaleric acid (oxime) on DB-1701. Neither pair of compounds were resolved on the similar packed column phases (not shown).

Isovaleric acidemia

In isovaleric acidemia, a deficiency of isovaleryl-CoA dehydrogenase prevents the conversion of isovaleryl-CoA to methylcrotonyl-CoA. In Fig. 4, the chromatograms from an isovaleric acidemia patient exhibit baseline resolution of the mono-, and di-TMS isovalerylglycines from capric acid (an internal standard). When large amounts of isovalerylglycine were present in the specimens we were unable to resolve isovalerylglycine from the capric acid on packed columns but we were able to on the capillary columns.

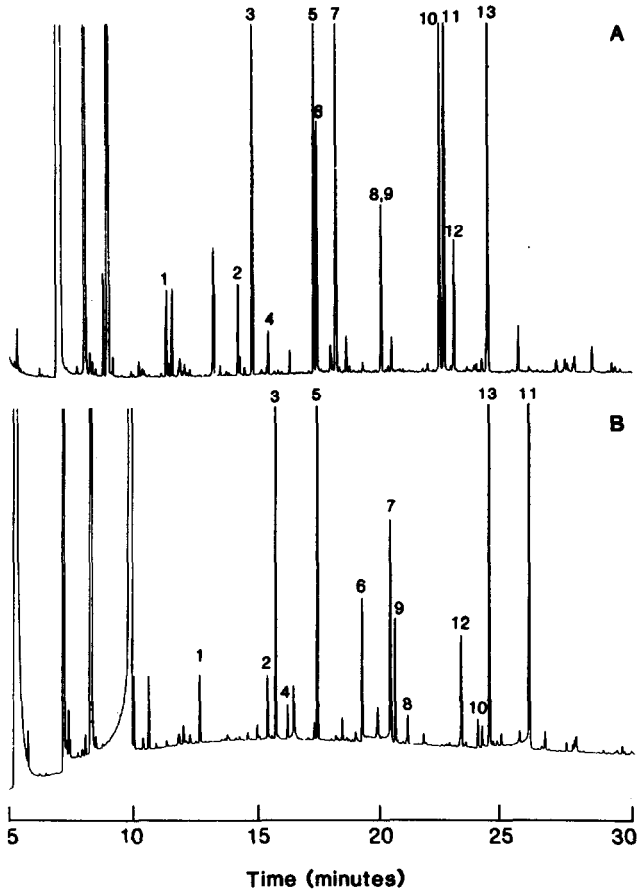

Fig. 4. DB-1 (A) and DB-1701 (B) urinary organic acid profiles of a patient with isovaleric acidemia. Compounds identified are: 1 = oxalic acid, 2 = phosphoric acid, 3 = succinic acid, 4 = fumaric acid, 5 = capric acid, 6 = isovalerylglycine (II), 7 = isovalerylglycine (I), 8 = *p*-hydroxyphenylacetic acid, 9 = 2-ketoglutaric acid (oxime), 10 = hippuric acid (II), 11 = hippuric acid (I), 12 = citric acid, 13 = pentadecanoic acid.

DISCUSSION

In recent years, GC has gained increasing acceptance as an analytical tool for diagnosing organic acidurias [1]. In these inherited disorders, abnormal amounts of specific metabolites accumulate in the urine. Identification of these compounds has routinely required that the gas chromatograph be coupled to a mass spectrometer. In a recent study, Tanaka and coworkers [4, 5] were able to diagnose 23 inherited disorders using GC alone. In their procedure, specimens were chromatographed on two packed columns of different stationary phases. Organic acids were identified according to a specific pair of MU values from the two columns.

We have successfully employed the procedure of Tanaka and coworkers [4, 5] in our laboratory. It has been necessary on a portion of the specimens, however, to confirm identities of poorly resolved compounds by GC—MS. Since publication of this procedure in 1980, bonded-phase fused-silica capillary columns with extraordinary numbers of theoretical plates have been introduced. Bonded phases have also shown a great degree of thermal stability. Considering the problems of resolution encountered with packed columns, we developed a dual-column GC method based on bonded-phase capillary columns.

Using this dual-column capillary method, we established an MU table and found less variability in the MU values of standards such that better identifications were possible. The increased resolution and sensitivity of the capillary columns permitted excellent identification of primary as well as secondary metabolites important in organic acidurias.

These results indicate a significant improvement, compared to packed-column methods, in the separation of constituents from urine when subjected to GC on bonded-phase capillary columns. In our laboratory, this new method has now replaced the procedure using packed columns. We have developed a reliable and accurate method for the diagnosis of organic acidurias and for all practical purposes GC—MS confirmation is not needed for routine study of organic acidurias.

ACKNOWLEDGEMENTS

We gratefully acknowledge the assistance of Kelly Fregien for performing the GC—MS studies, and the help of Kerry Schwichtenberg and Siri Kavanaugh for the data analysis for preparation of the methylene unit table.

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