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Note

Urinary organic acids: retention indices on two capillary gas chromatography columns

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Since organic acidurias are disorders whose clinical features are variable and not specific, the diagnosis of these inborn errors of metabolism requires identification of characteristic urinary metabolites, mainly carboxylic acids [1].

The high cost and expertise involved in gas chromatography–mass spectrometry (GC–MS), which is the technique of choice, limit the number of laboratories that can provide reliable identification of organic acids. However, detection of abnormal urinary profiles and recognition of the well defined organic acidurias have been claimed by some authors to be feasible using gas chromatography (GC) alone [2–4]. Nevertheless, the complexity of the urinary organic acid fraction demands to have recourse (a) for each peak, to a characteristic pair of retention indices, expressed in methylene units (MU), obtained on two columns of different polarity, in order to increase the reliability of the identification [3,5], and (b) to the high resolution of capillary GC [4,6].

The aim of this paper is to assess MU values and their standard deviations for 64 clinically important organic acids and three acylglycines, and to study briefly some possible causes of MU variations.

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EXPERIMENTAL

Instrumentation

The analyses were carried out on two Intersmat IGC 120 DFL gas chromatographs (Intersmat Instruments, Chelles les Coudreaux, France) equipped with a flame ionization detector and a SGE OCI-3 manual on-column injector (Scientific Glass Engineering, Ringwood, Australia). The chromatographs were coupled to an Enica 21 dual-channel integrator (Delsi Instruments, Suresnes, France). Two WCOT fused-silica capillary columns (25 m×0.32 mm I.D.) with chemically bonded phases were used: a CP Sil 5 CB (polydimethylsiloxane), 0.43 μm film thickness, from Chrompack International (Middelburg, The Netherlands) and an RSL 300 (polyphenylmethylsiloxane), 0.3 μm film thickness, from RSL Alltech-Europe (Eke, Belgium).

Standards and reagents

The organic acid standards and acetylglycine were obtained from Sigma (St. Louis, MO, U.S.A.), Serva (Heidelberg, F.R.G.) or Janssen (Beerse, Belgium). The internal standard (2-phenylbutyric acid) was from Janssen.

Propionylglycine and isovalerylglycine were kindly synthesized in our Institute by Dr. J. Hoyois according to Herbst and Shemin [7] and Bondi and Eissler [8], respectively. Hydroxylamine hydrochloride was purchased from Serva.

The silylating reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Pierce Europe (Oud-Beijerland, The Netherlands). The hydrocarbon standards (even numbers from C_{10} to C_{24}) were provided by Supelco (Bellefonte, PA, U.S.A.). All other reagents or chemicals were purchased from E. Merck (Darmstadt, F.R.G.) and were of analytical grade.

Extraction and derivatization

We adopted with some modifications the method described by Jakobs et al. [9]. The keto acids were first converted into oxime derivatives by incubating, at 60°C for 30 min, 1 ml of their aqueous solution (corresponding to 25–100 μg of free acid) with 1 ml of a 25 g/l aqueous hydroxylamine hydrochloride solution, the mixture being adjusted to pH 14.0 with 7.5 *M* sodium hydroxide. After cooling, the solution was acidified to pH 1.0 using 6 *M* hydrochloric acid. For the other compounds, 2 ml of aqueous solution (also corresponding to 25–100 μg of free acid) were directly acidified to pH 1.0.

To these acidified solutions, 25 μl of a 1 g/l aqueous internal standard solution and 1 ml of a saturated sodium chloride solution were added. The samples were then extracted three times for 5 min on a rotary mixer with 6 ml of ethyl acetate. After centrifugation the combined organic phases were dried on 1 g of anhydrous sodium sulphate for 5 min and evaporated under nitrogen at

50 °C in small Silli-vials using a Silli-Vap evaporator (Pierce Europe). The dry residue was taken up in 25 μl of chloroform and 100 μl of BSTFA + 1% TMCS, and the capped Silli-vials were heated for 30 min at 60 °C on a Silli-Therm heating module (Pierce Europe). A 0.2-μl sample of each final solution was injected.

Gas chromatography

GC was performed with a temperature programme from 60 to 280 °C at 6 °C/min, after an initial delay of 30 s at 60 °C. The temperature was finally maintained at 280 °C for 5 min. The detector temperature was 300 °C. Nitrogen at a linear velocity of 30 cm/s was used as carrier gas.

In order to determine the MU values, an even hydrocarbon standard solution (C_{10} to C_{24}) in hexane was co-injected with the final solutions. Organic acids were injected once a day for four to fifteen days.

RESULTS AND DISCUSSION

Table I lists the MU values and the between-day standard deviations we observed for the 67 compounds on the polydimethylsiloxane and the polyphenylmethylsiloxane phases.

When the hydrocarbon mixture is co-injected, the MU values are remarkably precise, in spite of the fact that the oven temperature is manually ordered and that the chromatographs used are not sophisticated. Indeed, the standard deviations range from 0.001 to 0.014, corresponding to absolute retention times of 0.13 to 1.78 s. Our MU values are slightly different from those previously quoted for polydimethylsiloxane phases [1,3,4].

The second phase we used was a polyphenylmethylsiloxane coating that differs from the OV-1701 and DB-1701 phases used by others [4,6] in the absence of cyanopropyl groups. This last type of phase (in our case a CP Sil 19 CB from Chrompack) gave, in our hands, less satisfactory results than the RSL 300 with respect to peak tailing.

As expected, we observed a slight but significant alteration of the selectivity when programming the oven temperature at different rates. For instance, methylsuccinic acid gives MU values of 13.234, 13.221 and 13.210 at 4, 6 and 8 °C/min, respectively. We found a similar alteration on changing the nitrogen linear velocity from 30 to 15 cm/s. The extent of these influences, however, is such that the inherent imprecisions of oven temperature programming and carrier gas flow regulation do not modify the MU values obtained when working at a given rate and a given gas velocity. Finally, we can point out that on-column injection gives satisfactory results for this purpose.

Examples of chromatograms obtained on CP Sil 5 CB are shown in Fig. 1.

In conclusion, owing to the high resolution of capillary GC and the excellent reproducibility of the MU values, tentative recognition of urinary organic acid

TABLE I

METHYLENE UNITS OF 64 ORGANIC ACIDS AND THREE ACYLGLYCINES

Compounds were analysed as TMS or oxime-TMS derivatives, on two capillary columns.

Peak No.	Compound	CP Sil 5 CB		RSL 300	
		Mean[a]	S.D.[a]	Mean[a]	S.D.[a]
1	Lactic acid	10.59	0.002	10.92	0.004
2	Caproic acid (hexanoic acid)	10.63	0.002	11.13	0.003
3	Glycolic acid	10.70	0.002	11.20	0.004
4	Oxalic acid	11.16	0.005	12.30	0.006
5	Glyoxylic acid (oxime)	11.21	0.002	11.94	0.006
6	2-Hydroxybutyric acid	11.29	0.003	11.62	0.004
7	Pyruvic acid (oxime)	11.47	0.003	12.14	0.004
8	3-Hydroxybutyric acid	11.60	0.003	12.00	—[b]
9	2-Hydroxyisovaleric acid	11.69	0.004	11.94	0.005
10	2-Ketobutyric acid (oxime)	11.87	0.004	12.52	0.004
11	Malonic acid	11.96	0.004	12.89	0.004
12	Methylmalonic acid	12.10	0.005	12.88	0.002
13	2-Ketoisovaleric acid (oxime)	12.14	0.005	12.79	0.003
14	Benzoic acid	12.30	0.003	13.54	0.009
15	2-Hydroxyisocaproic acid	12.41	0.004	12.71	0.004
16	Acetylglycine	12.53	0.002	14.56	0.005
17	Caprylic acid (octanoic acid)	12.58	0.004	13.12	0.005
18a	2-Keto-3-methylvaleric acid (L-oxime)	12.73	0.004	13.32	0.002
19	Ethylmalonic acid	12.78	0.002	13.50	0 002
20	Phenylacetic acid	12.79	0.004	14.24	0.005
18b	2-Keto-3-methylvaleric acid (D-oxime)	12.88	0.004	13.47	0.003
21	2-Ketoisocaproic acid (oxime)	12.89	0.004	13.43	0.004
22	Maleic acid	12.92	0.006	14.15	0.003
23	Succinic acid	13.07	0.005	14.00	—[b]
24	Methylsuccinic acid	13.22	0.003	14.00	—[b]
25	Propionylglycine	13.33	0.003	15.31	0.005
26	Glyceric acid	13.40	0.002	13.73	0.003
27	Fumaric acid	13.45	0.004	13.96	0.003
28	2-Phenylbutyric acid (internal standard)	13.65	0.004	14.94	0.004
29	Glutaric acid	13.95	0.005	14.91	0.003
30	3-Methylglutaric acid	14.18	0.003	15.03	0.002
31	Capric acid (decanoic acid)	14.53	0.005	15.07	0.007
32	Isovalerylglycine	14.62	0.003	16.44	0.005
33	Mandelic acid	14.72	0.002	15.92	0.004
34	3-Hydroxy-3-methylglutaric acid	14.76	0.007	15.62	0.007
35	Malic acid	14.97	0.005	15.52	0.006
36	Adipic acid	14.98	0.007	15.94	0.007
37	*o*-Hydroxybenzoic acid (salicylic acid)	15.05	0.002	16.19	0.004
38	Pyroglutamic acid	15.09	0.004	16.69	0.005
39	*m*-Hydroxybenzoic acid	15.59	0 006	16.64	0.003
40	Phenyllactic acid	15.82	0.004	16.87	0.006

TABLE I (continued)

Peak No.	Compound	CP Sil 5 CB		RSL 300	
		Mean[a]	S.D.[a]	Mean[a]	S.D.[a]
41	Pimelic acid	15.94	0.006	16.96	0.005
42	*m*-Hydroxyphenylacetic acid	15.97	0.005	17.28	0.006
43	*p*-Hydroxybenzoic acid	16.21	0.004	17.17	0.004
44	*p*-Hydroxyphenylacetic acid	16.28	0.004	17.56	0.004
45	2-Ketoglutaric acid (oxime)	16.31	0.002	17.18	0.005
46	Phenylpyruvic acid (oxime)	16.32	0.001	17.77	0.004
47	Lauric acid (dodecanoic acid)	16.48	0.005	17.01	0.006
48	Suberic acid	16.91	0.006	17.91	0.005
49	2-Ketoadipic acid (oxime)	17.17	0.006	18.00	–[b]
50	*cis*-Aconitic acid	17.49	0.008	18.45	0.009
51	Vanillic acid	17.54	0.004	18.91	0.007
52	Homovanillic acid	17.58	0.005	19.24	0.005
53	Gentisic acid	17.79	0.004	18.66	0.006
54	*p*-Hydroxymandelic acid	17.82	0.006	18.82	0.006
55	Azelaic acid	17.88	0.006	18.90	0.007
56	Hippuric acid	18.00	–[b]	20 75	0.012
57	Citric acid	18.39	0.014	18.87	0.006
58	Myristic acid	18.44	0.003	18.96	0.006
59	3-Indoleacetic acid	18.71	0.004	22.08	0.008
60	Vanilmandelic acid	18.79	0.004	20.12	0.007
61	*p*-Hydroxyphenyllactic acid	19.08	0.004	19.89	0.004
62	3,4-Dihydroxymandelic acid	19.38	0.005	20.17	0.006
63	*p*-Hydroxyphenylpyruvic acid (oxime)	19.38	0.002	20.63	0.007
64	Palmitic acid	20.41	0.004	20.92	0 003
65	*o*-Hydroxyhippuric acid	20.48	0.010	22.76	0.006
66	5-Hydroxyindole-3-acetic acid	21.68	0.004	>24	
67	Oleic acid	22.09	0.005	22.82	0.007
68	Stearic acid	22.38	0.006	22.88	0.009

[a]Mean and between-day standard deviation ($n=4$–15).
[b]Peak not resolved from corresponding hydrocarbon.

profiles can effectively be done by the dual-column method. Moreover, a sophisticated gas chromatograph is not needed, thus allowing this screening procedure to be performed in a non-specialized laboratory.

However, it must be borne in mind that the complexity of the urinary organic acid fraction should invite the analyst to confirm the results of the screening procedure by GC–MS.

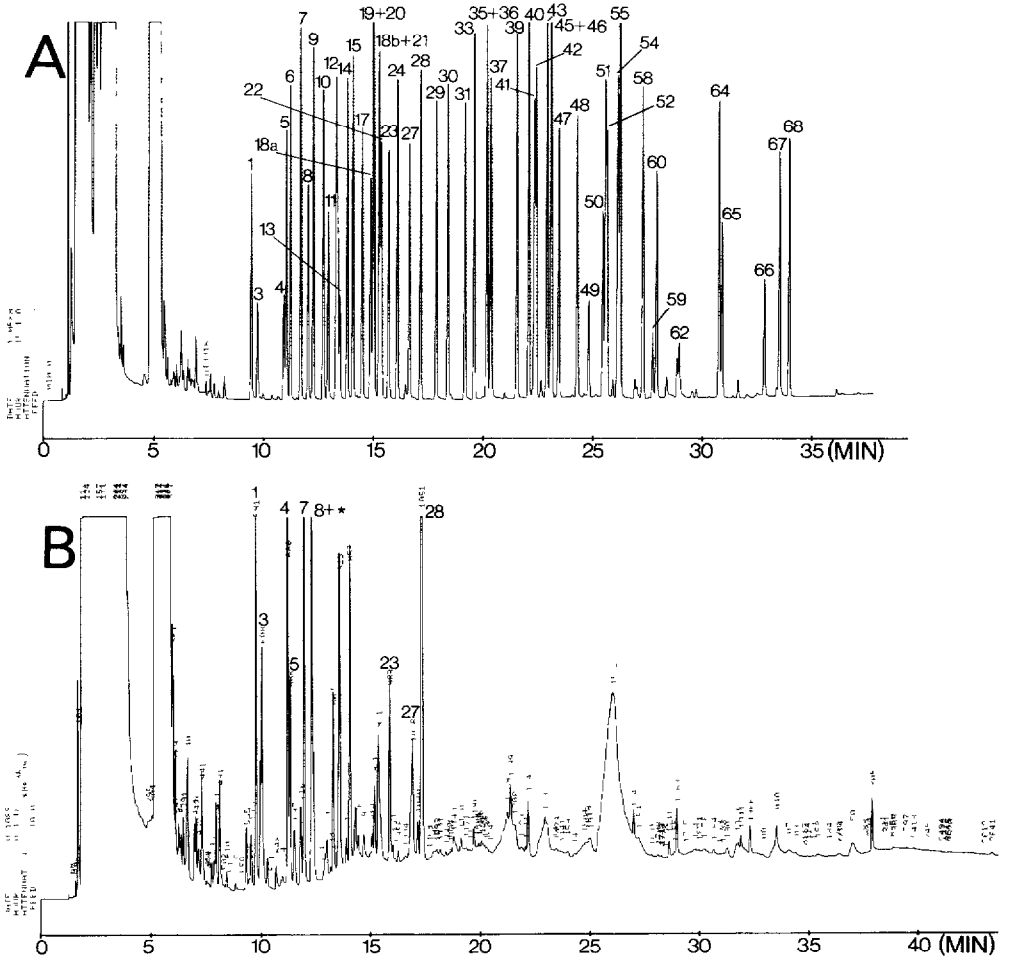

Fig. 1. Chromatograms obtained with a CP Sil 5 CB of TMS derivatives of organic acids extracted from (A) an aqueous standard solution and (B) a urine sample from a five-month-old child. For (A) 1 ml of aqueous solution containing 25–100 μg of each standard substance was treated and for (B) an aliquot of urine corresponding to 1 mg of creatinine was treated. Extraction, derivatization (with hydroxylamine hydrochloride and BSTFA + 1% TMCS) and operating conditions are as described in the text. Attenuations: (A) $64 \cdot 10^{-12}$ A/mV; (B) $32 \cdot 10^{-12}$ A/mV. Chromatograms shown were obtained without hydrocarbon co-injection. Peak assignments were made according to our MU values. Peak numbers refer to Table I; ⋆ = 3-hydroxyisobutyric acid.

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