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BASIC PROFILES OF ORGANIC ACIDS IN URINE

H.M. LIEBICH*

Medizinische Universitätsklinik, D-7400 Tübingen (F.R.G.)

and

C. FÖRST

Kernforschungszentrum, D-7500 Karlsruhe (F.R.G.)

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SUMMARY

Altogether 143 of the organic acids regularly occurring in urine of healthy individuals are identified as methyl esters by gas chromatography–mass spectrometry with respect to their complete chemical structures. They are classified as dicarboxylic acids, oxocarboxylic acids, hydroxycarboxylic acids, aromatic acids, furancarboxylic acids, nitrogen-containing acids and acid conjugates. By pre-fractionating the complex mixture of the total organic acids, peak overlap is minimized, and substances in low concentrations can also be detected and identified. The qualitative patterns of the urinary organic acids in the fractions are constant and reproducible, and in many cases a reliable identification of organic acids is possible by gas chromatography alone, using methylene units and separation on OV-1701 capillary columns.

INTRODUCTION

More than most of the other endogenous substances in biological samples, in particular urine samples, the chemical class of organic acids represents a very complex mixture of compounds in a broad range of concentrations. Originating from the metabolism of amino acids, carbohydrates, fatty acids and biogenic amines, and from ketogenesis, urinary organic acids are indicators for organic acidurias in conjunction with hereditary diseases [1–3], acquired metabolic disorders such as diabetes mellitus [4] and other diseases, e.g. kidney [5] and liver [6] diseases.

Numerous reports have been devoted to the analysis of certain groups [4,6–

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12] or the total profile [13–21] of organic acids applying gas chromatography–mass spectrometry (GC–MS). Because in many laboratories access to GC–MS instrumentation is limited, efforts have been made to recognize, identify and quantitate abnormalities in urinary organic acids by GC procedures alone. By separating the trimethylsilyl derivatives of the acids on two GC columns [22–26], identification is based on the methylene units (MUs). In a number of hereditary defects organic acidurias are characterized by very drastic changes of the acid profile, and the dual-column methods give satisfactory results. In the case of less pronounced abnormalities and low acid concentrations, interferences and peak overlap occur [25], which hamper identification and quantitation of the acids in the complex mixture.

Improvement may be achieved with a different approach. Instead of separating the total acid mixture on two columns, the acids are pre-fractionated prior to GC analysis [4]. The procedure is applied to establish base profiles of organic acids in normal urine, on the background of which comparative studies of abnormal profiles can be conducted.

EXPERIMENTAL

Samples

Urine samples were collected for 24 h from eight healthy individuals. After the collection period, the samples were either analysed within 10 h or stored at −20°C prior to analysis.

Sample preparation

The sample preparation, including deproteinization with 2-propanol, derivatization of the carbonyl groups with O-methylhydroxylamine hydrochloride, extraction by anion-exchange chromatography, methylation with diazomethane and pre-fractionation by thin-layer chromatography (TLC), has been previously described [4].

The sample preparation using deuterated reagents, especially deuterated diazomethane, and the synthesis of deuterated diazomethane have also been described [27].

Gas chromatographic and mass spectrometric analysis

The GC analyses were performed on a Model 3700 gas chromatograph with a flame ionization detector (Varian, Darmstadt, F.R.G.). The following GC conditions were used: 25 m × 0.2 mm I.D. fused-silica column, coated with OV-1701 (Scientific Glass Engineering, Weiterstadt, F.R.G.); carrier gas, nitrogen at 4 ml/min; column temperature, 40°C for 10 min, then programmed at 2°C/min to 230°C; injector block temperature, 250°C; sample size, 1 μl at a splitting ratio of 1:20.

For the GC–MS analyses, a combination of a Model 2700 gas chromato-

graph, CH5 mass spectrometer and Spectrosystem SS 100 computer (Varian MAT, Bremen, F.R.G.) and a Model TSQ 70 quadrupole mass spectrometer combined with a Model 3400 gas chromatograph (Finnigan MAT, Bremen, F.R.G.) were used. The mass spectra were recorded over the mass range m/z 15–450 by automatic, repetitive scanning. Helium was used as carrier gas. The GC conditions were the same as described for the GC separations.

The MUs were determined using a solution of hydrocarbon standards in hexane, containing carbon numbers from 7 to 30. The MUs were calculated by a previous method [22]. The standard mixture and the urinary samples were analysed separately under identical GC conditions. Coinjection was not performed because of peak overlap of standard and urinary compounds. To correct for possible inaccuracies caused by the separate injection procedure, the MUs of the acids were calculated within the GC profiles of the acid fractions of four healthy individuals. The MUs of a compound from the four runs differed by between 0 and 0.04 units, and the MUs listed in Table I represent average values. Prior to the determination of the MUs three of the samples were analysed by GC–MS.

Reference substances

The reference substances were either synthesized (marked with RS in Table I) or purchased (RS with index). Reference substances with index 1 were purchased from Fluka (Neu-Ulm, F.R.G.), with index 2 from EGA-Chemie (Steinheim, F.R.G.), with index 3 from E. Merck (Darmstadt, F.R.G.), with index 4 from Sigma Chemie (Munich, F.R.G.) and with index 5 from Ventron (Karlsruhe, F.R.G.).

The syntheses of 2-ethyl-3-oxohexanoic acid, *threo*-3-hydroxy-2-methylbutyric acid and 3-hydroxy-2-ethylpropionic acid have been described previously [4]. 4-Deoxyerythronic acid (*erythro*-2,3-dihydroxybutyric acid) was synthesized from allothreonine by diazotization with $Ba(NO_2)_2$ and reaction with concentrated sulphuric acid [28]. 4-Deoxythreonic acid (*threo*-2,3-dihydroxybutyric acid) was synthesized from threonine by diazotization with $Ba(NO_2)_2$ and reaction with concentrated sulphuric acid [28].

The N-acetylamino acids were prepared according to the procedure previously described [12].

2-Pyrroloylglycine was synthesized from 2-pyrrolecarboxylic acid and 3,4-dimethoxycinnamoylglycine from 3,4-dimethoxycinnamic acid. To 3 g of the acid, 50 ml of chloroform–benzene–dimethylformamide (45:45:10) and a solution of 2 g of dicyclohexylcarbodiimide in 5 ml of chloroform were added. The reaction mixture was kept at room temperature for 1 h and then centrifuged. The supernatant was treated with 200 mg of methyl glycinate hydrochloride and 200 μl of pyridine. The mixture was kept at room temperature for 12 h. Each glycine conjugate was purified by TLC under the conditions described for the urinary compound.

TABLE I

ORGANIC ACIDS IDENTIFIED AS METHYL ESTERS IN URINE OF HEALTHY INDIVIDUALS

Consecutive number	Peak number	Methylene unit	Fraction	Substance	Identification
1 Dicarboxylic acids					
1	13	10.67	2b	Malonic acid	RS 3
2	17	10.91	2a,2b	Methylmalonic acid	RS 2
3	24	11.68	2a	Ethylmalonic acid	RS 1
4	25	11.69	2b	Succinic acid	RS 2
5	30	11.92	2a,2b	Methylsuccinic acid	RS 1
6	32	12.38	2a	2,3-Methylenesuccinic acid	(29)
7	34	12.72	2b	Glutaric acid	RS 2
8	36	12.74	2a	Ethylsuccinic acid	A
9	39	13.00	2a,2b	3-Methylglutaric acid	RS 2
10	45	13.34	2a,2b	3-Methylglutaconic acid	RS 2
11	46	13.46	2a,2b	2,3-Methyleneglutaric acid	(29)
12	50	13.74	2a,2b	3-Methylglutaconic acid	RS 2
13	51	13.77	2a	2-Ethylglutaric acid	A
14	53	13.85	2a,2b	Adipic acid	RS 3
15	54	14.10	2a	2-Methyladipic acid	RS 2
16	58	14.26	2a,2b	3-Methyladipic acid	RS 1
17	59	14.31	2a	2,4-Dimethyladipic acid	(20)
18	61	14.47	2b	Muconic acid	RS 2
19	65	14.85	2b	3,4-Methyleneadipic acid	(8)
20	67	14.90	2a,2b	Pimelic acid	RS 3
21	71	15 26	2a	3-Methylpimelic acid	(20)
22	72	15.39	2a	2,4-Dimethylpimelic acid	(20)
23	75	15.57	2a	2,3-Methylenepimelic acid	A
24	79	15.96	2a	3,4-Methylenepimelic acid	(8)
25	81	15.99	2a	Suberic acid	RS 1
26	85	16.29	2a	3-Methylsuberic acid	(20)
27	87	16.60	2a	2,3-Methylenesuberic acid	A
28	92	16.89	2a	3,4-Methylenesuberic acid	(8)
29	96	17.02	2a	Azelaic acid	RS 1
30	98	17.28	2a	3-Methylazelaic acid	A
31	108	17.98	2a	3,4-Methyleneazelaic acid	A
32	109	18.00	2a	Sebacic acid	RS 2
33	113	18.37	2a	5-Decynedioic acid	(20)
34	118	18 95	2a	3,4-Methylenesebacic acid	A
2 Oxocarboxylic acids					
35	4	9.37	2a	Glyoxylic acid	RS 1
36	10	10.17	2a,2b	Pyruvic acid	RS 1
37	14	10.78	2a	2-Oxobutyric acid	RS 1
38	15	10.90	2b	3-Oxobutyric acid	RS 3
39	16	10.91	2a	2-Oxoisovaleric acid	RS 4
		11.06	2a		
40	20	11.39	2b	4-Oxobutyric acid	RS 4
		11.53	2b		
41	23	11.68	2a	3-Methyl-2-oxovaleric acid	RS 1
		11.82	2a		
42	31	11.96	2a	2-Oxoisocaproic acid	RS 1
43	44	13.29	2a	2-Ethyl-3-oxohexanoic acid	RS

TABLE I (continued)

Consecutive number	Peak number	Methylene unit	Fraction	Substance	Identification
44	56	14.11	2b	2-Oxosuccinic acid	RS 1
45	68	14.92	2b	2-Oxoglutaric acid	RS 3
		15.42	2b		
46	82	16.06	2b	3-Oxoadipic acid	RS 1
47	83	16.15	2b	2-Oxoadipic acid	RS 4
3 Hydroxycarboxylic acids					
48	1	8.24	3c,3d	Hydroxyacetic acid	RS 1
49	2	8.48	3a,3b	Lactic acid	RS 1
50	3	8.53	3a,3b	2-Hydroxyisobutyric acid	RS 2
51	5	9.44	3a	2-Hydroxybutyric acid	RS 1
52	6	9.90	3c,3d	3-Hydroxypropionic acid	RS 1
53	7	9.92	3a,3b	3-Hydroxyisovaleric acid	(20)
54	8	9.94	3b,3c	3-Hydroxybutyric acid	RS 3
55	9	9.96	2b,3a	2-Hydroxyisovaleric acid	RS 1
56	11	10.57	3b,3c	3-Hydroxyisobutyric acid	(29)
57	12	10.64	3a,3b	*threo*-3-Hydroxy-2-methyl-butyric acid	RS
58	19	11.01	3a	2-Hydroxy-3-methylvaleric acid	RS 4
59	21	11.49	3b,3c	2-Ethylhydracrylic acid	RS
60	22	11.63	3a	4-Methyl-γ-butyrolactone	(30)
61	26	11.71	4a	4-Deoxyerythronic acid	RS
62	28	11.76	4a	3-Deoxytetronic acid	A
63	33	12.42	4a	2-Deoxytetronic acid	A
64	35	12.74	3a,3b	2-Hydroxy-2-methylsuccinic acid	(9)
65	38	13.00	2b,3a	O-Methylmalic acid	RS 3
66	43	13.20	3b,3c	Malic acid	RS 3
67	47	13.55	3a	2-Hydroxy-2-ethylsuccinic acid	(9)
68	52	13.84	3a,3b	3-Hydroxy-3-methylglutaric acid	RS 1
69	55	14.10	2b,3a	2-Hydroxy-2-isopropyl-succinic acid	(9)
70	60	14.35	3b	2-Hydroxyglutaric acid lactone	RS
71	64	14.83	3a,3b	Tartaric acid	RS 3
72	94	17.00	3b,3c	Citric acid	RS 3
73	100	17.44	3b	Methylcitric acid	(9)
74	101	17.55	3b	Isocitric acid	(20)
75	102	17.57	3a	3-Hydroxy-3-(carboxymethyl)-adipic acid	(9)
4 Aromatic acids					
76	29	11.92	2a,2b,3a	Benzoic acid	RS 3
77	37	12.97	2a,2b,3a, 3b,3c	Phenylacetic acid	RS 1
78	42	13.16	2a	4-Methylbenzoic acid	RS 2
79	62	14.63	3a	Mandelic acid	RS 1
80	63	14.71	2a	3-Hydroxybenzoic acid (a)	RS 1
81	70	15.21	2a	4-Hydroxybenzoic acid (a)	RS 2
82	73	15.43	2a	2-Hydroxyphenylacetic acid (a)	RS 1
83	74	15.52	3a	Phenyllactic acid	RS 1
84	76	15.77	2a	3-Hydroxyphenylacetic acid (a)	RS 1
85	80	15.96	2a	4-Hydroxyphenylacetic acid (a)	RS 1
		18.20	2b,3a,3b	4-Hydroxyphenylacetic acid (b)	

(Continued on p. 6)

TABLE I (continued)

Consecutive number	Peak number	Methylene unit	Fraction	Substance	Identification
86	86	16.45	2a,2b,3a	Phthalic acid	RS 1
87	88	16.73	2a	3-Hydroxyphenylpropionic acid (a)	RS 5
88	103	17 65	2a	3-Hydroxycinnamic acid (a)	RS 2
89	105	17.70	3a,3b	4-Hydroxymandelic acid (a)	RS 4
90	106	17.78	2a,2b	Vanillic acid (a)	RS 1
91	110	18.01	2b,3a	Homovanillic acid (a)	RS 1
		18.25	2b	Homovanillic acid (b)	
92	114	18.44	3a,3b	4-Hydroxyphenyllactic acid (a)	RS 4
93	115	18 59	2a	4-Hydroxycinnamic acid (a)	RS 2
94	116	18 92	2b	4-Hydroxyphenylpropionic acid	RS 1
95	119	19.16	3a,3b	3-Hydroxyphenylhydracrylic acid (a)	RS
96	120	19.19	2a	4-Hydroxy-3-methoxyphenyl-propionic acid (a)	RS 1
97	126	20.17	3b	Vanillylmandelic acid (a)	RS 1
98	127	20.95	2a	4-Hydroxy-3-methoxy-cinnamic acid (a)	RS 1
5 Furancarboxylic acid					
99	18	10.99	2a	Furan-3-carboxylic acid	RS 1
100	27	11.73	2b	5-Methylfuran-2-carboxylic acid	(20)
101	78	15.85	2b	Furan-2,5-dicarboxylic acid	(20)
102	84	16.26	3b,3c	5-Hydroxymethylfuran-2-carboxylic acid	(20)
103	93	16.97	3a	*cis*-Tetrahydro-2,5-furan-diacetic acid	(11)
104	97	17 03	3a	*trans*-Tetrahydro-2,5-furan-diacetic acid	(11)
105	122	19 46	2a	3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid	(20)
106	128	21.28	2a	3-Carboxy-4-methyl-5-pentyl-2-furanpropionic acid	(20)
6 Nitrogen-containing acids					
107	40	13.00	2b	Pyrrole-2-carboxylic acid	RS 2
108	41	13.14	3b	Pyrazin-2-carboxylic acid	RS 2
109	48	13.64	3d	N-Acetylalanine	RS
110	49	13.68	3b	2-Picolinic acid	RS 1
111	57	14 19	3b	Pyrrole-3-carboxylic acid	A
112	66	14.85	3d	N-Acetylvaline	RS
113	69	15.02	2a,2b	Anthranilic acid	RS 1
114	77	15.80	3d	N-Acetylleucine	RS
115	91	16.77	4a	N-Acetylproline	RS
116	95	17.00	3c	Pyroglutamic acid	RS 2
117	99	17.33	3d,4a	N-Acetylaspartic acid	RS
118	104	17.68	3b	Pyridine-2,3-dicarboxylic acid	RS 1
119	111	18.14	3c	N-Acetylaminooctanoic acid	(31)
120	112	18.15	3d,4a	6-Methoxy-pyridine-3-carboxylic acid	RS 2

TABLE I (continued)

Consecutive number	Peak number	Methylene unit	Fraction	Substance	Identification
121	117	18.94	3d,4a	N-Acetylglutamic acid	RS
122	129	21.57	2b,3a	3-Indoleacetic acid	RS 3
123	130	21.76	3a	Kynurenic acid	RS 1
124	132	22 68	4a	N-Acetyltyrosine (a)	RS
125	133	22.72	3b	3-Indolecarboxylic acid	RS 4
126	136	23 90	4a	3-Indolelactic acid	RS 2
127	137	24.15	4a	3-Indolehydracrylic acid	A
128	143	28 46	4a	N-Acetyltryptophan	RS
7 Acid conjugates					
129	89	16.74	3b,3c	3-Methylcrotonylglycine	(32)
130	90	16.77	3b,3c	Tiglylglycine	(32)
131	107	17.83	3b,3c,3d	Furoylglycine	(20)
132	121	19.40	3a	N-Methylhippuric acid	(20)
133	123	19.46	3b,3c	Picolinoylglycine	RS
134	124	20.02	2b,3a,3b, 3c,3d	Hippuric acid	RS 3
135	125	20.08	4a	2-Pyrroloylglycine	RS
136	131	22.33	3b	2-Hydroxyhippuric acid (a)	RS 2
137	134	22 93	3b,3c,3d	3-Hydroxyhippuric acid (a)	RS
138	135	23.40	3c,3d,4a	4-Hydroxyhippuric acid (a)	RS
139	138	25 12	4a	N-Phenylacetylpyroglutamic acid	(20)
140	139	25.28	3b,3c,3d	N-Phenylacetylglutamic acid	(20)
141	140	25 65	3d,4a	4-Hydroxy-3-methoxy-hippuric acid (a)	(20)
142	141	26.80	4a	4-Hydroxy-3-methoxy-cinnamoylglycine	RS
143	142	26.83	4a	N-Phenylacetyl-N-methylglutamine γ-lactam	(20)
8 Artifacts produced by the analytical procedure					
	a	9.57	3d	N,N-Dimethylalanine	RS
	b	10 21	3d	N-Methylvaline	RS
	c	10 46	3a	N,N-Dimethylvaline	RS
	d	11.12	3c,3d	Phosphoric acid	RS 1
	e	11 17	3d	N-Methylleucine	RS
	f	11.21	3d	N-Methylisoleucine	RS
	g	11 44	2b	N,N-Dimethylleucine	RS
	h	11.44	2b	N,N-Dimethylisoleucine	RS
	i	11.87	3b,3c	N,N-Dimethylthreonine	RS
	j	13.53	3b	N,N-Dimethyl-S-methylcysteine	RS
	k	13.63	3b,3c	N,N-Dimethylaspartic acid	RS
	l	14.00	3d	Pyrazole-3-carboxylic acid	(33)
	m	14 46	3b	N,N-Dimethylmethionine	RS
	n	14.72	3c	N,N-Dimethylglutamic acid	RS
	o	15.11	3b	2-Methylpyrazole-3-carboxylic acid	(33)
	p	15.60	3d	N-Methylphenylalanine	RS
	q	15.85	3a,3b	N,N-Dimethylphenylalanine	RS

(Continued on p. 8)

TABLE I (continued)

Consecutive number	Peak number	Methylene unit	Fraction	Substance	Identification
	r_1	16.44	3a	Methylaconitic acid	RS
	r_2	16 92	3a	Methylaconitic acid	RS
	r_3	17.24	3a	Methylaconitic acid	RS
	r_4	17.43	3a	Methylaconitic acid	RS
	s	18.87	3b,3c	N,N-Dimethyltyrosine	RS
	t_1	21.26	3a,3b	Tetramethyluric acid	(20)
	t_2	21.46	3a,3b	Tetramethyluric acid	(20)
	t_3	22.08	3b,3c	Tetramethyluric acid	(20)
	t_4	22.52	3b,3c	Tetramethyluric acid	A
	t_5	23.71	3d,4a	Tetramethyluric acid	A
	u	23.89	3d	N-Methyltryptophan	RS
	v	24.04	3d	N,N-Dimethyltryptophan	RS

The N-methylated and N,N-dimethylated amino acids were synthesized according to the procedure described previously [27]. The methylaconitic acid isomers were prepared from aconitic acid by reaction with diazomethane under the conditions described for the methylation of the organic acids.

Underivatized reference compounds were transformed into methyl esters and methyl esters/O-methyloximes, respectively, according to the procedure described for the urinary acids.

RESULTS AND DISCUSSION

Organic acids in normal urines

Profiles of the organic acid derivatives from normal urine within the TLC fractions are shown in the chromatograms of Figs. 1–3. The qualitative patterns of corresponding fractions of the urinary acids from different normal individuals are similar and constant.

In four out of eight urine samples the organic acids were identified by GC–MS analysis; in the other four samples the identification was achieved by GC alone, on the basis of the MUs. The peak numbers and peak indices of the chromatographic profiles refer to Table I, which lists 143 organic acids classified into dicarboxylic acids, oxocarboxylic acids, hydroxycarboxylic acids, aromatic acids, furancarboxylic acids, nitrogen-containing acids and acid conjugates (groups 1–7).

Table I also shows artifacts produced by the analytical procedure. They include acids with methyl groups introduced by the reaction with diazomethane, acids formed during the preparation procedure and phosphoric acid (group 8). The peak numbers of the compounds within each group of Table I, including group 8, are listed in the order of increasing MU. Identical peak numbers are

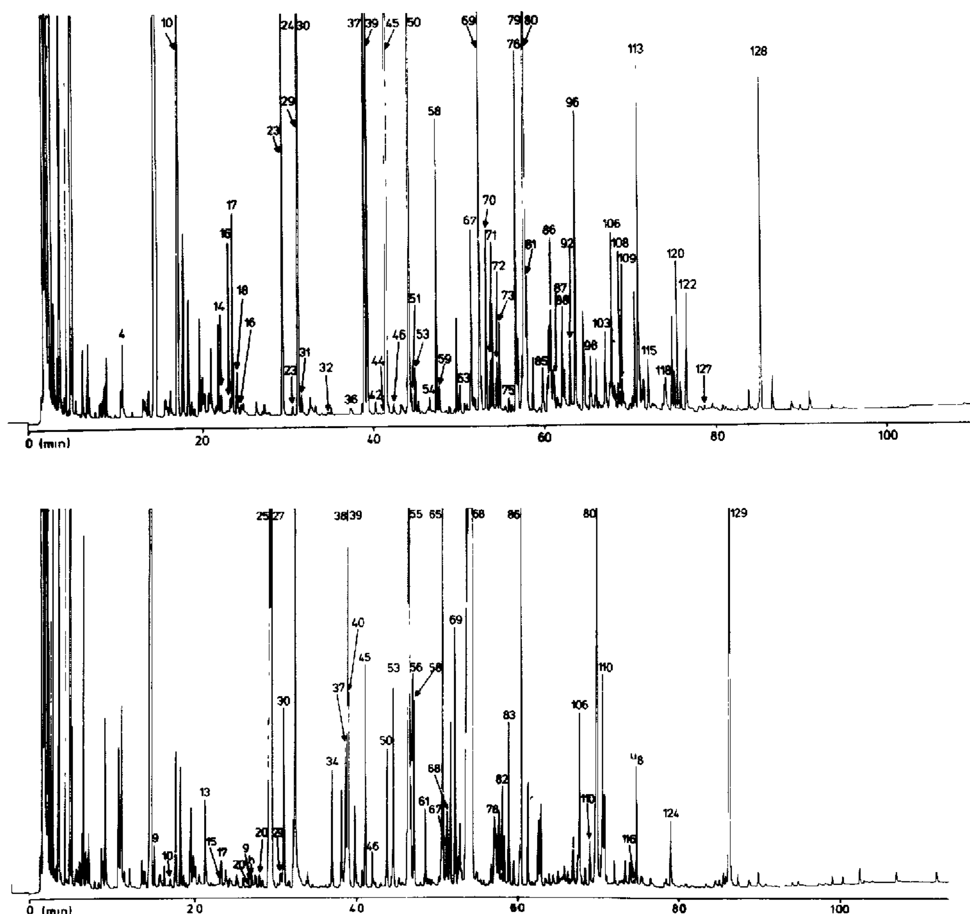

Fig. 1. Fractions 2a (top) and 2b (bottom) of the derivatives of the organic acids in urine of a healthy individual.

given for the *syn–anti* isomeric peaks of the O-methyloxime derivatives of the oxocarboxylic acids (group 2). The peak number listed refers to the first isomeric peak. Analogously, identical peak numbers are given to the phenolic acid derivatives with the free phenolic OH group and the phenolic OH group methylated by diazomethane, respectively (group 4). The phenolic acid derivatives of group 4 and group 7 are labelled with substance indices [(a) methylated at the phenolic OH group by diazomethane as derived from the results of the experiments using deuterated diazomethane for sample preparation; (b) free phenolic OH group].

The identification of the organic acids of Table I is based on the mass spectra and the MUs of the urinary compounds and of reference substances (RS).

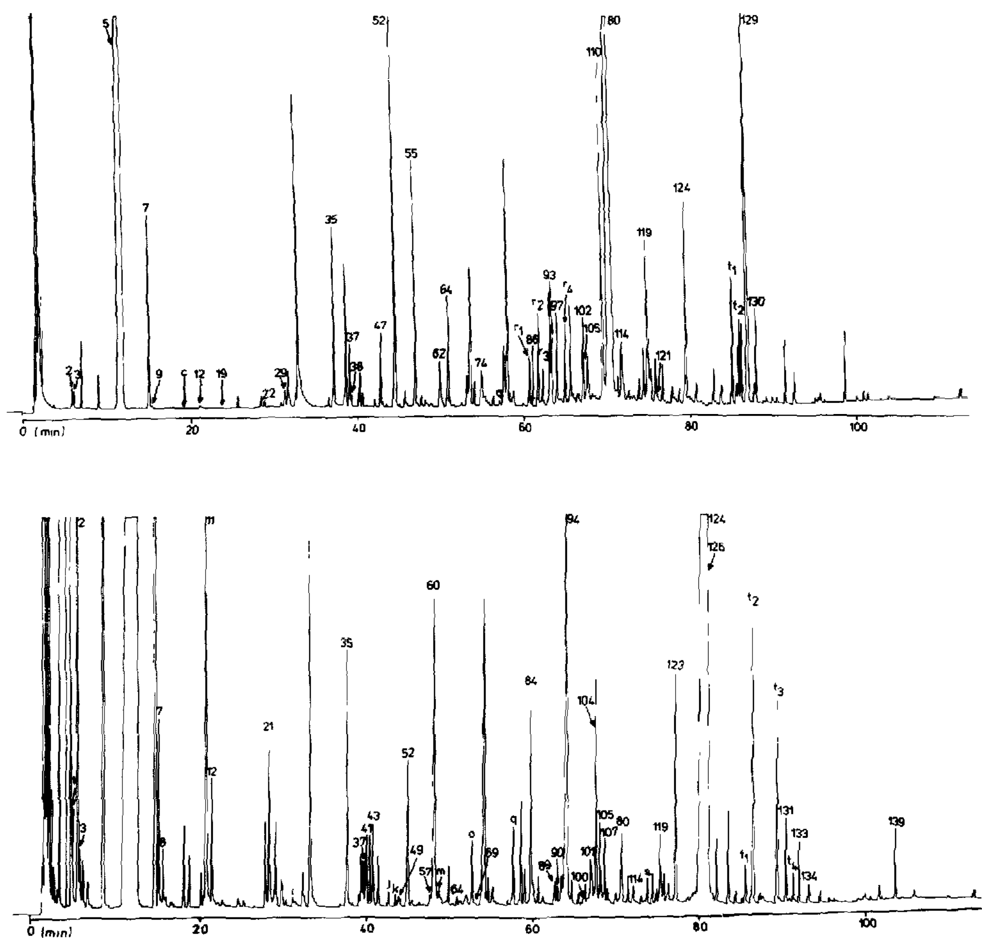

Fig. 2 Fractions 3a (top) and 3b (bottom) of the derivatives of the organic acids in urine of a healthy individual.

Identification on the basis of reference spectra from the literature are indicated by reference to the literature. Identification by analogy (marked with A), is based on the comparison with the GC–MS data of homologous or otherwise analogous urinary organic acids.

Distribution of the organic acids in the TLC fractions

The organic acids appear in TLC fractions 2a–4a (Fig. 4) according to increasing polarities. Fractions 2a and 2b (Fig. 1) contain mainly dicarboxylic and oxocarboxylic acids. Besides, most of the aromatic acids and the furancarboxylic acids occur in fraction 2a.

In fractions 3a–3d, monohydroxycarboxylic, nitrogen-containing acids and

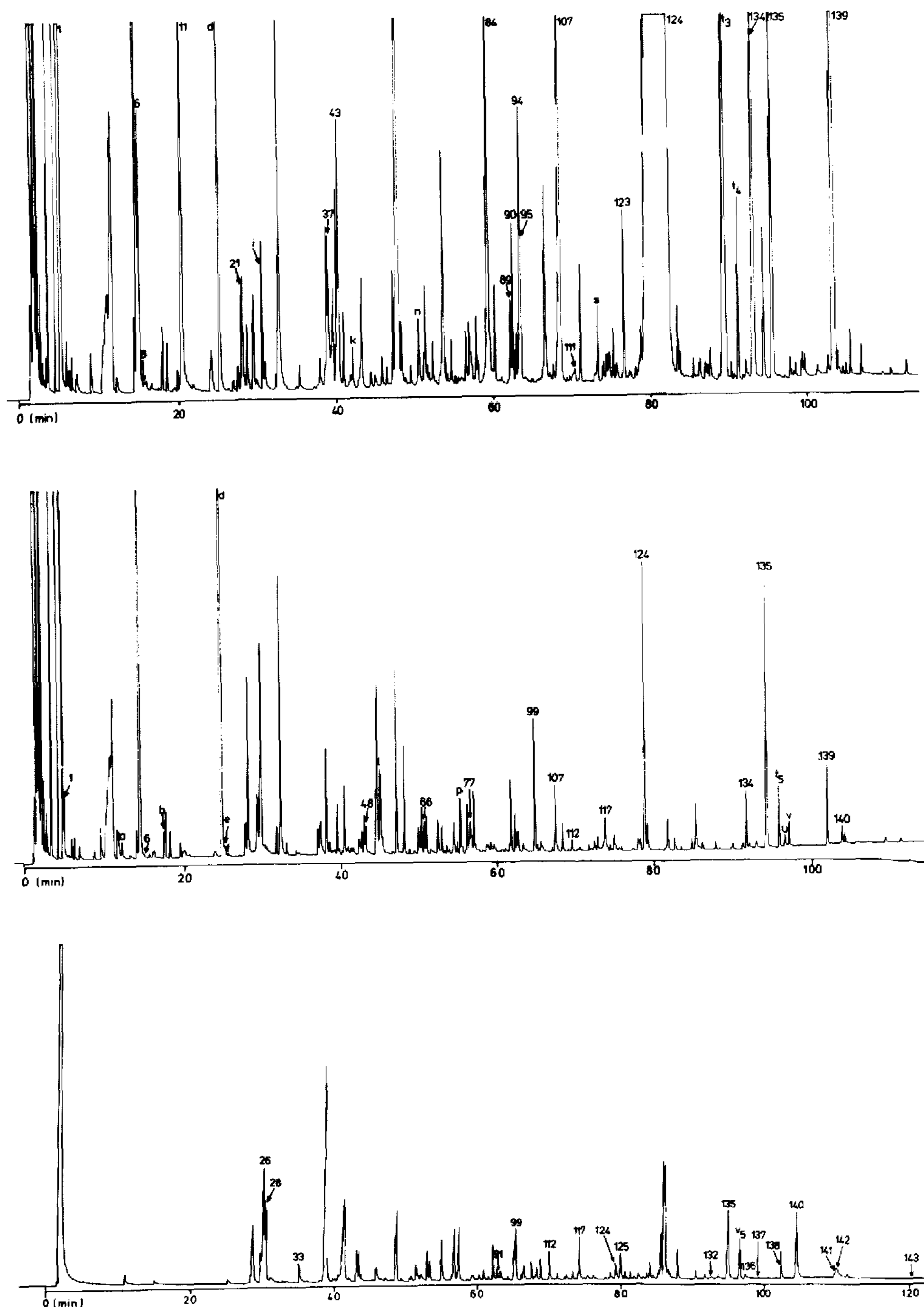

Fig. 3. Fractions 3c (top), 3d (middle) and 4a (bottom) of the derivatives of the organic acids in urine of a healthy individual.

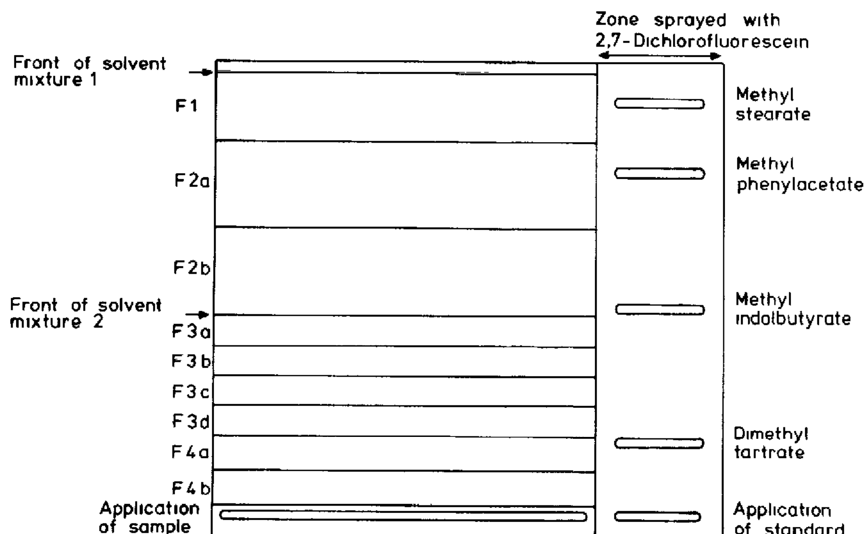

Fig. 4 Pre-fractionation by TLC

acid conjugates predominate. The monohydroxycarboxylic acids [4] are part of all four fractions 3a–3d (Figs. 2 and 3). In the group of nitrogen-containing acids, the N-acetylamino acids are enriched in fraction 3d [12], whereas the others are distributed over a wider range. The acid conjugates appear mainly in fractions 3b–3d with hippuric acid at a maximum in 3c. The hydroxyhippuric acid derivatives are part of 3b–3d and appear in these fractions according to the increasing polarities of the 2-, 3- and 4-hydroxy metabolites.

The N-methylated amino acid derivatives listed (group 8 in Table I) are enriched in fraction 3d. The N,N-dimethylated amino acids are constituents of 3a–3d except for the N,N-dimethylated leucine and isoleucine, which appear in fraction 2b [27].

In fraction 4a (Fig. 3) dihydroxycarboxylic acids, nitrogen-containing acids and acid conjugates are found.

Fraction 1 contains fatty acids, which have not been further investigated, whereas in fraction 4b no organic acids were identified.

Identification of the organic acids

More than 140 of the organic acids regularly occurring in urine of healthy individuals are identified as methyl esters by GC–MS with respect to their complete chemical structure. Partially identified components are not included in this report. Systematic studies of the mass spectrometric fragmentation of several classes of organic acids have been described elsewhere, e.g. oxocarboxylic acids and monohydroxycarboxylic acids [4], furancarboxylic acids [11] and N-acetylamino acids [12].

Methylation of the acids with diazomethane is easy to perform and yields a quantitative reaction. The resulting methyl esters give more characteristic mass spectra than trimethylsilyl derivatives. However, since diazomethane may principally react with phenolic OH groups, carbonyl compounds, olefinic double bonds and amino groups, identification of some organic acids may be complicated by the formation of artifacts. Their concentrations are low when short reaction times are chosen for diazomethane.

To differentiate between possible artifacts from diazomethane and the original compounds, we used deuterated reagents, in particular deuterated diazomethane. The experiments show that for the 2,3-methylenedicarboxylic acids and 3,4-methylenedicarboxylic acids (Table I, group 1) the introduction of the methylene group by diazomethane can be excluded. These results are not in agreement with those of other workers [34] discussing the 2,3-methylenedicarboxylic acids as artifacts from α,β-unsaturated dicarboxylic acids by cycloaddition of diazomethane to the double bond.

The phenolic acids (Table I, group 4) are identified in the form of the O-methyl ether derivatives. With the exception of 4-hydroxyphenylacetic acid and homovanillic acid, the phenolic OH groups of all other aromatic derivatives are completely methylated by diazomethane.

In the case of the N-methylated and N,N-dimethylated amino acids (Table I, group 8), the experiments with deuterated diazomethane prove that N-methylation and N,N-dimethylation are caused by the reaction of diazomethane with the amino group [27].

GC analysis of the organic acids

Based on the GC–MS analysis, a comprehensive overview of the acidic metabolites excreted in the urine of healthy individuals is established. As a result of the pre-fractionation of the total organic acid sample by TLC, peak interferences are reduced, substances in low concentrations are also detected and identified and a detailed profile analysis is possible. Without pre-fractionation a complete analysis of the very complex mixture of organic acids is hard to achieve, even with high-efficiency capillary columns. The constancy and reproducibility of the qualitative pattern within corresponding fractions of all samples allow in many cases the reliable identification of the acids on the basis of their MUs alone. Based on these findings, GC analyses of selected urinary acids from pathological samples are possible. Controls by GC–MS can be restricted to ambiguous identifications.

With the dual-column techniques applied to the total acid samples, vast changes of the urinary acid composition in hereditary disorders can be recognized. The separation achieved with total organic acid samples [25,26] does not appear sufficient for the analysis of more subtle abnormalities in other metabolic diseases. Better separations are obtained with pre-fractionation. On the other hand, the pre-fractionation lengthens the analytical procedure and

renders quantitations of the organic acids more difficult because a number of substances occur in several fractions. However, because of a strict standardization of the method, pathological patterns of the acids are easily recognized and classified when compared with the base profiles of organic acids. For quantitative determination of selected organic acids, such as oxocarboxylic and hydroxycarboxylic acids, the number of fractions may be reduced [35] in order to avoid distribution of a substance between several fractions.

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