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Note

Gas chromatographic–mass spectrometric characterisation of unsaturated dicarboxylic acids in urine

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Dicarboxylic aciduria is a condition sometimes encountered during routine urine screening for inborn errors of metabolism [1] and may be due to ketosis, a transient condition in some neonates [2] or a number of enzyme deficiencies mainly involving fatty acid metabolism or transport. Unsaturated dicarboxylic acids have also been reported in some of these conditions. These compounds are usually detected using gas chromatography (GC) and/or gas chromatography–mass spectrometry (GC–MS). Unfortunately, little information regarding the position of unsaturation in these dicarboxylic acids is obtained with these techniques. With non-polar columns, many isomeric unsaturated organic acids will co-elute and may not be adequately separated from other fatty acid metabolites, usually 3-hydroxydicarboxylic acids. The mass spectra of the isomeric unsaturated compounds are often very similar and the double bond position is not apparent. Although some detailed structural studies have been undertaken in isolated cases [3–6] there is generally little information available regarding the structure or occurrence of unsaturated dicarboxylic acids.

This report describes a technique suitable for the identification of unsaturated organic acids in urine and its application to the identification of a series of unsaturated dicarboxylic and 3-hydroxydicarboxylic acids in the urine of subjects with abnormal fatty acid metabolism.

EXPERIMENTAL

Urine samples

Samples for study were taken from random urine specimens submitted for routine screening for inborn errors of metabolism. Three groups were studied: (i) control subjects ($n = 7$): no abnormalities detected during routine screening; (ii) subjects with ketosis ($n = 12$): ketones present in urine by diptest; (iii) subjects

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with medium-chain acyl coenzyme A dehydrogenase (MCAD) deficiency ($n=3$): detected during routine screening and subsequently confirmed by enzyme assays; urine specimens were obtained during episodes of metabolic decompensation.

Chemicals

3,3-Dimethylglutaric acid, adipic acid, suberic acid, sebacic acid and Sephadex DEAE A-25 were obtained from Sigma (St. Louis, MO, U.S.A.); potassium permanganate, sodium hydroxide and barium hydroxide were analytical grade from BDH (Poole, U.K.); sodium sulphite was from Merck (Darmstadt, F.R.G.); bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was from Pierce (Rockford, IL, U.S.A.).

Derivatisation

For each urine specimen, an unoxidised and a potassium permanganate-oxidised profile was obtained. Unsaturated organic acids were identified by comparison of the two profiles (Fig. 1). The process of derivatisation is shown in Fig. 2. Potassium permanganate oxidation of unsaturated organic acids leads to the formation of diol derivatives which, when trimethylsilylated and subjected to MS give ions indicative of the position of unsaturation [7].

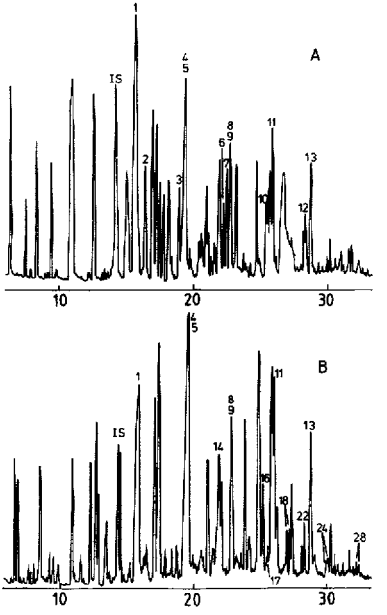

Fig. 1 Reconstructed ion chromatograms of (A) unoxidised and (B) potassium permanganate-oxidised urine obtained from a patient with MCAD deficiency IS = internal standard Refer to Tables I and II for peak identities

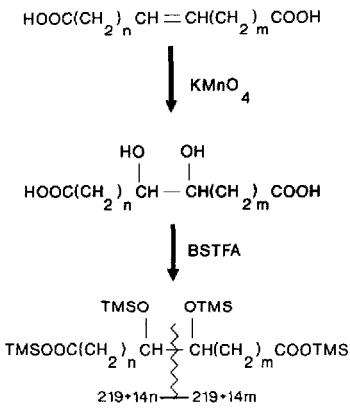

Fig 2 Formation of diol-TMS derivatives of unsaturated dicarboxylic acids following potassium permanganate oxidation Indicated mass fragments were used to determine positions of unsaturation.

Potassium permanganate oxidation

Urine (1 ml), 100 μl of an aqueous internal standard (3.5 m*M* 3,3-dimethylglutaric acid) and 400 μl of 0.1 *M* sodium hydroxide were mixed and cooled in ice. A 400-μl volume of 0.05 *M* potassium permanganate solution was added and the solution stood in ice for 5 min. If the purple colour disappeared a further 400 μl of potassium permanganate solution were added and the solution stood on ice for a further 5 min. Sodium sulphite (0.2 *M*) was added dropwise until the purple colour had disappeared and the precipitate was removed by centrifugation.

Sephadex DEAE A-25 chromatography

The supernatant from the oxidation step or a 1-ml aliquot of unoxidised urine containing 100 μl of internal standard solution was treated dropwise with saturated barium hydroxide until no more white precipitate was formed. This removed excess sulphite and sulphate which otherwise interfered in subsequent steps. The precipitate was removed by centrifugation, the supernatant was adjusted to pH 7 with 1 *M* acetic acid and the organic acids were extracted on Sephadex DEAE A-25 [8]. After eluting the organic acids, the eluates were dried by overnight lyophilisation.

Trimethylsilyl (TMS) derivatives were formed by incubating the lyophilisates for 30 min at 60°C with 50 μl of BSTFA. This mixture was then analysed directly by GC–MS.

Gas chromatography–mass spectrometry

Data were collected on a Finnigan 1020 GC–MS system. GC conditions were as follows: 25 m × 0.22 mm I.D. BP1 column, 0.25 μm film thickness (SGE, Melbourne, Australia). Injection volume was 0.5 μl with a splitless injection time

of 40 s. The injector temperature was 300°C and the GC oven was held at 80°C for 1 min, then ramped to 300°C at 6°C/min. The mass spectrometer was scanned from *m/z* 100 to 600 in 2 s and in some cases from *m/z* 200 to 700 in 2 s. Molecular masses of derivatised compounds were inferred from M − 15 ions which are intense in TMS derivatives [9]. It was necessary to inject relatively large amounts of derivative onto the column in order to obtain spectra of some of the minor urine constituents. In some cases this resulted in overloaded GC peaks of some of the major constituents and partial overlapping with other peaks. In these situations, the purity of spectra obtained was checked by examining ion profiles across the peak.

Some urine specimens were analysed in duplicate to assess the reproducibility of the method. The pattern of unsaturated metabolites identified was qualitatively the same in both cases.

RESULTS

The diol derivatives eluted later in the gas chromatogram than their parent compounds and were well separated from interfering peaks that were present in the unoxidised urine profiles. In addition, the formation of diol derivatives enabled most of the isomeric unsaturated dicarboxylic acids to be adequately resolved on the capillary column used

TABLE I

GC–MS CHARACTERISTICS OF DICARBOXYLIC ACIDS FOUND IN URINE AS TMS DERIVATIVES

Dicarboxylic acid[a]	RRT[b]	M^+	Major ions > *m/z* 100[c]
1. C_6 (adipic)	1 093	290	111(100), 141(50),147(58),159(14),172(24),275(11)
2. Δ^2C_6	1.150	288	109(32),139(28),147(100), 155(23),198(11),273(11)
3. Δ^xC_8	1.329	316	109(42),117(54),136(100),147(70),185(19),217(10),257(3),301(4)
4 $3OHC_6$	1.351	378	109(45),129(48),147(100),203(20),233(10),247(21),363(8)
5. C_8 (suberic)	1 358	318	129(90),149(72),169(100),187(86),303(26)
6 Δ^xC_{10}	1 557	344	117(64),119(100).137(40),147(43),164(62),254(6),329(6)
7. $3OH\Delta^xC_8$	1 575	404	155(30),147(100),167(20),189(41),233(49),244(34),389(3)
8 $3OHC_8$	1.592	406	137(59),147(100),169(57),185(24),233(22),391(4)
9 C_{10} (sebacic)	1 597	346	117(79),129(99),149(100),215(96),331(67)
10 $3OH\Delta^xC_{10}$	1 781	432	117(24), 147(100),189(31),217(23),233(39),272(8),342(2),417(2)
11 $3OHC_{10}$	1.816	434	147(100),169(32),189(25),233(31),274(8),303(13),329(6),419(5)
12. $3OH\Delta^xC_{12}$	1.982	460	129(49),147(100),189(63),191(49),233(70),300(16),445(1)
13. $3OHC_{12}$	2.011	462	129(18),147(100),189(19),233(30),315(6),331(11),405(5),447(4)

[a] Examples of abbreviations used Δ^3C_6 = hex-3-ene-1,6-dioic acid, Δ^xC_{10} = decene-1,10-dioic acid (position of unsaturation unknown), $3OH\Delta^5C_{10}$ = 3-hydroxy-dec-5-ene-1,10-dioic acid.

[b] Retention time relative to 3,3-dimethylglutaric acid TMS derivative

[c] Percentage intensities of ions given in parentheses

Preliminary experiments showed that the diol derivatives were too polar to be efficiently solvent-extracted. Anion-exchange extraction on Sephadex DEAE A-25 gave improved recoveries of the diol derivatives and allowed correspondingly smaller volumes of urine to be used.

GC–MS characteristics of the TMS derivatives of the major dicarboxylic acids observed in the unoxidised urine profiles are shown in Table I. Saturated dicarboxylic acids were identified against standards; 3-hydroxysebacic acid was identified from a published mass spectrum of the TMS derivative [10]. Mass spectra were not available for the other 3-hydroxydicarboxylic acids but the mass spectra and relative retention times are consistent with the structures in Table I. The GC–MS characteristics of the diol-TMS derivatives of the unsaturated dicarboxylic acids are given in Table II.

The unsaturated dicarboxylic acids were characterised as follows:

(i) Observation of a peak in the unoxidised profile with retention time slightly shorter and a molecular weight 2 m/z less than the corresponding saturated dicarboxylic acid.

(ii) Absence of this peak in the oxidised urine profile.

TABLE II

GC–MS CHARACTERISTICS OF UNSATURATED DICARBOXYLIC ACIDS AS DIOL-TMS DERIVATIVES

Dicarboxylic acid[a]	RRT[b]	M^+	Major ions > m/z 100 [c]
14 Δ^2C_6	1 515	466	129(100),247(75),292(22),361(9),451(4)
15 Δ^2C_8[d]	1 724	494	147(100),185(33),275(10),292(15)
16 Δ^3C_8	1 749	494	129(88),147(60),171(100),261(73),306(5),389(4),479(1)
17 Δ^4C_8	1 765	494	129(100),157(35),247(55),389(2),479(1)
18 3OHΔ^5C_8	1 874/1 888	582	129(70),147(80),259(100),306(3),384(2),477(1)
19 Δ^2C_{10}[d]	1 920	522	213(50),292(100),303(80)
20 Δ^3C_{10}	1 945	522	199(65),289(100),306(100),417(17)
21 Δ^4C_{10}	1 953	522	129(80),185(61),247(100),275(26),327(6),417(2)
22 Δ^5C_{10}	1 970	522	129(100),171(80),261(55),327(2),507(1)
23 3OHΔ^7C_{10}[d]	2 077	610	287(100),306(50),377(20)
24 3OHΔ^5C_{10}	2.083/2 094	610	129(90),171(70),259(80),261(65),415(1),505(1)
25. 3OHΔ^6C_{10}	2.086	610	129(100),247(50),273(45),363(3),415(2),505(1)
26. Δ^3C_{12}	2.136	550	129(60),171(40),261(60),289(15),306(55),317(100),445(10)
27. 3OHΔ^6C_{12}	2.252	638	185(50),273(100),275(40),363(20)
28 3OHΔ^5C_{12}	2 259/2 268	638	147(70),259(100),289(20),369(5),443(3),533(1)
29 3OHΔ^7C_{12}	2 275	638	129(100),147(65),261(50),287(25),306(10),377(5),491(1)
30 3OHΔ^9C_{12}[d]	2 280	638	129(100),306(20),315(15),405(5)

[a] See Table I, footnote *a*.

[b] Retention time relative to TMS derivative of 3,3-dimethylglutaric acid.

[c] Percentage intensities of ions given in parentheses

[d] Tentative identification

(iii) Appearence of one or more peaks in the oxidised profile with molecular weights 178 *m/z* greater than the corresponding unoxidised acids and with greater retention time.

(iv) Unsaturated hydroxy acids were identified as 3-hydroxylated from the observation of an ion at *m/z* 233 [9].

The position of unsaturation was determined by observation of ions due to cleavage between adjacent trimethylsiloxy groups in the oxidised profile as shown in Fig. 2. Unsaturated 3-hydroxydicarboxylic acids produced an analogous cleavage with one ion having a mass increment of *m/z* 88 (OTMS substitution). Major ions at 90 *m/z* less than these cleavage ions were also frequently observed due to the loss of TMSOH (trimethylsilanol). For the derivatives of Δ^2-unsaturated dicarboxylic acids the expected ion of mass *m/z* 219 was of low intensity. Instead, an intense ion of mass *m/z* 292 was observed due to the expected cleavage and migration of a TMS group. Such re-arrangement products have previously been observed for the TMS derivatives of 2,3-dihydroxycarboxylic and 2,3-dihydroxydicarboxylic acids [9]. A similar rearrangement ion was observed for Δ^3-unsaturated dicarboxylic acids, leading to an ion of mass *m/z* 306.

TABLE III

FREQUENCY OF DETECTION OF UNSATURATED DICARBOXYLIC ACIDS IN GROUPS STUDIED

Dicarboxylic acid[a]	Frequency (%)		
	Normal ($n=7$)	Ketotic ($n=12$)	MCAD ($n=3$)
Δ^2C_6	0	100	100
Δ^2C_8[b]	0	0	66
Δ^3C_8	0	75	66
Δ^4C_8	0	42	100
Δ^2C_{10}[b]	0	0	66
Δ^3C_{10}	0	50	66
Δ^4C_{10}	0	33	33
Δ^5C_{10}	0	42	33
Δ^3C_{12}	0	8	33
$3OH\Delta^5C_8$	0	58	66
$3OH\Delta^5C_{10}$	0	17	33
$3OH\Delta^6C_{10}$	0	17	33
$3OH\Delta^7C_{10}$[b]	0	17	33
$3OH\Delta^5C_{12}$	0	17	33
$3OH\Delta^6C_{12}$	0	25	33
$3OH\Delta^7C_{12}$	0	17	0
$3OH\Delta^9C_{12}$[b]	0	8	33

[a] See Table I, footnote *a*

[b] Tentative identification.

Diol-TMS derivatives of the 3OHΔ^5-dicarboxylic acids were found to give double peaks on GC. As a result of the accepted *cis* mechanism of permanganate oxidation of a double bond, a second centre of asymmetry is introduced into the molecule, producing two diastereomers which are potentially separable on GC. It has been observed that the separation of diastereomeric pairs on non-polar GC columns decreases as the distance between asymmetric centres in the molecule increases [11] which would explain why double peaks were not observed for the diol-TMS derivatives of other 3-hydroxy unsaturated dicarboxylic acids with double bonds further removed from the 3-hydroxy group.

All the unsaturated dicarboxylic acids detected were monounsaturated. No polyunsaturated dicarboxylic acids were detected in the unoxidised profiles and the levels of the unsaturated dicarboxylic acids were qualitatively lower than the corresponding saturated dicarboxylic acids. Determination of the *cis–trans* configuration about the double bonds was not attempted. Table III shows the frequencies at which the various unsaturated dicarboxylic cids were observed in the groups studied.

DISCUSSION

A relatively simple method is presented for the characterisation of unsaturated dicarboxylic acids in urine. Unlike previous methods, small volumes of urine may

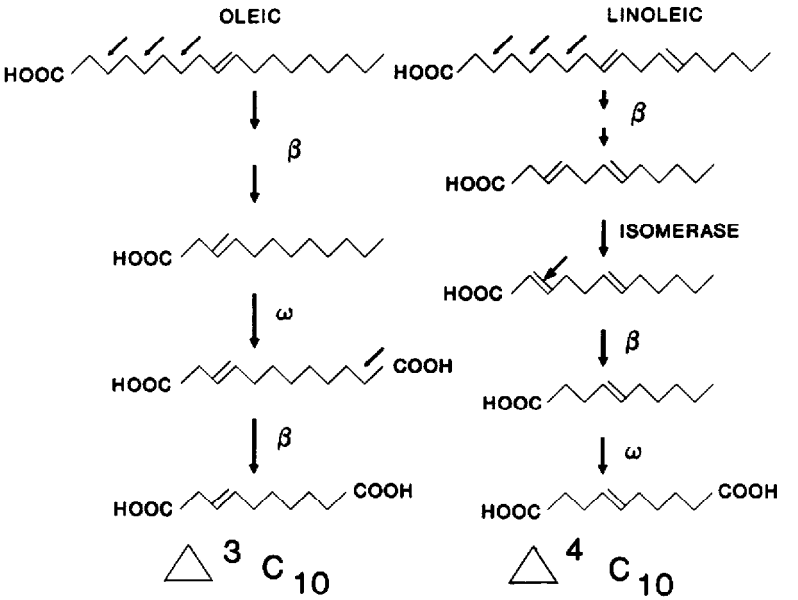

Fig 3. Speculative metabolic pathways for the formation of unsaturated dicarboxylic acids with even and odd Δ positions Diagonal arrows indicate site of action for β-oxidation (removal of an acetyl moiety), ω indicates formation of a dicarboxylic acid by ω-oxidation Isomerase indicates the enzyme Δ^3-*cis*-Δ^2-*trans*-enoyl coenzyme A isomerase.

be used (2 ml) and extensive sample preparation is not required, making the method suitable for investigation of newborns and critically ill patients.

Some of the unsaturated dicarboxylic acids observed have previously been characterized in urine [3–5]. Although unsaturated 3-hydroxydicarboxylic acids have been reported [10] the double bond positions do not appear to have been determined previously. The unsaturated dicarboxylic acids, like the saturated dicarboxylic acids, are most likely due to fatty acid oxidation as they were only observed in the ketotic group (increased fatty acid oxidation) and the MCAD group (block in fatty acid oxidation leading to the accumulation of intermediary products of fatty acid metabolism).

The origin of the unsaturated dicarboxylic acids may be explained in terms of ω-oxidation and repeated cycles of β-oxidation of longer-chain unsaturated fatty acids (Fig. 3). It is possible to explain the unsaturated dicarboxylic acids with an odd numbered Δ position as metabolites of oleic acid (Δ^9). The unsaturated dicarboxylic acids with an even numbered Δ position may be metabolites of linoleic acid ($\Delta^9\Delta^{12}$) that has had the Δ^9 double bond removed through an Δ^3-*cis*-Δ^2-*trans*-enoyl coenzyme A isomerase step. The saturated and unsaturated 3-hydroxydicarboxylic acids probably originate from intermediates formed during the β-oxidation process that have undergone a similar chain shortening process [10].

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