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REVIEW

METABOLIC PROFILING WITH GAS CHROMATOGRAPHY—MASS SPECTROMETRY AND ITS APPLICATION TO CLINICAL MEDICINE

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1. INTRODUCTION

Metabolic profiling is a multicomponent chromatographic analysis of body fluids and tissues. The aim of metabolic profiling is to correlate normal and disease states with certain characteristic features of chromatographic profiles

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and to study the difference between normal and pathological metabolism. Metabolic profiling can lead to the discovery of previously unknown disease entities and metabolic disorders. In a number of diseases, metabolic profiling with a gas chromatograph—mass spectrometer—computer (GC—MS—COM) system has become a critical means for clinical diagnosis.

Several approaches have been taken in the development of methods for the identification and the quantitation of components in biological complex mixtures. High-performance liquid chromatography (HPLC) and gas chromatography (GC) have been used in the study of urinary metabolites.

The advantages of HPLC are (1) derivatization of the compound is not needed; (2) heat-labile and high-molecular-weight compounds can be analysed; and (3) each compound can be collected easily without decomposition. One disadvantage of HPLC at present is the difficulty of identifying compounds due to incomplete interfacing with a mass spectrometer. The advantages of GC are (1) the compounds can be identified by MS, because of complete interfacing with MS; and (2) the open-tubular capillary column usually provides better separation than with HPLC. The disadvantages of GC are (1) derivatization is usually needed; (2) heat-labile and high-molecular-weight compounds cannot be analysed; and (3) collection of each compound is usually difficult.

Identification of individual compounds only by the absolute or relative retention time is often questionable in the analysis of extremely complex mixtures. Currently, the method of choice for the analysis of organic acids, volatiles, polyols and steroids involves the use of a GC—MS—COM system. Metabolic profiles are obtained as reconstructed ion chromatograms, and the individual compounds are identified by comparing the mass spectra and relative retention times with those of reference compounds.

The object of this review is to survey the methods for metabolic profiling with GC—MS and the clinical applications of the technique in the investigation of several diseases.

2. ISOLATION METHODS FROM URINE

2.1. Organic acids

The isolation of organic acids from urine is currently accomplished either by solvent extraction [1—5] or by anion-exchange chromatography [6—10]. Solvent extraction is performed by extraction of acidified, salt-saturated urine with ethyl acetate and/or diethyl ether. The advantages of the solvent-extraction method are (1) simple and rapid sample preparation; (2) efficient extraction of aromatic acids; and (3) minimal extraction of phosphoric acid and sulphuric acid [11,12]. The disadvantages of the method are (1) extraction of neutral compounds such as glycerol and urea; and (2) inefficient extraction of polyhydroxy acids [11,12].

In DEAE-Sephadex anion-exchange chromatography, neutral and basic compounds are washed through the column first and the acidic components are eluted with aqueous pyridinium acetate. In order to remove large amounts of sulphuric acid and phosphoric acid, barium hydroxide precipitation should be performed before anion-exchange chromatography [12]. The advantages of

anion-exchange chromatography are (1) extraction of more organic acids, including polyhydroxy acids; (2) no extraction of neutral compounds, such as glycerol and urea; and (3) more precise extraction than with solvent extraction [12]. The disadvantages of the method are (1) the time required for lyophilization of the eluate; (2) the extraction of phosphoric acid even after barium hydroxide precipitation; (3) poor recovery of tricarboxylic acids, such as citric acid and aconitic acid, due to precipitation with barium hydroxide; (4) a tendency for aldonic acids to lactonize; and (5) difficulty in drying the sample completely [11].

In routine clinical use, the isolation of organic acids by solvent extraction is better because it is faster. Ion-exchange chromatography, however, must be used in studies with polyhydroxy acids. Alternative approaches for isolation of organic acids have been reported.

Organic acids can be isolated using solid-phase extraction tubes (Jetubes) filled with an inert cellulose gauze matrix [13]. Acidified urine is transferred to the tube. The organic acids are eluted with a diethyl ether—ethyl acetate (1:1) mixture. The eluate is dried and evaporated. The advantages of this method are (1) simple and rapid sample pretreatment; and (2) recovery and reproducibility, for most organic acids, equal to or better than solvent extraction.

Organic acids are isolated from urine and amniotic fluid by adsorption of the acids on silica gel [14]. After acidification of the sample, the organic acids are adsorbed onto the silica gel. The silica gel is packed into a column, and the organic acids are eluted with a *tert.*-amyl alcohol—chloroform—diethyl ether (1:0.8:0.2) mixture under pressure in a nitrogen atmosphere. The advantages of the method are (1) prior deproteinization is not required; (2) recovery of organic acids, including citric acid, is better than with solvent extraction; and (3) extraction of phosphoric acid and sulphuric acid is not marked. However, the recovery of organic acids by the silica gel method was not compared with that by DEAE-Sephadex anion-exchange chromatography.

Organic acids are isolated from amniotic fluid by liquid partition chromatography [15]. The column is packed with silicic acid hydrated with sulphuric acid. After rinsing the column with 10% 2-methyl-2-butanol in chloroform, the sample is placed on the column and eluted with this solvent. The advantages of liquid partition chromatography are (1) prior deproteinization is not required; and (2) separation of the desired acids from unwanted acids, such as lactic acid which is normally present in a high concentration in amniotic fluid. The recovery of organic acids and the reproducibility, however, have not yet been determined.

Organic acids can be extracted by the Extrelut column (Merck) [16]. The acidified urine is poured into the column and the acids are eluted with ethyl acetate. The advantages of the method are (1) prior deproteinization is not required; (2) quick extraction time; and (3) better reproducibility than with solvent extraction. One drawback of the method is the inefficient extraction of polar acids, such as polyhydroxy acids.

These alternative approaches for the isolation of organic acids, however, have not been widely used.

Oximation [1,9,12], methoximation [8,14] or ethoximation [7,17] of keto acids before isolation of organic acids increases the recovery of these unstable acids.

Before GC—MS analysis, further separation of the extracted organic acid fraction is sometimes performed by thin-layer chromatography [18—22], silicic acid column chromatography [19,23], or HPLC [24], to detect minor components.

2.2. *Short-chain fatty acids*

Short-chain (C_2—C_8) fatty acids are volatile and therefore require special isolation methods. They are usually analysed without derivatization by GC—MS, and their isolation methods include steam distillation [25—28], vacuum distillation [28—31], partition chromatography on silicic acid [32], solvent extraction [33—36] and a direct injection method [37,38]. The recovery and precision of vacuum distillation were found to be superior to those of steam distillation [28]. Vacuum distillation is a faster and simpler method than steam distillation.

Short-chain fatty acids are also extracted using partition chromatography on silicic acid. This isolation method yields faster results than with the distillation methods. By this method, lactic acid and methylmalonic acid do not interfere with the analysis of short-chain fatty acids. The recovery of short-chain fatty acids from aqueous solutions was 65—150% and the precision measured as the coefficient of variation was 1—27% [32]. The recovery and precision were comparable to those of the vacuum distillation method.

Short-chain fatty acids are isolated from acidified, salt-saturated urine by diethyl ether extraction. The collected organic layer is alkalinized with methanolic sodium hydroxide and evaporated to dryness [33]. Although the solvent-extraction method is simple and rapid, the extract contains interfering compounds such as lactic acid and methylmalonic acid.

The direct injection method is a very rapid sampling procedure. After addition of the internal standard, 1 ml of urine is adjusted to pH 1, and a 1-μl aliquot is injected directly onto the GC column [38]. The life span of the column, however, will become very shortened due to the contamination of non-volatile compounds. The recovery and precision of the analysis are not determined.

2.3. *Volatiles*

The volatiles include ketones, aldehydes, alcohols, furan and pyrrole derivatives, other heterocyclic compounds, isocyanates, isothiocyanates, sulphides and hydrocarbons with 1—12 carbons and low boiling points ($<300°C$). Volatiles are thermally stable and analysed by GC—MS without derivatization.

Isolation techniques include (1) dynamic head-space sampling, with adsorption onto a porous polymer; (2) solvent extraction; (3) a transevaporator sampling procedure; and (4) a direct injection technique.

Dynamic head-space sampling has been applied to the analysis of urine [39—47], serum [48—51], tissue [52] and cerebrospinal fluid [53], and is best suited to the analysis of samples of unrestricted volume. The sample volume range is from 25 to 250 ml. Ammonium sulphate is added to the sample to increase the volatility. The mixture is heated to 90°C and stirred with a mag-

netic stirrer while helium is passed over the sample. The volatiles are carried away with the helium through a water-cooled condenser into a glass tube containing Tenax GC (a porous polymer of 2,6-diphenyl-*p*-phenylene oxide). The sampling time is usually 1 h. The technique is semiquantitative but reproducible. One disadvantage of the technique is the large sample volume required for analysis. The technique is not suited to the analysis of serum, since the volume required is too large for clinical examination. A gas-phase stripping apparatus was developed to improve the recovery of the volatiles from small volumes of biological fluids [54]. With this apparatus, helium is introduced from the base and passed through the sample to provide maximum surface contact between the gas and the liquid phases. The sample is contained in a glass tube packed with glass beads to avoid excessive formation of foam. A water-cooled condenser is attached to the glass tube, and a Tenax trap is attached to the condenser. The glass tube is heated to 55°C and the sampling time is usually 30 min. Even with this apparatus, a sample volume of several millilitres is required.

A solvent-extraction technique has been applied to the analysis of urine [55—57], plasma [57—59], tissue [59], breast milk and amniotic fluid [57]. The volatiles are extracted with a small volume of solvent such as diethyl ether. An advantage of the microscale solvent-extraction technique is the small sample volume, from 20 μl to 2 ml, required for the analysis. The disadvantages are (1) very pure solvents should be used; (2) evaporation of the solvent causes a loss of extracted volatiles with low boiling points; and (3) no one solvent is satisfactory for extraction of the diverse range of volatiles.

A transevaporator sampling technique was developed for microscale sampling, and has been applied to the analysis of urine, serum, cerebrospinal fluid, amniotic fluid and breast milk [60—64]. The transevaporator sampling apparatus contains a microcolumn packed with Porasil E (pore silica gel). The sample is injected into the microcolumn, which retains water, high-molecular-weight compounds and inorganic salts. The transevaporator is used in two modes: (1) modified head-space and (2) solvent extraction. In the modified head-space mode, helium is passed through the microcolumn to carry away the volatiles, which are then collected on a Tenax trap. In the solvent-extraction mode, helium is used to force the solvent, e.g. 2-chloropropane, through the microcolumn, thereby extracting the volatiles which are then collected on a glass bead trap. An advantage of the transevaporator sampling technique is the small sample volume ranging from 5 to 500 μl.

A direct injection method has been applied to the analysis of volatiles in plasma and cerebrospinal fluid [65]. A 100-μl sample is injected directly, water is removed in a condenser at 0°C, and volatiles are subsequently trapped and concentrated on a small, cooled, precolumn packed with Tenax GC [65] or a stainless-steel capillary tube immersed in liquid nitrogen [66]. The recovery of low-molecular-weight volatiles using liquid nitrogen is improved over the method using Tenax. The direct injection method is simple, rapid and quantitative for the analysis of volatiles in body fluids.

2.4. Sugars and polyols

Sugars and polyols are isolated from urine by deionization through a cation-

exchange resin and an anion-exchange resin [67—69]. A mixed resin such as an Amberlite MB-3 column is useful for isolating sugars and polyols. After evaporation of the eluate, the isolated polyols are usually converted to trimethylsilyl (TMS) [70,71] or acetylated derivatives [68,69,72,73].

When urine contains a considerable amount of glucose, glucose is removed by treating the urine with glucose oxidase and catalase and allowing oxygen to bubble through the solution. Gluconate formed during the procedure is removed by shaking with ion-exchange resins [68]. Since this method is time-consuming and laborious, and the enzymes should be removed before deionization, this method for the removal of glucose is not widely used.

Polyols have been isolated from urine [67,68], serum or plasma [67,68,70—74], and cerebrospinal fluid [69,74].

3. METHODS FOR ISOLATION FROM PROTEIN-CONTAINING FLUIDS AND TISSUES

Organic acids and polyols are usually extracted from serum or other protein-containing fluids after deproteinization. Deproteinization is usually carried out by membrane ultrafiltration [67,75,76] or by precipitation with ethanol [71], methanol [73], isopropanol [21], dichloromethane [77] or perchloric acid [68,69]. Membrane ultrafiltration through a cone filter [67,72] is a simple deproteinization method. The membrane filter should be washed with distilled water before use as it contains considerable amounts of glycerol. If the membrane filtration method is used, the serum ultrafiltrate obtained is free of protein-bound organic acids. In order to obtain a large amount of plasma ultrafiltrate, Thoma et al. [76] used a two-step ultrafiltration method. The interfering cell-wall components and proteins were removed by ultrafiltration using a filter with rather large pores, followed by a second ultrafiltration with a filter having smaller pores. The advantages of this method are (1) the system can separate acids not only from plasma and serum but also from whole blood; and (2) a large amount of ultrafiltrate can be obtained. The disadvantages of the method are the time and effort it requires. To extract total organic acids from protein-containing fluids, the method of choice for deproteinization is precipitation with an organic solvent such as ethanol [71]. The precipitated protein is removed by centrifugation and the supernatant is concentrated to remove the organic solvent.

The deproteinized aqueous solution is treated in the same way as urine, or directly evaporated [73,75]. Although the direct evaporation after deproteinization is a rapid and reproducible method, the residue contains a variety of compounds such as organic acids, polyols and amino acids.

Ng et al. [77] isolated organic acids from serum by deproteinization with dichloromethane and further extraction with dichloromethane. They identified a number of long-chain dicarboxylic acids in serum. By using membrane ultrafiltration instead of dichloromethane precipitation for deproteinization of serum, these less polar protein-bound acids could hardly be detected.

Liebich et al. [21] isolated organic acids from uraemic plasma by deproteinization with isopropanol and then by anion-exchange chromatography. They found a marked increase of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid in the uraemic plasma as compared with normal serum. The less polar and

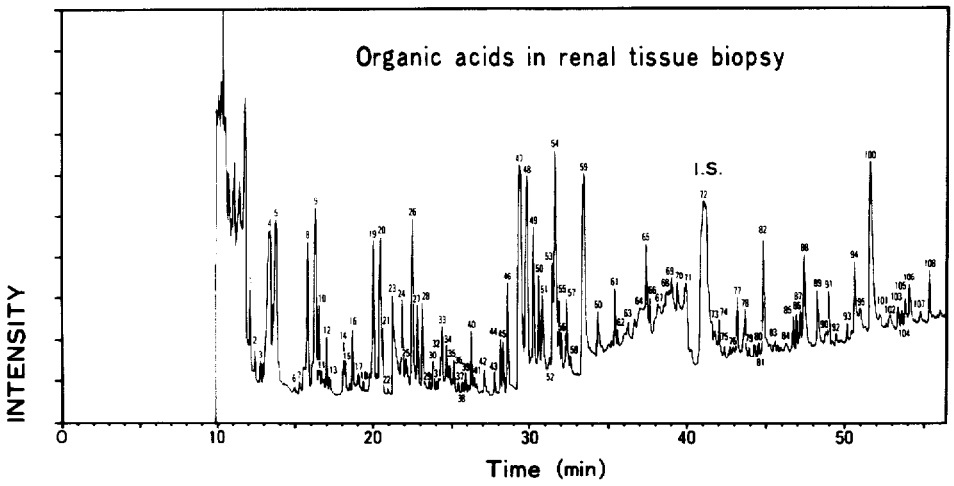

Fig. 1. Gas chromatogram of methoxime-TMS derivatives of organic acids isolated from 1 mg of renal tissue (wet weight). The renal tissue was obtained by biopsy from a patient with chronic glomerulonephritis to determine the histological type of the glomerular lesion. GC conditions: OV-101 open-tubular glass capillary column (30 m), splitless injection, 70—250°C, 3°C/min. The peaks were identified as follows: 3 = pyruvic acid, 4 = lactic acid, 5 = 2-hydroxyisobutyric acid, glycolic acid, 8 = 2-hydroxybutyric acid, 9 = 3-hydroxypropionic acid, 12 = 3-hydroxybutyric acid, 19 = 4-hydroxybutyric acid, 20 = diethylene glycol (artifact), 25 = phosphoric acid, 26 = glycerol, 27 = 4-hydroxy-2-butenoic acid, 28 = succinic acid, 30 = methylsuccinic acid, 33 = 2-methylglyceric acid, 34 = glyceric acid, 36 = nonanoic acid, 37 = 4-deoxyerythronic acid, 42 = glutaric acid, 45 = 3-deoxytetronic acid, 47 = 2-deoxytetronic acid, 59 = 2,3-dideoxypentonic acid, 71 = isosaccharino-1,4-lactone. (Reproduced from ref. 80.)

protein-bound acids may be undetectable if membrane ultrafiltration is used instead of isopropanol precipitation.

Short-chain fatty acids are usually isolated from serum or other protein-containing fluids without prior deproteinization by steam distillation [25,27, 28], vacuum distillation [31], solvent extraction [36] or direct injection [37,38]. However, deproteinization with perchloric acid is used to isolate the short-chain fatty acids by silicic acid column chromatography [32]. Deproteinization yielded poor recovery of the short-chain fatty acids, probably due to their coprecipitation with protein during deproteinization [31].

Volatiles are isolated from serum or other protein-containing fluids without deproteinization, and the samples are treated in the same way as urine.

The organic acids in tissues are extracted after homogenization and deproteinization with ethanol. After centrifugation, the supernatant is concentrated to remove ethanol. The resulting solution is treated in the same way as urine. Organic acids in heart muscle [78,79], kidneys [80,81] and several other tissues [82] were analysed by GC—MS. Since the GC—MS profiling method is highly sensitive, extremely small samples obtained by biopsy from patients can be analysed (Fig. 1).

4. DERIVATIZATION METHODS

Organic acids are usually converted to TMS [2,5,11,13,15], oxime-TMS [1,4,10,12], methoxime-TMS [9,14], ethoxime-TMS [8,17] or methyl ester

[16,18—20,24] derivatives. Trimethylsilylation is easy to perform, and the derivatives are well separated on a GC column and usually provide $(M-CH_3)^+$ ion peaks. For routine clinical use, trimethylsilylation may be the preferred method. However, TMS derivatives are not stable on storage, and the mass fragmentation of TMS derivatives is often difficult to interpret and sometimes does not provide enough information to elucidate the structure of new compounds.

tert.-Butyldimethylsilylation of organic acids is reported to have good analytical potential [83]. Compared with TMS derivatives, *tert.*-butyldimethylsilyl derivatives offer the advantages of (1) much higher hydrolytic stability; (2) better separation on GC; and (3) an intense molecular-weight-indicative peak $(M-57)^+$. The disadvantages of *tert.*-butyldimethylsilylation are (1) fewer mass spectral data for organic acids; (2) increased retention time on GC, particularly for polyhydroxy acid; and (3) difficult silylation of the hydroxyl group with steric hindrance.

Methylation with diazomethane is easy to perform, the derivatives are stable and their mass fragmentation provides sufficient information for structural identification [20]. Diazomethane reacts with carboxylic groups and phenolic hydroxy groups and may react with carbonyl compounds and olefinic double bonds [84]. Methylation of hydroxy groups, such as in hydroxydicarboxylic acids, may be recognized. Methylation with diazomethane also provides side-reactions such as N-methylation and N,N-dimethylation of amino acids [85]. Methylation with diazomethane is not suited to the analysis of polyhydroxycarboxylic acids, since the hydroxy groups are not methylated and the derivatized compounds do not pass through the GC column [16]. Identified methoxy ring-substituted aromatic acids are often difficult to interpret, because phenolic hydroxy groups can be methylated. The methoxy group in the aromatic acid may be a methylated hydroxy group or a natural methoxy group. By using deuterated diazomethane, the methylated aromatic hydroxy group can be discriminated from the natural methoxy group.

TMS ether—methyl ester derivatization of organic acids is sometimes used to identify unknown compounds [23]. For example, if the retention time of a methylated compound changes after trimethylsilylation, the compound contains hydroxy or other functional groups.

Dicyclohexyl ester derivatization of aliphatic dicarboxylic acids is used to identify and quantitate dicarboxylic acids in the urine of patients [86]. The dicyclohexyl esters are stable for days at room temperature, and their electron impact (EI) mass spectra exhibit a prominent $(acid+H)^+$ ion and an $(acid+H-18)^+$ ion for selected ion monitoring (SIM). However, the derivatization method is not widely used.

Keto acids are more readily determined as oxime-TMS [1,4,10,12], methoxime-TMS [9,14], ethoxime-TMS [8,17] or methoxime-methyl ester [21,22] derivatives. Oximation, methoximation or ethoximation of short-chain keto acids before solvent extraction or ion-exchange chromatography can prevent the loss of these unstable acids. Methoxime-TMS or ethoxime-TMS are particularly preferable, because the derivatized keto acids usually provide $(M-15)^+$ and $(M-31)^+$ or $(M-45)^+$, suggesting molecular ions and the presence of keto groups. Aliphatic and aromatic 2-keto acids can be analysed as O-tri-

methylsilylquinoxalinol derivatives [87—90]. 2-Keto acids are unstable, and decompose, dimerize and decarboxylate easily. O-TMS-quinoxalinol derivatization is applied to the analysis of phenylpyruvic acid and 4-hydroxyphenylpyruvic acid [87] and 2-ketoglutaric acid [88—90]. Since the O-TMS-quinoxalinols are stable and their EI mass spectra exhibit intense molecular ions, the derivatization method is suited for the quantitation of 2-keto acids.

5. PROFILING WITH A GAS CHROMATOGRAPH—MASS SPECTROMETER—COMPUTER SYSTEM

5.1. Profile analysis

The concept of metabolic profiling using GC—MS was first proposed by Horning and Horning [91]. They applied this method to the analysis of steroids, organic acids and drug metabolites in urine. These metabolic profiles were used in various ways, including studies of pathological conditions and drug metabolism, as well as in human developmental studies.

Metabolic profiling analysis by GC—MS can detect and identify a number of metabolites simultaneously. Sweeley and co-workers have developed an automated metabolic profiling method for organic acids using a GC—MS—COM system [10,92,93]. Identification and quantitation of compounds are performed by a computer program (MSSMET). GC retention indices of peaks are obtained from simultaneous analysis of hydrocarbons. This program uses a reverse library search procedure, which tries to find characteristic fragment ions of given metabolites in their predetermined retention index ranges. The relative intensity of the characteristic fragment ion is used for quantitation. This completely automatic profiling method can detect and quantitate over 150 metabolites in a single sample and has been applied to the analysis of urinary organic acids in healthy subjects and patients with neuroblastoma, alkaptonuria and juvenile diabetes [92,93]. Mizuno et al. [94] developed another automatic GC—MS—COM system, which uses a 'Diagnosis Computer Program" for analysis of urinary organic acids. In this program, the profile of urinary organic acids from a patient with an inherited metabolic disorder is compared with a normal profile. Metabolic disorders are diagnosed by identification and quantitation of abnormal acids in the urine. The identification of the acids is performed by a library search. Using this program, 26 inherited metabolic disorders can be diagnosed. The number of peaks detected in the gas chromatograms are often too many to be identified completely. McConnell et al. [43] developed threshold logic units, a form of non-parametric pattern recognition, to distinguish between normal and pathological profiles of gas chromatograms and to designate the most significant profile components in the distinction between normal and pathological profiles. By this pattern recognition method, identification need only be performed for those compounds found to be significant in the distinction.

Metabolic profiling has been used with great success, especially in the field of inherited metabolic diseases [95—98]. So far, more than 30 new organic acidurias were discovered by GC—MS, as listed in Table 1. The altered metabolism in the diseases, as well as normal metabolism, has been elucidated by

TABLE 1

ORGANIC ACIDURIAS THAT CAN BE DIAGNOSED BY GC—MS PROFILING ANALYSIS

Organic acidurias	Increased acidic metabolites in urine and/or serum	Reference
Carnitine deficiency	Adipic acid, pimelic acid, heptenedioic acid, suberic acid, octenedioic acid	103
Dihydrolipoyl dehydrogenase (E_3) deficiency	Lactic acid, 2-hydroxyisovaleric acid, 2-ketoisocaproic acid, 2-hydroxyisocaproic acid, 2-ketoglutaric acid, 2-hydroxyglutaric acid, 2-hydroxybutyric acid	104
Dihydropyrimidine dehydrogenase deficiency	Uracil, thymine	105,106
Ethylmalonic-adipic aciduria	Ethylmalonic acid, adipic acid, hexanoylglycine, methylsuccinic acid, glutaric acid	107,108
Fumaric aciduria	Fumaric acid	109
Glutaric aciduria type I	Glutaric acid, glutaconic acid, 3-hydroxyglutaric acid, adipic acid	110,111
Glutaric aciduria type II	Glutaric acid, isobutyric acid, isovaleric acid, 2-methylbutyric acid, butyric acid, ethylmalonic acid adipic acid, suberic acid, sebacic acid, 3-hydroxyisovaleric acid, methylsuccinic acid, 2-hydroxyisobutyric acid, 2-hydroxyglutaric acid, lactic acid, isobutyrylglycine, isovalerylglycine, 2-methylbutyrylglycine	3,33,112
D-Glyceric aciduria	D-Glyceric acid	113
L-Glyceric aciduria (primary hyperoxaluria type 2)	L-Glyceric acid, oxalic acid	114
Glyceroluria	Glycerol	115,116
4-Hydroxybutyric aciduria	4-Hydroxybutyric acid, succinic semialdehyde	4,117,118

TABLE 1 (continued)

Organic acidurias	Increased acidic metabolites in urine and/or serum	Reference
2-Hydroxyglutaric aciduria	2-Hydroxyglutaric acid	119
3-Hydroxy-3-methylglutaryl CoA lyase deficiency (3-hydroxy-3-methylglutaric aciduria)	3-Hydroxy-3-methylglutaric acid, 3-methylglutaconic acid, 3-hydroxyisovaleric acid, 3-methylglutaric acid, 3-methylcrotonylglycine	120—122
4-Hydroxyphenylacetic aciduria	4-Hydroxyphenylacetic acid	123,124
Isovaleric acidaemia	Isovaleric acid, isovalerylglycine, 3-hydroxyisovaleric acid, 4-hydroxyisovaleric acid, mesaconic acid, methylsuccinic acid, 3-hydroxyisoheptanoic acid, N-isovalerylglutamic acid, isovaleryl-β-D-glucuronide, N-isovalerylalanine, N-isovalerylsarcosine	125—133
Jamaican vomiting sickness	Ethylmalonic acid, methylsuccinic acid, glutaric acid, adipic acid, suberic acid, sebacic acid, 3-hydroxyisovaleric acid, 4-octenedioic acid, 4-decenedioic acid, butyrylglycine, isovalerylglycine, hexanoylglycine	134,135
2-Ketoadipic aciduria	2-Ketoadipic acid, 2-hydroxyadipic acid, glutaric acid, 2-ketoglutaric acid	136,137
2-Ketobutyrate dehydrogenase deficiency	2-Hydroxybutyric acid	138
2-Ketoglutaric aciduria	2-Ketoglutaric acid	96
3-Ketothiolase deficiency (2-methyl-3-hydroxybutyric aciduria)	2-Methyl-3-hydroxybutyric acid, 2-methylacetoacetic acid, tiglylglycine, 2-methylglutaconic acid	139,140,178
Lactic aciduria	Lactic acid, pyruvic acid, 2-hydroxybutyric acid, acetoacetic acid, 3-hydroxybutyric acid, 2-ketoglutaric acid, C_6—C_{12} dicarboxylic acids, monoenic C_{10}—C_{14} dicarboxylic acids	141—149

(Continued on p. 324)

TABLE 1 (continued)

Organic acidurias	Increased acidic metabolites in urine and/or serum	Reference
Maple syrup urine disease	2-Hydroxyisovaleric acid, 2-ketoisovaleric acid, 2-hydroxy-3-methylvaleric acid, 2-keto-3-methylvaleric acid, 2-hydroxyisocaproic acid, 2-ketoisocaproic acid, 2-ketoglutaric acid	3,150,151
3-Methylcrotonyl CoA carboxylase deficiency (3-methylcrotonylglycinuria)	3-Hydroxyisovaleric acid, 3-methylcrotonylglycine, 3-methylcrotonic acid	152,153
Methylmalonic aciduria	Methylmalonic acid, 3-hydroxyvaleric acid, 3-ketovaleric acid, methylcitric acid, 3-hydroxypropionic acid, 2-methyl-3-hydroxybutyric acid, 2-methylacetoacetic acid, tiglylglycine, 2-methylglutaconic acid, lactic acid, 3-hydroxybutyric acid	3,15,154—157,178
3-Methylglutaconic aciduria	3-Methylglutaconic acid, 3-hydroxyisovaleric acid, 3-methylglutaric acid	158—160
Mevalonic aciduria	Mevalonic acid	161
Multiple carboxylase deficiency	3-Hydroxyisovaleric acid, 3-methylcrotonylglycine, tiglylglycine, 3-hydroxypropionic acid, methylcitric acid, lactic acid	3,162,163
Non-ketotic dicarboxylic aciduria	Adipic acid, suberic acid, sebacic acid, decenedioic acid, suberylglycine, hexanoylglycine, octanoylglycine, decanoylglycine, 5-hydroxyhexanoic acid, 7-hydroxyoctanoic acid, 9-hydroxydecanoic acid	164—170
Ornitine transcarbamylase deficiency	Uracil, orotic acid	171
Orotic aciduria	Orotic acid	172
Phenylketonuria	Phenylacetic acid, phenyllactic acid, phenylpyruvic acid, phenylacetylglutamine, phenylethyleneglycol	3,173,174
Primary hyperoxaluria type 1	Oxalic acid, glycolic acid, glyoxylic acid	175

TABLE 1 (continued)

Organic acidurias	Increased acidic metabolites in urine and/or serum	Reference
Propionic acidaemia	Propionic acid, methylcitric acid, 3-hydroxypropionic acid, 2-methylacetoacetic acid, 2-methyl-3-hydroxybutyric acid, 3-ketovaleric acid, 3-hydroxyvaleric acid, 2-methyl-3-ketovaleric acid, 2-methyl-3-hydroxyvaleric acid, 2-hydroxy-2-methylsuccinic acid, propionylglycine, tiglic acid, tiglylglycine, 2-methylbutyrylglycine, 2-methylglutaconic acid, lactic acid, glutaric acid, 3-hydroxyisovaleric acid, 3-methylglutaconic acid, 3-hydroxy-3-methylglutaric acid, 3-ethyl-3-hydroxyglutaric acid, maleic acid, methyl ethyl ketone	2,3,5,176–181
Pyroglutamic aciduria	Pyroglutamic acid	182
Refsum's disease	2,6-Dimethylsuberic acid, 3-methyladipic acid, (phytanic acid)	183–185
Tyrosinaemia	4-Hydroxyphenylacetic acid, 4-hydroxyphenyllactic acid, 4-hydroxyphenylpyruvic acid, succinylacetone, succinylacetoacetate, 4-keto-6-hydroxyheptanoic acid	186,187

metabolic profiling of organic acids as reviewed by Jellum [99]. Currently, the use of metabolic profiling has become routine in the diagnosis of these inborn metabolic disorders.
by Jellum [99]. Currently, the use of metabolic profiling has become routine in the diagnosis of these inborn metabolic disorders.

Normal urinary metabolic profiles have also been intensely investigated to find out more about various types of physiological metabolism [7,100,101]. Spiteller and Spiteller [20] detected some 500 acidic compounds in normal urine and characterized ca. 200 compounds by their mass spectra, including newly identified urofuran acids. They also identified ethyl, isopropyl and butyl derivatives of malic acid, 3-propyl-substituted 3-hydroxyglutaric acid, 3-hydroxy-3-(methylcarbomethoxy)-adipinic acid and two isomeric 2-methylcitric acids [102].

5.2. Structural identification of compounds

Identification of compounds is usually based on retention times (or other retention indices such as methylene units) and EI mass spectra. Tanaka et al. [188] reported retention indices, in terms of methylene units, on 10% OV-1

and 10% OV-17 columns of TMS derivatives for more than 155 important metabolic acidic compounds. These retention indices were useful to identify abnormal acids and to diagnose organic acidurias only by GC [3]. In those cases in which the fragment pattern of a compound is known, low-resolution EI-MS is sufficient. In these cases, the major fragments of the compounds are compared with the fragments already in a computer [189,190] or in a text. In those cases where a compound is unknown, chemical ionization (CI) and high-resolution EI analyses are often performed. The molecular weight of the compound is obtained by CI-MS [191], and the elemental composition of the fragment ions is obtained by high-resolution MS. Burlingame and co-workers developed a high-resolution GC—high-resolution MS system [192] and applied it to the analysis of urinary organic acids [193]. The identification of quinic acid and other compounds in normal urine by this high-resolution GC—high-resolution MS system demonstrated the potential application of this system in a combination with a normal GC—MS system. If needed, the derivatization method may be changed. For example, EI mass spectra of methylated derivatives are usually more informative for structural identification than those of TMS derivatives [20]. Deuterated silylating reagent or deuterated diazomethane [85] is sometimes utilized to interpret the fragmentation pattern. Alkyldimethylsilylating reagents such as dimethylethylsilylimidazole, *n*-propyldimethylsilylimidazole and *tert.*-butyldimethylsilylimidazole are also used to obtain information on molecular ion and mass fragmentation [194]. In cases where a compound cannot be identified by these methods, the compound is isolated by preparative GC and analysed by another form of instrumental analysis such as nuclear magnetic resonance (NMR) spectroscopy.

5.3. Quantitation of compounds

Quantitation of a compound is based on the ratio of the peak area or height of the compound and internal standard. The internal standard is usually a chemical analogue of the analyte with closely similar extraction and derivatization behaviour, isomers, or compounds labelled with stable isotopes. The ratio of the peak area or height is obtained from a total or reconstructed ion-monitoring chromatogram, mass chromatogram or SIM chromatogram [195]. High-resolution GC separation is usually used to quantitate compounds in biological complex mixtures from total or reconstructed ion-monitoring chromatograms. However, complete separation of the mixtures is often difficult, even with high-resolution GC. The preferred quantitation method for metabolic profiling is mass chromatography, which is used in the automated GC—MS—COM system [10,92,93]. The most sensitive method for quantitation is SIM. This method is, however, limited to quantitation of a few compounds and is not suited to metabolic profiling analysis. During analysis of biological extracts, this high sensitivity may not be realized due to interference from other mixture components, leading to a high background signal and overlapping peaks. High-resolution SIM achieves greater selectivity and increased signal-to-noise ratio [196].

5.4. Compounds interfering in profiling

GC—MS profiling analysis is often associated with interfering compounds

which include contaminants, metabolites of drugs or other exogenous compounds, and artifacts produced in the sample work-up, derivatization and GC analysis. Jellum described many artifacts and pitfalls in the GC—MS profiling of organic acids and short-chain fatty acids in his review [99]. Goodman and Markey [97] listed the artifact ions and their sources in the GC—MS profiling of organic acids. Ende and Spiteller [197] reviewed contaminants in mass spectrometry. Contaminants are compounds inadvertently introduced from the environment into a sample, and are present in organic solvents, plastic containers, plastic tubing materials, filter papers, stoppers, lubricants, sorbents, the liquid phase in GC, reagents, etc. Plastic containers contain a variety of plasticizers such as dialkylphthalates, esters of aliphatic dicarboxylic acids, trialkyl or triaryl esters of phosphoric acids, trialkyl citrates and butyl epoxystearate.

Brown et al. [198] detected acetyltributylcitrate and di(2-ethylhexyl)-phthalate in the acid fraction of urine from patients with undiagnosed metabolic disorders. These compounds are plasticizers arising from poly(vinyl chloride) transfusion tubing and plastic storage containers. An Extrelut column contains a variety of contaminants including phthalates, resin acids, long-chain saturated alcohols and aliphatic acids [199]. These contaminants are detected in the organic acid fraction of biological material after a work-up procedure with an Extrelut column. To distinguish the contaminants from the compounds originally present in a sample, a blank using the same reagents and materials in the same amounts as with the sample should be analysed.

Metabolites of drugs or other exogenous compounds cause difficulty in interpreting the GC—MS profiling data. For example, intravenous administration of calcium levulinate leads to urinary excretion of 4-ketopentanoic acid and 4-hydroxypentanoic acid [200]. These acids, with respect to retention times and mass spectra, closely resemble 2-methylacetoacetic acid and 2-methyl-3-hydroxybutyric acid, the two acids found in patients with 3-ketothiolase deficiency. Metabolites of exogenous compounds of dietary sources are excreted into the urine, obscuring the other diagnostically important metabolites. Patients fed with a synthetic diet flavoured with 3-ethoxy-4-hydroxybenzaldehyde excrete large amounts of 3-ethoxy-4-hydroxybenzoic acid and traces of 3-ethoxy-4-hydroxymandelic acid into the urine [201]. These metabolites obscure a useful portion of organic acid profile from consideration.

Kamerling et al. [202] identified (2-ethoxyethoxy)acetic acid in the urine of patients with a suspected metabolic disorder. The acid is assumed to be a metabolite of an exogenous precursor, 2-(2-ethoxyethoxy)ethanol. Patients fed with medium-chain triglycerides excrete large amounts of C_6—C_{10} dicarboxylic acids and considerable amounts of 5-hydroxyhexanoic acid and 7-hydroxyoctanoic acid [203]. The urinary profile of organic acids closely resembles that in non-ketotic dicarboxylic aciduria. Shinka et al. [204] detected benzoylalanine in the urine of hyperammonaemic patients treated with sodium benzoate. This abnormal compound is assumed to be derived from endogenous conjugation of benzoyl coenzyme A (CoA) with alanine.

Artifacts produced in the work-up procedure, derivatization and GC analysis often lead to misinterpretation of the GC—MS profiling data. Diazomethane used as a methylating agent may produce artifacts from unsaturated dicarbox-

ylic acids by reacting with the double bond in samples which are kept for some hours before GC—MS analysis [84]. With fumaric acid, the production of three artifacts is so rapid that methylation using diazomethane is not suited for its derivatization. Spontaneous decarboxylation by heat occurs at the injection port of the gas chromatograph. 3-Methylcrotonic acid and 3-methyl-3-butenoic acid were produced from 3-methylglutaconic acid, presumably at the injection port of the gas chromatograph [205,206]. Tanaka [207] reported artificial production of isovaleric acid from 2-ketoisocaproic acid. Butanone was formed by spontaneous decarboxylation of 2-methylacetoacetic acid in aqueous solution at room temperature [208].

Intestinal bacteria sometimes play a role in the formation of artifacts. Ketting et al. [209] detected lactyl lactate and succinyl lactate in the urine of patients screened for inherited metabolic diseases. Since urinary D-lactate is considered to be an exogenous product formed by intestinal bacteria, the occurrence of these compounds containing D-lactate suggests that the compounds are artifacts formed from bacterial D-lactate.

6. CLINICAL APPLICATION OF PROFILING

6.1. Uraemia

In patients with end-stage renal disease, many metabolites are retained in the blood, causing various uraemic symptoms. The clinical state of uraemic patients can be improved by dialysis of blood to remove toxic metabolic products that would normally be excreted by the kidney. Recently, GC—MS profiling analysis has been used to identify such abnormally accumulated metabolites in uraemic blood (Table 2).

Dowty et al [50] analysed low-molecular-weight volatile organics in the uraemic plasma before and after haemodialysis using high-resolution GC—MS. The volatile compounds were collected on Tenax GC adsorbent prior to analysis. About 100 volatiles could be monitored by the method, and several compounds, such as methyl mercaptan, 2-butanone, pyridine, dipropyl ketone and cyclohexanone, were found to be accumulated in the uraemic plasma.

Liebich and Wöll [51] used the same adsorbent to analyse the volatile compounds in the uraemic plasma by high-resolution GC—MS. They quantitated the concentration of total 4-heptanone by computer SIM and demonstrated that the concentrations were 10- to 40-fold higher in the serum of patients with chronic renal failure as compared with those in normal serum.

Bultitude and Newham [210] used gel permeation chromatography to analyse the non-volatile compounds in the uraemic plasma by GC—MS. Fractionation of the metabolites by gel permeation chromatography (Sephadex G-10) was followed by lyophilization and trimethylsilylation. They identified glycerol, erythritol, erythronic acid, 2-deoxyerythropentonic acid, arabitol, arabinonic acid, inositol and lactose, which appeared to be specific to or increased in the uraemic plasma. Gel permeation chromatography was used to remove the plasma protein and to give an initial fractionation of the metabolites. Since gel permeation chromatography is a laborious and time-consuming sample pretreatment procedure, it is not appropriate for routine metabolic profiling and quantitative determination.

TABLE 2

ACCUMULATED METABOLITES IN URAEMIC PLASMA IDENTIFIED BY MS

Accumulated metabolites		Reference
Volatiles	Methyl mercaptan, acetone, 2-butanone, chloroform, carbon tetrachloride, benzene, toluene, pyridine, dipropyl ketone, cyclohexanone, 4-heptanone	50,51
Organic acids	Erythronic acid, 2-deoxyerythropentonic acid, tartaric acid	75,210
	Glyceric acid, 2-methylglyceric acid, 4-deoxyerythronic acid, 4-deoxythreonic acid, 3-deoxytetronic acid, citramalic acid, malic acid, 2-hydroxyglutaric acid, 3-deoxy-2-C(hydroxymethyl)tetronic acid	
	2-Ketoglutaric acid	90
	Glutaric acid, 3-methylglutaric acid, 3-methylglutaconic acid, adipic acid, 3-methyladipic acid, 3,4-methyleneadipic acid, 2,4-dimethyladipic acid, pimelic acid, 3-methylsuberic acid, azelaic acid, 5-decynedioic acid	21
	3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid, 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid	21
	3-Hydroxybenzoic acid, 4-hydroxybenzoic acid, 2-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, vanillic acid, 4-hydroxymandelic acid, homovanillic acid, vanilmandelic acid	213,214
Conjugates	Phenylacetylglutamine, phenylacetylglutamic acid	16
	Furoylglycine, hippuric acid, 3-hydroxyhippuric acid, 4-hydroxyhippuric acid	21
Phenols	Phenol, *p*-cresol, catechol, resorcinol, hydroquinone, homocatechol, 2-methoxyresorcinol, 3-methoxycatechol, methoxyhydroquinone	215
Polyols	Glycerol, erythritol, threitol, arabitol, mannitol, sorbitol, chiroinositol, scylloinositol, myoinositol	67,75,210
Steroids	11β-Hydroxyetiocholanolone, 11-ketoetiocholanolone, 11β-hydroxyandrosterone, 11-ketoandrosterone, 3,16,17-trihydroxy-11-ketoandrostane, 3,16,17-trihydroxyandrostanes	221,222

Masimore et al. [211] used ion-exchange chromatography (DEAE-Sephadex) to separate acidic compounds from haemodialysate. The acids were eluted with 1.5 *M* pyridinium acetate, which can be removed from the acidic residue by lyophilization. They identified phosphoric acid, glucopyranurono(6→1)-lactone, citric acid, D-gluconic acid-δ-lactone, α-D-glucose and β-D-glucose in the haemodialysate as their TMS derivatives by GC—MS. However, their separation of the acids by ion-exchange chromatography was not complete, because glucose and other neutral compounds were detected in the GC profile as rather

high peaks, causing difficulty in detecting other acidic compounds. Since phosphoric acid was detected in the profile as a very high peak, barium hydroxide should be used before ion-exchange chromatography to remove a major portion of phosphoric acid. By removing phosphoric acid, the detection of the other minor acids will become much easier and the lifespan of the GC column much longer.

Schoots et al. [75] analysed the non-volatile compounds in the uraemic serum before and after haemodialysis by high-resolution GC—MS. They used a fast pretreatment procedure, which consists of ultrafiltration for the removal of proteins, evaporation and trimethylsilylation. Some twenty compounds, including urea, threonine, Δ-pyrrolidone-5-carboxylic acid, tartaric acid and fructose, were identified by use of EI-MS and CI-MS. They observed that haemodialysis resulted in a significant decrease in the concentration of many compounds and that the different components were not removed to the same extent. The sample pretreatment procedure used is fast and reliable for the analysis of many compounds with high serum levels, but it is difficult to identify many other minor components accumulated in the uraemic serum because of the interference by the large peaks of urea, phosphoric acid, glucose, etc. By the use of several profiling methods such as high-resolution GC—MS, isotachophoresis and HPLC, Schoots et al. [212] demonstrated that the uraemic ultrafiltrate fractions in the so-called middle molecular mass region (mol. wt. 500—2000), contained considerable amounts of substances of low molecular mass, such as carbohydrates, organic acids, amino acids and UV-absorbing solutes.

Niwa and co-workers [213—216] newly identified 2,4-dimethyladipic acid, catechol, resorcinol, hydroquinone, 2-methoxyresorcinol, 3-methoxycatechol, methoxyhydroquinone, xyloisosaccharinolactone and isosaccharinolactone in uraemic ultrafiltrate by the use of high-resolution GC—MS, and quantitated nine hydroxyphenolic acids and six dicarboxylic acids in uraemic serum by SIM. At present, 1 ml of serum ultrafiltrate is used for the routine profile analysis of organic acids by use of high-resolution GC with splitless injection (Fig. 2). Serum was ultrafiltrated through a cone filter (CF-25, Amicon) to remove plasma protein. Ethyl acetate was used to extract hydroxyphenolic acids with recoveries of $>70\%$ and dicarboxylic acids with recoveries of $>90\%$. Ethyl acetate (or diethyl ether) extraction is a fast sample pretreatment procedure that is suitable for the extraction of these less polar organic acids. The concentrations of nine hydroxyphenolic acids and five dicarboxylic acids in uraemic serum were higher than in normal serum. In particular, the concentrations of 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid were markedly increased in uraemic serum. These hydroxyphenolic acids are important as uraemic toxins because of their inhibition of platelet aggregation.

Pinkston et al. [16] analysed the organic acids in haemodialysate in the form of methyl esters by high-resolution GC—MS. They extracted organic acids using an Extrelut column, identifying some 70 compounds in the uraemic haemodialysate and comparing the profiles with those of normal urine. They noted a marked increase of N-phenylacetyl-α-aminoglutarimide in the uraemic haemodialysate, which was formed by heat-induced ring closure of phenylacetylglutamine. The same authors also noted an increase of the glutamic acid conjugate of phenylacetic acid in the haemodialysate.

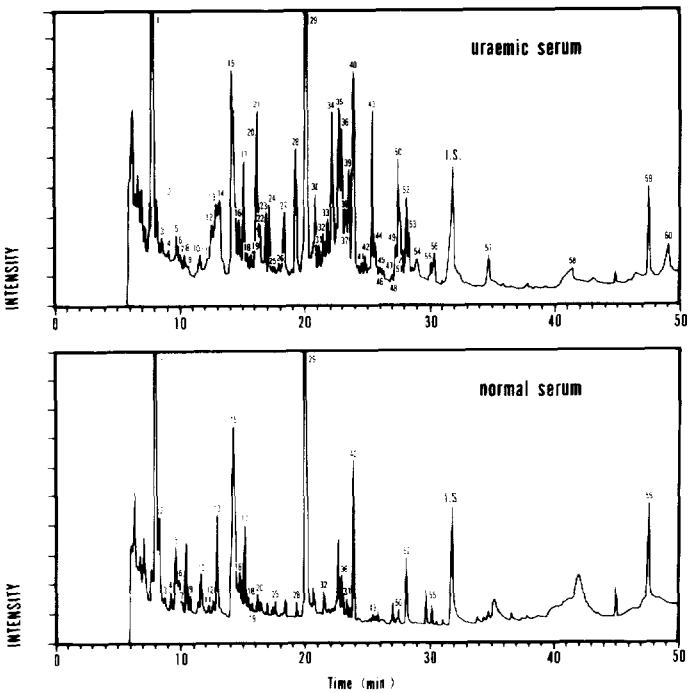

Fig. 2. Gas chromatograms of TMS derivatives of organic acids isolated from 1-ml volumes of uraemic serum ultrafiltrate (upper chromatogram) and normal serum ultrafiltrate (lower chromatogram). *p*-(*n*-Amyl)benzoic acid was added to the serum ultrafiltrate at a concentration of 5 μg/ml as an internal standard. GC conditions: OV-101 fused-silica capillary column (50 m), splitless injection, 120—290°C, 3°C/min. The peaks were identified as follows: 1 = lactic acid, 2 = glycolic acid, 6 = 3-hydroxypropionic acid, 8 = 3-hydroxyisobutyric acid, 9 = 2-hydroxy-2-methylbutyric acid, 10 = 2-deoxytetrono-1,4-lactone, 12 = 2-ethylhydracrylic acid, 13 = diethyleneglycol (artifact), 14 = urea, 15 = glycerol, 17 = succinic acid, 19 = methylsuccinic acid, 20 = 2-methylglyceric acid, 21 = glyceric acid, 22 = fumaric acid, 23 = 4-deoxyerythronic acid, 24 = 4-deoxythreonic acid, 28 = 3-deoxytetronic acid, 29 = 2-deoxytetronic acid, 33 = citramalic acid, 34 = malic acid, 39 = 3-methyladipic acid, 42 = erythronic acid, 43 = 2-hydroxyglutaric acid, 45 = pimelic acid, 49 = 4-hydroxybenzoic acid, 50 = 4-hydroxyphenylacetic acid, 53 = 3-deoxy-2-C(hydroxymethyl)tetronic acid, 56 = isosaccharino-1,4-lactone.

Liebich et al. [21] also investigated the organic acid profiles in plasma from chronic dialysis patients in the form of methyl esters by high-resolution GC—MS. They used isopropanol for deproteinization of the plasma. The level of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid was markedly increased in the uraemic plasma. Because of the high affinity of the lipophilic acid for plasma proteins, the acid was less dialysable as compared with urea, creatinine and hippuric acid. The plasma levels of 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid, 3-hydroxyhippuric acid and 4-hydroxyhippuric acid were also increased.

Accumulation of phenols in uraemic serum has been suspected to be responsible for uraemic symptoms, such as anaemia and impaired coagulation. Wengle and Hellström [217] quantitated the sulphate esters of phenol and *p*-cresol in uraemic serum by GC—MS. The concentrations of conjugated phenol and

p-cresol were found to be increased in uraemic serum. Niwa et al. [218] quantitated the concentrations of unconjugated and conjugated phenol, *p*-cresol, benzyl alcohol, catechol, hydroquinone, homocatechol and 2-methoxyresorcinol in uraemic serum using SIM. Concentrations of all these unconjugated and conjugated phenols were found to be markedly increased in the uraemic serum as compared with normal serum. Conjugated phenols existed mainly as sulphate esters.

Metabolic profiling was also applied for the analysis of polyols in uraemic blood, cerebrospinal fluid and urine. Pitkänen [68] analysed the polyols in serum and urine as acetylates by GC. He separated polyols by ion-exchange chromatography and found that the concentrations of erythritol, arabitol, mannitol, sorbitol and myoinositol were increased in uraemic serum. The concentration of myoinositol was also increased in uraemic urine. Servo et al. [74] analysed the polyols in uraemic cerebrospinal fluid using GC—MS and found that the concentrations of arabitol, mannitol and myoinositol were increased in patients with uraemia and that the concentration of 1-deoxyglucose (1,5-anhydroglucitol) was decreased in patients with uraemia. Niwa and co-workers [67,219] analysed the polyols in uraemic serum by high-resolution GC—MS. Many polyols, including 4-deoxythreitol, 4-deoxyerythritol, 5-deoxyxylitol, 5-deoxyarabitol, 2-deoxyribitol, 6-deoxymannitol, 6-deoxygalactitol, 6-deoxyallitol, 6-deoxygulitol, neoinositol, and chiroinositol, were newly identified in human urine. In patients with uraemia, the urinary excretion and the serum concentration of myoinositol, chiroinositol and scylloinositol were found to be increased. The serum level of 1-deoxyglucose was significantly decreased in uraemic patients. Metabolic profiling analysis was applied to amines in uraemic urine by Ohki et al. [220]. After acid hydrolysis of conjugated amines in urine, free amines were isolated by cation-exchange chromatography, evaporated, pentafluoropropylated and analysed using GC—MS. In uraemic patients, the urinary excretion of methylguanidine was increased, whereas the urinary excretion of the other eight amines was decreased in uraemic patients.

TABLE 3

INCREASED URINARY VOLATILES IN DIABETES MELLITUS

Increased urinary volatiles	Species	Reference
1-Ethanol, 1-propanol, 2-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol, 1-octanol, acetone, 2-pentanone, 2-heptanone, 4-heptanone, 3-hepten-2-one, cyclohexanone, pyrizine, pyrazine, methylpyrazine, 2,3-dimethylpyrazine, 2-methyl-6-ethylpyrazine, vinylpyrazine, 2,3,5-trimethylpyrazine, dimethylethylpyrazine, picoline, 2-methylthiofuran, dimethyl-tri-sulphate, 2-*tert.*-butylphenol, indole	Human	40—42,56,224
2-Heptanol, 3-hexanone, 2-penten-2-one, 5-hepten-2-one, 3-ethyl-4-methyl-2-hexanone, phenylacetone, 4-phenyl-2-butanone, acetophenone, pentanal, hexanal, nonanal, 2-nonanal, 2-acetylfuran, 5-methylfurfural, pyrrole, N-acetylpyrrole, 3-methyl-N-acetylpyrrole	Rat, mouse	46,225,226

Profiling has been widely used to study the metabolic disorder of steroids in various diseases. Ludwig and co-workers [221,222] applied this steroid profiling method to elucidate the altered metabolism of steroids in uraemic blood. Steroids were extracted from uraemic ultrafiltrate with an XAD-4 column, hydrolysed with helicase, purified with DEAP-LH-20 and then separated on Sephadex LH-20. After trimethylsilylation, the steroids were profiled using high-resolution GC—MS. Steroid fraction of uraemic ultrafiltrate contained the sulphates of 11β-hydroxyetiocholanolone, 11-ketoetiocholanolone, 11β-hydroxyandrosterone and 11-ketoandrosterone in high concentrations. In uraemic blood, androstenediol sulphate was detected as a major steroid, while in normal blood dehydroepiandrosterone sulphate was so detected. They also identified ten so far unknown steroids as components of blood [223] and identified 3,16,17-trihydroxy-11-ketoandrostane as a new type of natural steroid. The haemofiltrate analysed is well suited for the identification of minor steroids in blood, since it can be obtained easily in large amounts.

6.2. Diabetes mellitus

Several reports have indicated that the pattern of urinary volatile metabolites in patients with diabetes mellitus differs from that in normal patients. Table 3 lists the volatiles whose concentrations were increased in the urine of patients with diabetes mellitus. These volatiles include ketones, alcohols, furans, pyrroles and sulphur compounds, which were by-products of a wide range of metabolic processes. In most reports, volatiles were analysed by a head-space concentration method followed by high-resolution GC—MS. In the head-space concentration method, volatiles were purged from the heated urine (100°C) with high-purity helium and absorbed on the porous polymer Tenax GC. The porous polymer was contained in platinum microbaskets and subsequently encapsulated for injection. In a report by Liebich and Huesgen [56], 4-heptanone and 2-heptanone were extracted from urine with cyclohexane, and their concentrations were determined by GC—MS.

The aliphatic ketones are thought to arise from the decarboxylation of keto acids. The abnormally high levels of such compounds in the urine may be a result of the increased lipid catabolism. 4-Heptanone is considered to arise from 3-ketocarboxylic acid and 2-ethyl-3-ketohexanoic acid [47]. Indole is probably formed from tryptophan. The increased level of indole in the urine suggests the increased catabolism of protein in diabetes. The aliphatic aldehydes arise via a pathway related to fat metabolism. The increased levels of these compounds in the urine may be due to the increased lipid peroxidation. Acetophenone is known to arise from phenyl-β-ketopropionic acid, which is formed from phenylpropionic acid and possibly cinnamic acid.

Diabetic ketosis is known to be caused by accumulation of ketone bodies, namely 3-hydroxybutyric acid, acetoacetic acid and acetone. Table 4 lists the organic acids increased in the urine of patients with diabetic ketosis. Pettersen and co-workers [226,227] demonstrated that the urinary concentrations of adipic acid and suberic acid were increased in diabetic ketotic patients. These medium-chain dicarboxylic acids are formed by ω-oxidation and subsequent

TABLE 4

ORGANIC ACIDS INCREASED IN URINE OF PATIENTS WITH KETOACIDOSIS DUE TO DIABETES MELLITUS OR OTHER METABOLIC DISORDERS

Increased urinary acids	Reference
Adipic acid, suberic acid	226,227
Succinic acid	84
Lactic acid, 2-hydroxybutyric acid, 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid, 2-hydroxyisovaleric acid, 3-hydroxyisovaleric acid	146,231—233
3-Hydroxyvaleric acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, 5-hydroxyhexanoic acid, 2-hydroxy-2-methyllevulinic acid	228—230
3-Hydroxyoctanedioic acid, 3-hydroxyoctenedioic acid, 3-hydroxydecanedioic acid, 3-hydroxydecenedioic acid, 3-hydroxydodecanedioic acid, 3-hydroxydodecenedioic acid, 3-hydroxytetradecenedioic acid, 3-hydroxytetradecadienedioic acid	23

β-oxidation of long-chain dicarboxylic acids. Niwa and co-workers [228—230] identified 3-hydroxyvaleric acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, 5-hydroxyhexanoic acid and 2-hydroxy-2-methyllevulinic acid in the urine of diabetic patients with ketoacidosis as abnormal metabolites, and demonstrated that the urinary concentrations of lactic acid, 2-hydroxybutyric acid, 2-methyl-3-hydroxybutyric acid, 3-hydroxyisovaleric acid and adipic acid were elevated in diabetic ketoacidotic patients (Fig. 3). 3-Hydroxyvaleric acid may be formed from condensation of acetyl CoA and propionyl CoA, which is a metabolite of isoleucine, methionine and threonine. 3-Hydroxyhexanoic acid and 3-hydroxyoctanoic acid seem to be formed from 3-hydroxyhexanoyl CoA and 3-hydroxyoctanoyl CoA, respectively, as β-oxidation intermediates due to the impaired sequence of β-oxidation, which is caused by the relative deficiency of CoA and/or NAD^+ in mitochondria. 5-Hydroxyhexanoic acid seems to be formed by $\omega-1$ oxidation and β-oxidation of free fatty acids. The source of 2-hydroxy-2-methyllevulinic acid is not known, but the compound possibly derives from the condensation of pyruvic acid and acetone. The occurrence of the abnormal acids in diabetic ketotic urine described above is not specific to the diabetic state but rather relates to ketosis itself.

In patients with ketoacidosis and diabetic ketoacidosis, the urinary concentrations of 2-hydroxybutyric acid [146], 3-hydroxyisobutyric acid [231], 2-hydroxyisovaleric acid [232] and 3-hydroxyisovaleric acid [231,233] were reported to be increased.

Greter et al. [23] detected a number of aliphatic 3-hydroxy dicarboxylic acids in the urine of patients with ketoacidosis due to diabetes mellitus or undefined metabolic disorders. The major acid was 3-hydroxydecanedioic acid. Minor acids were 3-hydroxyoctanedioic acid, 3-hydroxyoctenedioic acid, 3-hydroxydecenedioic acid, 3-hydroxydodecanedioic acid, 3-hydroxydodecenedioic acid, 3-hydroxytetradecenedioic acid and 3-hydroxytetradecadienedioic acid. A combination of ω-oxidation and incomplete β-oxidation was suggested for the formation of the 3-hydroxy dicarboxylic acids from fatty acids.

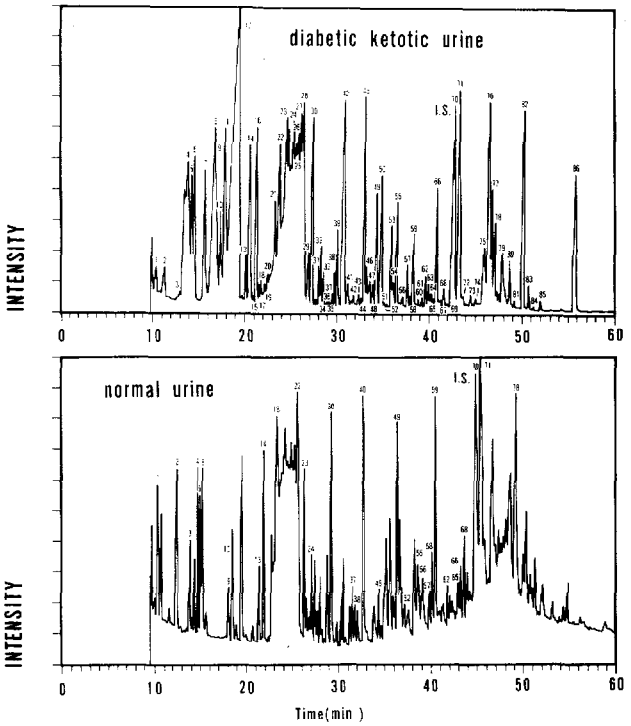

Fig. 3. Gas chromatograms of methoxime-TMS derivatives of organic acids in the urine of a diabetic patient with ketoacidosis (upper chromatogram) and a healthy subject (lower chromatogram). GC conditions: OV-101 open-tubular glass capillary column (30 m), splitless injection, 70—250°C, 3°C/min. The peaks were identified as follows: 4 = lactic acid, 5 = 2-hydroxyisobutyric acid, 6 = glycolic acid, 7, 8 = acetoacetic acid, 9 = 2-hydroxybutyric acid, 10 = *p*-cresol, 11 = 3-hydroxypropionic acid, 12 = 3-hydroxybutyric acid, 2-hydroxyisovaleric acid (minor component), 13 = 2-methyl-3-hydroxybutyric acid, 14 = 3-hydroxyisovaleric acid, 16 = 2-ethylhydracrylic acid, 17 = 3-hydroxyvaleric acid, 18 = diethyleneglycol (artifact), 21 = ethylmalonic acid, 22 = glycerol, 23 = succinic acid, 24 = methylsuccinic acid, 26 = 2-methylglyceric acid, 27 = glyceric acid, 28 = fumaric acid, 29 = 5-hydroxyhexanoic acid, 4-deoxyerythronic acid, 30 = 4-deoxythreonic acid, 38 = 3-deoxytetronic acid, 39 = 2-hydroxy-2-methyllevulinic acid, 40 = 2-deoxytetronic acid, 45 = adipic acid, 48 = 2,4-dimethyladipic acid, 49 = 3-methyladipic acid, 50 = 2,3-dideoxypentonic acid, 52 = 2-hydroxyphenylacetic acid, 53 = 2-ketoglutaric acid, 55 = 2-hydroxyglutaric acid, 56 = pimelic acid, 3-hydroxyphenylacetic acid, 57 = 3-hydroxy-3-methylglutaric acid, 58 = 4-hydroxybenzoic acid, 59 = 4-hydroxyphenylacetic acid, 62 = 3-deoxy-2-C(hydroxymethyl)tetronic acid, 64, 66 = 3-deoxypentonic acid, 67 = α-isosaccharino-1,4-lactone, 68 = β-isosaccharino-1,4-lactone, 70 = internal standard [*p*-(*n*-amyl)benzoic acid], 71 = homovanillic acid, 73 = azelaic acid, 76 = citric acid, 77 = 3-(4-hydroxyphenyl)hydracrylic acid. (Reproduced from ref. 228.)

With the use of animal models of diabetes mellitus, assessment of a slight difference in the excretion of urinary acids between normal and diabetic rats became possible. In diabetic urine, high levels of intermediates of glucose metabolism such as lactic acid, pyruvic acid, glyceraldehyde, dihydroxyacetone, 3-hydroxypropionic acid and glyceric acid were observed. The intermediates of the tricarboxylic acid cycle, phenolic compounds and aromatic acids were also found to be increased in diabetic urine [225].

Polyols in the physiological fluids of diabetic patients have been studied using the GC—MS profiling technique. In diabetic urine, the concentrations of mannitol and myoinositol were reported to be increased [68]. However, in diabetic cerebrospinal fluid, the concentration of myoinositol was decreased [74]. The increased urinary excretion of myoinositol is a result of glycosuria, since the renal glucose and myoinositol reabsorption mechanism is closely related.

Pitkänen [69] first identified 1-deoxyglucose in human cerebrospinal fluid, and showed that the concentration of the compound in cerebrospinal fluid was very low in diabetic patients. The compound was further detected in human serum [70,72]. In plasma of diabetic patients without insulin therapy 1-deoxyglucose was not detected, while with insulin therapy the compound became measurable in diabetic plasma. However, the concentration remained low compared with that in normal plasma [70].

Profiling of polyols in serum and glycosuric urine is difficult because of the large amount of glucose. In one study [68], glucose was removed by treating the samples with glucose oxidase and catalase, allowing oxygen to bubble through the solution. Gluconate formed during the process was removed by prolonged shaking with ion-exchange resins. The method is, however, laborious and time-consuming. Yoshioka et al. [71] prepared the polyols in plasma without the removal of glucose. Their sampling procedure consists of deproteinization, centrifugation and evaporation of the supernatant. Although this sampling procedure is rapid and simple, the detection of the other minor polyols, such as mannitol and sorbitol, may be hampered by the large peaks of glucose.

6.3. Dicarboxylic aciduria

Dicarboxylic aciduria often occurs during ketosis. In some patients, however, dicarboxylic aciduria occurs with mild or absent ketonuria. Increased urinary excretion of dicarboxylic acids has been described in a number of diseases. These include congenital lactic acidosis [143,144], glycogen storage disease types I and III [234], systemic carnitine deficiency [103], glutaric aciduria type II [33,112]. Jamaican vomiting sickness [134], hyperglycaemia [86], valproate administration [235], Reye's syndrome [9,77], Reye's-like syndrome [17,236—239], non-ketotic dicarboxylic aciduria [164—170] and ethylmalonic-adipic aciduria [107,108].

Dicarboxylic aciduria is often accompanied by single or recurrent episodes of hypoglycaemia, and is caused by a number of different defects of β-oxidation, which include: (1) short-chain acyl CoA dehydrogenase deficiency; (2) medium-chain acyl CoA dehydrogenase deficiency; (3) long-chain acyl CoA dehydrogenase deficiency; (4) multiple acyl CoA dehydrogenase deficiency; (5) carnitine deficiency; (6) riboflavin deficiency; and (7) carnitine palmitoyl transferase deficiency.

Table 5 lists the acids detected in the urine of patients with dicarboxylic aciduria with increased concentration.

Non-ketotic dicarboxylic aciduria is considered to be caused by medium-chain acyl CoA dehydrogenase deficiency, and the concentrations of C_6—C_{10}

TABLE 5

ORGANIC ACIDS INCREASED IN URINE OF PATIENTS WITH DICARBOXYLIC ACIDURIA

	Increased acids	Reference
Saturated dicarboxylic acids	Glutaric acid, adipic acid, pimelic acid, suberic acid, azelaic acid, sebacic acid, dodecanedioic acid*, tetradecanedioic acid*, hexadecanedioic acid*, octadecanedioic acid*	77,103,107,108, 165
Unsaturated dicarboxylic acids	Heptenedioic acid, 2-octenedioic acid, 4-octenedioic acid, 2-decenedioic acid, 3-decenedioic acid, 4-decenedioic acid, 5-decenedioic acid, 3-dodecenedioic acid, 5-dodecenedioic acid, 5-tetradecenedioic acid, tetradecadienedioic acid*, hexadecenedioic acid*, octadecenedioic acid*, octadecadienedioic acid*	17,77,143,144, 169
Branched dicarboxylic acid	Ethylmalonic acid, methylsuccinic acid	33,107,108,112, 166,241
3-Hydroxydicarboxylic acid	3-Hydroxysebacic acid	240
2-Hydroxy-dicarboxylic acid	2-Hydroxyglutaric acid	112
Glycine conjugates of acids	Butyrylglycine, isobutyrylglycine, isovalerylglycine, 2-methylbutyrylglycine, hexanoylglycine, octanoylglycine, decanoylglycine	107,108,112, 165—168,170,241
Short-chain carboxylic acids	Isobutyric acid, butyric acid, isovaleric acid, 2-methylbutyric acid, propionic acid, hexanoic acid, octanoic acid	33,169
Ψ-Hydroxycarboxylic acids	5-Hydroxyhexanoic acid, 7-hydroxyoctanoic acid, 9-hydroxydecanoic acid	167—170,242
Ψ-Ketocarboxylic acids	5-Ketohexanoic acid, 7-ketooctanoic acid	242
3-Hydroxycarboxylic acids	3-Hydroxyhexanoic acid, 3-hydroxyoctanoic acid	169,242
	3-Hydroxybutyric acid, 3-hydroxyisovaleric acid	17,33,112
2-Hydroxycarboxylic acids	Lactic acid, 2-hydroxybutyric acid, 2-hydroxyisobutyric acid, 2-hydroxyisovaleric acid	33,112
Phenolic acid	4-Hydroxyphenylacetic acid	17

*The acids were detected only in the sera of patients with Reye's syndrome [77].

dicarboxylic acids, Ψ-hydroxy fatty acids and glycine conjugates of acids were found to be increased in the urine of these patients. Dicarboxylic acids are considered to be formed from ω-oxidation of accumulated fatty acids in cytoplasmic reticulum followed by β-oxidation in peroxisomes. Ψ-Hydroxy fatty acids are thought to be formed from ω−1 hydroxylation of fatty acids in cytoplasmic reticulum followed by β-oxidation in peroxisomes. Hexanoylglycine seems to be formed by glycine conjugation of hexanoyl CoA in mitochondria.

Glutaric aciduria type II was found to be due to multiple acyl CoA dehydrogenase deficiency, and characterized by the urinary excretion of large amounts of glutaric acid and smaller, albeit abnormal, amounts of isobutyric acid, isovaleric acid, 2-methylbutyric acid, ethylmalonic acid, 2-hydroxyglutaric acid, lactic acid, isobutyrylglycine, isovalerylglycine and 2-methylbutyrylglycine [33,112]. The presence of hypersarcosinaemia and sarcosinuria may define a subtype of glutaric aciduria type II due to deficiency of an electron-carrier flavoprotein [112].

Ethylmalonic-adipic aciduria is characterized by the urinary excretion of large amounts of ethylmalonic acid, adipic acid and hexanoylglycine [107, 108]. Smaller, albeit abnormal, amounts of methylsuccinic acid, suberic acid, sebacic acid and decenedioic acid were also detected in the urine. Glutaric acid was occasionally detected in the urine. 3-Hydroxybutyric acid was not detected. Ethylmalonic-adipic aciduria was found to be due to the deficiency of activities of butyryl CoA, glutaryl CoA and isovaleryl CoA dehydrogenases [108].

Jamaican vomiting sickness was demonstrated to be caused by ingestion of hypoglycin A by Tanaka et al. [134]. Hypoglycin A inhibits several short-chain acyl CoA dehydrogenases including isovaleryl CoA dehydrogenase, *n*-butyryl CoA dehydrogenase and glutaryl CoA dehydrogenase. The urinary concentrations of ethylmalonic acid, methylsuccinic acid, glutaric acid, C_6—C_8 dicarboxylic acids, butyrylglycine, isovalerylglycine and hexanoylglycine were increased.

Reye's-like syndrome, with close similarities to Jamaican vomiting sickness, was reported by Chalmers et al. [17] and characterized by the urinary excretion of large amounts of C_6—C_{10} dicarboxylic acids, monoenic C_8—C_{12} dicarboxylic acids and 5-hydroxyhexanoic acid. The occurrence of large amounts of 5-hydroxyhexanoic acid may suggest that its precursor, hex-4-enoic acid, is a causative toxin. The possible toxin may inhibit the short-chain acyl CoA dehydrogenases.

Reye's syndrome is a childhood disease characterized by encephalopathy, fatty infiltration of the liver and kidney and mitochondrial change. The urinary concentrations of pyruvic acid and lactic acid were found to be elevated [9]. In Reye's syndrome, the accumulation of long-chain dicarboxylic acids was detected in the serum, which include four saturated dicarboxylic acids (dodecanedioic, tetradecanedioic, hexadecanedioic and octadecanedioic) and six unsaturated dicarboxylic acids (dodecenedioic, tetradecenedioic, tetradecadienedioic, hexadecenedioic, octadecadienedioic and octadecenedioic). The occurrence of the long-chain dicarboxylic acids seems to be the result of the increased ω-oxidation of long-chain fatty acids in the cytoplasma, or the impaired metabolism of ω-dicarboxylic acids formed in Reye's patients. The appearance of the acids, however, is not determined to be specific to Reye's syndrome.

Systemic carnitine deficiency also showed saturated and monoenic C_6—C_8 dicarboxylic aciduria [103]. The carnitine content of serum and tissue was low, and the dicarboxylic aciduria was thought to be due to decreased synthesis of carnitine, with subsequent impairment of fatty acid transport between cytoplasma and mitochondrial matrix. ω-Oxidation in cytoplasmic reticulum may be increased to compensate for the impairment of β-oxidation in mitochondria.

Congenital lactic acidosis with dicarboxylic aciduria was reported [143,144]. The urinary concentrations of lactic acid, C_6—C_{12} dicarboxylic acids and monoenic C_{10}—C_{14} dicarboxylic acids were found to be increased. Impaired β-oxidation at the acyl CoA dehydrogenation step was suggested as the basis for the metabolic disorder.

Dicarboxylic aciduria was reported to be associated with glycogen storage type I and type III [234]. The patients excreted large amounts of lactic acid, 3-hydroxybutyric acid and saturated C_6—C_{10} dicarboxylic acids into the urine. The occurrence of the dicarboxylic acids was suggested to be the result of inadequate glucogenesis with consequent enhancement of β-oxidation and ω-oxidation of fatty acids.

7. SUMMARY

Nowadays, metabolic profiling is widely applied in clinical medicine for the diagnosis and study of human diseases. The number of these applications and their diversity have increased rapidly in the past few years. This review summarizes recent advances in the methods for sample pretreatment and the clinical application of GC—MS to the study of uraemia, diabetes mellitus, dicarboxylic aciduria and other organic acidurias. High-resolution GC—MS is well suited to the profile analysis of metabolic disorders.

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