Journal of Chromatography, 143 (1977) 31—38
Biomedical Applications

CHROMBIO. 001

INVESTIGATION OF THE METABOLIC PATTERN IN MAPLE SYRUP URINE DISEASE BY MEANS OF GLASS CAPILLARY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

CORNELIS JAKOBS*, EIVIND SOLEM, JOHAN EK, KARIN HALVORSEN and EGIL JELLUM**

Institute of Clinical Biochemistry, Rikshospitalet, Oslo (Norway); Department of Paediatrics, Rikshospitalet, Oslo (Norway); and Department of Paediatrics, Sentralsykehuset i Akershus, Nordbyhagen (Norway)

(Received June 24th, 1976)

SUMMARY

Urine and serum from patients with maple syrup urine disease (MSUD) have been examined quantitatively and qualitatively using glass capillary gas chromatography in combination with mass spectrometry. During clinical episodes, patients with this disease were found to excrete increased amounts of the following metabolites in addition to the previously recognized branched-chain 2-keto and 2-hydroxy acids, lactate and 3-hydroxybutyrate: 2-hydroxybutyrate, 2-hydroxyisobutyrate, 3-hydroxyisovalerate, 3-hydroxyisobutyrate and 2-methyl-3-hydroxybutyrate. Most of the latter compounds seem to accompany ketoacidosis and lactic acidosis. The capillary column also separated the D- and L-forms of 2-keto-3-methylvalerate, and both isomers were, in contrast to earlier assumptions, present in the MSUD patients. The results clearly demonstrate that new information on the metabolic situation in well known disorders may be obtained by exploiting the high resolving power of capillary columns.

INTRODUCTION

Maple syrup urine disease (MSUD) has been the subject of numerous reports and review articles (see, e.g., refs. 1—5). The disease, which may have at least four distinguishable forms, depending inter alia on the residual enzyme activities [3, 6], leads to the accumulation and increased excretion in the urine of the branched-chain 2-keto and 2-hydroxy acids originating from leucine, isoleu-

*On leave from the Department of Paediatrics, Free University of Berlin, Kaiserin Auguste Victoria Haus, West Berlin, G.F.R.
**Address reprint requests to: Dr. E. Jellum, Institute of Clinical Biochemistry, Rikshospitalet, Oslo 1, Norway.

cine and valine. Urine from patients with MSUD normally also contains a considerable amount of 3-hydroxybutyrate, acetoacetate and lactate. Recent results have shown that ketoacidosis in general is accompanied by increased excretion of 3-hydroxyisovaleric acid [7], 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid [8] in addition to the dicarboxylic acids adipic and suberic acid [9]. Similarly, there is a correlation between the excretion of lactic and 2-hydroxybutyric acids [10]. It is thus evident that MSUD patients who also have ketoacidosis and lactic acidosis may excrete increased amounts of many different organic acids in their urine. The presence of these chemically similar carboxylic compounds makes the exact quantitative measurement of the MSUD branched-chain keto and hydroxy acids difficult. Gas chromatography (GC) on packed columns and combined gas chromatography—mass spectrometry (GC—MS) have recently been used for the study of these acids in MSUD patients [6,11,12].

However, even with the use of these methods, one may run into difficulties because the packed GC columns do not separate all of the acids that accumulate in the patients. Thus, on packed GC columns, several of the closely related keto and hydroxy acids have a tendency to be co-chromatographed. For instance, it is extremely difficult to separate the D-form of 2-keto-3-methylvaleric acid from 2-ketoisocaproic acid on any type of packed column.

The development of highly efficient glass capillary columns with 80,000—100,000 theoretical plates, combined with a mass spectrometer for absolute identification, opens up the possibility of separating and identifying all of the organic acids that occur in MSUD patients who are also suffering from ketoacidosis and lactic acidosis. In this paper, we describe the use of this technique in the re-investigation of the metabolic pattern in blood and urine from such patients before and after dietary treatment.

MATERIALS

The reference compounds DL-lactic, pyruvic, DL-3-hydroxybutyric, DL-2-hydroxyisovaleric, malonic, DL-2-hydroxy-*n*-valeric, methylmalonic, 2-ketoisovaleric, acetoacetic, L-2-hydroxy-3-methylvaleric, L-2-hydroxyisocaproic, DL-2-keto-3-methylvaleric and 2-ketoisocaproic acids (usually the sodium salts) were obtained from Sigma (St. Louis, Mo., U.S.A.). 2-Hydroxyisobutyric acid was obtained from Koch-Light (Colnbrook, Great Britain) and 2-ketobutyric acid from Fluka (Buchs, Switzerland). All acids were used without further purification. 2-Hydroxybutyric acid was synthesized from 2-ketobutyric acid by reduction with sodium borohydride (Fluka).

Sulphosalicylic acid, used for protein precipitation, was obtained from BDH (Poole, Great Britain). The extraction solvent (ethyl acetate) was obtained from Merck (Darmstadt, G.F.R.) and the reagents used for the preparation of derivatives [bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane and hydroxylamine hydrochloride] were purchased from Pierce (Rockford, Ill., U.S.A.). The glass capillary column for gas chromatography (25 m × 0.25 mm) was obtained from LKB (Stockholm, Sweden) and was coated with SE-30. Packed GC columns were also used and contained 10% OV-17 on Gas-Chrom Q, or 8% BDS on Chromosorb W. The solid support and stationary phases were obtained from Applied Science Labs. (State College, Pa., U.S.A.).

METHODS

Preparation of derivatives for gas chromatography

All keto, hydroxy and other organic acids were determined by GC of their corresponding trimethylsilyl and/or trimethylsilyl oxime derivatives. A modified method based on the procedure described by Sternowsky et al. [13] was used. To a urine sample or deproteinized serum sample (2 ml) was added malonic acid (0.1 mg) as internal standard. The mixture was acidified using 6 *N* hydrochloric acid. Saturated sodium chloride solution (2 ml) was added before extraction with ethyl acetate (three 5-ml volumes), and the organic phases were combined and evaporated to dryness in a rotary evaporator. The residue was dissolved in pyridine (50 μl) containing hydroxylamine hydrochloride (20 mg/ml). The mixture was kept at room temperature for 30 min, before addition of BSTFA (50 μl, containing 10%, v/v, of trimethylchlorosilane). After a further 30 min at room temperature, an aliquot was injected into the gas chromatograph. Aqueous mixtures of reference compounds were treated in an identical manner for comparison.

Gas chromatography

A Varian Model 2100 gas chromatograph, equipped with a hydrogen flame-ionization detector, was used. The instrument was fitted with the glass capillary column or packed columns as described above. The gas chromatograph was operated under the following conditions: injector temperature, 250°; detector temperature, 250°; column temperature, programmed from 80° to 110° at a rate of 0.5°/min. A splitting ratio of 1:12 was used and the gas flow-rate through the capillary column was adjusted to 1.2 ml/min. The number of theoretical plates was approximately 85,000.

Gas chromatography—mass spectrometry

In the combined GC—MS, one instrument consisted of a Varian 1440 gas chromatograph, a molecular separator of the glass-frit type (kept at 230°) and a single-focusing mass spectrometer, Type CH7 (Varian-MAT), normally operated with an ionization energy of 70 eV. The gas chromatograph was alternatively equipped with three different packed columns (2 m × 1/4 in. O.D.) filled with 10% OV-17 on Chromosorb Q, 8% BDS on Chromosorb W or 20% SE-30 on Chromosorb Q. Helium was used as the carrier gas (30 ml/min). Parts of this investigation were also carried out using a Varian 112 mass spectrometer fitted with a glass capillary column (SE-30, 25 m × 0.25 mm; LKB) connected directly to the ion source. Both GC—MS instruments were connected on-line to a computer system (Spectro System 100 MS; Varian-MAT).

Amino acid analyses

The free amino acid levels in urine and serum (deproteinized with sulphosalicylic acid, 40 mg/ml) were determined on a JEOL JLC-6AH Automatic Analyzer, fitted with an electronic integrator (JEOL). Lithium buffers were used.

Identification and quantitative analyses of the organic acids

The identities of the organic acids in the biological specimens were deter-

mined by comparison of the GC retention times and MS data with those of authentic reference compounds. The amount of an acid excreted in the urine was always related to the creatinine content (determined in a Technicon Auto-Analyzer). Correction factors, including both the degree of extraction and the relative responses in the flame-ionization detector, were determined by treating known amounts of the different organic acids and the internal standard (malonic acid) in the same way as the patient samples.

Patients

Samples were obtained from three patients with MSUD. One patient had MSUD of the persistent type (patient 1), the other two had intermittent types. Of these, patient 3 had the least severe clinical form. This 6-month-old girl was diagnosed for the first time during the present work. All patients were treated with a regular MSUD diet, which was administered and adjusted according to the serum leucine, valine and isoleucine levels.

RESULTS

Fig. 1 (bottom) shows the capillary gas chromatogram of a standard mixture of most of the organic acids expected to occur in patients suffering from MSUD combined with ketoacidosis and lactic acidosis. The hatched peaks refer to solvents, excess of reagents and reagent by-products. All acids were eluted as single GC peaks, except acetoacetic, 2-ketoisovaleric and 2-keto-3-methylvaleric acids. With the last acid two peaks were obtained owing to the separation of the L- and D-isomers. It should be noted that the D-2-keto-3-methylvaleric acid was well separated from 2-ketoisocaproic acid using the capillary column. The separation of these two compounds cannot be achieved using packed columns.

Fig. 1 (top and middle) shows the gas chromatograms of the urinary organic acids of patient 3 before and after dietary treatment. It can be seen that most of the abnormal carboxylic acids (except 2-hydroxyisovaleric acid) had disappeared after 2 days on the special diet.

Table I shows the amounts of organic acids in the urine samples of patients 1, 2 and 3 and the serum of patient 3. The values were calculated from the capillary GC data. It can be seen that both D- and L-2-keto-3-methylvaleric acid were present in nearly equal amounts and 2-hydroxyisovaleric acid was the dominating MSUD hydroxy acid, in agreement with earlier reports [6, 11, 12]. Also, 2-hydroxy-3-methylvaleric acid was present to a significant extent, whereas 2-hydroxyisocaproic acid occurred in much smaller amounts (Fig. 1 and Table I). When the ratios between the branched-chain keto acids and the hydroxy acids were calculated (Table I, last column), it was found that these values are entirely different for each pair of corresponding acids (see also ref. 6). It is also noteworthy that many organic acids known to be associated with ketoacidosis and lactic acidosis (2-hydroxybutyric, 3-hydroxyisobutyric, 2-methyl-3-hydroxybutyric and 3-hydroxyisovaleric acid) were present. In addition, the patients excreted significant amounts of 2-hydroxyisobutyric acid, which disappeared on dietary treatment (Fig. 1 and Table I). The most persistent metabolite, which was still excreted after 2 days on an MSUD diet, was 2-hydroxyisovaleric acid.

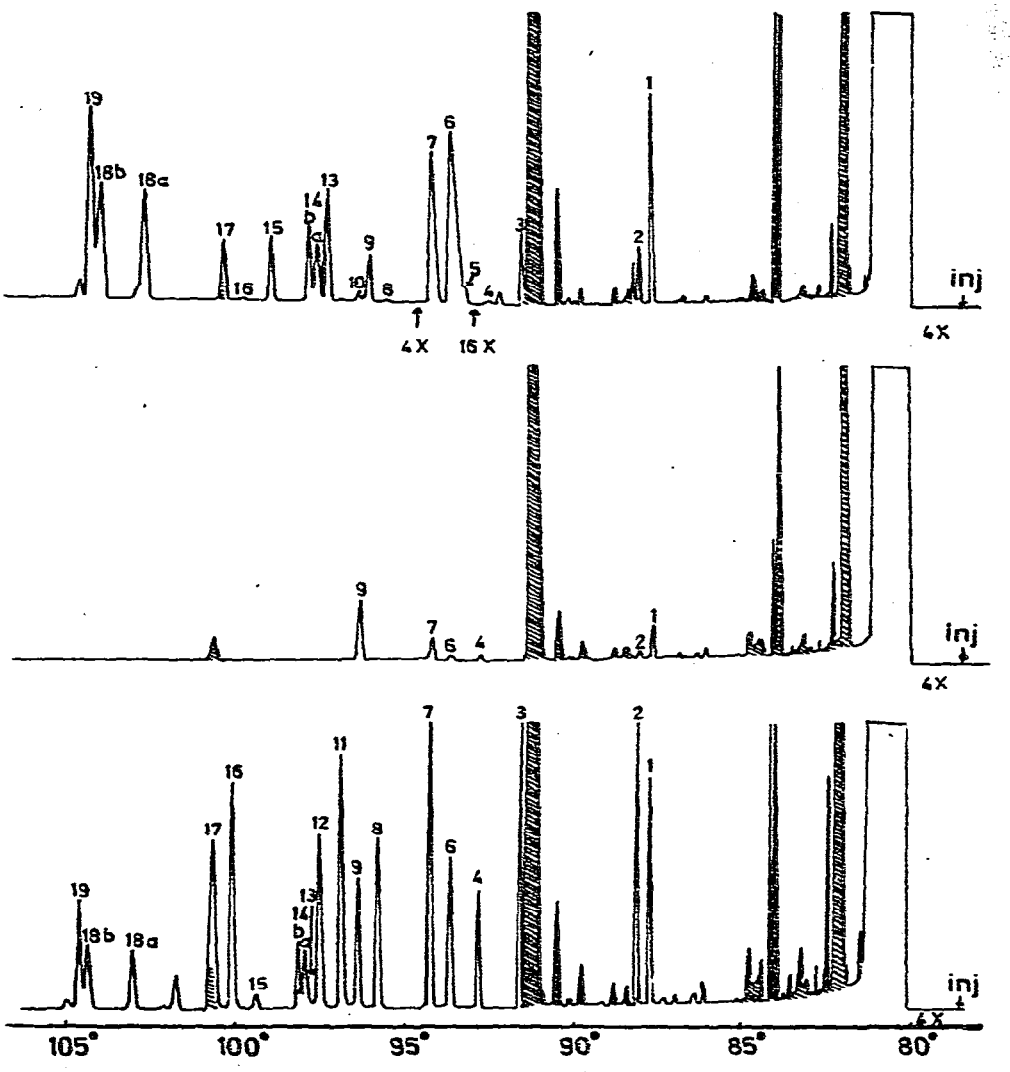

Fig. 1. Gas chromatographic separation of keto and hydroxy acids in the urine of a patient with maple syrup urine disease. Top chromatogram, patient 3 before dietary treatment; middle chromatogram, patient 3 after 2 days on diet; bottom chromatogram, a mixture of reference compounds. An SE-30 glass capillary column (25 m × 0.25 mm) was used. Peaks: 1 = lactic acid; 2 = 2-hydroxyisobutyric acid; 3 = 2-hydroxybutyric acid; 4 = pyruvic acid; 5 = 3-hydroxyisobutyric acid; 6 = 3-hydroxybutyric acid; 7 = 2-hydroxyisovaleric acid; 8 = 2-ketobutyric acid; 9 = malonic acid (internal standard); 10 = 2-methyl-3-hydroxybutyric acid; 11 = 2-hydroxy-*n*-valeric acid; 12 = methylmalonic acid; 13 = 3-hydroxyisovaleric acid; 14a and 14b = 2-ketoisovaleric acid; 15 = acetoacetic acid; 16 = 2-hydroxyisocaproic acid; 17 = 2-hydroxy-3-methylvaleric acid; 18a = L-2-keto-3-methylvaleric acid; 18b = D-2-keto-3-methylvaleric acid; 19 = 2-ketoisocaproic acid.

TABLE I

ORGANIC ACIDS IN THE URINE AND SERUM OF PATIENTS WITH MSUD

Organic acid	Urine value (mg/g creatinine) before treatment		Patient 3, female, 6 months					Average ratio of keto acid to hydroxy acid in the urine of the 3 patients before treatment
			Urine value (mg/g creatinine)			Serum value (mg/l)		
	Patient 1, male, 20 days	Patient 2, female, 2 years	Before treatment	1st day after start of diet	2nd day after start of diet	Before treatment	2nd day after start of diet	
Valine metabolites								
2-Hydroxyisovalerate	3144	3810	875	131	40	149	88	
2-Ketoisovalerate	816	753	330	Not detectable	Not detectable	13	Not detectable	0.26
Isoleucine metabolites								
2-Hydroxy-3-methylvalerate	420	438	70	Not detectable	Not detectable	5	Not detectable	
L-2-Keto-3-methylvalerate	1373	1275	279	Trace	Trace	19	4	6.66
D-2-Keto-3-methylvalerate	1200	1600	287	Trace	Trace	20	3	
Leucine metabolites								
2-Hydroxyisocaproate	91	27	3	Not detectable	Not detectable	Trace	Not detectable	
2-Ketoisocaproate	5100	612	496	Trace	Trace	90	11	45.5
Lactate	489	756	173	69	53	374	157	
Pyruvate	69	80	13	41	33	6	17	
2-Hydroxybutyrate	58	1366	65	14	Not detectable	15	8	
2-Hydroxyisobutyrate	204	44	38	14	13	60	19	
Acetoacetate	41	1051	170	28	Not detectable	19	Not detectable	
3-Hydroxybutyrate	3464	24900	2059	262	13	90	65	
3-Hydroxyisovalerate	107	181	122	14	Not detectable	6	Not detectable	
3-Hydroxyisobutyrate	120	150	110	11	Not detectable	Trace	Not detectable	
2-Methyl-3-hydroxybutyrate	40	50	20	Trace	Not detectable	Trace	Not detectable	

Numerous serum amino acid analyses were performed on the patients during the observation period of several months. Before treatment, the valine level was about twice, leucine about ten times and isoleucine six times the normal level. The alanine level, on the other hand, was reduced, being only one quarter of the normal value. Dietary treatment normalized the amino acid pattern.

DISCUSSION

Although MSUD is a disorder that has been known and studied for many years, the cause of the clinical symptoms still remains unclear. Recent theories suggest that the accumulated branched-chain keto acids, particularly 2-ketoisocaproic acid, competitively inhibit pyruvate decarboxylase in the brain, thereby causing damage [14, 15]. Quantitative analyses of the keto acids are of interest in examining this hypothesis and it is also of interest to study the accumulation and excretion of other organic acids. Various forms of chromatography, e.g., paper, thin-layer and, more recently, gas chromatography on packed columns [6, 11, 12], have been used for this purpose. Because the resolving power of these methods was not sufficient to separate and quantitate all of the closely similar metabolites that were likely to occur in MSUD patients, we have adopted highly efficient glass capillary columns, combined with mass spectrometry for identification purposes. The results showed that as many as twelve different metabolites in addition to the branched-chain keto acids accumulated in MSUD patients during clinical episodes. Most of the former metabolites were related to the ketoacidosis and lactic acidosis accompanying the clinical condition. The role of these new metabolites in the disease and the clinical picture is not known. It is not unlikely, however, that some of them may be toxic to the brain cells, for instance through enzyme inhibition, and that these metabolites also may contribute to the clinical symptoms.

In this context, the observation that some MSUD patients are clinically ill for at least 1 day after they stop excreting the traditional keto acids should be mentioned. The explanation might be that some of the metabolites other than the branched-chain keto acids have a longer half-life in the brain cells and thus prolong the clinical condition.

Apart from demonstrating that MSUD patients excrete elevated amounts of at least 15 different organic acids, it is of interest that both the D- and L-forms of 2-keto-3-methylvaleric acid accumulated in nearly equal amounts. Although the presence of both isomers has been postulated and can be explained in terms of in vivo transformation via enolization [16], previous methods, including GC, have failed to detect the D-form. The reason for this failure may be that on packed columns the D-compound is co-chromatographed with 2-ketoisocaproic acid. The amount of the latter is thereby determined with an erroneously high value, and this keto acid may therefore not necessarily be the major MSUD metabolite excreted, as is usually assumed [4]. Thus, two of the three patients excreted more of L- + D-2-keto-3-methylvaleric acid and much more (2—5 times) 2-hydroxyisovaleric acid than 2-ketoisocaproic acid (Table I).

The large amount of 2-hydroxyisovaleric acid in MSUD patients and the large differences in the ratio of keto acid to hydroxy acid (Table I), which have previously been observed by other workers [6] and are confirmed in this study,

may warrant some discussion. Most likely the hydroxy acids are formed enzymatically from their corresponding keto acids by the action of one or more dehydrogenases. One enzyme to be considered is lactate dehydrogenase (LDH), which is known to be of broad specificity. However, it is unlikely that this particular enzyme plays a significant role in the above conversion as the branched-chain keto acids that occur in MSUD are very poor substrates for LDH (unpublished results). Other dehydrogenases must therefore be considered. The entirely different ratios of, e.g., the ketoisovaleric—hydroxyisovaleric acid pair and of the ketoisocaproic—hydroxyisocaproic acid pair may indicate that not only one, but several, dehydrogenases are involved. Alterations in the NADH:NAD ratio cannot explain these findings as the redox situation within the same cells of a patient must necessarily be identical whether one is concerned with the reduction of 2-ketoisocaproic acid or of 2-ketoisovaleric acid. Further enzyme investigations are required, however, in order to give an adequate solution to the large variations in the proportions of keto to hydroxy acid, and factors such as isoenzymes, K_m values and product inhibition must also be considered.

The presence of a hitherto unrecognized metabolite in connection with MSUD, 2-hydroxyisobutyric acid, was demonstrated in all three patients. The metabolic origin and fate of this compound are not known, although one might speculate that it stems from α-oxidation of branched-chain fatty acids.

ACKNOWLEDGEMENT

The technical assistance of Mr. Arne Tiltnes is gratefully acknowledged.

REFERENCES

1 J.H. Menkes, P.L. Hurst and J.M. Craig, *Pediatrics*, 14 (1954) 462.
2 J. Dancis, M. Levitz and R.G. Westall, *Pediatrics*, 25 (1960) 72.
3 J. Dancis, J. Hutzler, S.E. Snyderman and R.P. Cox, *J. Pediat.*, 81 (1972) 312.
4 V.H. Auerbach and A.M. diGeorge, in F.A. Hommes and C.J. van den Berg (Editors), *Inborn Errors of Metabolism*, Academic Press, London, 1973, p. 337.
5 S.E. Snyderman, in W.L. Nyhan (Editor), *Heritable Disorders of Amino Acid Metabolism*, Wiley, New York, 1974, p. 17.
6 G. Lancaster, O.A. Mamer and C.R. Scriver, *Metabolism*, 23 (1974) 257.
7 S. Landaas, *Clin. Chim. Acta*, 54 (1974) 39.
8 S. Landaas, *Clin. Chim. Acta*, 64 (1975) 143.
9 J.E. Pettersen, E. Jellum and L. Eldjarn, *Clin. Chim. Acta*, 38 (1972) 17.
10 J. E. Pettersen, S. Landaas and L. Eldjarn, *Clin. Chim. Acta*, 48 (1973) 213.
11 M. Greer and C.M. Williams, *Biochem. Med.*, 1 (1967) 87.
12 D. Gompertz and G. H. Draffan, *Clin. Chim. Acta*, 40 (1972) 5.
13 H.J. Sternowsky, J. Roboz, F. Hutterer and G. Gaull, *Clin. Chim. Acta*, 47 (1973) 371.
14 P.M. Dreyfus and A.L. Prensky, *Nature (London)*, 214 (1967) 276.
15 C.L. McArthur, III, and J.A. Bowden, *Int. J. Biochem.*, 3 (1972) 193.
16 B. Halpern and G.E. Pollock, *Biochem. Med.*, 4 (1970) 352.