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The absolute configuration of urinary 5-hydroxyhexanoic acid — a product of fatty acid (ω-1)-oxidation — in patients with non-ketotic dicarboxylic aciduria

J.P. Kamerling[a,b], M. Duran[a], L. Bruinvis[a], D. Ketting[a], S.K. Wadman[a,*], J.F.G. Vliegenthart[b]

[a] *University Children's Hospital 'Het Wilhelmina Kinderziekenhuis', Nieuwe Gracht 137, 3512 LK Utrecht (The Netherlands) and* [b] *Department of Bio-Organic Chemistry, State University of Utrecht, Croesestraat 79, 3522 AD Utrecht (The Netherlands)*

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Summary

The absolute configuration of urinary 5-hydroxyhexanoic acid was determined by means of capillary gas-liquid chromatography of the *O*-D-2-phenylpropionylated methyl ester derivatives on SE-30 as stationary phase. In three patients with non-ketotic dicarboxylic aciduria and one patient on a diet containing excessive amounts of medium-chain triglycerides, ~70% of the L-isomer and ~30% of the D-isomer were found. On the basis of these results it is concluded that 5-hydroxyhexanoic acid is a 'normal' degradation product of fatty acids.

Introduction

Ketone bodies, which are formed from acetyl-CoA, the normal end product of fatty acid oxidation, are an important source of energy to the human body when the glycogen stores are depleted. The contribution of the gluconeogenesis in this respect appears to be limited. In normal human subjects the breakdown of fatty acids proceeds via β-oxidation. The resulting acetyl-CoA moieties condense to form acetoacetate which is converted to D-3-hydroxybutyrate. There is, however, a growing list of reports on subjects who are apparently unable to β-oxidize fatty acids properly [1–4]. The metabolism of fatty acids in these patients is shifted in part to ω-oxidation. In addition, varying amounts of 5-hydroxyhexanoic acid, 7-hydroxyoctanoic acid and 9-hydroxydecanoic acid have been found in urine samples from such

* To whom correspondence should be addressed.

patients as a reflection of (ω-1)-oxidation. The clinical condition associated with excessive excretion of 5-hydroxyhexanoic acid seems to be part of Reye's syndrome, at least in a number of cases [4,5]. Inhibition of acyl-CoA dehydrogenases, e.g. by hypoglycin A in Jamaican vomiting sickness [6], may have the same clinical and biochemical effect.

Little information is available on the mode of formation of 5-hydroxyhexanoic acid in humans. Knowledge of the absolute configuration would enable a comparison with mechanisms that have been suggested to be operative in several animal cell systems.

In this report we describe the determination of the absolute configuration of urinary 5-hydroxyhexanoic acid in patients showing an impaired β-oxidation of fatty acids. The relevance of this finding will be discussed in relation to the published data on (ω-1)-oxidation of fatty acids [7,8].

Methods and materials

Chemicals

DL-5-Hydroxyhexanoic acid was prepared from 4-acetylbutyric acid (Aldrich Europe, Beerse, Belgium) by reduction with sodium borohydride (2 h at room temperature) followed by neutralization with Dowex 50 × 8, H^+-form (25–50 mesh) and removal of boric acid by co-evaporation with methanol.

Because of the easy lactone formation of 5-hydroxyhexanoic acid, the compound was converted immediately into its Ba-salt, in which form it can be stored safely.

Methyl-D-5-hydroxyhexanoate (80% D) was prepared by anodic coupling of D-3-acetoxybutyric acid (80% D) [9–11] and succinic acid monomethyl ester [12], followed by alkaline hydrolysis and, after acidification and extraction, re-esterification with diazomethane in diethyl ether [7,13].

D-2-Phenylpropionyl chloride was synthesized as described by Hammarström and Hamberg [14]. To test the optical purity of this reagent, it was esterified with (-)-menthol and analyzed on a capillary column of SP-1000 at 160°C as reported earlier for other chiral organic acids [15]. It was found that the acid chloride contained 1% of the L-enantiomer.

Preparation of O-D-2-phenylpropionylated (D-PP) derivatives of methyl 5-hydroxyhexanoates

5-Hydroxyhexanoic acid (25 μmol) was dissolved in 1 ml ethyl acetate and esterified with diazomethane in diethyl ether. After removal of the excess of diazomethane by evaporation to dryness, the residue was treated with 100 μmol D-2-phenylpropionyl chloride in 200 μl dry benzene and 50 μl dry pyridine [14]. The mixture was kept at room temperature for 2 h. Subsequently, 2 ml benzene was added and the organic phase was extracted twice with 1 ml 1 mol/l $NaHCO_3$, followed by two washings with 1 ml water. The benzene layer was dried on anhydrous Na_2SO_4, and, after evaporation, the residue was dissolved in ethyl acetate for analysis by GLC and GLC-MS. Urinary ethyl acetate extracts were derivatized in the same way.

Quantitative analysis of urinary organic acids

Organic acids were extracted from urine with ethyl acetate and analyzed quantitatively by GLC of the corresponding pertrimethylsilyl derivatives as reported earlier [16]. For a quantitative analysis of urinary 5-hydroxyhexanoate the urine extract has to be silylated immediately after removal of the ethyl acetate by evaporation. It has been our experience that storage of the dry residue for 1 day leads to a loss of nearly 50%, probably due to lactone formation. Silylated samples can be stored up to a week without any adverse effects.

Capillary gas-liquid chromatography

The D-PP derivatives of methyl 5-hydroxyhexanoates and the urinary extracts treated with diazomethane and D-2-phenylpropionyl chloride were analyzed by capillary GLC on SE-30 (wall-coated glass capillary column, 25 m × 0.3 mm) using a Varian Aerograph 3700 instrument with a flame-ionization detector. The oven, injection-port and detector temperatures were 150°, 210° and 230°C, respectively. The carrier gas nitrogen flow-rate was 1.5 ml/min and the make-up gas nitrogen flow-rate 30 ml/min. For the injection a stream splitter with a ratio 1:10 was used.

Gas chromatography-mass spectrometry

The 75-eV mass spectra of the derivatized 5-hydroxyhexanoic acids were recorded on a Jeol JCG-20/JMS-D100/W-JMA combination using 3.8% SE-30 on Chromosorb W/HP, 100–120 mesh, as column material. The ion-source temperature was 150°C, the accelerating voltage 3 kV, the ionizing current 300 μA and the oven temperature 160°C.

Patients

Patient 1 was a 10-month-old boy who was admitted in a hypoglycemic subcomatose state following two days of diarrhoea and convulsions. Plasma glucose was 1.25 mmol/l and the gas chromatogram of urinary organic acids showed a major peak of 5-hydroxyhexanoic acid, together with adipic, suberic and unsaturated suberic acids. After treatment with i.v. glucose the patient recovered completely.

Patient 2 had had several attacks of hypoglycemia, accompanied by dicarboxylic aciduria. When she was put on a ketogenic diet she became comatose due to hypoglycemia. A urine sample collected in this period contained appreciable amounts of dicarboxylic and (ω-1)-hydroxy fatty acids. Analysis of urinary organic acids as their methyl ester derivatives revealed that this sample also contained *N*-suberylglycine and *N*-hexanoylglycine.

Patient 3 was a five-year-old mentally retarded girl who had been suffering from epilepsy (Lennox type) for 1 year. Her convulsions hardly responded to the usual drug therapy including valproic acid. Subsequently she was given a diet containing medium-chain triglycerides (MCT), an experimental therapy having proved its value in drug-resistent epileptics [17]. However, in this patient the 3-hydroxybutyrate

concentration failed to reach the required level of 2 mmol/l and there was no clinical response. Dicarboxylic acids prevailed in the urinary organic acid profile. Plasma carnitine was 17 μmol/l (normal 25–60).

Patient 4 was a two-year-old healthy boy whose transient dicarboxylic aciduria occurred after minor surgery anaesthesia.

Results

The capillary gas chromatogram of the D-PP derivative of methyl DL-5-hydroxyhexanoate on SE-30 as the non-chiral stationary phase is presented in Fig. 1a. The enantiomeric origin of the two peaks was deduced from co-chromatographic experiments with the D-PP derivative of synthetic methyl D-5-hydroxyhexanoate (80% D). Hence, the diastereomeric derivative of L-5-hydroxyhexanoic acid has a shorter retention time than that of D-5-hydroxyhexanoic acid. Both peaks could also be resolved on a packed column of 3.8% SE-30; they gave rise to the same mass spectrum (Fig. 2).

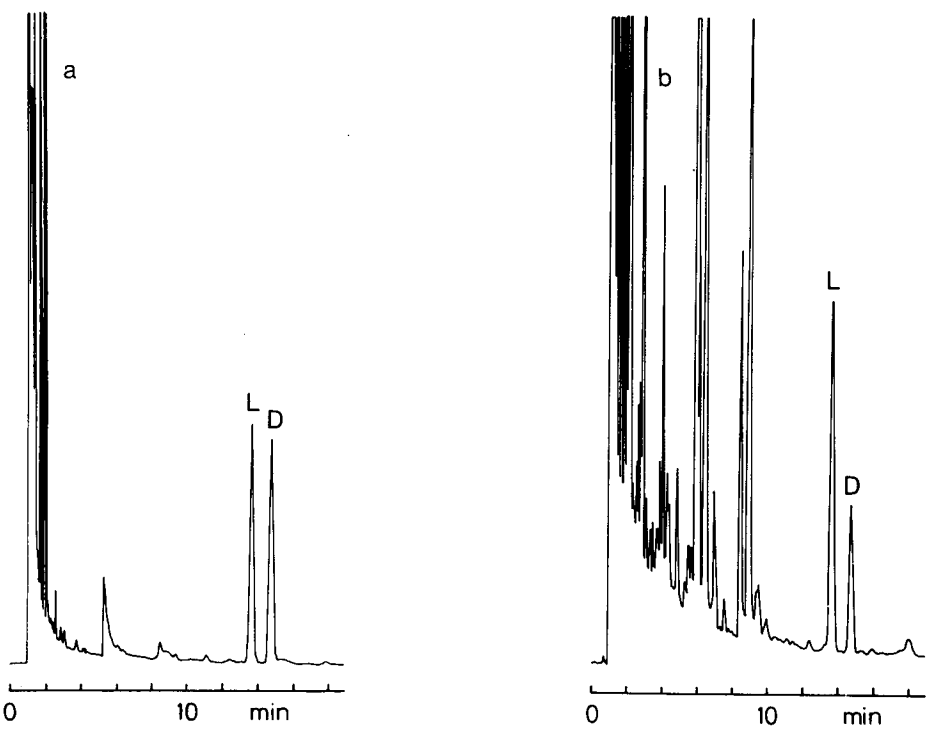

Fig. 1. (a) Capillary gas-liquid chromatography of the *O*-D-2-phenylpropionylated methyl ester derivatives of synthetic DL-5-hydroxyhexanoic acid on SE-30. (b) Characteristic chromatogram of a urine extract showing the ratio between L- and D-5-hydroxyhexanoic acid.

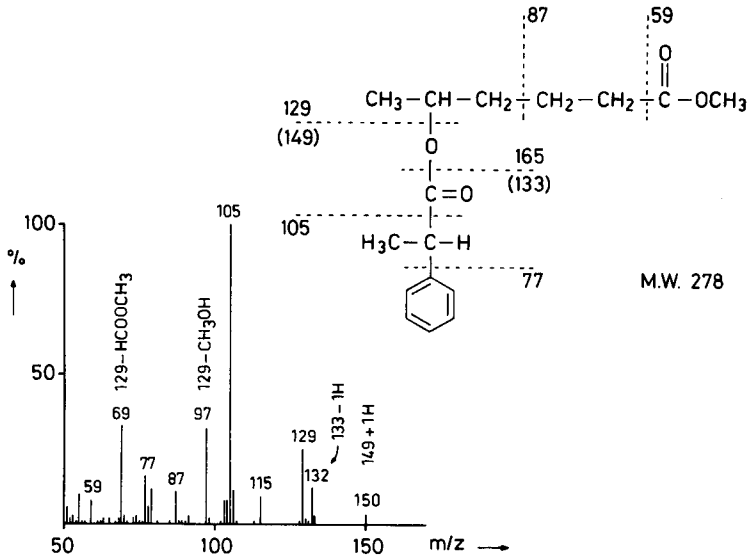

Fig. 2. Mass spectrum of the *O*-D-2-phenylpropionylated methyl ester derivative of DL-5-hydroxyhexanoic acid.

The excretion values of 5-hydroxyhexanoate in the patients and the distribution of the L- and D-enantiomers are shown in Table I. The L-enantiomer was predominant in all samples; the L/D-ratio was more or less constant. A typical example of a gas chromatogram showing the separation of the enantiomers in a patient's urine is given in Fig. 1b.

There does not seem to be a marked relation between the urinary 5-hydroxyhexanoate concentration and those of the dicarboxylic acids. In general the excretion of 5-hydroxyhexanoic acid was always found to be lower than that of adipic acid, the only exception being patient 1, whose adipic acid excretion amounted to 691 μmol/l, whereas 5-hydroxyhexanoate was 803 μmol/l. Subjects with a normal ability to β-oxidize fatty acids may produce 5-hydroxyhexanoate when fed a diet containing medium-chain triglycerides instead of ordinary fat.

TABLE I

RATIO OF URINARY L-5-HYDROXYHEXANOATE (L-5HH) TO D-5-HYDROXYHEXANOATE (D-5HH) IN PATIENTS WITH DICARBOXYLIC ACIDURIA

Patient	L-5HH (%)	D-5HH (%)	Total excretion (μmol/l)
1	71	29	803
2	74	26	515
3	71	29	2311
4	66	34	912

Urinary concentrations between 32 and 177 μmol/l were found in three children on such a diet. These values sharply contrast with those observed in patient 3, whose 5-hydroxyhexanoate levels reached 2.5 mmol/l on an MCT-diet.

So far 5-hydroxyhexanoate has been detected in all patients showing an increased ω-oxidation of fatty acids, either as a result of an apparently defective β-oxidation or following the ingestion of medium-chain triglycerides. Disappearance of 5-hydroxyhexanoate excretion (e.g. after stopping the MCT-diet) was always seen in combination with a disappearing adipate/suberate excretion.

Discussion

Capillary gas-liquid chromatography of diastereomeric derivatives of chiral hydroxycarboxylic acids on non-chiral stationary phases has proved to be a valuable approach for the determination of the absolute configurations of these acids [15,18,19]. The separation described here of D- and L-5-hydroxyhexanoic acids as their *O*-D-2-phenylpropionylated methyl ester derivatives could even be achieved by packed column GLC.

The majority of enzymic reactions taking place in the human body are highly stereospecific, thereby leading to the formation of one enantiomer only. This has been exemplified with 3-hydroxybutyrate possessing the D-configuration, whereas 2-hydroxybutyrate in humans is always in the L-form [19].

The ω- and (ω-1)-oxidation of fatty acids have been studied by various authors. These reactions have been shown to proceed mainly in the microsomal fraction of liver and kidney cells [7].

In vitro studies of (ω-1)-oxidation of some fatty acids have demonstrated the formation of two stereoisomers. When rat liver microsomes were incubated with decanoic acid, 75% of the resulting 9-hydroxydecanoate had the L-configuration, whereas 25% consisted of the D-form [7]. The same authors also studied the oxidation of lauric acid and found the respective 11-hydroxylated stereo isomers to occur in a ratio of 40:60. They presumed the L-stereo isomer to be the minor component [20]. The finding of the two enantiomers of 5-hydroxyhexanoate suggests that the in vivo (ω-1)-oxidation of fatty acids probably proceeds via the same mechanism(s) as tentatively deduced from in vitro experiments, i.e. a cytochrome P-450 mediated direct hydroxylation. We could not find evidence for any further metabolism of 5-hydroxyhexanoate.

The absolute amount of 5-hydroxyhexanoic acid produced by the human body is only small. The products of ω-oxidation, i.e. the dicarboxylic acids, were always of more importance than the (ω-1)-hydroxylated acids, with the exception of patient 1.

In addition to the well-known dicarboxylic aciduria resulting from the ingestion of medium-chain triglycerides [21], Shigematsu et al [22] recently described the urinary excretion of 5-hydroxyhexanoic acid by normal infants who were given a MCT-containing formula. It is therefore unlikely that 5-hydroxyhexanoic acid is a characteristic metabolite indicating some kind of intoxication, as was suggested by Chalmers et al [4]. Mamer's hypothesis that 5-hydroxyhexanoate production is a consequence of a deficiency of medium-chain acyl-CoA dehydrogenase [5] also has

to be rejected in view of its widespread occurrence.

We conclude that 5-hydroxyhexanoic acid is merely a by-product of fatty acid oxidation. It is produced in appreciable amounts only when the normal β-oxidation is malfunctioning or when excess fatty acid is available for extra-mitochondrial oxidation. Its finding may be a sensitive indicator for the detection of such conditions.

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