JOURNAL OF MASS SPECTROMETRY, VOL. 31, 633–638 (1996)

3-, 6- and 7-Hydroxyoctanoic Acids are Metabolites of Medium-Chain Triglycerides and Excreted in Urine as Glucuronides

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Three new metabolites of medium-chain fatty acid oxidation, 3-, 6- and 7-hydroxyoctanoyl β-D-glucuronide, were identified in the urine of six infants who were fed a diet enriched in medium-chain triglycerides (MCT). Glucuronides were extracted from the urine by organic solvent extraction with ethyl acetate and by solid-phase extraction on Sep-Pak C_{18} cartridges. The compounds of interest were also purified from the organic solvent extract by preparative one-dimensional thin-layer chromatography. Cleavage of the glucuronides was achieved by either alkaline hydrolysis or enzymatic hydrolysis with β-D-glucuronidase. The analyses of the trimethylsilylated derivatives were performed both by gas chromatography with flame ionization detection (GC/FID) and by gas chromatography/mass spectrometry (GC/MS). The structure of the hydroxyoctanoic acids was proved by comparison of their mass spectra with those of reference substances. Authentic 6-hydroxyoctanoic acid was synthesized. The presence of 6-hydroxyoctanoyl glucuronide shows that in addition to β-oxidation, ω-oxidation and (ω − 1)-hydroxylation, medium-chain fatty acids can be oxidized at the ω − 2 position. The conjugation of medium-chain hydroxymonocarboxylic acids with glucuronic acid has not been described in humans before.

KEYWORDS: hydroxyoctanoyl glucuronides; medium-chain fatty acid oxidation; omega oxidation; medium-chain triglyceride feeding; mass spectrometry

INTRODUCTION

Medium-chain triglyceride (MCT) oil is an edible oil composed of triglycerides with saturated fatty acid residues of chain length C_6–C_{12}. Medium-chain triglycerides were introduced in clinical nutrition in the 1950s and over the past 45 years they have been used effectively in the treatment of patients with disorders related to digestion, absorption or transport of conventional dietary fats (long-chain triglycerides (LCTs).[1] Moreover, they have been used in feeding formulas for low birth weight infants[2] and preterm neonates[3,4] to enhance fat absorption and to provide a concentrated energy source. Recently, a protective effect of MCT feeding in inherited long-chain fatty acid oxidation disorders has also been reported.[5] The widespread use of MCT in clinical nutrition, replacing LCT, is mainly based upon the efficiency of their intestinal absorption, transport to the liver and rapid hepatic oxidation. Recent findings suggest that medium-chain fatty acids (MCFA) can be incorporated in plasma lipids[6] and in the adipose tissue[7] or even converted into long-chain fatty acids.[8] MCTs are hydrolysed both faster and more completely than LCTs in the gut and the resulting free acids are then rapidly absorbed and transported to the liver via the hepatic portal circulation.[9] Medium-chain fatty acids are, unlike long-chain fatty acids, able to cross the double mitochondrial membrane independent of carnitine. In the mitochondrial matrix they are activated to Coenzyme A (CoA) esters, which are rapidly oxidized through β-oxidation. The resulting excess of acetyl-CoA can then follow different metabolic pathways, both in the mitochondria (Krebs cycle, ketogenesis) and in the cytosol (*de novo* synthesis of fatty acids and cholesterol).[1] Medium-chain fatty acids can also undergo β-oxidation in peroxisomes, which is similar to mitochondrial β-oxidation but under separate genetic control.[10] In addition, hepatic microsomes carry out ω- and (ω − 1)-oxidation, which require the involvement of cytochrome P450.[11–14] Under normal conditions the amount of either peroxisomal or microsomal oxidation is almost negligible.[1,2] However, when mitochondrial β-oxidation is either saturated by excessive delivery of fatty acids to the liver (diabetic ketoacidosis, normal fasting and feeding with MCT) or genetically impaired, the alternative fatty acid oxidation pathways are induced and MCFAs are oxidized both in liver peroxisomes and microsomes.[15,16] Under these conditions, large amounts of dicarboxylic and (ω − 1)-hydroxy acids are produced and readily excreted in urine.[13] In this respect, microsomal oxidation can be seen as a detoxification mechanism. The deacylation of acyl-CoA by thioesterases and the conjugation of acyl groups to glycine, carnitine and glucuronic acid are the

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CCC 1076–5174/96/060633–06

Received 8 August 1995
Accepted 8 February 1996

major detoxification systems in humans. These conjugation mechanisms lead to the formation of more polar compounds that can cross cellular membranes more easily than do the respective CoA esters being readily excreted in urine.[16] However, and despite the considerable number of known fatty acid conjugated compounds with carnitine and glycine, ester glucuronides of fatty acids have only been described for valproyl glucuronide,[17] isovaleryl glucuronide[18] and octanoyl glucuronide[19,20]

This paper describes the identification of 3-, 6- and 7-OH-octanoyl glucuronide, three unknown metabolites of MCT, in the urine of children under MCT-enriched diets. As far as we know, 6-OH-octanoic acid is the first ($\omega - 2$)-hydroxy acid to be described in humans.

EXPERIMENTAL

Synthesis of reference 6-hydroxyoctanoic acid

6-OH-octanoic acid was prepared by reaction of methyl adipate with 2-hydroxybutyric acid in the presence of sodium methoxide (the Kolbe reaction).[21] A 5.3 g amount of methyl adipate, 5.3 g of 2-hydroxybutyric acid and 0.5 g of sodium methoxide dissolved in 160 ml of methanol were placed in an electrolysis cell with two platinum electrodes. This solution was electrolysed with a current of 1.3 A until the solution became slightly alkaline (~2 h). Subsequently the mixture was concentrated by rotary evaporation and then an aliquot of 1 ml was submitted to alkaline hydrolysis with 1 ml of 11 M NaOH at 100 °C for 1 h. After cooling, the pH was adjusted to 1–2 with HCl and the compound of interest was extracted twice with ethyl acetate. After drying over anhydrous sodium sulphate and evaporation of the solvent, the extracted compounds were trimethylsilylated and then analysed by GC/MS.

7-OH-octanoic acid was obtained as a gift from Dr N. Gregersen (Aarhus, Denmark). 3-OH-octanoic acid has been synthesized previously in our laboratory.[22]

Biological samples

Urine samples were collected from six children who were given a diet enriched in MCT. Their ages varied between 1 and 10 months and the reasons for the MCT diet were, depending on each case, malabsorption, vomiting, gastroesophageal reflux, insufficient weight gain or convulsions. A fatty acid oxidation disorder was excluded in these children based on the lack of evidence of hypoketotic hypoglycaemia and the absence of liver or muscle disease. A healthy adult was submitted to an MCT loading test to emphasize the metabolic response to the MCT diet. The taste and smell of pure MCT oil are unpleasant; therefore, we had to limit the control experiment to one subject. MCT loading (1.5 g of MCT oil/kg) was performed after a 12 h overnight fast. Urine samples were collected 12 h before and in two 4 h collections after the loading test.

Sample preparation

Organic acids were extracted twice with two volumes of ethyl acetate from acidified urine (pH 1–2). The combined organic layers were dried over anhydrous sodium sulphate. The solvent was then removed by rotary evaporation at 35 °C under reduced pressure. The residue was trimethylsilylated with *N,N*-bis(trimethylsilyl)trifluoroacetamide, pyridine and trimethylchlorosilane (5:1:0.05, v/v/v) at 60 °C for 30 min, as described before.[19]

Enzymatic hydrolysis

Urine and the thin-layer chromatographically (TLC) isolated metabolites were incubated with 1.5×10^{-4} U of β-D-glucuronidase (EC 3.2.1.31, from *Escherichia coli*; Boehringer, Mannheim, Germany) at pH 6.9 and 37 °C for 16 h. The reaction mixture was acidified to pH 1–2, extracted with ethyl acetate and then analysed by GC/MS. Identification of 3-OH-octanoic[22] and 7-OH-octanoic acid was carried out by comparison with reference mass spectra. The reference mass spectrum for the identification of 6-OH-octanoic acid was achieved by the synthesis of the reference compound.

GC/FID analyses

Capillary GC analyses were performed with a Star 3400 instrument (Varian, Palo Alto, CA, USA) equipped with a WCOT fused-silica capillary column (25 m × 0.25 mm i.d.) coated with CP-Sil19 CB, film thickness 0.19 μm (Chrompack, Middelburg, The Netherlands), a flame ionization detector (FID), a Model 8100 autosampler and a Model DS 604 data system, A 1 μl volume of each sample was injected in the split mode (ratio 1:20) under the following conditions: initial oven temperature, 60 °C for 4 min, then programmed at 5 °C min^{-1} to 280 °C, the final temperature being maintained for 9 min. The temperatures of the injection port and the FID were 240 and 310 °C, respectively.

CG/MS analyses

For the GC/MS analyses we used two instruments: (A) a Hewlett-Packard (Avondale, PA, USA) Model 5890 Series II gas chromatograph interfaced with a Jeol (Tokyo, Japan) JMS-AX 505 W mass spectrometer in the chemical ionization (CI) mode with ammonia as the reagent gas and (B) a Varian Model 3700 gas chromatograph coupled to a Ribermag (Argenteuil, France) R10-10C quadrupole mass spectrometer in the electron impact (EI) mode.

In both instruments source temperature was kept at 250 °C and the ionization voltage was set at 70 eV in either the EI or CI mode. Data acquisition was processed with a scan range of 80–800. The chromato-

graphic conditions for each GC/MS combination were identical with those used in the GC/FID procedure.

Purification of urinary glucuronides

Thin-layer chromatography. The OH-octanoyl glucuronides were purified from the ethyl acetate extract by TLC. Silica sheets (10 × 10 cm, 0.1 mm layer, Alufolien; Merck, Darmstadt, Germany) were developed twice with *n*-butanol–acetic acid–water (2:1:1, v/v/v). The detection of glucuronides was performed by a colour reaction with orcinol–sulphuric acid at 105 °C for 3 min (40 mg of orcinol dissolved in a mixture of 4 ml of concentrated sulphuric acid and 80 ml of acetone). The compounds of interest were extracted from silica with water and then submitted to alkaline hydrolysis followed by extraction with ethyl acetate and subsequent analysis by GC/MS.[18,19]

Solid-phase extraction. Volumes of 5 ml of urine were passed through a Sep-Pak C_{18} cartridge (Waters–Millipore, Milford, MA, USA) previously washed with 5 ml of methanol followed by 5 ml of water. The cartridge was then washed again with 5 ml of water followed by 5 ml of hexane. The glucuronides were eluted with two 5 ml portions of methanol.[23] The methanolic extract was evaporated by rotary evaporation and the residue was tested either for the presence of intact glucuronides by GC/MS or for the presence of 3-, 6- and 7-OH-octanoate, also by GC/MS but after alkaline hydrolysis.

RESULTS

We studied urine samples from six children, aged 1–10 months, who were under a diet enriched in MCT oil. GC/FID and further GC/MS analysis of hydrolysed organic acids revealed the presence of an unknown compound in the urine of all the children. Therefore, in order to clarify whether it was a metabolite of MCT or not, a healthy adult was submitted to an MCT loading. Urinary organic acids from samples collected before and after the loading were analysed by GC/FID and GC/MS. Thus, in the sample collected before the loading no unknown compound could be detected at the position of the index peak either before or after alkaline hydrolysis. However, the organic acid profile from both samples collected after the loading showed the same metabolite as observed in the MCT-treated children, although in a larger amount. Thus, the unknown compound is probably a metabolite of MCT. This metabolite corresponds to peak 2 on the gas chromatogram, its retention time in our system being 23.43 min (Fig. 1).

On GC/MS analysis in the EI mode, the mass spectrum of this substance showed a fragment at *m/z* 289, suggestive of a molecular mass of 304 (compatible with hydroxylated octanoic acid). The base peak was at *m/z* 131, probably due to $[C_3H_6OTMS]^+$. Based on this fragmentation pattern, we hypothesized that its structure was identical with 6-OH-octanoic acid. In order to

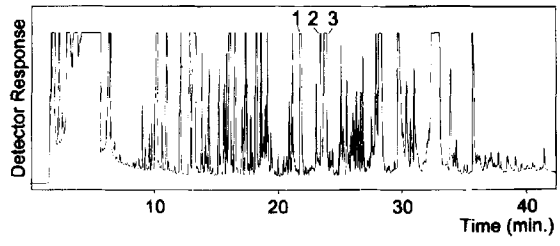

Figure 1. GC/FID of hydrolysed urinary organic acids (TMS derivatives) from the control after MCT loading. Peaks: 1 = 3-OH-octanoate + internal standard (3-phenylbutyrate); 2 = unknown; 3 = 7-OH-octanoate + adipate.

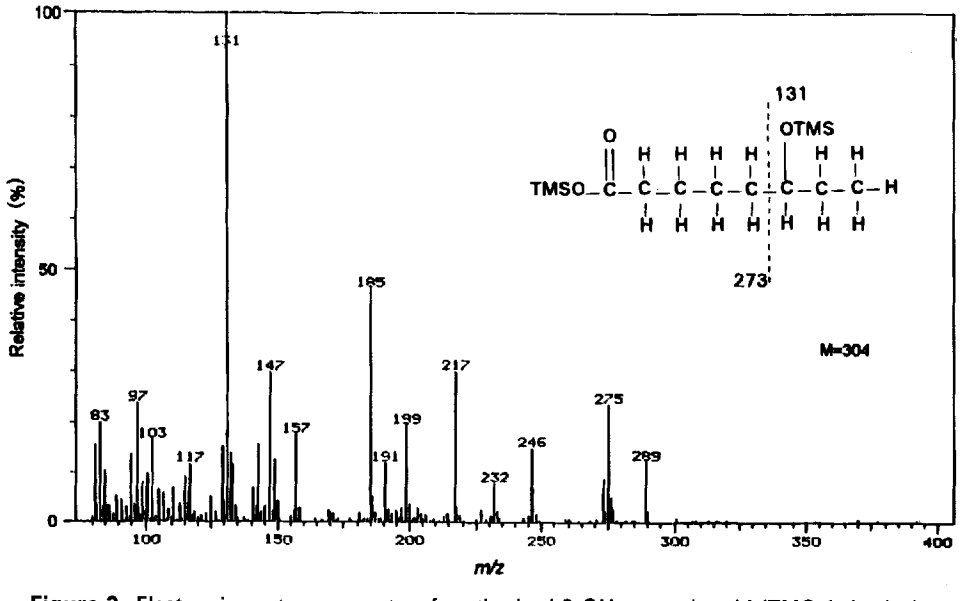

Figure 2. Electron impact mass spectra of synthesized 6-OH-octanoic acid (TMS derivative).

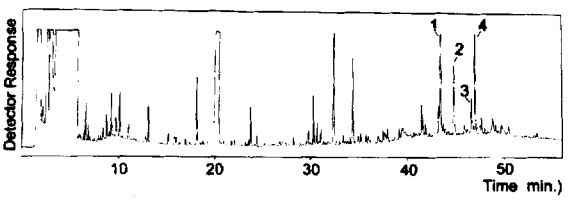

Figure 3. GC/FID of urinary organic acids (TMS derivatives) after solid-phase extraction. The sample was from a control subject after MCT loading. Peaks: 1 = octanoyl glucuronide; 2 = 3-OH-octanoyl glucuronide; 3 = 6-OH-octanoyl glucuronide; 4 = 7-OH-octanoyl glucuronide.

prove this hypothesis, we synthesized the reference substance, which eluted at the same retention time (23.45 min) and showed an identical mass spectrum (Fig. 2). The clear similarity of the GC retention times and the EI mass spectra of both synthesized 6-OH-octanoic acid and the unknown compound allowed us to conclude that they are identical substances.

As 6-OH-octanoic acid was observed in urine only after alkaline hydrolysis, it must mean that this substance was excreted in the form of a conjugate. Among the different conjugation mechanisms such as with glycine, carnitine and glucuronic acid, the last one seemed to be the most probable in accordance with those of other medium-chain fatty acids such as isovaleric acid[18] and octanoic acid.[19,20] Isolation of the acylcarnitine fraction from the urine and subsequent analysis of the acyl groups failed to detect hydroxyoctanoylcarnitine(s).

In order to establish the presence of glucuronides, we used solid-phase extraction with Sep-Pak C_{18} car-

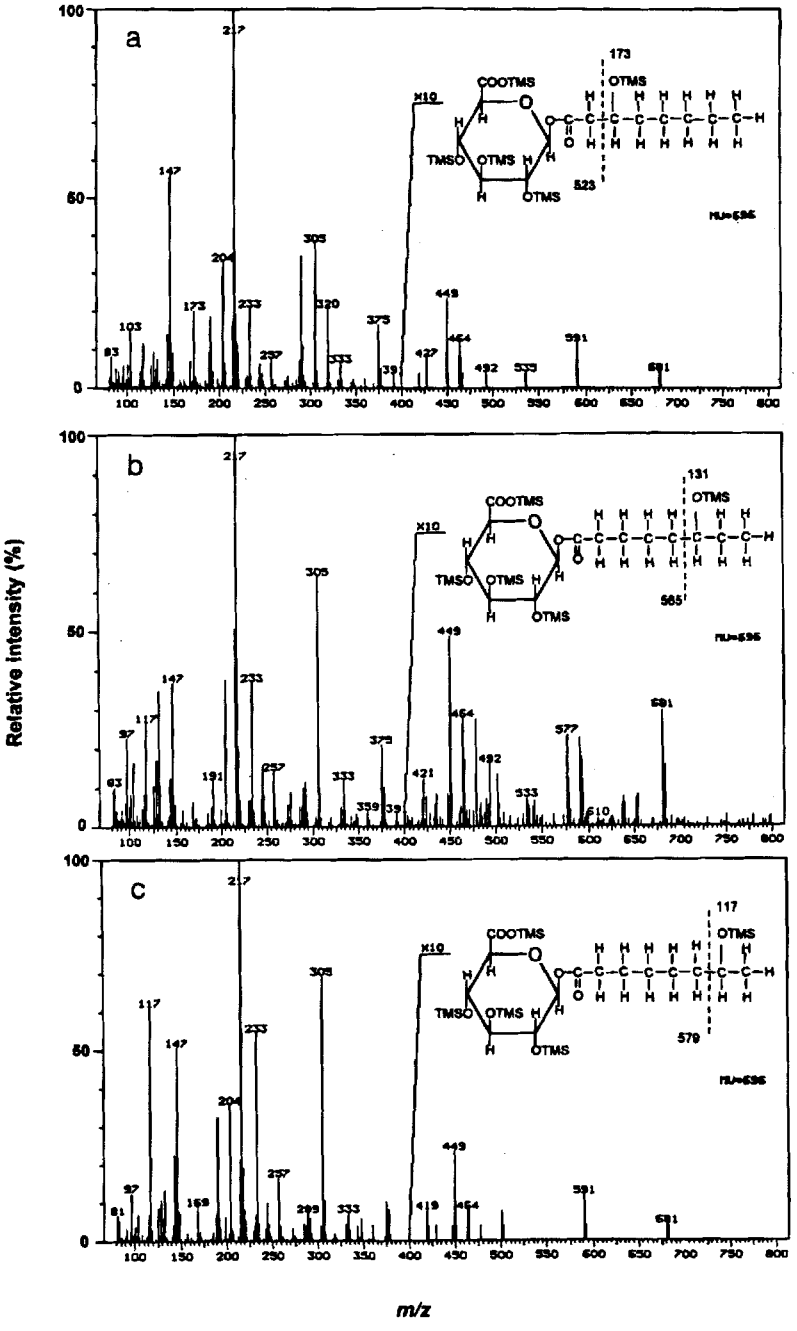

Figure 4. Electron impact mass spectra of (TMS derivatives) (a) 3-OH-octanoyl glucuronide (b) 6-OH-octanoyl glucuronide and (c) 7-OH-octanoyl glucuronide, isolated from urine of the control after the MCT loading test.

Table 1. Ammonia chemical ionization mass spectrometry of trimethylsilylated 3-, 6- and 7-hydroxyoctanoyl glucuronides in the urine of a control subject after an oral loading test with medium-chain triglycerides[a]

	Relative abundance (%)		
m/z	3-OH-	6-OH-	7-OH-
714	8	4	8
410	64	53	46
336		5	5
305	22	9	10
250	8		5
234	18	7	6
233	11	6	11
217	7	7	6
153			13
90	100	100	100

[a] Analytical conditions are given in the text.

tridges. Both GC/FID and GC/MS analyses of the eluted compounds now revealed three major peaks (Fig. 3). Their retention times in our system were 44.80, 46.67 and 47.00 min, respectively.

The EI mass spectra of all three substances showed a fragment at *m/z* 681, suggestive of a molecular mass of 696 (Fig. 4). This was subsequently confirmed by an analysis in the ammonia CI mode, which showed a peak at *m/z* 714 ($[M + 18]^+$ for all three compounds (Table 1). The fragmentation patterns obtained by EI/MS were suggestive of the presence of trimethylsilylated glucuronides. The ion at *m/z* 591 corresponds to $[M - CH_3 - TMSOH]^+$. The fragments observed at *m/z* 204, 217, 305 and 375 are derived from trimethylsilylglycone and are commonly seen in the mass spectra of trimethylsilylated glucuronides.[24,25] The OH-octanoyl fragment $[C_8H_{14}O_2TMS]^+$ was also present in all the mass spectra at *m/z* 215. A characteristic fragment ion was observed for the 3-, 6- and 7-OH-octanoyl moieties at *m/z* 173, 131 and 117, respectively. This represents α-cleavage of the molecule next to the C—O—TMS group. Furthermore, alkaline hydrolysis of the solid-phase extract led to the disappearance of all these compounds, which again proves their proposed structure. Enzymatic hydrolysis with β-D-glucuronidase would be the approach to prove that these three compounds are in fact β-D-glucuronides. For this purpose, we isolated the glucuronides by TLC and subsequent elution from the thin-layer sheets with water. When this extract was incubated with β-D-glucuronidase, further GC/MS analysis (TMS derivatives) revealed only 3-, 6- and 7-OH-octanoate. Thus we conclude that these compounds are excreted as β-D-glucuronides.

Neither 3-, 6- nor 7-OH-octanoyl glucuronide was synthesized and therefore a quantitative determination was not possible. Nevertheless, the relative abundances of the OH-octanoyl glucuronides excreted by the six children and the control were similar. The most abundant OH-octanoyl glucuronide excreted was always 7-OH-octanoyl glucuronide, followed by 3- and 6-OH-octanoyl glucuronide in smaller amounts.

The healthy adult control who was submitted to an MCT loading test with 80 g of medium-chain triglycerides excreted all the OH-octanoyl glucuronides in much higher quantities, as expected after the loading. Similar loading tests in two children suspected of having a non-specified defect of long-chain fatty acid oxidation gave comparable results.

DISCUSSION

We have identified three novel intermediates of medium-chain fatty acid oxidation, viz. 3-, 6- and 7-hydroxyoctanoyl-β-D-glucuronide. The structure of these substances was proved by comparison of their mass spectra with those of reference substances and by the results of hydrolysis experiments including one with β-D-glucuronidase. EI fragmentation of trimethylsilylated hydroxymonocarboxylic acids is characteristic: the base peak in the mass spectrum is the fragment representing cleavage next to the hydroxyl group, proximal to the carboxyl group. This was observed previously for a serious of 3-hydroxyfatty acids[22] and confirmed in the present work for octanoic acid with a hydroxyl group at position 6 or 7. The same fragmentation takes place in EIMS of the intact glucuronides. Under these conditions, the molecular ion cannot be detected; ammonia CIMS filled this gap.

Medium- and long-chain 3-hydroxymonocarboxylic acids are known to be conjugated with carnitine.[26] Nevertheless, conjugation of medium-chain hydroxymonocarboxylic acids with glucuronic acid in humans has not been described before. Although mitochondrial β-oxidation is the main pathway of fatty acid catabolism, extra-mitochondrial pathways located in peroxisomes and the endoplasmatic reticulum are available.[27] It is known that in microsomes monocarboxylic acids can be hydroxylated at either terminal (ω-position) or penultimate carbon [(ω-1) position] by an NADPH-dependent cytochrome P450 monooxygenase to form ω- and (ω-1)-hydroxy acids, that can be further oxidized to ω-keto acids and dicarboxylic acids. The products of microsomal oxidation can then be chain shortened, undergoing β-oxidation either in mitochondria or peroxisomes.[14]

This work suggests that ω- and (ω-1)-oxidation are not unique alternative metabolic pathways available in human microsomes for fatty acid oxidation. The GC/MS analysis of hydrolysed urines from MCT-feeding children revealed the presence of two hydroxyoctanoic acids whose metabolic origin is known to be mitochondrial β-oxidation (3-OH-octanoic acid) and microsomal (ω-1)-oxidation (7-OH-octanoic acid). Nevertheless, we also detected a hitherto unknown metabolite, 6-OH-octanoic acid, which provides evidence that medium-chain fatty acids in situations of overloaded mitochondrial β-oxidation (as in the case of MCT feeding) can be oxidized by the cytochrome P450 superfamily, not only at ω and ω-1 positions but also at the ω-2 position. (ω-2)-Hydroxylation has recently been described to be present in some bacteria. It has been reported that ω-1, ω-2 and ω-3 medium- and long-chain

monohydroxylated fatty acids are produced by an unusual bacterial cytochrome P450 enzyme that exhibits higher sequence identity (25%) with the CYP 4A class of mammalian enzymes than with other bacterial enzymes.[28] It has also been reported that (ω-2)-hydroxylated metabolites of arachidonic acid are produced by cytochrome P450 oxidoreductase from rabbits.[29]

In our studies, 6-OH-octanoic acid was the only (ω-2) -hydroxylated metabolite from MCT. We could not find evidence for the presence of 8-OH-decanoic acid or 4-OH-hexanoic acid, in either free or conjugated form. In this respect, it must be realised that octanoate is by far the most abundant fatty acid in MCT.

The fact that the accumulated 3-, 6- and 7-OH-octanoic acids are excreted not only in the free form but also as their respective β-D-glucuronides revealed an unknown conjugation mechanism for this kind of compound.

Glucuronidation is an extra-mitochondrial process catalysed by a membrane-bound microsomal multi-gene UDP-glucuronosyltransferase family, which is the principal route of elimination for a large number of drugs and other xenobiotics.[30,31] Conjugation with glucuronic acid confers greater polarity and water solubility on the aglycone, thereby facilitating excretion. In this respect, the formation of 3-, 6- and 7-OH-octanoyl β-D-glucuronide can be seen as a detoxification process. The fact that 6-OH-octanoic acid has been detected in non-hydrolysed urines only as 6-OH-octanoyl β-D-glucuronide seems to indicate that this metabolite has a high affinity to UDP-glucuronyltransferase.

MCT ingestion results in a prompt supply of medium-chain fatty acids to the liver cells, leading to an overloading of mitochondrial β-oxidation and subsequently to the activation of the alternative pathways. Its consequence is the accumulation of the partially oxidized metabolites generated by all the pathways involved in fatty acid oxidation metabolism. Thus some of these metabolites undergo glucuronidation in order to accelerate their excretion in urine. This seems to be the reason for the elimination of 3- (mitochondrial β-oxidation), 6- and 7-OH-octanoic acid (microsomal (ω-2) and (ω-1)-oxidation, respectively) as their corresponding β-D-glucuronides. As such, the formation of hydroxylated octanoate is a futile process in humans who have a normal β-oxidation capacity for medium-chain fatty acids. Octanoate is an efficient energy source and should not be disposed of in this way. No methods are available to prevent the microsomal hydroxylation.

In summary, this work has demonstrated that hydroxymonocarboxylic acids can undergo glucuronidation, all the 6-OH-octanoic acid found in the urine being excreted in the form of glucuronide. At the same time, our findings provide evidence that medium-chain fatty acids can be oxidized in microsomes at ω, ω-1 and ω-2 positions. The significance and the regulation of this pathway is not fully understood. More studies are needed for the complete elucidation of microsomal fatty acid oxidation and the significance of the metabolites produced.

Acknowledgement

Part of this work was made possible by a grant (BD 2589/93/ Programa Ciência) from the Junta Nacional de Investigação Científica e Tecnológica (Lisbon, Portugal) to Catarina C. G. Costa.

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