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Abnormal fatty acid metabolism in patients in hopantenate therapy during clinical episodes

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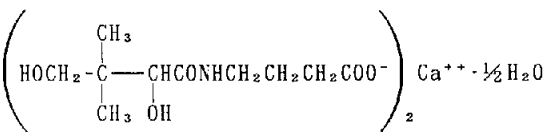
ABSTRACT

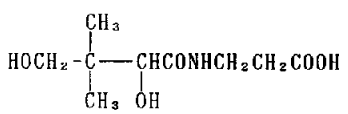
Calcium 4-(2,4-dihydroxy-3,3-dimethylbutyramido)butyrate hemihydrate (hopantenate), a cerebral metabolic enhancer used in Japan since 1978, is a homologue of pantothenic acid. Using mass spectrometry, we found urinary excretion of 4-hydroxydodecanedioic acid, 4-hydroxytetradecanedioic acid and a series of 2-hydroxydicarboxylic acids (C_8–C_{14}), in addition to a series of odd- and even-numbered dicarboxylic acids (C_5–C_{12}) and 3-hydroxydicarboxylic acids (C_8–C_{14}) in patients receiving hopantenate during episodes of Reye's-like syndrome. Our findings suggest that an acute intoxication associated with hopantenate occurs owing to pantothenic acid deficiency or the inhibition of CoA-requiring reactions during stress, *i.e.* infection, prolonged fasting, or malnutrition.

INTRODUCTION

Calcium 4-(2,4-dihydroxy-3,3-dimethylbutyramido)butyrate hemihydrate (hopantenate), a homologue of pantothenic acid, has been used as a cerebral metabolic enhancer for adults and children in Japan since 1978 (Fig. 1). Up to 1989, more than ten mentally retarded or autistic children and several senile



Hopantenate



Pantothenic Acid

Fig. 1. Molecular structures of hopantenate and pantothenic acid.

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patients with dementia or cerebral vascular disease were reported to have developed a Reye's-like syndrome during hopantenate therapy [1]. Although the incidence of such episodes is very low, hopantenate is presumed to be a pantothenic acid antagonist, and this acute intoxication seems to be due to pantothenic acid deficiency [2] or the inhibition of reactions requiring CoA. In the present study, we analysed the urinary organic acids of patients in hopantenate therapy during acute Reye's-like episodes, by means of capillary gas chromatography–mass spectrometry (GC–MS) and mass chromatography, in order to understand the abnormal metabolism in hopantenate intoxication.

EXPERIMENTAL

Subjects

Case 1. A two-year-old boy, the second child of healthy, non-consanguineous Japanese parents, was diagnosed as autistic during his second year of life. After 3 months therapy with 1.5 g/day of hopantenate, he developed a Reye's-like syndrome. We analyzed the patient's urinary acids during the acute episode and 1 month after his recovery.

Case 2. A 68-year-old woman with a history of cerebral infarction had been treated with hopantenate for one year and developed a Reye's-like syndrome. Urine samples were examined on admission and 1 month after her attack.

Chemicals

3-Hydroxymyristic acid and heptadecanoic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan). N,O-Bis(trimethylsilyl)trifluoroacetamide was obtained from Wako Pure Chemicals Industries (Tokyo, Japan) and N,O-bis-(nonadeutero-trimethylsilyl)acetamide was from Merck, Sharp & Dohme (Montreal, Canada). Other chemicals were obtained from commercial sources.

Analysis of organic acids in urine

Urine samples equivalent to 0.5 mg of creatinine, after addition of 20 μg of 3-hydroxymyristic acid and heptadecanoic acid as internal standards, were acidified to pH 1, and extracted three times with diethyl ether. The extracts were concentrated to dryness and trimethylsilylated with N,O-bis(trimethylsilyl)trifluoroacetamide or N,O-bis(nonadeutero-trimethylsilyl)acetamide for 1 h at 60°C, before analysis by GC–MS.

GC–MS was performed on a JEOL JMS DX-300 gas chromatograph–mass spectrometer supported by a JMA-3500 data acquisition system. The chromatographic separation was carried out on a MPS-50 fused-silica capillary column (25 m × 0.32 mm I.D., 1.0 μm film thickness, from Quadrex, CT, U.S.A.) using helium as a carrier gas at a column flow-rate of 0.8 ml/min. The injection temperature was 250°C, and the injection was carried out in the splitless mode. The oven temperature was started at 100°C and raised at 8°C/min to 290°C. Electron im-

pact MS parameters were: ionizing voltage, 70 eV; emission current, 0.3 mA; mass range, 50–650 u; ion-soure temperature, 220°C; acceleration voltage, 3 kV.

RESULTS

The profile of urinary organic acids from the child (case 1, shown in Fig. 2) and the adult patient (case 2) during the acute episodes of Reye's-like syndrome showed massive lactic aciduria. Ketone bodies and 2-hydroxybutyric acid were

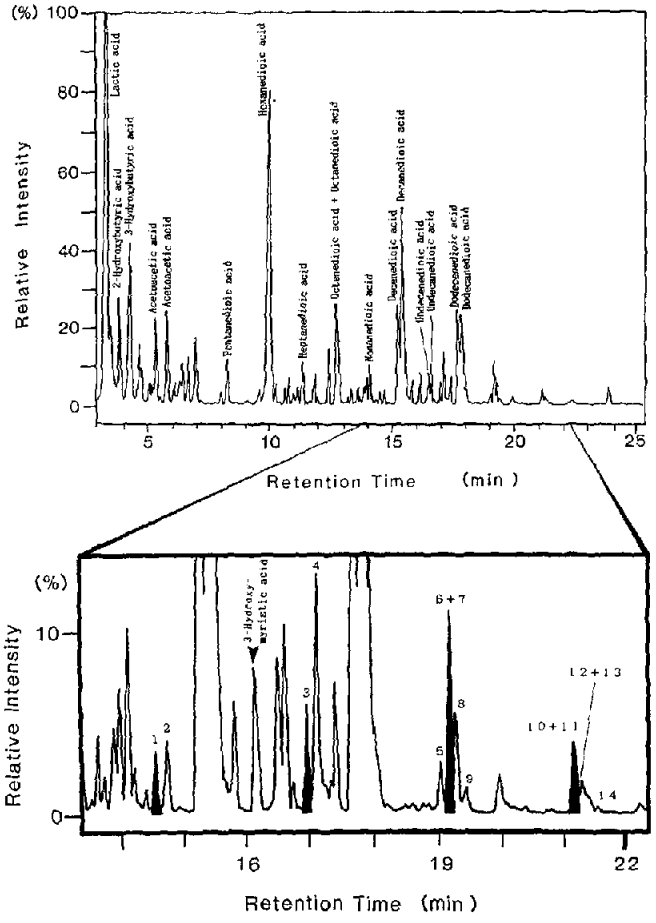
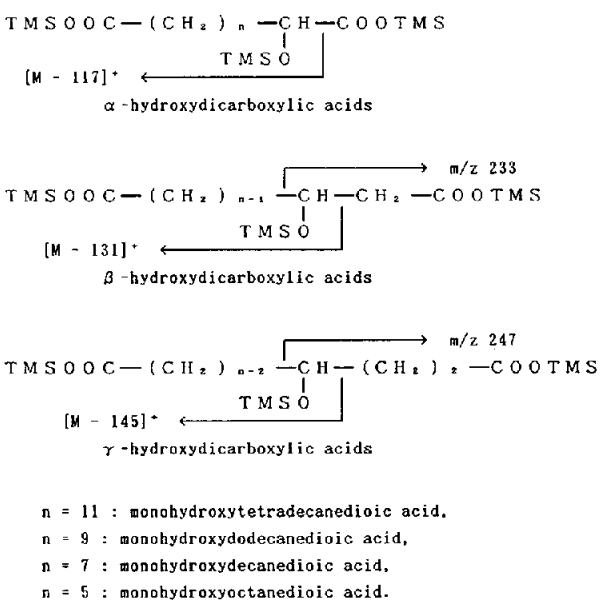


Fig. 2. RIC chromatogram of TMS derivatives of urinary organic acids in urine of a male infant (case 1) with acute clinical episode during hopantenate therapy. Peaks: 1 = 2-hydroxyoctanedioic acid; 2 = 3-hydroxyoctanedioic acid; 3 = 2-hydroxydecanedioic acid; 4 = 3-hydroxydecanedioic acid; 5 = 2-hydroxydodecenedioic acid; 6 = 2-hydroxydodecanedioic acid; 7 = 3-hydroxydodecenedioic acid; 8 = 3-hydroxydodecanedioic acid; 9 = 4-hydroxydodecanedioic acid; 10 = 2-hydroxytetradecenedioic acid; 11 = 2-hydroxytetradecanedioic acid; 12 = 3-hydroxytetradecenedioic acid; 13 = 3-hydroxytetradecanedioic acid; 14 = 4-hydroxytetradecanedioic acid.

also increased. Markedly increased excretion of even-numbered dicarboxylic acids with chain lengths of C_6–C_{12} was found. We noticed that odd-numbered dicarboxylic acids (C_5–C_{11}) were moderately increased and that some of the even- and odd-numbered dicarboxylic acids were accompanied by their corresponding unsaturated analogues. Increased excretion of a series of ($\omega-1$)-hydroxylated even-numbered fatty acids (C_6–C_{10}) and of 3-hydroxydicarboxylic acids (C_8–C_{14}) was also observed.

2-Hydroxydecanedioic acid was detected in the urine. This compound (peak 3 in Fig. 2) was eluted just before 3-hydroxydecanedioic acid (peak 4 in Fig. 2). The highest m/z value in the spectra obtained from both peaks was 419 $[M-CH_3]^+$. The first peak was identified as 2-hydroxydecanedioic acid tri-TMS because the most prominent ion was m/z 317, which corresponds to $[M-COOTMS]^+$, the α-cleavage ion of the TMS-ether (Fig. 3) [3]. The second peak was identified as 3-hydroxydecanedioic acid tri-TMS by the very intense peaks produced by α-cleavage of the TMS-ether, producing m/z 303 $[M-CH_2COOTMS]^+$ and m/z 233 $[TMSOCHCH_2COOTMS]^+$. The origin of these fragment ions is shown in Fig. 3, and has been reported previously [3].

2-Hydroxydodecanedioic acid was also detected (peaks 6 and 7 in Fig. 2). This compound was eluted just before 3-hydroxydodecanedioic acid (peak 8 in Fig. 2). A very intense peak was found at m/z 345 $[M-COOTMS]^+$ due to α-cleavage of TMS-ether (Fig. 3). The unkown compound (peak 9 in Fig. 2), which has the highest mass number at m/z 447 and is possibly $[M-CH_3]^+$, was eluted just after



n = 11 : monohydroxytetradecanedioic acid,
n = 9 : monohydroxydodecanedioic acid,
n = 7 : monohydroxydecanedioic acid,
n = 5 : monohydroxyoctanedioic acid.

Fig. 3. Comparison of molecular structures and typical fragmentations of trimethylsilyl derivatives of α-hydroxy-, β-hydroxy- and γ-hydroxydicarboxylic acids.

3-hydroxydodecanedioic acid. In the mass spectrum of the latter, two prominent ion peaks were observed at *m/z* 331 and *m/z* 233 due to α-cleavage of TMS-ether, but in that of the unknown compound, the prominent peaks were at *m/z* 317 and *m/z* 247, indicating that a hydroxyl group is located at the γ-position to a carboxyl group. The fragment peaks at *m/z* 204 and *m/z* 217, which are commonly observed in long-chain TMS derivatives of dicarboxylic acids [4], were also found. Thus we identified the unknown compound as 4-hydroxydodecanedioic acid.

Their homologues, 2-hydroxytetradecanedioic acid (peak 11 in Fig. 2) and 4-hydroxytetradecanedioic acid (peak 14 in Fig. 2), were also identified. They were eluted just before and after 3-hydroxytetradecanedioic acid, respectively. When the urinary acids were derivatized with N,O-bis(nonadeuterotrimethylsilyl) acetamide, the mass numbers of the characteristic ion peaks due to α-cleavage of the TMS-ether [3] shifted as expected in the mass spectra of 2-hydroxydicarboxylic acids and 4-hydroxydicarboxylic acids, as wel as in those of 3-hydroxydicarboxylic acids. Fig. 4 shows the mass chromatograms of TMS derivatives of monohydroxydicarboxylic acids with chain length C_{12} (*m/z* 447, $[M - CH_3]^+$) and C_{14} (*m/z* 475, $[M - CH_3]^+$). The ion peak at *m/z* 247 is commonly observed for 4-hydroxydicarboxylic acid tri-TMS due to α-cleavage of TMS-ether. Other α-cleavage ion peaks are found at *m/z* 317 and 345 in 4-hydroxydodecanedioic acid and 4-hydroxytetradecanedioic acid, respectively. The very intense ion peak at *m/z* 345 found at scan number 1157 is due to 2-hydroxydodecanedioic acid, and that at *m/z* 373 found at scan number 1278 is due to 2-hydroxytetradecanedioic acid. The two 3-hydroxydicarboxylic acids monitored by the ion peak at

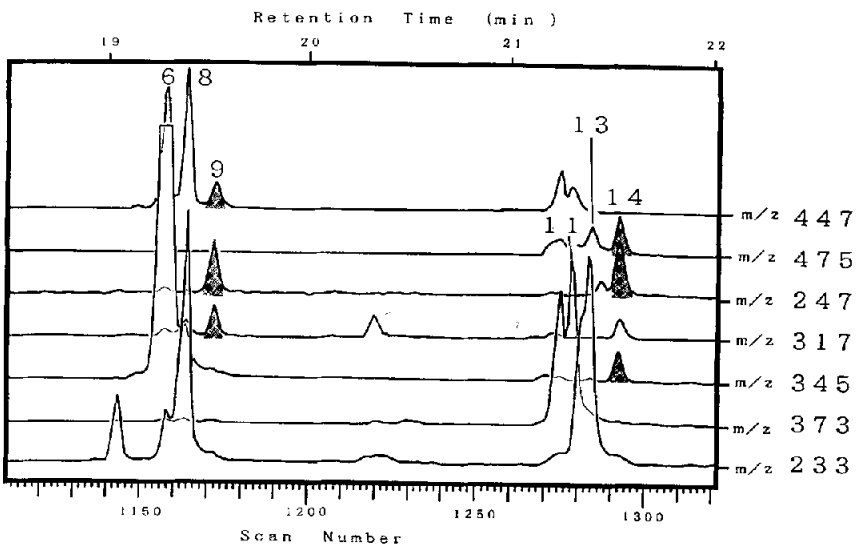


Fig. 4. Mass chromatograms of TMS derivatives of C_{12} and C_{14} monohydroxydicarboxylic acids. Numbers on the chromatograms indicate the corresponding peak numbers in Fig. 2.

m/z 233 were located between the 2- and 4-hydroxylated dicarboxylic acids, at scan numbers 1165 and 1283, respectively.

All 3-hydroxydicarboxylic acids and 2-hydroxydecanedioic acid were identified by the comparison of the mass spectra and retention times of the authentic standards reported [5,6]. The molecular structures of other 2-hydroxydicarboxylic acids and 4-hydroxydicarboxylic acids were confirmed, based on spectral interpretation and chromatographic behaviour. The existence of monounsaturated 4-hydroxytetradecanedioic acid was indicated by the presence of the prominent ions at *m/z* 247 and *m/z* 343 produced by α-cleavage, and the highest *m/z* value of 473.

The urinary organic acid profiles of both patients returned to normal after intensive care and withdrawal of hopantenate.

DISCUSSION

3-Hydroxylated medium- and long-chain dicarboxylic acids and their unsaturated homologues, identified first by Greter *et al.* [5] in 1980, are excreted in patients with abnormal fatty acid metabolism of various etiologies. The presence of 2-hydroxydecanedioic acid in human urine was first described in Zellweger syndrome and neonatal adrenoleukodystrophy [6], but the significantly increased excretion of this compound in human urine has not been reported under any other conditions. In the study of urinary organic acid profiles of patients with clinical episodes of Reye's-like syndrome during hopantenate therapy, a markedly increased excretion of 2-hydroxydodecanedioic acid, 2-hydroxyoctanedioic acid and 2-hydroxydecanedioic acid was found. In addition, we observed a moderately increased excretion of 4-hydroxydodecanedioic acid and 4-hydroxytetradecanedioic acid. It is known that the enzyme that hydroxylates at the α-carbon of the carboxyl group is present in microsomes in the kidney and brain. It does not require fatty acyl-CoA as the substrate nor lead to the generation of high-energy phosphate. Subsequent oxidation of α-hydroxy fatty acid results in shorter-chain-length odd-numbered fatty acids. Although long-chain 2-hydroxydicarboxylic acids have not been detected in the urine of patients with X-linked childhood adrenoleukodystrophy, it was demonstrated that peroxisomal very-long-chain fatty acyl-CoA synthase is deficient in this disease [7], which results in the accumulation of very-long-chain fatty acids. In Zellweger syndrome [8] and neonatal adrenoleukodystrophy [9], the oxidation of very-long-chain fatty acids is impaired, and the accumulated very-long-chain fatty acids may undergo α- and ω-oxidation.

The enzyme that hydroxylates at the γ-position of the carboxyl group of unsaturated fatty acids in mammals was suggested by Dupont and Mathias [10], although Huxtable and Wakil [11] failed to detect it in beef heart mitochondria [9]. One possible pathway for the formation of 4-hydroxydodecanedioic acid is initial ω- and incomplete β-oxidation of oleic acid to form 3-*cis*-dodecenedioic acid and

its subsequent hydration. Although the exact mechanism for the formation of 4-hydroxylated fatty acid derivatives is not clear, our findings demonstrated that γ-hydroxy fatty acids are formed in humans and that increased formation of γ-hydroxy fatty acids takes place in patients with acute Reye's-like syndrome during hopantenate therapy. After intensive care and withdrawal of hopantenate, both patients recovered with normalization of the urinary organic acid profiles. From the above results, we excluded the possibility of the involvement of known inherited metabolic disorders in our patients.

Hopantenate is a homologue of pantothenic acid, a constituent of CoA, and is formed by substitution of the β-alanine moiety of pantothenic acid with γ-aminobutyric acid (GABA). The present findings suggest that ω- as well as α-oxidation is enhanced in such patients owing to impaired β-oxidation of fatty acids in mitochondria and/or peroxisomes. Although the exact mechanisms for the impaired β-oxidation of fatty acids and the enhanced formation of 2-hydroxydicarboxylic acids and 4-hydroxydicarboxylic acids in hopantenate intoxication are not fully understood, it was suggested that hopantenate has the potential to diminish utilization of CoA or reduce absorption or transport of precursor pantothenic acid, and that this tendency may arise when extensive fat mobilization is induced under certain conditions.

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