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Rapid and sensitive detection of urinary 4-hydroxybutyric acid and its related compounds by gas chromatography–mass spectrometry in a patient with succinic semialdehyde dehydrogenase deficiency

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Abstract

We describe the rapid and sensitive detection of 4-hydroxybutyric acid, which is a marker compound for succinic semialdehyde dehydrogenase (SSADH) deficiency. Urinary 4-hydroxybutyric acid and 3,4-dihydroxybutyric acid were targeted, quantified by gas chromatography–mass spectrometry after simplified urease digestion in which lactone formation from γ-hydroxy acids is minimized. The recovery of 4-hydroxybutyric acid using this method was over 93%. 2,2-Dimethylsuccinic acid was used as an internal standard. The detection limit of this method was 1 nmol ml^{-1} for both 4-hydroxybutyric acid and 3,4-dihydroxybutyric acid. The urinary concentrations of 4-hydroxybutyric acid and of 3,4-dihydroxybutyric acid from the patient with an SSADH deficiency were 880–3628 mmol mol^{-1} creatinine (control; 3.3 ± 3.3 mmol mol^{-1} creatinine) and 810–1366 mmol mol^{-1} creatinine (control; 67.4 ± 56.2 mmol mol^{-1} creatinine), respectively. The simplified urease digestion of urine is very useful for quantifying 4-hydroxybutyric acid and its related compounds in patients with 4-hydroxybutyric aciduria.
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1. Introduction

Succinic semialdehyde dehydrogenase deficiency (4-hydroxybutyric aciduria: 4HBA-uria; McKusick 271980) is an inborn error of 4-aminobutyric acid (GABA) metabolism and the first patient was described by Jakobs et al. in 1981 [1]. Over 150 patients have been identified, and the clinical and biochemical findings have been summarized [2–4]. Large amounts of 4-hydroxybutyric acid accumulate in the urine, blood and cerebrospinal fluid (CSF) of patients with 4HBA-uria [5] and the diagnosis is usually based upon high concentrations of urinary 4HBA. Levels of β- and α-oxidation metabolites of 4HBA are also increased, and succinic semialdehyde or its metabolites should have a secondary inhibitory effect on fatty acid β-oxidation because 4HBA-uria is occasionally accompanied by medium-chain dicarboxylic aciduria [6].

Gas chromatography–mass spectrometry (GC–MS) is a sensitive and accurate means of measuring 4HBA in urine. Samples have historically been

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prepared by organic solvent extraction under acidic conditions, but lactonization of γ-hydroxy acids is enhanced during this procedure. Furthermore, the lactones may be lost during evaporation to dryness before derivatization because they are more volatile than free acids [5]. This poses a serious problem when detecting lower concentrations of 4HBA in 4HBA-uria. Such patients may be misdiagnosed with other conditions (such as deficient β-oxidation of fatty acids). The present paper describes the diagnosis of 4HBA-uria by targeting γ-hydroxy acids with low lactone formation using simplified urease digestion with GC–MS [7,8].

2. Experimental

2.1. Chemicals

We obtained 4-hydroxybutyric acid and 3-hydroxybutyrolactone from Tokyo Kasei Kogyo (Tokyo, Japan), and urease (type C-3: from Jack beans) from Sigma (St. Louis, MO, USA). The silylation reagents, N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), were purchased from Wako (Osaka, Japan).

2.2. Patient

The patient was the second child born to non-consanguineous Japanese parents. At 1 month of age, he developed severe hypotonia and ataxia. The urine at 65th days was screened by GC–MS for metabolic diseases. The amounts of 4HBA in his urine were elevated and he was diagnosed with 4HBA-urea. Urine, serum and cerebrospinal fluid (CSF) were collected at days 65, 109, 116 and 128. The treatment with vigabatrin, an irreversible inhibitor of GABA transaminase, was started at day 109 (20 mg/kg/day). It decreased urinary, serum and CSF concentrations of 4HBA. In terms of overt symptoms, only the incidence of seizures improved in this patient.

2.3. Sample preparation

Samples were prepared and derivatized as described previously [7,8]. In brief, 0.1 ml of urine was digested with 20 units of urease at 37 °C for 10 min. After adding 25 nmol of 2,2-dimethylsuccinic acid as the internal standard [8], the urine was deproteinized with 1 ml ethanol. The precipitate was removed by centrifugation, and then the supernatant was concentrated under reduced pressure and evaporated to dryness under nitrogen gas. The residue was trimethylsilylated using 100 μl of BSTFA plus 10% TMCS at 80 °C for 30 min, then 1 μl of the reaction mixture was analyzed by GC–MS. Serum and CSF samples were also prepared in the same manner. Urinary creatinine was enzymatically measured using a Beckman Synchron CX5CE autoanalyzer (Beckman Instruments, Fullerton, CA, USA).

2.4. Gas chromatography–mass spectrometry

Samples were analyzed using a QP-5000 gas chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a fused-silica capillary column (J&W DB-5MS, 30 m×0.25 mm×0.25 μm) under the conditions described in Ref. [8]. The temperature was programmed to increase at a rate of 17 °C/min from 60 to 325 °C, which was maintained for 10 min. Electron impact mass spectra were obtained by repetitive scanning at a rate of 0.25-s intervals from m/z 50 to 650. The internal standard for 4HBA and 3,4-dihydroxybutyric acid (2DT) quantitation was 2,2-dimethylsuccinic acid. We quantified 4HBA and 2DT by mass chromatography.

3. Results

Fig. 1 shows TIC chromatograms of urinary metabolites from the patient with 4HBA-uria. The upper panel (A) shows diethyl ether extraction of 0.5 ml of urine under acidic conditions and the lower (B) shows the urease digestion of 0.1 ml of urine. A large peak corresponding to 4HBA was apparent in both methods but the urease procedure was more sensitive. Medium-chain dicarboxylic acids (glutaric acid, adipic acid and suberic acid) were also excreted but were more sensitively (about two times) detected after organic solvent extraction. However, the urease method was very sensitive in detecting 3,4-dihydroxybutyric (2DT), 2,4-dihydroxybutyric (3DT) and 4,5-dihydroxyhexanoic acids. Fig. 2 shows mass

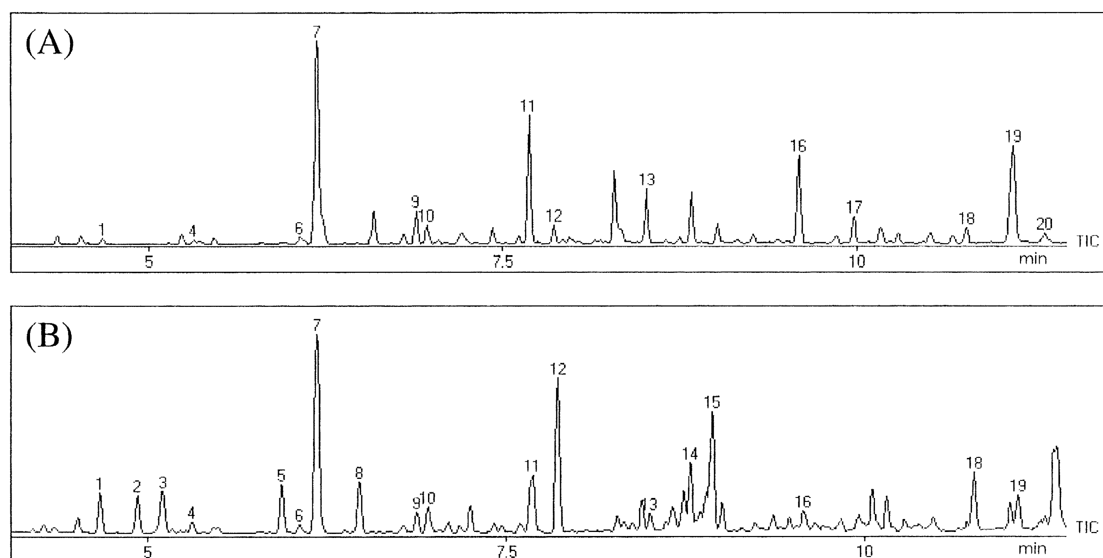

Fig. 1. Total ion currents of trimethylsilyl (TMS) derivatives of urinary metabolites in a patient with 4-hydroxybutyric aciduria. (A) Diethylether extraction; (B) urease digestion. Eluted peaks: 1 = glycolate; 2 = alanine; 3 = glycine; 4 = 3-hydroxypropionate; 5 = 3-amino-butyrate; 6 = 3-hydroxybutyrolactone; 7 = 4-hydroxybutyrate; 8 = phosphate; 9 = succinate; 10 = 2,2-dimethylsuccinate (I.S.); 11 = glutarate and 2,4-dihydroxybutyrate; 12 = 3,4-dihydroxybutyrate; 13 = adipate; 14 = 4,5-dihydroxyhexanoate; 15 = creatinine; 16 = 4-hydroxy-phenylacetate; 17 = suberate; 18 = citrate; 19 = hippurate; 20 = sebacate.

spectra of TMS derivatives of these metabolites. The recovery of 4HBA was over 93% under alkaline conditions, but only 14% under acidic conditions (Fig. 3). The greatest amount of 4HBA converted to its lactone form under acidic conditions but the lactone of 4HBA was not detected by the present method. We added 50 nmol of 4HBA to eight urine specimens that were extracted after urease digestion. The extraction reproducibility of 4HBA was 93–110% (average, 97%, RSD, 5.7%). The γ-lactone of 2DT appeared as a small peak in the urine of the patient with 4HBA-uria. The recovery of 2DT was 90% under alkaline conditions and 80% under acidic conditions. The amount of lactone generated from 2DT was less than that from 4HBA under acidic conditions and minimized in alkaline conditions (Fig. 4). Calibration curves for 4HBA and 2DT were constructed by comparing a selective ion peak area to that of the internal standard that was obtained by mass chromatography. An authentic solution of 2DT was generated from 3-hydroxybutyrolactone by adding 2 *M* sodium hydroxide. The internal standard was 2,2-dimethylsuccinic acid (DMS) and the quantitative target ion was m/z 231. The ions m/z 233 and m/z 321 were used for 4HBA and for 2DT, respectively. The calibration curves were $y = 0.033x - 0.033$ ($r^2 = 0.999$) for 4HBA and $y = 0.011x - 0.009$ ($r^2 = 0.999$) for 2DT at the range of 0–400 nmol. Fig. 5 shows a mass chromatogram of urinary 4HBA (m/z 233), 3DT (m/z 219), 2DT (m/z 321), 4,5-dihydroxyhexanoate (m/z 247) and DMS (IS, m/z231) from the patient. Fig. 6 shows a mass chromatogram of 4HBA and 2DT in serum and CSF from the patient. The detection limits of 4HBA and 2DT were 1 nmol ml^{-1}. The amounts of 4HBA and 2DT detected in the patient are shown in Table 1.

4. Discussion

The urinary excretion of 4HBA is suggested to be age-dependent, being high in younger, and low in older patients [5]. However, lower levels of urinary 4HBA (only 10-fold higher than controls) in neonatal patients have also been reported [9]. Interpretation of results is very difficult in such patients and the frequency of false-negatives can be high. Lactone formation interferes with the detection of low 4HBA

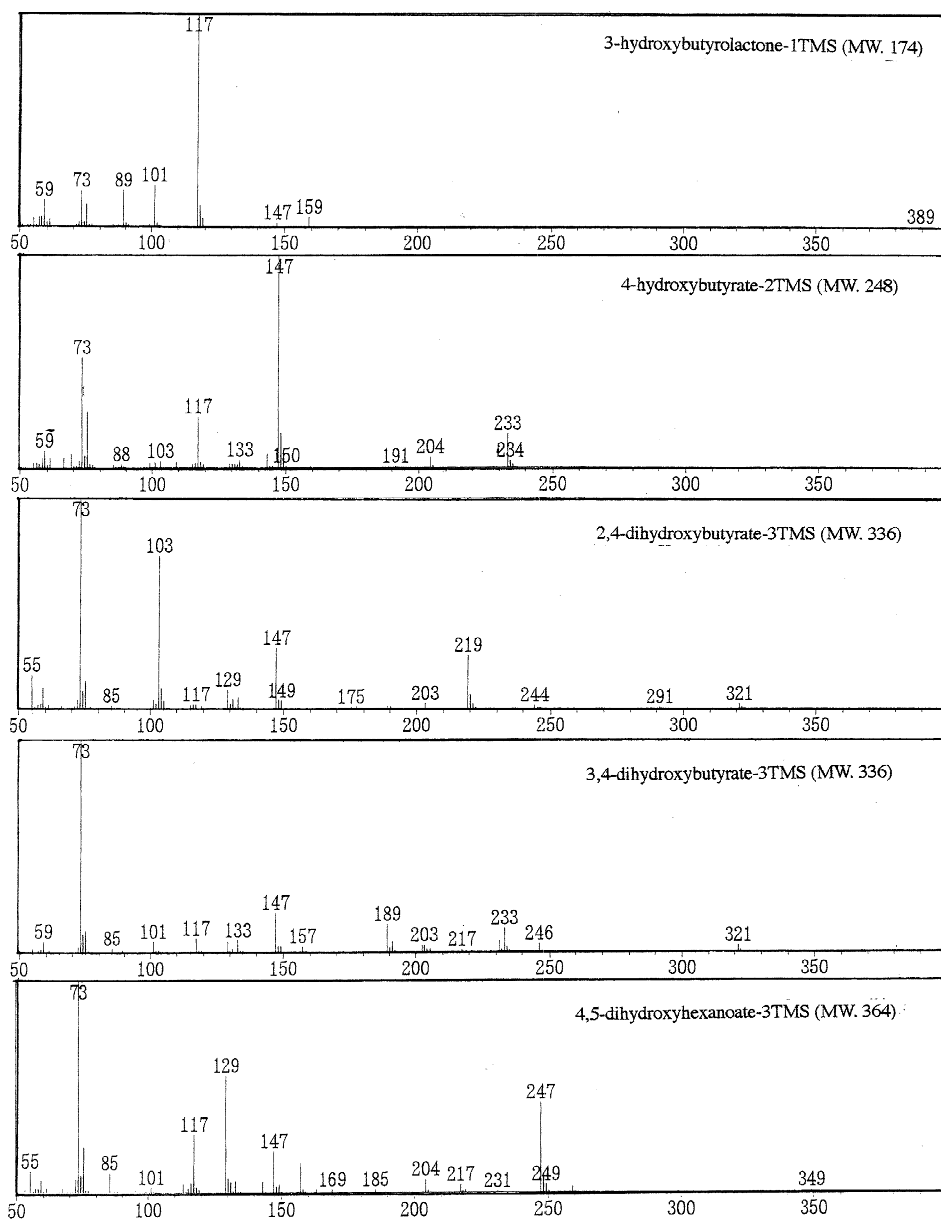

Fig. 2. Mass spectra of 4-hydroxybutyric acid and related compounds.

concentrations in organic solvent extraction methods [9]. Stable isotope dilution analysis of 4HBA prevents quantitative loss [5] and it is an excellent method, but it is quite complex. We developed a simpler means of detecting urinary metabolites using GC–MS combined with urease digestion [8]. In this method, urease decomposes urinary urea into ammonia and carbon dioxide, which produces an alkaline environment that appears to prevent lactone formation from γ-hydroxy acids. The recovery of 4HBA was over 93% using this method. We also confirmed that 2DT does not significantly convert to

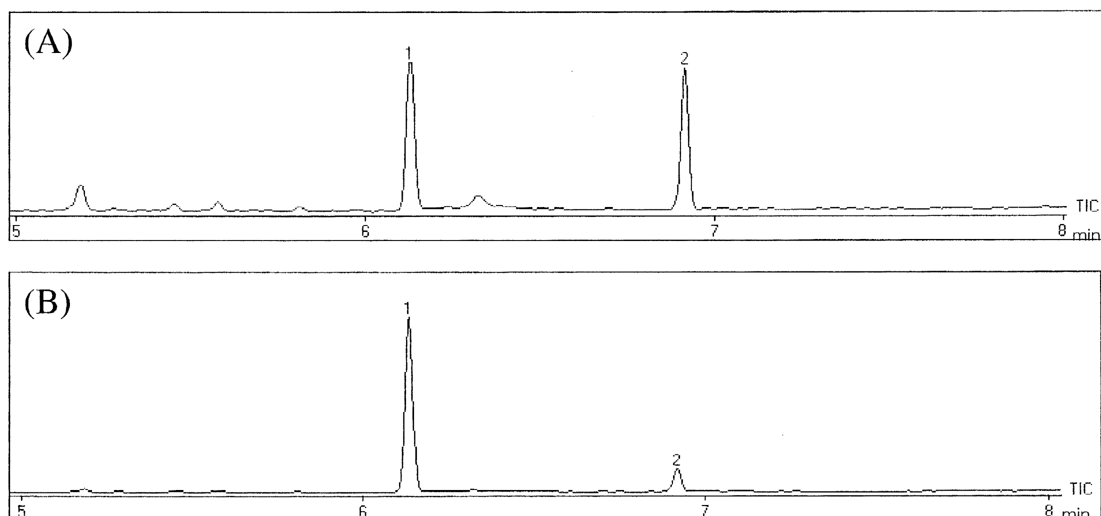

Fig. 3. Total ion currents of TMS derivatives of authentic 4-hydroxybutyrate under acidic (A) and alkaline (B) conditions. 1 = 4-hydroxybutyrate; 2 = 2,2-dimethylsuccinate (I.S.).

its lactone form under alkaline conditions. This method therefore allows the sensitive detection of low concentrations of γ-hydroxy acids.

We applied our urease method to a 2-month-old boy with 4HBA-uria and found that levels of urinary 4HBA were 380–860-fold higher than normal (880–3628 mmol mol^{-1} creatinine). Large amounts of 4HBA were also detected in patient's serum (2195–2918 μmol l^{-1}) and CSF (1910–2200 μmol l^{-1}), which were within the reported range [5]. Another

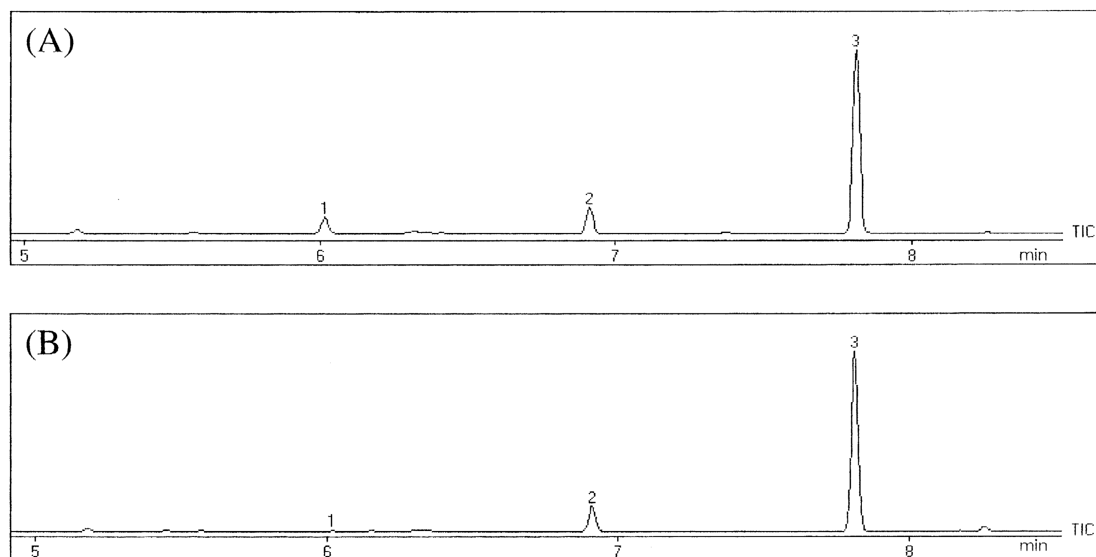

Fig. 4. Total ion currents of TMS derivatives of 3,4-dihydroxybutyrate in acidic (A) and alkaline (B) conditions. 1 = 3-hydroxybutyrolactone; 2 = 2,2-dimethylsuccinate (I.S.); 3 = 3,4-dihydroxybutyrate.

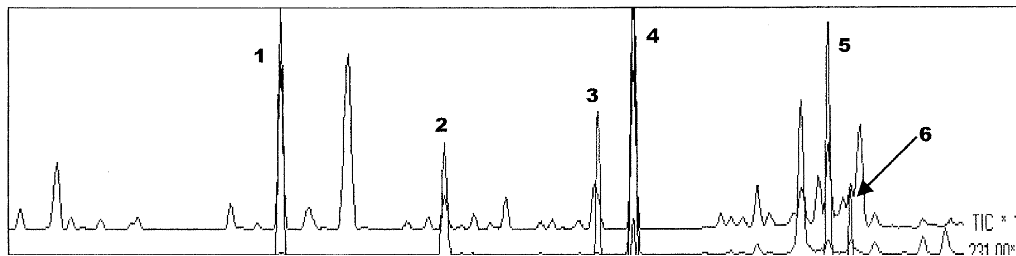

Fig. 5. Mass chromatogram of TMS derivatives of metabolites from urine of a patient with 4-hydroxybutyric aciduria. Targeted ions: m/z 233 for 4-hydroxybutyrate, m/z 321 for 3,4-dihydroxybutyrate, m/z 219 for 2,4-dihydroxybutyrate, m/z 247 for 4,5-dihydroxyhexanoate and m/z 231 for 2,2-dimethylsuccinate (IS). Peaks: 1 = 4-hydroxybutyrate; 2 = 2,2-dimethylsuccinate; 3 = 2,4-dihydroxybutyrate; 4 = 3,4-dihydroxybutyrate; 5 and 6 = 4,5-dihydroxyhexanoate.

characteristic finding was the excretion of 2DT, which is a normal component of urine that originates from the β-oxidation metabolite of 4HBA, although it may also arise from carbohydrate degradation [10]. Urinary concentrations of 2DT were always high in our patient (12–20-fold higher than the normal level). We also detected large amounts of 2,4-dihydroxybutyric acid (3DT, the α-oxidation metabolite of 4HBA) and 4,5-dihydroxyhexanoic acid (condensation product of succinic semialdehyde with a 2-carbon fragment [6]). These alternative metabolic pathways for the disposal of 4HBA are considered to be less important because very low excreted levels have been detected by organic solvent extraction [9]. However, the present analytical procedure always detected abnormally increased concentrations of

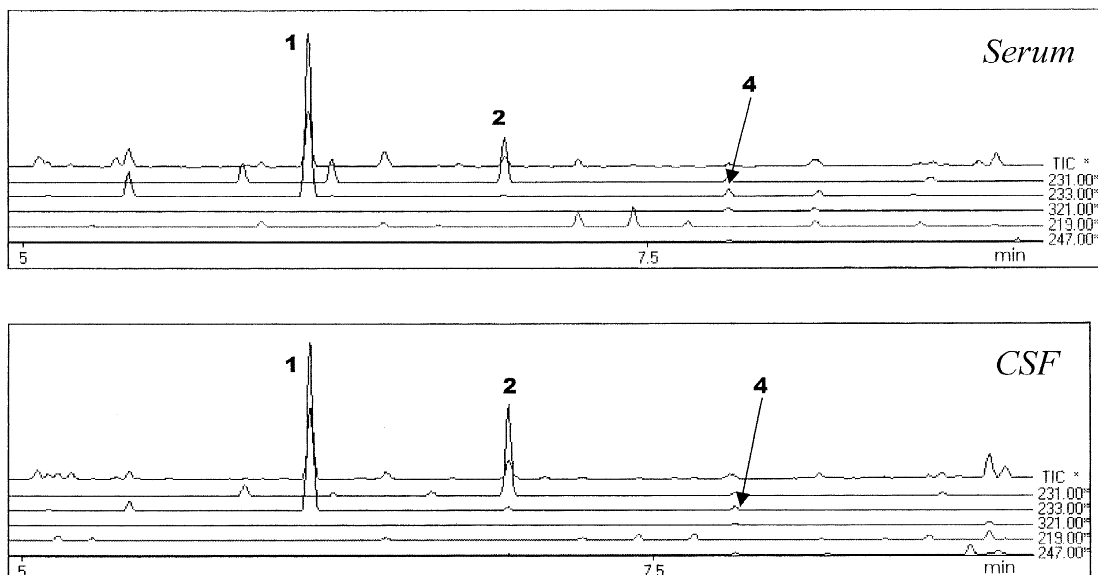

Fig. 6. Mass chromatograms of TMS derivatives of metabolites from serum and CSF of a patient with 4-hydroxybutyric aciduria. Targeted ions were same as those described in Fig. 5.

Table 1
Concentrations of 4-hydroxybutyrate and 3,4-dihydroxybutyrate in patient's urine, serum and CSF

	Urine (mmol/mol creatinine)		Serum (μmol l^{-1}) 4-Hydroxybutyrate	CSF (μmol l^{-1}) 4-Hydroxybutyrate
	4-Hydroxybutyrate	3,4-Dihydroxybutyrate		
Control	3.3±3.3 (n=13) (range 0–10.8)	67.4±56.2 (n=13) (range 0–213)	1.2±1.2 (n=8) (range 0–3.2)	
Patient Y.E				
65 days	880	810	–	–
109 days[a]	3630	1370	2920	2200
116 days[b]	1560	1020	2590	1910
128 days[c]	2200	1160	2110	1760
Gibson et al. (1990) [5]	116–7600 (control: 2.64±3.46)		98–1500 (plasma) (control: 1.09±2.87)	410–3100 (control: 0.98±1.17)

[a] Valproate (anticonvulsive agent) administration (100 mg/kg/day, 4 weeks).
[b] Vigabatrin administration (20 mg/kg/day, 1 week).
[c] Vigabatrin administration (20 mg/kg/day, 2weeks+30 mg/kg/day, 2 days).

secondary metabolites in the patient's urine, suggesting that alternative metabolic pathways of 4HBA are quite important for reducing 4HBA levels in body fluids. The present patient also excreted large amounts of glutaric, adipic, suberic and sebacic acids. However, dicarboxylic aciduria was variable and sometimes disappeared. Succinic semialdehyde or its metabolites may reflect secondary inhibition of mitochondrial fatty acid β-oxidation [6].

Urease digestion combined with GC–MS analysis enables sensitive and simultaneous detection of 4-hydroxybutyric acid, polyhydroxy acids (such as 2,4- and 3,4-dihydroxybutyric acid, and 4,5-dihydroxyhexanoic acid) and amino acids. This simple yet rapid procedure may lead to the correct diagnosis of 4HBA-uria and prevent false-negative diagnosis of this condition.

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