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Urinary organic acids in peroxisomal disorders: a simple screening method

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Abstract

Using GC–MS, we studied urinary organic acids in 20 Japanese patients with peroxisomal disorders, including Zellweger syndrome (ZS), neonatal adrenoleukodystrophy, and single deficiency of peroxisomal β-oxidation enzymes. Non-ketotic dicarboxylic aciduria with elevated sebacate/adipate molar ratio was observed in 19 of the 20 patients. Elevation of 2-hydroxysebacate and epoxydicarboxylic acids were seen in 13 and 18, respectively. Tyrosyluria was remarkable in all patients. In two ZS patients, we tracked the time course from birth to infancy, and all the above stated findings were detected, except for one sample. Urinary organic acid analysis is indeed useful for screening subjects with peroxisomal disorders.

Keywords: Peroxisomal disorders; Organic acids; Epoxydicarboxylic acid

1. Introduction

Peroxisomal disorder represents a class of inherited diseases in which peroxisomal functions including β-oxidation, plasmalogen synthesis, cholesterol and dolichol synthesis, or metabolism of peroxide, bile acids, glycolate, polyamine, phytanic acid or pipecholic acid, are impaired [1]. These are currently classified into two major categories [2]: (a) diseases in which the assembly of peroxisomes is defective, such as Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease, Zellweger-like syndrome, or rhizomelic chondrodysplasia punctata. There is an impairment of multiple peroxisomal function; and (b) diseases with a single defective peroxisomal enzyme, such as X-linked adrenoleukodyatrophy, D-bifunctional protein (DBP) deficiency [3], peroxisomal acyl-CoA oxidase (PAO) deficiency [4], or Refsum disease. Studies of these peroxisomal diseases have expanded understanding of the role of peroxisomes in cell metabolism, in addition to elucidating pathophysiology of the diseases.

The clinical presentation of peroxisomal disorders is commonly characterized by craniofacial dysmorphism, hearing impairment, hypotonia, seizures, developmental delay, hepatomegaly or renal cyst [1,2]. Most patients with ZS died within a few months after birth, while patients with NALD, or

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deficiency PAO, have milder symptoms and often survive beyond the first year of life [1].

The accumulation of serum very-long chain fatty acids (VLCFAs), expressed as an elevated ratio of C26/C22 or C24/C22, indicates a peroxisomal disorder, except in cases of Refsum disease or rhizomelic chondrodysplasia punctata [1]. Therefore, measurement of VLCFAs is useful to screen for peroxisomal disorders. To reach a precise diagnosis of each disorder, extended examinations such as measurement of phytanic acid, assay of peroxisomal enzymes including particle-bound catalase, acyl-CoA: dihydroxyacetone phosphate acyltransferase acyl-CoA oxidase, bifunctional protein or thiolase, morphological studies including immunofluorescent microscopy or electron microscopy, or molecular studies are required [5–7]. However, only a few laboratories are equipped to examine peroxisomal disorders. Hence, an accurate diagnosis is often established only after the patient has died.

Use of urine has not received much attention in screening for peroxisomal disorders, although there are reports in which mild dicarboxylic aciduria, tyrosyluria, elevation of 2-hydroxysebacate (2HS), or epoxydicarboxylic aciduria (EDA-uria) occurs in peroxisomal disorders [8–12]. We analyzed urine samples from 20 Japanese children with peroxisomal disorders, and the time course of urinary organic acids in two ZS patients was studied.

2. Materials and methods

2.1. Patients

Twenty Japanese children with peroxisomal disorders were studied: 15 with ZS, two each with NALD and DBP deficiency, and one with PAO deficiency. The diagnosis for all these patients had previously been established based on clinical symptoms, routine laboratory tests, measurement of VLCFAs, enzyme assays or morphological features [3].

2.2. Organic acid analysis

Urinary organic acids were analyzed by gas chromatography–mass spectrometry (GC–MS), after solvent extraction with ethyl acetate and diethyl ether, oximation, and trimethylsilyl (TMS) derivatization, as described [13]. Internal standards, 20 μg each of margarate (MGA) and tetracosane (C24), and/or 40 μg of tropate (TA) were added to urine samples containing 0.2 mg creatinine. Oximation, solvent extraction and TMS derivatization were then done.

For GC–MS analysis, we used a Shimadzu GCMS QP5000 (Shimadzu, Kyoto, Japan), and the column was a fused-silica DB-5 capillary of 30 m×0.25 mm I.D. with 1 μm film thickness (J&W Scientific, Folsom, CA, USA). The temperature program was started from 100 to 290°C at a rate of 4°C/min, with initial and final holding times of 1 and 10 min, respectively.

Quantification was based on mass chromatography with the automated GC–MS data processing system we developed [13]. The selective ions for quantification (Q-ion) and confirmation (C-ion), and methylene unit (MU) values of the compounds and internal standards are shown in Table 1. Index compounds

Table 1
Selected ions for quantification, and confirmation and methylene unit values for each compound determined

Compound	Q-ion	C-ion	MU
Dicarboxylic acid			
Adipate (Adi)	275	111	15.10
Suberate (Sub)	303	187	17.02
Sebacate (Seb)	331	215	18.99
Dodecanedioate (Dod)	359	243	20.89
Hydroxysebacate			
2-Hydroxysebacate (2HS)	317	391	20.59
3-Hydroxysebacate (3HS)	303	233	20.71
Aromatic acid			
4-Hydroxyphenyllactate (PHPL)	308	293	19.19
Epoxydicarboxylic acid			
3,6-Epoxydodecanedioate (E12DA)	201	174	21.71
3,6-Epoxytetracanedioate (E14DA)	201	174	23.61
Internal standard			
Margarate (MGA)	327	145	21.46
Tetracosane (C24)	99	67	24.00
Tropate (TA)	280	295	16.10

Abbreviations: Q- and C-ions=selected ions for quantification and confirmation, respectively; MU=methylene unit value on DB-5 capillary column.

used were adipate (Adi), suberate (Sub), sebacate (Seb), dodecanedioate (Dod), 2-hydroxysebacate (2HS), 3-hydroxysebacate (3HS), 4-hydroxyphenyllactate (PHPL), 3,6-epoxydodecanedioate (E12DA), and 3,6-epoxytetradecanedioate (E14DA).

2.3. VLCFA measurement

Serum VLCFAs were analyzed by capillary gas chromatography using a DB-1 column, as described [14]. The case 15 patient was the only one for whom serum VLCFAs were measured using another method [15]. The normal values of C24/C22 ratios of the former and latter were 0.606±0.125 and 0.22, respectively.

3. Results

3.1. Urinary organic acids

A total ion chromatogram (TIC) of urinary organic acids in the case 14 patient with ZS is illustrated in Fig. 1. This patient was most recently identified in our laboratory. Dicarboxylic aciduria, elevation of 2HS and PHPL, and EDA-uria were observed in the profile, as compared with findings in a normal control infant. The spectra of 2HS, 3HS, E12DA and E14DA taken in this analysis are illustrated in Fig. 2.

Urinary organic acid profiles of the 20 patients with peroxisomal disorders are summarized in Table 2. Dicarboxylic aciduria was defined as an elevation in at least one of the four dicarboxylic acids (Adi, Sub, Seb, or Dod), The molar ratio of Seb to Adi (C10/C6) was also compared. In 19 out of the 20, dicarboxylic aciduria with an elevated C10/C6 ratio was observed, albeit the degree being varied. In all cases other than that of ZS, dicarboxylic aciduria tended to be mild. An elevation of 2HS was observed in 13 of the 20. Tyrosyluria or elevation of PHPL was seen in all 20 and that in case 17 was remarkable at over three times the maximum value of controls. In three cases, including one each of NALD, DBP deficiency and PAO deficiency, the tyrosyluria was mild. Concerning EDA-uria, in 18 of the 20 cases, either or both of E12DA and E14DA were elevated. The amount of 3HS and Dod excretion varied and was not significant in peroxisomal disorders, in this study.

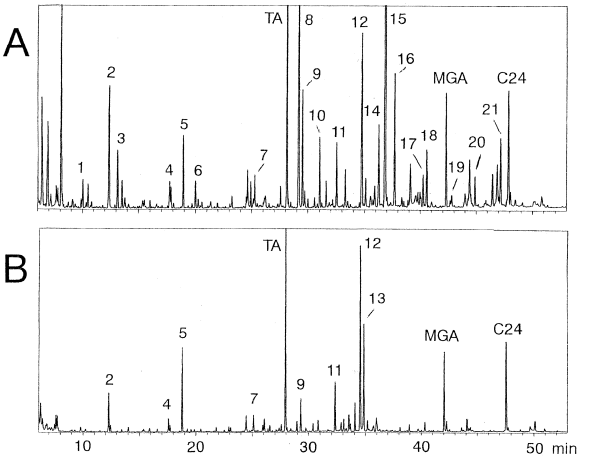

Fig. 1. Total ion chromatogram of urinary organic acids by GC–MS. Urine from the case 14 patient (A) and a normal control (B). Peaks: 1=lactate; 2=oxalate; 3=pyruvate; 4=phosphorate; 5=succinate; 6=fumarate; 7=adipate; 8=2-ketoglutarate; 9=*p*-hydroxyphenylacetate; 10=suberate; 11=aconitate; 12=citrate; 13=hippurate; 14=sebacate; 15=*p*-hydroxyphenyllactate; 16=*p*-hydroxyphenylpyruvate; 17=2-hydroxysebacate; 18=3-hydroxysebacate; 19=3,6-epoxydodecatenioate; 20=3-hydroxydodecanedioate; 21=3,6-epoxytetradecanedioate; TA=tropate (internal standard); MGA=margarate (internal standard), and C24=tetracosane (internal standard).

3.2. Serum VLCFAs

In all the 20 with peroxisomal disorders, the C24/C22 ratio of serum VLCFAs was significantly high, as shown in Table 2.

3.3. Serial observation of urinary organic acids

The time course of urinary organic acids could be followed in two (cases 1 and 2) of ZS. In both, as shown in Table 3, dicarboxylic aciduria had a high C10/C6 ratio, and tyrosyluria and EDA-uria were apparent. With one exception, 2HS was elevated in all samples. Only in the case 1 patient, a sample was taken shortly after birth (day 1), and there was no significant elevation of dicarboxylic acids, 2HS, or EDAs. However, the elevation of PHPL was striking in all samples, without exception.

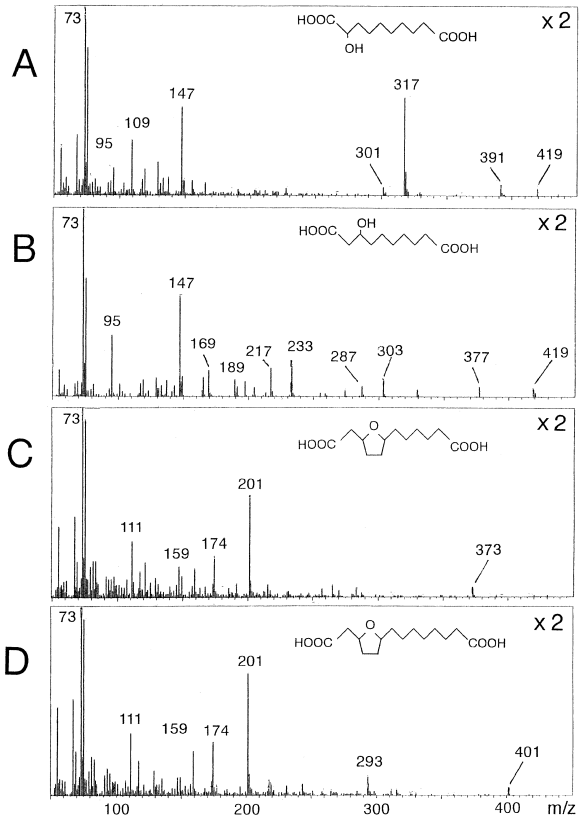

Fig. 2. Mass spectra of TMS derivatives. 2-Hydroxysebacate (A), 3-hydroxysebacate (B), 3,6-epoxydodecanedioate (C), and 3,6-epoxytetradecanedioate (D).

4. Discussion

Several markers in urinary organic acids analyzed by GC–MS proved useful for screening for peroxisomal disorders, including diseases with multiple and single enzyme defects, analyzing urinary organic acids from a total 20 patients with such disorders. The markers are (a) non-ketotic dicarboxylic aciduria with a high ratio of C10/C6, (b) tylosyluria, (c) elevation of 2HS and (d) EDA-uria. Usually, the diagnosis of peroxisomal disorders may require several tests including biochemical, histochemical, or molecular investigation as well as serum very-long-chain fatty acid measurement, and is not always easy. Recently, we developed a personal computer-based system of automated metabolic profiling and interpretation of GC–MS data for screening for organic acidemias (automated system) [13]. We added the GC–MS data including Q- and C-ions, and MU values of the above markers to the automated system to screen such disorders. Hence, peroxisomal disorders also became easily detectable with our system using GC–MS.

Non-ketotic dicarboxylic aciduria reflects a decreased capacity of mitochondrial and/or peroxisomal β-oxidation which could increase the availability of β-oxidation. It was reported that liver mitochondria from patients with peroxisomal disorders were structurally distorted and impaired [16]. Defects in peroxisomal β-oxidation result in a reduced function of mitochondria as well as the increased microsomal β-oxidation which leads to dicarboxylic aciduria. In peroxisomal disorders, β-oxidation of longer chain dicarboxylic acids is partially blocked and excretion of longer chain dicarboxylic acids such as Seb may be more prominent. Shimizu et al. reported that carnitine concentration and acylglycine excretion in patients with peroxisomal disorder were in a normal range [11]. Therefore, the mechanism of dicarboxylic aciduria seen in peroxisomal disorders is likely to differ from that seen in mitochondrial disorders.

Tyrosyluria is often observed in premature infants or sick neonatal babies. This condition is known as ‘‘transient neonatal tyrosinemia’’, otherwise, it is a sign of liver dysfunction. In peroxisomal disorders, tyrosyluria was observed even later than 4 weeks after birth and was the most consistent factor. However, the excreted amount of PHPL was usually milder in patients with a single enzyme defect such as DBP or PAO deficiency. Tyrosyluria should be stressed as being characteristic of peroxisomal disorders.

Elevation of 2HS in peroxisomal disorders is considered to be due to an increased α-oxidation resulting from the block in VLCFA metabolism [10]. An elevation of 3HS excretion was unlikely to be specific for peroxisomal disorders. It was reported that amounts of 3HS did non-specifically correlated with that of Adi [11]. EDA-uria is seen when castor oil is ingested, and in such cases, 3,6-epoxyoctanedioc acid excretion is prominent [17]. In our study, an increased excretion of longer chain EDAs

Table 2
Urinary organic acids and serum very-long chain fatty acid in 20 patients with peroxisomal disorders

Name	Age	Adi	Sub	Seb	(C10/C6)	2HS	PHPL	E12DA	E14DA	C24/C22 (serum)
Zelleweger syndrome										
Case 1	2 months	14.5	43.1	26.3	(1.8)	14.3	2367.7	14.9	31.3	1.715
Case 2	2 months	5.5	31.5	15.9	(2.9)	16.4	1004.4	85.8	173.0	2.403
Case 3	1 months	13.1	32.93	2.2	(2.5)	57.3	819.2	4.9	26.7	1.735
Case 4	5 months	89.1	55.2	68.1	(0.8)	53.2	1739.7	9.8	42.9	2.093
Case 5	3 months	71.5	335.0	130.4	(1.8)	20.5	1179.0	0.0	0.0	2.768
Case 6	6 months	37.3	34.8	19.2	(0.5)	161.8	474.1	2.5	6.6	2.700
Case 7	3 months	23.2	73.7	72.2	(3.1)	16.4	2184.0	0.0	10.6	1.687
Case 8	15 days	52.5	122.7	130.9	(2.5)	17.2	2906.9	5.1	59.5	1.819
Case 9	10 months	48.2	322.5	298.8	(6.2)	0.0	167.9	56.3	23.9	2.334
Case 10	3 months	0.1	21.0	9.7	(9.7)	0.0	85.4	41.8	23.3	1.785
Case 11	1 month	8.6	9.7	4.9	(0.6)	0.0	198.0	0.0	1.4	2.025
Case 12	5 months	17.8	34.7	22.7	(1.3)	0.0	479.1	7.7	4.9	1.830
Case 13	5 months	0.1	21.6	12.8	(12.8)	5.9	372.9	13.2	12.7	2.120
Case 14	4 months	25.6	28.6	35.6	(1.4)	17.8	246.3	0.0	9.9	1.872
Case 15	1 month	20.4	40.9	53.9	(2.6)	0.0	52.0	20.9	332.7	0.40*
Neonatal ALD										
Case 16	2 years	14.8	14.7	12.4	(0.8)	57.3	462.5	5.6	34.6	1.156
Case 17	2 years	10.9	22.0	37.8	(3.5)	12.1	20.3	1159.4	388.8	2.215
DBP deficiency										
Case 18	5 months	6.6	4.9	2.0	(0.3)	0.0	226.2	9.0	22.9	1.107
Case 19	11 months	134.1	61.9	21.8	(0.2)	2.7	22.5	0.0	3.7	1.521
PAO deficiency										
Case 20	3 years	9.4	27.4	25.7	(2.7)	0.0	15.8	5.0	4.2	1.197
Normal (n=30)		1.7–16.5	0.5–8.6	0.3–5.0	(<0.2)	<0.2	<9.2	<2.2	<1.6	0.606±0.125

Unit: urinary organic acid is mol/mol creatine; C24/C22, peak area ratio of tetra- to docosahexaenic acid on gas chromatography. *, the ratio value of this case (case 15) was measured according to the method of Igarashi et al. [15] (normal control, 0.22). ''0.0'' means ''undetectable''. Abnormal data are underlined. Abbreviations: C10/C6=urinary sebacate/adipate molar ratio; ALD=adrenoleukodystrophy; BFP=D-bifunctional protein; PAO=peroxisomal acyl-CoA oxidase; Adi, Sub, Seb, 2HS, PHPL, E12DA and E14DA as in Table 1.

like E12DA or E14DA was observed in children with peroxisomal disorders. Although the precise mechanism of the EDA-uria in peroxisomal disorders is unclear, it can be considered that peroxisomes play a role in EDA metabolism, and if so, then defective peroxisomes result in accumulation of EDAs.

Measurement of serum VLCFAs (C24/C22 ratio or C26/C22 ratio) is often used for screening for peroxisomal disorders. More convenient methods for VLCFA measurements using blood filter paper [18] or tandem mass spectrometry have been developed [19]. Careful evaluation of organic acids in the urine, as shown in our study, is a simple and effective approach to detect peroxisomal disorders as well as for serum VLCFA measurements.

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Table 3
Time course of urinary organic acids in two babies with Zellweger syndrome

	Age	Adi	Sub	Seb	(C10/C6)	2HS	PHPL	E12DA	E14DA
Case 1	28 days	16.7	81.6	28.6	(1.7)	22.7	1310.5	9.7	75.0
	28 days	31.5	61.9	52.8	(1.7)	27.7	1317.9	12.3	8.8
	62 days	58.9	228.2	262.0	(4.5)	32.0	761.5	24.9	69.7
	78 days	25.3	47.0	67.7	(2.7)	16.8	294.7	6.8	5.2
	98 days	76.1	104.6	155.4	(2.0)	8.7	68.9	8.1	19.2
Case 2	1 day	0.1	8.9	2.9	(2.9)	0.0	66.3	0.0	1.2
	5 days	9.3	19.0	6.5	(0.7)	36.8	130.6	14.8	3.9
	14 days	17.0	51.6	68.7	(4.0)	3.7	256.9	7.9	104.6
	19 days	10.3	74.0	18.6	(1.8)	23.9	126.9	8.3	3.6
	36 days	14.0	34.6	18.6	(1.3)	9.2	111.3	45.8	28.4
	43 days	12.7	19.8	18.0	(1.4)	35.4	45.1	33.2	27.3
	43 days	21.5	25.9	26.0	(1.2)	11.5	68.9	51.7	8.3
	46 days	17.2	47.7	36.8	(2.1)	24.7	96.9	78.4	93.2
	49 days	23.0	35.2	13.2	(0.6)	44.3	106.2	102.3	8.6
	61 days	13.3	51.3	24.7	(1.9)	16.6	266.1	198.6	12.4
Normal		1.7–16.5	0.5–8.6	0.3–5.0	(<0.2)	<0.2	<9.2	<2.2	<1.6

Units and abbreviations are the same as in Tables 1 and 2. Abnormal data are underlined.

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