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Application of optical isomer analysis by diastereomer derivatization GC/MS to determine the condition of patients with short bowel syndrome☆

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Abstract

To establish a method for separating the optical isomers of lactic acid, we modified the derivatization steps in our procedure for urinary mass-screening for inborn errors of metabolism. For chiral recognition, we chose *O*-trifluoroacetyl-(−)-menthylation derivatization instead of our previous method, trimethylsilyl derivatization, and the samples were then analyzed under GC/MS by capillary gas chromatography on a DB-5MS column. This method can be used to follow-up the condition of a patient with short bowel syndrome and to prevent onset and/or seizure. D-Lactic acid was also isolated from the urine of healthy controls as one of the main peaks in the chromatogram.

Keywords: Short bowel syndrome; Urinary D-lactic acid; Separation of enantiomers; *O*-trifluoroacetylated (−)-menthyl ester

1. Introduction

In the analysis of urinary metabolites, such as organic acids, amino acids, sugar monomers, and their related compounds, by gas–liquid chromatography (GC) in patients with suspected inborn errors of metabolism (IEMs), it is important to determine the absolute configurations of the metabolic products. This is because different optical isomers may originate from different metabolic pathways and reflect enzyme defects. Many IEMs in which a large quantity of L-lactic acid is excreted are known, but IEMs in which excess D-lactic acid is excreted have not been described. A heavy excretion of D-lactic acid can be the result of short bowel syndrome (SBS), which is not an IEM. D-Lactic acid is normally present in the blood of mammals at nanomolar concentrations, due to methylglyoxal metabolism, but millimolar D-lactic acid concentrations can arise from excess gastrointestinal microbial production [1]. Specific D-lactic acid assays are generally not available in clinical laboratories.

Methods for the chiral resolution of lactic acid using capillary electrophoresis with chiral additives or HPLC with a chiral column are reported [2–5]. The separation can also be achieved by derivatization GC/MS, which is thought to be superior to the other techniques in its absolute sensitivity and robustness.

In the literature, the resolution of racemic mixtures by GC has been achieved in two ways: (1) by separating the enantiomers using a chiral stationary phase and (2) by converting the enantiomers into diastereomers with a chiral reagent and then separating them using a non-chiral stationary phase. Gil-Av and Nurok [6] published a comprehensive review of this subject in 1974. Pollock and Jermany [7] separated the enantiomers of some *O*-acetylated 2-hydroxy acids of the 2-butyl, 3-methyl-2-butyl, and 3,3-dimethyl-2-butyl esters by GC. Kamerling et al. [8] reported separating diastereomers of the *O*-acetylated menthyl esters of lactic and glyceric acids by capillary GC; the samples were isolated from patients with lactic aciduria and primary hyperoxaluria 2 (PH2). We also reported separating the enantiomers of the *O*-acetylated glyceric acid of the 2-butyl esters by GC, which were isolated from patients with PH2 [9].

In this paper, we describe the highly sensitive separation of the diastereomers of the *O*-trifluoroacetylated (−)-menthyl ester of lactic acid, which was isolated from urine samples from

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patients with SBS and healthy controls, by capillary GC/MS on a DB-5MS column. This method may prove useful in the follow-up survey of the condition of patients with SBS and in onset and/or seizure prevention.

2. Experimental

2.1. Subjects

Urine and serum from two Japanese male patients with a diagnosis of SBS were used as the SBS samples. Urine specimens that were collected in our pilot study of newborn mass screening in Japan [10] were used as the newborn control group. Another control group was represented by samples that had been screened for the chemical diagnosis of IEMs, but in which no abnormality had been demonstrated.

Urine and serum specimens were received frozen in dry ice and were stored at −20 °C prior to analysis.

2.2. Chemicals

D-(−)-Lactic acid lithium salt (purity: 100% enantiomer) (D-lactic acid), (1*R*,2*S*,5*R*)-(−)-menthol (purity: >99%) ((−)-menthol) and Type C-3 jack-bean urease were from Sigma Chemical Company, Saint Louis, MO. Pyridine was from Nacalai Tesque Inc., Kyoto, Japan. Trifluoroacetic anhydride (purity: >99%) and acetyl chloride were from Aldrich, Milwaukee, WI. Other reagents were from Wako Pure Chemical Industry Ltd., Osaka, Japan.

2.3. Preparation and GC/MS analysis

Quantitative analysis of urinary and/or serum lactic acid in the D/L form was done using the routine method established by Matsumoto and Kuhara [11], in which the urinary metabolites are changed to their TMS derivatives. Separation of the enantiomers of lactic acid was achieved by a minor modification of the derivatization steps described in reference [11]. Thirty units of urease solution was added to the standards (D- and DL-lactic acids), serum samples (0.1 ml from various patient specimens), or urine samples (0.1–0.2 ml from healthy controls, or the appropriate dilution of patient specimens), and the reaction was run at 37 °C for 15 min. To this reaction mixture, di-methylsuccinic acid was added as an internal standard to a final concentration of 250 nmol/ml. The mixture was vortexed with 0.9 ml ethanol and spun for deproteination. The supernatant was evaporated under N_2 at 37 °C, and the residue was used for each derivatization method.

Urinary creatinine was assayed by enzymatic reaction on a Beckman Synchron CX5 Clinical System. The D- and L- or DL-lactic acid concentration was expressed in relation to creatinine (mmol/mol creatinine).

2.3.1. Derivatization of lactic acid to the *O*-trifluoroacetylated (−)-menthyl ester

The method of Kim et al. [12] for (−)-menthylation was followed with minor changes. One hundred microliters of (−)-menthol solution (200 μg/μl in ethyl acetate) was added to the residue, and the ethyl acetate was evaporated to dryness under a gentle stream of nitrogen at 37 °C. To the residue were added 60 μl of toluene and 5 μl of acetyl chloride. The mixture was then heated to 100 °C for 1 h. After heating, the excess agents were removed under a stream of nitrogen. The concentrate was then reacted with 100 μl of trifluoroacetic anhydride–acetonitrile (2:1) at 60 °C for 20 min. Subsequently, the excess reagents were removed by evaporation under a stream of nitrogen at 37 °C, and the residue was dissolved in chloroform for GC/MS analysis.

2.3.2. GC–MS analysis

An aliquot (1 μl) of derivatized sample was injected into a Hewlett-Packard model 6890/5973 gas chromatography-mass selective detector equipped with a fused silica capillary column (DB-5MS, 30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA), using an automatic injector with a split ratio of 30:1. The temperatures of the GC injector and of the interface line were 250 and 280 °C, respectively. To determine the absolute configurations of the *O*-trifluoroacetylated (−)-menthyl ester of lactic acid, the column oven temperature was programmed to increase from 60 to 320 °C at a rate of 17 °C/min. The mass spectrometer was operated in the electron ionization (EI) mode with the following source parameters: electron energy 70 eV, emission current 35 μA, electron multiplier 1.4 kV and source and detector temperature 230 and 150 °C, respectively. Selected ion monitoring (SIM) (dwell time 100 ms) was carried out to measure low-level samples of lactic acid, for example from healthy controls, and mass chromatography was used to measure high levels, *m/z* 50 to *m/z* 350, 4.72 scan/s.

3. Results

In Fig. 1, a portion of the total ion chromatogram (TIC) for the trimethylsilylated esters of DL-lactic acid (A) and the mass chromatogram of *m/z* 141 for the *O*-trifluoroacetylated (−)-menthyl esters of D- and L-lactic acid (B) isolated from patient urine are shown. The mass spectrum of authentic D-lactic acid is shown in Fig. 2. To calculate the D- and L-configuration ratio, we measured peaks representing the abundance of each fragment ion: *m/z* 141 (as the quantitative ion), 138, and 95 (as qualifier ions).

$$\text{D-Configuration ratio (\%)} = 100 \times \frac{\text{peak area of D-lactic acid}}{\text{sum of each peak area of D- and L-lactic acid}}$$

3.1. Reproducibility of the derivatization method

The reproducibility of the derivatization was checked for the D- and L-configuration ratios of DL-lactic acid by measuring artificial samples. Two artificial samples, one with a low level of D-lactic acid (S1) and one with a high level (S2) were prepared. We then prepared four specimens each with low and high levels of D-lactic acid. The GC/MS measurements were repeated four times, for a total of 32 measurements.

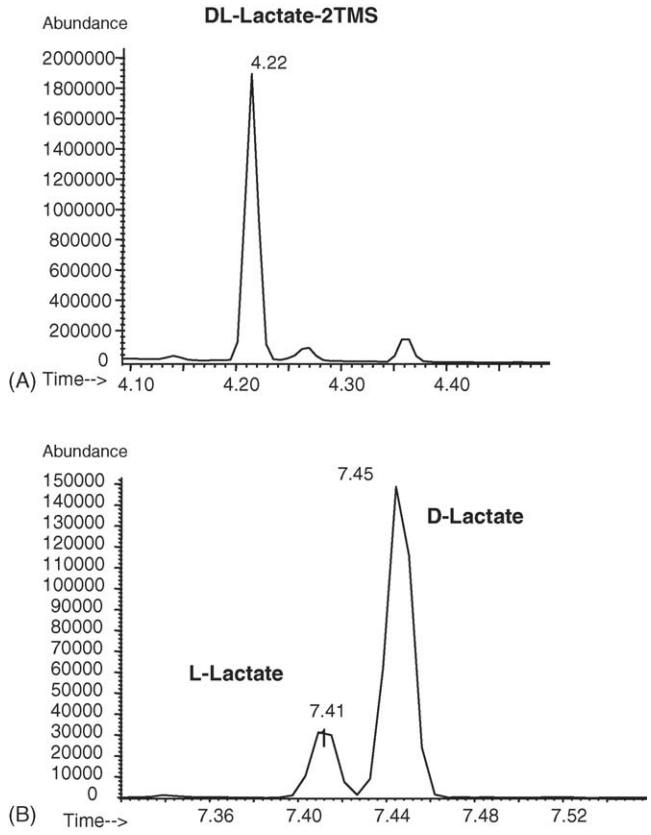

Fig. 1. TIC and mass chromatogram of a patient with short bowel syndrome in remission.
Enlarged portion of the TIC showing the DL-lactic acid peak (A), and the mass chromatogram of *m/z* 141 for lactic acid *O*-trifluoroacetylated (−)-menthyl ester (B).
The column oven temperature was programmed to rise from 60 to 320 °C at 17 °C/min.

The average, minimum, and maximum measurements are presented as the D-configuration (%) of the fragment ion of *m/z* 141 (Table 1).

The percentages of the low-level D-configuration sample of *O*-trifluoroacetylated (−)-menthyl ester varied from 14.6 to 15.1% (average ± S.D., 14.9 ± 0.15%; coefficient of variation (CV) (%), 1.02%). The percentages of the high-level D-configuration sample varied from 65.2 to 68.6% (average ± S.D., 67.3 ± 0.92%; coefficient of variation (CV) (%), 1.36%).

3.2. Correlation in the D-configuration ratio between the serum and urinary lactic acid

The correlation between the serum and urinary D-configuration lactic acid was checked by measuring eight pairs of specimens from eight different subjects (Fig. 3). The highest values were from the specimens isolated from the urine and serum of a patient with SBS in remission. The lowest values were those of a patient with unconfirmed L-lactic acidemia. The D-lactic acid configuration ratio in urine correlated with that in serum ($R^2 = 0.9138$).

Table 1
Reproducibility of derivatization of D-lactic acid, *O*-trifluoroacetylated menthyl ester

Groups	Samples	Intra-sample ($n = 4$ each)			Inter-sample ($n = 16$)				
		Average	S.D.	CV	Average	S.D.	CV	Min	Max
(A) *m/z* 141									
S1	1	14.7	0.13	0.86	14.9	0.15	1.02	14.6	15.1
	2	15.0	0.08	0.51					
	3	15.0	0.13	0.85					
	4	14.8	0.09	0.60					
S2	1	67.5	0.41	0.61	67.3	0.92	1.36	65.2	68.6
	2	66.2	0.79	1.20					
	3	67.1	0.43	0.64					
	4	68.3	0.24	0.35					
(B) *m/z* 138									
S1	1	19.1	0.22	1.14	21.2	1.32	6.22	18.8	22.6
	2	21.9	0.43	1.96					
	3	21.5	0.45	2.09					
	4	22.1	0.56	2.53					
S2	1	69.8	0.24	0.34	69.6	0.82	1.18	67.6	71.0
	2	68.8	0.90	1.30					
	3	69.6	1.25	1.80					
	4	70.7	0.40	0.57					
(C) *m/z* 95									
S1	1	21.1	0.38	1.80	23.4	1.44	6.16	20.7	25.1
	2	24.3	0.41	1.68					
	3	23.9	0.54	2.27					
	4	24.4	0.57	2.35					
S2	1	70.1	0.20	0.28	70.0	1.12	1.60	67.2	71.5
	2	68.6	0.99	1.45					
	3	69.9	1.62	2.32					
	4	71.3	0.27	0.38					

Two artificial samples, one with a low-level of D-lactic acid (S1; 17%) and one with a high level (S2; 70%) were prepared. The average, minimum, and maximum measurements are presented as the D-configuration ratio (%) of the fragment ion of *m/z* 141,138, and 95.

3.3. D-Configuration ratio of serum lactic acid in patients with various diseases

The D-configuration ratios of serum lactic acid isolated from patients with SBS, unconfirmed lactic acidemia, hypertyrosinemia, and from dialysis patients, were compared (Fig. 4). The ratios for patients with SBS were higher than those for any other patients. The next highest values were those of dialysis patients and one control. The lowest value was from a patient with L-lactic acidemia, but his L-configuration ratio was the highest of all the samples tested.

3.4. Clinical applications

The DL-lactic acid levels in the urine of the SBS patients in remission were 943 and 26 mmol/mol creatinine. D-Lactic aciduria is not known in any disorder other than SBS, which shows an abnormally high excretion of D-lactic acid. To detect abnormalities in the samples required determining the absolute configuration of lactic acid. However, because the absolute con-

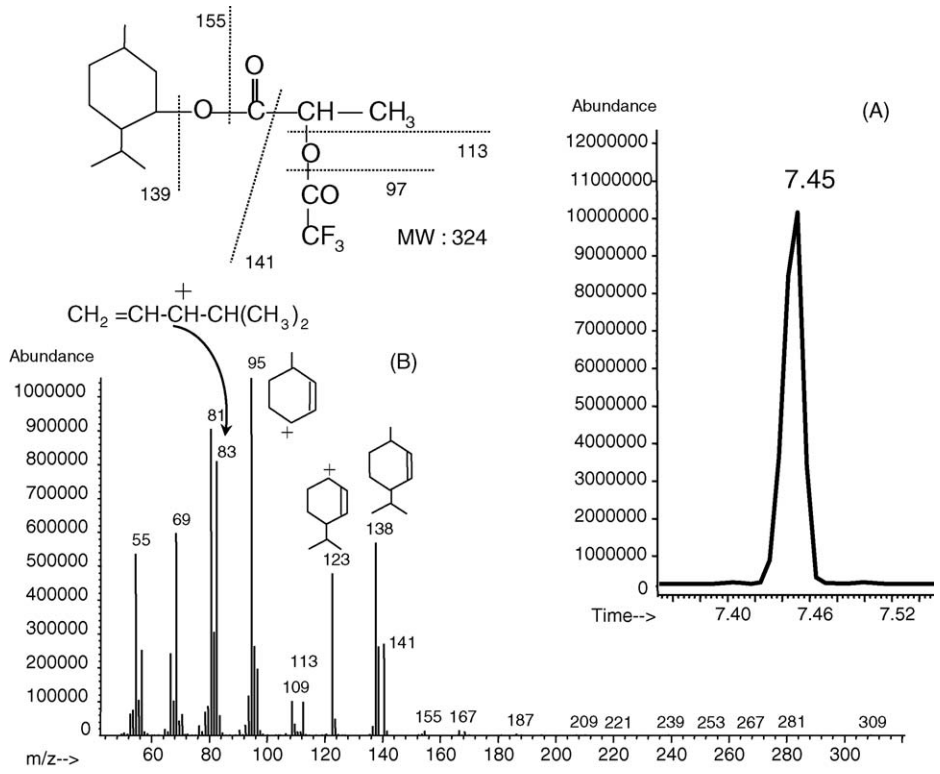

Fig. 2. Mass chromatogram (*m/z* 141) (A), mass spectrum (B), and the molecular formula of authentic D-lactic acid *O*-trifluoroacetylated (−)-menthyl ester.

figuration cannot be determined by the routine measurement using TMS derivatives, it was necessary to separate the enantiomers.

Lactic acid was isolated from the urine of a patient with SBS by the urease pretreatment method. After derivatization to the *O*-trifluoroacetylated (−)-menthyl ester, two peaks were found in the total ion chromatogram (Fig. 1B, right and Fig. 5A); the larger peak had the same retention time as the derivative of authentic D-lactic acid (Fig. 2A) (99% L and 1% D). In the two SBS patients in our study, the D-lactic acid levels detected in the urine were 771 and 10 mmol/mol creatinine. About 82 and 40% of the lactic acids had the D-configuration, respectively (Table 2).

In contrast, the DL-lactic acid concentration that we obtained from healthy controls varied from 2 to 134 mmol/mol creatinine. When lactic acid was isolated from the urine of healthy controls as the *O*-trifluoroacetylated (−)-menthyl ester derivative and analyzed for the absolute configurations, two main peaks were found in the mass chromatograms (*m/z* 141), and they had the same retention times as the derivatives of authentic D- and

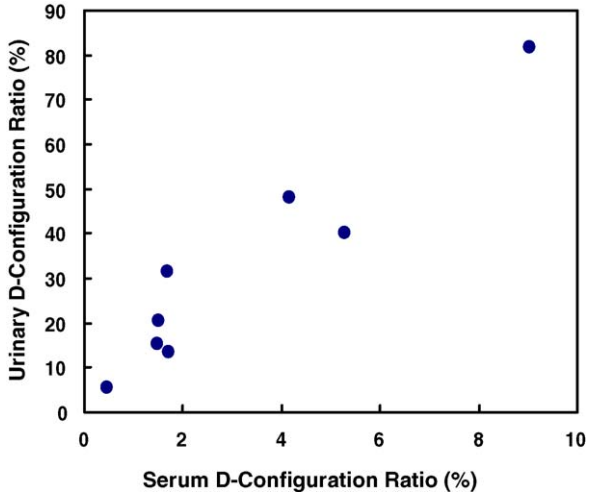

Fig. 3. Correlation between the serum and urinary lactic acid D-configuration ratios. The lactic acid was isolated from eight pairs of serum and urine samples from eight different subjects. To calculate the D-configuration ratio of lactic acid, the fragment ion of *m/z* 141 was used.

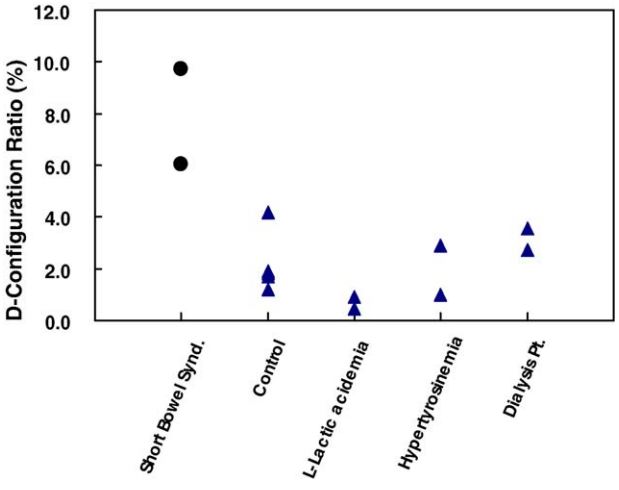

Fig. 4. D-Configuration ratio of serum lactic acid in patients with various diseases. The D-configuration ratio of serum lactic acid isolated from patients with short bowel syndrome, unconfirmed lactic acidemia, hypertyrosinemia, and from dialysis patients were plotted.

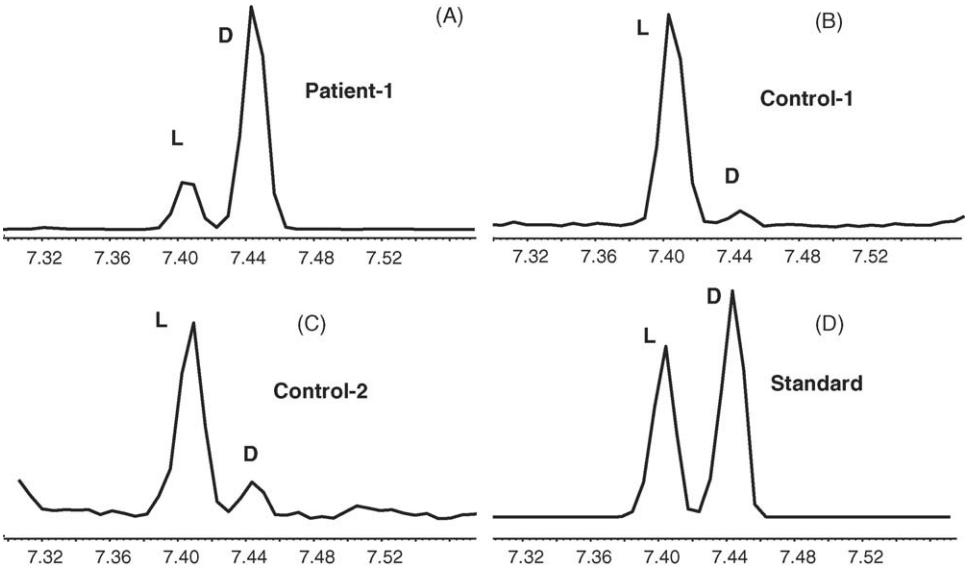

Fig. 5. Mass chromatograms (*m/z* 141) of urinary lactic acid *O*-trifluoroacetylated (−)-menthyl ester isolated from patients with short bowel syndrome in remission (A), healthy controls (B and C), and standard DL-lactic acid (D). Peak heights are normalized to full-scale detection.

Table 2
D-Configuration of lactic acid in urine from patients with short bowel syndrome in remission and control subjects

	Age	Sex	DL-Lactic acid levels (mmol/mol creatinine)	D-Lactic acid configuration (%)
Patient 1	2 years	Male	943	82
Patient 2	16 years	Male	26	40
Newborn baby group ($n=5$)	4–5 days		2–134	18.6 ± 11.6
Infant group ($n=5$)	5 months–13 years		35–94	19.7 ± 7.13

L-lactic acid (Fig. 5). The D-configuration ratio was widely distributed (range 6.2–35.7% D). No correlation was found between the D-configuration ratio and the DL-lactic acid urinary concentration, which was normalized to the creatinine concentration. There was no clear difference between the newborn group and the infant group (Table 2).

4. Discussion

As far as we know, this is the first report of determining the absolute configuration of lactic acids from the urine of healthy control subjects using only 200 μl of urine or urine extract from dried urine filter paper. In contrast, the absolute configuration of lactic acids mentioned in the literature has been limited to lactic acids isolated from the body fluids of patients. Kamerling et al. [8] reported the separation by capillary GC of diastereomers of the *O*-trifluoroacetylated (−)-menthyl esters of lactic and glyceric acids isolated from 5 ml of patient urine. Using this method, an even larger quantity of urine is necessary to separate the diastereomers of lactic acid in the urine of healthy controls than in the urine of patients, and it is unlikely that enough urine could be recovered from samples obtained as dried urine on filter paper. To achieve high sensitivity, we devised a pretreatment method for the sample and used GC/MS, which has a higher sensitivity than GC. We used urease pretreatment to isolate the lactic acid from the urine specimens instead of organic solvent extraction and the *O*-trifluoroacetylated (−)-menthyl ester as the derivatized form.

In the urine from the two patients (2 and 16 years old) with SBS in remission, about 82 and 40% of the lactic acids had the D-configuration, respectively. We have found only one published report, by Bongaerts et al., giving the values for the D- and L-lactic acid in the urine of patients with SBS, obtained by an enzymatic assay [13]. In their report, the D-configurations of an infant patient at the time of seizure and of each adult subject in remission were calculated by us to be 96–98 and 50–85%, respectively. Our analysis of their data also indicated that the level of D-lactic acid changed dramatically over 1 day in a patient with SBS: the D-configurations in this patient ranged between 55 and 93%. The D-configurations of urinary lactic acid in our patients were similar those calculated from the data of Bongaerts et al.

We examined the absolute configuration of lactic acid in the urine of healthy controls because there was no report of these values, as far as we could determine. Haschke-Becher et al. reported the healthy value of the urinary concentration of D-lactic acid by the enzymatic method [14], but not by the absolute configuration. It is reported that the concentration of D-lactic acid decreases with age, with the concentrations of children less than 1 year old being the highest [14]. However, our experiments showed no difference between the configuration ratios of D-lactic acid in the newborn baby group ($n=5$, 4–5 days old) and the infant

group ($n = 5$, 5 months to 13 years old). In addition, the configuration ratios of the D-lactic acid of 2 samples in 10 exceeded 30%, approaching the lower value found in one of the patients with SBS in remission.

D-Lactic acidosis is a rare metabolic occurrence in humans, but it is occasionally observed as a consequence of SBS. Patients who have had extensive resectioning of the small bowel, leaving behind a bowel <150 cm in length are at risk for various metabolic and nutritional disturbances and are classified as having SBS. D-Lactic acidosis in SBS was first described in 1979 [15]. Infection, ischemia, and trauma all result in significantly elevated D-lactic acid in the blood. Most of these conditions yield a D-lactic acid concentration (typically <1 mmol/L) that does not result in acidosis or neurological symptoms. Ewaschuk et al. reported that the use of D-lactic acid as a diagnostic aid in clinical practice would require the availability of a D-lactic acid assay [1]. The present study showed that the D-lactic acid configuration ratio in the urine correlated with that in the serum, and that the D-lactic acid measurement in urine is more sensitive and useful than that in blood.

The present study shows that the *O*-trifluoroacetylated (−)-menthyl ester was suitable for following-up the condition of patients with SBS and for onset and/or seizure prevention.

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