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Changes in urinary level and configuration ratio of D-lactic acid in patients with short bowel syndrome☆

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Abstract

The present study showed that the D-lactic acid configuration ratio in the urine rose earlier than that in blood or the urinary or blood D-lactic acid levels upon disease onset, and that the D-lactic acid measurement in urine is more sensitive and useful than that in blood. As this result, a prediction of a D-lactic acidosis may be possible. To simplify the procedure for detecting D-lactic acid, we first showed a correlation between the D-lactic acid configuration ratio in urine and blood, indicating urine could be used. To separate the optical isomers of lactic acid, we simplified our previous procedure. For chiral recognition, we chose *O*-acetyl-(−)-menthylation and analyzed the samples under GC/MS by capillary gas chromatography on a DB-5MS column. This procedure is less sensitive than the former method, but it is faster and simpler, requiring only one derivatization step. This method may be useful for predicting D-lactic acidosis in patients with short bowel syndrome.

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

1. Introduction

D-Lactic acidosis is a relatively rare neurologic syndrome that occurs in individuals with short bowel syndrome (SBS) or following jejunoileal bypass surgery for the treatment of obesity. 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]. Oh et al. [2] first described D-lactic acidosis in humans in 1979. However, the disorder is well known in veterinary medicine and was recognized in ruminants long before its presence was identified in humans.

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 [3–6]. 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 [7] published a comprehensive review of this subject in 1974. Pollock and Jermany [8] 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. [9] separated diastereomers of the *O*-acetylated menthyl esters of lactic and glyceric acids by capillary GC, using samples isolated from patients with lactic aciduria and primary hyperoxaluria 2. We also reported separating the enantiomers of the *O*-trifluoroacetylated lactic acid of menthyl esters by GC, in samples isolated from patients with SBS [10].

In this paper, we describe a faster and simpler method for determining D-lactic acid using the diastereomers of the *O*-acetylated-(−)-menthyl ester of lactic acid. This method was

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suitable for following up the condition of patients with SBS and detecting D-lactic acidosis onset, preventing possible seizures.

2. Experimental

2.1. Subjects

The urine and blood from two Japanese patients in whom SBS was diagnosed were used as SBS samples. Urine specimens collected from healthy volunteers were used as controls.

The urine and plasma specimens were used promptly or were stored at −20 °C prior to analysis.

2.2. Chemicals

9D-(−)-Lactic acid lithium salt (purity: 100% enantiomer) (D-lactic acid), (1R,2S,5R)-(−)-menthol (purity: > 99%) (−)-menthol) and Type C-3 jack-bean urease were from Sigma Chemical Company, Saint Louis, MO. Acetyl chloride was from Aldrich, Milwaukee, WI. *N,O*-Bis(trimethylsilyl)-trifluoroacetamide with 10% Trimethylchlorosilane (BSTFA) was from Pierce, Rockford, IL. 2,2-dimethylsuccinic acid (DMS) was from Fluka Chimie AG, Switzerland. Other reagents were from Wako Pure Chemical Industry Ltd., Osaka, Japan.

2.3. Preparation and GC/MS analysis

2.3.1. Extraction and derivatization of lactic acid

2.3.1.1. Separation of the enantiomers. Separation of the enantiomers of lactic acid from urine was achieved by a minor modification of the extraction and derivatization steps described previously [10,11]. Urine samples (0.2–1.0 ml) were acidified to pH 2 with 2 N HCl and then extracted with 3 ml of ethyl acetate. The organic layers were evaporated under a stream of nitrogen gas to reduce the volume.

The method of Kim et al. [12] for (−)-menthylation was followed with minor changes. One hundred microliters of (−)-menthol solution (200 mg/ml in ethyl acetate) was added to the extract, 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 solvent was removed under a stream of nitrogen at 37 °C, and the residue was dissolved in chloroform for GC/MS analysis.

2.3.1.2. Quantitative analysis. Quantitative analysis of urinary and/or serum lactic acid in the D/L form was performed using the routine method established by Matsumoto and Kuhara [13], in which the urinary or serum metabolites are changed to their TMS derivatives. Thirty units of urease solution was added to the standards (D- and DL-lactic acids), urine samples (0.1–0.2 ml, equivalent to 1 μmol creatinine, from healthy controls, or the appropriate dilution of patient specimens), or serum samples (0.05 ml from patients and controls), and the reaction was performed at 37 °C for 15 min. To this reaction mixture, dimethylsuccinic 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 to remove proteins. The supernatant was evaporated under N_2 at 37 °C, and the residue was derivatized with 100 μl of BSTFA at 80 °C for 30 min.

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

2.3.2. GC/MS analysis

2.3.2.1. Separation of the enantiomers. 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 the interface line were 250 and 280 °C, respectively. To determine the absolute configurations of the *O*-acetylated-(−)-menthyl ester of lactic acid, the column oven temperature was programmed to increase from 160 to 250 °C at a rate of 10 °C/min and then from 250 to 320 °C at a rate of 30 °C/min. The mass spectrometer was operated in the electron impact (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 60 ms) was carried out to measure low levels of lactic acid in urine, for example in samples from healthy controls or a patient with SBS in remission, and mass chromatography was used to measure high levels, *m/z* 50–350, at 4.72 scans/s.

2.3.2.2. Quantitative analysis. Quantitative analysis of the lactic acid TMS derivatives was performed using a routine method [13], and only the scan mode and a program condition were different from the measurement conditions used for the enantiomers. The conditions for the scanning mass range and programming of the column oven temperature were from *m/z* 50 to 650, and from 60 to 320 °C, respectively, at a rate of 17 °C/min. DMS was used as the internal standard for the quantitative analysis.

3. Results

The mass spectrum of authentic D-lactic acid is shown in Figs. 1 and 2 shows the mass chromatogram of *m/z* 87 for the *O*-acetylated-(−)-menthyl esters of D- and L-lactic acid (left) and of *m/z* 141 for the *O*-trifluoroacetylated (−)-menthyl esters of D- and L-lactic acid (right) isolated from urine specimens collected from one SBS patient at two different times (top versus bottom). To calculate the D- and L- configuration ratio, we measured the peaks representing the abundance of each fragment ion: *m/z* 87 (as the quantitative ion), 138, and 95 (as qualifier ions).

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

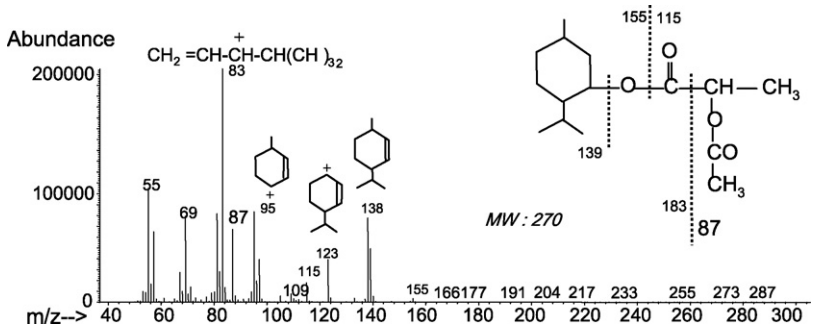

Fig. 1. Mass spectrum and structural formula of the *O*-acetyl-(−)-menthyl ester of D-lactic acid.

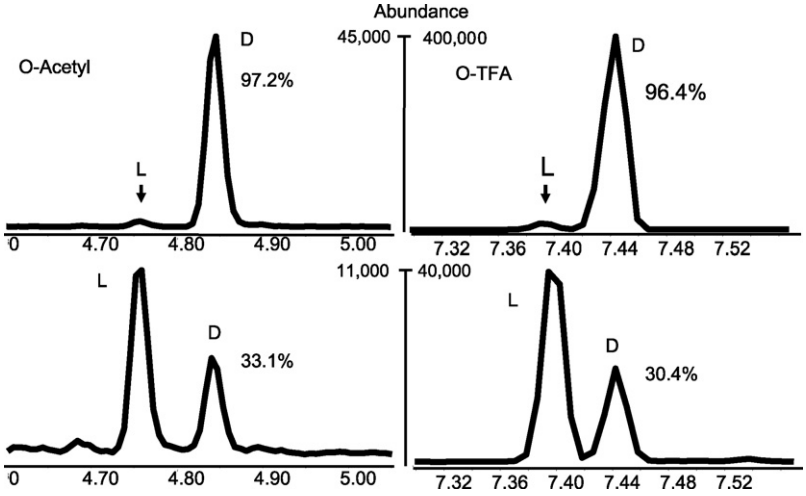

Fig. 2. Mass chromatogram (*m/z* 87) comparison of the absolute configuration ratios of lactic acid in two different urine specimens from a patient with SBS, derivatized with two different methods. With *O*-acetyl-(−)-menthylation (left), and *O*-trifluoroacetyl-(−)- menthylation (right).

Mass chromatograms of the same sample derivatized with the two different acylation procedures showed approximately the same D- and L-configuration ratio.

3.1. Correlation of serum and urinary lactic acid

In urine and serum the relation between the D-lactic acid configuration ratio and the $\log_{10}$ transformed level of D-lactic acid had a correlation coefficient (r) of 0.94 ($n = 104$) and 0.74 ($n = 52$), respectively.

Thirty-six pairs of urine and blood samples were divided into three groups, representing onset, treatment, and remission stages. Each measured value was plotted with the D-configuration ratio on the x-axis and the $\log_{10}$ transformed level of D-lactic acid on the y-axis (Fig. 3). In urine, the relationship between the configuration ratio and the transformed level of D-lactic acid showed a reasonably good correlation except during disease episodes. In serum, the same parameters also showed a reasonably good correlation except during remission.

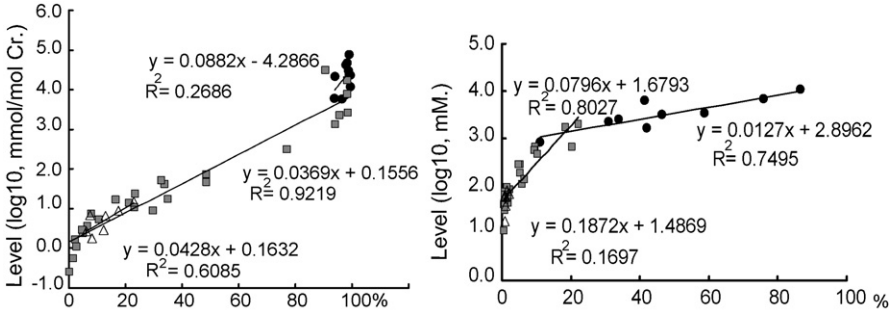

Fig. 3. Correlation between the D-lactic acid configuration ratio and the $\log_{10}$ transformed level of D-lactic acid in urine (left) and serum (right) from a patient with SBS. ●, episode stage; □, treatment stage; △, remission stage.

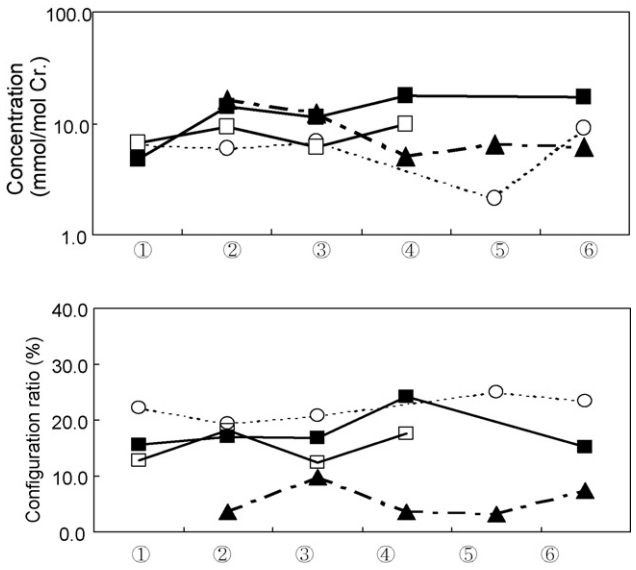

Fig. 4. Change in the level and absolute configuration of urinary D-lactic acid in patients with SBS in remission. Samples 1a and 1b were collected from Patient 1 on different days. ■, Patient 1a; □, Patient 1b; ▲; Patient 2; ◯, control. Sampling points were; ①, before breakfast; ②, after breakfast; ③, before lunch; ④, afternoon; ⑤, after supper; ⑥, before bed.

On the other hand, poor correlation was found for the D-configuration ratio between serum and urinary lactic acid, and the correlation of D-lactic acid levels between the serum and urine was also low.

3.2. Diurnal variation in the urinary levels and configuration ratios of D-lactic acid

Urine specimens were collected several times in 1 day from two SBS patients in remission and from a healthy control child. In Fig. 4, the changes in 1 day in the urinary levels (top) and configuration ratios (bottom) of D-lactic acid are shown. No remarkable changes were recognized in the values for samples obtained from the same individual within the same day. In this experiment, the configuration ratios of D-lactic acid for the healthy control were slightly higher than those of the SBS patients. However, the D-lactic acid levels were slightly lower in the control than in the patients.

A moderate elevation in the urinary D-lactic acid level could increase the configuration ratio from 20–30% to 90% within the same day (Fig. 5). When the urinary D-lactic acid level was higher than several thousand mmol/mol creatinine, the configuration ratio was maintained constantly at >95% (Fig. 6).

3.3. Clinical applications

D-Lactic acidosis has been defined as a metabolic acidosis accompanied by an increase in serum D-lactic acid to $\geqq$3 mM [14]. Because Patient 1 developed D-lactic acidosis, we continued to analyze the D-lactic acid levels and configuration ratio in this patient's urine and/or blood for several months. D-lactic acidosis was observed four times in this period, and the D-lactic acid level in the urine and serum ranged from 0.3 to 6400 mmol/mol creatinine and from 11 to 10,900 μmol/l, respectively. The configuration ratios for D-lactic acid ranged from 0.3 to 99.6% and from 0.6 to 96.1% in the urine and serum, respectively. Data from part of this period are shown in Fig. 6.

The clinical and biochemical characteristics of these patients will be published elsewhere.

4. Discussion

As far as we know, our previous report was the first to determine the absolute configuration of lactic acids from the urine of healthy control subjects using only 200 μl of urine or extracts from dried urine on filter paper [10]. In contrast, determination of the absolute configuration of lactic acids reported in the literature has been limited to larger samples isolated from the body fluids of patients. Kamerling et al. [9] reported the separation by capillary GC of diastereomers of the *O*-trifluoroacetylated

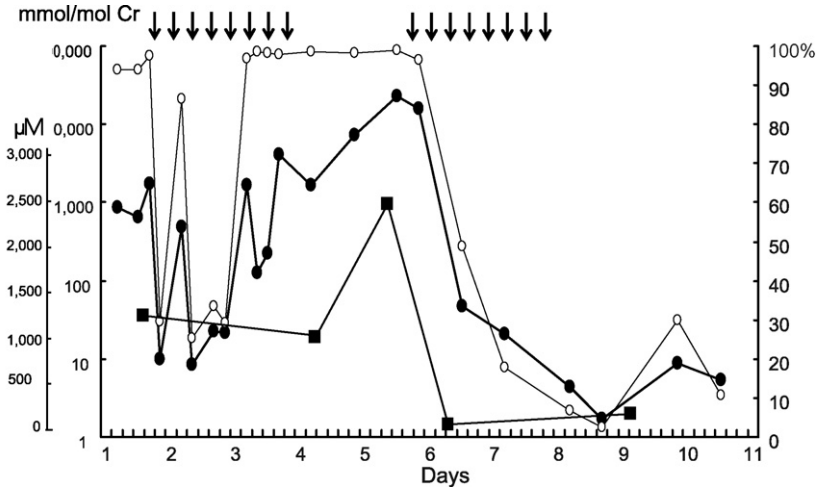

Fig. 5. Change in the urinary and blood levels and configuration ratio of D-lactic acid in a patient with SBS during an episode with a moderate increase in D-lactic acid levels. ●, Concentration of urinary D-lactic acid (mmol/mol creatinine); ◯, configuration ratio of urinary D-lactic acid (%); ■, concentration of blood D-lactic acid (μM). ↓, Administration of antibiotic.

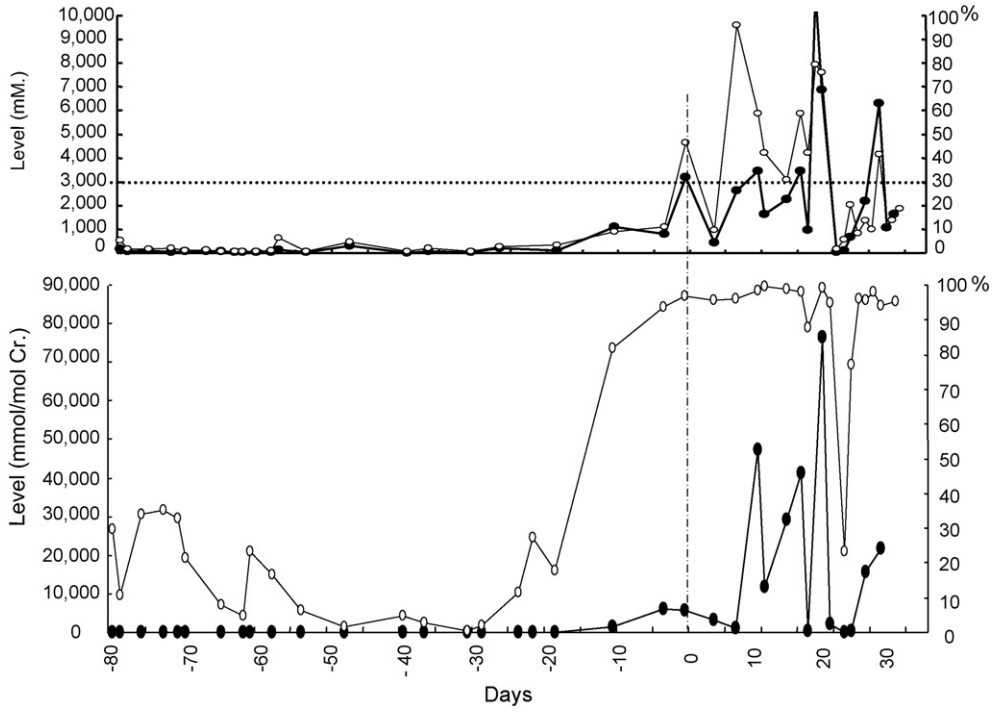

Fig. 6. Change in urinary and blood levels and configuration ratios of D-lactic acid in a patient with SBS before and during D-lactic acidosis (top, blood; bottom, urine). ●, Concentration of D-lactic acid (mmol/mol creatinine, urine; μM, blood); ◯, configuration ratio of D-lactic acid (%). The dashed line at the top shows the threshold level of D-lactic acidosis [14].

(−)-menthyl esters of lactic acid with other acids isolated from 5 ml of patient urine.

However, our previous method was not yet satisfactory for clinical application, because it was time consuming and not simple. Here, by omitting the *O*-trifluoroacetylation step, we shortened sample preparation time by more than 30 min. As a result, the detection sensitivity decreased, which we compensated for by increasing the sample volume and using SIM for the measurement.

No remarkable changes were recognized in values of samples obtained on the same day from the SBS patients in remission. Therefore, we may determine the state of a patient in remission from only one daily measurement.

Until now, it was thought that a prediction of D-lactic acidosis was impossible, since the blood D-lactic acid can rise suddenly within a day without any symptoms or measurable diagnostic signs. However, as Fig. 6 shows, before the patient entered a state of D-lactic acidosis, very high urinary D-lactic acid configuration ratios were observed continuously, for more than 2 weeks before the episode of lactic acidosis. Therefore, a continuous increase in the configuration ratio of urinary D-lactic acid observed during a weekly analysis could be a sign of D-lactic acidosis onset.

The correlation coefficient indicated a reasonably good correlation between the urinary D-lactic acid configuration ratio and the level of D-lactic acid in urine except during disease episodes. In serum, the same parameters also showed a reasonably good correlation except during remission.

On the other hand, a poor correlation between the D-configuration ratio of serum and urinary D-lactic acid level was found, and the correlation between the D-lactic acid levels in serum and urine was also low. Upon disease onset, the urinary D-configuration ratio began to increase before the blood D-configuration ratio or the urinary or blood D-lactic acid levels, and the urinary D-configuration ratio continued to rise along with the increases in the other parameters.

A possible explanation for the different behavior of D- and L-lactic acid in patients is as follows. It is well known that urinary L-lactic acid increases when the blood L-lactic acid exceeds a threshold level [1]. As D-lactic acid is poorly reabsorbed into the kidney, D-lactic acid generated by bacterial flora in the intestine seems to increase directly the D-lactic acid excretion in urine. As a result, the D-configuration ratio in urine increases rapidly. When the excretion of D-lactic acid in urine does not catch up to the rate at which D-lactic acid is generated in the intestine, the level of D-lactic acid in the blood may rise.

Ewaschuk et al. [1] 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. Our previous report was the first to focus on the configuration ratio of D-lactic acid. The present study showed that the measurement in urine is more sensitive and more useful than its measurement in blood.

Our results indicate that the routine prediction of D-lactic acidosis may be possible. More data will be necessary before it can be established as a system for predicting the condition of a patient.

5. Conclusion

The present study showed that the *O*-acetylated-(−)-menthyl ester of D- and L-lactic acid followed by GS/MS measurement

was a suitable procedure for following up the condition of patients with SBS and is a promising method for detecting the onset of D-lactic acidosis and possibly preventing seizures.

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