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Differential chemical diagnosis of primary hyperoxaluria type II Highly sensitive analysis of optical isomers of glyceric acid by GC/MS as diastereoisomeric derivatives

Yoshito Inoue*, Toshihiro Shinka, Morimasa Ohse, Tomiko Kuhara

Division of Human Genetics, Medical Research Institute, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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Abstract

We established a separation method for the optical isomers of glyceric acid in urine by modifying the derivatization steps of the procedure used for the screening and diagnosis. The trimethylsilyl derivatization step in the mass screening procedure was replaced by *O*-acetyl-(+)-2-butylation, and the samples were analyzed under equivalent GC/MS conditions by capillary gas chromatography on a DB-5MS column. This method can be applied to cases that show a high urinary concentration of glyceric acid to obtain a differential diagnosis of primary hyperoxaluria type II and D-glyceric aciduria easily. L-Glyceric acid was also isolated from the urine of healthy controls as one of the main peaks.
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Keywords: Primary hyperoxaluria type II; Urinary glyceric acid; Separation of enantiomers; *O*-Acetylated (+)-2-butyl ester

1. Introduction

In the analysis of urinary organic acids by gas–liquid chromatography (GC) in patients with suspected inborn errors of metabolism (IEMs), it is important to determine the absolute configurations of metabolic products in human urine. This is because different optical isomers may originate from separate metabolic pathways and reflect enzyme defects. For example, a heavy excretion of L-glyceric acid is the result of a D-glycerate dehydrogenase [EC 1.1.1.29] deficiency in primary hyperoxaluria type II (PH2) [1], and a heavy excretion of D-glyceric acid has been found in the urine of a patient with D-glycerate kinase [EC 2.7.1.31] deficiency [2].

In the literature, the resolution of racemic mixtures by GC has been achieved in two ways: (1) by separation of the enantiomers using a chiral stationary phase; and (2) by conversion of the enantiomers into diastereomers by a chiral reagent and then using a non-chiral stationary phase. Gil-Av and Nurok [3] published a comprehensive review on this subject in 1974. Pollock and Jermany [4] 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. [5] 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 PH2.

In this paper, we describe the highly sensitive separation of the diastereomers of the *O*-acetylated (+)-2-butyl ester of glyceric acid, which was isolated from urine samples collected from a patient with PH2 and healthy controls, by

* Corresponding author. Tel.: +81 76 286 2211x3602;
fax: +81 76 286 2312.
E-mail address: yosh@kanazawa-med.ac.jp (Y. Inoue).

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capillary GC/MS on a DB-5MS column. This method may prove useful in diagnosing certain diseases.

2. Experimental

2.1. Subjects

Urine from a Japanese male patient with a diagnosis of PH2, as were determined by calcium oxalate calculus and a heavy excretion of glyceric acid, was used for the samples from a typical PH2 case.

Urine specimens that were collected for a pilot study of newborn mass screening in Japan [6] were used as the newborn control group. Another age-matched 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 that was obtained as a dried sample on filter paper was extracted with distilled water, and used for sample pretreatment. Other urine samples were received frozen in dry ice and were stored at −20 °C prior to analysis.

2.2. Chemicals

L- and DL-glyceric acids, calcium salt, and pyridine were from Nakarai Tesque Inc., Kyoto, Japan. (*S*)-(+)-2-Butanol (purity: >98%) was from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. Acetic anhydride was from Aldrich, Milwaukee, WI. Type C-3 jack-bean urease was from Sigma, St. Louis, MO. Other reagents were from Wako Pure Chemical Industry Ltd., Osaka, Japan.

2.3. Preparation and GC/MS analysis

Quantitative analysis of urinary glyceric acid in the D/L form was done by the routine method established by Matsumoto and Kuhara [7], in which the urinary metabolites are converted to TMS derivatives. The separation analysis of the enantiomers of glyceric acid involved a modification only of the derivatization steps of the above-mentioned method. To the standards (L- and DL-glyceric acids) or urine samples (0.1–0.2 ml from healthy controls, or the appropriate dilution of patient specimens), 30 units of urease solution were added and the reaction was conducted at 37 °C for 10 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 of ethanol and spun for deproteination. The supernatant was evaporated under N_2 at 37 °C, and the residue was used for each derivatization method.

2.3.1. Derivatization of glyceric acid to the O-acetylated (+)-2-butyl esters

Butylation for the separation of diastereomers was achieved by adding 0.05 ml (*S*)-(+)-2-butanol and 0.05 ml acetyl chloride to the residue, and heating the samples at 40 °C for 30 min. Subsequently, the excess reagents were removed by evaporation under a stream of nitrogen at 37 °C. The residue was acetylated in 0.1 ml of pyridine–acetic anhydride (1:1) at 40 °C for 30 min. Finally, the solvent was evaporated in the presence of toluene and the residue dissolved in 0.1 ml chloroform for GC/MS analysis.

2.3.2. Derivatization of glyceric acid to the O-acetylated (−)-menthyl ester

The method of Kim et al. [8] 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 30 μl of toluene and 2 μl of acetyl chloride. The mixture was then heated at 100 °C for 1 h. The method of Kamerling et al. [5] for *O*-acetylation was followed with minor changes. After the removal of excess reagents, the concentrate was reacted with 100 μl of pyridine–acetic anhydride (1:1) at 100 °C for 30 min. Subsequently, the excess reagents were removed by evaporation under a stream of nitrogen at 37 °C in the presence of toluene, and the residue was dissolved in chloroform for GC/MS analysis.

2.3.3. 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, 0.25 μm × 0.25 mm × 30 m, J&W Scientific, Folsom, CA, USA), using an automatic injector with a split ratio of 30:1. To compare the extent to which glyceric acid had been derivatized to *O*-acetylated (+)-2-butyl and *O*-acetylated (−)-menthyl esters, the column oven temperature was programmed to increase from 60 to 320 °C at a rate of 17 °C/min. To determine the absolute configurations of the *O*-acetylated (+)-2-butyl ester of glyceric acid, the column oven temperature was held at 100 °C for 2 min, then programmed to increase to 165 °C at 6 °C/min, and then to 320 °C at 32 °C/min. Selected ion monitoring (SIM) (dwell times 80 ms) was carried out to measure samples with low glyceric acid concentration, for example, those from healthy controls, and mass chromatography was used to measure those with high levels, *m/z* 50–350, 4.72 scan/s.

3. Results

The total ion chromatograms (TICs) of the *O*-acetylated (+)-2-butyl and *O*-acetylated (−)-menthyl esters of the standard DL- and L-glyceric acid and patient urine are shown in Fig. 1. For menthylation, the samples contained five times more glyceric acid as was used for butylation. This resulted in a peak abundance that was almost the same for both.

The mass spectra of L-glyceric acid prepared by both methods are shown in Fig. 2. To calculate the D- and

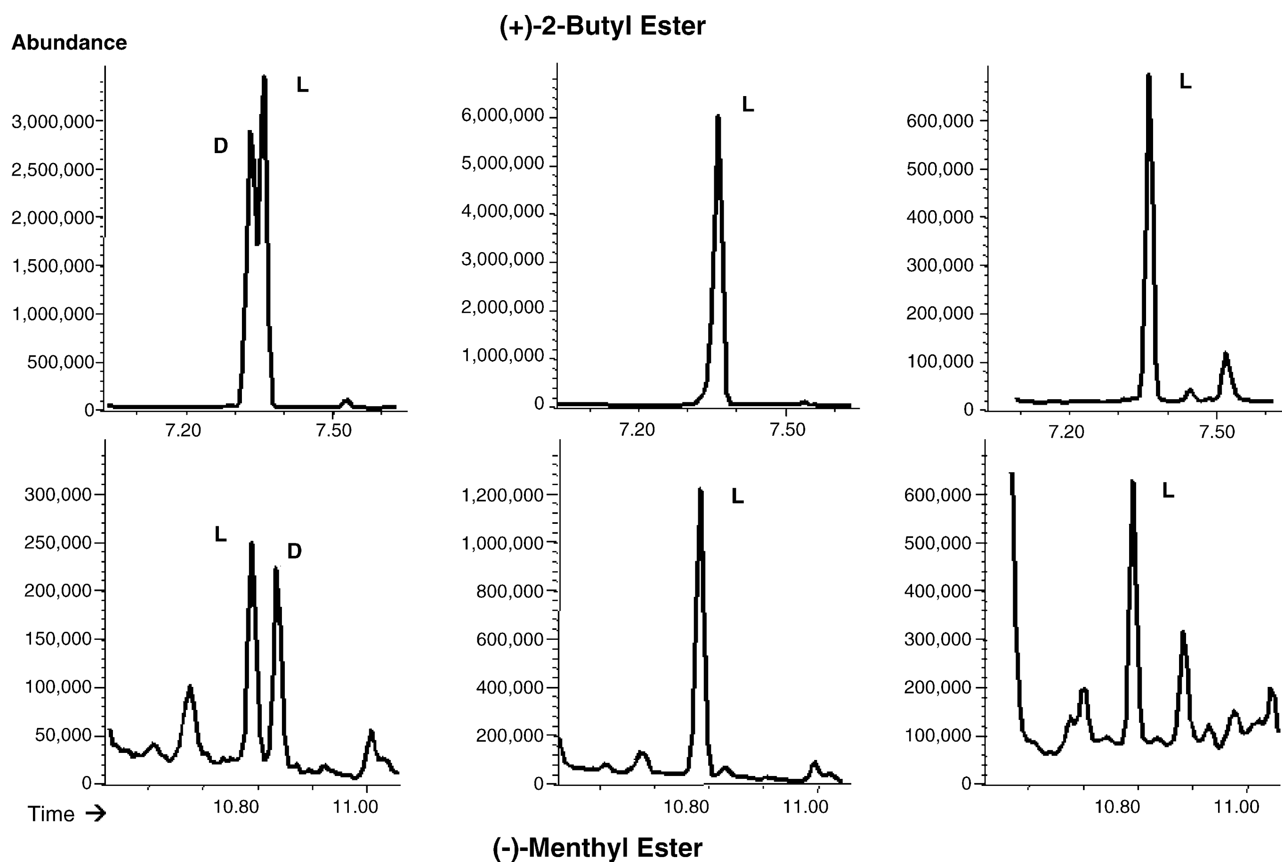

Fig. 1. TIC chromatograms of *O*-acetylated (+)-2-butyl (upper) and *O*-acetylated (−)-menthyl (lower) esters that were prepared from DL-glyceric acid (left), L-glyceric acid (center), or HP2 patient urine (right), respectively. All samples for menthylation were prepared using five times the quantity of glyceric acid in the starting samples as for butylation. The column oven temperature was programmed to rise from 60 to 320 °C at 17 °C/min.

L-configuration ratio, the fragment ions of *m/z* 173, 103, 131, and 86 were analyzed for the *O*-acetylated (+)-2-butyl ester and *m/z* 131, 103, 83, and 138 for the *O*-acetylated (−)-menthyl ester. There was no significant difference in the configuration ratios measured with the different fragment ions. Therefore, we chose the ions of *m/z* 173 and 131, respectively, for calculating the D- and L-configuration ratio of the *O*-acetylated (+)-2-butyl and *O*-acetylated (−)-menthyl esters.

3.1. Accuracy and precision of the derivatization method

The reproducibility of the derivatization was analyzed for the D- and L-configuration ratio of DL-glyceric acid by measuring three standard samples three times each. The percentages of the L-configuration of *O*-acetylated (+)-2-butyl ester varied from 51.5 to 53.8% (average ± SD, 52.2 ± 0.9%; coefficient of variation (CV), 1.80%).

To verify the stability of the D- and L-configuration ratio of the *O*-acetylated (+)-2-butyl and (−)-menthyl esters of DL-glyceric acid, samples kept at room temperature were measured on the day of preparation, and also on the 1, 2, 3, and 6 days later.

The L-configuration ratio of the *O*-acetylated (+)-2-butyl ester of DL-glyceric acid varied from 51.8 to 54.7% (average ± SD, 54.6 ± 1.5%; CV, 2.74%), and that of the *O*-acetylated (−)-menthyl ester varied from 54.2 to 54.3% (average ± SD, 54.2 ± 0.1%; CV, 0.13%). The stability over the 6-day time course of the *O*-acetylated (−)-menthyl ester was superior to that of the *O*-acetylated (+)-2-butyl ester (Table 1).

3.2. Clinical applications

The DL-glyceric acid concentration in the urine of the PH2 patient was found to be 360–1700 nmol/μmol creatinine. D-Glyceric aciduria is another disease that is known to show an abnormal increase in D-glyceric acid excretion. Because the absolute configuration of glyceric acid cannot be determined

Table 1
Stability of the L-configuration of *O*-acetylated (+)-2-butyl and (−)-menthyl esters of DL-glyceric acid

Derivatives	Average (%)	SD	CV (%)
O-Acetylated (+)-2-butyl ester	54.63	1.49	2.74
O-Acetylated menthyl ester	54.88	0.42	0.77

Three samples each were derivatized using each method, and measured more than three times. The L-configuration stability was calculated as the percentage of the peak area of the L-configuration to the total peak area of the L- and D-configuration.

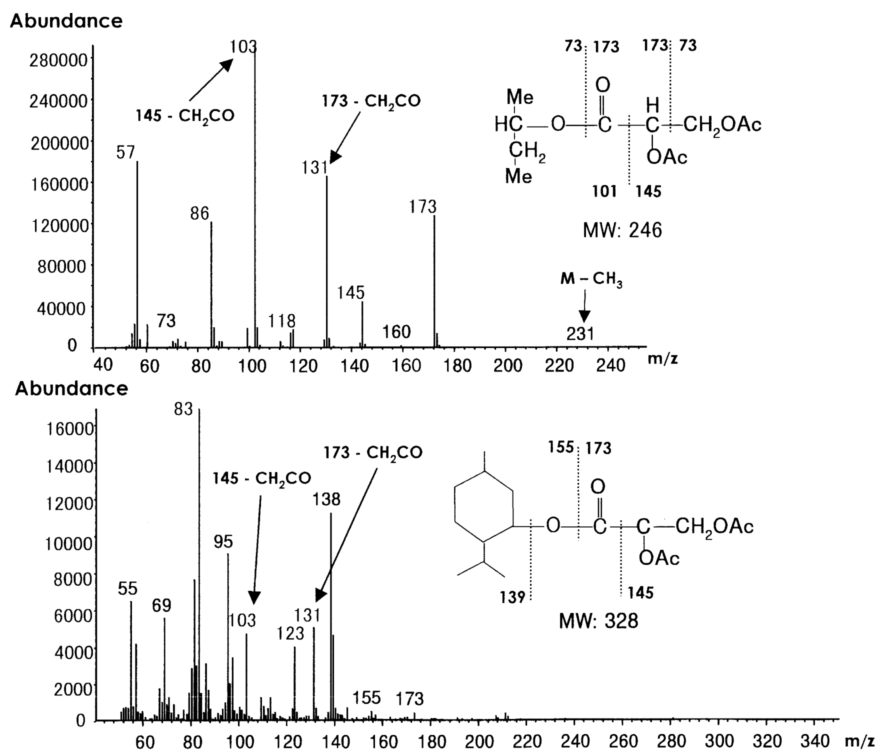

Fig. 2. Mass spectra of *O*-acetylated (+)-2-butyl (upper), and (−)-menthyl (lower) esters of L-glyceric acid.

by routine measurement using TMS derivatives, it is necessary to subject abnormal samples to a separation procedure that can determine the absolute configuration of the glyceric acid. In contrast, the DL-glyceric acid concentration measured in healthy controls in this study was 4–44 nmol/μmol creatinine (less than 2SD of the control value).

Glyceric acid was isolated from the urine of the patient with PH2 by the urease pretreatment method. After derivatization to the *O*-acetyl (+)-2-butyl ester, only one main peak was found in the total ion chromatogram (Fig. 1, right and Fig. 3(1)); it had the same retention time as the derivative of authentic L-glyceric acid (Fig. 1, left and center) (99% L and 1% D).

When glyceric acid was isolated from the urine of healthy controls as the *O*-acetyl (+)-2-butyl ester derivative and analyzed for the absolute configurations, two main peaks were found in the chromatograms, and they had the same retention times as the derivatives of authentic D- and L-glyceric acid (Fig. 3). The D- and L-configuration ratio was widely distributed (range 36–93% L and 7–64% D). No correlation was found between the configuration ratio and the glyceric acid urinary concentration, which was normalized to the creatinine concentration. It was difficult to perform the analysis with the glyceric acid *O*-acetylated (−)-menthyl ester from the same control urine samples, because the volume of usable urine was limited, except for the urine from the patient with PH2.

The clinical and biochemical characteristics of this patient and his family members will be published elsewhere.

4. Discussion

As far as we know, this is the first method for determining the absolute configuration of glyceric acids in the urine of healthy control subjects using only 100 μl of urine or extract from dried urine filter paper. The absolute configuration of glyceric acids mentioned in the literature has been limited to those isolated from the body fluids of PH2 patients. Here, we determined the absolute configuration of the glyceric acids in the urine of healthy control subjects. To achieve high sensitivity, we used urease pretreatment to isolate glyceric acid from the urine specimens and the *O*-acetylated (+)-2-butyl ester as the derivatized form.

Because most reports have focused on 2-hydroxy or 3-hydroxy acids, there were none giving the most suitable conditions for studying glyceric acid, a 2,3-dihydroxy acid. We expected that the methods of (−)-menthylation and/or (+)-2-butylation following the *O*-acetylation of 2-hydroxy acids would be relatively suitable. These methods were compared using standard compounds and patient urine. Kamerling et al. have reported the separation by capillary GC of diastereomers of the *O*-acetylated (−)-menthyl esters of lactic and glyceric acids isolated from patients. When this method was used, a larger quantity of urine was necessary to separate the diastereomers of glyceric acid from the urine of healthy controls than from the urine of patients, and it seemed unlikely that enough urine could be recovered from samples obtained as dried urine on filter paper. In an article from 1981, Kamerling et al. indicated that this (−)-menthylation

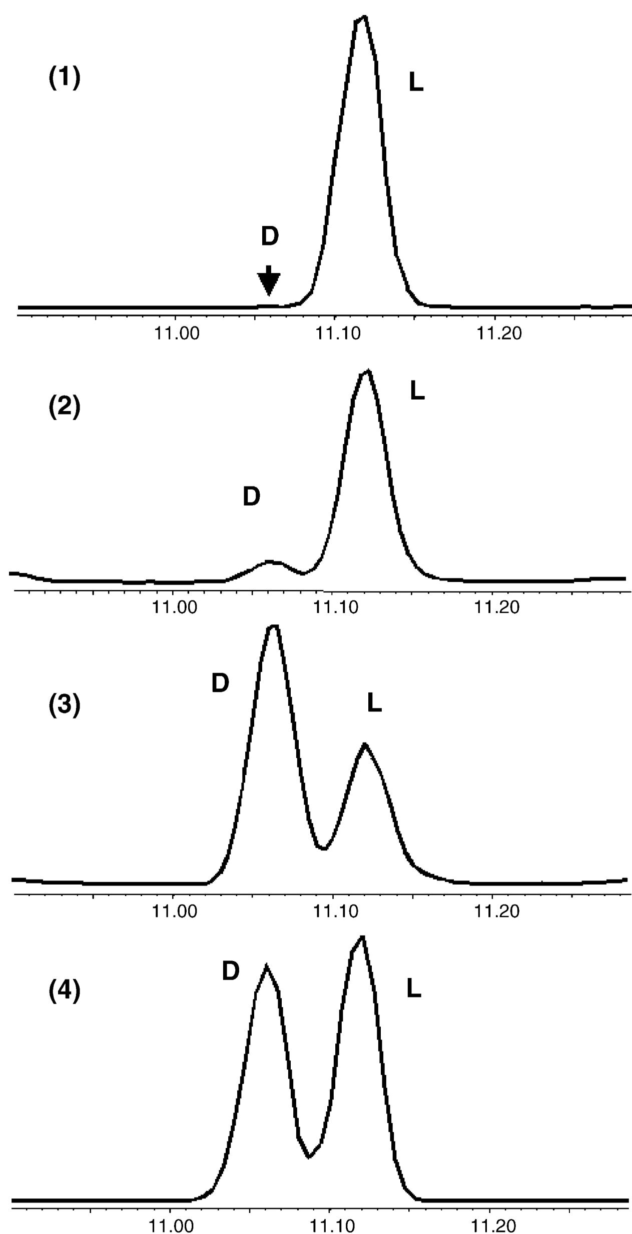

Fig. 3. Mass chromatograms (1 and 4) and SIM chromatograms (2 and 3) of target ion (m/z 173) of the O-acetylated (+)-2-butyl esters of D- and L-glyceric acid from PH2 patient urine (1), the healthy control with the maximum L-configuration in the control group (2), the healthy control with the minimum L-configuration in the control group (3), and standard DL-glyceric acid (4). Peak heights were normalized to full-scale deflection.

method was not well-suited for all groups of chiral hydroxy acids, and therefore other procedures had to be developed for the O-acetylated di-(−)-2-butyl ester of 2-hydroxyglutaric acid [9]. We have succeeded in measuring the diastereomers of glyceric acid in urine samples from healthy controls by our present procedure of (+)-2-butylation, although (S)-(+)-2-butanol is more expensive than menthol.

The esterification with menthol and acetylation of hydroxyl groups on C-2 and C-3 of glyceric acid requires a higher reaction temperature than does the (+)-2-butylation method. However, there was a clear difference in the quantity of the reaction products needed for the two methods. Using standard compounds and the urine sample from the patient with PH2 in this study, five times the volume of urine was needed for the (−)-menthylation method than for the (−)-2-butylation method to obtain similar results (Fig. 1). Moreover, it was difficult to detect the glyceric acid as the O-acetylated (−)-menthyl ester from urine samples dried on filter paper, which contained a limited usable volume of urine. Only the amount of sample from the patient was large enough for easy detection of this ester.

In the PH2 patient's urine, about 99% of the glyceric acids had the L-configuration, and there was no difference between this result and the results reported in the literature [5]. We examined the absolute configuration of glyceric acid in the urine of healthy controls, because it had not been reported previously as far as we know. These experiments showed a very high proportion of the L-configuration but a low overall concentration of glyceric acid in the urine of several healthy controls. Five of the 20 low-concentration samples from healthy controls had an L-configuration ratio greater than 90% (maximum 93%). However, the absolute configuration of glyceric acid in the low-concentration urine is outside the objectives of the differential diagnosis and does not give information influencing the diagnostic result. Nevertheless, it is useful here to show that this method has very high sensitivity.

The present study shows that the O-acetylated (+)-2-butyl ester was suitable for determining the absolute configuration of 2,3-dihyroxy acids, like glyceric acid, and useful for the differential diagnosis of PH2 and D-glyceric aciduria.

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