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ORIGINAL RESEARCH

Chiral liquid chromatography tandem mass spectrometry in the determination of the configuration of glyceric acid in urine of patients with D-glyceric and L-glyceric acidurias

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ABSTRACT: Glyceric acid is a highly polar chiral carboxylic acid that is usually not detected during routine organic acid analysis. Increased excretion is observed in two phenotypically distinct and rare inherited metabolic diseases, D-glyceric aciduria, and L-glyceric aciduria (also known as primary hyperoxaluria type 2). The determination of the exact configuration of the excreted glyceric acid is necessary for the accurate diagnosis of D-glyceric aciduria and for the differentiation between type 1 and type 2 primary hyperoxaluria. The separation of the two stereoisomers was achieved using a narrow-bore ristocetin A glycopeptide antibiotic silica gel bonded column. Triethylamine acetate at pH 4.1 with 10% methanol was used as mobile phase. The column was directly interfaced to a triple quadrupole tandem mass spectrometer and the electrospray ion source was operated in the negative ion mode. Three parent-to-daughter transitions were employed to specifically detect eluting glyceric enantiomers from essentially untreated urine samples. The two forms of glyceric acid were satisfactorily separated at 3.6 and 4.5 min. Application of the method led to the confirmation of three cases of D-glyceric aciduria from three different families. Two other cases are suspected to be L-glyceric aciduria but further confirmation is needed. The method allowed the detection of the glyceric acid stereoisomers in control urine where it was found without exception that L-glyceric was the predominate metabolite. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Glyceric acid (2,3-dihydroxypropionic acid) is a plant and mammalian metabolite that exists in two configurations, D(+) and L(−). Both enantiomers are intermediary metabolites normally excreted in trace amounts in urine. The two enantiomers are key biochemical markers for two rare inherited metabolic diseases, with different phenotypes. The first disease is D-glyceric aciduria, which was described first in 1974 and later detailed (Brandt *et al.*, 1974, 1976). They reported a case of non-ketotic hyperglycinemia (NKH) with increased excretion of D-glyceric acid in the urine and serum of a Serbian child from a non-consanguineous couple. This mentally retarded child showed the typical neurological symptoms of NKH, and excreted excessive amounts of D-glyceric in urine (usually not detectable in controls). In 1976, a second patient was studied of an Afghani origin, excreting excess D-glyceric acid, normal glycine with a different clinical picture of tachypnea, tachycardia, metabolic acidosis and neurologically normal (Wadman *et al.*, 1976). After these two cases had been described, several other cases came to light and the enzyme deficiency has been determined to be D-glycerate kinase, the enzyme involved in the conversion of D-glyceric to D-2-phosphoglycerate (Kølvraa *et al.*, 1984; Duran *et al.*, 1987; Fontaine *et al.*, 1989; Van Schaftingen, 1989; Bonham *et al.*, 1990).

The second disease entity involving glyceric acid is primary hyperoxaluria type 2 (PH2). Primary hyperoxalurias (PH) are two rare inherited defects characterized by overproduction and accumulation of oxalate in tissue (oxalosis). PH1, also called glycolic aciduria, is more common and is due to hepatic peroxisomal alanine-glyoxylate aminotransferase deficiency (Danpure and Jennings, 1986). Biochemically, the disease is characterized by deficient conversion of glyoxylic acid to glycine and increased oxidation to oxalate and glycolate with

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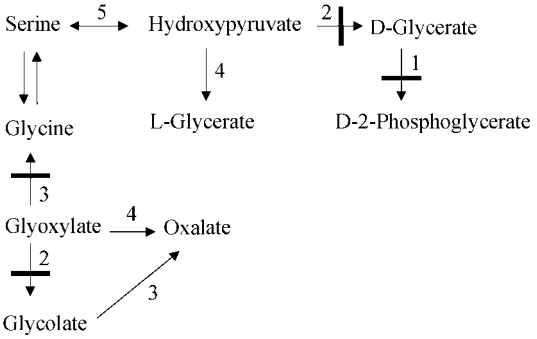
Abbreviations used: CID, collision-induced dissociation; FIA, flow injection analysis; MS1, static mass analyzer; NKH, non-ketoic hyperglycinemia; Z-OHG, DL-2-hydroxyglutaric acid; PH1, primary hyperoxaluria type 1; PH2, primary hyperoxaluria type 2.



Scheme 1. The metabolic pathways involved in D-glyceric aciduria, primary hyperoxaluria type 2 (PH2; L-glyceric aciduria), and primary hyperoxaluria type 1. Deficiency of D-glycerate kinase leads to D-glyceric acidemia (1). Deficiency of D-glycerate dehydrogenase/glyoxylate reductase leads to L-glyceric aciduria (2). Deficiency of alanine-glyoxylate aminotransferase leads to PH1 (3). The metabolic block of pathway 2 leads to reduction of hydroxypyruvate by lactate dehydrogenase to L-glycerate and oxidation of glyoxylate to oxalate by lactate dehydrogenase, glycolate oxidase and xanthine oxidase.

normal glyceric acid (see Scheme 1). PH2, also called L-glyceric aciduria, has had less than 30 cases reported worldwide (Chalmers *et al.*, 1984). The metabolic defect is due to deficiencies of D-glycerate dehydrogenase and glyoxylate reductase, leading to excessive urinary oxalate and excretion of L-glyceric acid, the latter being the cornerstone for the diagnosis of PH2. The disease characterized with urolithiasis or nephrocalcinosis with terminal renal failure is less prevalent than in PH1 (Kemper *et al.*, 1997; Mansell, 1995).

Determination of the configuration of glyceric acid is a necessary test for the differentiation of PH2 from D-glyceric aciduria and for the confirmation of these disorders, and the excretion of L-glyceric is important in differentiating PH2 from PH1. The aim of the work presented here is to develop a simple and direct method for the enantiomeric resolution to allow for the rapid and accurate diagnosis of glyceric aciduria patients.

EXPERIMENTAL

Chemicals. DL-glyceric, D-glyceric as hemi calcium salts, and triethylamine were purchased from Sigma Co. (St Louis, MO, USA). Methanol, acetonitrile and glacial acetic acid for HPLC were purchased from Fisher Scientific Co. (Fairlawn, NJ, USA).

Tandem mass spectrometry (MS/MS). MS/MS and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was carried out on a QuattroLC bench-top triple quadrupole tandem mass spectrometer (Micromass, Altrincham, UK) equipped with an electrospray ionization (ESI) Z-spray ion source. The system was interfaced with a Hewlett-Packard Model HP1100 binary HPLC pump connected to a Gilson Model 232XL autosampler and a Rheodyne injector. The injector was connected to the electrospray source using peak tubing.

MS/MS parameters. The product ion spectrum of DL-glyceric acid was acquired in negative-ion ESI using a capillary voltage of 3.0 kV, a cone voltage of 25 V, collision energy of 15 eV, and a gas pressure of 1.5×10^{-3} mbar. The $[M-1]^-$ ion for standard glyceric acid at *m/z* 105 was selected by a static mass analyzer 1 (MS1) and subjected to collision-induced dissociation (CID) and fragments were analyzed by a scanning MS2 in the range of 30–110 Da. For flow injection analysis of standards the ion source temperature was maintained at 125°C and the desolvation temperature was kept at 250°C. For LC-MS/MS analysis of standards or urine samples the ion source temperature was raised to 150°C and the desolvation temperature was maintained at 350°C. Analysis of urine samples was carried out in the multiple reaction monitoring mode (MRM) and it included three CID transitions at $105 \rightarrow 75$, $105 \rightarrow 57$ and $105 \rightarrow 45$. A dwell time of 0.25 s was used for each transition with two repeats each.

Chromatography. The mobile phase used for the enantiomeric separation for glyceric acid consisted of a mixture of 0.1% triethylamine adjusted to pH 4.1 with 5% acetic acid solution, and methanol (9:1 v/v). The column used was a Chirobiotic R column of 250 mm length × 2.0 mm i.d., 5 µm silica gel particles bonded to the macrocyclic glycopeptide ristocetin A (Advanced Separation Technologies, Whippany, NJ, USA). The flow rate was 0.3 mL/min with no split. The total run time was 10 min.

Determination of the configuration of urinary glyceric acid. Peak identification of the two configurations of glyceric acid was established by analyzing commercially available DL-glyceric and D-glyceric standards using chromatographic conditions described above. For analysis of urine from controls and patients a volume of urine was diluted 1:1 with mobile phase and filtered through a 0.45 µm membrane filter. A volume of the filtrate equivalent to 125 ng creatinine (1.1 nmol) was injected (2–10 µL) into the system from a microtiter plate. For samples with high creatinine value further dilution with mobile phase was carried out.

RESULTS AND DISCUSSION

Glyceric acid is a highly polar aliphatic carboxylic acid that has a small molecular weight and no chromophore. Its recovery from acidified urine by extraction with organic solvents is extremely poor. Dietzen *et al.* (1997) reported on the use of tetrahydrofuran for better recovery of glycolic and glyceric acids from urine. However, routine organic acid using diethylether and/or ethylacetate for extraction and analysis of derivatized extracts from controls by GC/FID or GC/MS usually fails to detect normal levels. Therefore, finding it in the GC/MS profile of organic acids is suggestive of either D-glyceric aciduria or primary hyperoxaluria type 2. In the latter, another pathognomonic metabolite is oxalic acid, albeit it may be difficult qualitatively to tell that oxalate is indeed high in urine without a quantitatively determining its

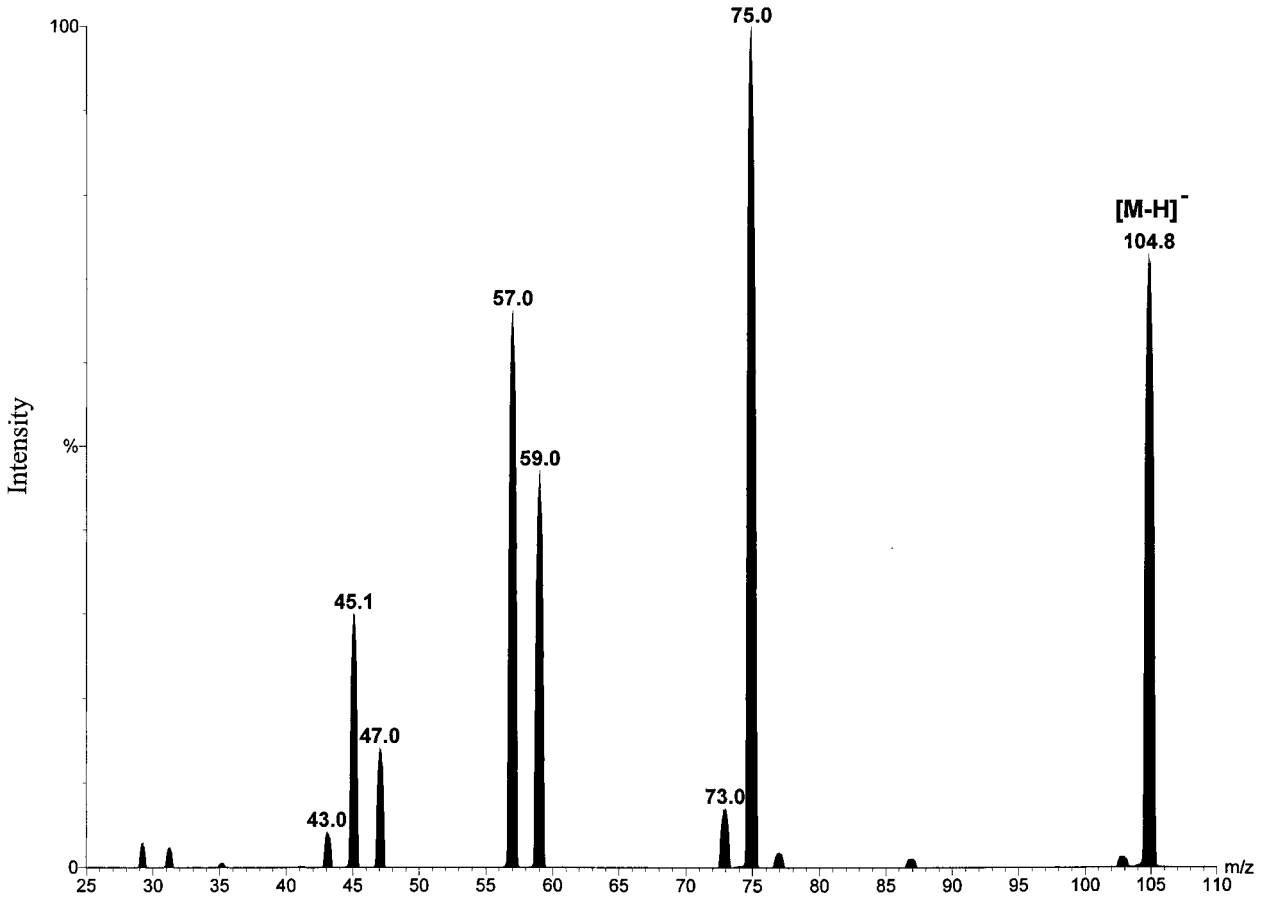


Figure 1. ESI-MS/MS product ion spectrum of *m/z* 105 $[M-H]^-$ for standard DL-glyceric acid obtained using collision energy of 15 eV and cone voltage of 25 V, and 1 s scanning time.

levels versus controls. Even in confirmed cases oxalate excretion showed significant variation (Helin, 1980). This makes the determination of the exact configuration of glyceric acid necessary for differentiation between PH1 and PH2 and for confirmation of the diagnosis of PH2. As matter of fact, in all PH2 cases and all D-glyceric cases described in the literature, the exact configuration of glyceric acid has been determined. This was mostly done by converting the glyceric acid enantiomers to the corresponding diastereomers through the formation of the O-acetylated menthyl ester derivative using (−) menthol and acetic anhydride, followed by GC or GC/MS analysis on an achiral column (Wadman *et al.*, 1976). An alternative approach was reported where the methyl esters of DL-glyceric acid were separated using a dual column GC analysis where the precolumn was achiral, and the main column was chiral fused silica capillary column coated with a film of heptakis (2,3-di-O-methyl-6-*O-tert* butyldimethysilyl)-*β*-cyclodextrin. This approach succeeded in the enantiomeric separation of the methyl esters of DL-lactic acid, DL-2-hydroxyglutaric acid (2-OHG), and DL-glyceric acid in urine of patients and an afghan hound (Kaunzinger *et al.*, 1996).

We recently succeeded in the development of chiral LC-MS/MS method for the direct separation of the underivatized enantiomers of 2-OHG (Rashed *et al.*, 2000). We therefore decided to investigate the possibility of direct chiral separation of DL-glyceric using tandem mass spectrometry. Similar to 2-OHG, glyceric acid circulates in blood and is excreted in urine as a carboxylate anion, and thus is ideally suited for electrospray analysis in the negative ion detection mode. However, because of the small molecular ion at *m/z* 105 $[M-H]^-$, we expected interference from background and from other urine constituents even by MS/MS analysis. To investigate this approach we optimized the conditions necessary for the detection of glyceric acid where a standard solution of the DL-mixture was injected in the flow injection mode (FIA). Figure 1 shows the product ion spectrum for underivatized DL-glyceric obtained by MS/MS analysis in negative ion mode. The spectrum shows the M-1 ion at *m/z* 105 and five pronounced fragments at *m/z* 75, 59, 57, 47 and 45. We used three of these fragmentation processes to develop a method for the selective detection of glyceric acid by setting up three MS/MS-MRM transitions of parent-to-

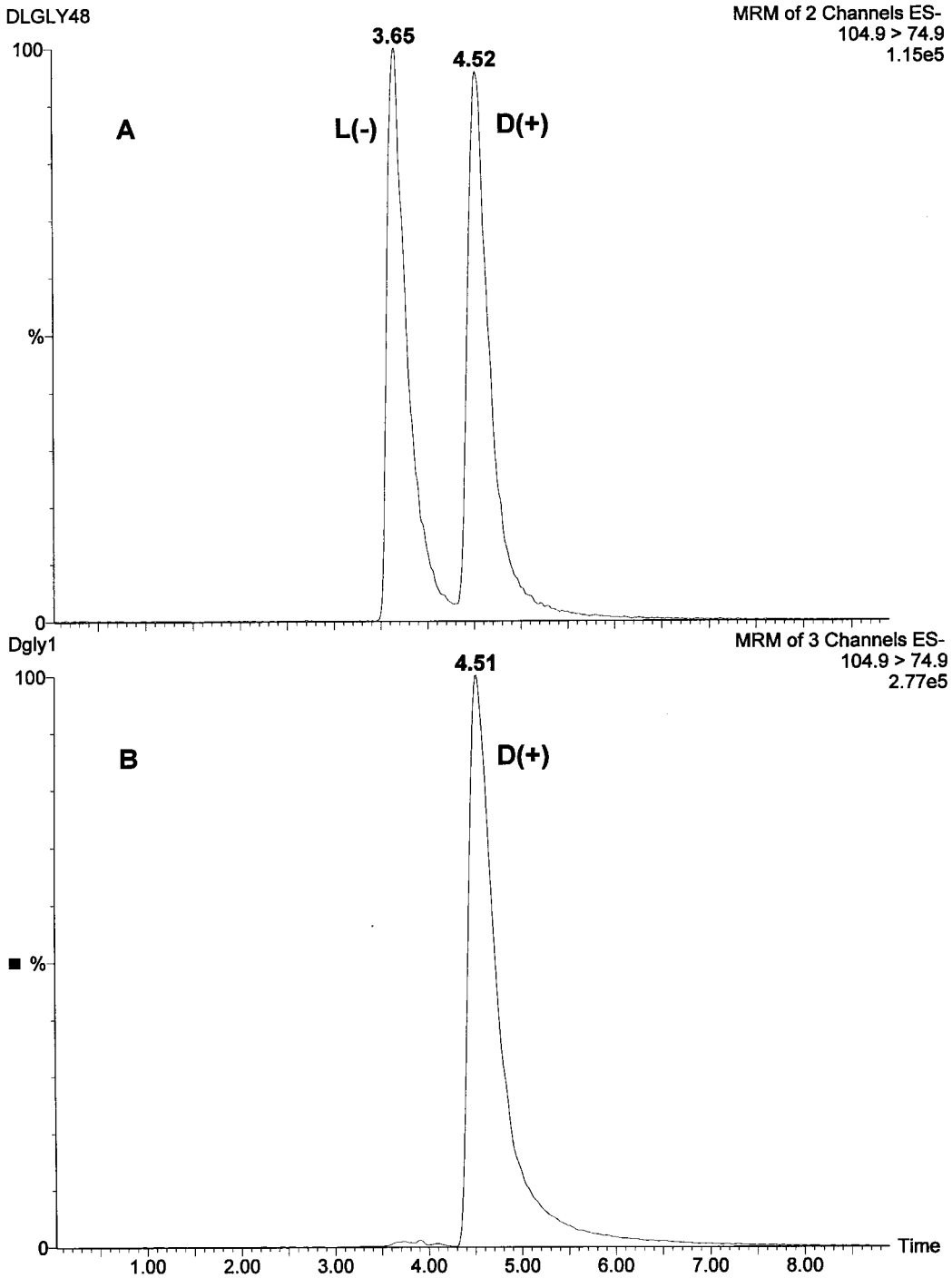


Figure 2. LC-MS/MS negative ion chromatograms obtained for the MRM transition *m/z* 105 → 75 using ristocetin A column. (A) A standard mixture of DL-glyceric acid; (B) a standard solution of D-glyceric acid.

daughter (precursor-to-product), 105 → 75, 105 → 57 and 105 → 45.

Attempts at chiral LC-MS/MS separation of glyceric acid were carried out using two narrow-bore chiral columns, ristocetin A (Chirobiotic R), and teicoplanin (Chirobiotic T) in the reversed-phase mode using a triethylamine acetate buffer at pH 4.1, pH 5.0, and pH 7.0 with either methanol or acetonitrile as organic modifiers. The teicoplanin column did not yield satisfactory separation under these conditions. On the other hand, the ristocetin column did separate the two enantiomers, particularly when using methanol as the organic modifier. The best separation was achieved using the ristocetin column and triethylamine acetate buffer at pH 4.1 with

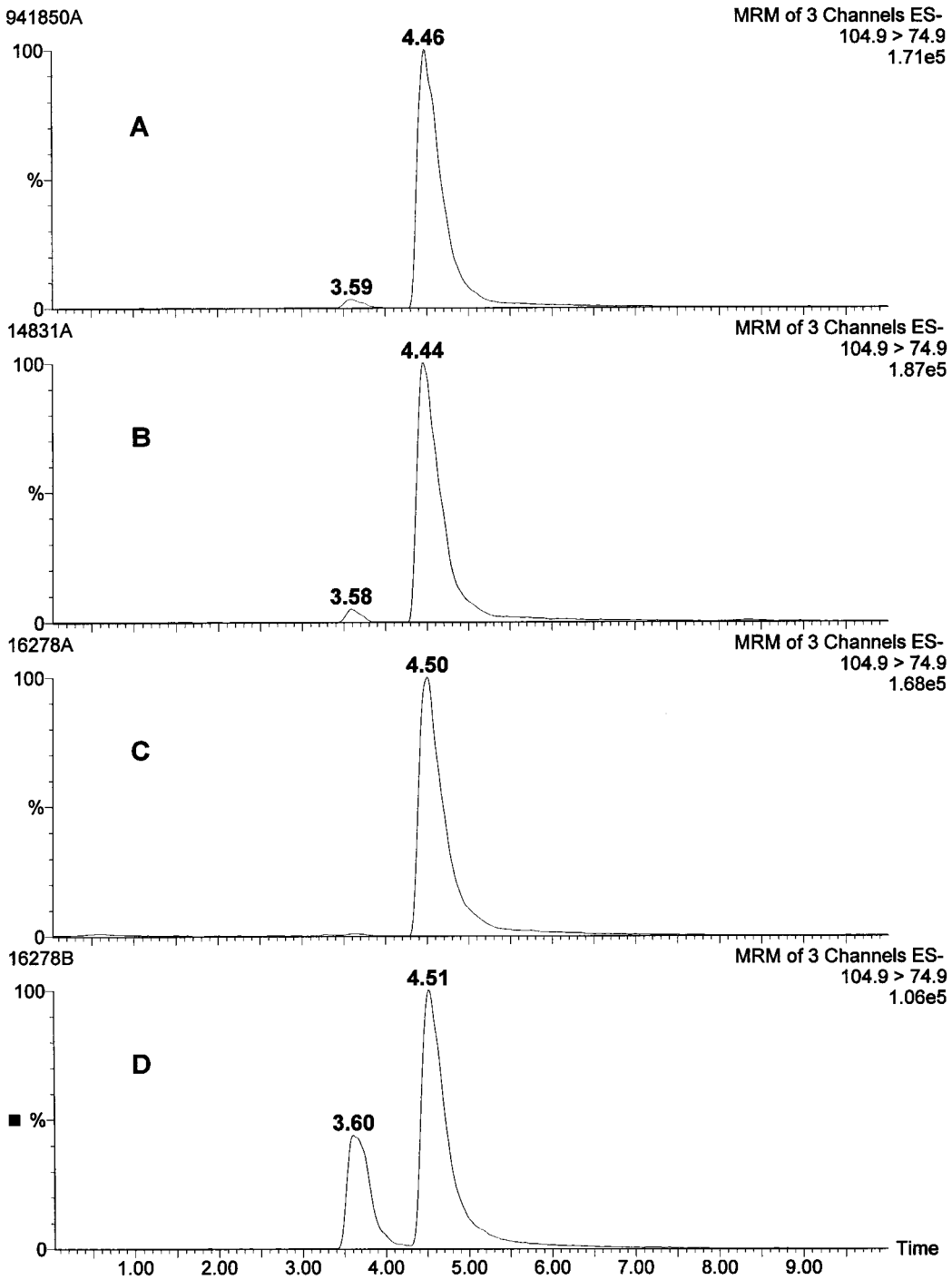


Figure 3. MRM profiles for the transition *m/z* 105 → 75 for (A) patient 1 urine; (B) patient 2 urine; (C) patient 3 urine; (D) patient 3 urine spiked with DL-glyceric acid standard solution.

10% methanol at flow rate of 0.3 mL/min. Decreasing the flow rate to 0.2 or 0.1 mL/min did not enhance the separation but only broadened the peaks and delayed their elution. The MS/MS conditions were thus optimized for such a relatively high flow rate by increasing the desolvation gas flow and increasing the desolvation temperature. Figure 2(A) shows the MRM transition *m/z* 105 → 75 for the separation of a standard solution of DL-glyceric where the L-form eluted at about 3.6 min, while the D-form eluted at about 4.5 min. Figure 2(B) shows the MRM transition *m/z* 105 → 75 for a standard solution of D-glyceric acid. Peak integration showed that the D- and L-stereoisomers gave the same peak area response.

Using conditions described above we analyzed urine

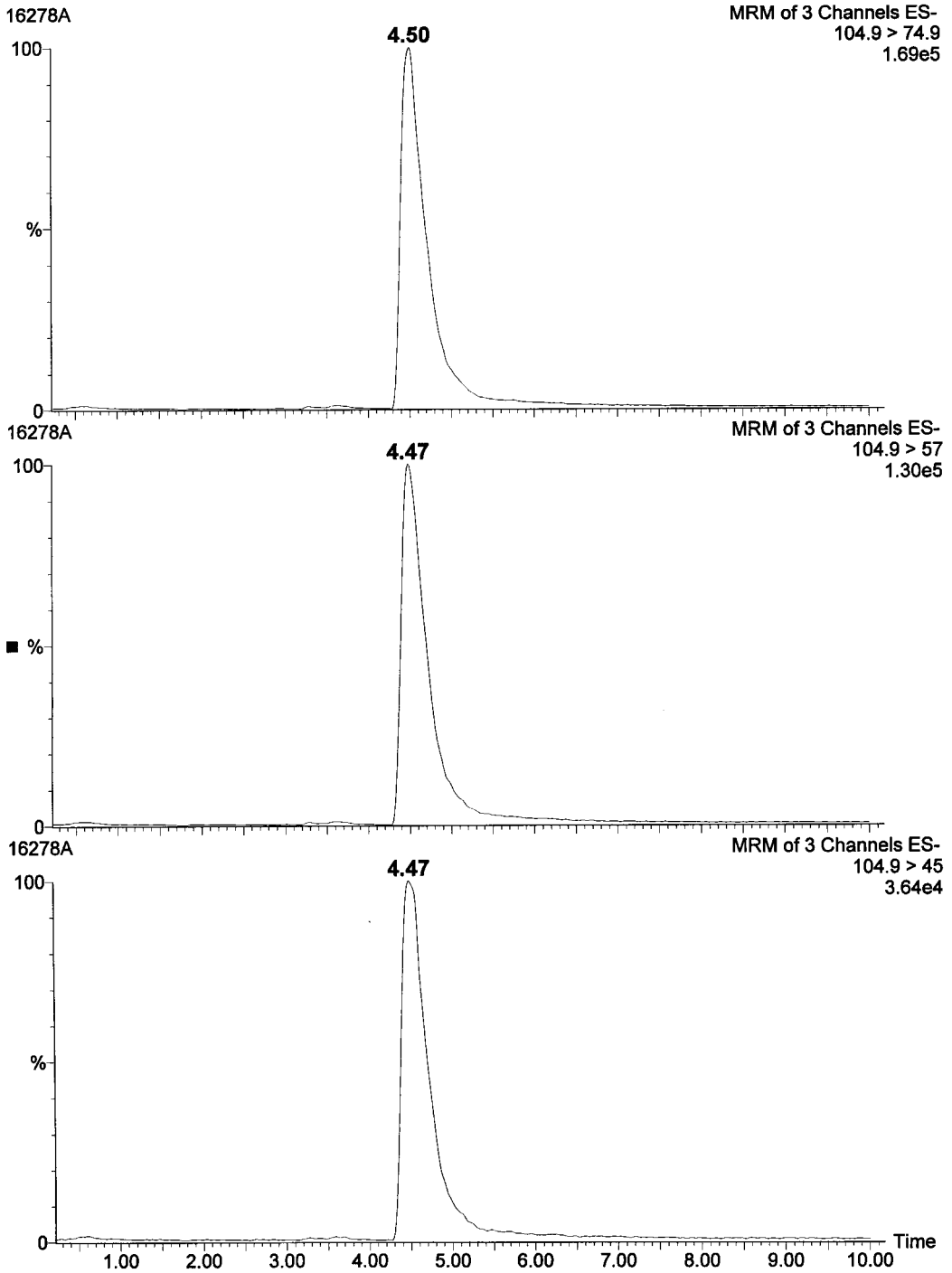


Figure 4. MRM profiles for the three transitions characteristic for D-glyceric acid at *m/z* 105 → 75, 105 → 57, 105 → 45 obtained in the urine of patient 3.

samples from five patients where glyceric acid elevation was detected by routine GC/MS analysis for organic acids. We also analyzed 23 control pediatric samples which were referred to us for metabolic screening. In all these urine samples, the organic acid profile was not remarkable and no glyceric acid was observed by GC/MS analysis. We first determined the creatinine value for all urine samples. All the samples were diluted 1:1 with mobile phase. We then analyzed several control samples to determine whether we could detect glyceric acid enantiomers in these samples. We found that a volume of control urine equivalent to 125 ng (1.1 nmol) of creatinine always showed peaks corresponding to either both D- and L-glyceric or at least one of the enantiomers

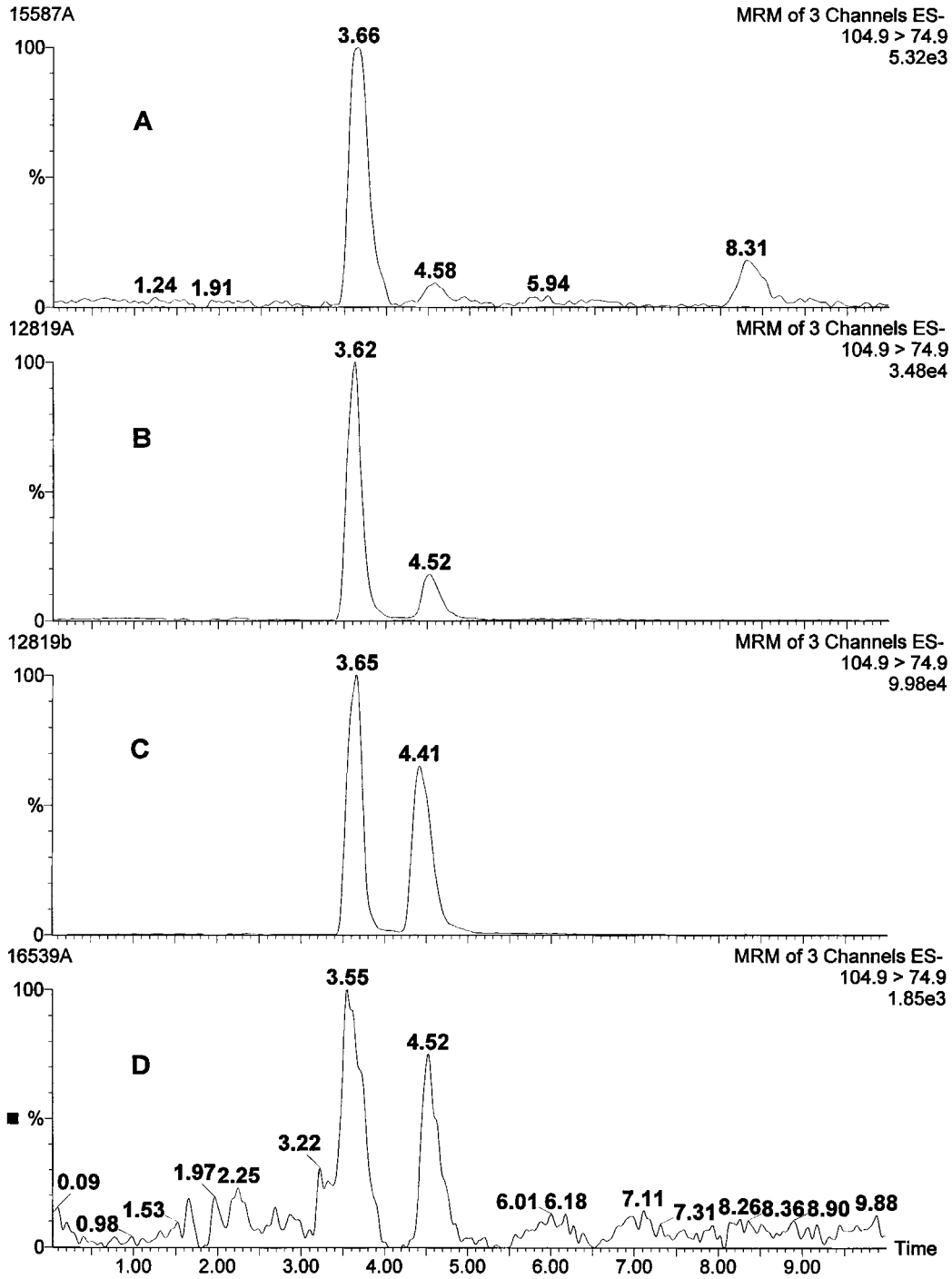


Figure 5. MRM profiles for the transition *m/z* 105 → 75 for (A) patient 4 urine; (B) patient 5 urine; (C) patient 5 urine spiked with DL-glyceric acid standard solution; and (D) a representative control urine profile.

with a signal-to-noise ratio >3. Accordingly, all control and abnormal urine samples were injected so that the volume injected was equivalent to 125 ng creatinine. For samples with high creatinine values, further dilution with mobile phase was necessary. The minimum volume injected was 2 μL and the maximum volume injected was maintained at less than 10 μL to avoid overloading the column. Despite this precaution we did observe signs of column overloading as evidenced by peak broadening and peak splitting in a number of control samples.

Figure 3(A–C) shows the profiles of the transition *m/z* 105 → 75 for patients 1–3, and Fig. 3(D) shows the profile from patient 3 spiked with DL-glyceric acid standard solution. The urine from all three patients

showed highly elevated D-glyceric with only a trace of the L-form. In all control urines, the L-form was higher than the D-form. The level of D-glyceric acid in patients' urine was at least two orders of magnitude higher than in controls. It is noteworthy that under the conditions used we observed no carry-over between samples.

Figure 4 shows three MRM profiles for the three transitions characteristic for the D-glyceric acid in the urine of patient 3. It is clear that all three transitions coincided and showed no interferences in that region of the profile. Figure 5(A) and (B) shows the profiles for the MRM transition m/z 105 → 75 for urine from patients 4 and 5. Both profiles show elevated L-glyceric acid with little D-glyceric. Figure 5(C) shows the MRM profile for urine from patient 5 spiked with standard DL-glyceric acid. Figure 5(D) shows a representative control urine profile. The level of L-glyceric in these two patients was clearly higher than all control samples but was not 'qualitatively' as pronounced, as was the case for the D-glyceric aciduria cases. Therefore, it may be important to investigate the possibility of using this approach to develop a quantitative assay for L-glyceric acid and to carry further work with well-characterized PH2 cases to further validate the utility of this approach to differentiate PH1 from PH2.

In conclusion, we described a simple, rapid, and specific LC-MS/MS method for the direct enantiomeric separation and detection of the stereoisomers of glyceric acid using a macrocyclic glycopepetide ristocetin A antibiotic column. Sample preparation was very simple and analysis time fairly short. The method allowed the detection of normal levels of D- and L-glyceric acid in control urine. The method was clearly useful for the confirmation of three cases of D-glyceric aciduria and for differentiating this disease from PH2. Further work is needed to determine its utility in differentiating PH2 from PH1.

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