A New Method for the Chiral HRGC Assay of L-2-Hydroxyglutaric Acid in Urine

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Summary

L-2-Hydroxyglutaric aciduria is a recently discovered inherited neurometabolic disease for which diagnostic identification of the right enantiomer is required. A method is described where alkyl esters of both L- and D-2-hydroxyglutaric acids are prepared without side lactonization. Esterification is achieved at room temperature by reaction with appropriate alkyl chloroformates. This method avoids lactonization, in contrast to esterification with alcohols in acidic media or with diazomethane. The identity of the derivatives is established by HRGC-FTIR and HRGC-MS. Chiral HRGC separation with capillaries coated with 1(*R*)-*trans*-*N*-*N*'-1,2-cyclohexylenebisbenzamideoligodimethyL-siloxane and with a cyclodextrin is compared. The method is adequate for quantitative determinations.

1 Introduction

Analysis of organic acids in urine of patients suspected of inborn errors of metabolism has become an important tool in clinical chemistry. Its importance is demonstrated by the discovery of multiple metabolic disorders in the past ten years principally due to the introduction of HRGC and HRGC-MS techniques. L-2-Hydroxyglutaric aciduria is an inherited neurometabolic disease with clinical, biochemical and neuroimaging characteristics. The first case was described by Duran *et al.* [1] in 1980 and since then about a dozen cases have been reported. The diagnosis of this metabolic disease depends upon HRGC screening of the urinary organic acid analysis profile, generally *via* the trimethylsilyl derivatives. Characteristic is the presence of a large peak of 2-hydroxyglutaric acid. However, the absolute configuration of the acid must be known, as L-2-hydroxyglutaric aciduria and D-2-hydroxyglutaric aciduria [2] are different diseases. The GC analysis of L-2-hydroxyglutaric acid is difficult for two reasons: (i) derivatization of pure enantiomers of 2-hydroxyglutaric acid is difficult due to the ease of lactone formation and (ii) lack of an appropriate chromatographic system for enantiomer separation. Analytical determination of absolute configuration of 2-hydroxyglutaric acid enantiomers is currently achieved through the preparation of di-2-(*R*)-butyl esters of the *O*-acetyl derivative. The diastereomers are separated on achiral columns; however, lactone formation is a significant side reaction that precludes use of the method for accurate quantitation purposes [1,3,4,9]. Enzymatic [5], NMR [6], and reversed phase HPLC methods with copper amino acid complexes as eluents [7] have been reported in food chemistry and biochemistry but they are not practical for routine work in clinical chemistry.

In this work we have studied a method for derivatization and separation of 2-hydroxyglutaric acid enantiomers by chiral HRGC. Esterification is achieved at room temperature by reaction with appropriate alkyl chloroformates. This method avoids lactonization, in contrast to esterification with alcohols in acidic media or with diazomethane. Identity of the derivatives is established by HRGC-FTIR and HRGC-MS. Fused silica capillaries coated with a new chiral siloxane copolymer, 1(*R*)-*trans*-*N*,*N*'-1,2-cyclohexylenebisbenzamideoligodimethylsiloxane [10] or with a commercial cyclodextrin are used for separation of the enantiomers. The method affords reliable quantitative data.

2 Materials and Methods

Instruments. Gas chromatography was performed in a Carlo Erba Instrument of the Mega Series, Model 5300 equipped with a FID detector, a split-splitless injector, and a fused silica capillary (column A), 30m × 0.25 i.d., coated with cyclodex-B, d_f = 0.25 μm (CDX-B J&W Scientific, Folsom, CA, reference no. 1122532). Alternatively (column B) a home-made fused silica capillary coated with CH42, a 1(*R*)-*trans*-1,2-*N*,*N*'-cyclohexenylbisbenzamidedimethylsiloxane copolymer described by Lee *et al.* [10], 20 m × 0.20 mm i.d., d_f = 0.25 μm was used. The carrier gas was hydrogen, P_i = 70 kPa; injector temperature 250 °C; detector temperature 300 °C. Oven temperature for column A: initial temperature 120 °C for 2 min then programmed at a linear heating rate of 2 °/min up to 170 °C; for column B: programmed from 160 °C to 200 °C at a linear heating rate of 1.5 °/min. HRGC-MS measurements (EI, 70 eV, 1.5 s/decade) were performed with a Shimadzu instrument, Model QP-1000, equipped with a direct interface. Ion source temperature 250 °C. Multiplier 2,500 V. For GC-MS screenings of urine samples a ion trap detector (ITD) from Finnigan Mat was used. Peak integration and quantitative calculations were made with a computing integrator from Shimadzu, Model CR3 A, equipped with a floppy disk drive and a CRT monitor. HRGC-FTIR experiments were performed with a Nicolet instrument, Model 5SXC, equipped with a 100 μl light-pipe and a MCT detector. Transfer line and light-pipe temperatures 200 °C. Velocity of acquisition 1 interferogram/s.

Chemicals. Standards of L- and D-2-hydroxyglutaric acids (sodium salts) were obtained from Sigma (Bellafonte, USA). Alkyl chloroformates were from Fluka AG (Buchs, CH). All solvents were p.a. grade from E. Merck (Darmstadt). All solvents were

distilled before use. Stock solutions of L- and D-2-hydroxyglutaric acids (sodium salts) in water (100 mg/100 ml) were prepared and kept in the refrigerator.

2.1 Derivatization

Methylation with diazomethane. Into a Teflon-lined screw-cap derivatization vial were measured 100 µl of stock solutions of each 2-hydroxyglutaric acid enantiomers and the solvent eliminated by lyophilization. The residue was dissolved in 50 µl methanol and 600 µl of an ethereal solution of diazomethane was added stepwise under ice cooling, until the yellow color persisted. The solvent was evaporated and the residue dissolved in 100 µl of CH_2Cl_2. From this solution 1 µl aliquots were used for chromatography

Esterification with ethyl chloroformate. A volume of urine corresponding to 5 µmoles of creatinine was measured into a glass tube and acidified with 2M HCl until pH 1. The solution was saturated with NaCl and extracted with 5 ml of ethyl acetate. The tube was centrifuged, the supernatant transferred into a tube containing anhydrous sodium sulfate and filtered. The process was repeated 2 times. The clear solutions were collected, the solvent dried under a gentle stream of nitrogen and the dry residue stored in a refrigerator for further use. To the residue was added 100 µl of stock solution of D-2-hydroxyglutaric acid and the solvent evaporated. The residue was dissolved in 1.5 ml of ethyl acetate. Aliquots of 150 µl of this solution were derivatized as follows: after evaporation of the solvent under a light stream of nitrogen the residue was dissolved in water:ethanol:pyridine (60:32:8) and 5 µl of ethyl chloroformate were added. The solution was shaken in a vortex for 5 s. Keeping this time is crucial. After that time 100 µl of a 1% solution of ethyl chloroformate in $CHCl_3$ is added and the mixture is shaken again for 5 s. The organic layer is used immediately for chromatography from which 1 µl aliquots were injected. Aliquots of stock solutions of the standards (100 µl) were derivatized in the same way. For the formation of 2-chloroethyl esters the ethyl acetate residue was dissolved in 25 µl of water:pyridine (2:1), and 75 µl of acetonitrile:chloroethanol (2:3) and 5 µl of 2-chloroethyl chloroformate were added. The mixture was shaken for 5 s. After that time 5 µl of 2-chloroethyl chloroformate and 100 µl of chloroform were added, the mixture shaken for 5 s, and the organic layer used for chromatography.

3 Results and Discussion

Figure 1 shows a typical HRGC-MS profile of urinary organic acids as TMS derivatives, from a patient suspected of L-2-hydroxyglutaric aciduria. The chromatographic profile is immediately recognized by the presence of an excessive amount of 2-hydroxyglutaric acid. Usually the analytical diagnostic L-2-hydroxyglutaric aciduria is based on the preparation of *O*-acetyl 2(*R*)-butyl esters. Separation and identification of the diastereomers is achieved by HRGC and HRGC-MS. However, the derivatization reaction may have some detrimental effects in the analysis, principally if quantitative data are required. 2-Hydroxyglutaric acid is very prone to lactonization. Even very mild reaction conditions may lead to appreciable lactone formation. This tendency is enhanced under acidic reaction conditions and with temperature. Other possible side reactions in acidic media are solvolysis and partial racemization. The presence of extraneous

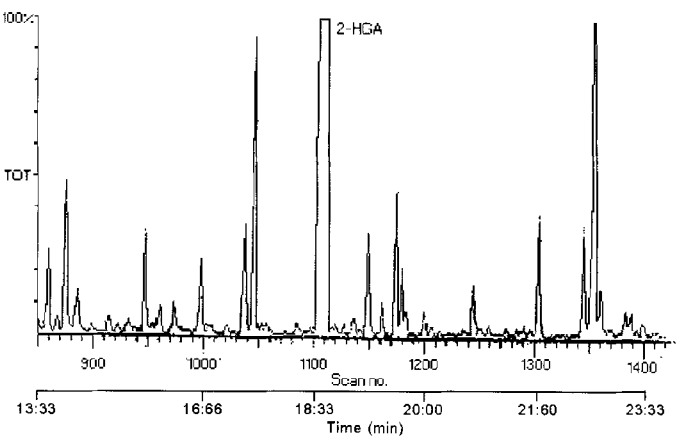

Figure 1. Partial total ion chromatogram (ITD) of urinary organic acid profile (TMS derivatives) in a case of 2-hydroxyglutaric aciduria. Column SPB-1, 60 m × 0.32 mm i.d. Oven temperature: isothermal at 100 °C for 5 min, increased at a linear heating rate of 5 °/min up to 260 °C, then kept isothermal for 10 min.

peaks in samples [1,8] may arise from these side reactions. In order to try to circumvent these difficulties we have tried a new procedure, based in the preparation of methyl esters with diazomethane in methanol. Enantiomer separation is achieved with a fused silica capillary coated with a 1(*R*)-*trans*-*N*,*N*'-1,2-cyclohexenylbisbenzamideoligosiloxane [10]. **Figure 2** shows an acceptable separation of both enantiomers but two relatively intense extraneous peaks corresponding to the expected side reactions are observed.

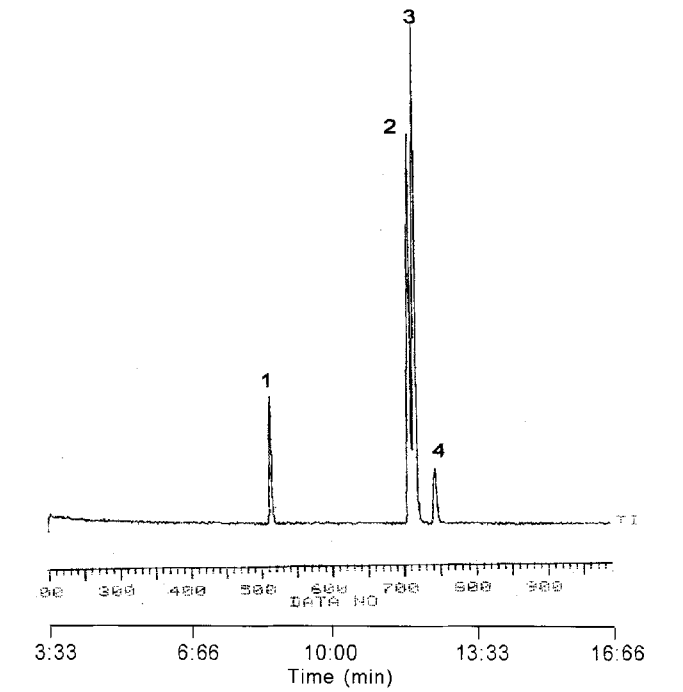

Figure 2. Separation of 2-hydroxyglutaric acid (2-HGA) enantiomers (GC-MS, QP-1000, see experimental part) after esterification with diazomethane in methanol on a fused silica capillary coated with a 1-(*R*)-*trans*-*N*,*N*'-1,2-cyclohexenylbisbenzamideoligosiloxane (column B). Peaks: 1 – 2-HGA γ-lactone; 2 – D-2-HGA; 3 – L-2-HGA; 4 – *O*-methyl 2-HGA dimethyl ester.

More classical derivatization procedures based on preparation of *O*-acyl esters were also unsuccessful in obtaining clean chromatograms. Such were the cases of *O*-pentafluoropropanoyll and *O*-hexafluorobutanoyl isobutyl ester derivatives. Moreover, with these derivatives the enantiomers could not be resolved.

We were more successful with the preparation of alkyl esters of 2-hydroxyglutaric acid by reaction with appropriate chloroformates in the presence of basic catalysts such as pyridine, 3-picoline, and *N*-methylpiperidine. This approach has been used by others for derivatization of fatty acids [11,12], and amino acids [13]. The reaction is fast and occurs at room temperature. However, in order to be effective, it is recommended that the technique be followed very exactly as described in the experimental part. We found that small deviations from the recommended reaction time leads to extraneous peaks with consequent low relative response factors. The L- and d-2-hydroxyglutaric acid ethyl esters are well separated in the cyclodextrin column (**Figure 3**). Although separation in column B (see experimental) is also achieved (Figure 2), lower selectivity is observed and the elution order is inverted with the D isomer eluting before the L. There is no noticeable formation of secondary products under the derivatization conditions used. It should be noted, however, that the derivatives are very unstable upon storage. It is essential that their preparation takes place immediately before chromatography.

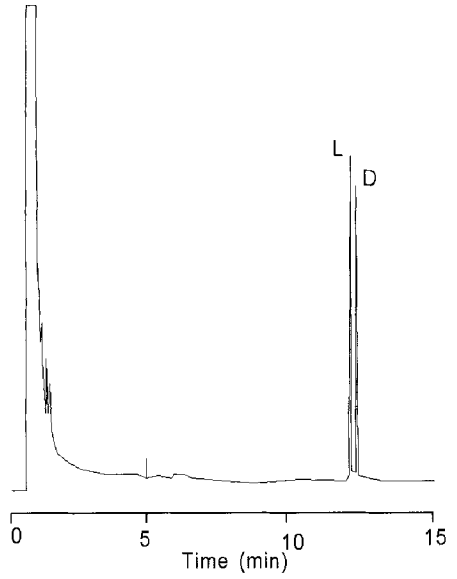

Figure 3. Chiral HRGC separation of L- and D-enantiomers standards of 2-hydroxyglutaric acid as their ethyl esters on a cyclodextrin capillary (column A, see experimental part).

Identity of the derivatives was confirmed by HRGC-MS. The mass spectra of the ethyl esters show a characteristic fragmentation pattern: *m/z* 204 (M^+, 5%), 159 ($M^+ - 45$, 28%), 158 ($M^+ - 46$, 2-HGA γ-lactone ethyl ester, 3%) 131 ($M^+ - COOC_2H_5$, 43%), and 85 (158-$COOC_2H_5$, 100%). The mass fragmentation pattern observed for the 2-chloroethyl derivatives is also consistent with the expected structure: 273 (M^+), 152 ($M^+ - 121$, $ClCH_2CH_2OCO\text{-}CH_2$), 121, 63 (100%, CH_2CH_2Cl). This was further confirmed by HRGC-FTIR. The IR spectrum of the 2-chloroethyl esters, as an example, show characteristic absorption at 1770 cm^{-1}, 1396 cm^{-1}, 1276 cm^{-1}, and 1251 cm^{-1}.

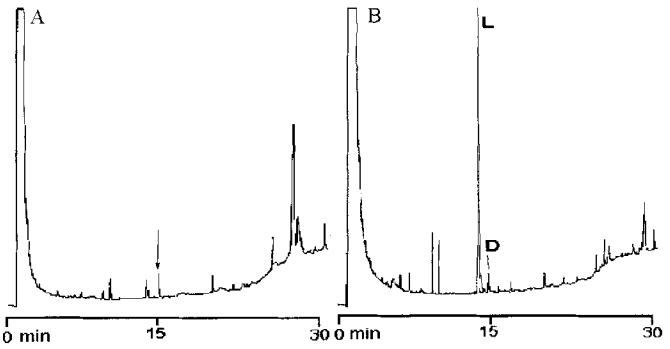

Figure 4. Chiral HRGC on a cyclodextrin capillary (column A) of: A – a blank urine; B – A urine from a patient suffering from L-hydroxyglutaric aciduria. 2-Hydroxyglutaric acid enantiomers analyzed as the corresponding diethyl esters. Conditions as in the experimental part.

Figure 4 shows the chromatograms corresponding to a blank urine from a healthy subject (A) and a urine from a patient suspected of L-2-hydroxyglutaric aciduria (B) after derivatization and chromatography with the cyclodextrin capillary. A very intense peak corresponding to L-2-hydroxyglutaric acid unequivocally confirms diagnostic of the disease. Although no noticeable racemization was observed with standards of pure enantiomers, a small peak with a retention time similar to that of the D isomer can be seen in real samples, representing about 2–3% of a total enantiomeric composition. These low levels of the accompanying peak lie far below the error of the method (8%) thus allowing the use of the D-enantiomer as an ideal internal standard for the quantitative assay of L-2-hydroxyglutaric acid in urine (enantiomeric labeling).

The method of enantiomeric labeling was tested for quantitative determination of L-2-hydroxyglutaric acid in urine of patients with L-2-hydroxyglutaric aciduria syndrome. The values were compared with the ones obtained by a GC-MS classical method (**Table 1**). The results are in good agreement with those obtained by less specific routine GC-MS methods of 2-hydroxyglutaric assay in urine through the TMS derivatives with 3-phenylbutyric acid as internal standard. Recoveries were 100–107% (CV = 8%, $n = 4$).

Table 1. Determination of 2-hydroxyglutaric acid in urine of patients presenting L-2-hydoxyglutaric aciduria syndrome by enantiomeric labeling. Comparison with values obtained in clinical screening by a non-specific GC-MS method. Values are in μmol/mmol creatinine.

Patient	GC-MS	Enantiomeric labeling	*n*	C.V.
Male 1	1420	1157	9	0.05
Male 2	780	870	8	0.04
Female 1	920	1200	6	0.08
Female 2	1370	1255	9	0.04
Female 3	850	779	6	0.03

4 Conclusions

A new procedure for the derivatization of 2-hydroxyglutaric acid is described. The method is based on the preparation of dialkyl esters by reaction with the corresponding alkyl chloroformates. No racemization is observed under the conditions used. The derivatives are adequate for HRGC separation on chiral columns. A new chiral liquid phase (CH42) developed by Lee *et al.* [10], and a commercial cyclodextrin capillary achieve separations of both 2-hydroxyglutaric acid enantiomers with good resolution. Unequivocal identification and quantitative analysis are thus possible in a very short time. In the field of 2-hydroxyglutaric acidurias the present method represents an important advance in the laboratory diagnosis of these inborn metabolic diseases. It is rapid, exempt of side products, and allows unequivocal determination of absolute configuration of 2-hydroxyglutaric acids. A minor disadvantage is that the derivatization procedure must be performed very accurately. Chromatography must take place immediately after derivative formation.

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