Journal of Chromatography, 143 (1977) 117—123
Biomedical Applications

CHROMBIO. 020

DETERMINATION OF THE CONFIGURATIONS OF LACTIC AND GLYCERIC ACIDS FROM HUMAN SERUM AND URINE BY CAPILLARY GAS—LIQUID CHROMATOGRAPHY

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(Received May 20th, 1976)

SUMMARY

The separation of the enantiomers of lactic and glyceric acids can be achieved by capillary gas chromatography on SP-1000 using the corresponding O-acetylated menthyl esters. The structures of the derivatives were proved by proton magnetic resonance spectroscopy and mass spectrometry. The method has been used for the determination of the absolute configurations of lactic and glyceric acids isolated from serum and urine from different patients.

INTRODUCTION

Nowadays, inborn errors of metabolism are intensively studied and there is a growing interest in the determination of the absolute configurations of metabolic products in human blood and urine. Different optical isomers may originate from separate metabolic pathways and may reflect different enzyme defects. For example, excretion of *S*-glyceric acid is the result of *R*-glycerate dehydrogenase deficiency in hyperoxaluria type II [1]., while *R*-glyceric acid has been found in a patient with the clinical symptoms of non-ketotic hyperglycinaemia [2]. Other examples recently investigated are *R*-methylsuccinic acid [3] and *S*-3,4-dihydroxybutyric acid [4]. The occurrence of *R*- and *S*-β-aminoisobutyric acid in man is well known [5], but the origin of the *S*-enantiomer has not yet been explained.

In our hospital, we were confronted with a child suffering from glyceric aciduria and another with an unexplained form of lactic aciduria. These patients prompted us to develop a gas chromatographic (GC) method for the determination of the absolute configurations of simple organic acids of this type.

In the literature, the resolution of racemic mixtures by GC has been achieved in two ways: (1) separation of the enantiomers with the use of a chiral stationary phase and (2) conversion of the enantiomers into diastereomers by a chiral reagent and the use of a non-chiral phase. Recently, Gil-Av and Nurok [6] published a comprehensive review on this subject. Pollock and Jermany [7] have achieved the separation of the enantiomers of some O-acetylated 2-hydroxy acids by GC of the 2-butyl, 3-methyl-2-butyl and 3,3-dimethyl-2-butyl esters. *S*-Lactic acid has been applied as an esterifying agent by several investigators for the separation of chiral alcohols, including menthol [6].

In this paper, we describe the separation of the diastereomers of the O-acetylated menthyl esters of lactic and glyceric acids by capillary GC on SP-1000, together with some recent clinical applications.

EXPERIMENTAL

Chemicals

R(+)-Glyceraldehyde, *S*(−)-glyceraldehyde and *R,S*-sodium lactate were purchased from Fluka (Buchs, Switzerland), *R,S*-calcium glycerate and (−)-menthol [(1*R*, 3*R*, 4*S*)-*p*-menthan-3-ol] from Aldrich Europe (Beerse, Belgium) and *R*-lithium lactate and *S*-lactic acid from Sigma (St. Louis, Mo., U.S.A.). *R*- and *S*-barium glycerate were synthesized by oxidation with HgO of *R*(+)- and *S*(−)-glyceraldehyde, respectively [8]; the salts were obtained as waxy masses with $[\alpha]_D^{20}$ values of +6.1° (*c* 1.8; H_2O) and −4.3° (*c* 4.0; H_2O), respectively. The purities of both compounds were tested by GC of the pertrimethylsilyl (TMS) derivatives on 5% GESE-52 [9]; they had the same retention time as the TMS derivative of *R,S*-glyceric acid. Their identity was further proved by gas chromatography—mass spectrometry (GC—MS) of the TMS derivatives. Capillary GC of the O-acetylated menthyl esters showed that during the oxidation reaction some racemization had occurred (*R*-barium glycerate, 88% *R* and 12% *S*; *S*-barium glycerate, 83% *S* and 17% *R*) (*cf.*, ref. 8). In general, free acids were obtained by treatment of the corresponding salts with Dowex 50-X8 (H^+) in water.

Synthesis of O-acetylated menthyl esters of lactic and glyceric acids

(−)-Menthol (300 mg) was added to 1—5 mg of dry hydroxy acid and esterified at 110° for 2 h by bubbling dry HCl gas through the solution [10]. Subsequently, the excess of HCl and menthol was removed by a gentle stream of nitrogen at 110°. To avoid possible losses of the volatile menthyl ester of lactic acid, it is advisable not to remove menthol completely. The residue was acetylated in 1 ml of pyridine—acetic anhydride (1:1) at 100° for 30 min. Finally, the solvent was evaporated in the presence of absolute ethanol and the residue dissolved in chloroform.

Capillary gas—liquid chromatography

A Varian Aerograph 2740-30-01 gas chromatograph equipped with a flame-ionization detector and a glass capillary column (25 m × 0.3 mm I.D.) coated with SP-1000 (LKB, Stockholm, Sweden) as stationary phase was used. For direct on-column injection (0.1—0.2 μl) without a stream splitter, a Pasteur pipette (length 140 mm, I.D. 1 mm) was used as the inlet, connected to the column with shrinkable PTFE. As the outlet to the detector, a second pipette (length 110 mm, I.D. 0.5 mm) was used. The carrier gas (nitrogen) flow-rate was 1 ml/min and the make-up gas (nitrogen) flow-rate was 30 ml/min. The injection port temperature was 200° and the detector temperature 220°; the oven temperature was 150° for lactic acid and 200° for glyceric acid.

Proton magnetic resonance spectroscopy

The proton magnetic resonance (PMR) spectra of the menthyl esters of *R,S*-lactic acid and *R,S*-glyceric acid as well as those of (—)-menthol, *R,S*-lactic acid and *R,S*-glyceric acid were recorded at 60 MHz with a Varian EM-360 spectrometer in CD_3OD as solvent and tetramethylsilane as internal standard at room temperature.

Gas chromatography—mass spectrometry

The 75-eV mass spectra of the O-acetylated menthyl esters of *R,S*-lactic acid and *R,S*-glyceric acid were recorded on a Jeol JGC-20 KP/JMS-D100/W-JMA combination at an ion-source temperature of 150°, an accelerating voltage of 3 kV and an ionizing current of 300 μA. As the column material, 5% GESE-52 on Chromosorb W AW DMCS, 100—120 mesh (HP), was used. The oven temperature was dependent on the sample.

Isolation of lactic and glyceric acids from urine or serum

Lactic and glyceric acids were extracted from urine with ethyl acetate as follows [11]. A 5-ml volume of saturated NaCl solution was added to 5 ml of urine, the mixture was acidified with concentrated HCl to pH 1—2 and subsequently extracted twice with 20 ml of ethyl acetate. The total organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. Glyceric acid was isolated from serum as follows. A 1-ml volume of serum was deproteinized with 9 ml of ethanol and the supernatant was evaporated in vacuo. The residue, dissolved in 1 ml of water, was applied to a DEAE-Sephadex A-25 anion-exchange column (6 × 0.7 cm) equilibrated with 20 ml of 0.5 *M* pyridinium acetate buffer. After washing with 10 ml of water, the organic acids were eluted with 15 ml of 1.5 *M* pyridinium acetate buffer [12]. Finally, the eluate was lyophilized.

RESULTS

To confirm the derivatization procedure, PMR spectroscopy was carried out after menthylation of the free acids and GC—MS after O-acetylation of the various esters. The following PMR data were obtained:

(a) Menthyl ester of *R,S*-lactic acid:

δ = 1.36 ppm (4.6 Hz), doublet,	3 H	lactic acid unit
δ = 4.22 ppm (4.6 Hz), quartet,	1 H	lactic acid unit
δ = 0.6—2.2 ppm,	18 H	menthyl unit
δ = 4.7 ppm, multiplet,	1 H	menthyl unit

(b) Menthyl ester of *R,S*-glyceric acid:

δ = 3.76 ppm (4 Hz), doublet,	2 H	glyceric acid unit
δ = 4.20 ppm (4 Hz), triplet,	1 H	glyceric acid unit
δ = 0.6—2.2 ppm,	18 H	menthyl unit
δ = 4.7 ppm, multiplet,	1 H	menthyl unit

The mass spectra of the O-acetylated menthyl esters of *R,S*-lactic acid (M = 270) and *R,S*-glyceric acid (M = 328) are presented in Figs. 1 and 2, respectively. In both instances the original unnormalized spectra showed very small peaks at *m/e* [M + 1]. The most intense peaks in the spectra are related to the fragmentation pattern of the menthyl unit.

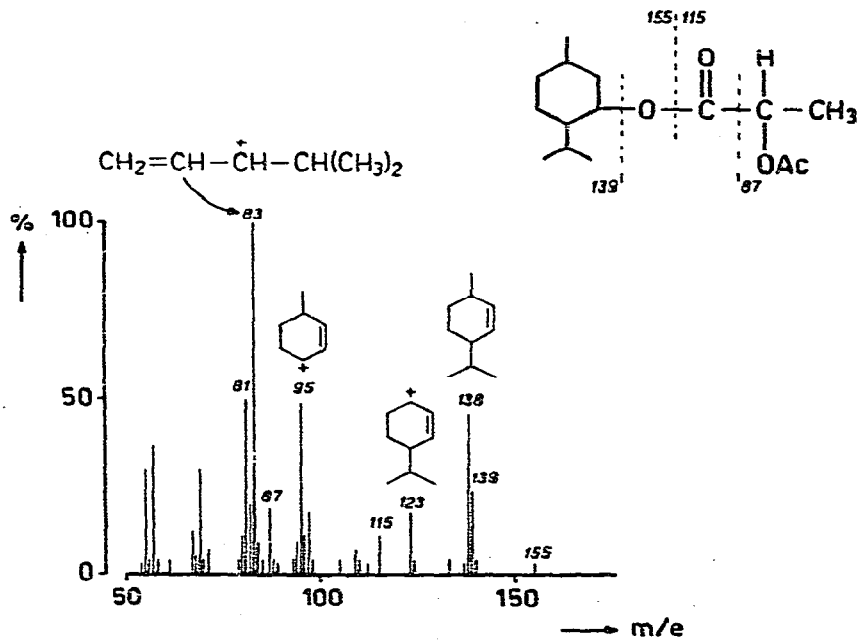

Fig. 1. Mass spectrum of the O-acetylated menthyl ester of *R,S*-lactic acid.

The gas chromatogram of the O-acetylated menthyl ester of *R,S*-lactic acid on SP-1000 is shown in Fig. 3a. The two peaks were identified by co-chromatography with the derivatives of the *R*- and *S*-enantiomers. Analysis of the commercially available *R*-lactic acid yielded 98.4% *R* and 1.6% *S*, while the *S*-enantiomer gave 99.4% *S* and 0.6% *R*. The origin of the contaminating optical antipodes is unknown; they might be intrinsic or be formed during the derivatization. For lactic acid, the resolution factor (the ratio of the retention times of configurational isomers) R_R/R_S was found to be 1.08.

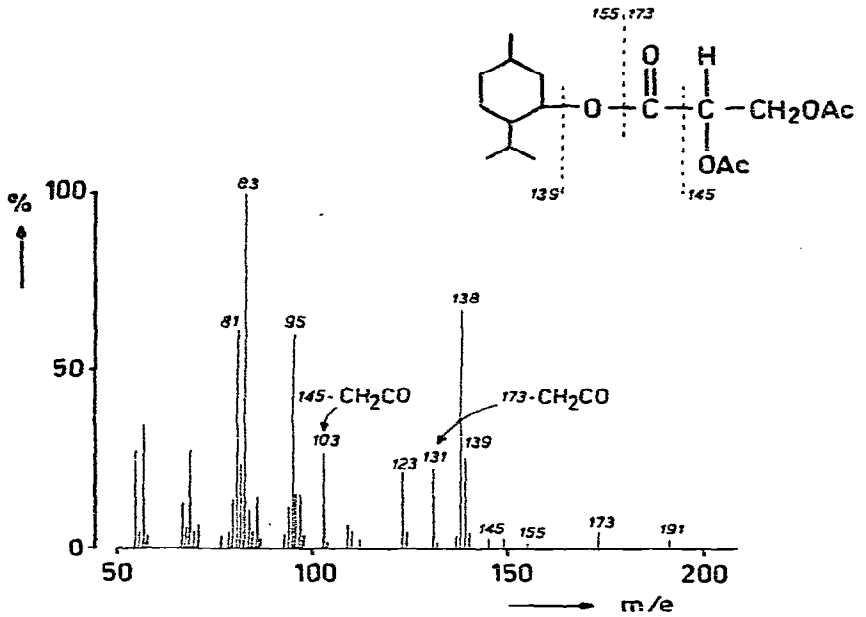

Fig. 2. Mass spectrum of the O-acetylated menthyl ester of *R,S*-glyceric acid.

The gas chromatogram of the O-acetylated menthyl ester of *R,S*-glyceric acid is shown in Fig. 3b. These peaks were identified in the same way as described for lactic acid. In this instance a resolution factor $R_R/R_S = 1.04$ was found.

The method has been applied to the determination of the absolute configurations of lactic and glyceric acids, present in large amounts in the urines and sera from some patients with unexplained metabolic disorders. Two applications are given below.

(1) A patient with lactic aciduria, but without concomitant hyperlactataemia

The lactic acid was isolated from urine by extraction with ethyl acetate. The extract was not further purified. After derivatization, only one main peak was found in the gas chromatogram, which had the same retention time as the derivative of *R*-lactic acid (Fig. 3c) (98.6% *R* and 1.4% *S*). Normally, only *S*-lactic acid occurs in human urine. *R*-Lactic acid was permanently present in this patient.

(2) A patient with glyceric acidaemia

The glyceric acid was isolated from urine by extraction with ethyl acetate and further purified by two-dimensional preparative paper chromatography. After derivatization, only one main peak was found in the gas chromatogram (Fig. 3d), having the same retention time as the authentic *R*-glyceric acid derivative (97.0% *R* and 3.0% *S*). The glyceric acid isolated from the serum was also the *R*-enantiomer (98.0% *R* and 2.0% *S*).

The clinical and biochemical aspects of both patients will be published elsewhere.

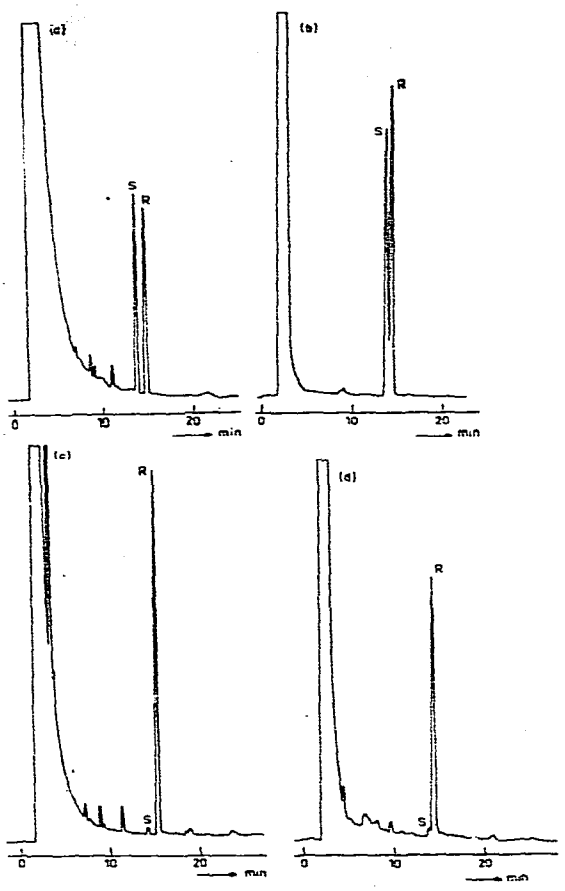

Fig. 3. Gas chromatograms of the O-acetylated menthyl esters of (a) synthetic *R,S*-lactic acid (150°), (b) synthetic *R,S*-glyceric acid (200°), (c) *R*-lactic acid isolated from urine (150°) and (d) *R*-glyceric acid isolated from urine (200°) on a glass capillary column (25 m × 0.3 mm I.D.) coated with SP-1000 as stationary phase (WCOT). The nitrogen flow-rate was 1 ml/min.

DISCUSSION

For the determination of the absolute configurations of lactic and glyceric acids, several methods have been applied in the literature. Firstly, it is possible to measure the optical rotation of the free acids or of suitable derivatives such as the salts or esters. The specific rotation values, $[\alpha]_D$, of the free acids are as low as 2—3° in water and are therefore not suitable for analytical purposes. The specific rotation values of the salts and alkyl esters are higher. Another disadvantage of the polarimetric method is the necessity to purify the isolated material highly. For these reasons, when only small amounts of material are available, the measurement of the optical rotation can give misleading results.

Secondly, in view of the high stereospecificity, the enzymes *R*- and *S*-lactate

dehydrogenase and *R*-glycerate dehydrogenase can be applied in order to establish the configurations of the acids. These methods can be used for small amounts of material. However, *R*- and *S*-lactate dehydrogenases do not only react with the natural substrate lactic acid, but also to some extent with other 2-hydroxy acids such as glyceric acid [13]. Ambiguous results may be obtained when mixtures of these acids are present in the materials to be analyzed. Therefore, it is necessary to remove any contaminating material which might also react with the enzymes. It should be noted that *R*-glycerate dehydrogenase does not react with *R*-lactic acid [14].

The technique presented here has been shown to be suitable for the configurational analysis of lactic and glyceric acids from physiological fluids, even when relatively small amounts of material are available. It is unnecessary to purify the starting material highly; simple isolation procedures are sufficient. Moreover, lactic and glyceric acids can be determined simultaneously.

ACKNOWLEDGEMENT

This investigation was supported in part with financial aid from Het Praeventiefonds.

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