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GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS AS THEIR DIASTEREOMERIC DERIVATIVES, ILLUSTRATED WITH 2-METHYLBUTYRIC ACID

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SUMMARY

A gas–liquid chromatographic method has been developed to separate on a small scale (acid samples as small as 2 mg) the enantiomers of 2-methylbutyric acid and to estimate a few per cent of one isomer as a contaminant in the other. Both the α-methylbenzyl ester and the α-methylbenzyl amide give a satisfactory resolution of the enantiomers. Chromatographic conditions in the order of importance are the final column temperature, the rate of temperature programming and the initial column temperature.

INTRODUCTION

2-Methylbutyric acid is one of the common metabolic intermediates occurring in living organisms and its natural enantiomer is the (*S*)-(+)-isomer[1]. In the course of a study on the metabolism of branched-chain fatty acids undertaken in this laboratory, it has become necessary to study the unnatural isomer, (*R*)-(−)-2-methylbutyric acid. Therefore, the accurate estimation of both isomers in a mixture has become essential. A polarimetric method can estimate the contamination of one isomer in the other to an extent of 10% or more, but lower amounts are difficult to determine accurately. There have been a number of reports of the enantiomeric separation of chiral compounds by gas–liquid chromatography (GLC)[2]. Thus this paper describes a GLC method that permits the accurate estimation of individual enantiomers of 2-methylbutyric acid and provides a general strategy applicable to other enantiomers.

EXPERIMENTAL

Chemicals

(*R,S*)- and (*S*)-(+)-2-methylbutyric acids, (*R,S*)-2-methylbutyryl chloride, (*S*)-(+)-butan-2-ol, (*R*)-(−)-octan-2-ol and (*R*)-(+)-α-methylbenzyl alcohol were obtained from Aldrich (Milwaukee, WI, U.S.A.). (*R*)-(+)-α-Methylbenzylamine was supplied by Fluka (Hauppauge, NY, U.S.A.). 1,3-Dicyclohexylcarbodiimide from

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Eastman-Kodak (Rochester, NY, U.S.A.) was purified by distillation under vacuum. Other chemicals used were the best grade commercially available.

Derivatization

Derivatives used for this study are shown in Fig. 1.

(*R,S*)-2-Methylbutyryl chloride was used to prepare the respective esters or amide from (*S*)-butan-2-ol, (*R*)-octan-2-ol, (*R*)-α-methylbenzyl alcohol or (*R*)-α-methylbenzylamine in the presence of pyridine[3]. A typical preparation is described below. (*R*)-Octan-2-ol (44 μl) was added to (*R,S*)-2-methylbutyryl chloride (30 μl) in pyridine (44 μl). To this mixture, benzene (60 μl) was added and vortexed. After 30 min at room temperature, *n*-hexane (500 μl) was added and vortexed. The hexane layer was removed. This extraction procedure was repeated two more times. The three hexane extracts were combined and used for GLC.

1,3-Dicyclohexylcarbodiimide was used to prepare an amide from (*S*)-2-methylbutyric acid and (*R*)-α-methylbenzylamine[4]. (*S*)-2-Methylbutyric acid (1 mmol, 101.1 mg) in 1 ml of tetrahydrofuran was added to a test-tube containing the equiv-

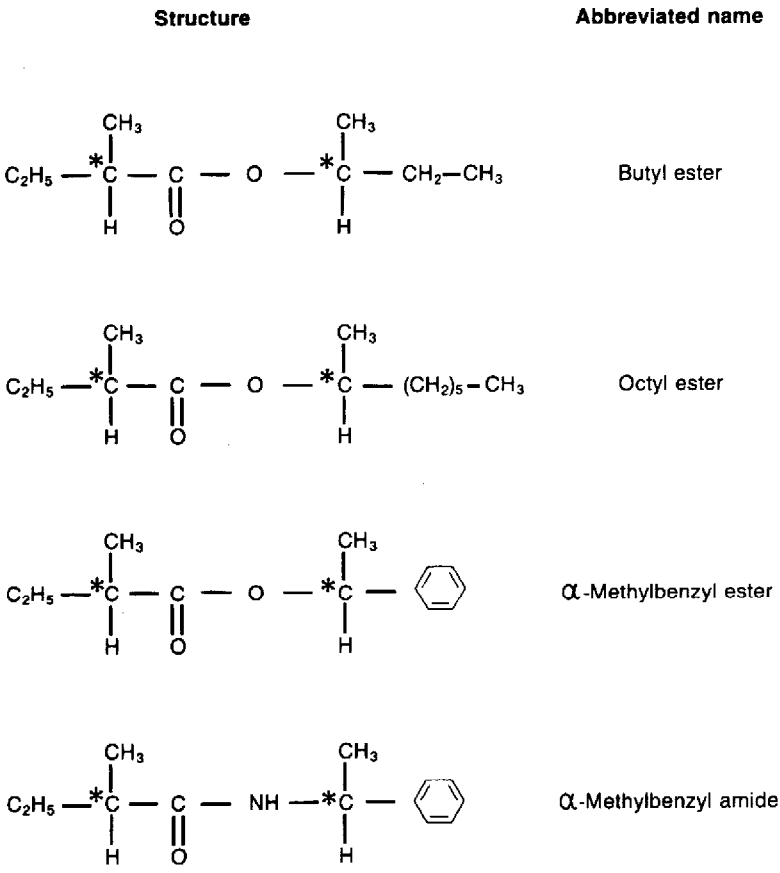

Fig. 1. Chemical structures of enantiomeric derivatives of 2-methylbutyric acid. Asymmetric carbon atoms are indicated by asterisks.

alent amount of dicyclohexylcarbodiimide (206.3 mg) and 2 ml of tetrahydrofuran in an ice-bath and mixed. (*R*)-α-Methylbenzylamine (1 mmol, 121.2 mg) was added to the chilled mixture and left for 24 h at room temperature. Ethyl acetate (3 ml) was added to the mixture and the solution filtered. The solvent was evaporated and the white crystals were dissolved in 3 ml of ethyl acetate and used for GLC. Similarly, an ester was prepared from (*S*)-2-methylbutyric acid and (*R*)-α-methylbenzyl alcohol.

All derivatives were identified by use of a Finnegan-Mat 4500 quadrupole gas chromatograph–mass spectrometer. Electron-impact spectra were used routinely. Chemical ionization spectra were used only for the determination of molecular weights.

Gas–liquid chromatography

A Hewlett-Packard Model 5830A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Avondale, PA, U.S.A.) was used throughout the study. Two fused-silica capillary columns (30 m long) were used: DX-1 (dimethylpolysiloxane–polyethylene glycol, 90:10) and DB-225 (cyanopropylmethyl–50% methylphenylpolysiloxane, 50:50) purchased from J. & W. Scientific (Rancho Cordova, CA, U.S.A.).

Standard conditions were as follows: initial column temperature, 65°C; rate of temperature programming, 38°/min; analysis completed at 245°C.

RESULTS AND DISCUSSION

Experimental strategy

Commercial capillary columns routinely available in the laboratory were chosen for this study rather than uncommon and expensive chiral columns. The investigation proceeded by the following sequence:

(a) Screen chiral reagents for the diastereomeric separation of 2-methylbutyric acid

(b) Select a suitable column to achieve good resolution of diastereomeric isomers

(c) Maximize the resolution by varying the chromatographic conditions

Diastereomeric derivatives of 2-methylbutyric acid

Esters are most commonly used in the diastereomeric separation of enantiomeric acids[2]. Three chiral alcohol reagents, (*S*)-butan-2-ol, (*R*)-octan-2-ol and (*R*)-α-methylbenzyl alcohol, were used for this purpose. The equivalent chain lengths[5] of the butan-2-ol ester and the octan-2-ol ester were 6.15 and 9.68 respectively on the DB-225 column and 6.62 and 10.34 respectively on a DX-1 column. No diastereomeric separation of either ester was observed. The α-methylbenzyl ester, however, was separated into two overlapping peaks, giving equivalent chain lengths of 11.60 and 11.65, and a resolution of 0.60 on the DB-225 column. This result indicates that a phenyl group, but not an alkyl group, attached to the asymmetric carbon of the chiral reagent contributes to the resolution of diastereomeric esters of 2-methylbutyric acid.

The possibility that this rule for esters may be applicable to amides as well was examined by the use of α-methylbenzylamine. The amide of (*R*)-α-methylbenzylamine

with 2-methylbutyric acid was resolved into two peaks giving equivalent chain lengths of 18.05 and 18.10, and a resolution of 0.77 on a DB-225 column. The chiral alkylamines, (*S*)-butan-2-amine and (*R*)-octan-2-amine, are commercially unavailable so that the diastereomeric separation of 2-methylbutyric acid with these amines was not examined. However, judging from the results with esters, the separation is unlikely to occur.

The diastereomeric separation of 2-methylbutyric acid was achieved as either its ester of (*R*)-α-methylbenzyl alcohol or its amide of (*R*)-α-methylbenzylamine. Because the latter is much less expensive than the former, it was used for further study.

An analytical procedure for the derivatization of 2-methylbutyric acid with (*R*)-α-methylbenzylamine on a small scale was investigated. The use of 1 mmol (101.1 mg) of acid as described in the Experimental section could be scaled down to 0.02 mmol (2 mg) of acid and one-two thousandth of the derivatized sample was sufficient for GLC analysis. The derivatization reaction on this scale took 2 h to initiate, judging by the formation of the insoluble urea by-product, whereas with $\geqslant$ 0.05 mmol of acid the reaction started immediately. The reaction mixtures were left for 24 h at room temperature.

Selection of columns

In general, the separation of two chemically similar compounds is achieved best on a column whose polarity is similar to that of the compounds: for example, alkanes on a methylsilicone column, methyl esters on a polyester column and alcohols on a polyglycol column. The diastereomeric amides of 2-methylbutyric acid with (*R*)-α-methylbenzylamine gave equivalent chain lengths of 13.67 and 13.73, and a resolution of 0.76 on the DX-1 column. Thus the resolution of the diastereomeric amides on the DB-225 column was no different from that on the DX-1 column. Similarly, the difference in equivalent chain length of the diastereomeric amides on the two columns was identical (0.05). Either column could be used for this separation, but because of the general rule mentioned above, the DB-225 column was chosen for further study.

Optimization of chromatographic conditions

Initially the retention temperature for the two diastereomeric α-methylbenzyl amides were determined under standard conditions as 240.3 and 240.7°C. The effect of the final temperature on the resolution of the diastereomeric amides was then studied by lowering it from 240 to 180°C, while keeping the initial temperature at 145°C and the rate of temperature programming at 3°C/min. The resolution was greatly improved as follows: 0.38 at 240°C, 0.58 at 220°C, 0.77 at 200°C, 0.86 at 190°C and 0.98 at 180°C (Fig. 2). The initial column temperature effects the band width of sample formed at the injection end of the column. The higher the temperature, the broader is the band width, resulting in a broader peak at the column end. Lowering the intial temperature from 175 to 135°C, while keeping the final temperature at 180°C, resulted in no appreciable increase in resolution as follows: 0.94 at 175°C, 0.92 at 165°C, 0.94 at 155°C, 0.96 at 145°C and 0.99 at 135°C. Lowering the rate of temperature programming from the standard of 3 to 0.5°C/min, while keeping the intial and final temperatures at 145 and 180°C, respectively, moderately increased the resolution as follows: 0.94 at 3°C/min, 1.02 at 2°C/min, 1.03 at 1°C/min and 1.07

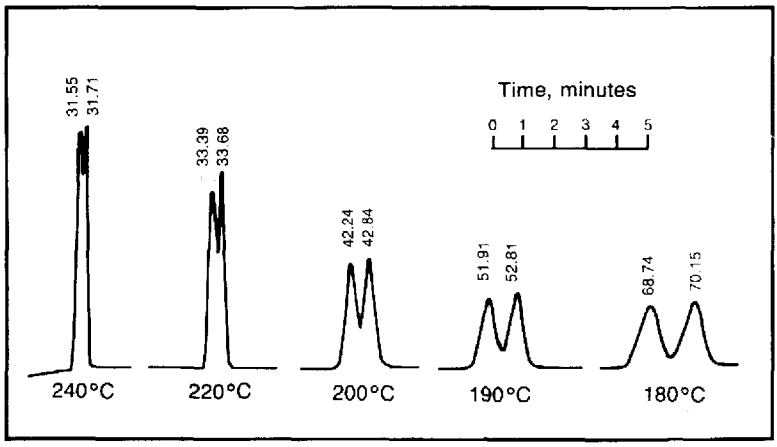

Fig. 2. Effect of final column temperature on the resolution of (*R*)-2-methylbenzyl amides of 2-methylbutyric acid. Retention times and final temperatures are shown at the top and the bottom of individual peaks, respectively.

at 0.5°C/min (Fig. 3). The resolution at ∞°C/min, which is equivalent to isothermal at 180°C, was 0.94.

When considering the resolution, the time required to complete the analysis and the cost of chiral reagents, the enantiomeric separation of 2-methylbutyric acid may best be achieved as its (*R*)-α-methylbenzyl amide on the DB-225 column initially at 145°C, increasing the temperature at a rate of 1°C/min to 180°C and maintaining the latter until completion of the analysis, which takes approximately 80 min.

The two peak components of the 2-methylbenzyl amide (Figs. 2 and 3) were identified by comparing their retention times with that of the authentic sample. The second peak gave a retention time identical to that of the amide prepared from (*S*)-2-methylbutyric acid and (*R*)-α-methylbenzylamine. Thus they have identical structures. Hence the first peak is due to the amide of (*R*)-2-methylbutyric acid and (*R*)-α-methylbenzylamine.

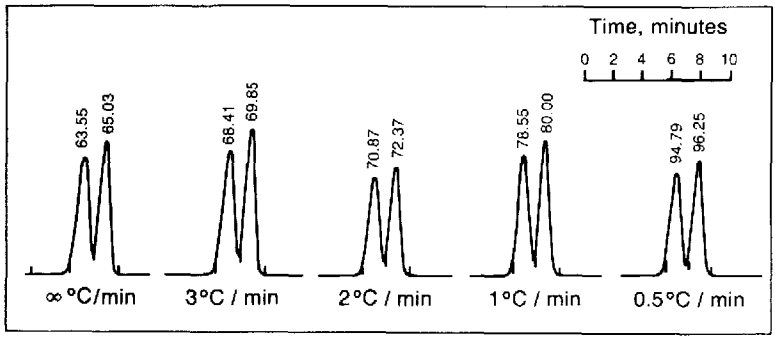

Fig. 3. Effect of rate of temperature programming on the resolution of (*R*)-α-methylbenzyl amides of 2-methylbutyric acid. Retention times and rates of temperature programming are shown at the top and the bottom of individual peaks, respectively.

The first peak, as seen in Figs. 2 and 3, was slightly broader than the second peak on the DB-225 column (a polar column), whereas the two peaks on the DX-1 column (a non-polar column) were very similar (not shown here). This indicates that the (*R*)-(*R*)-α-methylbenzyl amide is slightly more polar than the (*S*)-(*R*)-α-methylbenzyl amide. The two peaks for the α-methylbenzyl esters, however, showed identical shapes on both columns. Thus the polarity of the two diastereomeric esters is very similar. Table I summarizes these observations. The two theoretical plate numbers for the DB-225 column as measured with the amides are different, whereas those with the esters are very similar.

The final temperature was also important for the resolution of diastereomeric α-methylbenzyl esters. The retention temperatures of the esters on the DB-225 column were 192.4 and 193.1°C, respectively. These temperatures were used to estimate a range of final temperatures to be studied. Lowering the final temperature from 165 to 140°C, while keeping the initial temperature at 135°C and the rate of temperature programming at 3°C/min, greatly improved the resolution as follows: 0.60 at 165°C, 0.91 at 150°C and 1.01 at 140°C (Fig. 4).

A number of asymmetric compounds have been separated by GLC as their enantiomers under much less stringent conditions; even on a short packed column and in a short time[2]. In most cases, both the asymmetric compounds and the chiral reagents include a bulky group such as a phenyl group to enhance resolution. The ratio of the retention times of the two enantiomers is around 1.05 to 1.15. In the present case, only chiral reagents with a bulky group can be chosen. The ratios of the retention times of the two enantiomers were small, in the range of 1.006 to 1.023 (Figs. 2–4, Table I). Thus very stringent GLC conditions are required to achieve the near-baseline separation.

The difficulty of resolving enantiomeric isomers may be realized by looking at the retention characteristics expressed in terms of the equivalent chain length. In this laboratory, two peak components differing by 0.10–0.13 in their equivalent chain lengths, such as iso and anteiso fatty acids methyl esters with the same number of carbon atoms, have been routinely resolved on a long packed column or a support-coated open-tubular column with $\geqslant 12\,000$ theoretical plates[6]. Here the difference in

TABLE I

THEORETICAL PLATE NUMBER AND RESOLUTION OF THE AMIDE AND ESTER OF 2-METHYLBUTYRIC ACID

*Sample**	*Peak*	*Retention time (min)***	*Resolution*	*Theoretical plate number*
Amide	1	63.55	0.94	22 000
	2	65.03		32 700
Ester	1	29.43	0.90	38 800
	2	29.97		40 200

* The amide sample used contained (*R*)-2-methylbutyryl-(*R*)-α-methylbenzyl amide and (*S*)-2-methylbutyryl-(*R*)-α-methylbenzyl amide, and they were eluted in this order. The ester sample contained (*R*)-2-methylbutyryl-(*R*)-α-methylbenzyl ester and (*S*)-2-methylbutyryl-(*R*)-α-methylbenzyl ester, and they were eluted in this order.

** The DB-225 column was operated isothermally at 180°C for the amide and 150°C for the ester.

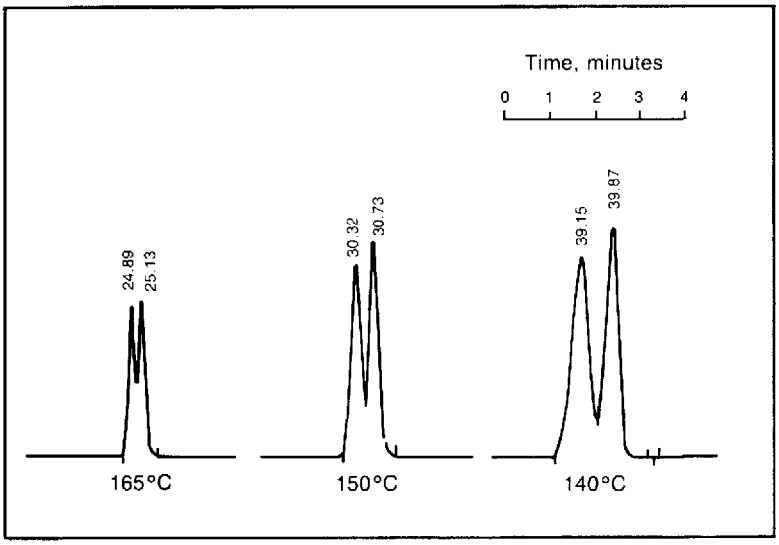

Fig. 4. Effect of final column temperature on the resolution of (*R*)-α-methylbenzyl esters of 2-methylbutyric acid. Retention times and final temperatures are shown at the top and the bottom of individual peaks, respectively.

equivalent chain length is only 0.05–0.06, requiring a much more efficient column under optimized conditions.

Application

A sample mixture of 2-methylbutyric acid isomers, prepared from 5 mg of the (*R*,*S*)-acid and 45 mg of the (*S*)-acid, was derivatized with (*R*)-α-methylbenzylamine. The analysis of the amide sample was carried out on the DB-225 column under optimum conditions: the column temperature was initially 145°C, then raised at a rate of 1°C/min to 180°C and kept at that temperature until completion of the analysis.

The average content of (*R*)-acid in three such samples with duplicated analyses was 5.9 ± 0.29% of the total acid. The expected value is 5.0%, suggesting that a slight overlap of the major (*S*)-acid peak contributes to this discrepancy and that a standard curve should be prepared to correct for it.

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