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Note

Separation of *cis* and *trans* isomers of hydroxycyclohexanecarboxylic acids by gas–liquid chomatography

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The gas–liquid chromatographic retention time of a given fatty acid is altered by the presence of substituents, the degree of change depending on the nature of the substituent and its position in the chain. This change has been extensively used for tentative identification of unknown fatty acids by gas–liquid chromatography[1].

During a study on microbial metabolism of cyclohexanecarboxylic acid in this laboratory, it became necessary to separate and estimate *cis* and *trans* isomers of 2-, 3-, and 4-hydroxycyclohexanecarboxylic acids. The method of characterization was similar to that used for fatty acids where a series of normal fatty acid methyl esters served as internal standards for measurement of the equivalent chain length[2]. Usually, the methyl esters of hydroxy acids gave adequate resolution, but where this was not the case, the trimethylsilyl derivative was used. This paper reports these results.

EXPERIMENTAL

Chemicals

Cis and *trans* isomers of 2-, 3-, and 4-hydroxycyclohexanecarboxylic acids were prepared from the corresponding benzoic acid derivatives[3–6]. In all cases, the purity of the products, determined by the methods described here, was more than 99%. Diazald was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Standard fatty acid methyl esters with eight to sixteen carbons were obtained from Applied Science Labs. (State College, Pa., U.S.A.). N-Trimethylsilylimidazole was from Pierce (Rockford, Ill., U.S.A.).

Gas–liquid chromatography

Two 6 ft. × 1/8 in. O.D. stainless-steel columns were used, one packed with 2.5% SE-30 on Chromosorb G (100–120 mesh) (Applied Science Labs.), and the other packed with 7% ethylene glycol adipate polymer on Chromosorb W (100–120 mesh) (Applied Science Labs.).

The gas–liquid chromatograph used was a Model GC-2000R with a dual hydrogen flame detector (Microtek Instruments, Baton Rouge, La., U.S.A.). The columns were operated isothermally at 120° and 130°, or under temperature-programmed conditions where the temperature was kept at 100° for 2 min, then

raised 4°/min to a final temperature of 200°. The retention time was measured by a Model 3370B GC digital integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

Samples of hydroxy acids were methylated with diazomethane[7]. In some cases, the esters were treated with N-trimethylsilylimidazole in N,N'-dimethylformamide (1 g/5 ml) at room temperature overnight. The treated samples were injected without further treatment. It was feared that the injected excess of N-trimethylsilylimidazole would react with active hydrogens of the ethylene glycol adipate polymer column, resulting in changing retention times. However, within limited use (40 injections) no such problem occurred.

Equivalent chain length was determined with normal C_8 to C_{16} fatty acid methyl esters (Applied Science Labs.) as internal standards. In the isothermal runs, this was obtained by interpolation of the logarithms of the net retention times of the compounds and of the standard methyl esters[8]. In the temperature-programmed runs, this was obtained by interpolation of the net retention times, rather than the logarithms[9].

Retention temperature was calculated from the retention times of the compounds based on temperature readings taken at about 120° and 190° in the linear portion of each run. Since the potentiometric thermometer was readable only to about ±1°, the absolute values are less accurate than the differences used in calculating the equivalent chain length. The high degree of linearity of the program is indicated by the fact that the retention time intervals between successive even-numbered normal fatty acid methyl esters were 4.15, 4.20 and 4.12 min.

RESULTS AND DISCUSSION

Separation of isomers of hydroxy acids was very poor on the SE-30 column. With neither 3-hydroxy acid nor 4-hydroxy acid were the pairs of *cis–trans* isomers separated. The retention temperatures were 126° and 127°, and the equivalent chain lengths 9.50 and 9.55 for the 3-hydroxy and the 4-hydroxy acids, respectively. A pair of *cis–trans* isomers of 2-hydroxy acid were separated giving 118° and 119° for their retention temperatures and 8.68 and 8.76 for equivalent chain length, respectively. Because of this poor performance, further study using the SE-30 column was not carried out.

The chromatograms shown in Fig. 1 were produced under temperature-programmed conditions using the polymer column. The *cis–trans* isomers of the 2-hydroxy acid were well separated. The *cis–trans* isomers of the 3-hydroxy acid were nearly completely resolved. The *cis–trans* isomers of the 4-hydroxy acid, however, were not separated at all. They both positioned at the same place as the *trans*-3-hydroxy acid peak (the right-upper portion of Fig. 1).

The equivalent chain lengths of three pairs of the isomers of the hydroxy acids given in Table I show a more quantitative aspect of the resolution. Among the *cis* isomers, the equivalent chain length of 2-hydroxy acid was significantly smaller than those of 3- and 4-hydroxy acids. This was also true in the case of the *trans* isomers. This is expected since the close association of an alcoholic and an acidic group in 2-hydroxy acid allows for much hydrogen bonding, hence a much more compact, less ionic molecule resulting in a smaller equivalent chain length.

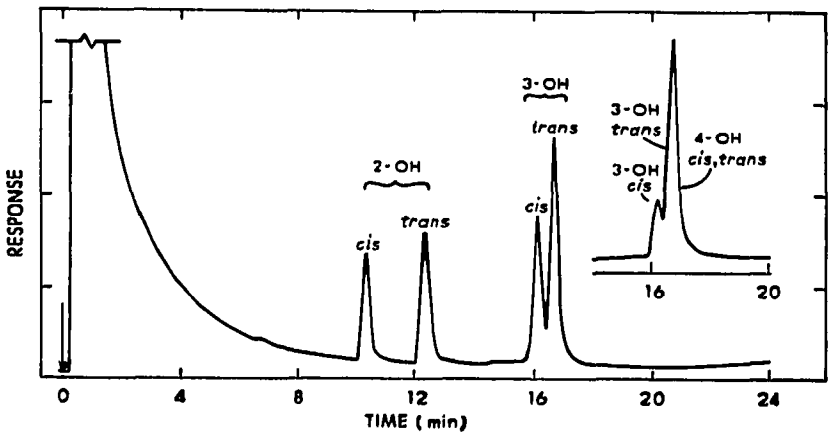

Fig. 1. Gas–liquid chromatography of *cis–trans* isomers of hydroxycyclohexanecarboxylic acids on the ethylene glycol adipate polymer column. The column temperature was kept at 100° for 2 min, then programmed at a rate of 4°/min to a final temperature of 200°.

TABLE I

EQUIVALENT CHAIN LENGTH AND RETENTION TEMPERATURE OF HYDROXYCYCLOHEXANECARBOXYLIC ACID METHYL ESTERS MEASURED UNDER TEMPERATURE-PROGRAMMED CONDITIONS

Methyl ester of hydroxy acid	*Equivalent chain length*			*Retention temperature (°C)*	
	Cis	*Trans*	*Difference (trans − cis)*	*Cis*	*Trans*
2-Hydroxy	12.02	12.98	0.96	131	142
3-Hydroxy	14.87	15.13	0.21	158	160
4-Hydroxy	14.98	14.98	0.00	159	159

The *cis* and the *trans* isomers of both 2- and 3-hydroxy acids were well separated. As indicated by the differences shown in the third column, the separation was better in the case of the isomers of 2-hydroxy acid than in the case of the isomers of 3-hydroxy acid. Isomers of 4-hydroxy acid were not separated at all.

Table II shows equivalent chain lengths of six isomers measured isothermally at 120° and 130°. Harris and Habgood[9] showed how the temperature-programmed equivalent chain length could be related to the isothermal values over the temperature range of the program; as a rough guide they suggested that the programmed value should be identical to the isothermal value for a temperature 30° to 50° below the retention temperature. The limited temperature range examined here does not justify a detailed comparison but on the approximate basis the programmed values of Table I are a little higher (perhaps one or two tenths of a unit) than would be expected from the isothermal values; the *cis–trans* differences, however, are in good agreement.

TABLE II

EQUIVALENT CHAIN LENGTH OF HYDROXYCYCLOHEXANECARBOXYLIC ACID METHYL ESTERS MEASURED AT 120° AND 130°

Methyl ester of hydroxy acid	*Column temperature (°C)*	*Equivalent chain length*					
		Before silylation			*After silylation*		
		Cis	*Trans*	*Difference (trans − cis)*	*Cis*	*Trans*	*Difference (trans − cis)*
2-Hydroxy	120	12.00	12.96	0.96	9.93	9.96	0.03
	130	12.12	13.03	0.91			
3-Hydroxy	120	14.69	14.96	0.27	10.20	11.13	0.93
	130	14.66	14.93	0.27			
4-Hydroxy	120	14.87	14.87	0.00	10.84	11.07	0.23
	130	14.92	14.92	0.00	10.78	11.04	0.26

It was thought that the introduction of a bulky group into the hydroxyl group of monohydroxycyclohexanecarboxylic acids would aid the separation of their *cis–trans* isomers, particularly in the case of the 4-hydroxy acid where the isomers were not resolved. Good separation was, indeed, obtained after trimethylsilylation, as shown in the right half of Table II. Trimethylsilylation of the 4-hydroxy isomers reduced the equivalent chain lengths about 4 units, as compared with those of the original esters, but the difference between equivalent chain lengths of the isomers is now more than 0.2, resulting in complete separation. Similarly, the separation of the 3-hydroxy isomers was greatly increased. In the case of the 2-hydroxy isomers, however, the trend is reversed; the isomers were not separated at all after trimethylsilylation. A possible explanation is that the trimethylsilyl group is too large to fit into the *cis* conformation of 2-hydroxy acid (equatorial–axial). Thus the *cis* isomer is converted during silylation to the *trans* isomer (equatorial–equatorial or axial–axial).

Trimethylsilylation is extensively used to increase the volatility of such compounds as amino acids and carbohydrates so that they can be analyzed by gas–liquid chromatography. The present work shows that in some cases, at least, it can also be used to improve the separation of difficultly resolvable isomers.

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