Journal of Chromatography, 287 (1984) 202–208
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,446

Note

High-performance liquid chromatography analyses of isomeric monoenoic and acetylenic fatty acids

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(Received November 23rd, 1983)

We recently described a method for the rapid preparation of phenacyl derivatives of fatty acids and the analyses of natural and standard mixtures by high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column at nanogram sensitivity[1]. Although several kinds of derivatives had been used earlier to permit the HPLC analysis of fatty acids using the more sensitive ultraviolet (UV) detector, our report appeared to be the first to demonstrate the quantitative analysis of fatty acids by HPLC. The present report is an extension of our earlier work with the phenacyl derivatives. The data show that a large number of isomeric octadecenoates and octadecynoates can be resolved by HPLC. A preliminary report of this work has appeared[2].

MATERIALS AND METHODS

Isomeric octadecynoates

The isomeric octadecynoates (Δ2, 3, 4, 5, 6, 7, 8, 10, 12, 13 and 14) were synthesized in this laboratory using a number of established procedures[3–7]. Generally, the lithium or sodium acetylide of a 1-alkyne was condensed with the appropriate omega bromoacid. Because long-chain omega bromocids were not available, the Δ12, Δ13, and Δ14 octadecynoates were synthesized in a three-step route: alpha-omega dihaloalkanes were condensed with the appropriate lithium acetylide, cyanide was added to form the nitrile, followed by hydrolysis to yield the acid. The Δ2, Δ3, and Δ4 octadecynoates were prepared by the addition of carbon dioxide, ethylene oxide and propylene oxide, respectively, to the Grignard's of the appropriate 1-alkynes. The alcohols of the latter two were oxidized to the acid. Because high-purity (more than 99%) 1-alkynes (Farchan, Willoughby, OH, U.S.A.), omega bromoacids (Aldrich, Milwaukee, WI, U.S.A.) and alpha-omega dihaloalkanes (Sapon, Bloomsbury, NJ, U.S.A.) were used, purification was simplified. Because only 1-alkynes were reactive, positional isomers were not produced and the presence of only one chain length in the other reagents assured a high percentage of the desired octadecynoate isomer. The minimal amount of purification needed was accomplished by thin-layer chromatography (TLC), preparative gas–liquid chromatography (GLC), HPLC, distillation or a combination of two or more of these methods. Details of the synthesis and purification procedures will be published elsewhere.

0021-9673/84/$03.00

Isomeric octadecenoates

The geometrical and positional octadecenoates were prepared from the octadecynoates by selective partial hydrogenation. The *cis*-octadecenoates were prepared by using the Lindlar catalyst[8] whereas the *trans*-octadecenoates were prepared by using metallic lithium in liquid ammonium[9]. These selective reductions gave geometrical isomer purity of more than 95%, and double bond migration was generally less than 5% as determined by ozonolysis and GLC of the aldesters[10–12]. Oleic, elaidic, *cis*-vaccenic and *trans*-vaccenic acids of high purity were purchased from Nu Chek Prep, Elysian, MN, U.S.A. Analysis of isomeric octadecenoates and octadecynoates by capillary GLC on polar and non-polar columns before and after hydrogenation[13], by silver ion TLC[14], and by ozonalysis and GLC of the pyrolysis products[10–12] indicated all the isomers were more than 95% pure and most were more than 99%.

Other materials

α-Bromoacetophenone was purchased from Aldrich and purified by distillation as described earlier[1]. Other common organic reagents used were usually reagent grade or better and purified before use. HPLC-grade glass-distilled solvents were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). Water was purified as described previously[1].

Derivative preparation and HPLC analysis

The free fatty acids were derivatized by heating for 15 min in a sealed culture tube containing an acetone solution of α-bromoacetophenone and triethylamine as described earlier[1]. The reaction mixture was spotted on a TLC plate containing silica gel G, developed in hexane–diethyl ether (80:20), the phenacyl derivative band eluted from the absorbent and analyzed. This is an optional step not normally required for routine analysis.

Analysis were carried out using an IBM Model 9533 liquid chromatograph (IBM, Danbury, CT, U.S.A.) equipped with a variable-wavelength detector as described previously[1]. Analyses were made at 242 nm and the detector signals were quantitated by a Spectra-Physics Minigrator (Spectra-Physics, Santa Clara, CA, U.S.A.). All analyses were carried out on an IBM 250 × 4.5 mm I.D. column packed with 5-μm octadecyl-bonded spherical silica. Most analyses were made isocratically using acetonitrile–water (75:25) for octadecynoates and 85:15 for octadecenoates at 2 ml/min.

RESULTS AND DISCUSSION

The retention times of several isomeric octadecynoates phenacyl derivatives, relative to palmitate, are given in the third column of Table I. A typical chromatogram depicting the resolution of several phenacyl octadecynoate isomers is shown in Fig. 1. Except the Δ2 isomer, all the isomeric acetylenic fatty acids had retention times shorter than palmitate. Both the retention times (Table I) and Fig. 1 indicate that as the triple bond is moved from the carbonyl group toward the center of the molecule, the solubility in the mobile phase increases resulting in earlier elution. As the triple bond then passes the Δ12 position progressing toward the terminal methyl group, the solubility of the isomers in the mobile phase decreases slightly. This results

TABLE I

RELATIVE RETENTION TIMES OF GEOMETRICAL AND POSITIONAL OCTADECENOATE ISOMERS AND POSITIONAL OCTADECYNOATE ISOMERS

Position of double or triple bond	*Relative retention times**		
	Octadecenoates		*Octadecynoates*
	Cis	*Trans*	
2	0.832	0.796	1.130
3	0.698	0.721	0.865
4	0.682	0.732	0.839
5	0.645	0.701	0.763
6	0.606	0.654	0.660
7	0.590	0.641	0.614
8	0.582	0.635	0.588
9	0.572	0.622	N.D.**
10	0.566	0.619	0.551
11	0.569	0.611	N.D.
12	0.563	0.609	0.541
13	0.568	0.618	0.545
14	0.579	0.619	0.552

* The retention times of the octadecenoates were relative to stearate with a retention time of 41.7 min, whereas the retention times of the octadecynoates were relative to palmitate with a retention time of 37.8 min. The monoenic and acetylenic isomers were analyzed under identical conditions except for the proportions of solvents: octadecynoates and octadecenoates were analyzed with acetonitrile–water (80:20) and (85:15), respectively.

** N.D. = Not determined.

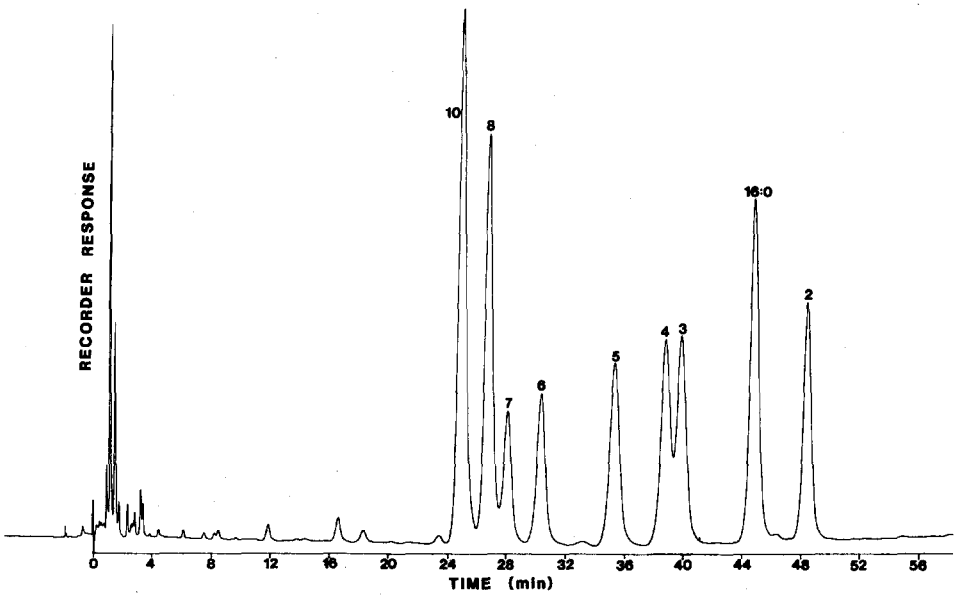

Fig. 1. A chromatogram showing the separation of isomeric octadecynoic acids as phenacyl derivatives by HPLC. Analysis was made on a 250 × 4.5 mm I.D. octadecyl column with an isocratic solvent flow-rate (2.0 ml/min) of acetonitrile–water (75:25). Except for palmitate (16:0), the numbered peaks represent the position of the triple bond, relative to the carboxyl group, in the acyl hydrocarbon chain.

in the incomplete resolution of the octadecynoate isomers with the triple bond located between the $\Delta 10$ and $\Delta 14$ positions.

The retention times of several geometrical and positional octadecenoate phenacyl derivatives, relative to stearate, are given in Table I. Chromatograms depicting the resolution of several positional isomers of the *cis*- and *trans*-octadecenoate phenacyl derivatives are shown in Figs. 2 and 3, respectively. The *trans* isomers were generally eluted after the corresponding *cis* isomers. The exception is the $\Delta 2$ isomer where the *trans* isomer eluted ahead of the *cis* isomer. This reversed order of elution is illustrated in Fig. 2 where the *trans* $\Delta 2$ and $\Delta 3$ isomers were included in the mixture of the *cis* isomers. The order of elution of the positional isomers in *cis* and *trans* series followed much the same order of the isomeric octadecynoates. As the double bond moved from near the carboxyl toward the terminal methyl group, the solubility in the mobile phase increased until the $\Delta 12$ position, and then decreased slightly. In apparently the only other case where isomeric *cis*- and *trans*-octadecenoates have been examined by HPLC, Svensson *et al.*[15] reported that isomers with the double

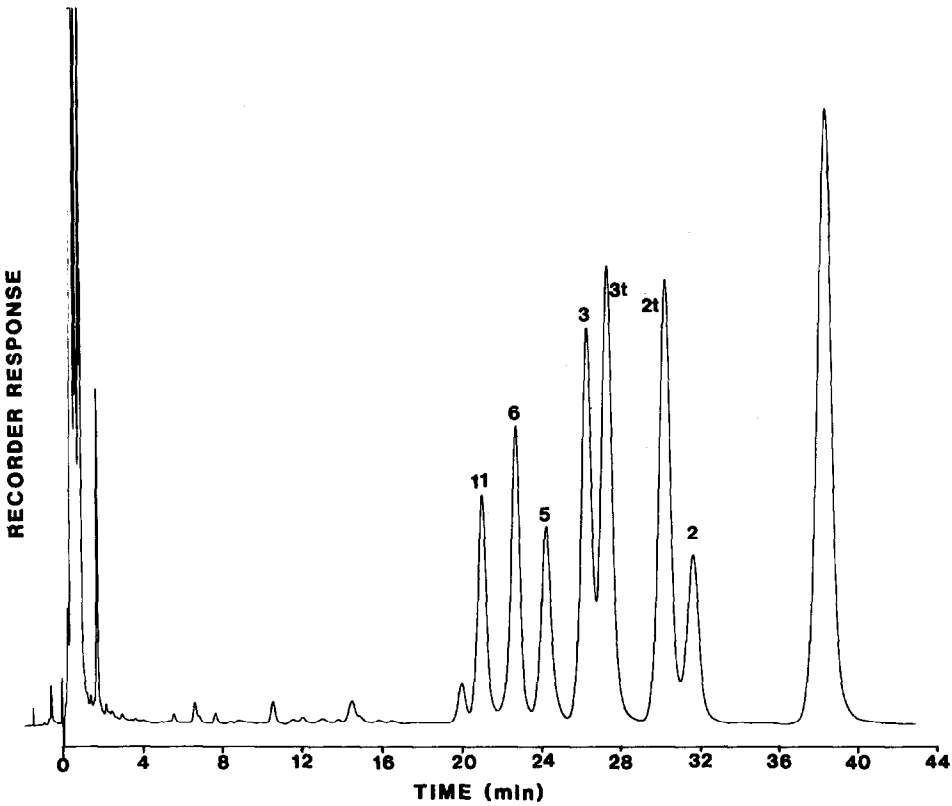

Fig. 2. A typical chromatogram showing resolution of some isomeric *cis*-octadecenoates as phenacyl esters by HPLC. Analysis was made on 250 × 4.5 mm I.D. octadecyl column with an isocratic flow-rate (2.0 ml/min) of acetonitrile–water (85:15). Except for stearate (18:0), the numbered peaks represent the position of the double bond, relative to the carboxyl group in the acyl hydrocarbon chain. Two *trans* isomers (3t, 2t) were included to show their relation to the *cis* isomers and the reversal of the elution order of the *trans*-$\Delta 2$ isomer.

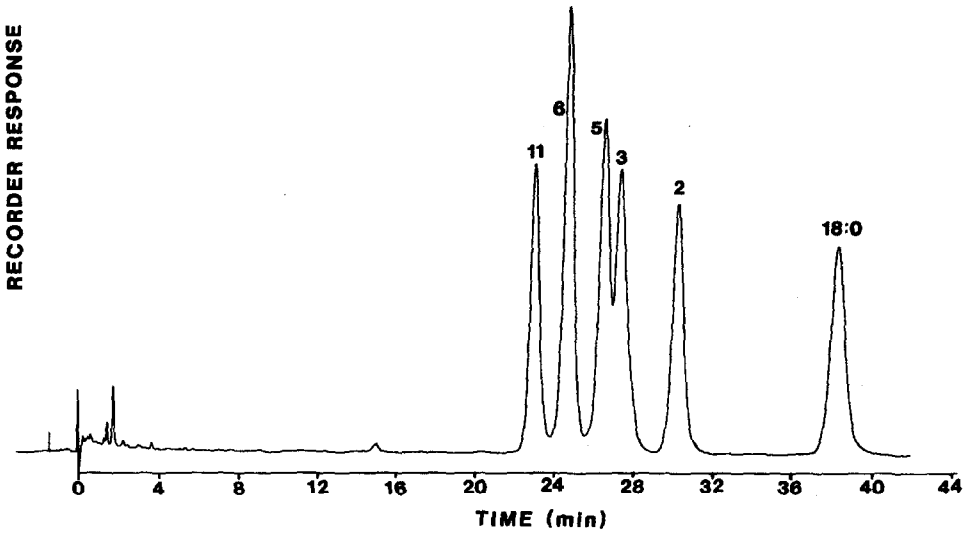

Fig. 3. A representative chromatogram showing the resolution of some isomeric *trans*-octadecenoates as phenacyl derivatives by HPLC. Analysis conditions were the same as given in Fig. 2. Numbered peaks indicate the position of the double bond, relative to the carbonyl carbon in the hydrocarbon chain. Stearate is labeled 18:0.

bond near the center of the methyl ester hydrocarbon chain exhibited the shorter retention time. They also noted that the *trans*-methyl esters had longer retention times than the corresponding *cis* isomers. These investigations did not examine the *cis* or *trans*-$\Delta 2$ isomers or the *trans*-$\Delta 3$ or -$\Delta 4$ isomers, therefore they were unable to observe the reversing of the elution order of the *cis*- and *trans*-$\Delta 2$ isomers observed in this study.

When the retention times of the positional *cis*- and *trans*-octadecenoate isomers and the octadecynoates isomers are plotted, relative to the position of the double or triple bond, some interesting observations were made (Fig. 4). At first it appeared that the $\Delta 3$ isomers were out of place, relative to the other isomers, which prompted us to repeat the synthesis, purification, and analysis. The data were nearly identical with the earlier data. After closer examination it became clear that the $\Delta 3$ isomers were behaving as expected, but the retention times of all the $\Delta 2$ (except *trans*), $\Delta 4$ and $\Delta 5$ isomers were being disturbed. When scale molecular models of the $\Delta 2$, $\Delta 3$, $\Delta 4$, and $\Delta 5$ isomers were assembled it became apparent that the unexpected behavior of the $\Delta 2$, $\Delta 4$ and $\Delta 5$ isomers resulted from the close proximity and interaction of the carbonyl oxygen with the pi bonds of the monoenoic and acetylenic linkages. The molecular models also showed that structures of the *trans*-Δ-3 and *cis*-$\Delta 3$-octadecenoates and the $\Delta 3$-octadecynoates do not permit the carbonyl oxygen and the pi bonds interaction. Actually, the behavior of the *cis*-$\Delta 2$ isomer does not involve the interaction of the pi bond with the carbonyl oxygen, but rather the interaction between the carbonyl oxygen and the hydrogen atoms on carbon number four which we have termed "non-classical" hydrogen bonding[13]. This hydrogen bonding provides for a resonance structure that reduces the polarity of the molecule causing it to spend more of its time associated with the C_{18} hydrocarbon chain bonded to the

silica, thus giving rise to the longest retention time of the monoenoic esters. One can evoke the argument that it is the conjugation of the carbon oxygen pi bond with $\Delta 2$-monoenoic pi bond that produces the longer than expected retention times, but this argument can be quickly squelched by the fact that the *trans*-$\Delta 2$-octadecenoate retention time is altered little if any (Fig. 4, Table I). Although the conjugation between the pi bonds of the carbonyl oxygen and the *cis*- or *trans*-$\Delta 2$ double bonds does not appear to have a significant effect on the retention times of the octadecenoates, the very long retention time of the $\Delta 2$-acetylenic ester (Table I and Fig. 4) may be attributable to conjugation. This suggests that the resonance hybrid between the carbonyl oxygen and the triple bond at the $\Delta 3$ position is much stronger than between a double bond. The altered retention times (longer) of the $\Delta 4$- and $\Delta 5$-monoene and acetylenes are more difficult to explain than the $\Delta 2$ isomers. The logical explanation appears to be that the interaction between the carbonyl oxygen and the double or triple bond reduces the polarity of the molecules. It is the nature of the interaction that is difficult to explain. One might expect the carbonyl oxygen to carry a partial negative charge, which would cause it to be attracted to an electrophilic center such as the acetylenic bond, but not the double bond which is consisted to be nucleophilic.

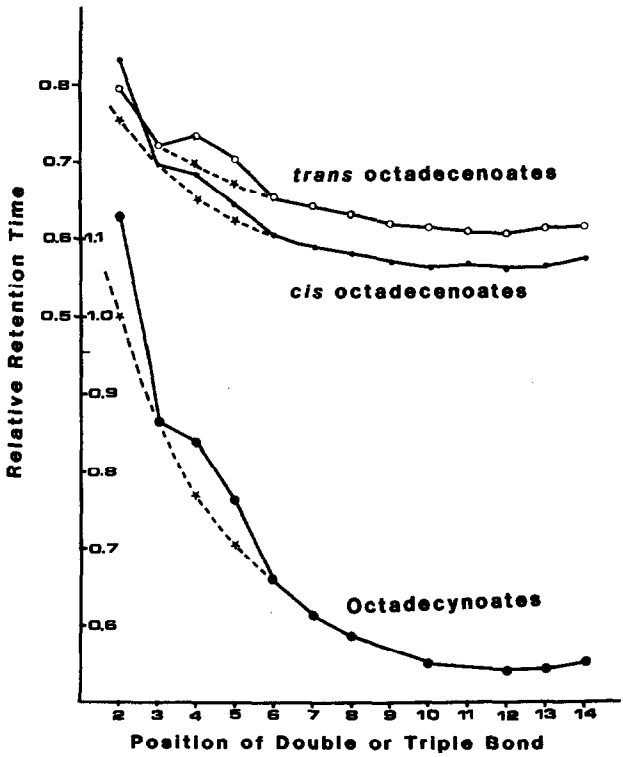

Fig. 4. A plot of the position of the triple bond in octadecynoates *versus* the retention times relative to palmitate. Also shown are plots of the position of double bond in the octadecenoates *versus* the retention times relative to the stearate. The dashed line represents an estimation of the retention times that should occur for the $\Delta 2$, $\Delta 4$ and $\Delta 5$ positional isomers when the retention times are corrected for interactions between the carbonyl oxygen and the unsaturation.

This appears somewhat inconsistent since the effect on the retention times was all in the same direction (longer) for both octadecenoates and octadecynoates, with the latter being retained more strongly (Fig. 4). Perhaps the interaction of the nucleophilic oxygen of the carbonyl group is with a nucleophilic site on the carbon atoms caused by a distortion of the pi-electron cloud.

If one follows the dashed lines through the 3-position to the 6-position in Fig. 4, a smooth curve is obtained in each case which should represent the effect of the position of double or triple bond on the retention times when the interactions between the carbonyl oxygen and the unsaturation are discounted. The longer retention times of the octadecenoates and octadecynoates with the double or triple bonds nearer the carbonyl group are predictable. When the double or triple bond is closer to the carbonyl group there is more hydrocarbon chain available to interact with the C_{18} hydrocarbon chains bound to the silica. When the double or triple bonds are near the center of the molecule there is less interaction and the molecules are carried along in the mobile solvent phase, and as a result short retention times are observed.

Although the proposed explanations may not elucidate fully the interactions between the positions of the unsaturation in the solutes and the bound stationary phase of the column, they do represent a starting point where hypotheses can be tested. A more complete understanding of the relations between solutes and the stationary phase could lead to the development of tailor-made columns for specific separations.

ACKNOWLEDGEMENTS

This work was supported by a research grant from Cotton, Inc. Thanks to Mr. David Porterfield for technical assistance and to Mrs. Robin Bronson for typing.

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