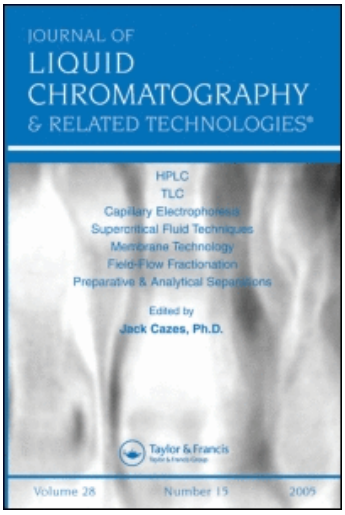

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RECYCLING HPLC OF p-BROMOPHENACYL ESTERS OF SATURATED C_{35-56} FATTY ACIDS FROM *MYCOBACTERIUM TUBERCULOSIS* H37Ra ON A SILICA COLUMN

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ABSTRACT

The p-bromophenacyl esters of saturated C_{35-56} fatty acids from *Mycobacterium tuberculosis* H37Ra were separated according to structural classes on a silica column by high performance liquid chromatography (HPLC). The sample was cycled five times during HPLC. Highly purified C_{35-38} esters were obtained by this method. Further HPLC fractionation on a reverse-phase column (C_{18}-bonded silica) gave complete separation of most of the remaining fractions. By combining mass spectrometry with HPLC separations, many of the fatty acid esters were tentatively identified.

INTRODUCTION

Mycobacterium tuberculosis H37Ra contains a complex mixture of C_{26-56} fatty acids (1). These acids have functional groups

such as double bonds, cyclopropane rings, and a methoxy group and might be precursors of mycolic acids (1-3). Mycolic acids are β-hydroxy fatty acids substituted at the α-position with a long aliphatic chain. These acids are a prominent part of the cell wall structure of the mycobacteria, nocardiae and corynebacteria (4,5). The structures of the α-mycolic acids from *M. tuberculosis* H37Ra are now known (6).

A C_{36} fatty acid from the H37Ra strain which contains two cyclopropane rings was structurally related to the α-mycolic acids (7). Other fatty acids might be similarly related. We wish to purify and determine the structures of these acids, but they are complex mixtures that have defied complete resolution. We have now developed a high performance liquid chromatography (HPLC) method to resolve the major C_{35-38} fatty acids as their p-bromophenacyl esters on a silica column. Complete separation of most of the other esters was achieved by combining the adsorption and reverse-phase modes of HPLC.

MATERIALS

Spectroquality distilled-in-glass ethylene dichloride, hexane, acetonitrile, p-dioxane, chloroform and methanol were purchased from Burdick and Jackson Laboratory. The p-bromophenacyl ester derivatization kit was purchased from Applied Sciences Laboratory, Inc.

METHODS

Growth of Bacteria

M. tuberculosis H37Ra was grown at 37°C in glycerol-alanine-salts medium in a New Brunswick 28-liter fermentor (1).

Isolation of the Long-Chain Saturated Fatty Acids

The general scheme used to isolate and purify the long-chain fatty acids from *M. tuberculosis* H37Ra was previously described

(8). Purified fatty acids (900 mg) were obtained from 511 g (harvested weight) of cells. This sample was derivatized to the p-bromophenacyl esters (9) and fractionated on a silicic acid-$AgNO_3$ column (Hi-Flosil-Ag, Applied Sciences Laboratory, Inc.) to yield the saturated esters. Sephadex LH-20 column chromatography of the fraction yielded the saturated C_{35-56} esters (60.1 mg).

HPLC was performed using an instrument containing the following components: two Waters Model 6000A solvent delivery systems, a Waters Model 660 solvent programmer, Waters Model U6K universal liquid chromatograph injector, and a Perkin-Elmer variable wavelength detector (Model LC-55). For the separation of the esters by the recycling mode, six 4 mm x 30 cm μPorasil columns (Waters Associates, Inc.) were placed in a series. The solvent system used was ethylene dichloride-hexane (35:65, v/v) at a flow rate of 1 ml/min. Separation of the esters was also performed in the reverse-phase mode using a 4 mm x 30 cm μBondapak C_{18} column (Waters Associates, Inc.). The solvent systems used were: p-dioxane-acetonitrile (3:7, v/v) for the C_{35-40} esters; p-dioxane-acetonitrile (1:1, v/v) for the C_{40-56} esters; and a linear gradient of 0-60% p-dioxane in acetonitrile over a period of 90 min. for the mixture of C_{35-56} esters. The flow rate was 1 ml/min.

Nuclear Magnetic Resonance and Mass Spectral Analysis

^1H Fourier transform nuclear magnetic resonance (NMR) spectra were determined with a Bruker Model HX-90E spectrometer at 90 MHz. Mass spectra were obtained on an AEI Model MS 902 mass spectrometer under the following conditions: ionization potential, 70 ev; source temperature, 150-170°C. Samples were introduced at the inlet with a probe. The size and the number of apparent double bonds were determined by mass spectrometry (MS).

RESULTS AND DISCUSSION

Analysis by NMR Spectroscopy and Reverse-Phase HPLC

NMR analysis of the p-bromophenacyl esters of saturated C_{35-56} fatty acids from *M. tuberculosis* H37Ra revealed chemical shifts characteristic of a cis-cyclopropane ring (0.60 and -0.30 ppm), a methoxy proton (3.36 ppm), and the p-bromophenacyl ester protons (7.85, 7.76, 7.67 and 7.57 ppm). This sample was devoid of the normal olefinic protons. Analysis of the sample on a μBondapak C_{18} column in the reverse-phase mode (Fig. 1) revealed a series of fatty acids from C_{35-56}. The major components were

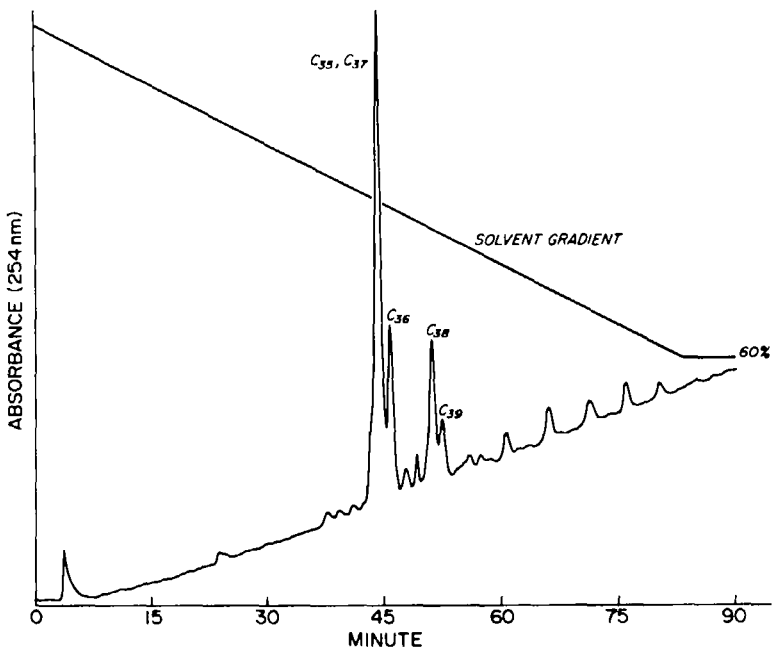


FIGURE 1. HPLC of saturated C_{35-56} fatty acid esters on a reverse-phase column. Column: 4 mm x 30 cm μBondapak C_{18}. Solvent: a linear gradient of 0-60% p-dioxane in acetonitrile was programmed over a period of 90 min.

C_{35-39} esters, but MS showed that each peak was a mixture of several esters.

HPLC on a Silica Column

When the saturated C_{35-56} esters (8.1 mg) were fractionated by recycling HPLC using six μPorasil columns in a series, purification of the C_{35-38} esters was achieved (Fig. 2). During the first cycle, peaks 1-4 were recycled and peaks 5-7 were collected. On the fourth cycle, peak 4 was shaved off on the back side of the peak. Peaks 1, 2a, 2b, 3a, 3b and 4 were recovered during the fifth cycle. Each of the recovered peaks (peaks 1-7) were analyzed by both MS (Table I) and analytical reverse-phase HPLC (Figs. 3-4). Structure I shows the diagnostic MS fragmentation of a p-bromophenacyl ester used to identify the recovered peaks. The MS peaks included the molecular ion M or in the methoxy-ester, M-32; M-79/81 (or M-32-79/81); M-213/215 (or M-32-213/215).

HPLC peak 1 was a complex mixture of the large esters (C_{43-49}). A C_{37} ester giving M(or M-32) values of m/e 740 and 742 also appeared in this fraction. We are presently unable to assign a structure for this ester. The major components were C_{43} and C_{45} esters (Structure I) and a C_{49} ester containing a methoxy group (Structure II). Baseline separation of most of the components in peak 1 were observed when the sample was chromatographed on the reverse-phase column. A separate analysis of the front slope of peak 1 (not illustrated in Fig. 2) showed it to contain the C_{50-56} esters.

HPLC peak 2a revealed a series of C_{41-44} esters (Structure I) with the C_{41} ester being the most prominent. Another series of C_{45-47} esters appeared and the C_{46} and C_{47} esters which contain a methoxy group were the major components (Structure III). Reverse-phase HPLC separated the two series and the C_{41}, C_{42}, C_{43}, and C_{44} components of one series, but separation of the C_{45}, C_{46}, and C_{47} in the second series was poor.

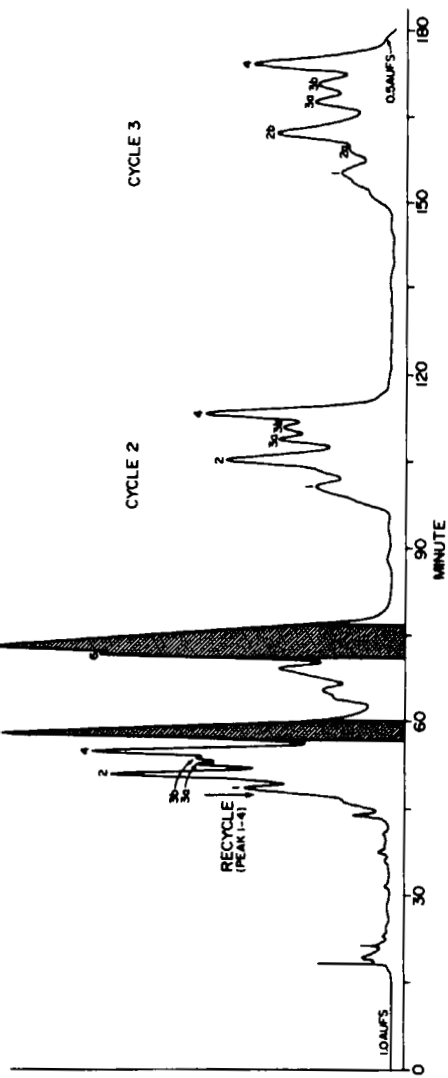


FIGURE 2. HPLC separation of the saturated C_{35-56} fatty acid esters on a silica column in the recycling mode. The shaded peaks were collected. Columns: six 4 mm x 30 cm μPorasils in series. Solvent: ethylene dichloride-hexane (35:65, v/v). Sample load: 8.1 mg. Peaks 1-4 were recycled.

TABLE I. Mass Spectral Identification of the Major Components in the Silica Column HPLC Fractions.

HPLC peak	m/e						No. of carbon	Structure	Assignment a+b+c	x
	M	M-32	M-79/81	M-32-79/81	M-213/215	M-32-213/215				
1	826,828	-	747	-	613	-	C_{43}	I	35	-
	854,856	-	775	-	641	-	C_{45}	I	37	-
	-	894,896	-	815	-	698	$C_{49}OCH_3$	III	39	2
2a	798,800	-	719	-	585	-	C_{41}	I	33	-
	-	852,854	-	773	-	656	$C_{46}OCH_3$	III	36	2
	-	866,868	-	787	-	670	$C_{47}OCH_3$	III	37	2
2b	756,758	-	677	-	543	-	C_{38}	I	30	-
3a	742,744	-	663	-	529	-	C_{37}	I	29	-
3b	742,744	-	663	-	529	-	C_{37}	I	29	-
4	714,716	-	635	-	501	-	C_{35}	I	27	-
5.	728,730	-	649	-	515	-	C_{36}	I	28	-
6	768,770	-	689	-	555	-	C_{39}	II	29	-
7	740,742	-	661	-	527	-	C_{37}	II	27	-

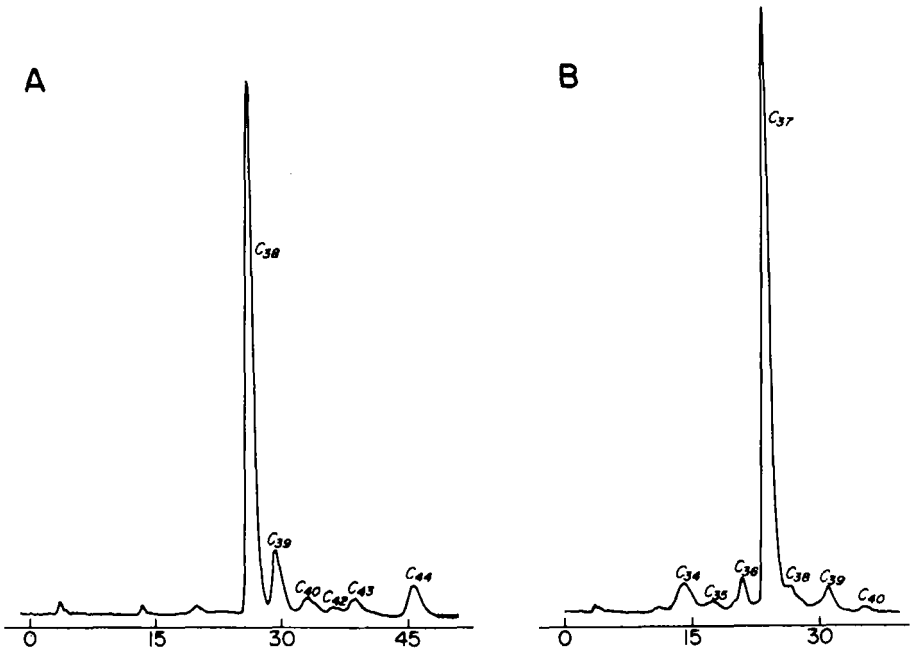


FIGURE 3. Reverse-phase HPLC of fractions 2b (A) and 3a (B). Column: 4 mm x 30 cm μBondapak C_{18}. Solvent: p-dioxane-acetonitrile (3:7, v/v). Absorbance was followed at 254 nm.

HPLC peak 2b was primarily a C_{38} ester with a Structure I assignment (Fig. 3A). This series appeared to include C_{39} and C_{40} esters. A minor series was also detected which contained the C_{42}, C_{43}, and C_{44} esters whose structures are not known. In a separate analysis, this minor series was found to be the most prominent component in the emerging shoulder fraction of peak 2b (cycle 5 of Fig. 2).

HPLC peak 3a was a C_{37} ester with trace amounts of C_{34}, C_{35}, C_{36}, C_{38}, C_{39}, and C_{40} esters in the series (Fig. 3B). HPLC peak 3b was also a C_{37} ester primarily with minor amounts of C_{35}, C_{36}, and C_{38} esters (Fig. 4A). HPLC peak 4 was a C_{35} ester with trace amounts of C_{33}, C_{36}, and C_{37} esters (Fig. 4B). We suggest Structure I for the esters in peaks 3a, 3b, and 4.

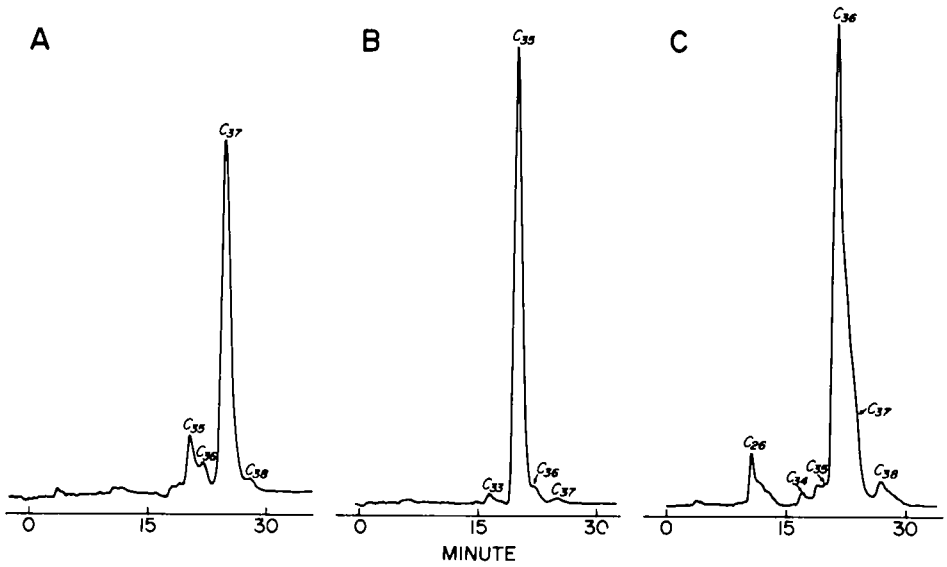
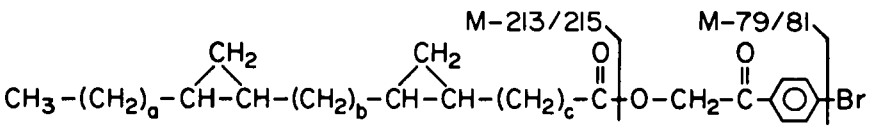
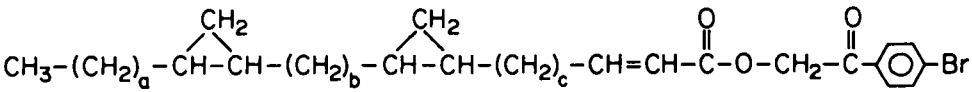


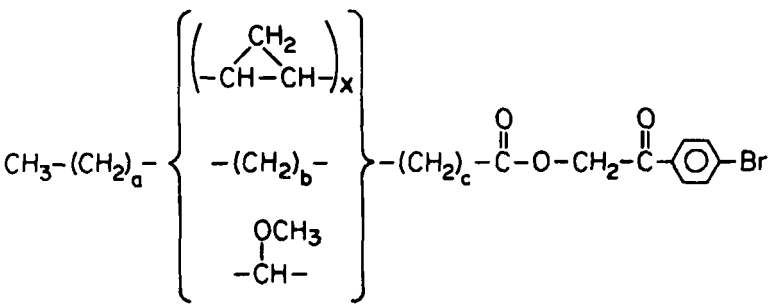
FIGURE 4. Reverse-phase HPLC of fractions 3b (A), 4 (B), and 5 (C). Conditions were same as in Figure 3.



Structure I



Structure II



Structure III

HPLC peak 5 was a C_{36} ester with a small amount of C_{26} ester (presumably a normal fatty acid ester) and trace amounts of C_{34}, C_{35}, C_{37}, and C_{38} esters (Fig. 4C). The C_{36} ester was represented by Structure I where a=17, b=10, and c=1 (7). HPLC peak 6 contained a C_{39} ester, whereas HPLC peak 7 contained a C_{37} ester. The minor component of this series was a C_{38} ester. The C_{37} ester was represented by Structure II where a=17, b=10, and c=0 (2). The peaks appearing between peaks 5 and 6 in Fig. 2 were not identified. MS did not give the diagnostic p-bromophenacyl ester fragmentations.

The C_{39} and C_{37} esters (peaks 6 and 7, respectively) each contained a double bond at the Δ^2 position as shown by Structure II. Gunstone et al. (10) showed that a cis-Δ^2-$C_{18:1}$ fatty acid ester migrated like a saturated ester when chromatographed on a thin layer of silica impregnated with $AgNO_3$. One would expect a similar property on a silicic acid-$AgNO_3$ column. The Δ^2-unsaturation was also found in the unsaturated long-chain fatty acids of the H37Ra strain (8). This represents a new series of fatty acids that contains the α,β-unsaturation. It is structurally similar to the phthienoic acids found only in the virulent strains of *M. tuberculosis* (11).

Four different C_{37} esters appeared in the chromatogram. The C_{37} esters of peaks 3a and 3b had the same general structure (Structure I), but since HPLC on a μPorasil column resolved the two esters, they must have subtle structural differences. The structure of the C_{37} ester in peak 1 is not known.

Recycling of the saturated C_{35-36} esters from the H37Ra strain on a silica column effected the following (Fig. 2): a) resolved the minor peak 1 into two shoulders, b) resolved peak 2 into two peaks -- 2a and 2b, c) further separated peaks 3a and 3b, and d) revealed a shoulder in peak 2b. This study illustrated that only one ester in each structural series is usually the major component while the others are trace components. The relative abundance of these major components is: C_{37} (peak 7) > C_{36} > C_{39} > C_{35} > C_{38} > C_{37} (peak 3a) > C_{37} (peak 3b).

CONCLUSIONS

This study showed that by combining the adsorption and reverse-phase modes of HPLC, it was possible to resolve the complex mixture of the long-chain saturated fatty acids of *M. tuberculosis* H37Ra into their individual components. Such purified esters are suitable for detailed structural studies. The silica column appeared to separate the esters according to structural classes, as well as allowing some separation according to size. The reverse-phase column separated the esters according to either the hydrocarbon chain length or the polarity.

ACKNOWLEDGMENTS

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