Separation of neutral lipids and free fatty acids by high-performance liquid chromatography using low wavelength ultraviolet detection

James G. Hamilton[1] and Karen Comai

Department of Pharmacology, Hoffmann-La Roche, Nutley, NJ 07110

Summary Normal phase, isocratic high-performance liquid chromatography methods are described for the separation of neutral lipid and fatty acid classes using low wavelength detection. Prior to high-performance liquid chromatography, methods were developed and are described for the separation of phospholipids from neutral lipids and fatty acids using small (600 mg) silica Sep-Paks™. Recoveries of cholesteryl esters, triglycerides, fatty acids, and phospholipids from the silica columns were greater than 95%. Two mobile phases are described for lipid class separation by high-performance liquid chromatography. The first mobile phase, hexane–2-propanol–acetic acid 100:0.5:0.01, resulted in incomplete separation of cholesteryl ester and triglyceride but excellent separations of fatty acids and cholesterol. The second mobile phase, hexane–n-butyl chloride–acetonitrile–acetic acid 90:10:1.5:0.01, resulted in complete separation of the four lipid classes. This mobile phase also separated individual triglycerides and fatty acids based on the number of double bonds. Recoveries of radiolabeled lipids for the four lipid classes from high-performance liquid chromatography was greater than 95% with both mobile phases.—**Hamilton, J. G., and K. Comai.** Separation of neutral lipids and free fatty acids by high-performance liquid chromatography using low wavelength ultraviolet detection. *J. Lipid Res.* 1984. **25:** 1142–1148.

Supplementary key words triglyceride • cholesteryl ester • cholesterol • fatty acid • silica Sep-Paks™

The development of mobile phases for high-performance liquid chromatography (HPLC) of lipids has been slow considering the wealth of available information on thin-layer and column chromatographic separation techniques for both polar and nonpolar lipids (1). A major reason for this slow progress has been the lack of adequate detection techniques for lipids. Although the best detectors for HPLC are spectrophotometers, which have reached a high level of sophistication, most lipids do not have specific UV-absorbing properties but absorb generally in the 190 to 210 nm range. If this UV range is used to monitor HPLC separation of lipids, the problem of severe restriction of mobile phase solvents compatible with this UV range arises. Traditional lipid chromatographic solvents such as ethyl ether and chloroform are of limited value because of their strong UV-absorbing properties. Additionally, ethyl ether forms peroxides during chromatographic procedures resulting in climbing baselines, again limiting its usefulness.

Some advances have been made in reversed phase separation of lipids because solvents such as methanol, acetonitrile, and water can be used in mobile phases due to their low absorption in the 190 to 210 nm range

Abbreviations: HPLC, high-performance liquid chromatography; UV, ultraviolet.

[1] To whom reprint requests should be addressed.

(1). However, because of the nature of reversed phase HPLC, it is useful only after lipids have been separated into specific lipid classes (1). Without prior column or thin-layer separation into lipid classes, reversed phase HPLC leads to significant overlap of cholesteryl esters and triglycerides (2, 3) and phosphatidylcholines with phosphatidylethanolamines (4).

This report describes in detail normal phase HPLC techniques for quantitative separation of neutral lipid classes and fatty acids. These isocratic HPLC separations are performed within 30 min.

MATERIALS AND METHODS

Lipids were extracted from rat, rabbit, and human sera, from rat liver, and from cultured rat aortic smooth muscle cells by the method of Bligh and Dyer (5). Extracts were stored under nitrogen at −20°C.

Radiolabeled rat liver phosphatidylcholine was prepared by intraperitoneal injection of 5 mCi of [^{3}H]choline chloride (New England Nuclear, Boston, MA) into a rat. After 24 hr, the rat was killed and the liver (16.2 g) was homogenized and extracted using the Bligh and Dyer method (5). The phospholipid fraction was obtained by separation from neutral lipid and fatty acids using a silica Sep-Pak™ column (see below). There were 2.5 × 10^8 dpm recovered in the phospholipid fraction.

HPLC was performed using a Waters Associates (Milford, MA) Model 660 solvent programmer with two Model 6000 A solvent delivery pumps, a model U6K injector, and a model 450 variable wavelength detector operated at 206 nm. Prepacked μPorasil columns (30 cm × 3.9 mm) were obtained from Waters Associates. The mobile phase flow rate was 2.0 ml/min. All injections for quantitative measurements were made with a TP #5250 syringe (Unimetrics Corporation, Anaheim, CA). All samples for HPLC separations were dissolved in the solvent that was used as the mobile phase. The HPLC system was maintained at room temperature, approximately 22°C.

Lipid standards were purchased from Nu-Chek Prep (Elysian, MN) and stored in sealed vials at −20°C. All solvents were HPLC grade and were purchased from Burdick and Jackson (Muskegon, MI). The chloroform used in these studies was preserved with 1.0% ethanol. Silica Sep-Pak™ columns (600 mg silica) were purchased from Waters Associates (Milford, MA). Tri[1-^{14}C]-oleoylglycerol (49 mCi/mmol), [U-^{14}C]palmitic acid (403 mCi/mmol), and L-α-phosphatidyl [2-^{14}C]ethanolamine, dioleoyl (40 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Cholesteryl [1-^{14}C]oleate (56.6 mCi/mmol) and cholesterol [1,2,6,7-^{3}H(N)] (79 Ci/mmole) were purchased from New England Nuclear (Boston, MA). When necessary these radiolabeled lipids were repurified by HPLC prior to use.

RESULTS

Separation of lipids on silica Sep-Pak™ columns

Lipid extracts from the Bligh and Dyer extraction procedure were evaporated to dryness under nitrogen, taken up in 2.0 ml of chloroform–acetic acid 100:1 and applied to silica Sep-Pak™ columns for separation of phospholipids from neutral lipids and fatty acids. The vessel containing the lipid extract was washed once with 2.0 ml of chloroform–acetic acid 100:1 and the solvent was added to the Sep-Pak™ column. The column was eluted further with an additional 12 ml of chloroform–acetic acid 100:1. The column eluant, consisting of the first 2 ml, the 2-ml wash, and the 12 ml of chloroform–acetic acid 100:1 was designated as fraction 1 of the column separation. The next eluting solvent, consisting of 5 ml of methanol–chloroform 2:1, was passed through the column (fraction 2). Fraction 3 consisted of 5 ml of methanol–chloroform–water 2:1:0.8. Fraction 4 consisted of an additional 5 ml of methanol–chloroform–water 2:1:0.8.

Neutral lipids and free fatty acids were eluted exclusively in fraction 1 by the chloroform–acetic acid 100:1 solvent system (**Table 1**). Approximately 93% of the phosphatidylethanolamine was eluted in fraction 2 by methanol–chloroform 2:1. Phosphatidylcholine, along with the remaining phosphatidylethanolamine, was eluted in fraction 3 by the methanol–chloroform–water 2:1:0.8 solvent system (Table 1).

TABLE 1. Recovery of lipids from silica Sep-Pak™ chromatography[a]

Lipid Class	% Recovery			
	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Cholesteryl ester	98.4 ± 0.5	<0.1	<0.1	<0.1
Triglyceride	99.2 ± 0.6	<0.1	<0.1	<0.1
Fatty acid	99.8 ± 0.5	<0.1	<0.1	<0.1
Cholesterol	98.0 ± 0.9	<0.1	<0.1	<0.1
Phosphatidylethanolamine	<0.1	>92	>7	>0.1
Phosphatidylcholine	<0.1	<0.1	99.4 ± 0.8	<0.1

[a] Lipids were extracted from human serum using the method of Bligh and Dyer (5). Radiolabeled lipid standards were added separately to an equivalent of 100 μl of human serum lipid to determine recovery. Results are expressed as the mean ± SD for six separate determinations for each lipid class. Fraction 1 solvent system is chloroform–acetic acid 100:1, 16 ml; fraction 2 solvent system is methanol–chloroform 2:1, 5 ml; fraction 3 and 4 solvent systems are methanol–chloroform–water 2:1:0.8, 5 ml each.

For quantitative recovery experiments, the following radiolabeled lipids were added to lipids extracted from human serum (equivalent to 100 μl of human serum): 0.02 μCi of [^{3}H]cholesteryl oleate, 0.1 μCi of [^{14}C]-cholesterol, 0.02 μCi of [U-^{14}C]palmitic acid, 0.02 μCi of tri[1-^{14}C]oleoylglycerol, 0.04 μCi of [^{14}C]phosphatidylcholine, and 0.04 μCi of [^{14}C]phosphatidylethanolamine. The column separation technique described above was performed six times for each individual radiolabeled lipid added to serum lipids and for combined radiolabeled lipids in serum lipids.

Table 1 gives the results of the recovery of phospholipids separated from neutral lipids and fatty acids on silica Sep-Pak™ columns. All the neutral lipid and fatty acids were recovered in fraction 1 solvent system consisting of a total of 16 ml of chloroform–acetic acid 100:1. The recoveries of the separate neutral lipid species and fatty acids were greater than 98% in all cases. No significant neutral lipid radioactivity or mass was detected in fraction 2 solvent system consisting of 5 ml of methanol–chloroform 2:1. This fraction contained 93% of the phosphatidylethanolamine. Rat liver phosphatidylcholine was eluted in fraction 3 solvent system consisting of 5 ml of methanol–chloroform–water 2:1:0.8. The recovery of phosphatidylcholine and phosphatidylethanolamine was greater than 99% (Table 1). No significant radioactivity was eluted from the column by passing an additional 5 ml of methanol–chloroform–water 2:1:0.8 through the column (fraction 4).

For further manipulation of the phospholipids in fraction 3, it was determined that these phospholipids could be reextracted into chloroform by the addition of 2.0 ml of water and 1.5 ml of chloroform for each 5.0

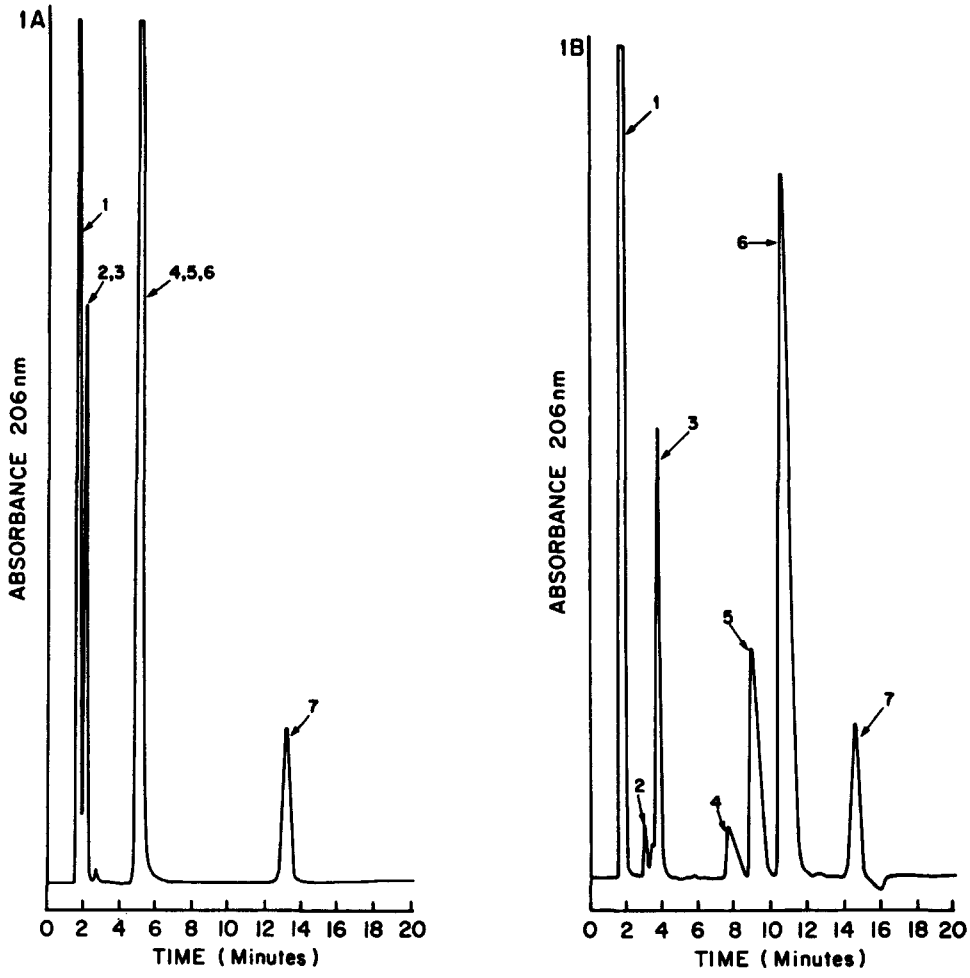

Fig. 1. HPLC separation of standard cholesteryl esters, triglycerides, fatty acids, and cholesterol. Column: 30 cm × 3.9 mm μPorasil; flow rate: 2.0 ml/min; detection: 0.2 A.U.F.S. at 206 nm; mobile phase: 1A, hexane–2-propanol–acetic acid 100:0.5:0.1; 1B, hexane–n-butyl chloride–acetonitrile–acetic acid 90:10:1.5:0.01. Standard compounds (peak number): 1, 25 μg each of cholesteryl oleate, cholesteryl linoleate, cholesteryl arachidonate, and cholesteryl docosahexaenoate; 2, 25 μg of triolein; 3, 25 μg of trilinolein; 4, 25 μg of oleic acid; 5, 25 μg of linoleic acid; 6, 25 μg of arachidonic acid; 7, 25 μg of cholesterol.

ml of fraction 3. The phospholipids were quantitatively recovered in the lower chloroform layer (Table 1).

Separation of neutral lipids and fatty acids by HPLC

Two mobile phases were developed to separate neutral lipids and free fatty acids by normal phase HPLC. Mobile phase 1 consisted of hexane–2-propanol–acetic acid. The amount of 2-propanol in the mobile phase was adjusted to optimize the separation of lipid classes. Mobile phase 2 consisted of hexane–n-butyl chloride–acetonitrile–acetic acid. With mobile phase 2, the amount of acetonitrile was adjusted to optimize separation of lipid classes.

Fig. 1 shows the HPLC chromatograms of the neutral lipid and fatty acid standards utilizing the two described mobile phases. The four cholesteryl esters that made up the cholesteryl ester standard (oleate, linoleate, arachidonate, and docosahexaenoate, 25 μg each) emerged as a single peak with both mobile phase 1 and mobile phase 2 (peak 1 in Figs. 1A and 1B). Triglycerides (triolein and trilinolein, 25 μg each) emerged as a single peak with mobile phase 1 (peak 2, 3, Fig. 1A) but as two discrete peaks with mobile phase 2 (triolein, peak 2 in Fig. 1B and trilinolein, peak 3 in Fig. 2B).

Although cholesteryl esters and triglycerides emerged as discrete peaks with mobile phase 1, they were incompletely separated from each other. With mobile phase 2, cholesteryl esters and triglycerides were completely separated from each other (Fig. 1B). Additionally, there was some separation of triolein from trilinolein (Fig. 1B).

Fatty acids (oleic, linoleic, and arachidonic acid, 25 μg each) emerged as a single peak with mobile phase 1 (peak 4, 5, 6, Fig. 1A). In contrast, these fatty acids were separated when chromatographed using mobile phase 2. Oleic acid emerged first (peak 4, Fig. 1B) followed by linoleic acid (peak 5, Fig. 1B) and arachidonic acid (peak 6, Fig. 1B). Cholesterol (25 μg) emerged as a single peak (peak 7, Figs. 1A and 1B) with both mobile phases.

Human serum lipids were separated on HPLC using the two mobile phases. Human serum was extracted using the Bligh and Dyer method (5) and separated on a silica Sep-Pak™ (Table 1). Fraction 1, containing the

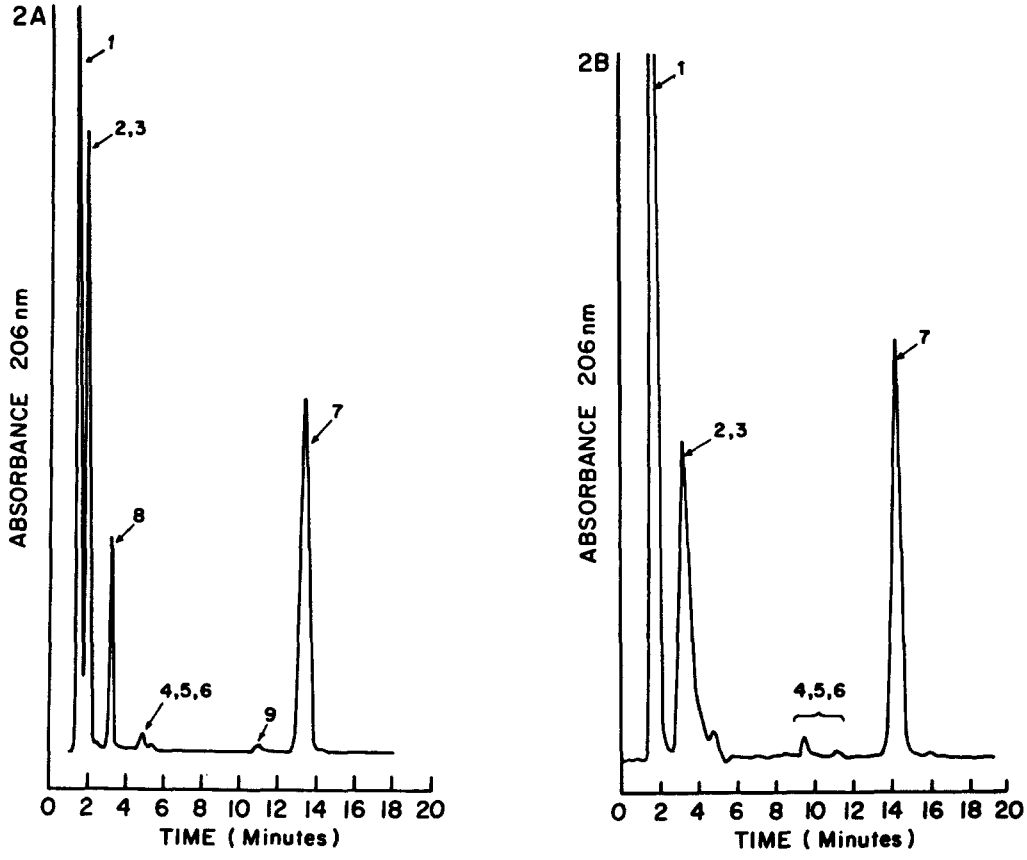

Fig. 2. HPLC separation of neutral lipid fraction from 100 μl of human serum extract. Column: 30 cm × 3.9 mm μPorasil; flow rate: 2.0 ml/min; detection; 0.2 A.U.F.S. at 206 nm; mobile phase; 2A, hexane–2-propanol–acetic acid 100:0.5:0.01; 2B, hexane–n-butyl chloride–acetonitrile–acetic acid 90:10:1.5:0.01. Peak identity: 1, cholesteryl esters; 2, 3, triglycerides; 4, 5, 6, fatty acids; 7, cholesterol; 8, 9, unknown.

neutral lipid and free fatty acids, was chromatographed by HPLC. As observed with the lipid standards, human serum cholesteryl esters and triglycerides were not separated using mobile phase 1 (**Fig. 2A**). With mobile phase 2, serum cholesteryl esters and triglycerides were separated (Fig. 2B). The level of serum free fatty acids appeared low. Serum cholesterol emerged with mobile phase 1 and mobile phase 2 where expected (Figs. 2A and 2B).

To determine lipid recoveries from the HPLC, radiolabeled lipids were added to the lipid extract of human serum as described in the section on lipid separation on silica columns. A programmable fraction collector was utilized to collect HPLC fractions. Using mobile phase 1 (hexane–2-propanol–acetic acid 100:0.5:0.01), cholesterol and free fatty acids were separated quantitatively from cholesteryl esters and triglycerides within 15 min (Fig. 2A). Triglycerides and cholesteryl esters eluted simultaneously between 1 and 2.75 min, fatty acids eluted between 5 and 6 min, and cholesterol eluted between 12.5 and 14.5 min. Recoveries of lipid utilizing this isocratic mobile phase were excellent, being greater than 95% for all lipid classes (**Table 2**). Using mobile phase 2 (hexane–n-butyl chloride–acetonitrile–acetic acid 90:10:1.5:0.01), the cholesteryl esters (radiolabeled cholesteryl oleate) were completely separated from triglycerides (radiolabeled triolein) with recoveries of each greater than 97% (Table 2). Fatty acid (radiolabeled palmitic acid) and cholesterol (radiolabeled cholesterol) were separated as expected and recoveries of each were greater than 96% (Table 2).

The versatility and capacity of the isocratic mobile phase 2 system are demonstrated in **Figs. 3A** and **3B,** which show separations of serum neutral lipids and fatty acids from normal and cholesterol-fed rabbits. Rabbit sera were extracted (5) and the phospholipid were removed by silica Sep-Pak™ chromatography as described earlier. In spite of the high cholesterol content of the hypercholesterolemic rabbit serum, the separation of lipid classes was complete. The triglycerides observed in the normal rabbit serum appeared greater than the triglycerides in hypercholesterolemic rabbit serum.

An additional example of lipid separation from tissue is shown in **Fig. 4A** and **B.** Rat liver neutral lipids and fatty acids (from 200 mg of rat liver) were separated using mobile phases 1 and 2.

TABLE 2. Recovery of lipids from HPLC

Lipid Class[a]	% Recovery	
	Mobil Phase 1[b]	Mobil Phase 2[c]
Cholesteryl ester	96.9 ± 1.0	98.0 ± 0.6
Triglyceride	97.8 ± 0.7	97.2 ± 0.7
Free fatty acid	95.5 ± 0.6	96.6 ± 0.9
Cholesterol	96.1 ± 0.6	97.7 ± 0.8

[a] Lipids were extracted from human serum using the method of Bligh and Dyer(5). Radiolabeled lipid standards were added separately to an equivalent of 100 μl of human serum lipid collected as fraction 1 from the silica Sep-Pak™ column.

[b] Mobile phase 1: hexane–2-propanol–acetic acid 100:0.5:0.01. The results are the mean ± SE for six determinations for each lipid class.

[c] Mobile phase 2: hexane–n-butyl chloride–acetonitrile–acetic acid 90:10:1.5:0.01. The results are the mean ± SE for six determinations for each lipid class.

DISCUSSION

Silica Sep-Pak™ columns were used to prepare the lipid extracts for HPLC. The use of these small columns effectively removed phospholipids, other polar lipids, and nonlipid components before HPLC. Once the polar lipids were removed, samples then were injected onto the HPLC every 20 min for separation of neutral lipid and fatty acid classes. Additionally, both phosphatidylcholine and phosphatidylethanolamine were recovered quantitatively from the Sep-Pak™ columns. Methanol–chloroform 2:1 eluted 93% of the phosphatidylethanolamine. The remaining phosphatidylethanolamine and phosphatidylcholine eluted with methanol–chloroform–water 2:1:0.8. Optimization of this separation or the fate of other polar lipids such as phosphatidylserine, phosphatidylinositol, cardiolipin, sphingomyelin, or lysophospholipids has not as yet been investigated.

The cholesteryl esters emerged in the breakthrough volume from the HPLC column with either mobile phase 1 or 2. Therefore, all other compounds less than or equally polar to the cholesteryl esters would be contained in this first peak. These types of compounds would include hydrocarbons, such as squalene and carotene, and simple esters such as wax esters. The hydrocarbons and wax esters could be separated further by normal phase HPLC using less polar solvents than in mobile phases 1 and 2 described in the present report or by using reversed phase columns. The cholesteryl esters from fraction 1 could be quantitated by gas–liquid chromatography or by hydrolysis followed by quantitative HPLC or gas–liquid chromatography of the fatty acids and cholesterol. This has been done for the cholesteryl esters of cultured smooth muscle cells (data not shown).

The mobile phase that uses hexane–2-propanol–acetic acid (mobile phase 1) can be used to separate compounds into groups based on polarity of functional groups. With this solvent system, carbon chain length and double bonds have little influence on separations, i.e., all fatty acids emerge as a single peak and are separated from alcohols which also emerge as a single peak. This mobile phase offers the advantage of collecting peaks containing

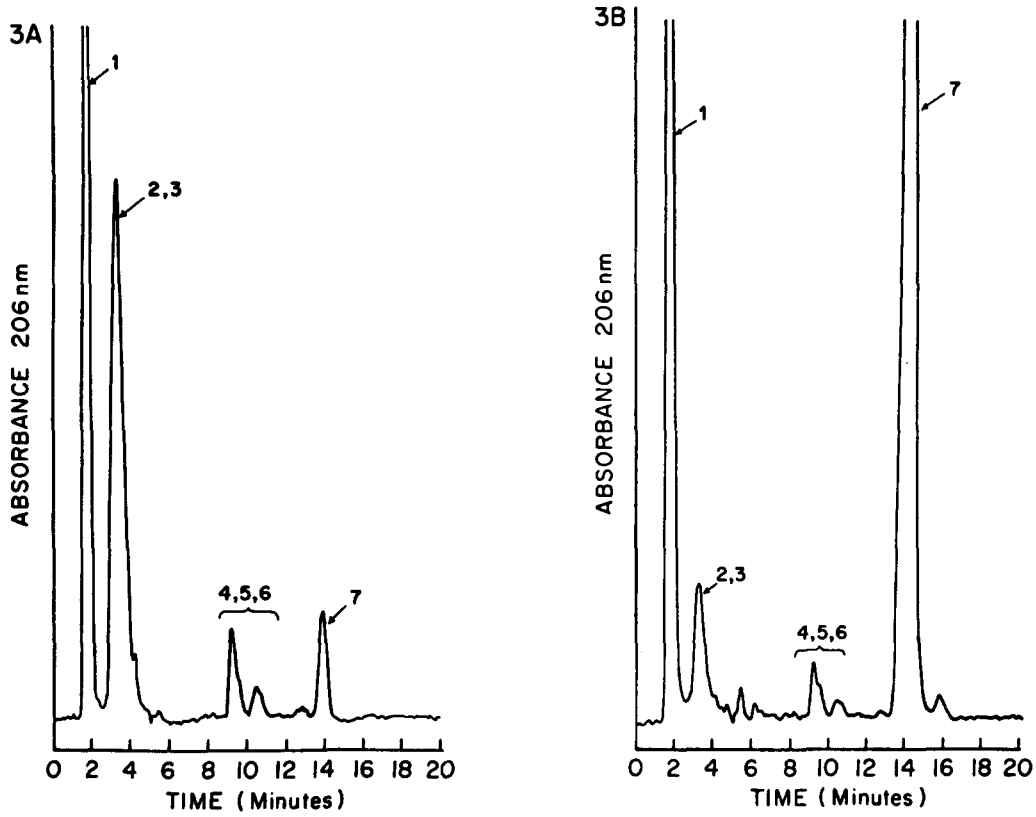

Fig. 3. HPLC separation of neutral lipid fraction from 100 μl of rabbit serum extract. Column: 30 cm × 3.9 mm μPorasil; flow rate: 2.0 ml/min; detection: 0.2 A.U.F.S. at 206 nm; mobile phase: hexane–n-butyl chloride–acetonitrile–acetic acid 90:10:1.5:0.01. 3A, Serum from a rabbit with a total cholesterol of 35 mg/dl. 3B, Serum from a rabbit with a total cholesterol of 800 mg/dl. Peak identities, same as in Fig. 2.

individual lipid classes and then other techniques can be employed to further separate the individual compounds. The primary disadvantage of mobile phase 1 is the poor separation between cholesteryl esters and triglycerides. However, mobile phase 2 containing hexane–n-butyl chloride–acetonitrile–acetic acid was developed to separate these two lipid classes. The separation is accomplished because the cholesteryl esters, regardless of double bonds and chain length, are not retained on the HPLC column; whereas the double bonds in triglycerides and fatty acids appear to retard the emergence of these lipids as shown in Fig. 1B. Additionally, highly unsaturated fatty acids [i.e., 22:5 (n-3), 22:5 (n-6), and 22:6 (n-3)] emerge near the cholesterol peak with mobile phase 2 (data not shown) which probably explains the apparent higher absorption peak for cholesterol in Fig. 4B. This effect seems to be similar to that of silver ions on the adsorption of compounds with double bonds to silica (6). The retention of these lipids is probably due to the acetonitrile in mobile phase 2 which has been shown by others to quench the effect of silver ion-loaded columns in reversed phase HPLC systems (7).

Acetonitrile is not generally used in normal phase mobile phases because it is insoluble in saturated hydrocarbons. In the current work, n-butyl chloride was used to make a single phase system with hexane and acetonitrile. n-Butyl chloride was chosen over chloroform or dichloromethane because of the desire to use low wavelength (206 nm) ultraviolet detection. n-Butyl chloride in hexane at concentrations greater than 15% could not be used due to absorbance at 206 nm.

For separations of compounds where low wavelength ultraviolet detection is not necessary, acetonitrile as the polar component in a normal phase separation may give some unique separations involving both double bonds and carbon chain length.

Recently, a mobile phase using isooctane–tetrahydrofuran–formic acid was reported to give "baseline" separations of cholesteryl esters and triglycerides from rat liver (8). However, the authors did not rigorously test this system and it appeared to be no better than the mobile phase 1 system described in the present work. Additionally, mobile phase 2 consisting of hexane–n-butyl chloride–acetonitrile–acetic acid gave complete separations of cholesteryl esters and triglycerides as determined with radiolabeled cholesteryl oleate and radiolabeled triolein (Table 2).

Quantitation of lipids using low wavelength detection

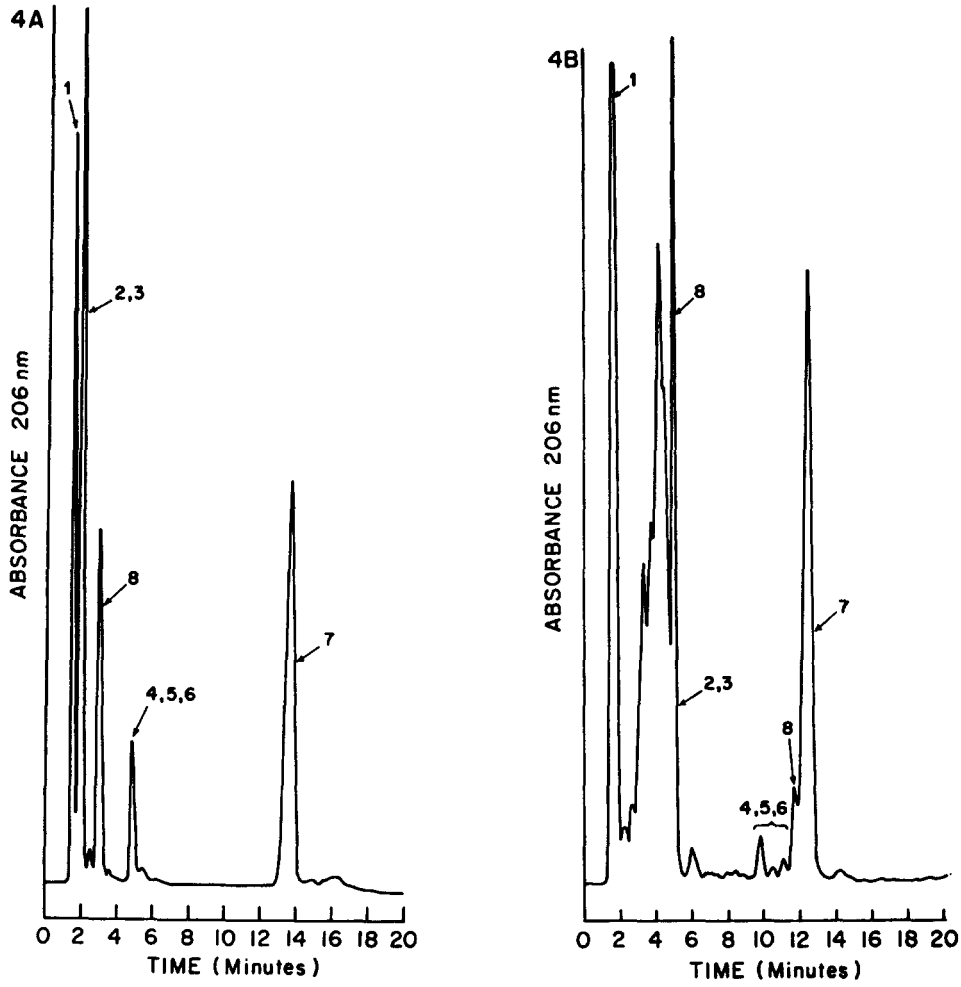

Fig. 4. HPLC separation of neutral lipid extract from 100 mg of rat liver. Column: 30 cm × 3.9 mm μPorasil; flow rate: 2.0 ml/min; detection: 0.2 A.U.F.S. at 206 nm; mobile phase: 4A and 4B same as in Fig. 1. Peak identity, same as in Fig. 2.

is not possible unless the number of double bonds is constant and known (1). This problem of quantitative determination at low wavelength is illustrated in Fig. 1B, where 25 μg each of oleic, linoleic, and arachidonic acid yielded curves with different areas.

The authors wish to thank Mrs. M. Johnson, Miss P. Manion, and Miss C. Muscara for their expertise in assembling and typing this manuscript.

Manuscript received 27 April 1984.

REFERENCES

1. Aitzemüller, K. 1982. Recent progress in the high performance liquid chromatography of lipids. *Prog. Lipid Res.* **21:** 171–193.
2. Carroll, R. M., and L. L. Rudel. 1981. Evaluation of a high-performance liquid chromatography method for isolation and quantitation of cholesterol and cholesteryl esters. *J. Lipid Res.* **22:** 359–363.
3. Kuksis, A., L. Marai, and J. J. Myher. 1983. Strategy of glycerolipid separation and quantitation by complementary analytical techniques. *J. Chromatogr.* **273:** 43–66.
4. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1983. Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. *J. Lipid Res.* **23:** 190–196.
5. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
6. De Vries, B. 1963. Quantitative separations of higher fatty acid methyl esters by adsorption chromatography on silica impregnated with silver nitrate. *J. Am. Oil Chem. Soc.* **40:** 184–186.
7. Powell, W. S. 1981. Separation of icosenoic acids, monohydroxyicosenoic acids, and prostaglandins by high-pressure liquid chromatography on a silver ion-loaded cation-exchange column. *Anal. Biochem.* **115:** 267–277.
8. Greenspan, M. D., and E. A. Schroeder. 1982. Separation and detection of neutral lipids and free fatty acids in a liver extract by high-performance liquid chromatography. *Anal. Biochem.* **127:** 441–448.