

## Evaluation of a high-performance liquid chromatography method for isolation and quantitation of cholesterol and cholesteryl esters

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**Summary** A rapid method for the separation and quantitation of unesterified cholesterol and cholesteryl esters by high-performance liquid chromatography is described. All of the cholesteryl esters typically present in plasma were resolved on Zorbax ODS reversed-phase columns using a linear gradient of water (3%–0%) in acetonitrile–tetrahydrofuran 65:35 (v/v) as eluting solvent. Results obtained by this method show good agreement with chemical and gas–liquid chromatographic methods for quantitation of cholesterol and determination of cholesteryl ester percentage compositions. High-performance liquid chromatography provides the only available method for the separation and quantitation of individual cholesteryl esters from lipid extracts of biological samples. — **Carroll, R. M., and L. L. Rudel.** Evaluation of a high-performance liquid chromatography method for isolation and quantitation of cholesterol and cholesteryl esters. *J. Lipid Res.* 1981. **22:** 359–363.

**Supplementary key words** gas–liquid chromatography · low density lipoprotein · African green monkeys

The relationship between cholesterol metabolism and atherosclerosis and the physiological role of cholesteryl esters in the genesis of the disease have resulted in numerous attempts to isolate and quantitate individual cholesteryl esters. Characterization of cholesteryl esters has generally been based on the study of cholesteryl ester fatty acids by gas–liquid chromatography and of cholesteryl esters directly by argentation thin-layer chromatography. However, these methods are time-consuming and do not preparatively separate the individual esters typically present in the plasma of most animals.

Direct analysis of cholesteryl esters is preferable in all instances and is a necessity when studying esters isotopically labeled in the cholesterol moiety. In the present study, we have examined the use of reversed-phase high-performance liquid chromatog-

raphy (HPLC) for the isolation and quantitation of cholesterol and individual cholesteryl esters in total lipid extracts of isolated plasma lipoproteins. With this technique, isolation and quantitation of unesterified cholesterol and individual cholesteryl esters is possible permitting direct analyses of biological samples after lipid extraction.

## EXPERIMENTAL METHODS

### Materials

Cholesterol and cholesteryl ester standards were purchased from Applied Science Division, Milton Roy Company, State College, PA, Suppelco, Bellfonte, PA, and Serdary Research Laboratories, London, Ontario, Canada. All solvents used were HPLC grade purchased from Fisher Chemical, Co., Fairlawn, NJ.

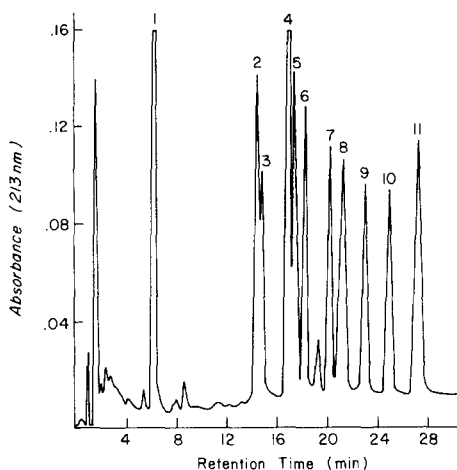
### Instrumentation

The HPLC analyses were carried out using a Dupont Model 850 liquid chromatograph equipped with a Dupont Model 837 variable wavelength spectrophotometer (Dupont Instruments, Wilmington, DE) attached to a Hewlett Packard Model 3380A integrating recorder (Hewlett Packard Instruments, Avondale, PA). Three chromatographic columns were used in series, a 5 cm × 2.1 mm stainless steel guard column packed with CO:Pell ODS (Whatman, Inc., Clifton, NJ), a 15 cm × 4.6 mm Zorbax ODS column, and a 25 cm × 4.6 mm Zorbax ODS column (Dupont Instruments, Wilmington, DE). These columns were found to have a lifetime in the range of 6 months to 1 year during daily usage.

### Methods

Adult male African green monkeys of the vervet subspecies were fed diets containing 40% of calories as fat. Fasting blood samples were collected as previously described (1). Lipoprotein samples were isolated by ultracentrifugation and separated by chromatography on Bio-Gel A-15 m agarose, 200–400 mesh (Bio-Rad Laboratories, Richmond, CA) as described by Rudel et al (2). Total lipid extracts of the lipoprotein samples were prepared by extraction with chloroform–methanol 2:1 (v/v) after the method of Folch, Lees, and Sloane Stanley (3). Cholesteryl heptadecanoate (50 μg) was added to aliquots of lipoprotein total lipid extracts containing 85–500 μg of cholesteryl ester. The lipoprotein extracts were prepared for HPLC analysis by evaporating the extraction solvent and redissolving the lipid in 50 μl of tetrahydrofuran–acetonitrile 80:20 (v/v). This 50-μl

Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; LDL, low density lipoprotein; VLDL, very low density lipoprotein.



**Fig. 1.** HPLC separation of cholesterol and cholesteryl ester standards. A 50- $\mu$ l sample containing 60  $\mu$ g of unesterified cholesterol and approximately 400  $\mu$ g of cholesteryl ester standards (see text) was applied to the column and eluted as described in Methods. Peak identification: (1) unesterified cholesterol, (2) cholesteryl linolenate, (3) cholesteryl arachidonate, (4) cholesteryl linoleate, (5) cholesteryl palmitoleate, (6) cholesteryl myristate, (7) cholesteryl oleate, (8) cholesteryl palmitate, (9) cholesteryl heptadecanoate, (10) cholesteryl stearate, (11) cholesteryl erucate.

volume was injected into the liquid chromatograph. The percent recovery of cholesteryl heptadecanoate was used to correct for loss of sample which occurred during the HPLC analysis.

HPLC solvents consisted of acetonitrile-tetrahydrofuran 65:35 (v/v) and double-distilled water. Cholesterol and cholesteryl esters were eluted from the column using a linear water gradient. The water concentration in acetonitrile-tetrahydrofuran was reduced from 3% to 0% over a 20-min period at 37°C with a solvent flow rate of 2.0 ml/min. Cholesterol and cholesteryl esters eluting from the column were detected by their ultraviolet absorption at 213 nm.

A Hewlett Packard Model 3380A integrating recorder was used to measure peak areas for cholesterol and individual cholesteryl esters at concentration ranges of 10–400  $\mu$ g at a full scale recorder sensitivity of 0.16 absorbance units. The mass of cholesterol and individual cholesteryl esters in the lipoprotein samples was calculated from standard curves of mass versus peak area by the following formula:

Sample Mass

$$= \left( \frac{\text{Mass of Standard}}{\text{Peak Area of Standard}} \right) \times \text{Peak Area of Sample}$$

$$\div (\% \text{ Recovery of Internal Standard})/100$$

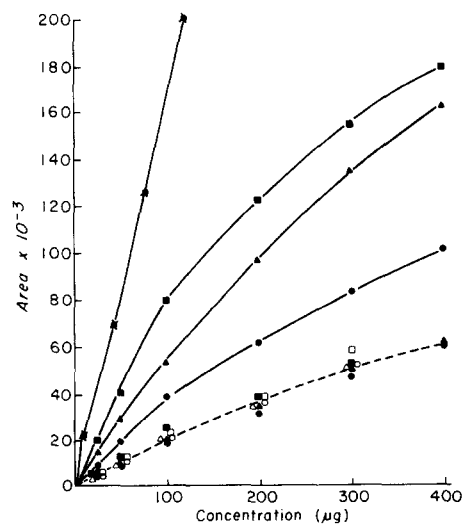
Chemical analysis of cholesterol and esterified cholesterol was accomplished by a method using

orthophthalaldehyde as described by Rudel and Morris (4).

Cholesteryl esters (100–200  $\mu$ g) for GLC analysis were isolated by thin-layer chromatography on silica gel H using a solvent system consisting of hexane-diethyl ether-acetic acid 75:25:1 (v/v/v). The cholesteryl esters were eluted from the silica gel with 10 ml of chloroform and 8-ml aliquots of the chloroform were dried under nitrogen at 60°C. Methyl esters of the cholesteryl ester fatty acids were prepared by a slight modification of the method of Glass (5). Cholesteryl esters were dissolved in 2 ml of methanol-benzene 60:40 (v/v) containing 4 g/dl of sodium hydroxide and 15 mg/dl of phenolphthalein and incubated at 45°C for 60 min. After incubation, the solution was neutralized by addition of 1 ml of 2N methanolic-HCl, 1.5 ml of water was added, and the methyl esters were extracted with 5 ml of hexane. The methyl esters were analyzed using a Bendix Model 2500 gas-liquid chromatograph equipped with a flame ionization detector. A silanized glass column (2 m  $\times$  4 mm) of 10% EGSS-Gas Chrom P (100–120 mesh) was used at a column temperature of 180°C. Conditions used were patterned after the method of Kuksis (6).

## RESULTS

A typical chromatogram of a mixture of cholesterol and cholesteryl ester standards is shown in **Fig. 1**.



**Fig. 2.** The relationship between peak area and mass of individual cholesteryl ester standards. The lines represent right to left: ---- cholesteryl myristate (●), cholesteryl palmitate (▲), cholesteryl palmitoleate (■), cholesteryl heptadecanoate (○), cholesteryl oleate (△), cholesteryl stearate (□), — free cholesterol (●), cholesteryl linoleate (▲), cholesteryl linolenate (■), and cholesteryl arachidonate (⊗).

The mixture contains 60  $\mu\text{g}$  of cholesterol, cholesteryl linoleate, and cholesteryl palmitate, 50  $\mu\text{g}$  of cholesteryl palmitoleate, cholesteryl oleate, cholesteryl heptadecanoate, and cholesteryl erucate, 10  $\mu\text{g}$  of cholesteryl linolenate, and 5  $\mu\text{g}$  of cholesterol arachidonate in an injection volume of 50  $\mu\text{l}$ . Relative to these amounts, the data in Fig. 1 suggest that the ultraviolet absorption of cholesterol and cholesteryl esters is dependent on both the length and the degree of unsaturation of the fatty acid in the ester. To allow quantitation of cholesterol and cholesteryl esters in samples, standard curves of mass versus peak area were prepared for each compound as shown in Fig. 2. The linear portion of each of the curves was from 0–100  $\mu\text{g}$ . The apparent extinction coefficients for cholesterol and for a number of cholesteryl esters over the concentration range of 0–100  $\mu\text{g}$  are shown in Table 1. The correlation coefficients for the lines that the extinction coefficients represent were greater than 0.99 over this concentration range. The reproducibility of retention volumes over a series of 26 sample injections was measured and the coefficient of variation ranged from 1.97% for free cholesterol to 2.23% for cholesteryl stearate.

The applicability of the HPLC assay method for cholesteryl ester determinations in biological samples was evaluated using total lipid extracts of isolated serum lipoproteins. Samples from animals fed either butter or safflower oil as dietary fat were examined so that the two methods could be compared on samples with markedly different cholesteryl ester compositions. Chromatograms of total lipid extracts of LDL from animals fed butter or safflower oil are shown in Figs. 3 and 4. The chromatograms indicate that marked changes in cholesteryl ester composition were produced by the change in dietary fat. Cholesteryl oleate and the more saturated esters were higher in butter-fed animals and cholesteryl linoleate predominated in safflower oil-fed animals. Marked changes were also produced in other peaks (U) with

TABLE 1. Apparent extinction coefficients for cholesterol and cholesteryl esters at 213 nM

Compound	$\mu\text{g}/\text{unit area} \times 10^{-3}$
Cholesterol	2.58
Cholesteryl myristate	3.83
Cholesteryl palmitate	3.98
Cholesteryl palmitoleate	3.17
Cholesteryl heptadecanoate	4.14
Cholesteryl stearate	4.29
Cholesteryl oleate	4.60
Cholesteryl linoleate	2.20
Cholesteryl linolenate	0.68
Cholesteryl arachidonate	0.51

Values in the table represent slopes of linear regression lines over the concentration ranges described in Methods.

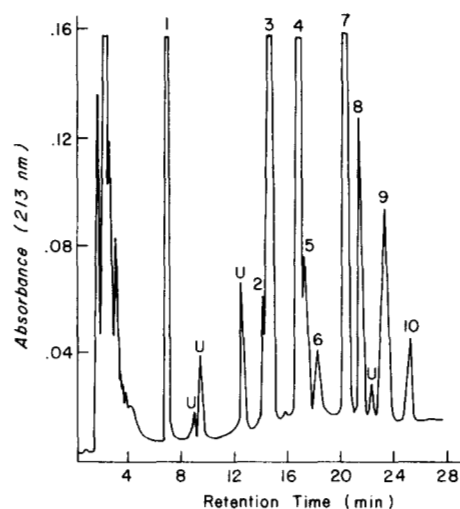


Fig. 3. HPLC separation of unesterified cholesterol and cholesteryl esters derived from lipid extracts of LDL. Plasma lipoproteins were isolated from a fasted adult African green monkey maintained on a diet containing 40% of calories as butter fat. Lipids were extracted with chloroform–methanol 2:1, and an aliquot containing approximately 200  $\mu\text{g}$  of total cholesterol was prepared and chromatographed as described in Methods. The identification of peaks 1–10 are the same as those for Fig. 1. Peaks labeled U contain cholesterol but have not been identified.

changes in diet, although these compounds have not yet been identified. The cholesteryl ester compositions obtained by HPLC were compared with those obtained by GLC analysis of cholesteryl ester fatty acids to assure the accuracy of the technique. The comparison (Table 2) of the HPLC and GLC methods

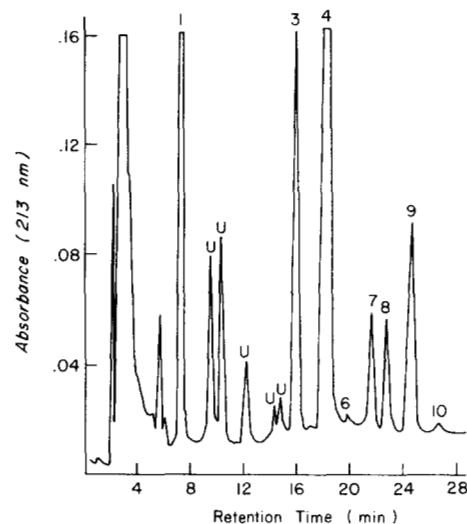


Fig. 4. HPLC separation of unesterified cholesterol and cholesteryl esters derived from lipid extracts of LDL. Plasma lipoproteins were isolated from a fasted adult male African green monkey maintained on a diet containing 40% of calories as safflower oil. Lipids were extracted with chloroform–methanol 2:1, and an aliquot containing approximately 200  $\mu\text{g}$  of total cholesterol was prepared and chromatographed as described in Methods. The identification of peaks 1–10 is the same as those for Fig. 1. Peaks labeled U contain cholesterol but have not been identified.

TABLE 2. Comparison of cholesteryl ester percentage compositions of standards and LDL samples determined by GLC and HPLC

	N	Fatty Acid	Cholesteryl Ester Percentage (w/w)							
			14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Standards	3	GLC	17.06 ± 0.95	12.33 ± 0.08	10.54 ± 0.25	11.26 ± 0.20	11.42 ± 0.42	11.13 ± 0.25	8.72 ± 0.16	17.64 ± 0.44
		HPLC	19.85 ± 1.39	12.50 ± 0.35	10.63 ± 0.07	11.11 ± 0.37	10.83 ± 0.44	10.55 ± 0.10	8.25 ± 0.29	16.28 ± 0.11
Saturated Fat	12	GLC	1.94 ± 0.14	15.23 ± 0.54	7.12 ± 0.45	5.60 ± 0.34	31.59 ± 1.02	37.35 ± 1.18	0.53 ± 0.16	1.40 ± 0.47
		HPLC	4.85 ± 0.50	13.26 ± 0.50	6.44 ± 2.04	5.67 ± 0.51	31.26 ± 0.81	35.07 ± 2.05	0.87 ± 0.04	4.95 ± 0.42
Unsaturated Fat	12	GLC	N.S.	8.13 ± 0.43	N.S.	1.32 ± 0.05	9.25 ± 0.50	79.98 ± 0.90	N.S.	0.80 ± 0.29
		HPLC	N.S.	7.58 ± 0.30	N.S.	0.74 ± 0.08	10.09 ± 0.46	79.17 ± 0.72	N.S.	1.80 ± 0.27

Values in the table represent mean ± SEM. The values for the saturated and unsaturated fat groups were determined using lipid extracts of LDL isolated from plasma obtained from fasted adult male African green monkeys. The saturated and unsaturated diets contained 40% of calories as butter fat and safflower oil, respectively. N.S. indicates that the amount of ester present in the sample was too low for accurate quantitation.

for determining cholesteryl ester compositions using the same standards and lipid extracts of lipoproteins from butter- and safflower oil-fed animals shows that both methods give similar results. Comparability in percentage distribution among the main cholesteryl ester components was generally within 1%. Higher values for cholesteryl arachidonate and cholesteryl myristate were obtained in butter-fed animals by the HPLC method.

The separation afforded by this method also permits the quantitation of cholesterol and cholesteryl esters, which was compared to the cholesterol and cholesteryl ester quantitation obtained by chemical analysis, **Table 3**. Aliquots of the same lipid extracts from 16 different LDL samples were taken for analysis by both methods. Agreement was excellent between the methods as indicated by comparison of mean, range, and standard error; no statistically significant differences were found between the results obtained by the two methods.

## DISCUSSION

As indicated in Fig. 1, good resolution of cholesterol and individual cholesteryl esters was obtained in approximately 30 min. The operating conditions used in this study were chosen to minimize analysis time while giving adequate resolution of individual esters. Further resolution of cholesteryl linolenate and arachidonate can be obtained by increasing the water content of the eluting solvent; however, small increases in water concentration greatly increase the analysis time. The data in Fig. 2 indicate that good linearity was found over the range of 0–100 µg for individual cholesteryl esters. It was difficult to obtain reproducibility for the peak areas containing less than 10 µg of the saturated esters. It appears feasible to quantitate amounts in the 100–400 µg range as long as the lack of linearity is taken into account. Above the 400 µg level, the saturated cholesteryl esters such as cholesteryl stearate were insoluble in the injection

TABLE 3. Comparison of cholesterol values obtained by HPLC and *O*-phthalaldehyde assay procedures

	N	<i>O</i> -phthalaldehyde			HPLC		
		Mean	Range	SEM	Mean	Range	SEM
Free cholesterol (µg)	16	24.58	13.72–36.48	1.53	25.40	15.62–36.47	1.46
Ester cholesterol (µg)	16	85.30	50.48–129.22	5.73	85.65	49.12–124.48	5.77
Total cholesterol (µg)	16	109.87	64.20–165.70	7.15	111.05	64.74–157.88	7.15

Values in the table represent assays of plasma LDL samples from fasted adult African green monkeys. The ester cholesterol values for the HPLC procedure were obtained by dividing the sum of the individual cholesteryl ester masses by 1.7 to convert cholesteryl ester to ester cholesterol.

solvent. For some of the LDL samples from animals fed the butter diet, individual cholesteryl esters were recovered after the HPLC separation and were subjected to further analysis by GLC and infrared spectroscopy. The results indicated that the material eluted in individual peaks (peak 2 to peak 10) contained pure cholesteryl ester. Some lipoprotein samples from butter-fed animals contained small amounts of cholesteryl elaidate. When present, cholesteryl elaidate elutes just after cholesteryl oleate and appears as a shoulder on the cholesteryl oleate peak.

The absorbance of the cholesteryl esters at 213 nm varies with chain length and the number of double bonds in the fatty acid side chain. We chose 213 nm for detection of cholesterol and cholesteryl esters because this wavelength is just above the UV cutoff of tetrahydrofuran. Attempts to substitute solvents with lower UV cutoffs such as hexane, cyclohexane, heptane, and isopropanol for tetrahydrofuran did not yield adequate separation of the esters.

Chromatograms of total lipid extracts of LDL from animals fed either butter (Fig. 3) or safflower oil (Fig. 4) indicate that good resolution of cholesterol and cholesteryl esters is obtained from biological samples. This occurred in situations in which the cholesteryl ester compositions were quite different, indicating the utility of the method. In addition to the samples shown here, we have chromatographed total lipid extracts of plasma, liver, lymph chylomicrons, and lymph VLDL. The limiting factor in obtaining suitable quantitation of cholesterol and cholesteryl esters directly from biological samples is the triglyceride concentration. Triglycerides interfered with quantitation from total lipid extracts, and these samples required separation of the cholesteryl esters and triglycerides prior to HPLC analysis of cholesteryl esters. The cholesteryl ester patterns and the types of changes in the pattern that we have seen using HPLC have been analogous to those reported in the literature (7).

In summary, data has been presented showing that HPLC provides a simple and rapid method for the preparative separation and quantitation of cholesterol and individual cholesteryl esters from biological samples. ■■

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