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DETERMINATION OF FREE, TOTAL, AND ESTERIFIED CHOLESTEROL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for measuring free, total, and esterified cholesterol in blood serum in which reversed-phase liquid chromatography is used and the eluate is monitored at 200 nm. The sample for total cholesterol is prepared according to the Abell-Kendall procedure, and for free cholesterol an extract of serum—isopropanol (1:5, v/v) is used. The column is a μBondapak C_{18}, 10 μm, and the mobile phase for total cholesterol is isopropanol—acetonitrile (50:50, v/v); for free cholesterol, it is isopropanol—acetonitrile—water (60:30:10). An approximation of the free cholesterol, triglycerides, and individual cholesteryl esters is obtained from single chromatograms of isopropanol extracts of serum if the first mobile phase is used. In a comparison study with the Abell-Kendall method for total cholesterol, the correlation is excellent and the precision is acceptable.

INTRODUCTION

Measuring serum cholesterol is one of the most frequently performed assays in the clinical laboratory, for which a wide variety of methods [1] are available and used. The classical method is a colorimetric assay based on the photometric measurement of the color formed when cholesterol reacts with a Lewis acid [2—4]. However, because of the hazards associated with using the strong acid medium in which the color is formed and the increasing emphasis on measuring both the free and esterified cholesterol, alternate methodologies have been and are being developed. Most of the newer methods are based on enzymatic hydrolysis and oxidation [5—7] or on chromatographic analysis [8—15]. The enzymatic reactions are followed by colorimetric [6] or electrochemical analysis [7], and the chromatography may be either gas—liquid [8—12] or thin-layer [13—15].

Although liquid chromatography (LC) has been used in analyses of lipids in general [16], its specific application in cholesterol methodology has been limited because cholesterol and related compounds absorb very little UV radiation in the wavelength range in which most UV detectors used in LC operate. Consequently, LC has been used in assays in which column chromatography is followed by chemical analysis of the collected chromatographic fractions [17—19] or in which the eluate is monitored on-line with other types of detectors. These detectors include the refractive index detector [20, 21], the moving-wire flame-ionization detector [22—25], and a laser infrared detector [26]. However, there are problems associated with these instruments, including slow analysis time, and lack of sensitivity.

With recent advances in LC-detector technology, high-performance spectrophotometers with low-volume flow cells are now commercially available that allow chromatographic eluates to be monitored at wavelengths as low as 200 nm. By coupling a high-performance reversed-phase chromatographic column and such a detector and by eluting with the appropriate solvents, we are now able to monitor photometrically at 200 nm and to measure cholesterol as it is eluted from the chromatographic column. In this paper, we describe the development and evaluation of an LC procedure to determine free and total cholesterol in blood serum and to calculate esterified cholesterol as the difference between them. This technique may also perhaps be used to measure individual cholesteryl esters.

MATERIALS AND METHODS

*Reagents**

Standard Reference Material (SRM 911a) cholesterol was purchased from the National Bureau of Standards (NBS) (Office of Standard Reference Materials, Washington, D.C. 20234). The cholesteryl esters were purchased from Sigma St. Louis, Mo., U.S.A.; the glycerides from P-L Biochemicals, (Milwaukee, Wisc., U.S.A.). Spectrophotometric grade isopropyl alcohol (IPA) and acetonitrile (CH_3CN) were obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.). Water was deionized, distilled in a glass apparatus, and filtered through a membrane with pores of 0.22 μm.

Preparation of standard solutions

A stock solution of cholesterol standard (10.00 g/l; 25.86 mmole/l) was prepared by dissolving 100.0 mg of the NBS cholesterol in 10 ml of isopropanol (IPA). The solution was then dispensed in 1-ml aliquots into small (1.7 ml) glass vials with Teflon™-lined caps and stored at −13°. Working standards were prepared from an aliquot of the stock solution which was thawed and diluted as necessary to cover the desired range of cholesterol concentrations. Stock solutions of each of the standards containing cholesteryl esters were prepared by dissolving a given quantity of ester in a specified volume of IPA: cholesteryl arachidonate, 5 mg/5 ml (1.00 g/l; 1.485 mmole/l); cholesteryl

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

linoleate, 5 mg/5 ml (1.00 g/l; 1.541 mmole/l); cholestery oleate, 5 mg/10 ml (0.50 g/l, 0.768 mmole/l); and cholesteryl palmitate, 5 mg/10 ml (0.50 g/l; 0.800 mmole/l). A working solution of standards of cholesterol and the esters was prepared by combining the required volume of each of the stock solutions and diluting to 875 μl with IPA so that this final volume contained the following concentrations: cholesterol, 0.12 g/l (0.310 mmole/l); cholesteryl arachidonate, 0.07 g/l (0.103 mmole/l); cholesteryl linoleate, 0.30 g/l (0.462 mmole/l); cholesteryl oleate, 0.10 g/l (0.154 mmole/l); and cholesteryl palmitate, 0.04 g/l (0.080 mmole/l). A 50-μl aliquot of this solution was then injected onto the column for a chromatographic analysis of the standards.

Samples

Frozen serum from individual patients and from serum pools was obtained from the Metabolic Biochemistry Branch of the Clinical Chemistry Division at the Centre for Disease Control (CDC). The pooled samples have been used in standardization programs and cover a wide range of cholesterol concentrations. Fresh samples of serum were obtained from patients visiting the CDC Public Health Service Clinic.

Sample and standard preparation

For the total cholesterol determination, serum was saponified and extracted according to the Abell-Kendall (AK) method [4]. Five ml of alcoholic KOH (6 ml of 33% KOH in H_2O, diluted to 100 ml with absolute alcohol) was added, with a Cornwall syringe, to 0.5 ml of serum in a 20 × 150 mm glass test tube equipped with a Teflon™-lined cap. The tubes were incubated at 45° for 60 min and then allowed to cool to room temperature. After the addition of 5 ml of water to each tube, it was cooled to room temperature, 10 ml of hexane were added to each, and the tubes were shaken mechanically for 10 min. A 4-ml aliquot of the hexane layer was pipetted into a 16 × 130 mm glass test tube and evaporated in a 45° oven at a reduced pressure of 20—28 p.s.i. The residue was then dissolved in 800 μl IPA and the extract used for sampling for injection onto the column of the chromatograph. In the comparison studies, a 4-ml aliquot of the hexane extract was also evaporated for analysis by the AK method.

For the free cholesterol determination, 100 μl of serum was vortexed with 500 μl of IPA for 2 min, centrifuged, and the supernatant removed. This supernatant was used for sampling for the chromatographic analysis of free cholesterol and to obtain a profile of the individual cholesteryl esters and other lipids.

The standards for determining total cholesterol were prepared by adding 5 ml of alcoholic KOH to 0.5 ml of each of the standards, which contained 1.00, 2.00, 3.00 and 4.00 g cholesterol per l (respectively, 2.60, 5.17, 7.76 and 10.34 mmole cholesterol per l), and then incubating, extracting, evaporating, and dissolving the residue as described above. IPA dilutions of the stock standard containing cholesterol were used in measurements of free cholesterol.

Chromatographic system

The system used for the chromatographic analyses included a Varian Model

4200 liquid chromatograph (Varian, Walnut Creek, Calif., U.S.A.) equipped with a Waters Model U6K injector (Waters Assoc., Milford, Mass., U.S.A.); a Waters μBondapak C_{18}, 10 μm, reversed-phase column (30 cm × 4 mm I.D.) preceded by a precolumn (5 cm × 2 mm I.D., packed with Waters C_{18}/Porasil B, 37–75 μm); a Varian Vari-Chrom variable wavelength detector; and a Varian Model A-25 recorder. A Varian Model CDS 101 integrator was used to record the peak area electronically from the detector signal.

Different mobile phases were used for the chromatographic measurement of total and free cholesterol. To measure total cholesterol, an isopropanol—acetonitrile (50:50, v/v) mobile phase (Solvent A) was used and isopropanol—acetonitrile—water (60:30:10, v/v) mobile phase (Solvent B) for nonesterified (free) cholesterol. Solvent A was used also to obtain a profile of cholesterol, triglycerides, and cholesteryl esters.

Procedures

Chromatography. To measure total and free cholesterol, respectively, aliquots of either the AK (30 μl) or IPA (50 μl) extracts were injected onto the chromatographic column. Cholesterol was eluted at a flow-rate of 1 ml/min and a pressure of either 600–1000 p.s.i. (Solvent A) or 1500 p.s.i. (Solvent B), and dectected by monitoring the column eluate at 200 nm. The cholesterol was quantitated by comparing the peak areas (expressed as counts and printed by the integrator) of the eluted cholesterol of the sample with those of the cholesterol standards. The standards and the samples were analyzed during the same half-day.

Calibration. Aliquots of either the AK (30 μl) or IPA (50 μl) extracts of the cholesterol working standards were chromatographically analyzed with Solvent A (Solvent B for free cholesterol determination), and a peak area measured for each of the cholesterol standards. A response factor was obtained from either the 3.00 g/l (7.76 mmole/l) standard or from the reciprocal of the slope of the standard curve.

Total cholesterol. A 30-μl aliquot of each of the AK extracts was chromatographically analyzed with Solvent A, and the resultant cholesterol peak area was multiplied by the appropriate factor to obtain the total cholesterol content of each sample. Although either Solvent A or B can be used as the eluting solvent, Solvent A is preferable because the analysis can be done more quickly with it.

Comparison of HPLC method with the AK method. Several of the serum pools were assayed for their total cholesterol content by the HPLC method and a manual version of the AK method (MAK). An AK extract was prepared for each sample and analyzed in duplicate with both methods.

Free cholesterol. A 50-μl aliquot of the IPA extract of unsaponified serum was analyzed chromatographically with Solvent B, and the cholesterol peak area was multiplied by the appropriate response factor to obtain the free cholesterol content of each sample. Between chromatographic runs, 1 ml of IPA was injected to remove other lipids, and the column was equilibrated with Solvent B for at least 10 min.

Esterified cholesterol. To determine the esterified cholesterol content, both the total and free cholesterol content of each sample were chromatographically

measured, and the esterified portion was calculated as the difference between the two. An approximation of the free and specific ester content of a serum sample can be obtained by comparing the chromatogram of a single extract of the serum with Solvent A and that of the standard containing cholesterol and cholesteryl esters. We call such a chromatogram a lipid profile.

Lipid profiles. Fifty μl of the IPA extract of (unsaponified) serum was chromatographically analyzed with Solvent A. Fifty μl of the standard containing cholesterol and cholesteryl esters was analyzed on the same day. The eluates from several analyses of serum were collected in fractions corresponding to the peaks recorded on the chart. These fractions were analyzed for cholesterol (Liebermann-Burchard reaction) and triglycerides (periodic acid oxidation followed by diacetyl acetone and ammonia) with the Technicon AutoAnalyzer II (AAII) [27] and with a direct-probe mass spectrometer.

RESULTS AND DISCUSSION

Fig. 1 illustrates a series of typical chromatograms obtained when the standard solutions and sera were analyzed by high-performance liquid chromatography (HPLC) for their total cholesterol content. Chromatograms A and B are replicates obtained from the 3.00 g/l (7.76 mmole/l) cholesterol standard, and peaks C—F represent four samples of serum which contained 2.65, 3.91, 4.00, and 1.53 g/l (6.86, 10.12, 10.35, and 3.96 mmole/l, respectively) of total cholesterol.

As demonstrated in Fig. 1, approximately 8 min are required per chromatographic run, with cholesterol being cleanly resolved and eluted in approximately 6 min.

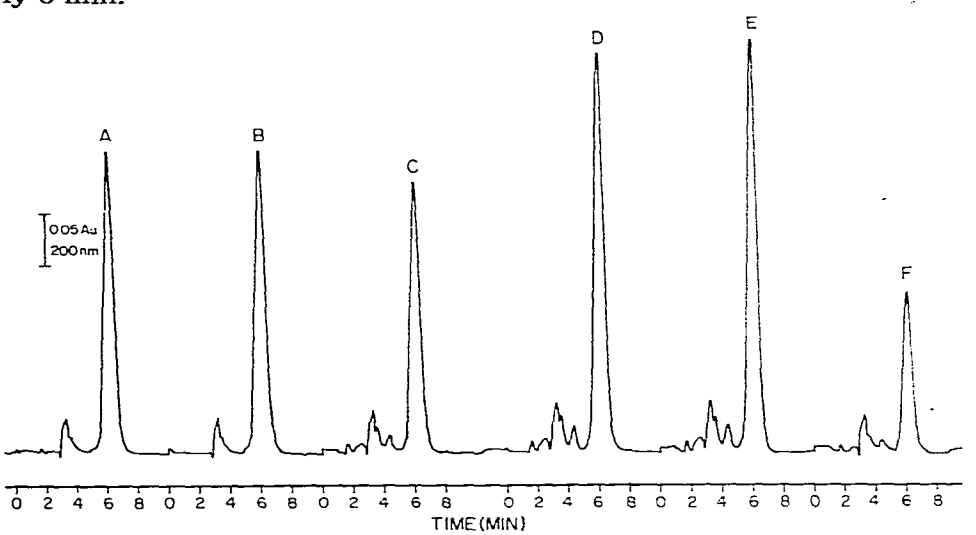

Fig. 1. Separation and measurement of total serum cholesterol by HPLC. Chromatograms A and B represent 3.0 g/l (7.76 mmole/l) cholesterol standards. Chromatograms C, D, E, and F represent samples of serum that contain 2.65, 3.91, 4.00, and 1.53 g/l (6.8, 10.12, 10.35, and 3.96 mmole/l) of total cholesterol, respectively. Chromatographic conditions: mobile phase: IPA—CH_3CN (50:50, v/v); pressure, 500 p.s.i.; flow-rate, 1 ml/min; 0.5 a.u.f.s.; band width, 8 nm; wavelength, 200 nm; column, μBondapak C_{18}; injection volume, 30 μl.

When peak areas expressed as integrator counts are plotted against the quantity of cholesterol injected or as the equivalent cholesterol concentration expressed as mg/dl, the data indicate that the method is linear over a concentration range of 0.25—5.00 g/l (0.65—12.93 mmole/l) of cholesterol. The lower limit of detection with the method is 0.25 μg of cholesterol at a detector sensitivity of 0.1 a.u.f.s. This is equivalent to a serum cholesterol concentration of 0.03 g/l (0.008 mmole/l).

The reproducibility of the HPLC method for measuring total cholesterol is shown in Tables I and II in which are summarized the precision data obtained

TABLE I

REPRODUCIBILITY OF CHROMATOGRAPHIC DETERMINATION OF TOTAL SERUM CHOLESTEROL: CHOLESTEROL STANDARDS WITH AND WITHOUT SAPONIFICATION AND EXTRACTION

	Cholesterol concentration (mg/dl)	Precision			
		Within-day		Day-to-day	
		n	C.V. (%)	*n*	C.V. (%)
Standards in IPA	100	5	1.58		
	200	5	0.65	15*	1.24
	300	5	0.55		
	400	5	0.45		
Standard processed by saponification and extraction	300	8	1.62	50**	2.02

*Three days of five replicate analyses each.
**Ten days of five replicate analyses each.

TABLE II

COMPARISON OF ANALYTICAL RESULTS OBTAINED BY ANALYZING SERUM POOLS FOR TOTAL CHOLESTEROL CONTENT BY BOTH HPLC AND MANUAL AK METHODS

n = number of days × 2 (duplicate samples were run each day).

Pool number	*n*	Analytical Results			
		HPLC		Manual AK	
		Cholesterol concentration (mg/dl)	Precision (C.V.%)	Cholesterol concentration (mg/dl)	Precision (C.V.%)
1	20	163	2.94	158	1.90
2	20	250	2.52	250	1.48
3	20	295	1.68	297	2.38
4	12	135	2.72	132	1.63
5	12	185	2.49	181	1.37
6	12	239	2.70	236	2.26
7	12	282	2.35	284	1.04
8	12	331	2.70	336	1.15
Mean		235	2.51	234	1.65

for standard cholesterol solutions and serum pools, respectively. In Table I, data are included for cholesterol standards analyzed directly with HPLC and for standards that had been processed prior to HPLC by the AK saponification and extraction procedures. With this data, we estimated not only the imprecision resulting from the chromatography, but also the combined error resulting from sample processing plus chromatography. As shown in Table I, the within-day imprecision of the chromatography step is inversely related to cholesterol concentration and ranges from 0.45% (expressed as coefficient of variation, C.V.) at a cholesterol concentrationof 4.00 g/l (10.35 mmole/l) to 1.58% at the 1.00 g/l (2.59 mmole/l) level. Day-to-day imprecision of the chromatography step is slightly higher than within-day variation (e.g., 1.24% vs. 0.65% at the 2.00 g/l (5.17 mmole/l) level. As expected, there were slight increases in analytical variation when the standards were processed by the saponification and extraction procedures prior to HPLC analysis. Table II indicates that the day-to-day variation of the AK method (mean C.V. = 1.65%) was slightly less than that of the HPLC method (mean C.V. = 2.51%).

For comparison purposes, five pooled serum samples were assayed for their total cholesterol content with both the HPLC and AK methods. As demonstrated in Fig. 2, the results obtained from the HPLC and the AK methods correlate (r = 0.998) and compare well. The data in Table II also indicate good correlation (r = 0.9997). Table III shows the results of HPLC determination of the free and total cholesterol in 20 samples of individual human sera. The

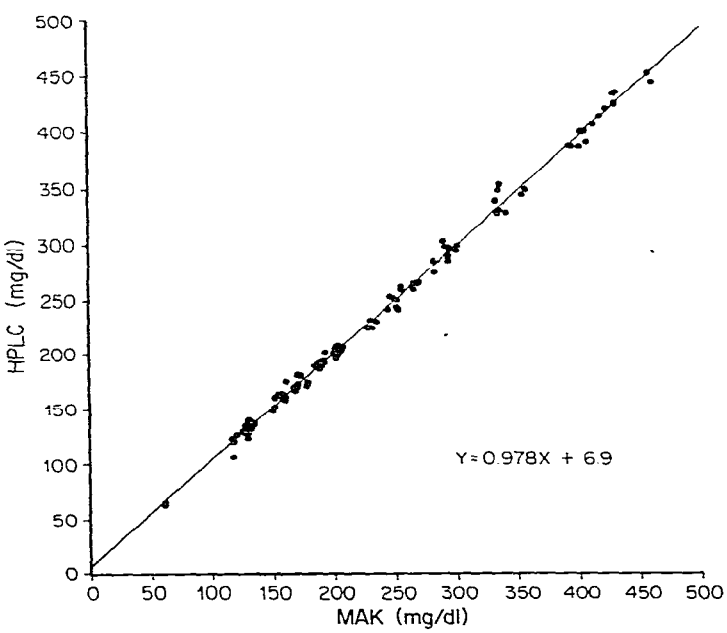

Fig. 2. Correlation between analytical values obtained for HPLC and AK analyses of human serum samples for their total cholesterol content. The 102 points represent duplicate analyses of equal aliquots of 51 samples of serum. Correlation coefficient = 0.998; standard error of estimate = 5.96.

TABLE III

RELATIVE PERCENTAGE OF FREE AND ESTERIFIED CHOLESTEROL IN HUMAN SERA DETERMINED BY HPLC

Sample No.	Cholesterol content (mg/dl)		Free (%)	Esterified (%)
	Total*	Free**		
1	299	61	20.4	79.6
2	299	56	18.7	81.3
3	230	54	23.5	76.5
4	314	65	20.7	79.3
5	279	56	20.1	79.9
6	131	27	20.6	79.4
7	205	37	18.0	82.0
8	202	62	30.6	69.4
9	217	64	29.5	70.5
10	237	58	24.5	75.5
11	355	96	27.0	73.0
12	217	59	27.2	72.8
13	230	58	25.2	74.8
14	158	36	22.8	77.2
15	192	45	23.4	76.6
16	194	48	24.7	75.3
17	242	56	23.1	76.9
18	297	78	26.3	73.7
19	301	84	27.9	72.1
20	242	66	27.9	72.1
Mean	242	58	24.1	75.9
Range	131—355	27—96	18—31	69—82

*Mobile Phase A.
**Mobile Phase B.

esterified cholesterol was calculated as the difference between the two. The percentage of free cholesterol relative to the total amount of cholesterol ranged from 20—31%, which is a range comparable to that of 17—39% reported in previous studies [13, 28].

Several compounds interfere in various cholesterol analyses [1]. For example, bilirubin interferes in the Liebermann-Burchard method [2, 3] if it is not removed before colorimetric analysis is performed, and it interferes also in the enzymatic method [1]. When we used the HPLC method, we found bilirubin did not interfere because it was removed in the AK extraction for the total cholesterol, and it was eluted from the column in the void fraction (3 min) before the cholesterol was eluted (6 min) in the free cholesterol determination. We found that vitamins A and D also did not interfere because both were eluted before cholesterol.

Steroids with a structure similar to cholesterol which are also potential interferences include: 5-cholestan-3β-ol (cholestanol), 5-cholest-7-ene-3β-ol (Δ7-cholestenol) and cholesta-5,7-diene-3β-ol (7-dehydrocholesterol). Cholestanol does not interfere in the HPLC method since it lacks the double bond present in cholesterol and thus would not absorb and be detected at 200 nm. In Solvent A, the latter two steroids co-elute with cholesterol and thus could inter-

fere in the measurement of total cholesterol. However, with this technique and appropriate solvents the steroids can be resolved.

Fig. 3 is representative of a series of chromatograms obtained from the HPLC analysis of unsaponified serum with Solvent A. Chromatogram I was obtained from the standard solution of cholesterol and cholesteryl esters, and chromatograms II and III were obtained from two serum samples. Approximately 20 min were required per chromatographic run with the compounds eluting in the following order: cholesterol (B), cholesteryl arachidonate (E), cholesteryl linoleate (F), cholesteryl oleate (G), and cholesteryl palmitate (H). There was a very small peak after the palmitate which co-chromatographed with cholesteryl stearate. Triglycerides in the eluates corresponded to peaks C and D and contributed also to peak E (cf. Fig. 4). Cholesterol was not resolved well with Solvent A if triglycerides were present in relatively high concentration, but Solvent B (which did resolve cholesterol and was used when free cholesterol was measured) did not produce satisfactory chromatograms of the cholesteryl esters.

Fig. 4 shows a chromatogram of an analysis similar to those in Fig. 3 but with bar graphs above the chromatogram which illustrate the percentage of glyceride in each fraction relative to the total amount of glycerides and the percentage of cholesterol in each fraction relative to the total amount of cholesterol. The known compounds that co-chromatographed with the fractions are as in Fig. 3. Direct-probe mass spectrometry of fractions collected during the chromatography of serum showed the molecular ion for cholesterol

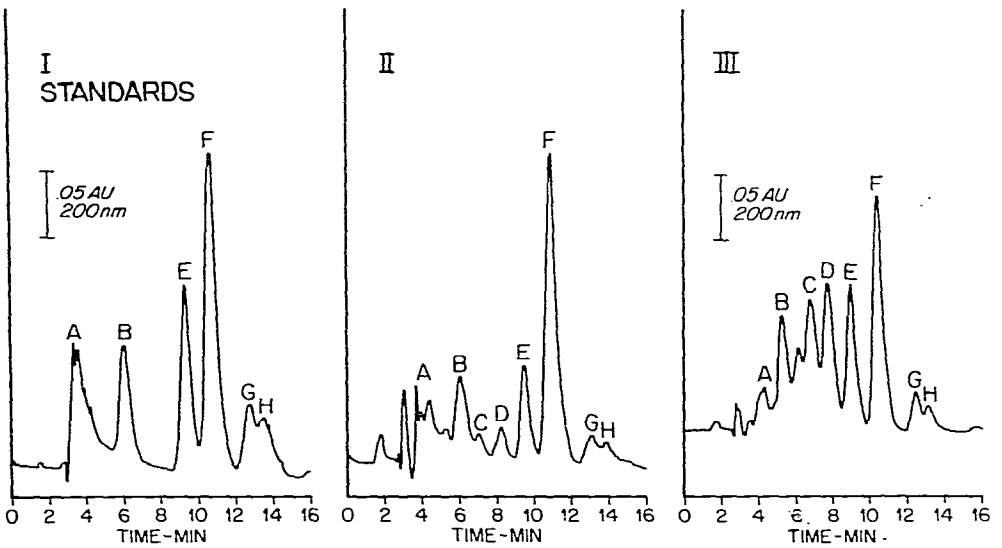

Fig. 3. Separation of cholesterol, triglycerides, and cholesteryl esters by HPLC. Chromatogram I represents a standard solution containing known quantities of the following materials in the 50-μl injection: cholesterol, 6 μg (B); cholesteryl arachidonate, 3.5 μg (E); cholesteryl linoleate, 15 μg (F); cholesteryl oleate, 5 μg (G); and cholesteryl palmitate, 2.5 μg (H). Chromatograms II and III represent 50 μl of 1:5 serum—IPA extracts. Peaks C and D represent unidentified glycerides and peak E represents unidentified glycerides and cholesteryl arachidonate. Chromatographic conditions are the same as those listed in Fig. 1.

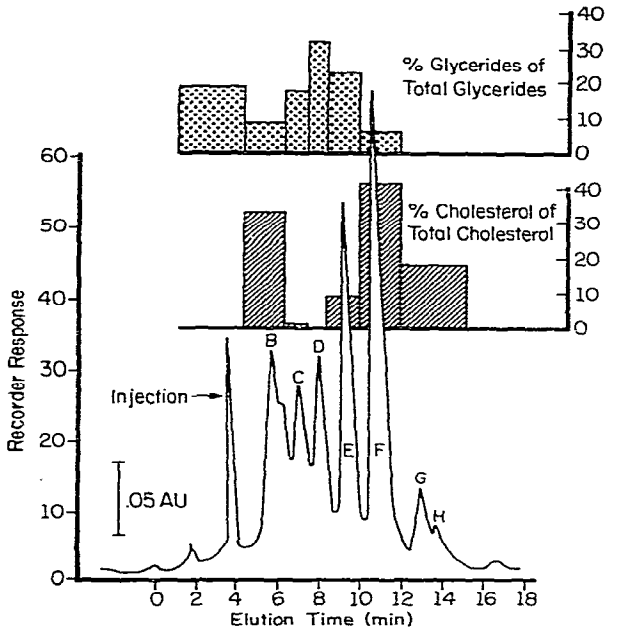

Fig. 4. Comparison of the chromatogram of 50 μl of a 1:5, serum—IPA extract with the analysis of the fractions of the eluate for triglycerides and cholesterol. The peaks are cholesterol (B); glycerides (C,D); glycerides and cholesteryl arachidonate (E); cholesteryl linoleate (F); cholesteryl oleate (G); and cholesteryl palmitate (H). Bar graphs above the chromatogram indicate the percentage of glycerides in the corresponding peak relative to the total amount of glycerides in all the peaks and the percentage of cholesterol in the corresponding peak relative to the total amount of the cholesterol in all the peaks. The bar widths represent the fraction collected during the elution of the underlying peaks. Chromatographic conditions are the same as in Figs. 1 and 3.

in fraction (peak) B, and that the cholesteryl esters in fractions (peaks) F, G, and H were, respectively, cholesteryl linoleate, cholesteryl oleate, and cholesteryl palmitate. Specific glycerides were not identified in peaks C, D, and E, but triolein and 1,2-dipalmitoylolein, which are eluted about the same time as the components of fraction E, do not co-chromatograph with any of the fractions collected from the chromatographic analysis of human serum. The approximately 20% of the total glycerides which (top bar graph, Fig. 4) is eluted before cholesterol is presumably a phospholipid-containing fraction, because we did not use the Zeolite-Lloyd reagent [27] in our glyceride analyses. Monoglycerides and diglycerides were also eluted before cholesterol.

In order to obtain a semiquantitative lipid profile of a serum, we compared the peak areas in a chromatogram (as II and III, Fig. 3) with the areas in a chromatogram of a standard (as I, Fig. 3). Cholesteryl oleate and palmitate concentrations thus determined ranged from 0.09 to 0.31 g/l (mean 0.25) and from 0.04 to 0.11 g/l (mean 0.09), respectively. A similar quantitation for cholesteryl linoleate indicated a range of 0.51—1.86 g/l (mean 1.35), probably heightened by the approximately 8% of the triglycerides present in this fraction (Fig. 4). The observed values for these cholesteryl esters were comparable to

those reported for the distribution of fatty acids in cholesteryl esters from human sera [28—30]. The values for free cholesterol similarly determined (Fig. 3) ranged from 0.26 to 0.96 g/l (mean 0.6 g/l) and were similar to those obtained with the HPLC method (Solvent B) for free cholesterol except that sera with high levels of triglycerides had erroneously high values for free cholesterol because the triglycerides were resolved poorly with Solvent A.

The HPLC method provides analytical results which compare favorably with those obtained with the widely used AK method and yields information about free, total, and esterified cholesterol from one easily prepared extract. Concentrated acid reagents are not used with HPLC and the metabolite is not destroyed and can be recovered for further study. An additional advantage is that with even as little as 50 μl of serum it is possible to measure total, free, and esterified cholesterol.

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