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DETERMINATION OF FREE AND ESTERIFIED CHOLESTEROL IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND OPTICAL ACTIVITY DETECTION

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SUMMARY

A separation and detection scheme is presented for the determination of free, esterified and total cholesterol in human serum. Separation is accomplished by reversed-phase high-performance liquid chromatography and the eluate is monitored by the laser-based optical activity detector. The method is simple, accurate and has the advantage of specificity and selectivity when compared with the many methods commonly used.

INTRODUCTION

Cholesterol is a major component of all mammalian plasma membranes. Though it is vital to cell growth and survival [1, 2], there is a statistically significant correlation between elevated serum cholesterol levels and cardiovascular diseases in general and atherosclerosis [3] in particular. Cholesterol is found in two forms in the serum: either as free cholesterol, or esterified with long-chain fatty acids such as palmitic acid. Typically about 75% of the serum cholesterol is esterified [4]. This ratio has been shown to be related to hormones [5], diet [6], toxic conditions such as ethanol poisoning [7], and disorders like familial lecithin:cholesterol acyltransferase deficiency [8].

Numerous analytical procedures are available and are being used for the measurement of serum cholesterol because of its clinical significance. The most commonly used methods are photometric and are based on the color reaction of cholesterol with acid reagent [9]. The Libermann—Burchard method or a modification of it [10] has been the most popular. Disadvantages of the colorimetric methods include temperature dependence, light sensitivity, color instability as well as the manipulation of corrosive reagents [4]. The first step in the newer enzymatic method involves the catalyzed hydrolysis of th

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cholesterol esters by cholesterol ester hydrolase to generate free cholesterol for subsequent analysis [11]. This method is highly accurate but is mainly for total serum cholesterol determination.

Chromatographic methods are capable of separating free and esterified cholesterol. Gas—liquid [12, 13], thin-layer [14, 15], and, most recently, high-performance liquid chromatography (HPLC) using a variable-wavelength UV detector [16] are now available. Although cholesterol can be detected at 200 nm where double bonds and other functional groups absorb energy, the choice of solvents becomes severely limited due to the transparency requirement in this region. Also more interfering compounds would absorb and be detected at this short wavelength. A particularly serious problem is the interference from triglycerides [16]. This makes good separation and accurate quantitation difficult.

We describe here an alternative procedure for the reversed-phase HPLC separation and determination of free and esterified cholesterol as it is found in human serum by on-line monitoring of the optical rotation of the eluate. The combined use of simple extraction, direct sample injection, HPLC and optical activity detection offers a sensitive and specific method for the profiling and quantitative analysis of serum cholesterol and its esters.

EXPERIMENTAL

Chromatography

Separation was performed on a 25 cm × 4.6 mm, 10 μm C_{18} column (Alltech, Deerfield, IL, U.S.A.). Samples were eluted with a tetrahydrofuran—water (76:24, v/v) mobile phase at a flow-rate of 0.5 ml/min and a pressure of about 4.5 MPa. All injections were made through a 200-μl sample loop at a conventional injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7010). To reduce any pressure fluctuations caused by the pump (Milton Roy, Riviera Beach, FL, U.S.A., Model 196-0066-001) at the detector, we used a commercial pulse-dampener (Handy & Harman, Norristown, PA, U.S.A., Model Li-Chroma-Damp II) in conjunction with a pressure gauge (Alltech, Model 9228).

Materials

Cholesterol and cholesteryl esters were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade tetrahydrofuran was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Water was deionized locally. Leder Norm normal clinical chemistry control serum was obtained from Lederle Diagnostics (American Cyanamid, Pearl River, NY, U.S.A.). Reference serum for automated procedures was purchased from Sigma (Cat. No. R3626). The serums from the two commercial sources were reconstituted with diluents as instructed by the manufacturers. Fresh serum from a healthy individual was obtained locally. For each 2.4 ml of serum sample, 7.6 ml of tetrahydrofuran was added drop by drop with vigorous stirring. The mixture was then centrifuged for 20 min at 11,000 *g* in a clinical centrifuge. The clear and yellowish liquid phase was thus separated from the residue and was used as the sample without further treatment. The prepared samples were then injected directly using the chromatographic and detection conditions described in this section.

Optical activity detection

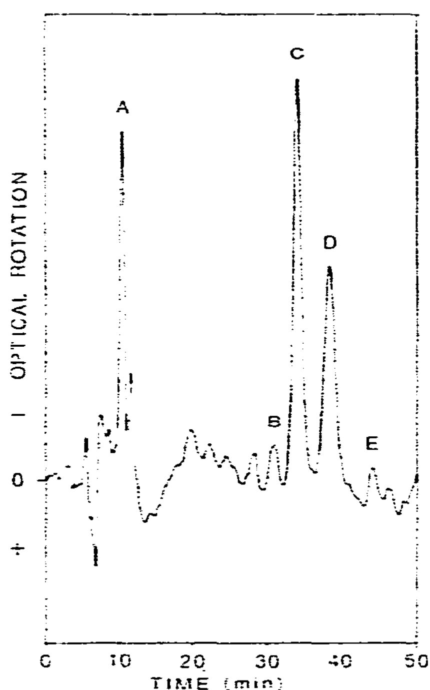
The basic arrangement for an optical activity detector for HPLC has been reported earlier [17, 18]. In this work the laser was operated at 514.5 nm. The laser power was maintained at about 20 mW at the flow cell by replacing one of the mirrors with a partially reflective optical flat. The system was operated without laser intensity stabilization. Instead, a longer time constant of 1.0 sec was used at the lock-in-amplifier without sacrificing performance. A flow cell with an internal volume of 200 μl was used. Optical alignment was made easier by the 1.6 mm diameter cell, which is about 1.5 times the size of the laser beam.

Laser heating causes changes in birefrigence in the polarizing crystals as well as the cell windows. Also, when the two polarizers are crossed, the light entering the second polarizer is rejected almost totally. The rejected light heats up the surrounding cement which holds the polarizing crystal in position. The thermal relaxation in the cement causes the crystal to rotate minutely and randomly deviates from the best extinction. Furthermore, with the flow cell in place the beam diverges due to the thermal lens effect [19] in the organic mobile phase. The expanded beam size reduces the effectiveness of extinction. These three effects combine to increase the residual depolarized light through the crossed polarizers and in turn the noise level. Since both heating and thermal lensing effects are laser-power dependent, lower laser powers are most desirable, up to the limit of photon statistics. So, in this work we used reduced power at 20 mW which proved to be satisfactory, and not the high powers used in the earlier work [17, 18].

Calculation of the concentrations of free and esterified cholesterol was straightforward using the procedures described in previous work [18], which eliminates the need for having available high-purity standards. In using the simple triangular approximation, the accuracy was limited by the measurement of peak base which was defined through extrapolation. Coupled with uncertainties in the specific rotations (α) at this laser wavelength and this solvent, a maximum error of $\pm$ 10% was assessed on our calculated results. This is confirmed by injecting single-component solutions of known concentration for each compound studied.

RESULTS AND DISCUSSION

Figs. 1 and 2 show chromatograms from the reconstituted serum and the freshly extracted natural serum, respectively, analyzed by the above procedure. Test solutions of individual cholesterols in tetrahydrofuran were used to determine the retention times under the same experimental conditions. HPLC retention times, the sign as well as the magnitude of the individual specific rotation, the possible distribution and structural considerations were the criteria for peak identification. Five major cholesterol peaks were identified: (A) cholesterol and cholestanol; (B) cholesteryl linolenate and arachidonate; (C) cholesteryl palmitoleate and linoleate; (D) cholesteryl palmitate and oleate; (E) cholesteryl stearate. In general the order of elution followed that obtained in earlier work using reversed-phase separation [16]. Obviously, other unidentified peaks are present in the chromatograms, but their signal levels are substantially below those of the main peaks.



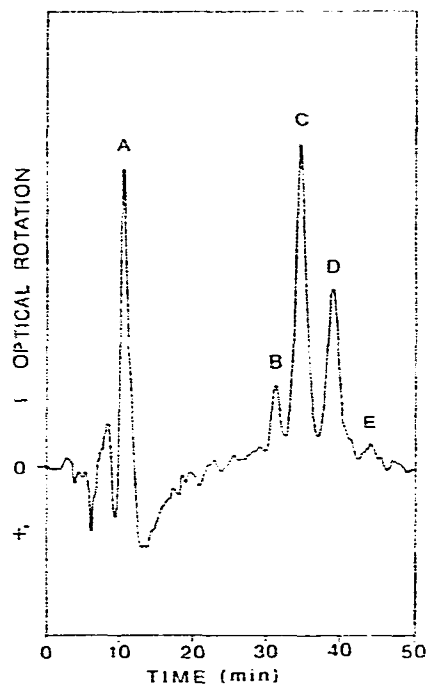


Fig. 1. Separation of cholesterol and cholesterol esters in reconstituted serum (Leder Norm). Peaks: A, cholesterol and cholestanol; B, cholesteryl linolenate and arachindonate; C, cholesteryl palmitoleate and linoleate; D, cholesteryl palmitate and oleate; E, cholesteryl stearate. Mobile phase, tetrahydrofuran—water (76:24, v/v); flow-rate, 0.5 ml/min.

Fig. 2. Separation of cholesterol and cholesterol esters in natural human serum (local). Peaks and conditions as in Fig. 1.

The structural difference between cholesterol and cholestanol is the presence of an isolated double bond between carbons 5 and 6. The small structural and molecular weight differences between these two compounds do not lead to a significant difference in the partition coefficients to permit a clean HPLC separation in our isocratic elution system. The dip (Fig. 1) and shoulder (Fig. 2) on the descending side of peak A are due to the appearance of cholestanol during the elution of cholesterol. This identification was established by the retention times and the signs of the optical rotations. The larger negative rotation of cholesterol ($[\alpha]_D^{20} = -39.5$) [20] was partially offset by the smaller positive rotation of cholestanol ($[\alpha]_D^{20} = +24.2$) [20]. A similar situation existed with three other pairs, peaks B, C, and D. Within each pair, despite the structural difference between the fatty acid chains, the change in polarity still did not manifest sufficient difference in partition coefficients for good separation. Between the two components in the same peak, the polarity increase due to one additional double bond was effectively offset by the decrease in polarity caused by the existence of two more methylene groups in the fatty acid chain.

Admittedly, the selected experimental conditions did not allow clear separation of every individual component. Further, not all the literature values of individual specific rotation, the common detector response factor, are available

for the cholesteryl esters. It is still possible to quantitate the combined contribution of those esters. Because the chiral centers are all in the cholesterol part, it is fair to assume similar values for the specific rotations of those cholesteryl esters considering the structural resemblance between them. The identical value of −24 is listed [21] for the specific rotation of cholesteryl palmitate and oleate. We thus take the liberty of using the same value for all components in peaks B, C, and D. The calculated serum cholesterol level is shown in Table I, using the method of triangular approximation discussed earlier. Again, the reliability of this procedure is confirmed by injecting standard solutions of the individual compounds.

The percentage of free cholesterol relative to the total amount of cholesterol is within the range of 17—39% reported in previous studies [22, 23]. For the purpose of quantitative comparison, an analysis of reference serum (Sigma, Cat. No. R3626) was performed. The total cholesterol determined by our HPLC method using optical activity detection was 135 mg/dl. A 130 mg/dl total cholesterol value based on classical Liebermann—Burchard method was provided on the data sheet for this sample. This indicates that our results are at least comparable to those determined colorimetrically. The total serum cholesterol determined by simple summation of the individual cholesterols is well within the normal range of 150—250 mg/dl [2].

The fatty acids of cholesterol esters have been separated and quantitated by various chromatographic methods [24]. Approximate distribution of the fatty acids of the cholesterol esters of normal serum, expressed as percent of total, is shown in Table II. The fatty acids of the seven cholesterol esters identified in our work make up about 96% of the total. Assuming the total cholesterol esters determined in this work to be only 96% of the total and calculating the approximate distribution of the cholesterol esters as percent of total according-

TABLE I

CONCENTRATION OF FREE AND ESTERIFIED CHOLESTEROL IN SERUM

Peak	Compound	Fatty acid*	Concentration in serum (mg/dl)**	
			Leder Norm	Local
A	Cholesterol Cholestanol		47	38
B	Cholesteryl linolenate Cholesteryl arachidonate	18:3 20:4	7	20
C	Cholesteryl palmitoleate Cholesteryl linoleate	16:1 18:2	76	86
D	Cholesteryl palmitate Cholesteryl oleate	16:0 18:1	56	58
E	Cholesteryl stearate	18:0	8	8
	Total cholesterol		194	210
	Free cholesterol (% of total)		24	18
	Esterified cholesterol (% of total)		76	82

*n:m refers to fatty acid with n carbons and m double bonds.
**All concentrations are ± 10%.

TABLE II

COMPARISON OF THE APPROXIMATE DISTRIBUTIONS OF CHOLESTEROL ESTERS AND THE FATTY ACIDS OF CHOLESTEROL ESTERS

Esterified fatty acid	Percentage of total fatty acid*		Percentage of total esters	
			Leder Norm	Local
Linolenic	4	10	5	11
Arachidonic	6			
Palmitoleic	6	49	50	48
Linoleic	43			
Palmitic	10	34	36	33
Oleaic	24			
Stearic	3	3	5	5
Others	4	4	(4)**	(4)**

*From ref. 24.
**By difference.

ly, Table II can be constructed for comparison. It is clear that the distribution of the cholesterol esters closely matches that of their corresponding fatty acids detached from the parent esters as expected. With some work, it should be possible to separate peaks B—D into the individual components, by for example introducing silver ions. However, unless future clinical studies show different implications for saturated versus unsaturated cholesterol esters, separation may not be necessary.

One major advantage of optical activity detection is its inherent selectivity. Compounds that do not possess optical activity simply will not interfere with analysis even if they elute with analytes we are interested in. This is especially useful when physiological fluids are being analyzed. Triglycerides are present in serum in relatively high concentration ranging from 29 to 134 mg/dl [24], and tend to interfere with HPLC serum cholesterol analysis when UV detection was monitored at 200 nm [16]. About 60% of the triglycerides are simple triglycerides [25] which do not possess asymmetric carbon and will elude optical activity detection. Among the 40% mixed triglycerides, only those with different fatty acid substituents on carbons 1 and 3 have the possibility of showing optical rotation since the carbon 2 then becomes asymmetric. Since the majority of the fatty acid substitutents come from long-chain fatty acids with similar chain lengths, the small structural differences among the three substituents surrounding the asymmetric carbon should only cause insignificant, if any, optical rotation. The combined effect of low optical activity and small quantity in their distribution makes their interference negligible in the region of interest. The numerical results presented verified this assumption. Small amounts of mono- and diglycerides (less than 2% of the total glycerides [24] have appreciable polarity because of their free hydroxyl group and were eluted before cholesterol. 7-Dehydrocholesterol has been identified in human serum and apparently is present in concentration ranging from 5 to 40 mg/dl with 20 mg/dl as the median. This optically active compound is a potential interference for free cholesterol. Since about 80% is esterified [24], the spreading

of the peaks by gradient elution should greatly reduce its interference. Even as is, deconvolution techniques can provide individual concentrations for cholesterol and 7-dehydrocholesterol, since the two have retention times that are distinctly different, though only slightly. Δ7-Cholesterol comprises about 1% of total cholesterol, therefore its interference on free cholesterol is within the possible error estimated in our method. Through the use of a higher efficiency column and gradient elution, improved separation could be achieved and potential interferences reduced.

In summary, we report here a straightforward procedure for the simultaneous determination of free and esterified cholesterol in human serum. Simple extraction, direct injection, HPLC and the use of an absolute standard d.c. Faraday cell [18], offer great convenience in cholesterol determination. The results indicate that this scheme appears to be accurate and precise and to suffer less from interfering substances than colorimetric, enzymatic or UV detection. We expect this technique to be of major importance in the calibration of other analytical methods in clinical studies.

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