Simultaneous determination of triglycerides and cholesterol esters in serum by infrared spectrophotometry

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SUMMARY With the use of a high-resolution grating infrared spectrophotometer, it is possible to analyze mixtures of cholesterol esters and triglycerides on the basis of the difference in their ester carbonyl absorption frequencies. In the analysis of serum lipids, the preliminary removal of phospholipids is accomplished by a simple batch adsorption on silicic acid. Infrared absorbance measurements are made on carbon tetrachloride solutions of the unadsorbed lipids, using stationary frequency settings at 1745 cm^{-1} and 1730 cm^{-1} (approximate band peaks of triglycerides and cholesterol esters, respectively). The influence of the minor lipid constituents in this mixture is considered, and the errors from this cause are shown to be negligibly small in most circumstances. The over-all accuracy is estimated to be about $\pm 5\%$.

TRIGLYCERIDES and cholesterol esters are two of the principal lipid classes found in serum. Measurements of either of these lipid components are of fundamental importance in many areas of lipid research.

The determination of serum cholesterol is quite commonplace, and the analytical methods employed are so numerous and varied that no attempt will be made to review them here. Basically the same methods are used to determine cholesterol esters. Serum triglycerides, on the other hand, have in the past presented a more difficult analytical problem, and their determination is much less frequently carried out. In older methodology, the estimation of triglycerides required measurements of total lipids, cholesterol, cholesterol esters, and phospholipids. The latter three components were subtracted from the total, and the difference was taken as triglycerides. In recent years, both chromatographic and direct methods have been developed Chromatographic procedures, however, are in general poorly suited for the routine handling of large numbers of samples. In the direct methods, glycerol is determined after hydrolysis of a lipid fraction from which phospholipids have been excluded. Various procedures have been described by Van Handel and Zilversmit (1), Carlson and Wadström (2), Jover (3), and Mendelsohn and Antonis (4).

Earlier efforts in this laboratory to apply infrared spectrophotometry to lipid analysis led to a satisfactory determination of triglycerides, provided they were first separated from cholesterol esters and phospholipids by chromatography (5). In another procedure (6), the total lipid extract from serum was analyzed directly, without chromatography, whereby values were obtained for total lipid, total esterified fatty acids, total phospholipids, and total cholesterol. The estimation of triglycerides by difference from these measurements is subject to the same compounding of errors as the older chemical methods. The development of an infrared method for triglycerides that is both simple and reliable has resulted from the use of a grating instrument which provides both higher resolving power and greater wavelength (or frequency) precision than the sodium chloride prism spectrometer with which the earlier methods were evolved.

The essence of the method is indicated in Fig. 1, which shows the ester carbonyl absorption bands of triolein and cholesterol oleate. The compounds are at approximately equivalent fatty acid concentrations in CCl_4. The absorption maxima are separated by about 13 cm^{-1}, and the frequency settings selected for measurement are at 1745 cm^{-1} and 1730 cm^{-1}. From calibration data for the two compounds at these two frequencies, it is possible to derive two simple linear equations which express the

composition of the mixture in terms of measured absorbances.

For serum lipids, it is necessary to remove phospholipids, which also contain ester groups absorbing in this region (1735 cm^{-1}). The separation may be accomplished in a variety of ways, including those that have been used in connection with chemical determinations. The procedure that will be described is a simple batch adsorption on silicic acid. The remaining mixture, on which infrared measurements are made, contains principally triglycerides and cholesterol esters, together with small amounts of unesterified cholesterol and free fatty acids. It will be shown below that these minor constituents, in the amounts ordinarily present, contribute negligibly small errors.

METHODS

The instrument used was a Perkin-Elmer Mode 1421 dual-grating spectrophotometer. Standard NaCl absorption cells, nominal thickness 1.0 mm.

Reagent grade solvents were used throughout. Evaporation of solvents was carried out on a hot plate at about 50°, using a current of nitrogen.

Extraction of Lipids

Extraction is carried out on 1.0 ml aliquots of serum using chloroform–methanol 2:1 (v/v) and the method of Sperry and Brand (7). Any procedure which extracts neutral lipids quantitatively without degradation would be satisfactory. Failure to extract phospholipids completely is of no consequence. The lipid extract is taken to dryness under nitrogen and redissolved in 0.5 ml of chloroform.

Adsorption of Phospholipids

Approximately 50 mg[1] of silicic acid (Bio-Rad Laboratories, Richmond, Calif., specially prepared for the chromatography of lipids) is measured into a 2 dram (8 ml) screw-cap glass vial, which has been calibrated and scribed at a volume of 6.0 ml. (The screw cap should be lined with polytetrafluoroethylene to prevent contamination by plasticizer from the ordinary liner.) Chloroform, 0.5 ml, is added to wet the silicic acid. The lipid sample, dissolved in 0.5 ml of chloroform, is transferred with a capillary dropping pipette to the vial containing the silicic acid. The sample vial is rinsed two or three times

[1] The maximum amount of serum phospholipids that can be adsorbed by 50 mg of silicic acid is about 4 mg. Only in rare sera is this level exceeded if the volume extracted is 1 ml. When extremely hyperlipidemic sera or lipids from other sources are to be analyzed, either the size of the lipid sample should be adjusted in accordance with this limitation or a larger quantity of silicic acid may be used. In order to allow for possible extremes, it may be preferable to use 100 mg of silicic acid at all times.

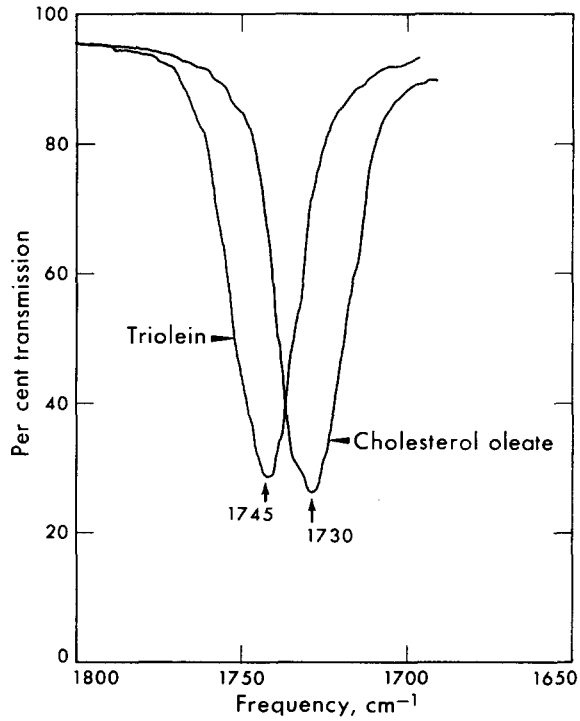

FIG. 1. Carbonyl absorption bands of triolein and cholesterol oleate. Solutions in CCl_4: triolein, 3.11 mg/ml; cholesterol oleate, 7.16 mg/ml. Cell thickness, 1.0 mm. Frequency scale expanded to 5 times normal.

with small volumes of acetone, the rinsings being combined with the sample in the scribed vial. The vial is then filled to the 6.0 ml mark with acetone and tightly capped. The contents are thoroughly mixed by inversion and rotation. After about 10–15 min, the mixing process is repeated, and the silicic acid is then allowed to settle for at least 30 min. (Presumably the silicic acid may be centrifuged down instead, but a minimum contact time has not been established.) Five milliliters of the clear supernatant solution are then transferred to a clean dry vial and evaporated to dryness under nitrogen. (This measurement may also be accomplished with a scribed vial, which is filled to the 5.0 ml mark by transferring the solution with a pipette.) To ensure complete removal of acetone from the sample, the vial is rinsed down with about 1 ml of $CHCl_3$, which is then evaporated, again under nitrogen.

Infrared Measurements

The dried aliquot of unadsorbed lipids is dissolved in an accurately measured volume of carbon tetrachloride, so as to give a total concentration in the range of 3–5 mg/ml. (A calibrated 1.0 ml gas-tight syringe is a convenient measuring device.) The volume required is usually between 0.6 and 1.2 ml. If no prior estimate of the amount of lipid is available, a trial may be made at a volume of 0.80 ml. If the measured absorbances are

TABLE 1 ANALYSIS OF PREPARED MIXTURES OF TRIOLEIN AND CHOLESTEROL OLEATE

Sample	Component	Weight in mg		% Error
		Known	Found	
1	TG	2.34	2.26	−3.4
	CE	3.91	4.08	+4.4
2	TG	2.67	2.70	+1.1
	CE	8.16	8.24	+1.0
3	TG	7.24	7.45	+2.9
	CE	6.63	6.95	+4.8
4	TG	14.86	15.0	+0.9
	CE	4.18	4.40	+5.3
5	TG	2.70	2.65	−1.9
	CE	4.54	4.52	−0.4
6	TG	0.21	0.21	0.0
	CE	4.27	4.30	+0.7
7	TG	0.41	0.37	−9.8
	CE	4.69	4.64	−1.1
8	TG	0.62	0.59	−4.8
	CE	4.75	4.56	−4.0

TG = triglyceride.
CE = cholesterol ester.

not within the range 0.2–0.6, it may be desirable or necessary to repeat the measurements at a different concentration.

The absorption cell is filled with the solution, and a duplicate cell is filled with carbon tetrachloride. The cells are placed in the sample and reference beams, respectively, of the spectrophotometer, and absorbances are measured at 1745 and 1730 cm^{-1}. The slit width is 0.25 mm, corresponding to a slit program setting of 2 × 1000 on this instrument.

Since at least one of the selected frequencies will ordinarily occur on a steeply sloping side of the resultant absorption band of the mixture, it is necessary to set the frequencies manually and record the absorbances at stationary scale positions. Settings can be made manually to about 0.1 cm^{-1}.

With the equations given in the next section, the measured absorbances are used to calculate concentrations of triglycerides and cholesterol esters in the CCl_4 solution. These are multiplied by the volume of CCl_4 to given weights (in milligrams, if milligrams/milliliter is the concentration unit). If the initial lipid extract is the total from 1 ml of serum, the calculation may be written as follows:

$$\text{mg/100 ml serum} = (\text{concn in } CCl_4) \times (\text{vol of } CCl_4) \times 6/5 \times 100$$

TABLE 2 OVER-ALL REPRODUCIBILITY AND COMPARISON WITH RESULTS OBTAINED BY COLUMN CHROMATOGRAPHY (SERUM LIPIDS)

Sample	Component	Two-Component Infrared	Chromatography Infrared
		mg/ml of serum	
1	TG	1.66, 1.58, 1.61, 1.58	1.69, 1.71
	CE	2.93, 2.67, 2.72, 2.68	2.58, 2.64
	TL	7.29, 7.36, 7.31, 7.43	7.22, 7.28
2	TG	0.75, 0.78, 0.71	0.78, 0.77
	CE	2.80, 2.80, 2.58	2.72, 2.61
	TL	6.17, 6.36, 5.98	6.23, 6.36
3	TG	5.20, 5.12, 5.24, 5.19	5.03, 4.70
	CE	3.12, 3.30, 3.39, 3.25	3.01, 2.86
	TL	11.83, 12.33, 12.09, 12.61	11.75, 10.89

TG = triglycerides.
CE = cholesterol esters.
TL = total lipids.

Calibration

Various triglycerides (tripalmitin, triolein, safflower oil, and mixed triglycerides from serum) have been shown to have their absorption maxima at the same frequency, 1742 ± 0.5 cm^{-1}. Similarly, for cholesterol palmitate, oleate, linoleate, and mixed serum cholesterol esters, the peak is at 1728.5 ± 0.5 cm^{-1}. The choice of 1745 and 1730 cm^{-1} for measurement instead of the precise peaks is somewhat arbitrary and has no effect on the analysis except to alter the absorption coefficients slightly. The absorption coefficients (absorbance/concentration) are theoretically constant per mole of carbonyl. Thus, in comparing calibrations for different triglycerides (or cholesterol esters) based on concentration by weight, the coefficients are found to be inversely proportional to molecular weights. Following a common practice, the molecular weight of oleic acid is taken as a reasonable approximation of the average molecular weight of mixed serum fatty acids and its esters are, therefore, considered to be directly applicable as standards.

Using pure triolein (Hormel Foundation) and pure cholesterol oleate (Applied Science Labs.), absorption coefficients are obtained for triglycerides and cholesterol esters at each of the two selected frequencies, 1745 and 1730 cm^{-1}. This is done in the customary way, i.e., by measuring the absorbances of several solutions of different concentrations (1–4 mg/ml for triglycerides; 2–7 mg/ml for cholesterol esters). Calibration data are plotted in Fig. 2. The absorption coefficients have been found to decrease slightly with increasing concentration; but if average values are used, the variations over the working range are within about ±3%. This has been taken as an adequate approximation to the conditions of Beer's law.

The Beer's law expression for two independent components becomes a two-term linear equation. For absorbances measured at two frequencies, there are two such equations as follows:

$$A_{1745\ cm^{-1}} = 0.165\ C_{TG} + 0.019\ C_{CE}$$

$$A_{1730\ cm^{-1}} = 0.040\ C_{TG} + 0.080\ C_{CE}$$

where A = absorbance, C = concentration (mg/ml), TG = triglycerides, and CE = cholesterol esters. The

numerical factors are the absorption coefficients (A/C) determined by calibration in a particular cell. Solving these equations to obtain expressions for concentrations in terms of absorbances,

$$C_{TG} = 6.44\ A_{1745} - 1.53\ A_{1730}$$

$$C_{CE} = -3.22\ A_{1745} + 13.27\ A_{1730}.$$

This pair of equations is applied in the analysis of samples, using absorbances measured as described in the previous section. The numbers given here should be considered as illustrative. A calibration should be carried out for the spectrophotometer, the operating conditions, and the cells that are to be used.

Blanks

Blanks should be run from time to time, using distilled water or saline in place of serum. Following the complete procedure, the results are calculated as apparent triglycerides and cholesterol esters in milligrams. Experience in this laboratory has given blank values for triglycerides ranging from 0.01 to 0.03 mg. For cholesterol esters, they have been from 0.03 to 0.08 mg. Relative to the quantities usually found in 1 ml serum samples, these blanks represent from about 1 to 4% of either component. If one component should be exceptionally low, the relative error due to the blank would be correspondingly higher.

The frequency of running blanks may be dictated by conditions. If consistently low enough to be insignificant, they may be neglected. High values indicate that a source of contamination needs to be identified and eliminated, e.g., by redistillation of solvents.

RESULTS

The two-component infrared analysis was tested using prepared mixtures of triolein and cholesterol oleate. Some typical results are given in Table 1.

As an evaluation of the over-all method, some analyses were carried out on replicate samples of serum, starting with extraction and proceeding through the adsorption step to the infrared measurements. The results were compared with cholesterol ester and triglyceride values for the same serum obtained by silicic acid chromatography and infrared analysis. These data are given in Table 2. Corresponding weights of initial lipids have been included in the table to show that the extremes of variation in the individual components reflect (in most instances) weight deviations in the total lipid extract. In the two-component analysis, no blank corrections have been applied.

Tables 1 and 2 show net performance. In addition, the underlying assumptions on which the analysis is based

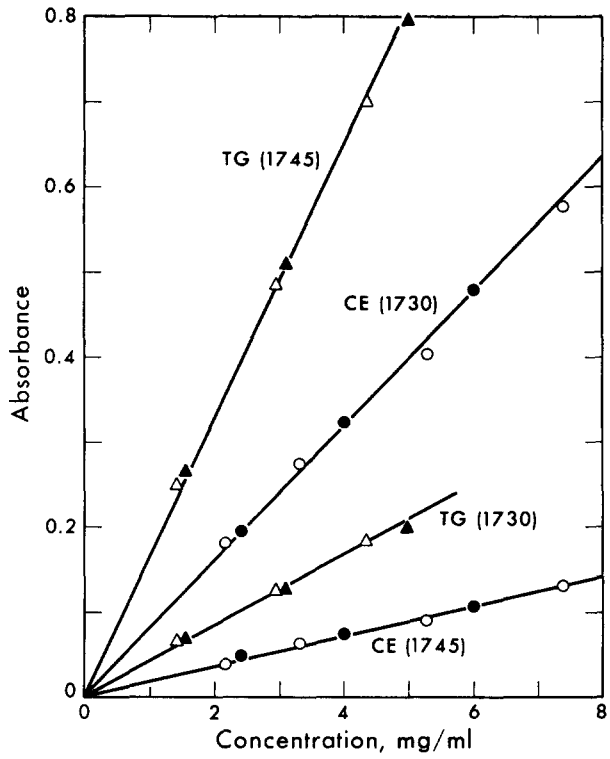

FIG. 2. Calibration curves for triolein (TG) and cholesterol oleate (CE). CCl_4 solutions; cell thickness, 1.0 mm. Frequencies, in cm^{-1}, are given in parentheses. Open and solid data points represent two separate calibrations approximately 5 months apart.

have been tested in several specific respects. In order that the mixture subjected to infrared measurement may be treated as a two-component system, two requirements must be met. These are:

(*a*) The silicic acid adsorption must provide a clean separation, removing all of the phospholipids and none of the components to be measured.

(*b*) The contributions of other unadsorbed lipids to the measured infrared absorbances must be negligibly small.

Effectiveness of Adsorption

With respect to the first requirement, the accumulated experience of many workers has left little doubt as to the efficacy of the silicic acid separation when carried out on columns. The use of silicic acid in batch adsorption has been reported by Marks et al. (8), and by Van Handel (9). In our experiments, we have varied the amount of silicic acid over a range from 30 to 100 mg with no significant difference in results. Use of either acetone or chloroform also gave substantially equivalent answers.

An experiment was performed to show that quantitative recovery of triglycerides and cholesterol esters is achieved in the adsorption procedure. Two prepared

mixtures were analyzed, each in quadruplicate, starting with the adsorption step. In two of each set, the silicic acid was omitted. Results of the analyses with silicic acid agree within 2% with those of the analyses in which no silicic acid was used.

Except in the case of one unusual serum (which contained over 700 mg of phospholipid per 100 ml, see footnote 1), the infrared spectra of the unadsorbed lipids have shown no discernible evidence of the presence of phospholipids. Calculations made from absorbance measurements indicate that in general the amount of phospholipid that can be present is so small that any resulting error in the analysis is less than 5%. In a few instances, the adsorbed phospholipids have been recovered by elution with methanol and either weighed or measured by infrared absorption. The amounts obtained have been in good agreement with those from column separation of duplicate samples.

Effect of Minor Unadsorbed Constituents

Unesterified cholesterol is present in the mixture of unadsorbed lipids that is subjected to infrared measurement, but since it has no carbonyl group its absorption at the frequencies of measurement is expected to be small. From calibration data it has been determined that amounts of cholesterol up to five times normal will contribute absorbance less than the error of measurement; clearly, this component can be ignored.

Small amounts of free fatty acids are also present in the mixture analyzed. The effect on the analysis can be estimated from calibration data, if the amount of fatty acid is known. Assuming a range of 10–30 mg/100 ml for normal serum and that the extraction procedure is quantitative for fatty acids, the calculated errors are 0.02–0.06 mg in triglycerides and 0.03–0.08 mg in cholesterol esters. Mixtures containing added fatty acids, in amounts corresponding to about 15 and 50 mg/100 ml of serum, were analyzed. At the higher level, the error in the calculated value for either component is approximately 0.1 mg. This corresponds to less than 5% for a normal range of cholesterol ester values, and 5–10% for triglycerides. It may be inferred that except in instances where unusually high fatty acid content is combined with a low triglyceride level, errors in both components are expected to be less than 5%.

It is possible to take account of free fatty acids by including them in the analysis as a third component. This requires an additional absorbance measurement at 1708 cm^{-1}, the fatty acid absorption peak. Calibration of all three components at all three frequencies is necessary, and the matrix calculation involves three simultaneous equations in three unknowns. A calculation of this type has been carried out for a random set of eight serum samples, and the results have been compared with those of the two-component calculation. The range of discrepancies for triglycerides is 0.00–0.03 mg, and for cholesterol esters, 0.00–0.05 mg. The average error in the two-component analysis, as a result of ignoring free fatty acids, is +1.9%.

The measurement of fatty acids in this way is not, however, very accurate, mainly because the amount present is very small in comparison with the major components of the mixture. Under the conditions of the analysis, small absorbance values are obtained at the fatty acid frequency, and the relative error in measurement becomes significant. Furthermore, the extraction procedure used here is not assumed to be appropriate for a fatty acid analysis. The values obtained in the eight serum samples mentioned above ranged from 8 to 19 mg/100 ml. These are within the expected range for normal serum, but they may be in error by as much as a factor of 2. This does not invalidate the assessment of errors in the two-component analysis, however.

Diglycerides and monoglycerides are other minor lipid constituents which are not excluded by silicic acid adsorption. Carlson and Wadström (10) have reported that these forms comprise about 5% (by weight) of the total glycerides in normal serum. Their absorption maxima are both at the same frequency as that of triglycerides; hence, they are included in the measurement of that component. If the analysis is considered to be specifically for triglycerides, a small positive error is inherent. If alternatively it is regarded as being for total glycerides, the error is negative (and much smaller) because of slight differences in absorptivities.

Effect of Oxidation

The known secondary products of autoxidation include carbonyl compounds (11). Such compounds, if present in lipid mixtures being analyzed by this method, would contribute errors. It has been assumed that with fresh serum samples and careful handling to limit exposure of lipids to air and heat, such errors can be kept at a negligible level. On long term storage of serum (frozen under nitrogen) and pure triolein (under nitrogen at room temperature), small changes in absorptivity have been observed which may be consequences of oxidation.

DISCUSSION

The method described here presents several possibilities for variations in procedure and for extending the scope of its applicability. It seems probable that any of several combinations of adsorbent (silicic acid, Florisil, zeolite) and solvent (chloroform, acetone, ether) would be satisfactory for separating the phospholipids. The rubber dialysis technique of van Beers et al. (12) should also serve this purpose. An extremely simple analysis would

result from the use of a direct extraction procedure, as described by Van Handel and Zilversmit (1), and by Mendelsohn and Antonis (4).

If a total lipid extract is obtained and the adsorption is carried out either batchwise or on a column, the phospholipid fraction can be recovered and measured by any suitable means. It is also possible that the analysis of the unadsorbed fraction can be extended to include the free fatty acids and unesterified cholesterol, although these components are usually present in relatively small amounts and high accuracy cannot be expected.

With the instrument and cells used here and only minor modifications in procedure, it should be possible to perform this analysis on 0.4 ml of serum. It seems reasonable to expect that it can be done on as little as 0.05 ml of serum by using microcells. Limitations at that level will arise from measurement of small volumes and from contaminations.

The resolving power required for this method is considerably less than that of the grating used. Some data have been obtained which indicate that a calcium fluoride prism is satisfactory. While it cannot be stated categorically at this point that sodium chloride prisms have insufficient resolving power, the concomitant difficulty of achieving sufficiently precise wavelength settings may preclude the use of most prism instruments. This needs to be tested.

Simpler and less expensive grating spectrometers are available, and some types undoubtedly have the necessary capability. It seems quite likely that some such instrument may serve as the basis for development of an automated system for routine performance of this analysis.

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