Determination of cholesterol and cholesterol esters

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Overview

Chemical determinations of cholesterol have been performed in both clinical and research laboratories for many decades. The classical, colorimetric Leiberman-Burchard reaction, described before the turn of the century, has spawned many modifications in efforts to overcome three major problems: 1) the presence of bile pigments and non-cholesterol sterols which contribute to false-high values; 2) the differing color intensities developed by free and esterified cholesterol, and 3) inadequate sensitivity. (Detailed reviews and critical discussions have been written.[1,2] The modification of Leiberman-Burchard called the "$FeCl_3$ Method,"[3] and the reaction of cholesterol with the aromatic aldehyde, *o*-phthalaldehyde,[4,5] which is based upon the Kamarowsky reaction,[6,7] remain useful and economical chemical methods. Since the advent of enzymatic methods,[8-11] the use of chemical procedures has declined rapidly; however, it is important to note that commercial secondary calibrators for auto-analyzers in use today often are standardized against these older chemical methods, as are new methods arising from specific research considerations.[10-14] The ease and sensitivity of the enzyme assay justifies its wide acceptance for most applications.

Historically, improvements in methodology to quantitate cholesterol were aimed at reaching a better understanding of cholesterol metabolism in blood, while more recently this technology has been applied to the study of other fluids, such as bile, and various tissues from different animal species. Extraction of hydrolyzed tissue into organic solvents[15] has aided this quest. The need to distinguish unesterified cholesterol (UC) from esterified cholesterol (EC) and to identify the fatty acyl chains of these esters prompted two developments. One method relies upon the precipitation of UC as its digitonide; total cholesterol (TC) is measured similarly following alkaline hydrolysis,[16,17] and EC is calculated from the difference (TC-UC). The other more recent method separates the major neutral lipid classes by silica chromatography, usually high performance liquid chromatography (HPLC), or thin layer chromatography (TLC). The latter approach will be described below. When interfering compounds are present, such as very high triglyceride (TG) levels or other sterols which are reactants in both the chemical and enzymatic assays, gas liquid chromatography (GLC) becomes the method of choice.[18]

This paper focuses on basic manual methods appropriate for the wide variety of samples generated within research laboratories that can easily be handled by skilled technicians with emphasis on accuracy as the most important feature.

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Solvents used are HPLC grade; general reagents are AR grade. Distilled, deionized water is used. Purities of lipid standards are checked by TLC and/or GLC and their concentration in organic solvents is quantitated gravimetrically by microbalance following solvent evaporation. Solvent extracts of lipids are stored in teflon-lined screw-capped tubes under an atmosphere of N_2 at $-20°C$. A final wrap of Teflon (PTFE) tape will prevent most instances of solvent evaporation.

The use of dichromate-sulfuric acid mix is discouraged in glassware washing since it may leave a Cr^{3+} residue. Ordinary laboratory detergents work well providing that a thorough rinse ensues. Glassware is twice rinsed with small amounts of an organic solvent before use.

Lipoprotein and tissue extractions

Lipoprotein fractions from plasma

Addition of EDTA to blood is recommended because this will chelate heavy metals and retard lipid oxidation.

For the precipitation of apoB-containing lipoproteins, a combination of dextran sulfate and $MgCl_2$ is most often used today because it is compatible with both chemical and enzymatic assays and because this reagent mix is inexpensive. Varying the ratio of dextran sulfate:Mg^{2+} allows HDL subclasses to be separated. The outcome is not affected by temperature (4°C to 23°C) or centrifugation conditions. Precipitation is best performed on the day of venipuncture as storage of plasma samples with subsequent precipitation may undervalue HDL cholesterol. Details and critical reviews have been written and the following reagent list and procedure is taken largely from these references.[19,20]

Materials. Dextran sulfate (Mwt 50,000 ± 5,000) is purchased from Genzyme Labs (Cambridge, MA, USA) and stored in a desiccator at 4° C.

1. Preservative solution: Dissolve 5.0 g NaN_3, 50 mg Gentamicin sulfate, and 100 mg chloramphenicol in water and adjust to 100 ml with water.
2. Dextran sulfate, 20 g per liter: Dissolve 2.0 g dextran sulfate in 80 ml water and adjust to pH 7.0 with HCl. Transfer quantitatively to a 100 ml volumetric flask, add 1.0 ml of preservative solution and bring to the mark with water. Store at 4° C.
3. Dextran sulfate, 40 g per liter: Dissolve 4.0 g dextran sulfate as above, add 1.0 ml of preservative solution; volume to 100 ml. Store at 4° C.
4. $MgCl_2 \cdot 6H_2O$, 1.0*M*: Dissolve 20.3 g $MgCl_2 \cdot 6H_2O$ in 80 ml water and adjust with NaOH to pH 7.0. Dilute to 100 ml. Store at 4° C.
5. $MgCl_2 \cdot 6H_2O$, 2.0*M*: Dissolve 40.6 g $MgCl_2 \cdot 6H_2O$ in 80 ml water as described above and dilute to 100 ml. Store at 4° C.
6. Combined reagent A: Mix equal volumes of dextran sulfate (20 g/l) and $MgCl_2$ (1.0*M*). Alternatively, this may be purchased as "HDL cholesterol reagent" from Sigma Chemicals cat. #352-3 (St. Louis, MO, USA) and prepared by adding 5 ml H_2O to the lyophilized contents of the vial.
7. Combined reagent B: Mix 1 volume of dextran sulfate (40 g/l) with 3 volumes of $MgCl_2$ (2.0*M*). Alternatively, this may be prepared from the Sigma reagent by adding 1.0 g $MgCl_2 \cdot 6H_2O$ and adjusting to a final volume of 5.3 ml with H_2O.

The above reagents A and B are stable at 4°C for at least 4 months when prepared directly; the alternative, reconstituted Sigma reagent, is stable for 6 weeks.

Procedure for separation of lipoprotein fractions from plasma.

1. Total HDL: To 1.0 ml of serum or EDTA plasma (room temperature) in a 1.5 ml microfuge tube, add 100 µl of combined reagent A and mix well. Let stand for 10 min. Centrifuge 10,000 rpm in a Microfuge (Beckman) for 1–2 min or a low-speed centrifuge at 1500 rpm for 30

min. Remove an aliquot from the clear supernatant containing HDL and transfer it to a second labeled vial. Calculation of HDL concentration must include a factor of 1.1 to correct for dilution.

Note. Hypertriglyceridemic samples will form a turbid supernatant which may be removed and thus HDL samples clarified by passage through a 0.2 μm filter; or a second precipitation may be performed after dilution of the sample with 1.0 ml of 150 mM NaCl solution followed by another 100 μl of the combined reagent. The sample is centrifuged again and a second dilution factor is included in the calculation.

2. HDL Subclasses: Transfer 0.5 ml of clear supernatant (containing total HDL) to a transparent centrifuge tube. Add 50 μl of combined reagent B; mix well and allow to stand at room temperature for 15 min. Centrifuge as above. Remove an aliquot for analysis of HDL_3. Calculations include a total dilution factor of 1.21.

$$HDL_2chol = \text{total HDLchol} - HDL_3chol \quad (1)$$

Note. If TG measurements have been made, VLDLchol and LDLchol may be estimated for most samples using the Friedewald equation[21,22]:

$$VLDLchol = TG \times F \quad (2)$$

When units of cholesterol and TG are mg/dl, F = 0.2; when they are mmol/L, F = 0.45.

$$LDLchol = \text{plasma chol} - HDLchol - VLDLchol \quad (3)$$

When plasma samples from cholesterol-fed rabbits are analyzed, the apoB aggregates float into a tight band due to the presence of EC-rich β-VLDL. In these instances, HDL is found in the infranate (unpublished observations).

Tissue extraction

Solvent extraction is necessary to release cholesterol from tissue. Small aliquots of minced or homogenized tissue are extracted according to either Folch[23] or Bligh and Dyer.[24] Red blood cells are extracted according to Rose and Oklander.[25] Large aliquots of tissue may be minced, lyophilized, and further powdered by chopping with a razor blade prior to lipid extraction. Aliquots of the final chloroform layer are stored at −20° C under N_2 in tubes with Teflon-lined caps for later analysis, preferably within 8 weeks.

Hydrolysis for total cholesterol using alcoholic KOH

Cholesterol esters are best hydrolyzed with ethanolic KOH. We have abandoned the use of cholesterol ester hydrolase in our enzyme assay system, and this for the following reasons: Cholesteryl arachidonate, common in tissues from the rat, is a poor substrate for enzymes from a variety of sources.[26] Cholesteryl stearate (m.p. 83° C[27]) and other high-melting esters may escape enzymatic hydrolysis because they are poorly solubilized. Proprietary calibrators, geared to human serum or plasma samples may not be appropriate. Furthermore, bilirubin, which interferes with the chemical reaction, and which in the enzymatic method competes with the dye-mix as an acceptor of hydrogen peroxide in the formation of biliverdin, is destroyed by alkali.[2] Interfering proteins and triglycerides also are eliminated. However, many non-cholesterol sterols accompany cholesterol throughout this procedure.

Lipid extracts dried under N_2, tissue homogenates, serum, or plasma may be assayed. Radio-labeled tracers may be used to monitor recovery. The following procedure, for 0.1 ml of plasma, has been modified from Abell[15]:

Procedure for hydrolysis of cholesterol esters. Freshly prepare 0.6 N KOH in ethanol by adding 10 ml of 33% aqueous KOH to 90 ml of 95% ethanol. Add 2.0 ml of this reagent to the sample, cap well, and heat in a water bath at 60° C for 30 min–1 hour (longer times for tissue). Cool. Add 4.0 ml of hexane and mix well. Add 3.0 ml H_2O. Extract vigorously. Centrifuge at 1500*g* to clarify layers. Remove the hexane layer or an aliquot of appropriate size.

Separation of unesterified cholesterol and cholesterol esters by thin layer chromatography

Materials. Silica Gel G or H TLC plates are purchased from Analtech, Inc. (Newark, DE, USA) and washed overnight in $CHCl_3$:CH_3OH (1:1). Activation of plates is not required.

Procedure for TLC of solvent extracts. TLC of solvent extracts of tissue is performed on silica gel G or H plates; standard lanes with UC and cholesterol oleate are included. Plates are developed in a hexane:ethyl ether:glacial acetic acid (70:30:1::v:v:v) system in ~40 min. Bands are visualized by brief exposure to iodine vapor.[28] The cholesterol region ($R_f \sim 0.2$), is scraped into tubes, eluted with $CHCl_3$:CH_3OH (2:1) and centrifuged to remove silica. This elution step is repeated twice. After evaporation of solvent under N_2, the samples are assayed for cholesterol.

It is usual to determine TC (following hydrolysis) and UC, and calculate the value of EC by difference. From this difference, the mass of cholesterol esters may be estimated by multiplying EC by 1.68 (the F.Wt. of cholesteryl oleate/F.Wt. of cholesterol) as an average value.

Chemical methods for measuring cholesterol in extracts

Total cholesterol is determined following hydrolysis. Unesterified cholesterol is eluted from silica gel G or H plates. Interfering substances include non-cholesterol sterols that are extracted along with cholesterol or co-migrate with cholesterol on TLC plates.

$FeCl_3$ modification of Leiberman-Burchard[3]

Principle. The basic mechanisms of the Leiberman-Burchard reaction have been discussed.[2,29]

Materials. Cholesterol is purchased from Nu Check Prep., Inc., (Elysian, MN, USA) or other reliable source.

1. Calibration curve: 50–250 μg cholesterol range. Standards prepared in glacial acetic acid may be used directly. If an organic solvent is used, this must be removed by evaporation.
2. $FeCl_3$ solution: 1 g $FeCl_3 \cdot 6H_2O$ in 10 ml glacial acetic acid. This solution is stable at −20° C for ~ 1 year.
3. Working solution: 1:100 dilution of $FeCl_3$ solution in 18 *M* H_2SO_4. This solution should be clear. If a precipitate is observed, it should be discarded.

Procedure for $FeCl_3$ method (25° C). To sample, standard, and blank tubes, add glacial acetic acid to a final volume of 1.5 ml and mix carefully. Add 1.0 ml of the working solution slowly by allowing it to run down the side of the tube and form 2 layers. Then mix very well. Allow to cool in the dark (~45 min) and read the absorbance at 560 nm.

o-*phthalaldehyde reagent*[4,5]

Principle. The mechanism of the condensation of sterols with aromatic aldehydes has been discussed.[7]

Materials.

1. Calibration curve: 10–50 μg cholesterol range.
2. Freshly prepare *o*-phthalaldehyde (Sigma) reagent by dissolving 50 mg/100 ml glacial acetic acid.

Procedure for *o*-phthalaldehyde method. This is performed on ice to prevent charring: Add 2.0 ml of *o*-phthalaldehyde reagent to tubes containing standards and samples (from which organic solvents have been evaporated) and to the blanks. Mix well and allow to stand for 10 min on ice. Add 1.0 ml 18*M* H_2SO_4 by careful layering. Mix immediately and thoroughly. Read the absorbance at 550 nm within 10–90 min.

Enzyme method for cholesterol determination[9-11]

TC is determined following alkaline hydrolysis; UC is assayed from either aqueous samples or solvent extracts.

Principle. Unesterified cholesterol is oxidized by cholesterol oxidase to cholest-4-en-3-one, with the simultaneous production of stoichiometric amounts of H_2O_2. In the presence of peroxidase, the H_2O_2 is coupled to 4-aminoantipyrene (AAP) and 2,4-dibromophenol (DBP) to yield a quinoneimine dye which has a maximum absorbance at 500 nm.

Interfering substances include β-OH Δ4 and Δ5 sterols, bilirubin, and other acceptors of H_2O_2.

We have modified the method of Allain as follows:

1. Cholesterol oxidase is from Streptomyces for both cost and efficiency.[20,31]
2. Phenol is replaced by 2,4-dibromophenol which develops a more intense color.[32]
3. Carbowax-6000 is eliminated.
4. The reaction is carried out in 20% 2-propanol (*Table 1*).
5. Cholesterol ester hydrolysis is not used.

Materials

Cholesterol oxidase (Streptomyces), peroxidase (horseradish, Type II), and Na cholate are from Sigma. AAP and DBP are purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Styrene or acrylic cuvets are from Sarstedt (Princeton, NJ, USA).

Cholesterol calibration curve in 2-propanol: Range from 0.5–40 μg (~2–100 nmol). Blanks and standards for total cholesterol determinations are subjected to the same hydrolytic procedure as the samples; blanks and standards for UC are not "hydrolyzed." (*Figure 1*)

Double strength dye-buffer mix: In 480 ml 100 m*M* sodium phosphate buffer pH 7.0, dissolve 0.16 g AAP and 0.24 g DBP. Solution may be slow.

Table 1 Effect of 2-propanol on color development

Volume % of 2-propanol[a]	% of Control[b] color
0	46
2.5	82
5.0	84
10	89
20	100
30	93
40	66
50	28

[a] Balance:water
[b] 20% 2-propanol is used as control value.

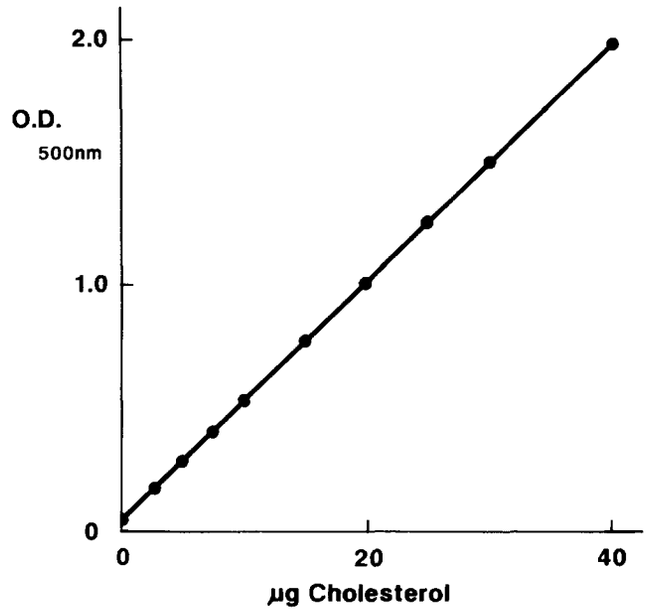

Figure 1 Cholesterol calibration curve for enzyme assay. Y = 0.0500(± 0.004 OD units/µg) + 0.026(± 0.026 OD units); mean (SD); *n* = 23 assays over a one-year period

Adjust the pH to 7.0 if needed and bring to 500 ml with phosphate buffer. Divide into convenient aliquots. This solution is stable at −20° C for at least 6 mos. Aliquots may be thawed several times (discard if discolored).

Dissolve each of the following reagents in 100 mM phosphate buffer pH 7.0: cholesterol oxidase to give 80 m Units in 5 µl; peroxidase to give 2 units in 10 µl; 520 mg of Na cholate in 10 ml. These solutions are stable at −20° C for at least 6 mos.

Prepare freshly solution A (for samples evaporated from organic solvent), and/or solution B (for aqueous samples).

A: Per assay tube,

0.45 ml double strength dye-buffer mix
0.3 ml H_2O
0.05 ml Na cholate solution
80 m Units of cholesterol oxidase (5 µl)
Final volume 1.0 ml

B: Per assay tube,

0.45 ml double strength dye-buffer mix
0.05 ml Na cholate solution
0.2 ml 2-propanol
80 m Units of cholesterol oxidase (5 µl)
Final volume 1.0 ml

Procedure for enzyme assay

(Aqueous samples and samples obtained from solvent extraction may be included within the same assay.)

Assemble in test tubes:

1. To standards, adjust volume to 200 µl with 2-propanol and mix well. Add 800 µl of solution A and mix again.
2. To samples evaporated from organic solvents, add 200 µl of 2-propanol and mix well. Add 800 µl of solution A and mix again.
3. To aqueous samples, adjust volume to 300 µl with water and mix. Add 700 µl of solution B and mix again.
4. Transfer to acrylic or styrene cuvets and read, against water, the initial OD at 500 nm. Add 2 Units of horseradish peroxidase (10µl) to each

cuvet. Cover with Parafilm and mix by inversion. After 20 min at 25° C, read the final OD at 500 nm.

5. Subtract final OD from initial OD and read the difference values from the calibration curve. Whereas each sample is in a unique cuvet, variances due to the cuvets, turbidity, and the like are eliminated in the difference calculations.

The final concentrations of the reaction mix are: Phosphate buffer pH 7.0, 50 mmol/L; AAP 0.8 mmol/L; DBP, 1.2 mmol/L; Na cholate, 6.0 mmol/L; cholesterol oxidase, 80 U/L; peroxidase, 2000 U/L; and 20% 2-propanol.

Notes:

1. In addition to phosphate buffer,[11] other publications list Tris,[33] or PIPES,[34] and pH ranges from 6.7 to 8.0 have been used successfully.
2. The inclusion of Triton X-100[34,35] diminishes the color response (*Table 2*).
3. The cholesterol oxidase concentration may be lowered to 40 U/L if the final reading is delayed beyond 40 min.
4. Na cholate may be replaced by an equi-molar amount of either Na taurodeoxycholate or Na taurocholate.
5. We have measured UC in rat lymph chylomicrons and in large emulsion particles which have TG:UC ratios of ~ 60:1 (w:w), without first purifying the UC by either solvent extraction or TLC,[36] by working at the low end of the standard curve (*Figure 2*). Agreement with GLC methods has been excellent. However, if the TG:UC mass ratio exceeds 90:1, turbidity becomes unmanageable, and GLC becomes the method of choice.
6. A semi-automatic method has been proposed for research laboratories using auto-diluters and an ELISA reader.[13]
7. When requirements of sensitivity fall into the pico-mole range as they do when samples are from cultured cells, fluorogens are used as H_2O_2 acceptors.[33,37,38]

Gas liquid chromatography for determination of cholesterol and fatty acyl chains of cholesterol esters (wide bore with flame ionization detector and automatic integration of peak areas)

GLC for sterols

When substances which interfere with either the chemical or enzymatic assays (e.g., very high TG values or non-cholesterol sterols), or when sample values are below 0.5 μg, GLC becomes the method of choice for cholesterol determinations. Samples need not be purified by TLC. A disadvantage is that, in the absence of an auto-injector, GLC is more time-consuming than chemical or enzymatic methods, especially when large numbers of samples are to be analyzed.

Materials

Stigmasterol is purchased from Matreya, Inc., formerly Supelco Lipids (Pleasant Gap, PA, USA); a 2', either 2 or 4 mm internal diameter glass

Table 2 Effect of addition of Triton X-100 on color development

% Triton X-100 (g/dl)[a]	% of Control color
0	100
0.1	80
0.5	75
1.0	65
2.5	50

[a] Final concentration in the assay mixture

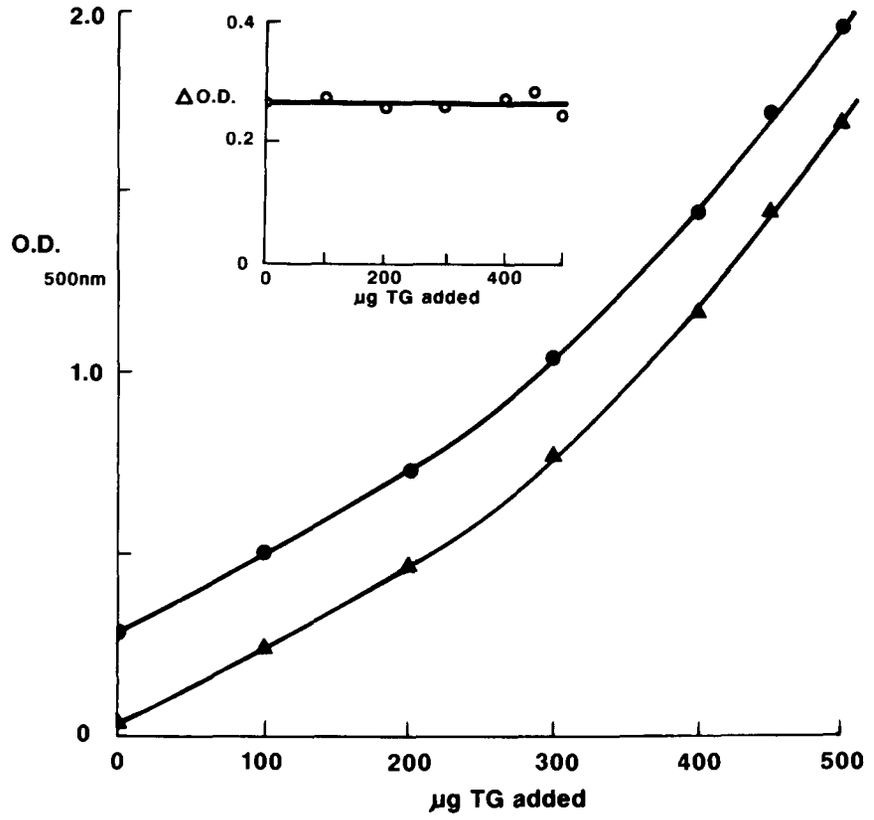

Figure 2 Effect of triglyceride addition on analysis of unesterified cholesterol. 5 μg of cholesterol were placed into each assay tube. Triolein was added in a range of 0–500 μg. Δ OD (initial); Θ OD (final). Inset shows the difference: OD (final) − OD (initial)

GLC column, and 3% SE30 on 80/100 mesh Supelcoport packing are purchased from Supelco, Inc. (Bellefonte, PA, USA).

GLC conditions:

Nitrogen carrier gas at 40 ml/min.
Oven temperature between 240°–270° C (isothermal).
Injector and detector temperatures at 300° C.

1. Cholesterol and stigmasterol stock solutions, each at 1 mg/ml, are prepared in CH_2Cl_2 or hexane. Determine the precise concentrations by microgravimetry of evaporated aliquots.
2. Working standards: Using convenient volumes of the stock solutions, prepare mixtures of cholesterol and stigmasterol in the following approximate ratios: 1:4, 1:2, 1:1, 2:1, and 4:1. Compute the exact ratios from the volumes of the stock solutions used. Label tubes accordingly. These solutions are stable at −20° C under an atmosphere of N_2 for at least 1 year. Solvent evaporation will not change the ratio of components.

Calculations: For each standard, calculate a factor, F, from the integrator printout which accounts for column variation and integrator response:

$$F = (\text{area\% cholesterol/area\% stigmasterol})/\text{std known ratio.}$$

$$\text{ave } F = \Sigma F/5 \text{ for 5 standards.}$$

$$\text{Expect ave } F = 1.05\ (\pm\ 0.02\ \text{SD}),\ n = 5 \qquad (4)$$

3. Preparation of internal standard: The stock solution of stigmasterol can serve as the internal standard solution also. Use an amount of stigmas-

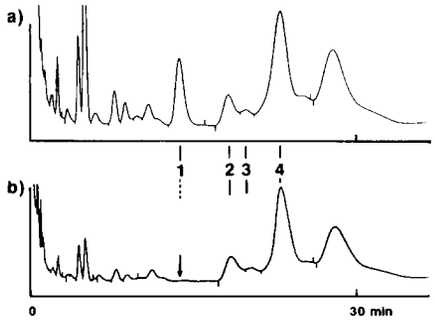

Figure 3 Cholesterol determination in the presence of plant sterols from safflower oil by GLC on SP-2250. 1) cholesterol; 2) campesterol; 3) stigmasterol; and 4) β-sitosterol. The upper panel shows the combined sterols, and the lower panel shows a similar amount of plant sterols in the absence of cholesterol

terol similar to the amount of cholesterol expected in the sample. Add the internal standard as early in the processing as possible, preferably prior to extraction.

4. Remove $CHCl_3$ and resuspend samples in hexane or CH_2Cl_2 prior to injection onto the GLC column. Expect a sample runtime of ~ 12 min.
5. Calculation for unknown: for each sample, calculate a ratio from the integration printout:

$$R = \text{area\% chol} / \text{area\% stig}$$

$$\mu g \text{ cholesterol in unknown sample} =$$

$$(R \times \mu g \text{ stigmasterol in sample})/\text{ave F} \quad (5)$$

Other GLC methods

When small amounts of cholesterol must be measured in the presence of plant sterols,[39] the use of both chemical and enzymatic assays is precluded because the plant sterols interfere strongly. Further, stigmasterol cannot be used as an internal standard for GLC in this instance, and an alternative standard, cholestane, can interfere with an early unknown peak (*Figure 3*). In this case, injections of precise amounts of cholesterol generate a calibration curve, and the following GLC conditions are observed:
Either a 2′ or 3′ glass column, 2 mm inner diameter, packed with 3% SP2250 on 100/120 mesh Supelcoport (Supelco).
Nitrogen flow at 30 ml/min.
Oven temp between 240–270°C (isothermal).
Injector and detector temps at 300°C.
Unknown values are read from the calibration curve.

GLC for identification of fatty acyl chains of cholesterol esters[30]

Materials

A 6′ glass column with a 2 mm inner diameter; GP 5% DEGS-PS on 100/120 Supelcoport packing; and BF_3-Methanol 12% (w+/w+) are purchased from Supelco Inc.
GLC conditions:

Oven temp between 170–190°C (isothermal).
Injection port and detector at 250°C.
N_2 flow rate: 20 ml/min.
(Optimum oven temperature and/or flow rate may differ slightly from the values listed.)

Reference standards: We have found that the methyl ester mix #GLC 63-A (Nu Chek, Elysian, MN, USA) is a sufficient basic reference for most applications. Other reference mixtures such as PUFA No. 1 or PUFA No. 2 (Matreya, Inc.) are helpful when polyunsaturated esters are present in samples. Methyl ester standard mixtures may also be compiled as required. In all cases, standards are dissolved in hexane or CH_2Cl_2 to a final concentration of ~5 μg total mass per μl. The percentage distribution of the component methyl esters is read from the integrator printout and compared to the supplier's specifications. Retention times are noted for comparision with sample unknowns.

Internal standard for quantitation: Quantitation is performed by adding a known amount of cholesteryl heptadecanoate (Nu Chek Prep) to the sample as internal standard, preferably before organic extraction and TLC. The amount of standard added is usually 10–20% of the expected mass of total cholesterol esters in the sample, commonly 25–200 μg. If the mass of cholesterol esters is small, a carrier, such as cholesteryl nervonate (24:1) is useful because methyl nervonate elutes late from the GLC column and integration may be halted before this ester reaches the detector.

Procedure for GLC of FAME

1. TLC is performed and the EC band is eluted from the silica gel as described above. Transmethylation of EC in the presence of silicic acid (i.e., without elution) is not always reliable.[40] Other bands, for example TG, free fatty acid, or phospholipid may be treated similarly.
2. Dry the samples thoroughly under a stream of N_2 in culture tubes. Water must be excluded.
3. Transmethylation is performed by adding in sequence to each tube:

   0.3 ml dry benzene or toluene (to solubilize cholesterol esters)
   0.3 ml dry methanol
   0.35 ml boron trifluoride in methanol

Cap the tubes with Teflon-lined caps and wrap the caps with Teflon tape. Heat in an oven or heating block for 45 min at 100° C, checking after the first 5 min for leaks. Cool the tubes and add 1.5 ml H_2O. Add 5 ml of hexane and extract vigorously. Centrifuge if necessary to clarify the layers. Transfer the hexane layer to a clean test tube and re-extract the aqueous layer with an additional 2 ml of hexane. Combine the hexane washes and evaporate under N_2. Resuspend the residue in hexane or CH_2Cl_2 to a final concentration of ~5 μg/μl.

Notes: Alternative methods of transmethylation have been discussed in detail.[41]

Alternative methods for cholesterol determination using HPLC have been discussed recently in this journal[42] as has the use of GLC capillary columns for FAME.[43]

Alternative literature[44]

Final comment

Selection of the optimal method for measuring cholesterol depends upon: sample source, frequency of analysis, required sensitivity, interfering compounds, and available instrumentation.

The chemical methods are useful when assays are performed infrequently and/or sample values are high, so that a dilution step, a source of error, is not needed. Only a spectrophotometer is required. The caustic reagents used in the chemical methods are disadvantageous, as is the separation of UC and CE by silica chromatography.

The enzymatic method is chosen for more frequent measurements of many samples whose values are within a 0.5 to 40 μg range (visible spectrometry) or a picomole range (fluorimetry). Silica chromatography usually is not required and in most instances UC may be determined directly from aqueous samples in the presence of EC when a hydrolysis step is omitted.

GLC methods, wide bore or capillary, expand the range of sensitivity below 0.5 μg for cholesterol and find universal use for measuring FAME. UC may be measured directly after solvent extraction and interfering sterols may be eliminated by virtue of the column's separating ability.

This selection of methodologies has been culled from a rich literature. Vanzetti, in 1964,[1] wrote that by 1935 over 150 modifications of the Leiberman-Burchard reaction had been published. The review by Zak, written in 1977,[2] lists 238 references. This paper was not intended as an exhaustive review. Simply, it is a description of methods that we have found most useful over the years and that are applicable to a wide variety of samples.

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