A simple method for the determination of serum glycerides, free cholesterol, and cholesterol esters using a binary solvent system

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SUMMARY By partitioning the lipids obtained from 0.1 ml of serum in a binary system formed of 87% ethanol and petroleum ether, the total amount of glycerides, free cholesterol, and cholesterol esters present in the serum is quantitatively determined.

By three partitions carried out with 3 ml of the upper phase and 1 ml of the lower phase of the aforesaid binary system, a phospholipid-free petroleum ether solution containing the total amount of the glycerides present in the serum is obtained. The glyceride content of this solution is determined by glycerol analysis.

Three partitions of serum lipids carried out with 1 ml of the upper phase and 5 ml of the lower phase of the same binary system lead to a complete removal of free cholesterol. The cholesterol ester content of the serum is thus measured by a cholesterol determination on the final petroleum ether upper phase. The free cholesterol content of the serum is measured by a cholesterol determination carried out on the combined lower phases isolated during the cholesterol ester determination.

As previously reported (1), glycerides are easily separated from polar lipids by partitioning the mixture in a binary solvent system formed by mixing equal volumes of 87% ethanol and petroleum ether. Glycerides are recovered from the upper phase, while phospholipids are distributed with a partition ratio (amount in lower phase/amount in upper phase for equal volumes) of about 6. Further studies on the partition ratios of free and esterified cholesterol in the aforesaid binary system have shown that cholesterol esters are also recovered quantitatively from the upper phase of the same binary system, while free cholesterol is distributed with a partition ratio of about 0.6 (equal volumes).

On the basis of these findings a simple method has been devised for the accurate determination of serum glycerides, free cholesterol, and cholesterol esters, requiring very small quantities of serum, i.e., quantities depending only upon the sensitivity of the spectrophotometric tests employed.

PROCEDURE

Extraction of the Lipids

A sample of 0.1 ml of serum is pipetted into a tube containing 2 ml of absolute ethanol–petroleum ether 1:1 (v/v) and the tube is placed for about 1 min in a boiling water bath. (Petroleum ether of bp 40–70° was used throughout this investigation.) After cooling at room temperature, 6 ml of petroleum ether are added and the contents of the tube are mixed by gentle swirling. Water (1 ml) is added, the mixture is gently swirled and allowed to stand at room temperature for 2 to 3 min. The upper phase of the binary system thus formed is then transferred quantitatively into a 10 ml volumetric flask, using small portions of petroleum ether, and adjusted to volume with the same solvent.

Glyceride Determination

Three milliliters of the petroleum ether lipid extract are transferred into a tube about 1 cm in diameter; 1 ml of 98% ethanol–petroleum ether 4:1 (v/v) is added and the contents of the tube are swirled gently. Then 0.1 ml of water is pipetted into the tube, mixed well, and allowed to stand at room temperature for 2 to 3 min. The lower phase of the binary system thus formed is removed with a syringe and discarded. This treat-

ment of the petroleum ether extract is repeated twice more. The final petroleum ether solution is evaporated to dryness at 60–70° and the glyceride content of the serum is then determined by the Van Handel, Zilversmit and Bowman procedure (2), i.e., saponification of the residue, oxidation of the resulting free glycerol to formic acid and formaldehyde, and colorimetric determination of a formaldehyde-chromotropic acid complex.

In the course of the present investigation the Van Handel, Zilversmit, and Bowman method was modified in two ways, namely: (*a*) a solution of higher arsenite concentration (2.0 M) was used, as this lowers the optical density of the blank (3), and (*b*) glycerol served as a standard instead of tripalmitin. The glycerol standard is prepared by taking 0.2 ml of an alcoholic glycerol solution containing 27 μg of glycerol per ml through the method described, starting with the saponification step.

An aqueous glycerol stock solution containing 297 μg of glycerol per ml is prepared by weighing dried (in vacuo) glycerol, and dissolving it in water. Fresh alcoholic glycerol working solutions containing 27 μg of glycerol per ml are prepared each time by mixing 1 ml of the aforesaid stock solution with 10 ml of ethanol; 0.2 ml of this working solution corresponds to 0.06 μmoles of glycerides.

Although glycerol is rather hygroscopic, it can readily be obtained in a pure form after prolonged drying in vacuo, and if some attention is given to carrying out the weighing procedure as quickly as possible in a dry atmosphere, the aforesaid procedure does not lead to erroneous results. This was tested by volumetric glycerol determinations (4) carried out on the glycerol stock solution.

Total Cholesterol Determination

A 1 ml sample of the original petroleum ether lipid extract is transferred to a centrifuge tube and the solution is evaporated to dryness at 60–70°. The total cholesterol content of the serum can then be determined using any of the standard methods (5–7). The following procedure, which is basically a combination of the methods devised by Abell et al. (5) and Searcy et al. (7), has been found very satisfactory: 0.5 ml of a freshly prepared alcoholic potassium hydroxide solution (2%) is added to the dried sample and the tube is incubated at 37–40° for 55 min. After cooling to room temperature, 2 ml of petroleum ether are added and mixed well with the contents of the tube. Next, 0.5 ml of water is added and after mixing well by swirling the tube is centrifuged until two clear layers have been formed. The lower layer is then removed with a syringe and discarded, and the petroleum ether extract thus obtained is taken to dryness at 60–70°. The residue is dissolved in 1.5 ml of glacial acetic acid saturated with ferrous sulfate (7); 0.5 ml of concentrated sulfuric acid is added and the contents of the tube are thoroughly mixed. Optical densities are measured after 10 min at 490 mμ against a reagent blank. Standards are prepared starting with the saponification step.

TABLE 1 COMPARISON OF GLYCERIDE DETERMINATIONS ON EIGHT SERA CARRIED OUT BY THE PRESENT METHOD AND BY THE CHROMATOGRAPHIC PROCEDURE OF BLANKENHORN, ROUSER, AND WEIMER (10)

Serum	Chromatographic Method	Present Method
	mg glyceride-glycerol/100 ml of serum	
1	8.8	8.8
2	7.4	7.5
3	15.4	15.5
4	20.6	20.8
5	15.6	16.7
6	7.3	7.4
7	10.8	11.4
8	19.4	20.6

Cholesterol Ester Determination

Two milliliters of the original petroleum ether lipid extract are transferred into a centrifuge tube and reduced in vacuo to about 1 ml. Next, 5 ml of 87% ethanol (previously equilibrated with an equal volume of petroleum ether) are added to the tube using a rapid delivery pipette, the mixture is immediately swirled and allowed to stand at room temperature for 2 to 3 min. The lower phase of the binary system thus formed is removed with a syringe. This treatment of the petroleum ether extract is repeated twice more. The final petroleum ether solution is taken to dryness at 60-70°, and the quantity of cholesterol esters present in the residue is then determined as described above (see total cholesterol determination).

Free Cholesterol Determination

The united lower phases obtained during the cholesterol ester determination (see above) are evaporated to dryness and the free cholesterol content of the serum is then determined as described above (see total cholesterol determination).

RESULTS AND DISCUSSION

A virtually complete recovery of the glycerides and cholesterol (free and esterified) present in serum is obtained by the ethanol–petroleum ether extraction technique here described. This was verified by comparing the data resulting from analyses carried out on lipid extracts obtained from the same sera both by the extraction procedure here described and by the chloro-

form–methanol 2:1 (v/v) extraction technique described by Sperry and Brand (8).

During the glyceride determination the binary system is best made by adding 0.1 ml of water to the ethanol–petroleum ether mixture, instead of adding 1 ml of the preequilibrated lower phase. By this technique inversion of the tube in order to mix its contents is not required, and losses of the extract caused in stoppers are avoided. As shown by our previously reported data (1), small alterations in the alcohol–water ratio of the binary system used do not change its separating power to an appreciable extent.

Lipid phosphorus could not be detected by a Bartlett determination (9) in the final petroleum ether extracts obtained during the glyceride determinations. As the sensitivity of the Bartlett method is of the order of 0.05 μg of phosphorus, the isolated glycerides could not have contained more than 1–2% of phospholipids.

The glyceride contents of eight sera determined in triplicate by the present method agreed closely (Table 1) with the results obtained by the Florisil method of Blankenhorn, Rouser, and Weimer (10).

The standard deviation of a single glyceride determination was found to be 0.3 mg of glyceride-glycerol per 100 ml of serum; this was calculated from the data obtained by determining the glyceride content of 40 human sera in duplicate.

Tripalmitin, dipalmitin, and monopalmitin solutions in petroleum ether were subjected to the partition treatment of the glyceride determination, and the glycerol contents of the final petroleum ether phases were determined. Glycerol recoveries ranged in all cases from 97 to 101%. This indicates that the total amount of serum glyceride-glycerol is measured according to the method described.

Large amounts (2–3 ml) of serum samples were extracted according to the above procedure, and free and esterified cholesterol were isolated from the extracts, by both the digitonin method (11) and the present one. The isolated fractions of esterified cholesterol were analyzed according to Searcy and Bergquist (7). The results thus obtained were in good agreement.

The standard deviations of a single determination of total, free, and esterified cholesterol using the present method were found to be 3.0–3.5 mg of cholesterol per 100 ml of serum. These values were calculated from the data obtained by analyzing 34 human sera in duplicate. The results of these analyses, expressed in mg/100 ml, ranged as follows: total cholesterol 127–264, free cholesterol 51–85, and esterified cholesterol 95–179.

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