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Notes on Methodology

A method for the estimation of blood glycerides employing florisil*

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» A rapid, reliable method for the direct determination of glycerides in human blood is required for many clinical studies. A suitable method is described in this report. A column of Florisil® (magnesium silicate) is employed to remove phospholipids from a lipid extract of plasma or serum, glycerides are then saponified, and glycerol determined by periodate oxidation. Methods based on similar principles have been developed by Carlson and Wadstrom (1), who have employed a column of silicic acid to separate glycerides from phospholipids, and by Van Handel and Zilversmit (2), and Cheng and Zilversmit (3), who have used zeolites to remove phospholipids by one-stage liquid-solid distribution. Radin *et al.* (4) have used Florisil for the recovery of cerebrosides free of phospholipid, while Carroll (5) has reported the separation of cholesterol, cholesterol esters, triglycerides, diglycerides, and monoglycerides on Florisil.

A glycerol standard solution is prepared from reagent grade glycerol. Specific gravity is determined by comparing the weight of glycerol and water delivered from a constant volume syringe. The water content of glycerol is then calculated (6) and a working standard solution (60 μg/ml) prepared. Solutions made with freshly opened glycerol and freshly distilled water remain unchanged for 18 months at 4° to 8°, and 2 months at room temperature if protected from contaminating molds.

Four per cent alcoholic potassium hydroxide solution (95% ethanol) is prepared each week, protected from air and light, and discarded when brown or turbid.

Chromotropic acid solution is prepared by dissolving 1 g of 4,5-dihydroxy-2,7-naphthalene disulfonic acid disodium salt (practical grade, Distillation Products, Rochester, New York) in 100 ml of water. The solution is filtered and made to 500 ml with 11 M reagent grade sulfuric acid. Chromotropic acid is stored in brown, glass-stoppered reagent bottles. Different lots usually produce blanks having different absorbencies that increase slowly as solutions age. Freshly made chromotropic acid should not have an absorbency greater than 0.045 when read at 570 mμ in a 1-cm cell against distilled water.

Ten normal sulfuric acid, 0.1 M sodium periodate solution, and 1.0 M sodium arsenite solution are prepared as directed by Lambert and Neish (7).

All solvents are reagent grade and all are redistilled in glass apparatus except for anhydrous reagent grade diethyl ether (peroxide-free, purchased in cans).

The water content of Florisil[1] (60 to 100 mesh) is determined by bringing an aliquot to constant weight at 105°. Enough water is then added as a fine spray from a syringe fitted with a No. 25 needle while shaking Florisil in an Erlenmeyer flask to give 40 mg of water per gram of adsorbent. This water content was selected after trial with anhydrous Florisil and adsorbent containing 20, 40, and 70 mg/g indicated that 40 mg/g was the minimum water content for essentially complete retention of lipid phosphorus with rapid and complete elution of glycerides.

Acetone-alcohol 1/1 (v/v) is used for extraction of lipids; the method has been found to yield similar results with chloroform-methanol 2/1 (v/v), and ethanol-ether 3/1 (v/v) extracts. One milliliter of serum or plasma is sprayed by forceful ejection from a calibrated syringe through a No. 25 needle into 25 ml of acetone-alcohol in extraction tubes, which are then closed with polyethylene screw caps (Belart Division of A. S. Aloe, Los Angeles, California; not attacked by acetone-alcohol). The tubes are shaken mechanically for 10 min-

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[1] Floridin Co., Tallahassee, Fla.

utes and then centrifuged briefly to pack precipitated protein. Six milliliter aliquots are withdrawn, dried under a stream of air at 55°, and taken up in 3 ml of a mixture of petroleum ether (60°–75°) and diethyl ether 95/5 (v/v).

Funnel tubes 300 x 4 mm (I.D.) are used for columns (Kimble, No. 46185, Owens-Illinois Glass Co., Owens, Illinois). These are plugged with surgical cotton previously extracted twice with chloroform. Florisil (700 to 800 mg) is packed loosely to give a height of approximately 125 mm. The petroleum ether-diethyl ether solution of lipid is then transferred to the bed and a small volume (1 to 2 ml) of the same solvent is used to effect quantitative transfer of the charge onto the column. Next, 8 ml of chloroform-acetone-water 99/99/2 (v/v) is added and collected with the charging solution in a 25-ml volumetric flask. Twelve columns can be run simultaneously. Two columns are not charged; petroleum ether-diethyl ether and chloroform-acetone-water solutions are passed through each into two volumetric flasks which are later used for the glycerol standard and reagent blank.

The effluents in volumetric flasks are taken to dryness under a stream of air at 55° for 15 minutes (it is important to remove the solvent completely). Alcoholic KOH (0.25 ml) and 95% ethanol (2.5 ml) are added. Flasks are gently shaken and placed in a 55° water bath for 30 minutes. Next, 0.25 ml of 10 N sulfuric acid and 2.5 ml of water are added. One milliliter of 0.1 M sodium periodate solution is added, and exactly 5 minutes later 1 ml of 1 M sodium arsenite solution is added. Iodine liberated in the reaction produces a yellow or brown color which fades rapidly, but may not disappear completely, until flasks are made to volume with water. After solutions are made to 25 ml volume, 2-ml aliquots are withdrawn, mixed with 10 ml of chromotropic acid solution, placed in a 100° bath in the dark for 30 minutes, removed, and read at 570 mμ. A blank solution and a glycerol standard are carried through saponification and periodate oxidation in flasks previously set aside. Repeat samples for color development can be drawn up to 36 hours later and aliquots that vary in size from 1 to 3 ml can be reacted with 10 ml of chromotropic acid.

TABLE 1. TRIPLICATE GLYCERIDE-GLYCEROL DETERMINATIONS ON 10 SERUM EXTRACTS

Serum Extracts	Assay Number 1	2	3
	mg/100 ml		
1	10.5	11.9	10.7
2	17.5	16.6	16.7
3	15.6	17.2	17.7
4	10.9	11.7	11.1
5	0.3	1.5	1.1
6	11.1	11.9	11.1
7	13.9	14.3	14.3
8	16.6	18.0	17.6
9	10.8	10.0	9.8
10	14.8	14.9	15.1

Triplicate assays performed on 10 serum extracts are shown in Table 1. Calculated from these data, the standard deviation of a single determination is 0.6 mg glyceride-glycerol per 100 ml serum. The average glyceride-glycerol content is 12.8 mg/100 ml, a value in satisfactory agreement with those previously reported by others (1, 8).

The removal of phospholipid from 16 serum extracts containing 12.3 to 29.3 μg phosphorus (average 20.1 μg) was studied. No detectable phosphorus could be found in 10 extracts after passage through Florisil. From 0.01 to 0.13 μg (average 0.07 μg) of apparent phosphorus was found in six column effluents. The apparent amount in the eluates was not related to the amount of phosphorus charged, and was at the lower limit of the accurate range of the sensitive method employed (9).

Recovery of added glycerides was studied with six serum extracts. One milliliter of serum was extracted, as described above, and 1 ml each of mono-, di-, and triglyceride solutions containing 5 μg of glycerol per ml was added to 3 ml of the extract. The total amount of glycerol in each mixture was approximately that expected from 6 ml of serum extract. Duplicate recoveries averaged 101%. Similar results were obtained with the extract from serum 5 (Table 1), indicating that the low glyceride-glycerol level found without additions for this sample was not in error.

Florisil appears to have several advantages over previous substances used to adsorb phospholipids from serum extracts. The ease with which Florisil can be handled constitutes a major advantage over silicic acid. Minimal preparation of Florisil yields a reproducible adsorbent readily packed into long and narrow columns to give increased resolution with rapid, reproducible flow rates. Quantitative transfer of the charge is readily performed with funnel tubes, and after the charge is adsorbed, the entire volume of wash solution can be added so that columns do not require further attention. These columns have been found to be very suitable for multiple, precise assays.

The advantage of Florisil over the zeolite Duocil is that it allows recovery of all glyceride classes without passing significant amounts of phosphorus. In our hands, only a variable proportion (1% to 40%) of monopalmitin added to human serum extracts was recovered from Duocil activated as described by Van

Handel and Zilversmit (2). Eluates from Duocil prepared in this manner also contained significant amounts of phosphorus (0.6% to 6.0% of the charge). Because Carlson and Wadstrom (10), Hirsch and Ahrens (11), and Mead and Fillerup (12) have presented evidence that partial glycerides can occur in human blood, it seems important to be certain that all glyceride classes are included when blood is assayed for glyceride-glycerol. In addition, when sera with extremely low glyceride-glycerol content, such as serum 5 (Table 1), are assayed, the presence of small amounts of phospholipid may introduce major analytical error. Florisil columns as described here have been found to pass so little phospholipid that the error introduced into glyceride-glycerol determinations will not exceed 0.12 mg/100 ml serum, while all glyceride classes are recovered quantitatively.

In the present method periodate oxidation for glycerol determination is performed by the method of Lambert and Neish (7), as modifications for the determination of smaller amounts of glycerol have not been required. The original method appears to be more reliable for routine applications. The present method differs from other direct blood glyceride-glycerol methods in that a glycerol standard solution is used. Glycerol can be readily obtained in pure form and standardized by weight, thus avoiding uncertainty that may be encountered in the use of triglyceride standards.

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