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The microtitration of total fatty acids of serum, with notes on the estimation of triglycerides *

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SUMMARY

A method is described for the determination of total fatty acids of serum by microtitration. The method is designed to permit also the determination of cholesterol and lipid phosphorus, and of triglycerides by difference. One ml. of serum is sufficient for duplicate determinations of total fatty acids, lipid phosphorus, and cholesterol. The essential steps include extraction of serum lipids, saponification, extraction and microtitration of the liberated fatty acids. A possible modification which may be useful in the estimation of triglycerides is described. Recoveries of pure fatty acids and triglycerides were 97 per cent complete. Short chain fatty acids and intermediates of carbohydrate metabolism were not detected by this method.

Fatty acids normally represent about 50 per cent of the total serum lipids. The fatty acids occur chiefly in phospholipids, esters of cholesterol, and in triglycerides, but small moieties also are present as di- and monoglycerides and as free fatty acids bound to albumin. No single lipid constituent accurately represents the status of the other lipids. For clinical as well as research purposes a lipid analysis is not complete unless the three major classes of lipids are measured. The concentration of total fatty acids provides an index of total lipids in terms of their common denominator, and when determined with cholesterol and phospholipids, permits by calculation estimation of triglycerides. The latter are of significance in coronary artery disease, diabetes, and other metabolic disorders as well as in the physiological transport of fat (1 to 4). Since chemical reactions are best expressed as taking place between equivalent weights rather than absolute weights of compounds, the concentration of total fatty acids is most usefully expressed in terms of chemical equivalents rather than absolute weight. A need thus exists for a practical yet accurate method for determining total fatty acids as equivalents, preferably a method sharing as many steps as possible with the chemical procedures necessary for determination of cholesterol and phospholipids.

Titrimetric determination of total fatty acids after hydrolysis of serum lipids permits direct estimation of total fatty acids as equivalents. With modern micro apparatus 4 ml. of serum seems disadvantageous and excessive for duplicate determinations of cholesterol, phospholipids, and fatty acids measured by the Man and Gildea modification of the Stoddard and Drury method (5).

The present method provides a technique for the microtitrimetic determination of total fatty acids of small amounts of serum. The method is of sufficient simplicity to permit its use as a routine procedure, yet accurate enough for use as a research tool. It was designed to permit also the determination of cholesterol by the method of Abell *et al.* (6) and of phospholipids by the Sperry method (7). One ml. of serum is sufficient for duplicate determinations of these three substances, although less than two-tenths of a ml. of serum is actually used for duplicate titrations of total fatty acids alone.

The lipids are initially extracted by the method of Folch *et al.* (8) with certain modifications. There follows a saponification procedure similar to, but more intensive than, that recommended by Abell *et al.* for total cholesterol (6). Following acidification of the saponified lipids, the liberated fatty acids as well as cholesterol are extracted by shaking with petroleum ether or hexane. The final step is the direct microtitration of the fatty acids in a hexane-alcohol two-phase system similar to that which has been described

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by Dole (9) for the titration of "nonesterified fatty acids."

METHODS

Reagents.

Chloroform (reagent grade), redistilled twice weekly.

Methanol (reagent grade), redistilled twice weekly.

Hexane (reagent grade). The Fisher product is used without further purification.

Absolute ethyl alcohol (reagent grade). U.S. Industrial Chemical Company absolute alcohol is used without further purification.

Chloroform-methanol (exactly 2:1 v/v).

Alcoholic potassium hydroxide: 6 cc. of 33 per cent potassium hydroxide are added to 94 cc. of absolute alcohol daily (6).

1 per cent phenolphthalein in 95 per cent alcohol.

1.8 N hydrochloric acid.

Nile blue indicator: 0.02 per cent aqueous Nile blue A solution is washed four or five times with hexane, then diluted 1:10 with absolute alcohol (10).

Standard 0.02 N sodium hydroxide.

Extraction. All determinations are carried out in duplicate. The procedure for a single determination is described. One half ml. of serum (or plasma) is pipetted into a 50 ml. round bottom glass-stoppered centrifuge tube.[1] Exactly 10 ml. of 2:1 chloroform-methanol are added to the serum with constant and vigorous swirling during the addition. The centrifuge tube is then stoppered, sealed with a drop of water, and shaken gently with a back-and-forth motion for 1 minute. The chloroform-methanol-serum mixture is allowed to stand at room temperature with occasional gentle agitation for 1 hour. Ten ml. of distilled water are then added gently down the side of the tube. The tube is stoppered without further shaking and allowed to stand in the refrigerator overnight, or for several days if desired. A reagent blank, included in each run, is prepared by the addition of 10.5 ml. distilled water to 10 ml. chloroform-methanol and treated the same way as the sera throughout. After 15 hours the mixture has separated into a lower chloroform phase and an upper aqueous phase which contains impurities of the original mixture soluble in methanol and water. The precipitated proteins are largely concentrated as a disk between the upper and lower phases.

Most of the lower chloroform phase is removed through a long metal cannula[2] or long hypodermic needle attached to a 10 ml. hypodermic syringe. The cannula is passed carefully through the aqueous phase and the protein precipitate by sliding it down the wall to the bottom of the centrifuge tube. About 5.5 ml. of the 6.67 ml. of clear chloroform extract can be removed and transferred to a test tube from which aliquots are then taken. At this stage it is convenient to remove 1 ml. for lipid phosphorus determination (7) if desired. From the remaining chloroform extract, 4 ml. are measured into a clean 50 ml. glass-stoppered centrifuge tube for determination of cholesterol and total fatty acids.

Saponification. The chloroform is readily evaporated from the unstoppered centrifuge tubes by placing the tubes in a large vacuum desiccator equipped with a sand bath which has been preheated to 50°C. The desiccator is evacuated by a water aspirator pump; 45 minutes is usually sufficient to bring the extracts to dryness. Within a few minutes of the completion of the evaporation (the lipids must not be left dry) 5 ml. of alcoholic potassium hydroxide and 1 drop of phenolphthalein are added to the dried lipid extract in the centrifuge tubes. The tubes are incubated in a water bath at 80°C for 1 hour. Approximately 5 ml. of water are then added to each tube. After 10 more minutes in the water bath, 1 ml. of 1.8 normal aqueous hydrochloric acid is added to the warm hydrolysate and the mixture swirled. This is a little more than enough hydrochloric acid to neutralize the potassium hydroxide and should decolorize the phenolphthalein and precipitate the liberated fatty acids as a fine cloud.

When the mixture has cooled to room temperature, exactly 10 ml. of hexane are added to each tube. The tube is stoppered, sealed with a drop of water, shaken vigorously for 1 minute, and centrifuged for 5 minutes at about 2000 rpm. The clear upper hexane phase then contains virtually all of the liberated fatty acids and the cholesterol now as free cholesterol.

If cholesterol is determined, an appropriate aliquot (5 ml. is satisfactory) may now be pipetted from the upper hexane phase for cholesterol determination by the method of Abell *et al.* (6). Then 3 ml. are pipetted from the remaining hexane phase into a 15 ml. centrifuge tube for determination of total fatty acids. Rubber bulb Propipettes® have been found useful in removing the aliquots from the upper phase without disturbing the lower phase.

Microtitration of Total Fatty Acids. Now 3 ml. of Nile blue indicator are added to the 3 ml. of hexane extract in a 15 cc. centrifuge tube. This two-phase system, an upper hexane phase containing the fatty acids and a lower alcohol phase containing the indicator, is titrated directly by adding 0.02 normal NaOH from a microburette. The 1 ml. Gilmont ultramicroburette

[1] Corning centrifuge tubes #8424 are excellent.

[2] Becton-Dickinson stainless steel laboratory cannula, #B-D 1250NR.

has been found satisfactory. For about 2 minutes before and also during the titration a stream of nitrogen, which has been through a solution of 300 ml. of indicator with 40 ml. of 33 per cent NaOH to remove carbon dioxide, is bubbled through the hexane and indicator through a fine glass tube leading to the bottom of the centrifuge tube. This serves to mix the two phases and to exclude carbon dioxide from the air. The end point is indicated by a change in color from blue to pink.

Calculations. The concentration of total fatty acids in meq. per l. is calculated according to the following formula:

$$\text{Total fatty acids in meq./l.} = (\text{ml. of 0.02 N NaOH} - \text{blank}) \times \frac{1000}{\text{ml. serum extracted}} \times 0.02 \times \frac{\text{total chloroform}}{\text{chloroform aliquot}} \times \frac{\text{total hexane}}{\text{hexane aliquot}}$$

Example: 0.5 ml. of serum was extracted with 10 ml. of 2:1 chloroform methanol. After removal of the methanol, 4 ml. of the 6.67 ml. of chloroform were removed for total fatty acid and cholesterol determination, and the solvent evaporated. After saponification, and addition of 10 ml. of hexane, 3 ml. of the hexane were measured for titration of total fatty acids. The unknown required a titer of 0.0755 ml. of 0.02 N sodium hydroxide; the blank gave a titer of 0.0080 (the blank is almost entirely due to the indicator and is constant from day to day). Using the above formula, it was determined that the concentration of total fatty acids in the serum was 15.0 meq. per l.

$$(0.0755 - 0.0080) \times \frac{1000}{0.5} \times 0.02 \times \frac{6.67}{4} \times \frac{10}{3} = 15.0 \text{ meq./l.}$$

Calculation of Triglycerides. The triglyceride concentration expressed as milliequivalents of triglyceride fatty acids per liter is calculated by subtracting the fatty acids of cholesterol esters and phospholipids from the total fatty acids.

The cholesterol is assumed to be 72 per cent esterified (11, 12). This ratio varies between the narrow limits of 68 to 76 per cent in the sera of normal persons (11). The assumed value of 72 per cent would therefore be within 6 per cent of the actual value. Although small increases in the ratio of esterified cholesterol to total cholesterol are common in diabetic acidosis and essential hyperlipemia, the triglycerides are also increased so that any error incurred by the use of the above formula would be small compared to the total triglycerides (4). In the presence of liver disease or obstructive jaundice, however, the ratio of esterified cholesterol to total cholesterol may be greatly reduced (13) and the free cholesterol should be measured rather than assumed. Unless the serum is obviously icteric, the above formula can be used with impunity.

The phospholipid fatty acids are estimated by assuming that 20 per cent of the phospholipids have one fatty acid molecule per atom of phosphorus (sphingomyelin) and that the remaining 80 per cent have two fatty acid molecules for every phosphorus atom (lecithin and cephalins) (11). The assumption regarding sphingomyelin has been borne out by Phillips' recent report (14) that sphingomyelin constitutes 18 per cent of normal serum phospholipids.

The fatty acids not accounted for as cholesterol esters or phospholipids are assumed to be derived from triglycerides, recognizing that a small fraction of these are present as free fatty acids or as mono- and diglycerides (9, 10, 15). The following formula is used, the derivation of which has been discussed previously (11):

$$\text{Triglyceride fatty acids in meq./l.} = \text{total fatty acids in meq./l.} - (\text{cholesterol fatty acids} + \text{phospholipid fatty acids}),$$

where

$$\text{cholesterol fatty acids in meq./l.} = \frac{10 \times 0.72 \times \text{total cholesterol in mg./100 ml.}}{386}$$

and

$$\text{phospholipid fatty acids in meq./l.} = \frac{10 \times [(0.80 \times 2) + 0.2] \text{ lipid phosphorus in mg./100 ml.}}{31}$$

RESULTS

Reproducibility. The difference between duplicate titrations was usually less than 0.0020 ml. of 0.02 N sodium hydroxide. In the entire range of total fatty acids tested, the mean difference between duplicate determinations was 0.40 meq. per l. $\pm$ 0.37 meq. per l. (or 1.98 $\pm$ 1.4 per cent). As practice is gained with the microburette, this error is rarely exceeded.

Recoveries. The completeness of recovery of fatty acids was tested by carrying out the entire procedure on solutions containing known concentrations of pure fatty acids or triglyceride.[3] The material to be tested was dissolved in hexane in known concentrations. An appropriate volume of the hexane solution was meas-

[3] Obtained from The Hormel Foundation, Austin, Minn.

ured into a 50 ml. centrifuge tube and the solvent removed *in vacuo*. To the dried lipid was added 0.5 ml. of water if the material was to be recovered alone, or 0.5 ml. of serum if it was to be recovered from serum. The results were calculated as concentration in water (or serum) and are thus directly comparable with the results of analyses of sera.

The recoveries of palmitic acid and triolein, alone or added to serum, were respectively 97.9 ± 2.6 per cent, and 98 ± 3.7 per cent. Lauric acid was also 97 per cent recovered. The concentrations tested ranged from 8 to 100 meq. per l., a range encompassing all normal and most abnormal sera. In concentrations below 8 meq. per l., recoveries were less complete. Since aliquots of the same size were used for all determinations, the method has applicability over a wide range of concentration without need to adjust the size of aliquots. Recovery of fatty acids with shorter chain length than lauric was not investigated because to date these have not been found in appreciable amounts in serum (16). The possibility that short-chain organic acids might interfere with the method was ruled out by analyzing aqueous solutions of pyruvic, citric, aceto-acetic, succinic, and lactic acids in concentrations between 100 and 200 mg. per 100 ml., higher than are likely to occur in serum. The values obtained were indistinguishable from the reagent blank.

As a further test of the method, fatty acid determinations were made on 60 sera which had been determined in the laboratory of Dr. Evelyn B. Man by the method of Man and Gildea (5). These sera were obtained from both normal persons and patients with a variety of disorders associated with abnormal lipids, including several cases of hypercholesterolemia and essential hyperlipemia. The fatty acid concentrations in these sera ranged from 6 to 100 meq. per l. and the titers of 0.02 N sodium hydroxide ranged from 0.027 to 0.45 ml. The mean difference between methods was 0.80 meq. per l., ± 1.0. When the difference between pairs was expressed as percentage of the total fatty acids, the mean difference was 3.6 ± 3.1 per cent. The positive deviations almost exactly equaled the negative deviations, so that the average algebraic difference was only 0.005 meq. per l.

If the lipid phosphorus is not determined, or is measured directly on serum, the total fatty acids can be measured by adding 5 ml. of the alcoholic potassium hydroxide directly to 0.5 ml. of serum and proceeding with the saponification and titration as described. The serum must be swirled during addition of the alcoholic potassium hydroxide, but further shaking only causes clumping of the protein and should be avoided.

The cholesterol method used here was that of Abell *et al.* (6), except that the serum lipids were extracted and dried prior to saponification, acidified, and extracted with hexane rather than petroleum ether after saponification. The extraction procedure was thought to be desirable prior to saponification in order to provide a common starting point for the determinations of total fatty acids, cholesterol, and phospholipids. Recovery of cholesterol added to serum prior to extraction was complete. In 15 sera analyzed by the method of Abell *et al.*, both with and without prior extraction, the cholesterol concentrations averaged 7 mg. per 100 ml. or 2.6 per cent higher in the extracted sera, although care was taken to treat the standards in exactly the same manner as the unknowns. This discrepancy has not been explained but is less than the error of the method (3 per cent).

A comparison between the cholesterol concentrations determined by the method cited here and by the gravimetric determination (17) from an alcohol-ether extract as conducted in the laboratory of Dr. Evelyn B. Man revealed that the gravimetric method gave somewhat lower values. The absolute amount of the discrepancy increased as the concentration of cholesterol increased; the relative discrepancy remained constant at about 7 to 9 per cent. This difference is small compared to the day-by-day fluctuations of serum cholesterol in a given individual. The lower gravimetric determinations may reflect mechanical errors of this technique, which may be greater now than before, since imported sintered glass funnels formerly used may have retained cholesterol digitonide more completely than the domestic funnels now in use.

Nonphospholipid Fatty Acids. The indirect estimation of triglycerides can be reduced in cumulative errors by eliminating the phospholipids, and thus any possible errors in assuming the fraction of total fatty acids contributed by the phospholipids. Advantage is taken of the absorption of phospholipids by silicic acid (18, 19) in the following adaptation of the method of Eder.[4]

The serum lipids are extracted by adding to 0.5 ml. serum 10 ml. of 2:1 chloroform-methanol, followed by 10 ml. of water, as described in the section on extraction. After standing overnight, about 5.5 ml. of the clear chloroform layer are transferred to a fresh 50 ml. centrifuge tube, with the aid of a syringe and long cannula, care being taken not to transfer any water with the chloroform. Approximately 0.5 g. of silicic acid[5] (¼ level teaspoon household measure is

[4] H. A. Eder. Unpublished method.

[5] Silicic acid (Mallinckrodt) 100 mesh, analytical reagent, "activated" by heating in an oven at 105°C for 24 hours.

convenient and measures about 0.44 g. silicic acid) is added to the chloroform extract through a funnel, with gentle agitation during the addition. The glass stopper is promptly inserted and after standing 15 minutes, with frequent brief, gentle agitation, the tube is centrifuged for 10 minutes. Exactly 4 ml. of the clear chloroform supernatant is pipetted into a fresh 50 ml. centrifuge tube. The subsequent evaporation, saponification, acidification, extraction with hexane, and measurement of aliquots for cholesterol and total fatty acid determination, are identical with the procedures described in the section on methods, except that 50 minutes is sufficient for saponification. The fatty acid titer represents the nonphospholipid fatty acids and is composed of triglyceride and cholesterol ester fatty acids. The triglyceride fatty acids are calculated by subtracting the cholesterol fatty acids (see above) from the nonphospholipid fatty acids.

The completeness of recovery of triglycerides by this method was assessed by analyzing solutions of pure triolein, varying in concentration from 8 to 60 meq. per l., and treated exactly as serum, and 96 to 100 per cent of the triolein was recovered. Ten per cent of free fatty acids added (as palmitic) was lost, but the contribution of free fatty acids to total fatty acids is so small that this loss would not have a detectable influence on the concentration of nonphospholipid fatty acids. Treatment with silicic acid did not influence the reagent blank.

Twenty sera of 20 different persons covering a wide range of concentrations of cholesterol, lipid phosphorus, and triglycerides were analyzed both with and without the addition of silicic acid to the chloroform extract. In none of the 20 was there detectable phosphorus in the extract treated with silicic acid. The cholesterol concentration was not changed by treatment with silicic acid. The triglycerides calculated by the two methods are shown in Table 1. There is good agreement between the two methods over the entire range examined. However, there is a distinct tendency for the triglycerides to be higher in the silicic acid method. This suggests either that there is more sphingomyelin present than assumed in the formula for calculating triglycerides, or that phospholipids are incompletely extracted or saponified during the determination of total fatty acids. In either event, the silicic acid method is probably the method of choice for future studies.

TABLE 1. LIPID VALUES OF TWENTY NORMAL AND ABNORMAL SERA: TRIGLYCERIDES CALCULATED IN THE CONVENTIONAL WAY BY DIFFERENCE AND AFTER REMOVAL OF PHOSPHOLIPIDS WITH SILICIC ACID

Serum	Cholesterol	Lipid P	Triglyceride Fatty Acids	
			Conventional	After Silicic Acid
	mg./100 ml.	*mg./100 ml.*	*meq/l.*	*meq./l.*
1	348	11.6	4.1	4.7
2	194	8.4	4.5	3.7
3	410	18.5	53.3	53.2
4	369	11.5	5.6	6.1
5	282	12.0	12.3	14.5
6	286	12.1	6.8	8.2
7	362	12.1	8.7	10.2
8	232	8.8	2.8	3.4
9	310	20.8	52.1	54.0
10	217	10.7	4.6	5.9
11	396	16.1	18.0	20.2
12	210	9.2	6.3	5.3
13	273	11.6	8.6	8.5
14	274	10.5	7.0	9.5
15	147	6.0	2.9	3.3
16	171	8.0	2.8	3.1
17	307	11.5	5.1	5.7
18	231	11.6	5.6	7.2
19	342	15.0	19.1	18.9
20	395	15.1	2.6	3.3

DISCUSSION

The extraction procedure described by Folch *et al.* (8) and adapted to serum by Sperry *et al.* (20) has been simplified in several respects. First, the separate addition of the chloroform and methanol and the heating of the extract (20) were not found necessary to ensure adequate extraction. It was, however, found essential to swirl constantly during, and for a brief period after, the addition of chloroform-methanol to the serum in order to ensure fine dispersion of the protein precipitate in the extraction mixture.

A second modification of the procedure of Folch *et al.* is the elimination of the filtration of the protein precipitate. The cold chloroform is of such great density that the proteins float to the top of the chloroform phase as the methanol diffuses into the upper aqueous phase.

A third modification avoids the removal of the aqueous phase and subsequent washings, as well as the reconstitution of the remaining chloroform with methanol and the necessity of adding solvent to a definite volume before taking aliquots. The modification is based on the assumption that all the chloroform originally added in the chloroform-methanol mixture is quantitatively restored by the removal of the

methanol into the aqueous phase or that traces of chloroform lost to the upper phase are balanced in volume by traces of water and methanol remaining in the lower phase. The measurement of volumes in a long 50 ml. glass-stoppered cylinder graduated in 0.02 ml. divisions, showed that a volume equal to the 20 ml. of chloroform theoretically present in 30 ml. of 2:1 chloroform-methanol mixture was restored by mixing with 30 ml. of water. Under these circumstances the evaporation of chloroform is evidently negligible, for the level of the chloroform water interface remained unchanged after standing at room temperature for 3 months. Changes in laboratory temperature between 20° and 30°C caused negligible volume changes.

Because of the possibility that serum free fatty acids, a small constituent of total fatty acids, might be incompletely recovered (21) unless the aqueous layer were acidified (22), dilute sulfuric acid and 0.5 M sodium acid phosphate buffer (pH = 4.02) were tried in place of water. In neither instance was the recovery of total fatty acids changed in the least.

The saponification is essentially that recommended by Abell *et al.* (6) for hydrolysis of cholesterol esters. Increasing the temperature from 38° to 80°C increased the yield of total fatty acids, presumably by improving the hydrolysis of the phospholipids. Increasing the strength of the alcoholic potassium hydroxide from approximately 2 per cent to 15 per cent or prolonging the time of saponification to 5 hours produced no further increase in total fatty acids. Acidification of the saponification mixture after incubation, necessary for extraction of the liberated fatty acids by hexane, made no difference in the cholesterol determination and did not contribute to the fatty acid reagent blank. The quantitative recovery of the hexane from the hexane-alcohol-water mixture was ascertained by the measurement of appropriate volumes in a graduated cylinder.

The ingenious two-phase titration system used by Dole for titration of free fatty acids (9) was satisfactory, and because the concentration of total fatty acids is so much greater than the concentration of free fatty acids, much smaller amounts of serum were required. The Nile blue indicator recommended by Gordon (10) gave a good end point. The lag in color change, presumably caused by the lag in transfer of fatty acids between phases, was minimized by the use of hexane instead of heptane and by increasing the volume of indicator to equal the volume of hexane solution. The titer of the blank was almost entirely contributed by the indicator, and constituted as much as 20 per cent of the titer of the unknown. Great care was taken, therefore, in the daily titration of the blank and in the accurate addition of the indicator. Lower blanks and better checks were obtained when carbon-dioxide-free nitrogen was allowed to bubble through the test tube for a few minutes prior to titration.[6]

Standardization of the alkali was carried out by weekly titration against dilutions of 0.1 N hydrochloric acid, and the correction factor thus obtained incorporated into each calculation. The burette was filled with sodium hydroxide from a small glass-stoppered bottle filled freshly each day, and flushed out with nitrogen before filling.

The present method has been found readily adaptable to the determination of lipids in lipoprotein fractions as well as in whole serum or plasma. It conserves time since little extra manipulation is required over that necessary for determining lipid phosphorus and cholesterol. When these determinations are also made, the present method compares favorably in both time and accuracy with existing methods, such as the hydroxylamine colorimetric method (22, 23). It might complement the triglyceride method of Van Handel and Zilversmit (24). It gives more chemically specific information than the gravimetric method of total lipid analysis (20), and avoids the assumptions necessary in the colorimetric total lipid method of Bragdon (25). Finally, it gives results closely comparable with those obtained by the method of Man and Gildea (5). Any future work done with the present method can therefore be compared with the studies of Man and Peters and their co-workers and can thus draw upon the wealth of information which has been given to the field of lipid investigation by these authors.

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