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A fluorimetric micro glycerol method and its application to the determination of serum triglycerides

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SUMMARY

A fluorimetric method has been developed for the estimation of glycerol in aqueous solution. It utilizes a series of reactions in which glycerol is heated with *o*-aminophenol in the presence of concentrated sulfuric acid and an oxidizing agent, to form 8-hydroxyquinoline which produces fluorescence on chelation with a divalent metal ion in alkaline solution. Experimental details are given for the estimation of serum triglycerides on phospholipid-free serum lipid extracts. The method can also be used for the estimation of phosphatide glycerol.

Recent interest in fat metabolism has created a demand for a specific routine method for the direct determination of serum triglyceride levels. While indirect methods which are based on the measurement of serum total fatty acids, cholesterol esters, and phospholipids are available, these require calculation of triglyceride levels by difference, and are unsatisfactory mainly because of the assumptions which have to be made, particularly of the proportion of fatty acids available in the phospholipids.

The application of more exact techniques has been rendered possible by the development of column chromatographic methods for the separation of triglycerides from other serum lipid components, but these tend to be too cumbersome and time-consuming for routine use. The recently published method of Van Handel and Zilversmit (1), with minor modifications, has proved very suitable for routine separation of phospholipids from other serum lipid components. Subsequent alkaline hydrolysis of the triglyceride extract according to Carlson and Wadström (2) allows for measurement of the liberated glycerol by the chromotropic-acid method of Lambert and Neish (3).

The micromethod to be described, which has been adapted from a spot test described by Feigl (4), is simple, accurate, and very sensitive. It utilizes a series of reactions based on the Skraup (5) quinoline synthesis, in which glycerol, liberated from triglycerides by alkaline hydrolysis, is heated at 140° with *o*-aminophenol in the presence of concentrated sulfuric acid and an oxidizing agent to form 8-hydroxyquinoline. The fluorescence produced by chelation of 8-hydroxyquinoline with a divalent metal ion in alkaline solution is utilized to provide a quantitative measure of the glycerol, and therefore of triglyceride concentration.

EXPERIMENTAL

Reagents. All reagents and solvents used were analytical reagent grade, with the exception of *o*-aminophenol, of which only a technical grade was available Light petroleum ether (b.p. 30°-60°) was redistilled before use. Diethyl ether and isopropyl ether were freed from peroxides by passage through a column of activated alumina (heated overnight at 170°) just prior to use.

Silicic acid: Silicic acid (Mallinckrodt; 100 mesh, suitable for chromatography) was size graded by sedimentation with distilled water. The fine particles, which constituted approximately 50% of the total, were separated, dried, and activated overnight at 170°.

Arsenic acid solution (0.6%): Fifty g of arsenic pentoxide are dissolved in 100 ml water, allowed to

stand for 3 to 4 days to form H_3AsO_4, and filtered if necessary. One ml of this stock solution is diluted to 100 ml with concentrated H_2SO_4.

o-*Aminophenol solution (1.6%):* Technical grade *o*-aminophenol is purified by sublimation at 170° in an atmosphere of nitrogen. The sublimed compound tends to oxidize rapidly at this temperature, and it is therefore either recrystallized from, or washed 3 times with, a small volume of isopropyl ether until a colorless solution is obtained when the pure white crystalline compound is dissolved in acetone. This pure product is stable indefinitely if stored in a brown, stoppered bottle. Immediately before use, 0.16 g of the pure *o*-aminophenol is dissolved in 10 ml acetone.

Mg^{++} solution (120 μg/ml): $MgSO_4 \cdot 7H_2O$ (0.12 g) is dissolved in distilled water and made up to 100 ml.

Triolein standards: A stock standard solution is prepared, containing 100 mg triolein (or equivalent amounts of other triglycerides) per 100 ml in chloroform. Dilute standards are prepared, containing 0.2 to 1.0 mg/ml, corresponding to the range 100 to 500 mg triglyceride per 100 ml serum when carried through the procedure.

Procedure. Activated silicic acid (1.2 g) is slurried in a glass-stoppered test tube with 1 ml of isopropyl ether, and 0.3 ml of serum added dropwise with shaking. A further 6.5 ml of isopropyl ether is then added, together with a few glass beads, and the mixture well shaken for half an hour. The silicic acid is allowed to settle, and a 5 ml aliquot of the supernatant extract (≡ 0.2 ml serum) is taken off into a glass-stoppered 15 ml conical centrifuge tube and evaporated to dryness on a hot-water bath using an air blower. One ml aliquots of the triolein standards are similarly evaporated to dryness.

To the dried extract is added 3 to 4 drops ether, 0.5 ml of methanol, and 3 drops of 2% methanolic KOH solution, and the mixture saponified for 30 minutes at 60° to 70°. Two drops of 6% methanolic acetic acid solution are then added, and the mixture evaporated just to dryness on a boiling water bath. Six ml of petroleum ether are added to the hot tube, followed by 0.5 ml of 10 N H_2SO_4, and the tube is stoppered, well shaken, and then centrifuged for 1 to 2 minutes. The petroleum ether layer is carefully pipetted off and discarded. Duplicate 0.1 ml aliquots of the aqueous glycerol phase are then treated as below.

One-tenth ml aliquots of the 1.6% *o*-aminophenol solution are pipetted into a number of test tubes fitted with glass stoppers, and the solvent evaporated, using an air blower. A 0.1 ml aliquot of the serum glycerol extract prepared above is added, followed by 0.4 ml of the 0.6% arsenic acid solution, and the mixture heated in a silicone oil bath at 140° for 15 minutes. The mixture is cooled in ice water, and 1 ml of the Mg^{++} solution is cautiously added with mixing. Five ml of 28% ammonia solution is carefully added, and the tube stoppered and well shaken.

A blank (0.1 ml of 10 N H_2SO_4 solution) and glycerol extracts (0.1 ml), derived as before from 1 ml aliquots of the standard solutions of triolein, are run simultaneously throughout the procedure.[1] After 5 to 10 minutes, aliquots of the above solutions are poured into Farrand fluorometer tubes, and the fluorescence produced by ultraviolet light is measured in a Farrand fluorometer, using aperture 6, with Corning 5874 (primary) and 2424 (secondary) filters. The fluorometer is set at 100% transmission with the highest standard (i.e., 200 μg corresponding to 500 mg/100 ml serum). Where low serum triglyceride concentrations are expected, lower concentration glycerol standards may be used for setting the instrument at 100% transmission, but in this case the blank reading will be higher, as shown in the Figures. It is important that all glassware should be scrupulously clean in order to obtain reproducible results.

RESULTS

The Fluorescence Reaction. The Skraup reaction is commonly used for the synthesis of quinoline derivatives from aromatic amines and glycerol in the presence of concentrated sulfuric acid and an oxidizing agent such as nitrobenzene or arsenic acid. Acrolein, formed by oxidation of glycerol, is probably an intermediate product in the reaction since it will also form quinoline derivatives. The use of *o*-aminophenol as the aromatic amine results in the formation of 8-hydroxyquinoline; however, a large excess of the reagent is required to ensure quantitative reaction with regard to glycerol. This leads to relatively high blanks as well as marked quenching of the fluorescence subsequently produced. Optimum conditions have nevertheless been established in which the influence of these two factors is minimized, and the graph of fluorescence against glycerol concentration is linear for the range 0 to 20 μg glycerol.

Triglycerides such as triolein, tripalmitin, tristearin, and trilinolein have all been found to give quantitative recoveries of glycerol after hydrolysis when checked

[1] Alternatively, glycerol standards, containing 2 to 20 μg of glycerol per 0.1 ml in 10 N H_2SO_4, can be used, since the hydrolysis of the triglyceride is quantitative. Satisfactory results are also obtained with aqueous glycerol solutions.

against glycerol standards. These results have also confirmed, as shown by Carlson and Wadström (2), that glycerol is not lost during the solvent evaporation after saponification. Triolein has been used as the standard for serum triglycerides, and its fluorescence curve is shown in Figure 1.

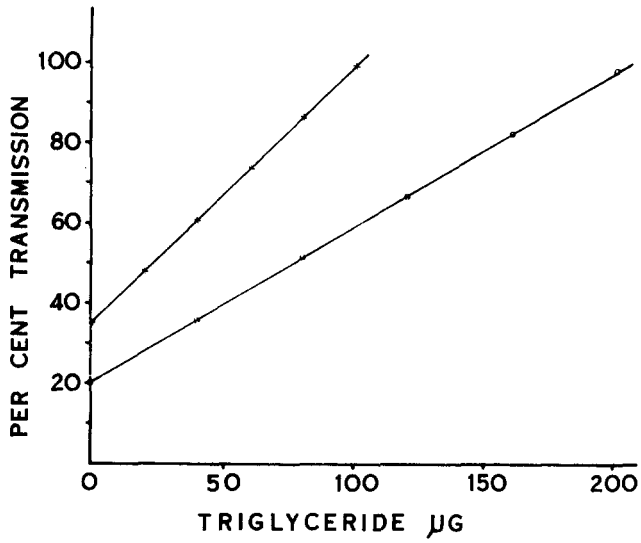

FIG. 1. Relationship between triglyceride concentration and intensity of fluorescence produced by pure *o*-aminophenol.
○——○——○; 200 µg standard set at 100% transmission.
x——x——x; 100 µg standard set at 100% transmission.

The precision of the fluorescence reaction has been determined on duplicate aqueous glycerol extracts derived from 20 different sera. The results indicate an average error of 3.9 mg for the range 17 to 363 mg/100 ml serum.[2] The error will also depend on the sensitivity of the fluorometer, since the range 20% to 100% transmission corresponds to 0 to 200 µg triglyceride when the instrument is set at 100% transmission with the 200 µg standard. As indicated in the procedure and shown in Figure 1, the error can be reduced by approximately one-third by setting at 100% transmission with the 100 µg standard. In this case, the range 35% to 100% transmission corresponds to 0 to 100 µg triglyceride. Since the method utilizes the glycerol liberated from triglycerides, small errors will be introduced by the presence of mono- and diglycerides which can occur up to about 5% to 10% in serum (6).

The influence of a number of serum components on the fluorescence reaction has been investigated. Inositol, glucose, and other hexose sugars do not affect the reaction. Serine, and bases such as choline and ethanolamine, also have no effect. Methanol and ethanol produce interfering fluorescence probably through their aldehydes; however, they are completely removed by evaporation after saponification of the triglycerides. Fatty acids and sterols, when heated with dehydrating agents such as concentrated sulfuric acid, produce interfering fluorescence, and are therefore removed with petroleum ether after saponification. Glycerophosphatides and sphingomyelin interfere in the reaction (see below), and are removed from the serum lipid extracts by silicic acid, as determined by the sensitive phosphorus assay procedure of Chen *et al.* (7). The concentration of free glycerol in serum is small relative to that of triglyceride, and its influence on the fluorescence would therefore be negligible. Moreover, addition of as much as 100 mg of glycerol or glucose to serum prior to extraction with silicic acid and isopropyl ether has had no influence whatsoever on the fluorescence reaction.

It has not been possible to assess directly whether any other serum components alter the serum blank or influence the amount of fluorescence. However, excellent correlation is obtained when the results of the fluorimetric method are compared with those of other procedures described later, and this indicates that any such interference is negligible.

Extraction of Nonphospholipid Serum Lipid Components. In their preparation of phospholipid-free serum extracts, Van Handel and Zilversmit (1) use Doucil and chloroform; however, filtration of the extract is necessary because of the high density of the solvent. Diethyl ether and isopropyl ether, which also extract nonphospholipids from silicic acid, do not require filtration since the silicic acid settles easily from the lower density ethers. Diethyl ether proved to be too volatile and losses were obtained at the shaking stage. These were prevented by the use of isopropyl ether (b.p. 67.5°), which has the additional advantage of having a very low mutual solubility with water.

Elution of all nonphospholipid serum lipid components by the isopropyl ether is quantitative. Cholesterol ester and free cholesterol estimations on the extracts by the method of Sperry and Webb (8), were not significantly different from values obtained on whole serum extracts.

The extraction procedure for triglycerides has been tested by recovery experiments. Triolein was added to serum by two methods: *Method 1.* Chloroform solutions of triolein were added to the stoppered test tubes, and the solvent blown off. Serum (0.3 ml) was added

[2] This has been calculated as the standard error of a single determination, S.D. $= \sqrt{\frac{\Sigma\Delta^2}{2N}}$, where Δ is the difference between two single tests performed on each sample, and N is the number of double determinations.

to the tubes and the mixture shaken. Silicic acid, premixed with a small amount of isopropyl ether, was then added and the mixture slurried with a glass rod. The remainder of the isopropyl ether, together with a few glass beads, was then added, and the mixture well shaken. Aliquots of serum were analyzed with and without the addition of triolein. *Method 2.* Chloroform solutions of triolein were added to larger (5 ml) serum aliquots, the solvent was blown off with N_2, and the mixture was well shaken for half an hour. Aliquots of serum were analyzed before and after the above addition. Recoveries of added triolein varied from 97% to 103% as shown in Table 1.

TABLE 1. RECOVERY OF TRIOLEIN ADDED TO DIFFERENT SERA

Serum	Added	Recovered	Recovered
	μg	*μg*	*per cent*
A (Method 1)	100	100	100
	150	155	103
	200	202	101
A (Method 2)	80	78.5	98
	40	39.6	99
	20	19.4	97
B (Method 2)	50	50.5	101
	100	97	97
	150	147	98
	200	204	102

Triglycerides were estimated fluorimetrically on duplicate aliquots of six serum samples. Results are shown in Table 2. The mean error between duplicate serum analyses was 4.1 mg/100 ml serum, not significantly different from the error on duplicate extracts of the same serum.

Comparison with Other Methods of Triglyceride Analysis. Triglycerides were estimated on 15 sera by the following three methods: (*a*) The proposed fluorimetric procedure. (*b*) By colorimetric estimation of ester groups using a modification (9) of the ferric hydroxamate procedure of Stern and Shapiro (10). This technique was carried out on the extract derived in (*a*) and also on whole sera extracted with Bloor solvent. Free and ester cholesterol were estimated on both extracts by the method described in the previous section (8). The mean molecular weight of the cholesterol ester fatty acids was assumed to be 280. On the whole serum extracts, triglyceride fatty acids were calculated according to the method of Thannhauser and Reinstein (11), serum phospholipids being estimated on the extracts according to the method of Fiske and Subbarow (12). (*c*) By the liberation and electrometric titration at pH 9.0 of fatty acids obtained from serum triglyceride fractions after chromatography of whole serum lipid extracts on silicic-acid columns according to Barron and Hanahan (13).

The results obtained by these methods are shown in Table 3. On the same isopropyl ether extract the fluorimetric method and the ester group method showed an average error of 4.1 mg/100 ml serum, not significantly different from the errors present in each method. Comparison of the fluorimetric and titrimetric methods produces a slightly higher average error of 5.4 mg/100 ml serum, while comparison against the ester group method on whole serum extracts shows the highest average error of 13.0 mg/100 ml serum. The latter method gives lower values than the other methods, probably because of an error in the assumption of the factor (0.69) for the calculation of phospholipid fatty acids (11).

Elution of free fatty acids by the isopropyl ether is also quantitative, and provides a simple method for their extraction free from other acidic serum components. Using larger serum aliquots the free fatty acids have been titrated directly on the isopropyl ether ex-

TABLE 2. ESTIMATION OF TRIGLYCERIDE CONCENTRATION ON DUPLICATE SERUM ALIQUOTS*

Specimen	Triglyceride Concentration (mg/100 ml serum)			
	1st Aliquot		2d Aliquot	
1	41	41.5	42	39.0
	42		36	
2	55	55.0	59	60.5
	55		62	
3	67	69.0	69	69.0
	71		69	
4	108	108.0	114	115.5
	108		117	
5	109	110.0	113	118.0
	111		123	
6	251	254.0	257	261.0
	257		265	

* S.D. of a single determination is 4.1 mg/100 ml serum.

TABLE 3. TRIGLYCERIDE CONCENTRATION BY DIFFERENT METHODS*

Specimen	Triglyceride Concentration (mg/100 ml serum)			
	A	B	C	D
1	82	80	84	78
2	275	265	283	236
3	17	14	20	0
4	160	163	165	150
5	240	233	251	225
6	263	270	273	253
7	122	121	123	118
8	75	73	75	70
9	55	57	63	38
10	363	359	347	323
11	260	264	265	241
12	266	268	277	247
13	327	312	333	315
14	168	166	163	162
15	122	122	125	118

* A: Fluorimetric procedure on isopropyl ether extract.
B: Ester group procedure on same isopropyl ether extract as in A.
C: Titrimetric method.
D: Ester group procedure on Bloor extract of whole serum.
Comparison A/B: S.D. of a single determination = 4.1 mg/100 ml serum.
Comparison A/C: S.D. of a single determination = 5.4 mg/100 ml serum.
Comparison A/D: S.D. of a single determination = 13.0 mg/100 ml serum.

tracts by electrometric titration at pH 9.0 (or with bromthymol blue) using alcoholic KOH. Recoveries of added palmitic and stearic acid have been quantitative. Results have compared favorably with those obtained by other methods.

Normal Values. Normal values obtained by the fluorimetric procedure for the White and Bantu population in South Africa were reported at the First Annual Congress of the Nutrition Society of Southern Africa, in November, 1959, and have recently been published (14). Mean fasting serum triglyceride levels for young White and Bantu males were 86 and 80 mg/100 ml serum, respectively.

DISCUSSION

The procedure recommended consists of a number of independent stages, each of which has been shown to be quantitative and highly selective. The initial extraction with silicic acid and isopropyl ether provides an efficient procedure for the extraction of the nonphospholipid serum lipid components. It has recently been shown by Cheng and Zilversmit (15), using Doucil in the analysis of rat plasma triglycerides, that the particle size of the adsorbent is an important factor governing the complete lysis of the protein-lipid bonds and adsorption of nonlipid serum components. We have similarly found it necessary to employ silicic acid having a particle size finer than 100 mesh, since larger particle sizes lead to low recoveries of the lipid components. Mallinckrodt silicic acid (100 mesh) has therefore been size graded before activation at 170°. We have subsequently used Baker Analyzed reagent grade silicic acid powder, of which not more than 7% is retained on a 100-mesh sieve, and found that recoveries were quantitative, neither the size-grading nor the activation at 170° being necessary.

The procedure for saponification of the triglycerides and isolation of the aqueous glycerol extract is almost identical to that of Carlson and Wadström (2) and the recovery experiments have confirmed that glycerol is not lost during evaporation of the methanol after saponification. For the acidification, 10 N sulfuric acid was used in order to maintain the water content of the final reaction mixture at a minimum.

As mentioned before, the fluorescence reaction is subject to a fairly high reagent blank produced by the large excess of *o*-aminophenol. It is therefore essential that the reagent be as pure as possible and absolutely colorless before use. The amount of oxidizing agent present is not as important a factor. The production of fluorescence with Mg^{++} ions will be affected only by a very large excess of the latter, which may cause precipitation of the Mg^{++}-8-hydroxyquinoline complex.

In attempting to purify the technical grade *o*-aminophenol (Kodak) by recrystallization from ether, a deep-red compound was isolated from the mother liquor after chromatographic separation on a silicic-acid column according to the following procedure: 50 g of technical grade *o*-aminophenol was refluxed for 1 hour with 500 ml of diethyl ether. Then 500 ml of petroleum ether (b.p. 30°-60°) was added, and the mixture cooled at −15° for half an hour. The residue was filtered off, and the clear supernatant was concentrated to a small volume (± 20 ml), and added to a silicic-acid column (100 g; previously activated at 170° and prepared in a 50:50 ether:petroleum ether mixture). Elution was carried out with this solvent mixture (which removed dissolved *o*-aminophenol) until no residue was obtained on evaporation of an aliquot of the eluate. Elution was then continued with pure ether, and a deep-red band began to move down

the column. The eluate containing this fraction was collected and evaporated to dryness under N_2 (yield: approximately 0.5 g). This impurity has a profound fluorescence quenching effect when added back to pure *o*-aminophenol. On its own, however, it proved to be a highly sensitive reagent for the quantitative estimation of glycerol. Four-tenths ml of a 0.0125% solution of this compound freshly prepared in the 0.6% arsenic acid solution, when added to the 0.1 ml glycerol extract and treated according to the procedure outlined above, produced an intense red fluorescence and a much lower blank reading (Fig. 2). In addition, the

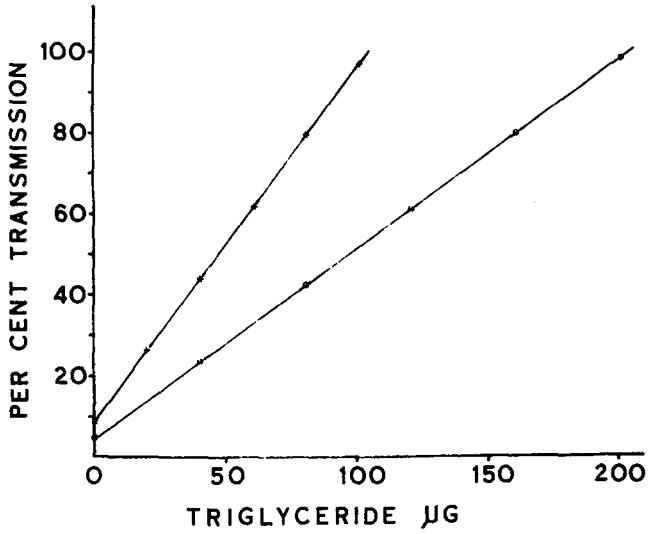

FIG. 2. Relationship between triglyceride concentration and intensity of fluorescence produced by red impurity obtained from technical *o*-aminophenol.
o——o——o; 200 µg standard set at 100% transmission.
x——x——x; 100 µg standard set at 100% transmission.

fluorescence produced in the ammoniacal solution did not require the presence of a divalent metal ion. The nature of this compound is not known, but current investigations suggest a highly polymerized product with the required polar reactive groups, since removal of either glycerol or the oxidizing agent from the reaction mixture did not produce fluorescence.

Application to the Estimation of Phosphatide Glycerol. The proposed method is particularly suited to the determination of glycerol in glycerophosphatides. Lecithins, cephalins, inositol phosphatides (containing inositol instead of a base in the glycerophosphatide), and lysolecithins obtained by fractionation of serum phospholipids on silicic-acid columns according to Hanahan *et al.* (16) have all given 1:1 glycerol:phosphorus molar ratios when analyzed for glycerol content according to the above procedure. The mild alkaline hydrolysis recommended in the procedure readily deacylates the fatty acid moiety of the glycerophosphatide resulting in the production of an equilibrium mixture of α- and β-glycerophosphate esters (17). It would appear, therefore, that both of these esters react quantitatively in the fluorescence reaction.

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