Quantitative determination of plasma free fatty acids and triglycerides by thin-layer chromatography

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SUMMARY The relation between the area of a spot and the amount of lipid contained in it after thin-layer chromatography has been applied to quantitative determination of plasma free fatty acids and triglycerides. Satisfactory results were obtained for both lipid classes with a standard deviation of ±8.1 and ±8.5% respectively.

KEY WORDS determination · plasma · free fatty acids · triglycerides · thin-layer chromatography

QUANTITATIVE ANALYSIS of lipids by elution from thin-layer chromatograms (1) usually requires milligram amounts. Densitometry on thin-layer plates has been successfully used for quantitative analysis by Zöllner et al. (2) for plasma cholesterol esters and by Blank et al. (3) for mixtures of mono-, di- and triglycerides, neutral and phospholipids. The relation of spot area to amount of materials after thin-layer chromatography (TLC) was first used by Seher (4, 5); Purdy and Truter (6) extended these observations and proposed that for TLC the square root of the spot area is a linear function of the logarithm of the weight of the substance contained in this area. The physicochemical basis for the relationship of area of spot to weight of material has been discussed in detail by Brenner et al. (7).

We have applied this procedure to the quantitative analysis of human plasma lipids, in particular free fatty acids (FFA) and triglycerides (TG).

Materials and methods. Plasma extracts were prepared and washed by the method of Folch et al. (8) and the lipids taken up in chloroform after evaporation of the solvent under nitrogen. For determination of TG the volume of chloroform solution was adjusted to equal the volume of plasma from which the lipids were derived; for FFA determination, the volume was one-quarter that of the plasma extracted.

Standards were prepared from pooled postprandial plasma as follows: lipids were extracted from three 4-ml aliquots and subjected to preparative TLC. The TG were eluted quantitatively with diethyl ether and weighed. FFA in the extract were measured according to the method of Dole and Meinertz (9) and the mean of eight determinations was taken as reference value. The TG concentration of the standard extract (in chloroform) was adjusted to 156 mg/100 ml by suitable dilution and the FFA concentration then made up to 200 mg/100 ml by addition of pure stearic acid. A postprandial plasma sample was taken for preparation of the standard, since the FFA content was very small and the amount of stearic acid to be added represented approximately 94% of the final amount. From this standard solution of FFA and TG two diluted standards (1:1 and 1:3 by volume) were prepared.

"Pyrex" glass plates (20 × 26 cm) were coated with Silica Gel G (E. Merck AG, Darmstadt, Germany) to a thickness of about 250 μ. The adsorbent was activated before use at 110° for 1 hr.

For quantitative determination of TG or FFA, 25 μl aliquots of plasma extracts of the appropriate concentration were applied as duplicate spots along the 20 cm side of a plate. As a rule, four unknowns were processed together with 25 μl of each of the three standard solutions, care being taken to produce spots of identical size at the baseline. The plate was developed in a mixture of petroleum ether (bp 30–60°)–diethyl ether–glacial acetic acid 82:18:1 (v/v/v); a saturated atmosphere was maintained in the tank by lining the walls with filter

TABLE 1 REPRODUCIBILITY OF THE SPOT SIZE METHOD ON TLC FOR DETERMINATION OF PLASMA FREE FATTY ACIDS AND TRIGLYCERIDES

	Mean ± SD	No. of Determinations
	mg/100 ml	
TG by spot size	155 ± 13.2	20
TG, gravimetric	156.6 ± 0.71	3
	μeq/liter	
FFA by spot size	377 ± 24.5	20
FFA, Dole and Meinertz (9)	470 ± 18.9	8

paper. After the solvent had risen to within 3 cm of the top, the plate was removed and briefly dried, and the lipid spots were made visible by spraying with sulfuric acid–water 1:1 (v/v) and charring on a hot plate at 250°.

The areas of the spots were conveniently measured by covering the plate with Scotch transparent tape (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) 8 inches in width. The border of the appropriate spots was then demarcated in ink on the surface of the Scotch tape (Fig. 1) and a photocopy made with a Verifax Signet Copier, Model B (Eastman Kodak Co., Rochester, N.Y.). The area corresponding to the spot was then accurately cut from the copy paper with a razor blade, and estimated by weighing the paper disks on a semimicro balance.

A standard curve was made for each plate by plotting on semilogarithmic paper the square root of the spot area against weight of lipid applied for the three standards. The TG or FFA contents of the unknown samples were then deduced from the areas of the appropriate spots, using the standard curve.

Results. Table 1 shows the results of 20 determinations of TG and FFA from the same plasma sample. The mean value for TG is compared with the control value obtained by gravimetric determination, as described above. The mean value for FFA from spot-size determination was 93 μeq/liter lower than the mean of eight determinations by the procedure of Dole and Meinertz (9).

There was generally good agreement for the TG content of 11 plasma samples, when determined in duplicate by gravimetry and by the present method (Table 2). There was again a tendency for FFA values determined by spot-size measurement to be lower than those obtained from the titration procedure, particularly at low FFA levels.

Figure 1 shows a typical TLC plate employed for determination of FFA, using plasma samples obtained before, during, and after exercise, from the same subject. The sequence of events is evident, with a moderately high initial level after a 15-hr fast, decrease of FFA concentration during strenuous exercise, and rebound rise during rest after cessation of work.

Figure 2 shows the potential application of TLC as a simple and fast screening procedure for abnormal plasma lipid levels by comparing the spots obtained from unknown samples with those from a standard plasma, as recently suggested by Sachs and Wolfman (10). Hypertriglyceridemia and hypercholesterolemia of all but very minor degrees are easily recognized and the plasma lipid levels may be determined subsequently using the same extract.

Discussion. The success of the method described depends initially upon complete extraction from plasma of TG and FFA. Unpublished work from this laboratory, using C^{14}-labeled lipids, has shown losses of these lipids during Folch extraction and washing (8) to be negligible.

The use of TLC during determination of TG and FFA has two advantages over other available methods. Firstly, an over-all picture of the total lipid composition of the plasma is obtained, and it is clear that only the desired component (TG or FFA) is being measured. Secondly, a single determination may be carried out for most plasma samples with only 25 μl (TG) or 100 μl (FFA) of plasma.

Important points during this procedure are as follows: TLC plates should be prepared and selected with care for uniformity of the silica gel layer. Samples and standards should be applied in the same volume (25 μl) of the same solvent (chloroform); a micro syringe (Hamilton Co., Whittier, Calif.) is recommended. Three different standards should be run with each plate so that a standard curve may be constructed for the particular conditions prevailing; the standard points should lie on or very close to a straight line when plotted as described. The area of the spots from the unknown samples should lie between the areas of the smallest and greatest standard spots, and

TABLE 2 COMPARISON OF SPOT SIZE METHOD WITH STANDARD METHODS

Subject No.	Triglycerides		Free Fatty Acids	
	Spot Size*	Gravimetry	Spot Size*	Titration
	mg/100 ml plasma		*μeq/liter plasma*	
1	228	231	450	460
2	103	92	445	466
3	47	46	267	318
4	125	117	372	375
5	128	110	763	698
6	50	51	110	185
7	77	86	223	270
8	115	126	170	290
9	506	532	637	691
10	114	120	310	355
11	349	339	1440	1530

* Mean of two determinations.

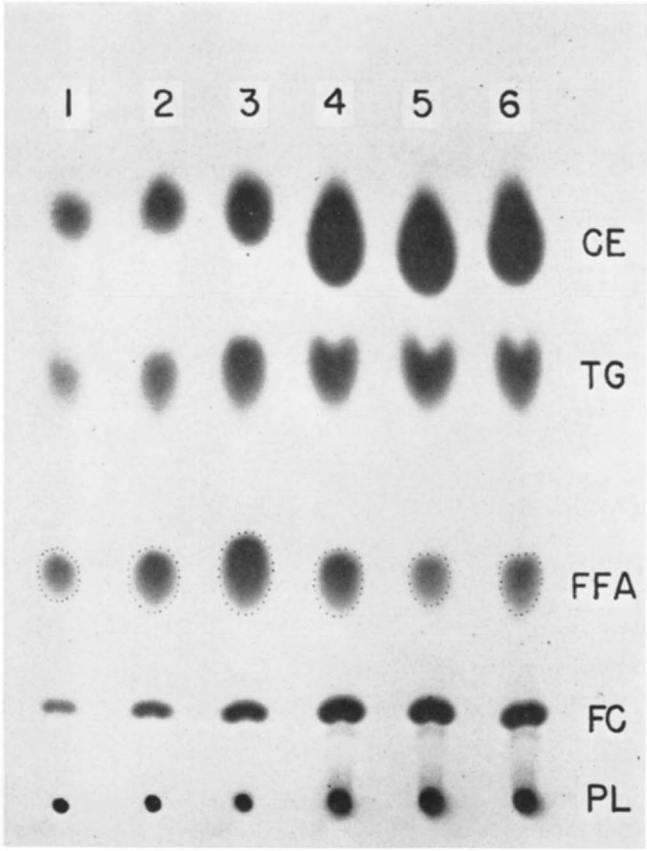

FIG. 1. TLC of plasma lipids, illustrating effect of exercise on free fatty acids. *1–3*, standard lipids; *4–6*, lipids from 100 μl of plasma taken before, during, and after vigorous exercise, respectively. Amount of lipid contained in FFA spots: *1*, 12.5 μg; *2*, 25 μg; *3*, 50 μg; *4*, 28 μg; *5*, 12 μg; *6*, 18 μg. CE, cholesterol esters; FC, free cholesterol; PL, phospholipids (at origin).

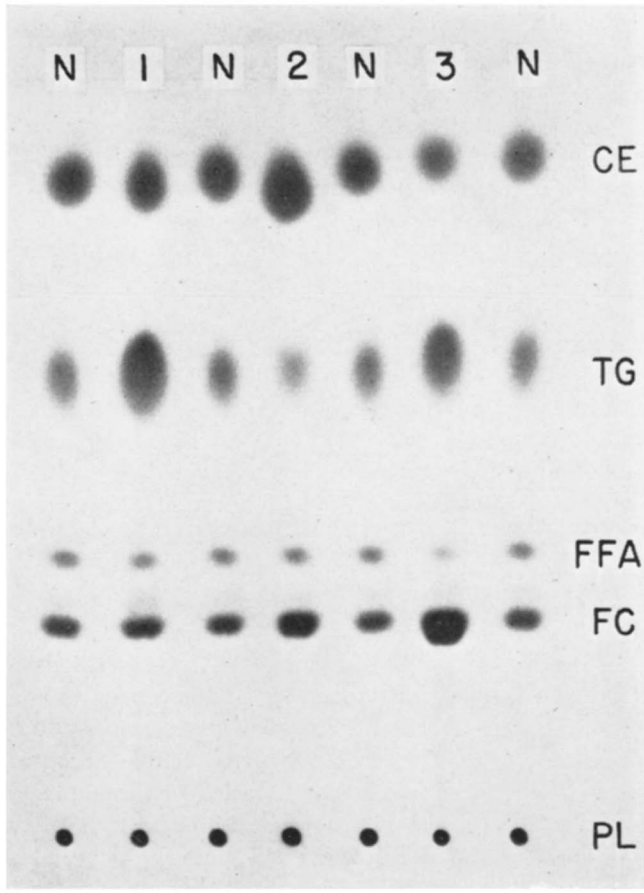

FIG. 2. Application of TLC in screening plasma samples. Diagnosis and plasma lipid levels (determined by chemical methods, in mg/100 ml) were as follows: *N*, normal: total cholesterol (TC), 174; TG, 82. *1*, hypertriglyceridemia: TC, 215; TG, 445. *2*, hypercholesterolemia: TC, 436; TG, 61. *3*, biliary cirrhosis: TC, 487; FC, 380; esterified cholesterol, 107; TG, 220.

therefore occasionally, adjustment of the concentration of an unknown may have to be made. Duplicate determinations are recommended.

Difficulties are encountered with FFA determination in the presence of markedly elevated plasma TG (> 500 mg/100 ml). Preliminary isolation of the FFA by preparative TLC may then be carried out, followed by a second chromatography and measurement of spot size.

The present method usually gives lower results for FFA than the titration procedure of Dole and Meinertz (9), particularly at low FFA levels (Tables 1 and 2). Possibly hydrolysis of phospholipids during the extraction procedure of Dole and Meinertz may be responsible for the discrepancy. Support for this was obtained by isolating FFA from a postprandial plasma by preparative TLC and titrating the FFA in the absence of phospholipids; good agreement with the spot-size determination of FFA was then obtained.

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