Journal of Chromatography, 146 (1978) 261—271
Biomedical Applications

CHROMBIO. 193

QUANTITATIVE HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY OF LIPIDS IN PLASMA AND LIVER HOMOGENATES AFTER DIRECT APPLICATION OF 0.5-μl SAMPLES TO THE SILICA-GEL LAYER

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(Received March 1st, 1978)

SUMMARY

Lipid profiles were determined by high-performance thin-layer chromatography (HPTLC) after direct application of 0.5 μl plasma from capillary blood to the silica-gel layer. Coefficients of variation for the fluorescence measurements were 2.1% for cholesterol, 1.5% for cholesterol esters, 2.8% for triacylglycerols, and 2.3% for phosphatidylcholine. The recovery of known amounts of lipid was 96—100%. A linear relationship between peak area and amount of lipid was found in the nmole range, corresponding to the amount of lipid in 0.125—0.75 μl Lipid-Trol, which served as the standard reference sample.

The plasma lipids of healthy subjects and of patients suffering from various illnesses were analyzed using reference methods and HPTLC. Identical values were obtained for cholesterol esters, triacylglycerols and phosphatidylcholine. Free cholesterol values determined by HPTLC were slightly lower (7%). The correlation between data obtained by reference methods and HPTLC was as follows: cholesterol, $r = 0.938$; cholesterol esters, $r = 0.964$; triacylglycerols, $r = 0.985$; phosphatidylcholine, $r = 0.938$. The separation and quantitation of liver lipids using HPTLC after direct application of the tissue homogenate to the silical-gel layer was carried out. Comparison with reference methods revealed that HPTLC gave higher cholesterol values (24%). The triacylglycerol concentrations, however, were identical under both methods and correlated satisfactorily ($r = 0.959$).

INTRODUCTION

Thin-layer chromatography (TLC) has been widely used for the determination of plasma lipids in various clinical situations [1—3]. However, lipid extraction prior to TLC is time-consuming and requires relatively large sample volumes. Therefore, efforts have been made to apply the plasma samples directly to the silica-gel layer. Whitner et al. [4], Buckley et al. [5], and Mantel et al. [6] succeeded in separating the neutral lipids of 10—20 μl serum using direct application of the sample to self-prepared thin-layer plates. With

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these systems, the phospholipids were not developed.

The direct application of 5—20 μl serum to commercial TLC plates has been described in a previous report [7]. Hydrochloric acid was used to denature the serum proteins on the plate. The quality of separation of the neutral lipids was satisfactory. The recovery of ^{3}H-label in free and esterified cholesterol of samples incubated with [^{3}H]-cholesterol prior to TLC was nearly 100%; the coefficient of variation was less than 2%. However, the phospholipids were not separated in this system.

It was the purpose of this study to develop a direct application procedure for quantitative high-performance TLC (HPTLC) in the nmole range. After the direct application of 0.5 μl capillary blood plasma to the silica-gel layer, the neutral and polar lipids were separated. Quantitation of the lipids was carried out by fluorescence measurement according to Segura and Gotto [8]. The results obtained for healthy subjects and various types of hyperlipidemic patients were found to correlate well with those determined by reference methods.

The same procedure was also applied to the separation of lipids in liver homogenates.

MATERIALS AND METHODS

HPTLC

HPTLC plates pre-coated with silica gel 60 without fluorescent indicator (10 × 20 cm; Merck, Darmstadt, G.F.R.) were used for nano-TLC.

Chemicals

All chemicals used were Merck analytical grade.

Reference methods

The results obtained by HPTLC were compared with the following reference methods: enzymatic determination of cholesterol [9]; enzymatic determination of triacylglycerols; alkaline hydrolysis of triacylglycerols and enzymatic determination of glycerol; phosphatide-phosphorus determination (Biochemical Test Combinations, from Boehringer, Mannheim, G.F.R.); cholesterol determination according to Liebermann—Burchard (Merck).

Standard reference samples

For standardization, commercial standard samples with known lipid concentrations were used: Lipid-Trol (Dade Div. Amer. Hosp. Supply Corp., Miami, Fla., U.S.A.); Precilip (Boehringer).

Instruments

For fluorescence measurement of the lipids separated by HPTLC, a Model KM 3 chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.) equipped with a mercury lamp was used. Emission was recorded with a Servogor S recorder (Metrawatt, Nuremberg, G.F.R.). In a few cases, electronic integration was carried out with the Autolab System I (Spectraphysics, Santa Clara, Calif., U.S.A.). A Desaga Uvis lamp (Heidelberg, G.F.R.) served for visual examination

of the chromatoplates at 366 nm. The enzymatic triacylglycerol determinations were carried out on the LKB reaction rate analyzer (Copenhagen, Denmark) in conjunction with a Hewlett-Packard calculator (Loveland, Colo., U.S.A.).

Preparation of the biological samples

Capillary blood was collected in heparinized capillaries and centrifuged in an hematocrit centrifuge. The plasma samples not immediately used for lipid analysis were frozen in the capillaries.

A 1-g sample of fresh rat liver was homogenized with purified sand in a mortar in the cold. The volume was made up to a total of 5 ml with Tris buffer, pH 7.4; 10 mmole/l. The homogenates were centrifuged for 10 min at 78 *g* and the supernatants subjected to lipid analysis.

Direct application of the samples

The HPTLC plates were purified overnight in solvent system I (see below); a longer purification period is not advisable. After evaporation of the solvents with a hair dryer, the plates were activated for 1 h at 110°. A 15-μl volume of absolute methanol was applied by means of a micro-pipette to the silica gel layer 1.2 cm from the lower edge of the plate. As soon as the moisture was absorbed by the silica gel, 0.5 μl plasma or liver homogenate were applied on the methanol spot. A 10-μl quantitative microlitre sample dispenser (Elevitch; Hamilton, Reno, Nev., U.S.A.) fitted with a disposable sample tip 1 1/8 in.; Corning, Palo Alto, Calif., U.S.A.) was used to apply the samples. The dispenser with the tip containing the sample, was held in an upright position and the sample was pushed out so that a drop hung on the tip; this known amount of the sample was then applied to the methanol spot (the tip was held in position for 2 sec). The dispenser was then removed without releasing the piston. Immediately afterwards, the plasma spot was covered with a few μl methanol. It is important that this second application of methanol does not exceed the diameter of the sample spot. The spots were then dried under a stream of cold air. A total of 14 samples could be applied to one HPTLC plate by this procedure.

Separation of lipids

The chromatogram was developed in a saturated chamber in solvent system I (chloroform—methanol—water, 65:30:5). The solvent front was allowed to migrate a distance of 3.7 cm from the lower edge of the plate (3.5 min). After evaporation of the solvents under a stream of cold air, this first run was repeated and the solvents were again evaporated. With solvent system II (*n*-hexane—diethyl ether—acetic acid, 80:20:1.5) the neutral lipids were developed by migration of the solvent front up to 1 cm from the top of the plate (20 min). The plates were dried under a stream of warm air for approximately 1 h until the odor of acetic acid could no longer be detected.

Solvent system I was used for the development of a total of 4 plates, solvent system II for 2 plates.

Detection

The method used was a slight modification of that described by Segura and Gotto [8]: the HPTLC plates were transferred to a sandwich chamber (Desaga, Heidelberg, G.F.R.) containing $(NH_4)HCO_3$ on the bottom; 0.5 g were used for the development of 1 plate and 1.0 g for 2—4 plates. The plates were positioned 3 cm from the bottom, and the chamber (sealed with high vacuum grease) was placed in an oven and heated for 10 h at 150°.

Fluorescence measurements

The fluorescence of the lipid spots was scanned across the axis of development with the Model KM 3 chromatogram spectrophotometer (excitation at 366 nm, emission at 430 nm). The peak areas were taken as the product of peak height and the half-width of the peak. In a few cases, integration was carried out electronically with the Autolab System I.

Standardization of the HPTLC procedure

Free and esterified cholesterol in the standard reference samples, Lipid-Trol and Precilip, were determined enzymatically [9]. Triacylglycerols and phosphatidylcholine were obtained by preparative TLC [10] and, after dissolving the lipids in 1% Tween 80, the enzymatic determination of the triacylglycerols was carried out using the LKB reaction rate analyzer; the phosphatidylcholine was ashed prior to phosphorus determination.

Liver homogenates

For reference analysis, cholesterol was obtained by preparative TLC and determined according to Liebermann—Burchard; the enzymatic determination was carried out in the homogenate. For triacylglycerol analysis, free and total glycerol in the homogenate were determined enzymatically.

Comparison of HPTLC with reference methods

The lipids of healthy subjects and of hyperlipidemic patients (newborns, children and adults) were analyzed by HPTLC and by reference methods. The phosphatidylcholine was estimated as described for the standard reference samples. The results were examined by linear-regression analysis.

RESULTS AND DISCUSSION

The separation of lipids after the direct application of serum or plasma to the silica-gel layer usually gives poor results, due to the proteins in the sample [11]. In the present study, the separation of the lipids after direct application of plasma samples to the HPTLC plate was sharp and reproducible (Fig. 1), with coefficients of variation of the fluorescence measurements less than 2.8% for the lipids investigated (Table I). This precision of determination is consistent with data reported by Mlekusch et al. [12, 13] for the fluorometric determination of lipids preceded by lipid extraction. The relationship between peak area and the amount of lipid was found to be linear in the nmole range, corresponding to the amount of lipid in 0.125—0.75 μl Lipid-Trol (Fig. 2). In our experience, most of the lipid profiles to be determined in a clinical laboratory can be obtained using 0.5 μl plasma.

In order to standardize the HPTLC procedure, the lipid concentrations of Lipid-Trol and Precilip were evaluated by reference methods. The lipid concentrations in Lipid-Trol served as standards on the HPTLC plates and Precilip was used for accuracy control. The recovery of the Precilip lipids on the HPTLC plates was 96—100% of the reference values (Table II).

In order to gain insight into the responses of different types of plasma samples, up to 93 samples from newborns, healthy adults and various types

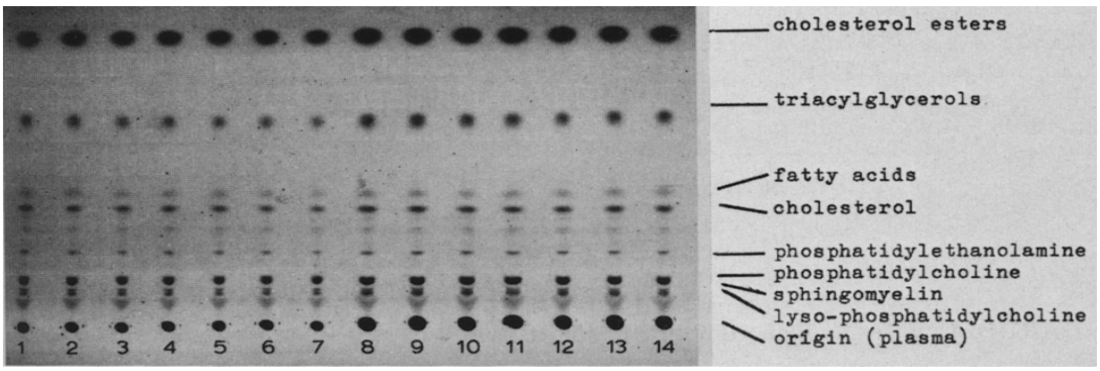

Fig. 1. Separation of plasma lipids by HPTLC (positions 1—7: 0.5 µl, positions 8—14: 1 µl Lipid-Trol). Visualization of the lipids with iodine vapor.

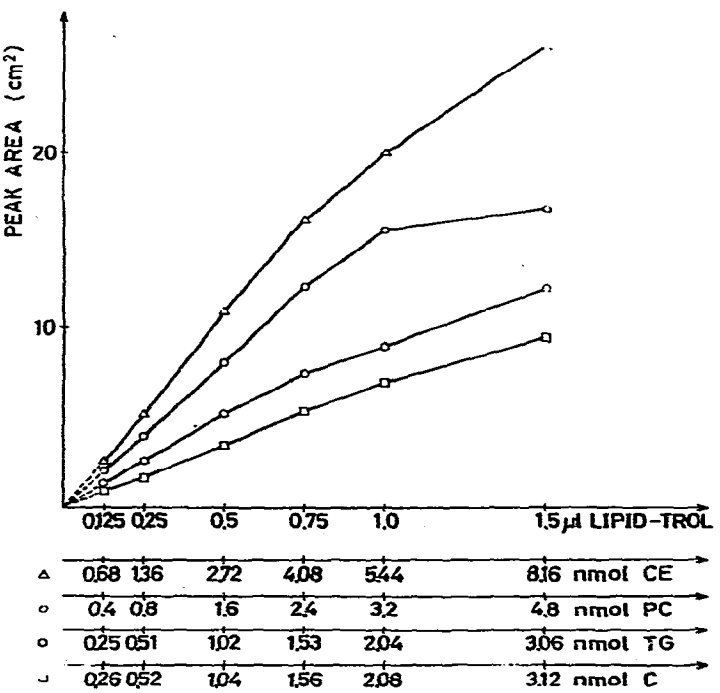

Fig. 2. Calibration curves for cholesterol esters (CE), phosphatidylcholine (PC), triacylglycerols (TG), and cholesterol (C).

TABLE I

RELATIVE RESPONSES OF LIPIDS AND PRECISION OF FLUORESCENCE MEASUREMENT

The lipids of Lipid-Trol were separated by HPTLC and the peak areas were taken as the product of peak height and half-width of the peak. The coefficients of variation (C.V., %) were calculated from 14 samples per HPTLC plate.

Lipid	Amount (nmole/spot)	Peak area (cm^2)	cm^2/nmole	C.V. (%)
Cholesterol	1.04	6.26±0.13	6.02	2.1
Cholesterol esters	2.71	15.74±0.23	5.82	1.5
Triacylglycerols	1.07	7.84±0.22	7.33	2.8
Phosphatidylcholine	1.6	8.7±0.2	5.4	2.3

TABLE II

STANDARDIZATION OF THE HPTLC PROCEDURE

The lipids of Lipid-Trol and Precilip were analyzed using the reference methods. The values obtained for Lipid-Trol served as standards and values of Precilip for accuracy control. Both standard reference samples were applied to each HPTLC plate. Mean values were obtained by analysis of the samples during 20 days. Numbers in parenthesis = coefficient of variation (%).

Lipids (mmole/l)	Lipid-Trol	Precilip		
	Reference method	Reference method	HPTLC	Recovery (%)
Cholesterol	2.08±0.10 (4.9)	0.87±0.08 (8.9)	0.87±0.07 (8.3)	100
Cholesterol esters	5.41±0.12 (2.3)	2.89±0.10 (3.4)	2.81±0.12 (4.3)	97
Triacylglycerols	2.14±0.08 (3.7)	0.84±0.03 (2.9)	0.81±0.03 (4.1)	96
Phosphatidylcholine	3.2 ±0.1 (3.2)	—	2.1 ±0.1 (4.8)	—

TABLE III

COMPARISON OF DATA OBTAINED FROM HUMAN SUBJECTS BY REFERENCE METHODS AND HPTLC

n = number of subjects investigated; P = error of probability according to t-test.

Lipids (mmole/l)	Reference method	HPTLC	P	n
Cholesterol	1.48±0.50	1.38±0.51	<0.0005	83
Cholesterol esters	3.35±1.16	3.30±1.05	<0.10	93
Triacylglycerols	1.03±0.66	1.05±0.68	<0.10	69
Phosphatidylcholine	2.7 ±0.6	2.7 ±0.5		22

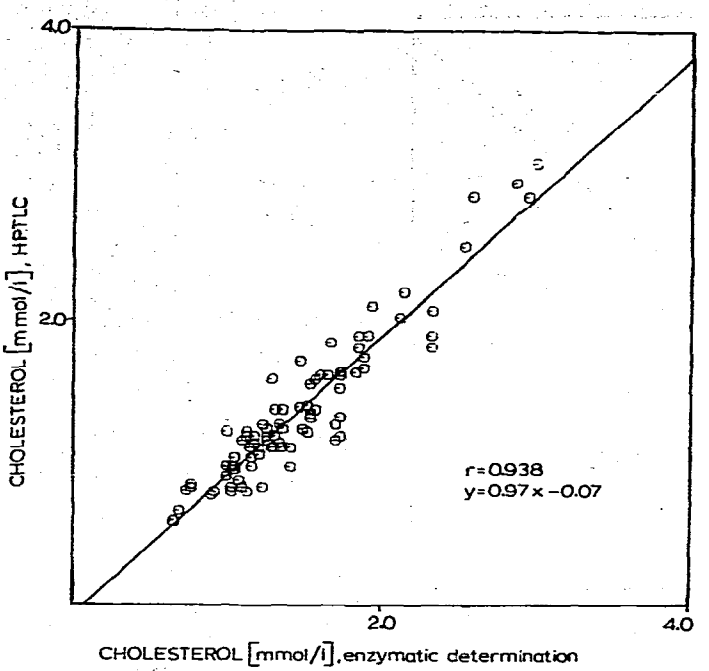

Fig. 3. Correlation between enzymatically-determined cholesterol and HPTLC cholesterol (n = 83); see Table III.

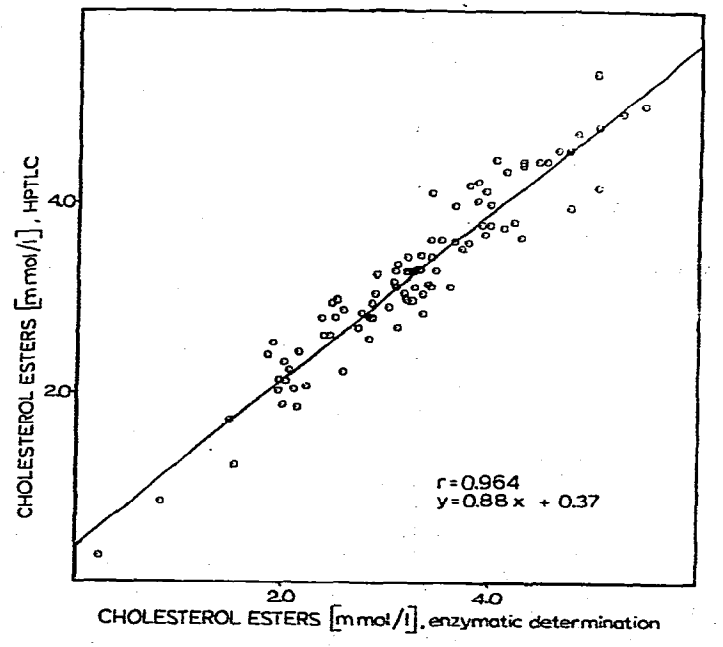

Fig. 4. Correlation between enzymatically-determined cholesterol esters and HPTLC cholesterol esters (n = 93); see Table III.

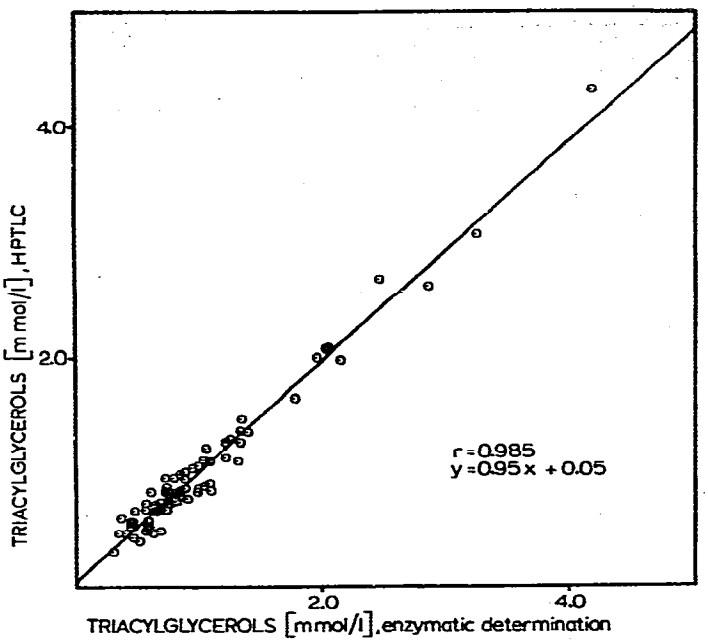

Fig. 5. Correlation between enzymatically-determined triacylglycerols and HPTLC triacylglycerols (n = 69); see Table III.

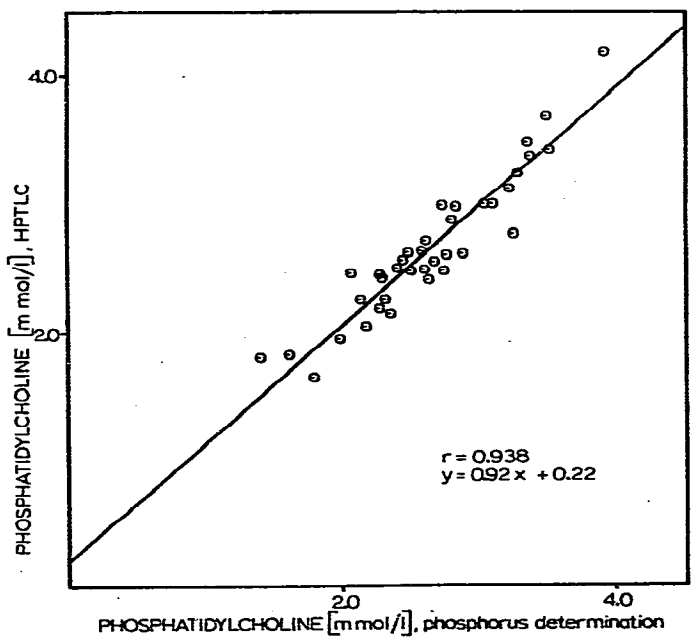

Fig. 6. Correlation between chemically-determined phosphatidylcholine and HPTLC phosphatidylcholine (n = 22); see Table III.

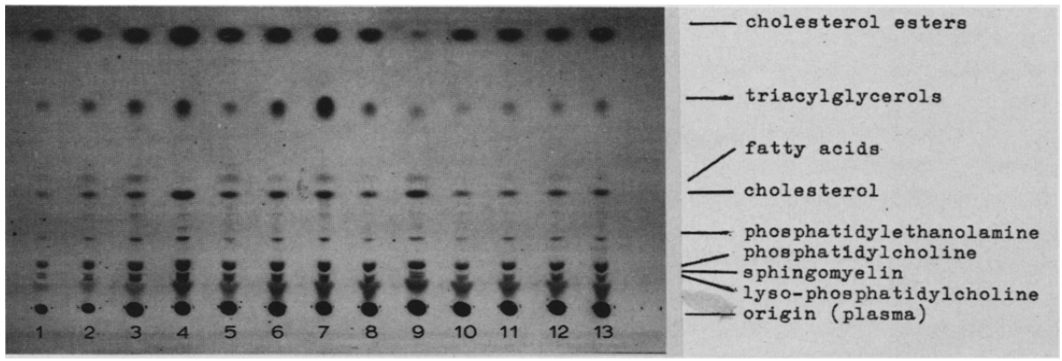

Fig. 7. Visual examination of lipid profiles of healthy subjects and different patients, compared with known lipid amounts in Lipid-Trol. The corresponding quantitative values are listed in Table IV.

of hyperlipidemic patients were analyzed by HPTLC and the results compared with those obtained using reference methods (Table III). The values obtained for cholesterol esters, triacylglycerols and phosphatidylcholine were identical. The HPTLC values for free cholesterol were 7% lower than those found by enzymatic determination. This result is possibly due to different fluorescent properties of sterols other than cholesterol present in the plasma, e.g. plant sterols. Linear-regression analysis of these data indicated good correlation between the reference methods and HPTLC for the lipids investigated (Figs. 3—6).

The lipid patterns of healthy subjects and various patients are presented in Fig. 7. By comparisons with the known lipid concentrations in Lipid-Trol

TABLE IV

DETERMINATION OF LIPIDS WITH REFERENCE METHODS

For visual examination, the lipids of 10 human subjects were separated by HPTLC and compared with known amounts of lipid in Lipid-Trol (see Fig. 7). The corresponding quantitative values (mmole/l) are listed here. (HLP = hyperlipoproteinemia.)

Position on HPTLC plate	Type of plasma sample	Cholesterol esters	Cholesterol	Triacyl-glycerols	Phosphatidyl-choline
1	Lipid-Trol	2.70	1.04	1.07	1.6
2	Lipid-Trol	5.41	2.08	2.14	3.2
3	Lipid-Trol	10.82	4.16	4.28	6.4
4	Type II HLP	17.3	5.4	2.46	—
5	Type II HLP	8.37	1.91	0.85	2.7
6	Type II HLP	8.68	2.53	2.52	3.5
7	Type IV HLP	8.94	2.97	7.36	3.5
8	Normal	4.48	1.81	1.19	2.8
9	Liver disease	0.64	3.44	0.85	—
10	Normal	3.27	1.29	0.43	2.2
11	Normal	4.01	1.55	0.58	2.7
12	Normal	3.80	1.66	0.92	2.8
13	Normal	4.28	1.32	0.92	2.5

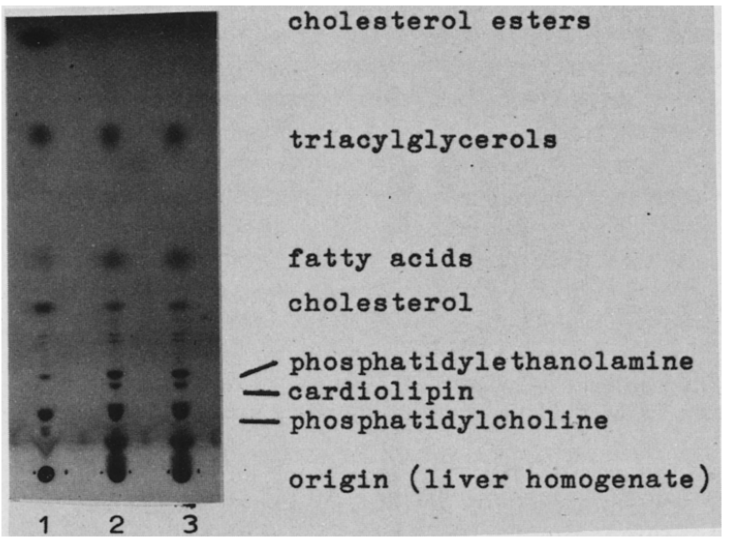

Fig. 8. Lipids of a liver homogenate (positions 2, 3), compared with Lipids-Trol (position 1).

present as standard on the HPTLC plate (positions 1—3), semi-quantitation of the lipids was possible. The values obtained by visual examination of the chromatoplate were in good agreement with data obtained by the reference methods (Table IV). One child (position 9) with liver disease associated with a severe icterus had practically no cholesterol esters, but had an increased free cholesterol content, reflecting the reduced lecithin—cholesterol acyltransferase activity found in liver parenchymatous damage [14, 15].

In order to ascertain whether this procedure can also be applied to lipid determinations of homogenized tissues, 0.5 µl of a liver homogenate were applied directly to the HPTLC plate and treated in the same manner as the plasma samples. As shown in Fig. 8, the separation of the lipids was as good as in the plasma samples. The coefficients of variation in the series (n = 10)

ABLE V

IPID DETERMINATION IN LIVER HOMOGENATES

iver samples from 6 rats were homogenized. For the Liebermann—Burchard reaction, cholester as obtained by preparative TLC. All enzymatic determinations were carried out on the hom nate. The data were examined by paired t-test statistics and linear-regression analysis.

ipids mole/g liver)	Procedure					
	Liebermann—Burchard	Enzymatic	HPTLC	Enzymatic: HPTLC		
				P	$y = bx + a$	r
holesterol	4.10±0.65	3.90±0.45	4.85±0.75	<0.0005	$1.44x - 0.16$	0.866
riacylglycerols	—	8.00±1.90	7.80±2.55	<0.30	$1.28x - 0.49$	0.959

were 3.3% for cholesterol and 5.7% for triacylglycerols. The concentrations of triacylglycerols and free cholesterol in liver homogenates from 6 rates were determined using the reference methods and HPTLC (Table V). The results obtained with the reference methods were consistent with data found by column chromatographic separation of lipids from rat liver [16]. Cholesterol values obtained with HPTLC were 24% higher than those found by enzymatic determination or the Liebermann–Burchard method. This difference was highly significant and the data showed relatively poor correlation (r = 0.866). These HPTLC values presumably reflect the existence in the liver of sterols with different fluorescence properties and/or the existence of sterols not detected by the reference methods. However, the relative measurement of liver sterols by HPTLC seems to be possible. The enzymatic and the HPTLC determination of triacylglycerols gave identical results and good correlation (r = 0.959). The results, although obtained from only 6 rat livers, suggest that at least the liver triacylglycerols can be estimated with satisfactory accuracy using the direct HPTLC procedure. Because of the very small quantity of tissue required, this method may be suitable for lipid analysis in tissue-biopsy material.

CONCLUSIONS.

With the procedure described in this report, the plasma lipids of 0.5-μl samples of capillary blood can be quantitated in the nmole range without lipid extraction prior to HPTLC. This makes the method suitable for routine analysis and large-scale studies.

Liver homogenates can be analyzed by the same procedure. This makes it suitable for rapid lipid determination on small amounts of biopsy material.

REFERENCES

1 V.P. Skipski, in G.J. Nelson (Editor), Blood Lipids and Lipoproteins, Wiley-Interscience, New York, 1972, p. 471.
2 L.A. Witting, in E.G. Perkins (Editor), Analysis of Lipids and Lipoproteins, American Oil Chemist's Society, Champaign, Ill., 1975, p. 90.
3 A. Kuksis, J. Chromatogr., 143 (1977) 3.
4 V.S. Whitner, O.T. Grier, A.N. Mann and R.F. Witter, J. Amer. Oil Chem. Soc., 41 (1965) 1154.
5 G.C. Buckley, J.A. Little, M.A. Antar and A. Csima, Clin. Chim. Acta, 23 (1969) 53.
6 M.J. Mantel, L.H.M. van Riel and A.F.K. Buys Ballot, Clin. Chim. Acta, 63 (1975) 297.
7 I.R. Kupke, Ninth International Congress on Clinical Chemistry, July 13–18, 1975, Toronto, Canada, p. 73; Clin. Chem., 21 (1975) 990.
8 R. Segura and A.M. Gotto, J. Chromatogr., 99 (1974) 643.
9 P. Roeschlau, E. Bernt and W. Gruber, J. Clin. Chem. Clin. Biochem., 12 (1974) 403.
10 I.R. Kupke, J. Mol. Cell. Cardiol., 4 (1972) 11.
11 J. Christiansen, J. Chromatogr., 123 (1976) 57.
12 W. Mlekusch, W. Truppe and B. Paletta, J. Chromatogr., 78 (1973) 438.
13 W. Mlekusch, W. Truppe and B. Paletta, Clin. Chim. Acta, 49 (1973) 73.
14 J.P. Blomhoff, S. Skrede and S. Ritland, Clin. Chim. Acta, 53 (1974) 197.
15 R. Kattermann, R. Achelis and D.I. Wolfrum, Acta Hepato-Splenol., 18 (1971) 153.
16 I.R. Kupke and W. Lamprecht, Hoppe-Seyler's Z. Physiol. Chem., 348 (1967) 17.