CHROM. 3730

Densitometric microquantitation of lipid classes separated by thin layer chromatography*

Application of densitometry to quantitation of lipids separated on thin-layer chromatography (TLC) described by BLANK *et al.*[1] became a powerful tool in analysis of both non-polar[2] and polar[3] lipids. By modification of that technique and of the excellent two-step TLC described by FREEMAN AND WEST[4] we were able to quantitate microquantities of lipids of amniotic fluid. We are not aware of another method that will allow such microquantitation with similar speed and relative simplicity. A broad outline of the procedure was reported before[5].

Materials

Reference compounds were obtained as follows: from Hormel Institute (Austin, Minn.): monopalmitin, dipalmitin, tripalmitin, palmitic acid, oleic acid, monoolein, triolein, cholesterol, cholesteryl stearate; from Applied Science Laboratories, State College, Pa.: palmitic acid, stearic acid, myristic acid, methyl palmitate, cholesteryl stearate, *n*-tetracosane, *n*-octadecane; from Supelco Inc, Bellefonte, Pa.: 1,2-dipalmitin and 1,3-dipalmitin. The following compounds were a generous gift of Dr. C. B. BARRETT of Unilever Research Laboratory, The Frythe, Welwyn, Herts., Great Britain: 1-monoolein, 1,3-diolein, 1,3-distearin, 1,3-dipalmitin. Chemical reagents were of analytical grade (Fisher Scientific, New York) but not redistilled.

Methods

Amniotic fluid collected from normal patients in the third trimester of pregnancy by abdominal amniocentesis or in labor by vaginal amniocentesis was centrifuged for 20 min at 3,000 r.p.m. (1500 *g*). The supernatant was kept at —20° until extraction. Fluids were not pooled. Lipids were extracted with 20 volumes of chloroform–ethyl alcohol (2:1, v/v) for 2 h at 40° with shaking. The filtered extract was brought to dryness under nitrogen in a Nutating evaporator (Zymel Corp., Ardsley Corp., New York). Non-lipid contaminants were removed by the method of FOLCH *et al.*[6] or of BIEZENSKI[7]. An aliquot of the extract was taken for lipid P determination[8]. Lipids were concentrated to 10–20 $\mu g/\mu l$. One microliter of the concentrate corresponded to about 0.1 ml of amniotic fluid. Serum lipids were extracted as described before[9].

Procedure

Neutral plates of Silica Gel G, 20 × 20 cm of 0.25 mm thickness were prepared in the usual manner. After drying in air the plate was cleaned by a prerun in Solvent I for 2 h. The plate was then dried in air for 20 min and activated in an oven at 110° for 20 min. Parallel lanes exactly 8 mm wide were drawn with a comblike instrument made with glass rods. Two to four microliters of the extract were applied 3.5 cm from the bottom of the plate with a Hamilton syringe in a series of dots across each lane along a line which fell short by 1 mm from either limiting line. The plate was developed in Solvent I consisting of diethyl ether–benzene–ethyl alcohol–acetic acid (40:50:2:0.2)[4]

* Supported by grants No. FR-05497 and No. HD 00441 from National Institutes of Health, U.S.P.H.S.

for 6.5 cm from the origin. This took approximately 13 min. After drying in air for 20 min the plate was placed in Solvent II consisting of petroleum ether–ethyl ether–acetic acid (90:10:1). The plate was developed for 6.5 cm more (13 cm in all from origin) which took 35–45 min. When dry the plate was sprayed lightly with 50% H_2SO_4 and placed on a metal hot plate till fumes disappeared. This took about 8 min. The surface temperature of the hot plate was approximately 260°. The surface temperature of the thin-layer plate was approximately 180°. Under the above conditions the plate burned evenly but plates of thinner glass gave density readings 5–10% higher.

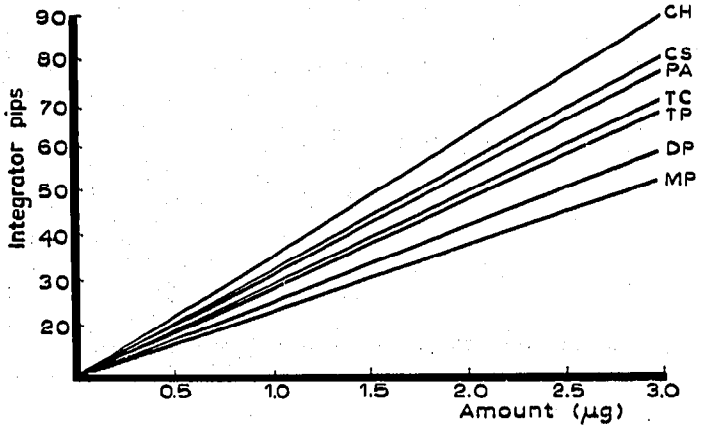


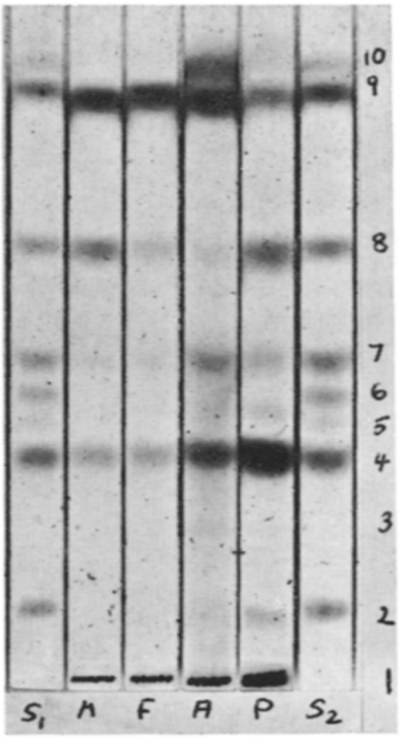
Fig. 1. Relation of amounts of reference compounds to densitometric readings of charred thin-layer plates. CH = Cholesterol; CS = cholesteryl stearate; PA = palmitic acid; TC = *n*-tetracosane; TP = tripalmitin; DP = dipalmitin; MP = monopalmitin.

Comparisons with reference compounds were essential due to specific burning characteristics (Fig. 1). These differences were only partly due to differences in carbon content and cannot be readily explained. Degree of fatty acid unsaturation appeared to be of little significance. The densities were determined essentially as described by BLANK *et al.*[1] by means of a Photovolt Densitometer Model No. 52C equipped with an automatic mobile stage, a Varicord Recorder Model No. 42B and an Electronic Integrator Model No. 49A. The collimating slit measured 0.1 mm × 6.0 mm. No filter was used. The width of the exchangeable apperture disc was adjusted to exactly 5 mm with black tape. Thus the slit was slightly narrower than the width of the streak. The instrument was set for 100% transmission on blank areas. The average number of integrator pips of each spot for the four lanes was converted into micrograms by comparison with reference compounds. Percentages and absolute amounts were then calculated from the microgram values. Density values obtained from phospholipids were not proportional to amounts applied and were disregarded.

Comment

The method as outlined was most suitable for amounts of about 1 μg of lipid in each spot. If any component exceeded grossly that amount it was necessary to run additional plates with diluted samples for better accuracy.

Separation of lipid classes of various tissues was satisfactory (Fig. 2). We found the same method of separation very suitable for preparatory purposes mainly because



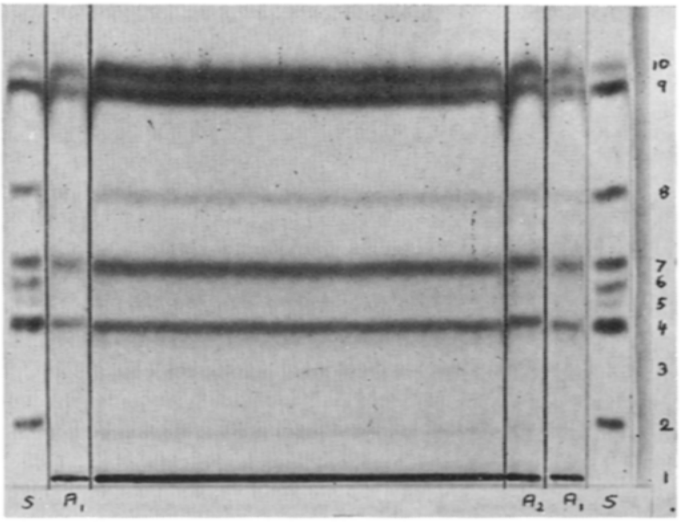


Fig. 2. Separation of lipid classes for densitometric quantitation by two-step thin-layer chromatography. S_1 = Reference compounds (2 μg each); M = pregnant woman (extract from 0.0015 ml serum); F = human cord blood (extract from 0.012 ml serum); A = human amniotic fluid (extract from 0.3 ml); P = human placenta (extract from 15.0 mg fresh tissue); S_2 = reference compounds (4 μg each). (1) Origin and phospholipids; (2) 1- and 2-monoglycerides; (3) unidentified in amniotic fluid; (4) cholesterol; (5) 1,2-diglyceride; (6) 1,3-diglyceride; (7) free fatty acids; (8) triglycerides; (9) cholesteryl esters; (10) hydrocarbons. Charred with 50% H_2SO_4.

Fig. 3. Preparatory thin-layer chromatography of human amniotic fluid. For details of technique see text. Plate thickness: 0.75 mm. Amount of lipid: 900 μg (5.6 ml amniotic fluid). S = Reference compounds (4 μg each); A_1 = extract from 0.225 ml amniotic fluid; A_2 = extract from 0.45 ml amniotic fluid. For identification of compounds see Fig. 2.

TABLE I

HUMAN AMNIOTIC FLUID LIPIDS

Non-polar lipids are determined by quantitative thin-layer densitometry as described in the text. Phospholipids are determined from lipid P × 25.

Class	*mg/100 ml ± 0.38*[a]	*% total lipids*
Phospholipids	3.99 ± 0.38[a]	29.3
Total non-polar	9.65 ± 0.48[b]	70.7
Monoglycerides	0.23 ± 0.02	1.7
Diglycerides	0.89 ± 0.11	6.5
Triglycerides	1.21 ± 0.12	8.9
Free cholesterol	1.40 ± 0.10	10.3
Cholesterol esters	2.00 ± 0.15	14.6
Hydrocarbons	1.75 ± 0.12	12.8
Free fatty acids	2.17 ± 0.13	15.9
Total lipids	13.64	

[a] Average of 43 fluids.
[b] Average of 53 fluids. Represents the sum of components.

each lipid migrated in a straight horizontal line (Fig. 3). Thus staining of outside guides with iodine was sufficient for streak localization.

Human amniotic fluid from patients in the third trimester showed presence of many lipids (Fig. 2). Phospholipids constituted the largest group, monoglycerides the smallest (Table I). Standard errors were occasionally high. This was probably due to the well known natural interfetal differences[10] rather than to methodological variations.

The procedure is particularly useful for microquantitation but we used it to equal advantage in determining the composition of placenta, liver, serum and other tissues.

Departments of Obstetrics and Gynecology,
Maimonides Medical Center, and
State University of New York, Downstate Medical Center,
Brooklyn, N.Y. (U.S.A.)

J. J. BIEZENSKI
W. POMERANCE
J. GOODMAN

1 M. L. BLANK, J. A. SCHMIT AND O. S. PRIVETT, *J. Am. Oil Chemists' Soc.*, 41 (1964) 371.
2 O. S. PRIVETT, M. L. BLANK AND W. O. LUNDBERG, *J. Am. Oil Chemists' Soc.*, 38 (1961) 312
3 L. GLUCK, M. V. KULOVICH AND S. J. BRODY, *J. Lipid Res.*, 7 (1966) 570.
4 C. P. FREEMAN AND D. WEST, *J. Lipid Res.*, 7 (1966) 324.
5 J. J. BIEZENSKI, W. POMERANCE AND J. GOODMAN, *Am. J. Obstet. Gynecol.*, in press.
6 J. FOLCH, M. LEES AND G. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
7 J. J. BIEZENSKI, *J. Lipid Res.*, 3 (1962) 120.
8 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
9 J. J. BIEZENSKI, *J. Chromatog.*, 36 (1968) 366.
10 J. J. BIEZENSKI, *Am. J. Obstet. Gynecol.*, in press.

Received August 5th, 1968.