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LIPID CONTENT OF HUMAN PLATELETS QUANTITATED BY THIN-LAYER CHROMATOGRAPHY IN COMBINATION WITH FLAME IONIZATION DETECTION

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SUMMARY

The lipid contents of human platelets from twenty-one healthy adults were analysed using thin-layer chromatography in combination with flame ionization detection.

The weight per cent of neutral lipids in human platelets was 14.6%, which consisted mainly of free cholesterol, that of phosphatidylethanolamine 24.9%, phosphatidylserine plus phosphatidylinositol 6.8%, phosphatidylcholine 35.2% and sphingomyelin 18.6%. Free cholesterol in 10^8 platelets was estimated as 7 μg and phospholipids as 46 μg from calibration standards. The reproducibility was satisfactory and the procedure could be performed quickly and simply.

INTRODUCTION

Dysfunctions of platelets play important roles in the pathogenesis of atherosclerosis [1—3]. Various properties of platelets including adhesiveness, aggregation, coagulation factors, prostaglandins, anti-platelet antibody and membrane glycoprotein, can now be measured in many clinical laboratories. In addition to these examinations, lipid analysis of platelets is required to investigate the role of lipids in the various functions of platelets. However, only a few papers concerning lipid analysis of human platelets have appeared, with technical difficulties demanding massive platelets beyond clinical applications.

This paper presents a simple and rapid method for the quantitative analysis of platelet lipids as a clinical and practical improvement, using a combination of thin-layer chromatography (TLC) and flame ionization detection (FID).

MATERIALS AND METHODS

Subjects

Platelets were obtained from 21 healthy adults (8 females and 13 males). The mean age, serum cholesterol and serum phospholipid of the subjects were 25 years, 162 mg/dl and 169 mg/dl, respectively. All of them were confirmed as having normolipidemia with normal platelet functions as regards aggregation, adhesiveness, volume, and malondialdehyde (MDA) release induced by arachidonic acid.

Preparation of platelets

Five millilitres of venous blood were taken from the subjects after overnight fasting using a siliconized tube containing 3.8% citrate. The blood was centrifuged at 100 *g* for 10 min at 23°C. The supernatant (platelet-rich plasma) was centrifuged for 10 min at 23°C. The platelets in the pellet were resuspended in 1.5 m*M* Tris-HCl buffer (pH 7.4) containing 0.15 m*M* NaCl, 5 m*M* KCl and 1.5 m*M* EDTA. After being rinsed twice with the same buffer, the platelets in the pellet were counted using a Hemalog 8 (Technicon Co., Tokyo, Japan). The number of platlets obtained from 5 ml of blood was approximately $1—8 \times 10^8$.

Analytical method for neutral lipids

Platelet lipids were extracted with chloroform—methanol (2:1, v/v) containing cholesterol acetate (MW 428, 10 mg/dl) as an internal standard. After the solvents were rinsed twice with saline, the top layer was discarded and the lower and intermediate layers were filtered through filter paper (Type 5A, Toyo Co., Tokyo, Japan). The solution of platelet lipids was evaporated in a water-bath at 40°C under reduced pressure. The dried samples were then dissolved in 200 μl of Folch's solution [4], and 3 μl of this solution were applied to a Chromarod for TLC. Development was in methanol for 10 mm from the point of application; the rod was then air-dried. Neutral lipids (cholesterol ester, cholesterol acetate, triglyceride, free cholesterol and phospholipid) were developed in *n*-hexane—diethyl ether (9:1, v/v) for 40 min at 20°C. The Chromarod was passed through a flame ionization detector after drying for 3 min at 110°C.

Thin-layer chromatography in combination with flame ionization detection

TLC was performed on a "Chromarod" (Iatron, Tokyo, Japan), a quartz rod 0.9 mm in diameter and 152 mm long, coated with silica gel. The rod was passed through a flame ionization detector (Iatron) after chromatographic development under the following conditions: hydrogen pressure 1.4 kg/cm^2, air flow-rate 2000—2500 ml/min, scanning speed gear 40 T, chart speed 240 mm/min, and recorder range 0.1 V. Each fraction, calibrated by flame ionization detector, was identified by using the purified standard such as cholesterol ester, triglyceride, free fatty acid and phosphatidylcholine (Sigma, St. Louis, MO, U.S.A.), and the values were quantitated by measuring the step height of the integrating signal in triplicate samples.

The height ratio of individual neutral lipid to internal standard (cholesterol acetate) was divided by the sum of the ratio and demonstrated as weight per cent.

For free cholesterol and phosphatidylcholine, calibration curves were obtained by adding various amounts of standard free cholesterol and phosphatidylcholine; the values of free cholesterol and phosphatidylcholine in platelets were expressed as μg per 10^8 platelets.

Analysis of phospholipids

Phospholipid in platelets was extracted by Folch's solution. The following procedures were the same as the procedures described above. Phospholipid was subfractionated in chloroform—methanol—water (60:30:3.5, v/v), containing 500 mg/dl of 2,3-di-*tert.*-butyl-1,4-methylphenol (BHT). Each fraction was identified using a standard mixture of phosphatidylcholine, sphingomyelin (Sigma), phosphatidylethanolamine (purified from *Escherichia coli*), phosphatidylinositol (purified from yeast), and phosphatidylserine (purified from bovine brain) (by courtesy of Prof. S. Nozima, School of Pharmacy, Tokyo University, Tokyo, Japan). Each fraction was expressed as a percentage by measuring the step height of the integrating curve of each fraction and calculating the weight per cent compared with that of total phospholipid.

RESULTS

Patterns of standard mixture of neutral lipids and of phospholipids

A standard mixture of neutral lipids was developed by TLC—FID (Fig. 1). Six peaks were separated clearly including cholesterol ester, triglyceride, free fatty acid, free cholesterol, phospholipid, and cholesterol acetate as internal standard. *n*-Eicosane, *n*-dodecylbenzene and lithocolic acid could also be de-

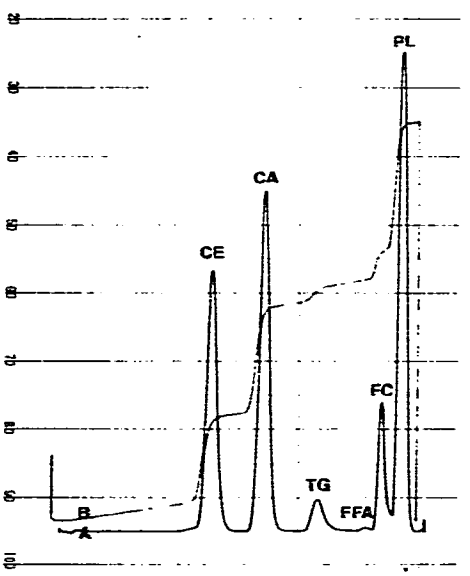


Fig. 1. Pattern of standard mixture of neutral lipids. CE, cholesterol ester; CA, cholesterol acetate; TG, triglyceride; FFA, free fatty acid; FC, free cholesterol; PL, phospholipid. B, integrating curve; A, differential curve.

tected by TLC—FID although cholesterol acetate was superior as internal standard in this system from the point of view of sensitivity and overlapping. A standard mixture of phospholipids could be clearly separated into five fractions which were identified as phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and sphingomyelin (Fig. 2).

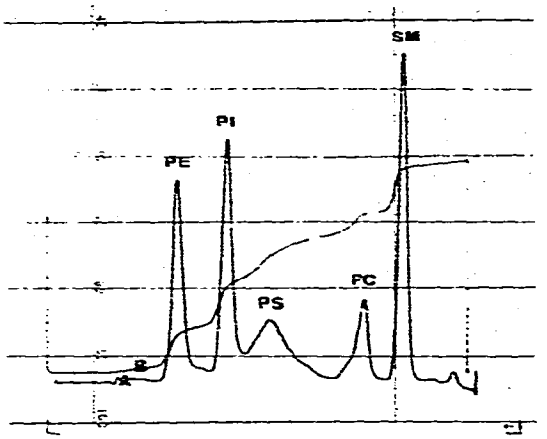


Fig. 2. Pattern of standard mixture of phospholipids. PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

Patterns of platelet lipids

Neutral platelet lipids were determined by TLC—FID as cholesterol ester, triglyceride, free fatty acid, free cholesterol, and phospholipid (Fig. 3).

The peaks of free cholesterol and phospholipid were sharp and high, al-

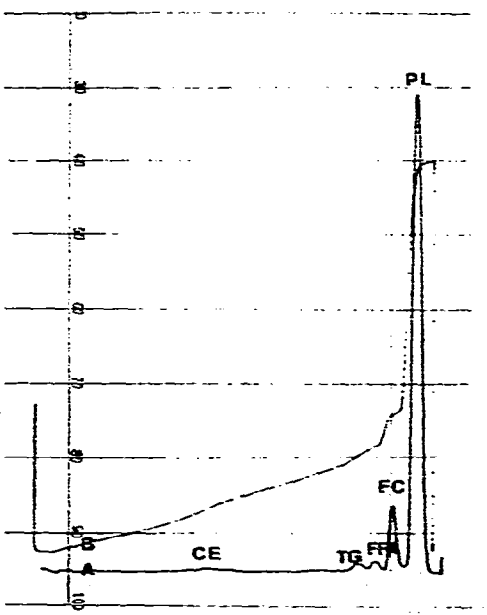
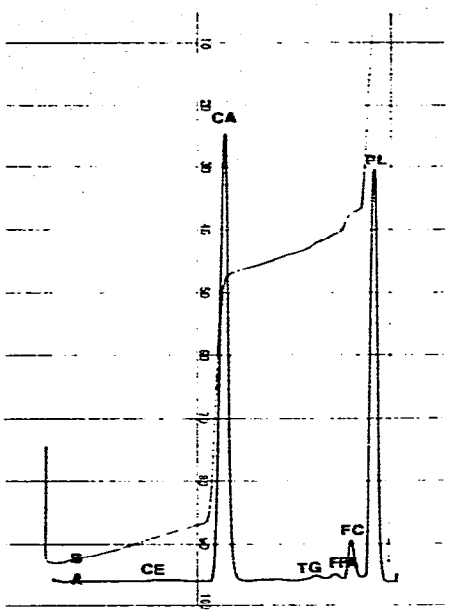


Fig. 3. Pattern of neutral lipids of platelets. Abbreviations as in Fig. 1.



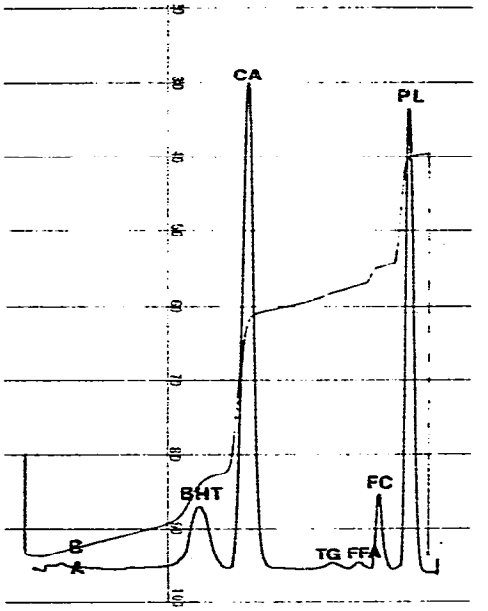


Fig. 4. Pattern of neutral lipids of platelets containing cholesterol acetate (CA) as internal standard. Abbreviations as in Fig. 1.

Fig. 5. Patterns of neutral lipids of platelets containing cholesterol acetate (CA) as internal standard and BHT as an antioxidant. Abbreviations as in Fig. 1.

though the peaks of cholesterol ester, free fatty acid, and triglyceride were lower and in many cases they were unrecognizable. The pattern of neutral lipids containing cholesterol acetate as an internal standard was no different from that without cholesterol acetate (Fig. 4). Neutral lipids of platelets with BHT added as an antioxidant and cholesterol acetate as an internal standard also separated into clear fractions without interference from one other (Fig. 5).

Platelet phospholipids were subfractionated into four peaks corresponding to phosphatidylethanolamine, phosphatidylinositol plus phosphatidylserine, phosphatidylcholine, and sphingomyelin (Fig. 6). Phosphatidylcholine and phosphatidylserine could not be separated clearly into two peaks by TLC—FID.

Reproducibility of the TLC—FID method

The reproducibility of the TLC—FID method was examined by analysing one sample of platelets ten times. The mean value and standard deviation of the weight per cent was calculated and the variation coefficients were 8.4% for free cholesterol, 37.0% for phosphatidylethanolamine, 45.0% for phosphatidylinositol plus phosphatidylserine, 11.1% for phosphatidylcholine and 3.5% for sphingomyelin.

Standard curves of free cholesterol and phosphatidylcholine

The standard curves of free cholesterol and phosphatidylcholine showed good linearity. The factor of each standard curve was $Y = 250.80\ X + 18$ for free cholesterol and $Y = 431.00\ X$ for phosphatidylcholine.

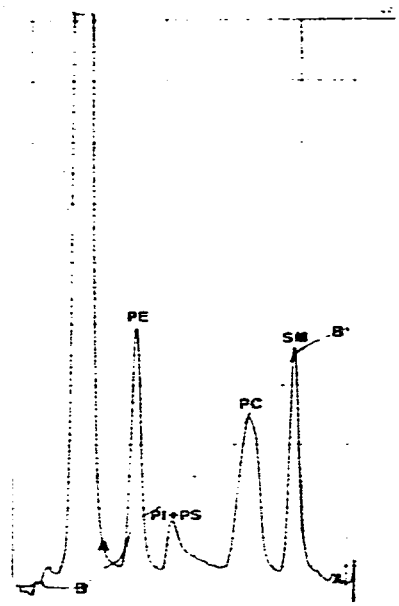


Fig. 6. Patterns of phospholipids of platelets. Abbreviations as in Fig. 2.

TABLE I

PERCENTAGE OF TOTAL LIPIDS IN WHOLE PLATELETS

	This paper (n=21)	Marcus et al. [5]
Neutral lipids	14.6±3.6	21.0
Phosphatidylethanolamine	24.9±4.5	20.0
Phosphatidylserine	6.8±3.4	8.0
Phosphatidylinositol		3.4
Phosphatidylcholine	35.2±3.1	31.4
Sphingomyelin	18.6±2.6	14.5
Lysolecithin	–	0.6
Cardiolipin	–	0.4
Gangliosides	–	0.5

TABLE II

ABSOLUTE AMOUNTS OF FREE CHOLESTEROL AND PHOSPHOLIPID AND PERCENTAGES OF PHOSPHOLIPID SUBFRACTIONS

	FC*	PL*	PE**	PI+PS**	PC**	SM**
This study	7.32±1.10	46.06±8.36	29.6±4.9	7.8±3.9	41.1±3.9	22.1±2.8
Shattil and Cooper [7]	11.22	34.44				
Nördoy and Rödset [10]		23.62	31.6	10.1	44.7	13.5
Marcus et al. [5]			27.0	15.1	38.4	17.0

*μg per 10^8 platelets.

**Percentage of each subfraction of phospholipids.

Lipid content of platelets

The values of platelet lipids were determined as weight percentages (Table I). Neutral lipids were about 15% and their major part consisted of free cholesterol. Phospholipids were approximately 80% and were subdivided into approximately 35% of phosphatidylcholine and 20% of phosphatidylethanolamine and sphingomyelin.

The values (μg) per 10^8 platelets calculated from the standard curve indicated also that 46 μg of phospholipid was major as compared with 7 μg of free cholesterol (Table II). Lysolecithin, cardiolipin and gangliosides could not be detected by TLC—FID.

DISCUSSION

It has been suggested that the lipid contents of platelets are important factors in inducing atherosclerosis through aggregation of platelets under hyperlipoproteinemic conditions [6, 7]. Vandamme and Vankerckhoven studied plasma lipid analysis by TLC—FID, and reported that the method correlated well with the conventional method of quantitating lipids in plasma [8]. Platelet lipid analysis in conjunction with plasma lipid analysis might provide useful information for evaluating the hyperaggregability of platelets in patients with hyperlipidemia.

A rapid and simple method to quantitate lipids in platelets is desirable for examining the possible role of platelet lipids in atherogenesis. The purpose of this study was to examine the validity of the TLC—FID method to quantitate platelet lipids, and to determine the absolute concentrations of the individual lipids in platelets from healthy adults.

As an internal standard for neutral lipids, *n*-eicosane, *n*-dodecylbenzene, lithocholic acid and cholesterol acetate were examined; cholesterol acetate was found to be most suitable since it did not overlap or interfere with coexisting peaks.

BHT added in the solvent as an antioxidant for neutral lipids was found to overlap completely the peak of cholesterol ester so that BHT was used only for the separation of phospholipids.

The TLC—FID patterns of human platelets demonstrated cholesterol ester, triglyceride, free fatty acid and free cholesterol as neutral lipids, and phospholipids containing phosphatidylethanolamine, phosphatidylinositol plus phosphatidylserine, phosphatidylcholine and sphingomyelin, but not lysolecithin, cardiolipin or gangliosides. The patterns of TLC—FID of these lipids in human platelets was compatible with that reported by Vandamme and Vankerckhoven [6].

The minimum amount detected by this TLC—FID method as a peak under the most sensitive conditions was 0.05 μg using cholesterol acetate. The peaks of the minor neutral lipids were usually low and were not necessarily detected as reliable peaks from 10^8 platelets. This would contribute to our finding of a lower percentage of neutral lipids compared to Marcus et al. [5].

Free cholesterol was quantitated most satisfactorily and reliably from the standard curve in this method. The lower amounts of free cholesterol found in our platelets compared to the data of Shattil and Cooper [9] obtained by gas—liquid chromatography were presumably due to the difference in the

methods, because there was no difference in the levels of plasma cholesterol and phospholipid between the two groups.

With regard to the phospholipid, the highest values were obtained in our study compared to others [7, 10]. We used phosphatidylcholine alone purified from egg yolk for the standard curve. There is a difference in the composition of the free fatty acids in phosphatidylcholine from egg yolk and that of platelets. The free fatty acid in phosphatidylcholine of egg yolk examined in our laboratory was C16:0, 38.4%; C18:0, 16.8%; C18:1, 18.4%; C18:2, 16.4%; whereas that of platelets reported by Marcus et al. [5] was C16:0, 34.1%; C18:0, 14.1%; C18:1, 27.0%; C18:2, 6.9%; C20:4, 12.0%; indicating an excess of C20:4 in platelets.

In addition, phosphatidylcholine is a major component of platelet phospholipids. These factors were considered to be a cause of the higher values for phospholipid found in our study.

As the subfraction of platelet phospholipids, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin were completely separated while phosphatidylinositol and phosphatidylserine were hardly separated from each other. This evidence may contribute to the lower amount of phosphatidylinositol plus phosphatidylserine and the larger amount of sphingomyelin found in our study compared to reported data [5, 10].

Trace amounts of lysolecithin, cardiolipin and gangliosides are known to exist in platelets, but they could not be detected by our method [5].

There are many advantages in employing the TLC—FID method to quantitate the lipid content of platelets. Firstly, concentration of different lipids can be determined in a single procedure. Since the molar ratio of free cholesterol to phospholipid has a significant role concerning the fluidity of platelet plasma membranes [7, 9], the values of the cholesterol/phospholipid molar ratio determined by this method are more reliable than those measured by conventional techniques which quantitate cholesterol and phospholipid separately.

Quantitation of the subfractions of phospholipids is feasible using the TLC—FID method. The Chromarod can be utilized repeatedly after it has passed through the flame ionization detector. Finally, it is very beneficial that the amount of sample required for chromatography of both neutral lipid and phospholipids is only 6 μl, since the remainder of the sample (194 μl) can be used for further lipid analysis such as fatty acids.

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