Acta Medica Okayama

Volume 28, *Issue* 6 1974 *Article* 3

DECEMBER 1974

The micromethod for determination of cholesterol, cholesteryl esters and phospholipids

Akinobu Okabe* Takeshi Katayama†

Yasuhiro Kanemasa‡

*Okayama University,
†Okayama University,
‡Okayama University,

The micromethod for determination of cholesterol, cholesteryl esters and phospholipids*

Akinobu Okabe, Takeshi Katayama, and Yasuhiro Kanemasa

Abstract

We examined the method for determining microquantities of lipids, including cholesterol, cholesteryl esters and phospholipids. A standard colorimetric procedure of cholesteryl esters was modified to accommodate a quantitative thin-layer chromatography. This method involved the following steps. (1) Separation of lipids by a thin-layer chromatography: Lipids were applied to Silica gel G plates. Plates were developed with petroleum ether-diethyl etheracetic acid (82: 18: 2, vIvIv). (2) Elution of cholesterol and its esters from scraped silica gel: After scraping the silica gel with adhered cholesterol and its esters, they were eluted with chloroform-methanol (4: 1, v,tv). In the case of phspholipids, the silica gel was calcified. (3) Colorimetric determination of the lipids: Cholesterol and its esters eluted from the silica gel were determined by the method of ZAK with ROSENTHAL'S color reagent directly and after saponification, respectively. Phospholipids were calculated from the phosphorous content determined by the method of KATES. On the basis of examination of recovery and analyses of lipids extracted from tissue, it was concluded that this method permitted a reliable estimation of microquantities of cholesterol, its esters and phospholipids from small amounts of biological materials.

---

*PMID: 4282002 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **28**, 403—410 (1974)

THE MICROMETHOD FOR DETERMINATION OF CHOLESTEROL, CHOLESTERYL ESTERS AND PHOSPHOLIPIDS

Akinobu OKABE, Takeshi KATAYAMA
and Yasuhiro KANEMASA

Department of Microbiology, Okayama University Medical School, Okayama, Japan (Director: Prof. J. Tawara)

Received for publication, May 13, 1974

Abstract: We examined the method for determining microquantities of lipids, including cholesterol, cholesteryl esters and phospholipids. A standard colorimetric procedure of cholesteryl esters was modified to accommodate a quantitative thin-layer chromatography. This method involved the following steps. (1) Separation of lipids by a thin-layer chromatography: Lipids were applied to Silica gel G plates. Plates were developed with petroleum ether-diethyl ether-acetic acid (82 : 18 : 2, v/v/v). (2) Elution of cholesterol and its esters from scraped silica gel: After scraping the silica gel with adhered cholesterol and its esters, they were eluted with chloroform-methanol (4 : 1, v/v). In the case of phspholipids, the silica gel was calcified. (3) Colorimetric determination of the lipids: Cholesterol and its esters eluted from the silica gel were determined by the method of ZAK with ROSENTHAL's color reagent directly and after saponification, respectively. Phospholipids were calculated from the phosphorous content determined by the method of KATES. On the basis of examination of recovery and analyses of lipids extracted from tissue, it was concluded that this method permitted a reliable estimation of microquantities of cholesterol, its esters and phospholipids from small amounts of biological materials.

Various procedures for quantitative estimation of cholesterol and cholesteryl esters have been published, since GRIGAUT (1) determined cholesterol by Liebermann-Burchard reaction in 1910. Among them should be mentioned the specific methods reported by SCHOENHEIMER *et al.* (2) and SPERRY *et al.* (3). These authors combined a simple chemical determination with purification by digitonin precipitation of free cholesterol. The simple but precise method was introduced by BADZIO *et al.* (4), who carried out extraction and separation on thin-layer chromatography (TLC). TLC methods were applied by many investigators to chemical analysis of cholesterol and other neutral lipids (5, 6, 7, 8). Although these are excellent methods of high specificity, these necessitate relatively large amounts of materials.

Recently the determination of cholesterol in a biologic membrane seems

to be significant because of the possible implication of the cholesterol in membrane fluidity as well as phospholipids (9, 10). There is need for a method that is sensitive enough to permit the examination of cholesterol from such small sources as plasma membranes.

The authors described in this paper a microdetermination method of cholesterol, its esters and phospholipids by TLC. The applicability of the procedure for microanalysis of tissue lipids was also demonstrated on lipid extracted from mouse liver.

MATERIALS AND METHODS

Reagents: All reagents used were of standard grade and were obtained from Wako Pure Chemical Industry, Ltd. and Katayama Chemical. Cholesterol was recrystallized three times from hot ethanol. Cholesterylstearate was found to be pure on TLC. Egg-lecithin was purified by preparative TLC. Cardiolipin and phosphatidylglycerol from *Staphylococcus aureus* were purified by silicic acid column chromatography (11). Chromatoplates measuring 20×20 cm coated 0.25 mm layer of Silica Gel G (Merk, Type 60) were prepared in the usual manner. The plates were activated at 110-120°C for 2 hours prior to use.

Preparation of Lipid from Mouse Liver: Mouse liver was homogenized in small amounts of chloroform-methanol (2:1, v/v). About 20-fold volumes of the solvent was added to homogenates. The extraction was performed by stirring overnight. The extract thus obtained was subjected to Folch's partition dialysis (12).

Chromtograpic Separation: Cholesterol, cholesterylstearate and unknown lipid mixture extracted from mouse liver were dissolved in chloroform to give concentration as 0.1, 0.2 and 1.0 mg/ml, respectively. One ml of each solution were measured into test tubes followed by evaporation to dryness. The lipids were redissolved in a minimum volume of chloroform and applied on a plate using a capillary to form a band 2 cm wide about 2.5 cm from the bottom of the plate. In order to transfer the remaining lipid residues onto the same part of the plate, the application was repeated two more times in the same manner. Care had to be taken to avoid damaging the surface of the silica gel. As for the unknown lipid samples, the gravimetric determination should be performed carefully. The weight of the lipid was determined after transferring to a preweighed small test tube (1-2gr.). One dimensional system was developed with the solvent mixture, petroleum ether (b.p. 30-60°C)-diethyl ether-acetic acid (82:18:2, v/v/v). The solvent front was allowed to move approx. one cm from the top of the plate. The plate was then air-dried and placed in iodine vapor to detect the lipid spots.

Extraction of Lipids from TLC: Cholestrol and cholesteryl esters fractions were scraped off with a razor blade to glass stoppered centrifuge tubes separately, while phospholipid fraction was into a Kjeldahl flask. The appropriate areas unstained with iodine were also removed to act blanks. Cholesterol and cholesteryl esters were eluted from the silica gel in the following ways. The

extraction was carried out with 5 ml of chloroform-methanol (4 : 1, v/v) by mechanical rotation at 40°C for 15 min. After centrifugating for 5 min. at 2000 rev/min., the eluates were removed with a disposal pipette. The elution procedure was repeated two more times and the combined eluates were brought to dryness.

Quantitation: Cholesterol was determined by the method of ZAK (13), except for the use of Rosenthal's reagent (14) as color reagent. Namely, three ml of glacial acetic acid were added to tubes containing cholesterol followed by the addition of two ml of Rosenthal's reagent. After standing at room temperature for 20 min., absorbance at 560 nm was read. Cholesteryl esters was converted to free form by hydrolysis with two ml of alcoholic potassium hydroxide solution (ethanol-2.5 g/dl of KOH, 19 : 1, v/v) at 60°C for 3 hours. The solusion was evaporated to dryness and the above-cited procedure was carried out. The phosphorus content of phospholipid fraction was determined by the method of KATES (15). The results were multiplied by 25 to give the phospholipid content.

RESULTS AND DISCUSSION

Chromatographic Separation: Fig. 1 shows a chromatographic separation of reference lipids applied individually or as a mixture and total lipids extracted from mouse liver. Cholesterol, cholesterylstearate, fraction 1 and fraction 6 gave positive sulfuric acid test (16). Cardiolipin, lecithin, phosphatidylglycerol and fraction 8 gave positive test with molybdenum blue reagents pray (17). As a result, this solvent system revealed a good separation of cholesterol, cholesteryl esters and phospholipids from each other. Fractions 3 and 4 were identified as triglyceride and free fatty acid, respectively, although these components were not included in quantitative analyses. Fractions 2, 5 and 7 were speculated to be methyl esters of fatty acid, diglyceride and monoglyceride, respectively, from the reports of MALINS *et al.* (18) and SKIPSKI *et al.* (19).

Recovery of Cholesterol after TLC: Eight separate analyses of 0.1 mg of cholesterol applied to TLC in a single experiment are shown in Table 1. The absorbance equivalent to 0.1 mg of cholesterol was calculated from eight determinations. The average per cent recovery was $98.2 \pm 2.2\%$ and proved that the extration procedure was sufficient for complete elution of cholesterol. The presence of the small amounts of silica gel contaminated into the eluate did not affect the determination, since a blank containing silica gel indicated no absorbance.

The Colorimetric Method for Determination of Cholesteryl esters: Cholesteryl esters were directly determined as cholesterol was. As shown in Fig. 2, cholesteryl esters gave far lower absorbance than equimolar quantities of cholesterol. Next, we investigated the method for determining cholesteryl esters. At first, the following procedure was adopted from a consideration

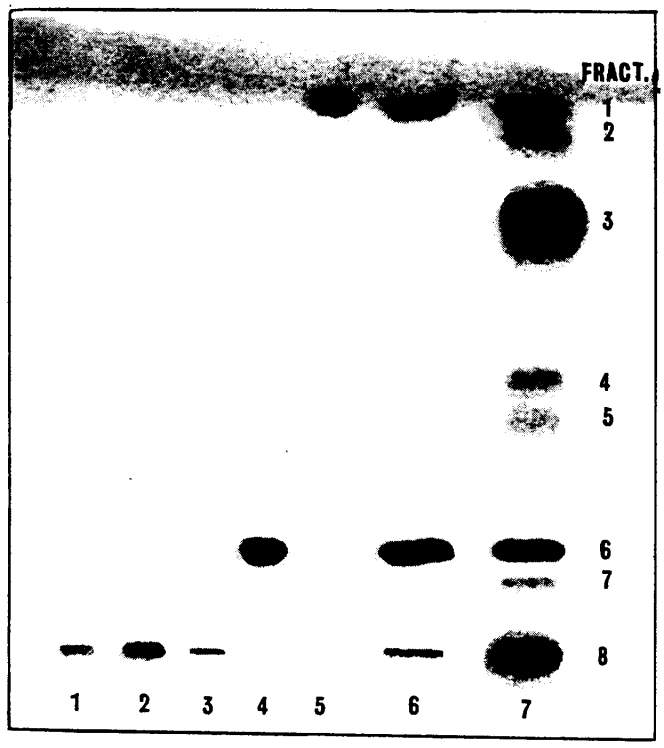

Fig. 1. Chromatogram of reference compounds and total lipid extracts from mouse liver. (1) cardiolipin; (2) phosphatidylglycerol; (3) phosphatidylcholine; (4) cholesterol; (5) cholesterylstearate; (6) mixture of (1)-(5); (7) total lipid extracts from mouse liver. Spots were detected for the purpose of illustration with 30% H_2SO_4 spray followed by charring. Fractions 1, 3, 4, 6 and 8 were identified as cholesteryl esters, triglycerides, free fatty acids, cholesterols and phospholipids. Fractions 2, 5 and 7 were not identified.

Table 1 Recovery of cholesterol and cholesterylstearate in eluates from silica gel after TLC

	0.1 mg of cholesterol		0.2 mg of cholesterylstearate	
	reference	after TLC	reference	after TLC
Absorbance	0.552	0.546	0.610	0.636
	0.547	0.534	0.626	0.609
	0.551	0.547	0.616	0.607
	0.549	0.542	0.599	0.616
	0.542	0.537	0.634	0.613
	0.548	0.539	0.628	0.615
	0.552	0.536	0.637	0.617
	0.549	0.527	0.624	0.628
Average	0.549	0.539	0.622	0.618
Average per cent recovery		98.2±2.2%		99.4±2.9%

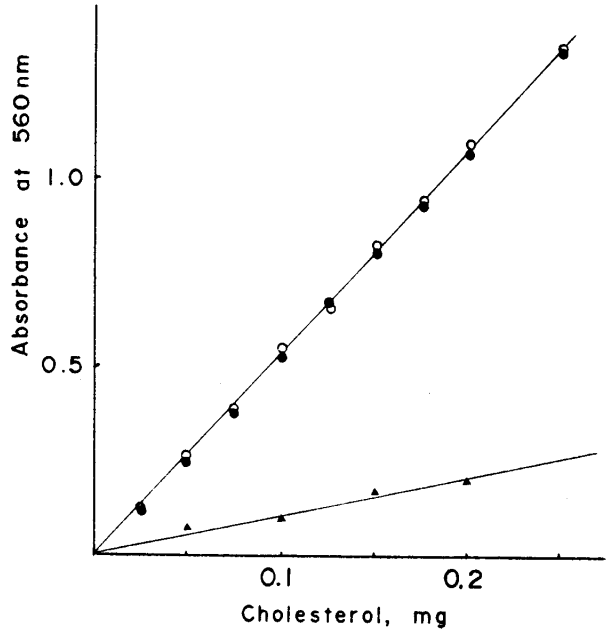

Fig. 2. Calibration curves for cholesterol (○——○), saponified cholesterylstearate (●——●) and unsaponified cholesterylstearate (▲——▲). Colorimetric procedures as described in the section of materials and methods. Cholesterylstearate was calculated in cholesterol equivalent.

of solubility of cholesteryl esters. Three ml of acetic acid were added to test tubes containing cholesteryl esters and then the tubes were placed in a boiling water for one min. Color reagent was added immediately after the tubes were removed, since the absorbance decreased with drop in temperature at the addition of the reagent. Consequently the calibration curve almost coincided with that of cholesterol. However, this method did not appear to be suitable for an accurate analysis because of hypochromic shift. Secondarily, the method used in determination of cholesterol was carried out after saponification. Fig. 3 represents the absorbance of 0.2 mg of cholesterylstearate hydrolyzed for different periods. It was found hydrolysis for three hours was adequate. As shown in Fig. 2 the calibration curve followed Beer's law through absorbance of 1.35 and revealed that equimolar concentrations of of cholesterol and its esters yielded color of equal intensity. Furthermore, the color remained stable for several hours.

ABELL *et al.* (20) adopted extraction with ether after saponification. They used 30% KOH/Ethanol solution as saponifying reagent. We omitted extraction and evaporated directly for the purpose of simplification of the

method. We lowered the concentration of KOH to 0.125% so as to reduce KOH residues after evaporation. Saponification was omitted in the methods of ZLATKIS *et al.* (21) and ROSENTHAL *et al.* (14). Saponification step appeared to be inevitable for the application of their methods to our system.

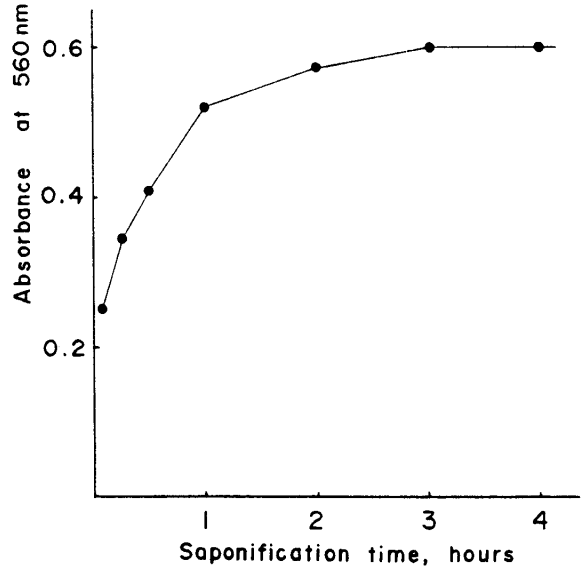

Fig. 3. The effect of sponification time on absorbance measurement of cholesteryl-stearate. Saponification was carried out at 60°C with 2 ml of alcoholic hydroxide solution (2.5 g/dl of KOH-ethanol, 1 : 19, v/v).

TABLE 2 CONTENTS OF CHOLESTEROL, CHOLESTERYL ESTERS AND PHOSPHOLIPIDS OF MOUSE LIVER AND BOVINE RED CELL GHOST

Lipids	Amounts in 1 mg of total lipid extract (μg)						
	Mouse liver						Bovine red cell ghost*
	1	2	3	4	5	average	
Cholesterol	41.8	42.2	44.1	43.9	41.2	42.6±1.5	287**
Cholesteryl esters***	21.0	21.5	21.7	21.7	22.0	21.6±0.6	trace
Phospholipids (P×25)	570	568	558	565	555	563±8	588

* Bovine red cell ghost was prepared by the method of HANAHAN (22).

** Average of two experiments on the same sample.

*** Calculated as cholesteryl oleate.

Recovery of Cholesterylstearate after TLC: On eight different 0.2 mg of cholesterylstearate we performed the procedure described above. The average per cent recovery was 99.4±2.9%.

Applicability of This Procedure: We examined the applicability of this procedure for microdetermination of cholesterol, its esters and phospholipids, using for an example lipids extracted from mouse liver. The estimated quantities of three lipid classes from five analyses are given in Table 2. The errors inherent in this method were within 3.5%. The method described here was also successfully applied for bovine erythrocyte membrane (Table 2). The obtained values are in good agreement with the data of GIER and VAN DEENEN (23).

SKIPSKI *et al.* (8) reported the method for analysis of neutral lipids by TLC. As to three lipid classes described in this paper, our method is more sensitive and simpler than their method. Microdetermination method of triglycerides and free fatty acids is now under investigation.

Acknowledgements: The authors are deeply indebted to Mr. M. HAYAMI, who kindly provided them with many helpful suggestions and discussions relating to this work. They also wish to thank Dr. M. INOUE of the Department of Biochemistry, Kumamoto University Medical School for help in the preparation of red cells ghosts, and to Miss NOZAKI for her skilled technical assistance.

The investigation was supported by a grant from the Sanyo Broadcasting Science and Culture Advancement Foundation and by a Grant-in-aid for Scientific Research from the Ministry of Education.

REFERENCES

1. GRIGAUT, A.: Procede colorimetrique de dosage de la cholesterine dans l'organisme. *Compt. rend. soc. biol.* **68**, 791, 1910
2. SCHOENHEIMER, R. and SPERRY, W.M.: A micromethod for the determination of free and combined cholesterol. *J. Biol. Chem.* **106**, 745, 1934
3. SPERRY, W.M. and WEBB, M.: A revision of the Schoenheimer and Sperry method for cholesterol determination. *J. Biol. Chem.* **187**, 97, 1950
4. BADZIO, T. and BOCZON, H.: The determination of free and esterified cholesterol in blood after separation by thin-layer chromatography. *Clin. Chim. Acta.* **13**, 794, 1966
5. ANGELICO, R., CAVINA, G., D'ANTONA, A. and GIOCOLI, G.: Fraction and determination of the lipid and steroid constituents of the adrenal glands of rats by means of thin-layer chromatography. *J. Chromatog.* **18**, 57, 1965
6. VACIKOVA, A., FELT, V. and MALIKOVA, J.: Chromatography of serum lipid fraction on a thin layer of Al_2O_3. *J. Chromatog.* **9**, 301, 1962
7. GLOSTER, J. and FLETCHER, R.F.: Quantitative analysis of serum lipids with thin-layer chromatography. *Clin. Chim. Acta.* **13**, 235, 1966
8. SKIPSKI, V.P., GOOD, J.J., BARCLAY, M. and REGGIO, R.B.: Quantitative analysis of simple lipid classes by thin-layer chromatography. *Biochim. Biophys. Acta.* **152**, 10, 1968
9. OLDFIELD, E. and CHAPMAN, D.: Dynamics of lipids in membranes: heterogeneity and the role of cholesterol. *FEBS. Lett.* **23**, 285, 1972
10. DE KRUYFF, B., DEMEL, R.A., SLOTBOOM, A.J., VAN DEENEN, L.L.M. and ROSENTHAL, A.F.: The effect of the polar headgroup on the lipid-cholesterol interaction: a monolayer and differential scanning calorimetry study. *Biochim. Biophys. Acta.* **307**, 1, 1973
11. YOSHIOKA, T., AKATSUKA, K., YAMAGAMI, A. and KANEMASA, Y.: A method of column chromatographic isolation of major phospholipid components of *Escherichia coli*. *Acta Med.*

Okayama **22**, 147, 1968

12. FOLCH, J., ASCOLI, I., LEES, M., MEATH, J. A. and LEBARON, F. N.: Preparation of lipid extracts from brain tissue. *J. Biol. Chem.* **191**, 833, 1951
13. ZAK, B., DICKENMAN, R. C., WHITE, E. G., BURNETT, H. and CHERNEY, P. J.: Rapid estimation of free and total cholesterol. *Am. J. Clin. Path.* **24**, 1307, 1954
14. ROSENTHAL, H. L., PFLUKE, M. L. and BUSCAGLIA, S.: A stable iron reagent for determination of cholesterol. *J. Lab. Clin. Med.* **50**, 318, 1957
15. KATES, M., ALLISON, A. G. and JAMES, A. T.: Phosphatides of human blood cells and their role in spherocytosis. *Biochim. Biophys. Acta* **48**, 571, 1961
16. JATZKEWITZ, H. and MEHL, E.: Thin layer chromatography of brain lipids; hydrolytic and breakdown products. *Z. Physiol. Chem.* **320**, 251, 1960
17. DITTMER, J. C. and LESTER, R. L.: A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5**, 126, 1964
18. MALINS, D. C. and MANGOLD, H. K.: Analysis of complex lipide mixtures by thin-layer chromatography and complementary methods. *J. Am. Oil Chemist's Soc.* **37**, 576, 1960
19. SKIPSKI, V. P., SMOLOWE, A. F., SULLIVAN, R. C. and BARCLAY, M.: Separation of lipid classes by thin-layer chromatography. *Biochim. Biophys. Acta* **106**, 386, 1965
20. ABELL, L. L., LEVY, B. B., BRODIE, B. B. and KENDALL, F. E.: A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195**, 357, 1952
21. ZLATKIS, A., ZAK, B. and BOYLE, A. J.: A new method for the direct determination of serum cholestrol. *J. Lab. Clin. Med.* **41**, 486, 1953
22. DODGE, J. T., MITCHELL, C. and HANAHAN, D. J.: The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem.* **100**, 119, 1963
23. GIER, J. D. and VAN DEENEN, L. L. M.: Some lipid characteristics of red cell membranes of various animal species. *Biochim. Biophys. Acta* **49**, 286, 1961