ACTA CHEMICA SCANDINAVICA 11 (1957) 1658—1662

Paper Chromatography and Colorimetric Determination of Free and Esterified Cholesterol in Very Small Amounts of Blood

PREBEN W. HANSEN and HENRIK DAM

Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark

A simple and rapid method for paper chromatography and colorimetric determination of cholesterol and total cholesterol esters in very small amounts of blood is described.

Cholesterol and cholesterol esters are separated within 20 min on paper with *iso*octane as mobile phase.

The quantitative determination after elution of the spots on the chromatogram is based on a modification of Zlatkis' color reaction. Equivalent amounts of cholesterol and cholesterol esters show the same optical density.

Total amounts of cholesterol and esters as low as 2.5 μg can be determined with an accuracy of 5 %.

In 1953 Zlatkis, Zak and Boyle [1] described a rapid and simple method for the determination of total cholesterol in blood. By the present modification of this method it is possible to determine cholesterol and its esters in amounts from 2.5 μg to 25 μg per 0.5 ml of reaction mixture.

The method of Zlatkis *et al.* is less specific than the methods of Liebermann-Burchard and Tschugaeff and frequently gives too high cholesterol values when used directly on plasma [2, 3].

We have, therefore, combined our modification of the method with a new and rapid paper chromatographic separation of cholesterol and its esters from other components which might interfere with the colorimetric determination.

EXPERIMENTAL

Reagents and Solvents

1. Standard cholesterol solution: 25.0 mg in 100 ml of chloroform.
2. Standard cholesterylacetate solution: 27.75 mg in 100 ml of chloroform.
3. Standard cholesterylpalmitate solution: 40.40 mg in 100 ml of chloroform.
4. Ferric chloride solution: 1.0 g in 10 ml of 99.5 % glacial acetic acid. (This solution can be kept for weeks).

5. Color reagent: 0.5 ml of the ferric chloride solution diluted to 25 ml with concentrated sulfuric acid. (The reagent has to be prepared just before the colorimetric determination is carried out).
6. Acetone-ethanol 1:1 (v/v).
7. 2,2,4-Trimethylpentane ("*iso*octane").
8. Chloroform.
9. Phosphotungstic acid solution: 10 % in ethanol.

Procedures

A. Preparation of sample for chromatography: 20 µl of plasma is pipetted by means of a constriction pipette (Carlsberg pipette [4]) into 300 µl of warm acetone-ethanol reagent in a 4.5 ml polyethylene-stoppered centrifuge tube. (In case the cholesterol is expected to be high the ratio of plasma taken to volume of extract should be reduced.) The tube is stoppered and placed for 5 min in a water bath at 80—85°C and then shaken violently for 1 min in a shaking apparatus (Microid Flask Shaker, Griffin & George Ltd., England). The tube is then centrifuged (approximately 1 min) and the supernatant liquid applied to the paper with a constriction pipette as described below. The precipitate is re-extracted twice with 100 µl acetone-ethanol reagent, every extract being applied to the paper.

B. Paper chromatography. Schleicher & Schüll 2043 b paper is cut out in sheets 15 cm long and 24.7 cm wide. Parallel to the longer edge of the paper three pencil lines are drawn at distances of 1, 2, and 12 cm from this edge. Between the 1 and 12 cm lines the paper is divided by means of a scalpel into 8 strips separated from each other by 1 mm open spaces.

The sheets are washed with *iso*octane by placing them for 5 min in the solvent in a glass bowl under slow motion. After drying the sheets are treated with chloroform in the same way.

By means of a constriction pipette the solution containing the cholesterol and cholesterol esters (from 2.5 to 25 µg) is applied as 1.5 cm streaks along the 2 cm line on each of the 6 strips in the middle. The first strip serves as a blank, whereas 100 µl from the cholesterol standard solution and 100 µl from one of the ester standard solutions are applied to the last strip. During application warm air is blown upward against the starting lines.

The paper is placed in the tank containing the mobile phase on the bottom. The tank must be completely saturated with vapor of *iso*octane before the chromatogram is started. Therefore, precaution must be taken to avoid vapor from escaping when the paper is brought into the tank. The chromatogram is started 5 min later and is run until the solvent front reaches the upper end of the strips. This requires about 20 min.

C. Elution of cholesterol and cholesterol esters. After chromatography the paper is removed from the tank and allowed to dry at room temperature for 1 min. The last strip is cut out and the spots are made visible by spraying with a solution of phosphotungstic acid (10 % in ethanol) followed by drying at 100°C for about 1 min. The zones on the other strips, including the blank, corresponding to the spots on the last strip, are cut out, placed in weighing glasses and extracted with 1.0 ml chloroform under gently shaking. The chloroform extracts are transferred to 1.5 ml test tubes with a constriction pipette, and the chloroform is evaporated by placing the tubes in a water bath at 70—80°C. The extraction procedure is repeated twice with 0.5 ml of chloroform.

While the chloroform extracts are evaporated, 20 µl of the cholesterol standard solution is pipetted into two test tubes and dried simultaneously with the eluates from the chromatogram. When dry the tubes are allowed to cool to room temperature, and subsequently 0.30 ml of glacial acetic acid is added. The tubes are stoppered and carefully shaken.

D. The colorimetric determination. From a Krogh-syringe [5] (mounted in a burette holder) 0.20 ml of the color reagent is added to the first of the tubes, and after rapid mixing with a small glass rod the tube is placed in a boiling water bath for exactly 1 min. The remaining tubes are treated similarly.

After cooling to room temperature in ice water the reaction mixtures are transferred to micro cuvettes with 1 cm light path and the optical density is measured at 530 mµ in a Beckman spectrophotometer.

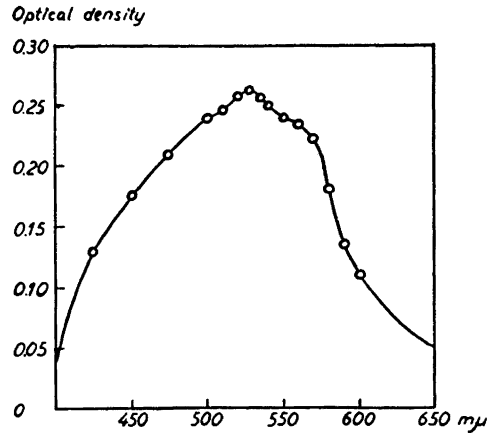

Fig. 1. Absorption curve for 5 μg cholesterol (free or esterified) per 0.5 ml reaction mixture.

DISCUSSION OF THE METHOD

The method of Zlatkis *et al.* consists in mixing a solution of cholesterol in 3 ml of glacial acetic acid with 2 ml of the color reagent, whereby a light brown color appears which changes to purple within a minute. This color shows a maximum at 560 mμ.

During our attempts to modify the method for determination of cholesterol and its esters in amounts from 2.5 μg to 25 μg per 0.5 ml (total) reaction mixture it was observed that by mixing the reagents as described by Zlatkis *et al.*, only the light brown color appeared. The temperature of the reaction mixture did not, because of the small volume, reach the level required for full development of the color (60—65°C)[6].

By placing the reaction mixture in a boiling water bath the light brown color changed to purple within a few seconds. After one minute in the boiling water bath followed by cooling in ice water the color obtained showed a maximum at 530 mμ for cholesterol as well as for cholesterol esters as shown in Fig. 1.

The color follows Beer's law in the concentration range examined, and equivalent amounts of cholesterol, cholesterylacetate and cholesterylpalmitate show the same optical density as it appears from Table 1.

The method of Zlatkis *et al.* was originally used on serum directly, but several authors [2,3] have stated that in this way the method often shows too high cholesterol values because of the interference of other compounds with the development of the color. It might be expected that combination of the method with a paper chromatographic separation of cholesterol and its esters from the interfering compounds would eliminate this error.

Several methods for paper chromatographic separation of cholesterol and its esters have been published [7-9], but most of these methods employ as mobile or stationary phase solvents which are difficult to remove from the

Table 1. Optical density at 530 mμ obtained with cholesterol and cholesterol esters per 0.5 ml reaction mixture (the total volume of the mixture).

Compound	μg/0.5 ml	Density	Density/μg cholesterol
Cholesterol	2.50	0.127	0.051
	5.00	0.260	0.052
	10.00	0.520	0.052
	15.00	0.782	0.052
	20.00	1.040	0.052
	25.00	1.320	0.053
Cholesterylacetate	2.78	0.130	0.052
	5.55	0.254	0.051
	11.10	0.512	0.051
	16.65	0.740	0.049
	22.20	0.995	0.050
	27.80	1.275	0.051
Cholesterylpalmitate	4.04	0.128	0.051
	8.08	0.255	0.051
	16.16	0.514	0.051
	24.24	0.762	0.051
	32.32	1.040	0.052
	40.40	1.290	0.052

Table 2. Recovery of cholesterol and cholesterol esters after chromatography of mixtures

Amounts in μg applied to the paper			Recovery			
Free cholesterol	Cholesterol as acetate	Cholesterol as palmitate	Cholesterol		Cholesterol as esters	
			μg	%	μg	%
2.5	10.0	15.0	2.49	99.6	25.08	100.3
5.0	10.0	10.0	4.98	99.6	19.50	97.5
10.0	5.0	10.0	9.87	98.7	14.33	95.6
15.0	5.0	5.0	14.60	97.4	9.55	95.5
20.0	0.0	5.0	19.12	95.7	5.15	103.1
25.0	2.5	0.0	24.75	98.9	2.59	103.9

Table 3. Comparison of the Sperry-Webb method with the present method.

Method of Sperry and Webb			Our method		
Free cholesterol	Esterified cholesterol	Total cholesterol	Free cholesterol	Esterified cholesterol	Total cholesterol
mg/100 ml					
46.00	91.20	137.20	43.35	89.30	132.65
39.30	69.90	109.20	35.50	67.35	102.85
47.80	75.50	123.30	45.40	75.00	120.40

paper after chromatography. When the chromatogram is eluted with chloroform these solvents will pass into solution and interfere with the development of the color.

We therefore tried to find a paper chromatographic procedure by which cholesterol and its esters could be separated on untreated paper and with a mobile phase which is easy to remove from the paper after chromatography. *Iso*octane fulfills this requirement, and with this solvent we were able to obtain clear separation of cholesterol and total cholesterol esters in 20 min. On Schleicher & Schüll 2043 b paper the R_F values for cholesterol and cholesterol esters were 0.35 and 0.80, respectively.

Table 2 shows the recovery after chromatography of mixtures of cholesterol, cholesterylacetate and cholesterylpalmitate.

Table 3 shows the results obtained with hamster plasma by the present method (0.02 ml plasma) and by the method of Sperry and Webb [10] (0.2 ml plasma).

By the present method determination of free and esterified cholesterol in plasma from 6 animals can be carried out within 5 h, whereas the same determination by the Sperry-Webb method requires 2 days.

REFERENCES

1. Zlatkis, A., Zak, B. and Boyle, A. J. *J. Lab. Clin. Med.* **41** (1953) 486.
2. Lovern, J. A. *Proc. Nutrition Soc. (Engl. and Scot.)* **15** (1956) 46.
3. Best, M., Van Loon, E. J., Wathen, J. D. and Seger, A. J. *Am. J. Med.* **16** (1954) 601.
4. Linderstrøm-Lang, K., and Holter, H. in Bamann, E. and Myrbäck, K. *Die Methoden der Fermentforschung*, Thieme, Leipzig, 1940. I p. 1132.
5. Krogh, A. *Ind. Eng. Chem., Anal. Ed.* **7** (1935) 130.
6. Fürst, V., Jr. and Lange, R. *Scand. J. Clin. Lab. Invest.* **6** (1954) 60.
7. Harrison, E. G. *Paper Chromatographic Study of Adrenal Cholesterol*, (Thesis) University of Illinois 1949.
8. Michalec, C. *Biochim. et Biophys. Acta* **19** (1956) 187.
9. See Michalec, C. *Naturwiss.* **42** (1955) 509.
10. Sperry, W. M. and Webb, M. *J. Biol. Chem.* **187** (1950) 97.

Received July 29, 1957.