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A METHOD FOR THE ESTIMATION OF FREE AND ESTERIFIED CHOLESTEROL INVOLVING THIN-LAYER CHROMATOGRAPHY*

J. DUTTA
Department of Chemistry, Lipid Chemistry, Bose Research Institute, Calcutta-700 009 (India)
A. BISWAS**
Department of Physiology, Calcutta University, Calcutta (India)
S. SAHA
Department of Chemistry, Bose Research Institute, Calcutta-700 009 (India)
and
C. DEB
Department of Physiology, Calcutta University, Calcutta (India)
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SUMMARY

A method for the estimation of both free and bound cholesterol in serum and plasma involving thin-layer chromatography has been developed. The serum or plasma is applied as a band directly on the plate. Extraction of lipid material from this band and the chromatographic separation of the above components are carried out on the same plate. The components are extracted from the adsorbent directly with acetic acid and measured colorimetrically using iron(III) chloride–sulphuric acid reagent.

INTRODUCTION

With increasing interest in the role of cholesterol and cholesterol esters as indicators of physiological and pathological processes, a number of methods has been proposed to replace the classical digitonin procedure[1] for their estimation in blood, which is not only lengthy but also requires the use of a large amount of plasma or serum. Most of these methods are based on the following general steps: extraction of free and esterified cholesterol from serum or plasma[2], separation of these two components by column[3,4] or thin-layer chromatography[5–7] (TLC) and their estimation by either colorimetry or densitometry. The Liebermann–Burchard or iron(III)

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** To whom correspondence should be addressed. Address: 29, Nilmoni Sarkar Street, Calcutta-700 050, India.

chloride–sulphuric acid[8] reaction is generally used for colour production in colorimetry. Even for the determination of the total cholesterol level, *i.e.*, both free and bound cholesterol in blood, the extraction step has been found to be unavoidable because otherwise spuriously high results are obtained owing to the formation of non-steroid chromophores in the above reactions.

An improved clinical method is presented in this paper for the determination of the cholesterol level in blood, separate values being obtained for the free and esterified forms, by TLC. The procedure involves the direct application of serum to the preparative TLC plate, the extraction and separation of cholesterol and its ester, which are carried out on the plate, and their final estimation by a modified Zlatkis method[9].

MATERIALS

Reagents other than the solvents and absorbent were all of analytical-reagent grade; the acetic acid was aldehyde free. The following solvents were used: chloroform (analytical-reagent grade), washed with water, dried over fused calcium chloride and re-distilled, 1% of absolute methanol being added as preservative; ethanol freed from aldehyde by distillation over potassium hydroxide pellets; diethyl ether freed from peroxides, dried and distilled; light petroleum (b.p. 60–80°), re-distilled. Silica gel G (E. Merck, Darmstadt, G.F.R.) was used as absorbent, and cholesterol and cholesteryl palmitate, used as reference compounds, were purchased from Applied Science Labs (State College, Pa., U.S.A.).

METHODS

Procedure with standard samples

Standard solutions of cholesterol (96.9 mg per 25 ml of ethanol) and cholesterol ester (105.5 mg per 25 ml of chloroform) were prepared; 60 ml of each standard solution were applied as bands on each 14 × 20 cm silica gel plate of 0.5-mm thickness, activated for 1 h at 110°. The plates were developed in a solvent-saturated chamber up to 15 cm from the spotting line. Light petroleum–diethyl ether–acetic acid (100:15:1.5) was used as the developing solvent.

The developed chromatograms were exposed to iodine vapour, the bands were outlined with a needle and the iodine was allowed to evaporate. The chromatograms were lightly sprayed with water and the moist bands scraped off into centrifuge tubes. The tubes were then heated for 10 min at 110° so as to remove water from the scraped-off material; 4 and 5 ml of acetic acid were added to each tube containing cholesterol and cholesterol ester, respectively, then mixed by stirring thoroughly and centrifuged for 15 min.

After centrifugation, 3 ml of the acetic acid extract were taken separately from both the cholesterol and cholesterol ester containing supernatants in the stoppered tubes and the cholesterol contents measured according to the modified method of Zlatkis[9]; 1.5 ml of the iron(III) chloride–sulphuric acid reagent were used for the development of the colour, and its optical density was measured at 570 nm with the aid of a Bausch & Lomb Spectronic 20 spectrophotometer. The cholesterol ester content was measured in terms of cholesterol.

$$\text{Free cholesterol (mg per 100 ml of serum)} = \frac{\text{Standard cholesterol (mg)} \times \text{O.D. of sample} \times 4 \times 100}{\text{O.D. of Standard} \times 0.1 \times 3}$$

$$\text{Esterified cholesterol (mg per 100 ml of serum)} = \frac{\text{Standard cholesterol (mg)} \times \text{O.D. of the sample} \times 5 \times 100}{\text{O.D. of Standard} \times 0.1 \times 3}$$

Two types of blanks were used: the reagent blank and plate blanks. Reagent blanks were obtained by adding 1.5 ml of the colour reagent to 3 ml of acetic acid. A plate blank was prepared by scraping off an area equivalent to the cholesterol or cholesterol ester bands from a clear bandless portion of the chromatogram and treating it as above. Optical density readings for the samples were obtained by subtracting the plate blank reading from those of the samples.

The same amount of standard samples as that applied to the plate was taken directly in separate tubes and the cholesterol contents were measured as above.

Procedure with serum samples

Blood samples of hospital patients were collected and assayed by the present procedure, the serum being separated from the blood as soon as possible after collection. The determination was either carried out immediately or the serum was stored at $-20°$.

A TLC plate prepared as before was divided lengthwise into three lanes that were 5, 5 and 2 cm wide; 0.05 ml of serum was applied evenly in the form of a band throughout the two 5-cm lanes, and the charged plate was dried in a desiccator over calcium chloride for 10 min. The plate was then subjected to two consecutive developments with the mixture chloroform–methanol (2:1) as solvent in a saturated chamber up to 1.5 cm from the starting line in each run (the plate was air dried before each run). This was done in order to extract the lipid material from the serum band. After extraction, a mixture of cholesterol and cholesterol palmitate was spotted on the middle of the 2 cm wide lane at a distance of 1.5 cm from the starting line.

The plate was then developed in a saturated chamber up to 16 cm from the starting line with the same solvent. The chromatogram was exposed to iodine vapour and the cholesterol and its ester bands were identified from the position of the reference spots. The cholesterol content of these two bands was estimated as before.

Procedure with added standards to serum samples

To evaluate accuracy and reproducibility of the method, known volumes of standard cholesterol and cholesterol ester solutions were applied on a plate followed by 0.05 ml of serum. The plates were treated in the same way as for the serum samples.

RESULTS AND DISCUSSION

Extraction of cholesterol and cholesteryl palmitate from the TLC plate

Table I shows the results of experiments performed to assess the amount of cholesterol and cholesterol ester that can be recovered from the absorbent. The results

TABLE I

ESTIMATION OF CHOLESTEROL AND CHOLESTEROL ESTER FROM CHROMATOGRAMS OF STANDARD SOLUTIONS

Calculations are according to ref. 10.

Sample	*Amount applied on plate (mg)*	*Amount obtained from plate* (mg)*	*Recovery (%)*
Cholesterol	0.079	0.078 ± 0.003	98.73 ± 3.74
	0.100	0.097 ± 0.001	97.00 ± 1.00
	0.121	0.116 ± 0.001	95.07 ± 0.83
Cholesteryl palmitate	0.133	0.127 ± 0.002	95.49 ± 1.50
	0.158	0.148 ± 0.002	93.67 ± 1.26
	0.169	0.169 ± 0.003	100.00 ± 1.77

* Mean value ± standard error of the mean (from four determinations).

indicate that the cholesterol content of both bands is within 10% of the results obtained by direct estimation of the same amount of the standard compounds.

Optimum conditions for the "on plate" quantitative extraction of lipids from serum sample

Application. From Fig. 1, the chromatogram of the same amount of serum sample but extracted under different conditions, it is clear that the conditions that enable a clean extracted band to be obtained are (i) even application of the sample throughout the lane; (ii) removal of some of the water from the serum band by desiccating the charged plate over fused calcium chloride for 30 min.

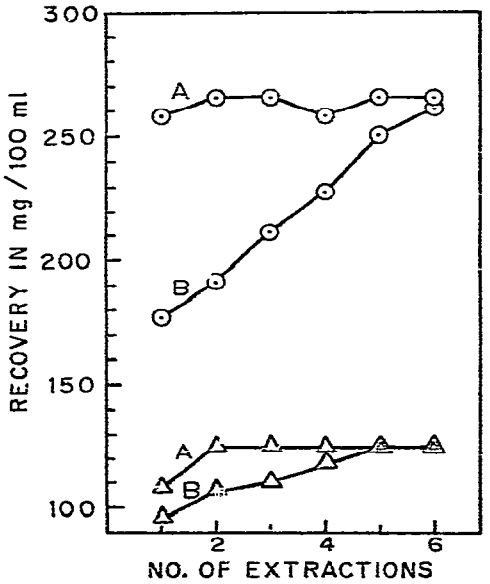

Fig. 1. Graphical representation of the amount of cholesterol and its ester *vs.* the number of extractions using two different solvent systems. Solvent: A, chloroform–methanol (2:1); B, ethanol–acetone (1:1). ⊙, cholesterol ester; △, cholesterol.

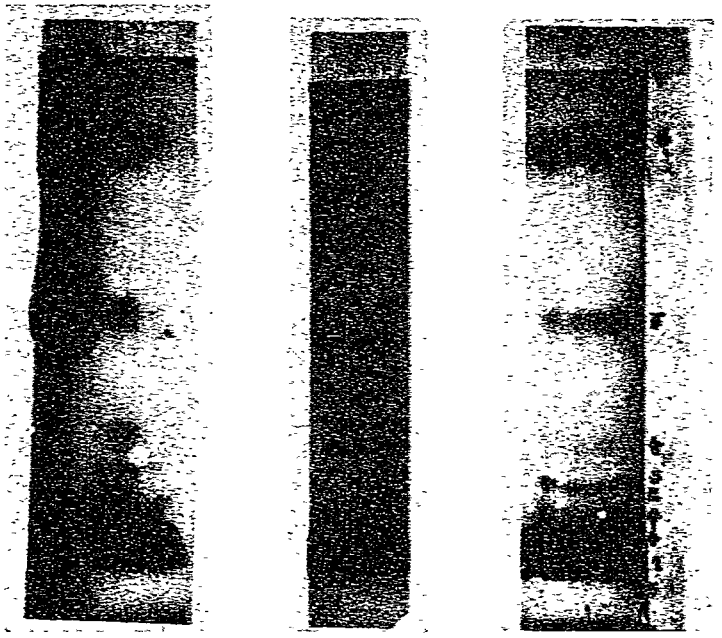

Fig. 2. TLC of the lipid materials of human serum. Extraction solvent, chloroform–methanol (2:1); separation solvent, diethyl ether–light petroleum–acetic acid (100:15:1.5); charring reagent, 20% ammonium sulphate–20% ammonium hydrogen sulphate (1:1). Charred at 225° for 15 min. A, serum applied without covering the entire width of the lane and desiccated before extraction; B, serum applied evenly throughout the lane and desiccated; C, serum applied evenly throughout the lane but not desiccated before extraction: 1 = line of application; ↔ = extraction up to 1.5 cm; 4 = upper band adjacent to that of the cholesterol; 5 = triglyceride; 6 = cholesteryl ester; 7 = solvent front.

TABLE II

SUITABILITY OF SOLVENTS DURING APPLICATION TO TLC PLATE OR TEST TUBE FOR THE EXTRACTION OF TOTAL CHOLESTEROL AND ITS ESTER FROM SERUM

Solvents: A, chloroform–methanol (2:1); B, ethanol–acetone (1:1).

Serum sample	*Extraction in tube*				*Extraction on plate*	
	Total lipid content (g) *		*Total cholesterol content (mg/100 ml)* **		*Total cholesterol content (mg/100 ml)* ***	
	A	*B*	*A*	*B*	*A*	*B*
I	0.012	0.017	125.40	128.42	124.44	85.36
II	0.011	0.015	155.21	201.76	194.00	139.68
III	0.016	0.018	178.48	194.08	186.24	154.79

* Serum (0.01 ml) mixed with 10 ml of solvent; heated at 60° for 10 min, centrifuged and total lipid weight from supernatant determined by gravimetric method.

** Colorimetric estimation of cholesterol content was made on known aliquot of above supernatant.

*** Serum (0.1 ml) applied to TLC plate was repeatedly extracted up to a distance of 1.5 cm, the total extraction area being scraped off for the estimation of cholesterol.

TABLE III

ESTIMATION OF CHOLESTEROL AND CHOLESTEROL ESTER IN SERUM SAMPLES WITH AND WITHOUT ADDED STANDARDS

Serum sample	*Level (mg/100 ml)*		*Amount of standards added to serum (mg/100 ml)*		*Enhanced levels obtained by the present method (mg/100 ml)*		*Recovery (%)*	
	Cholesterol	*Cholesterol ester*	*Cholesterol*	*Cholesterol ester*	*Cholesterol*	*Cholesterol ester*	*Cholesterol*	*Cholesterol ester*
I	44.5 ± 0.4*	161.6 ± 4	129.3	87.4	170.2 ± 6	244.2 ± 4	95.90 ± 3.46	96.18 ± 2.45
II	55.9 ± 1	168.3 ± 6	129.3	87.4	185.7 ± 3	248.4 ± 9	97.36 ± 1.73	94.37 ± 4.24
III	43.1 ± 1	159.5 ± 2	129.3	87.4	172.4 ± 4	244.2 ± 4	100.00 ± 1.00	97.82 ± 2.00
IV	51.9 ± 2.5	159.6 ± 5.7	129.3	87.4	191.5 ± 5	247.5 ± 9	90.11 ± 3.00	100.41 ± 4.24

* Mean ± standard error of mean (from four determinations).

Extraction solvents. The extractability of cholesterol and cholesterol ester from a serum band by the solvent, *i.e.*, chloroform–methanol (2:1) and ethanol–acetone (1:1), was studied. The results represented in Fig. 2 showed that, of the two solvents, the chloroform–methanol mixture is much more suitable and not more than two extractions with it are required for the complete removal of cholesterol and its ester from the serum band. It is interesting to note that the ethanol–acetone mixture, which is generally used for the extraction of lipid material, particularly cholesterol and its ester, from serum, has been found to be less efficient on a TLC plate (Table II).

Quantitative extraction from serum samples with known amount of added standards. Table III contains the results of experiments in which known amounts of cholesterol and cholesteryl palmitate were added to known volumes of serum samples, the levels of free and esterified cholesterol in which were known, and the enhanced levels were re-estimated. The results show over 95% recovery of the added materials.

The results of estimations on different serum samples by the present method and two other methods, *viz.*, the modified method of Zlatkis and the digitonin precipitation method are presented in Table IV. In the present method free and esterified cholesterol were determined directly and the total cholesterol content was obtained by addition, while in the digitonin procedure free and total cholesterol were determined directly and the amount of ester was calculated by difference; in the modified Zlatkis method only the total cholesterol value could be estimated.

Cholesterol values for different serum samples, as given by the digitonin procedure, are slightly lower than those obtained by the present procedure. This lower result might be due to loss of the floating precipitate during decantation. Results for the amount of total cholesterol obtained by the Zlatkis method were rather higher than those obtained by the present method; this discrepancy cannot be explained on the basis of the results of the present study.

Interference by other bands of serum lipid except cholesterol and its ester

The observed optical density values presented in Table V show that although in most instances bands other than those due to cholesterol and cholesterol ester pro-

TABLE IV

CHOLESTEROL VALUES (mg/100 ml) FOR INDIVIDUAL HUMAN SERUM

Serum sample	*Free cholesterol*		*Esterified cholesterol*		*Total cholesterol*		
	TLC method	*Digitonin method*	*TLC method*	*Digitonin method*	*TLC method*	*Digitonin method*	*King's method**
1	48.50 ± 3.2	42.11	123.13 ± 10.2	95.00	171.63 ± 10.21	149.54	174.63
2	39.42 ± 2.7	31.04	150.90 ± 16.2	131.86	190.30 ± 16.42	162.9	201.8
3	62.08 ± 3.3	51.84	131.92 ± 4.2	108.91	194.00 ± 6.16	160.75	201.76
4	32.64 ± 1.63	31.05	104.74 ± 4.9	97.17	137.38 ± 5.16	128.22	144.61
5	54.32 ± 3.6	38.88	155.20 ± 5.0	148.66	209.52 ± 4.42	187.54	234.76
6	38.19 ± 2.1	30.08	130.52 ± 6.7	125.12	168.69 ± 7.02	155.1	172.8
7	22.11 ± 1.3	16.59	110.57 ± 6.4	108.82	132.62 ± 6.52	125.31	172.17
8	81.09 ± 2.4	70.02	105.08 ± 4.8	103.20	186.29 ± 5.36	177.03	238.51
9	23.11 ± 1.4	18.43	66.34 ± 2.9	36.86	89.45 ± 3.22	75.29	96.69
10	65.96 ± 4.2	64.80	120.28 ± 10.8	113.40	186.24 ± 5.34	151.72	197.42

* Value obtained by applying King's method after extraction with ethanol–acetone (1:1)[9].

TABLE V

O.D. OF THE COLOUR PRODUCED FROM MATERIALS EXTRACTED FROM BANDS OTHER THAN THOSE DUE TO CHOLESTEROL AND ITS ESTER IN SOME SERUM SAMPLES

Serum sample	*O.D. due to material from the bands numbered from bottom to top of the chromatogram**					*O.D. of the plate blank*	*Cholesterol calculated from O.D. due to band 4 (mg/100 ml serum)*
	1	*2*	*4*	*5*	*7*		
V	0.02	0.02	0.02	0.09	0.02	0.02	30.00
VI	0.02	0.02	0.02	0.07	0.02	0.02	20.37
VII	0.03	0.03	0.03	0.15	0.03	0.03	46.56
VIII	0.03	0.02	0.02	0.08	0.03	0.03	23.67

* Numbering of the bands is the same as in Fig. 2.

duced no colour, significant O.D. values equivalent 20–45 mg of cholesterol were sometimes obtained from bands that had the same R_F value as a triglyceride. This indicates that higher results may be obtained in such instances if the cholesterol ester is not efficiently separated from this chromogenic agent. That the chromogen is not a steroid was proved by the fact that this band did not produce a colour specific for sterols when the chromatogram was sprayed with either antimony(III) chloride in acetic acid or iron(III)chloride in concentrated sulphuric acid. No further study to ascertain the nature of this chromogen was undertaken.

CONCLUSION

The present method can conveniently be used with as little as 0.05 ml of serum or plasma. No prior extraction of lipids is required and the possibility of interference from other chromogenic material has been avoided. From our experience, partially hemolysed samples can also be analysed without difficulty.

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