THE COLORIMETRIC ESTIMATION OF FREE AND COMBINED CHOLESTEROL IN SERUM

BY FUMIMASA YANAGISAWA AND MASAKI MIZOKOSHI

Research Institute for the Influences of Atomic Bomb, Hiroshima Branch of the National
Institute of Health of Japan, Hiroshima

(Received for publication, April 1, 1950)

This paper describes a simple and quick method for determination of free and combined cholesterol in serum. This method requires less hours to complete the determination as compared to the older method which requires at least twenty-hours. The total cholesterol is first determined by Zuckerman's method(1). Combined and free cholesterol are determined on the chloroform extract with the aid of digitonin. Free cholesterol is in the precipitates with digitonin and the combined cholesterol remains in solution. This method is quicker and more convenient than the method of Shoenheimer(2), Sperry(3), or Pijoan(4).

REAGENTS

5% sulfuric acid solution (5g of concentrated H_2SO_4 to 100 ml of H_2O).

Chloroform (analytical grade).

Concentrated sulfuric acid.

Acetic anhydride (analytical grade).

Acetic anhydride-sulfuric acid reagent. Mix cautiously 4 parts of cold acetic anhydride and 1 part of cold sulfuric acid (0-5°C) in a stoppered Erlenmeyer flask placed in ice. Use immediately after mixing (Kingsley's method (5)).

Digitonin solution (0.5mg digitonin dissolved in 100 ml of 50% alcohol).

Acetic acid-methyl alcohol solution (1 to 3 ratio by volume). Cholesterol standard (0.02mg per ml) 40mg of cholesterol is dissolved in chloroform and diluted exactly to 200 ml and 10 ml of this solution is diluted up to 100 ml with chloroform.

PROCEDURE

I. Total cholesterol.

To 0.3ml of blood serum in 25ml test tube is added 12ml of 5% sulfuric acid and exactly 15ml of chloroform from a burette. The tube is stoppered tightly with rubber stopper which has been soaked in chloroform for a half hour and dried on gauge. The content is then shaken on a shaking machine for a half hour and centrifuged. The water layer and the protein in the chloroform-water interface are removed by suction leaving the chloroform extract. If protein sticks to glass-wall, it is stirred with a glass-rod, and protein comes to the surface. Then it can be easily removed by glass-rod. For the determination of total cholesterol, 2ml of acetic anhydride-sulfuric acid is added to 5ml aliquot portion of chloroform extract and the mixture is shaken vigorously. After mixing, read the density of the color developed at 4 to 7 minutes' interval to obtain the maximum reading if the temperature of the reaction mixture is 25°C, at 10 to 15

minutes' interval at 20°C. The optical density is determined on a Evelyn photoelectric colorimeter using No. 620 filter.

II. Combined cholesterol.

To another 5ml aliquot portion of chloroform extract is added 1ml of digitonin solution and heated in a water bath at 70°C to complete dryness. Thereafter 5ml of warm chloroform is added and after 20 minutes filtered. The volume of the filtrate is adjusted to 5ml with chloroform and 2ml of acetic anhydride-sulfuric acid is added. The color is developed and determined as in the case of total cholesterol.

III. Free cholesterol.

To the remaining precipitate is added 2ml of warm acetic acid methyl alcohol solution and heated in a water bath at 70°C to complete dryness. To this is added 5ml of chloroform and shaken vigorously and filtered after 20 minutes. The volume of the filtrate is adjusted to 5ml with chloroform and added with 2ml of acetic anhydride-sulfuric acid. The color develops and its density is determined as in the case of total cholesterol.

STANDARD AND BLANK

The standard is read at the same time as the unknown. Add 1, 2, 3, 4, and 5ml of working cholesterol standard to photometer cuvettes and dilute each to 5ml with chloroform. Add 2ml of ice-cold fresh acetic anhydride-sulfuric acid, mix, and read the color as directed in "Procedure".

The blank consists of 2ml of acetic anhydride-sulfuric acid and 5ml of chloroform.

CALCULATION

The concentration of cholesterol in the unknown can be estimated by the standard curve. Since this curve is a straight line, the formula below will yield the cholesterol values in milligrams per cent when serum is used.

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times \text{mg in standard} = \text{mg per cent of cholesterol}$$

RESULTS

The results obtained are listed in Table 1. Theoretically the determined value of total cholesterol should be equal to the sum of combined cholesterol and free cholesterol, but practically the former is 1-5mg larger than the latter.

Table 1.

Sample	Total cholesterol	Combined cholesterol	Free cholesterol	Combined C. + Free C.
1	132	87	46	133
2	139	76	61	137
3	153	74	69	143
4	140	76	60	136
5	116	61	52	113

(The values are expressed in terms of milligrams of cholesterol in 100 ml of serum. Each value is the average of duplicates.)

Table 2 shows the result after adding the cholesterol standard solution to the serum.

Table 2.

Sample	Cholesterol added*	Total cholesterol	Combined cholesterol	Free cholesterol	Combined C. + Free C.
1	—	167	88	72	160
	100mg	263	97	152	249
2	—	143	87	53	140
	100mg	238	92	140	232
3	—	149	81	68	149
	50mg	192	87	101	188

* This amount of cholesterol is added to 100 cc of serum.

The result of comparison of the new method with Sperry's method is shown in Table 3.

Table 3.

Sample	Methods	Total cholesterol	Free cholesterol
1	New method	232	66
	Sperry's method	223	64
2	New method	153	74
	Sperry's method	148	72
3	New method	116	52
	Sperry's method	117	53

As shown in Table 3 each value of free cholesterol obtained by two methods is almost the same, and the result comparing this method with Pijoan's method is shown in Table 4.

Table 4.

Sample	Pijoan's method cholesterol	New method cholesterol
1	135	137
2	120	124
3	126	128
4	263	273
5	184	189

Pijoan's method is rather complicated and sometimes erroneous.

SUMMARY

By modifying the Zuckerman's method of cholesterol determination, the writer found a rapid estimation method of free and combined cholesterol.

REFERENCES

- 1) Zuckerman, J.L. and Natelson, S.: A convenient and rapid procedure for total cholesterol estimation using an acid celoroform extraction. *J. Lab. Clin. Med.*, **33**, 1322, 1948.
- 2) Schoenheimer, R. and Sperry, W.M.: Micromethod for determination of free and combined cholesterol. *J. Biol. Chem.*, **160**, 745, 1934.
- 3) Sperry, W.M.: A micromethod for the determination of total and free cholesterol. *Am. J. Clin. Path.*, Tech. Suppl., **2**, 91, 1938.
- 4) Pijoan, M. and Walter C.W.: A micromethod for the determination of blood cholesterol. *J. Lab. Clin. Med.*, **22**, 968, 1937.
- 5) Kingsley, G.R. and Schaffert, R.R.: Determination of free and total cholesterol by direct chloroform extraction. *J. Biol. Chem.*, **180**, 315, 1949.

Errata

Vol.	No.	Page	Line	
2	3	119	11	For 1924 read 1942.
"	"	121 (Table 1)	3	For Streptmicin read Streptomycin.
"	"	123 (Table 3)	12	For concetration read concentration.
"	"	124	6	For very read vary.
"	"	125	20	After "appear" insert "in the excretions than streptomycin. On this point, streptomycin appears".
"	"	129 (References 2)	5	For Japanese read English.
"	4	187	22	For 0.5mg read 5.0mg.
"	"	"	23	For and read any.