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# The Chromatographic Separation of Free and Combined Plasma Cholesterol

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The standard method of estimation of plasma cholesterol, free and combined (Schoenheimer & Sperry, 1934), involves the precipitation of cholesterol as the digitonide before and after hydrolysis. Separation and purification of free and esterified cholesterol in plasma extracts and direct estimation of the two fractions would greatly simplify the procedure. Trappe (1942) and Hess (1947) have demonstrated the ease with which the separation could be achieved by chromatography on activated alumina. Both workers, however, used eluents placed rather high in the series. These might be expected to elute other components of the plasma lipid fraction, thereby diminishing the effectiveness of the purification. Contamination of the cholesterol fractions was in fact noted by Trappe (1942). Re-investigation of the method of separation was desirable.

The problem of direct colorimetric estimation of the separated fractions was solved by Trappe (1942), by using a reaction in which esterification did not alter the colour production. This condition does not hold in the more commonly used Liebermann-Burchard procedure (see Sperry & Brand, 1943). For this reason Hess (1947) hydrolysed the esters before estimation. The separated fractions could, however, be directly estimated if the effect of esterification on the colour production were independent of the fatty acid group involved. The results of Yasuda (1936) suggested that this was the case and this approach to the problem was investigated further. It is known that cholesteryl esters in plasma are mainly derived from unsaturated acids, and recently Keegan & Gould (1953) have reported the isolation of cholesteryl oleate from plasma. Accordingly, both saturated and unsaturated esters were prepared and used as models.

## METHODS

### *Purification of reagents*

*Chloroform.* This was washed, dried over $CaCl_2$ and used freshly distilled.

*Sulphuric acid.* A.R. grade (British Drug Houses Ltd.).

*Acetic anhydride.* The A.R. grade was distilled at constant boiling point.

*Alumina* (Peter Spence). This was washed with water and methanol and dried to grade II activity (Brockmann & Schodder, 1941).

*Light petroleum.* A.R. grade, b.p. 60–80°.

*Benzene.* The A.R. grade was refluxed with conc. $H_2SO_4$ (30 ml./l.) for 30 min., washed with NaOH and water, dried over Na and distilled.

*Acetone.* This was refluxed with and distilled from $KMnO_4$, dried over $K_2CO_3$ and redistilled.

*Ethanol.* This was refluxed with and distilled from NaOH.

### *Standard solutions*

Cholesterol, m.p. 148°; cholesteryl acetate, m.p. 114°; cholesteryl palmitate, m.p. 80°; cholesteryl stearate, m.p. 79°; cholesteryl oleate, m.p. 41°. (All compounds recrystallized to constant m.p.; all m.p. corr.) The anhydrous solid (20–30 mg., as cholesterol) was dissolved in 100 ml. $CHCl_3$.

### *Colour reactions*

$H_2SO_4$-acetic anhydride reagent was prepared by adding, with mixing, 1 ml. $H_2SO_4$ to 20 ml. chilled acetic anhydride (at 0°) and again chilling for 10 min. It was used within 30 min. of preparation. Cholesterol, free and esterified, was estimated by the Liebermann-Burchard reaction. The cholesterol solution was evaporated to dryness and desiccated for at least 6 hr. over $H_2SO_4$ *in vacuo*. The residue was dissolved in 6 ml. $CHCl_3$, and 2 ml. $H_2SO_4$-acetic anhydride reagent were added with mixing. The colour was developed in the dark in a water bath at 25° for 10 min. and read within 5 min. in a Spekker photoelectric absorptiometer with Ilford filters no. 608 (690 mμ.).

### *Chromatography*

All solvent proportions are given as % (v/v). Standard proportions were cholesterol 0·3–0·5 mg., $Al_2O_3$ 200 mg. Chromatographic tubes were made from pipettes of 25 ml. capacity, cut through the bulb and having a stem 10–12 cm. long and 0·4 cm. int. diam. The $Al_2O_3$ was packed as a slurry in light petroleum (care was taken to exclude air) to a height of 3 cm. over a small plug of cotton wool in the tip of the pipette. The remaining part of the bulb and stem acted as a reservoir and was used to regulate the rate of flow to 20 ml./hr. Compounds applied to the column were dissolved in 0·5 ml. light petroleum and followed by three washes of 0·5 ml. of the same solvent. Unless otherwise stated, cuts of 5 ml. were taken.

### *Plasma extracts*

The acetone-ethanol reagent, 1:1, was freshly prepared. The extraction was carried out by a slight modification of the method of Schoenheimer & Sperry (1934). Plasma (0·2 ml.) was pipetted into 3 ml. warm acetone-ethanol reagent in a 10 ml. glass-stoppered centrifuge tube. The solution was heated to boiling point in a water bath and shaken for 2 min. After cooling, the tube was centrifuged and the supernatant liquid transferred to a second tube. The residue was re-extracted with 2 ml. acetone-ethanol reagent as above, and the combined supernatant liquids were evaporated to dryness in a water bath in a current of air.

## RESULTS AND DISCUSSION

### *The Liebermann-Burchard colour reaction*

Because of the controversy in the literature about the optimum conditions for the Liebermann-Burchard reaction of cholesterol esters, the following experiments were carried out (cf. Sperry & Brand, 1943).

*The effect of temperature on the rate of colour development.* Known volumes of cholesterol and cholesteryl stearate solutions were prepared for estimation as described above. The colour which developed during incubation at 15, 20, 25 or 30° was read at 5 min. intervals. As is shown in Fig. 1, the colour developed by the ester reached its maximum more quickly than that of the alcohol, at all temperatures examined. At 15° the colour in both cases was slow to develop, and did not reach a maximum even after 20 min. incubation, while at 30° the colour was unstable, reached a maximum within 5 min. and faded almost immediately. Between 20 and 25°, however, the rate of colour development appeared to be independent of temperature. It reached a maximum after 10 min. incubation and remained constant for at least a further 10 min. These results are, in general, in agreement with those of previous workers, and other esters of cholesterol gave values similar to those of the stearate.

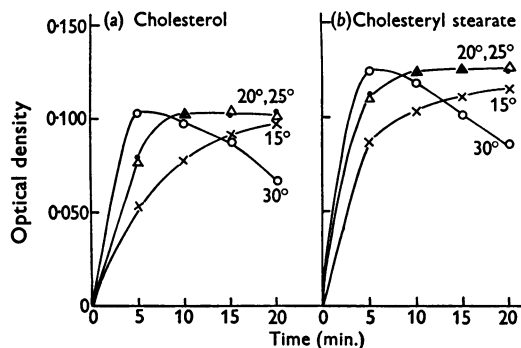


Fig. 1. The effect of temperature on the rate of colour development. (*a*) 200 μg. cholesterol were dissolved in 6 ml. $CHCl_3$ and 2 ml. acetic anhydride-$H_2SO_4$ mixture. The colour was read at time intervals after incubating at 15° (×—×), 20° (△—△), 25° (●—●), and 30° ○—○). (*b*) 304 μg. cholesteryl stearate (180 μg. cholesterol) were treated as (*a*).

*The colour developed by cholesteryl esters.* As is seen from Fig. 1, the intensity of colour developed by cholesteryl stearate is greater than that developed by an equivalent weight of the alcohol. The behaviour of other esters was therefore investigated. Samples of standard cholesteryl acetate, palmitate, stearate or oleate solutions were made up to 6 ml. with $CHCl_3$, 2 ml. of the $H_2SO_4$-acetic anhydride reagent were added, and the colour was developed in the dark at 25° for 10 min. The colour developed in each case was found to be independent of the esterifying acid and to be directly proportional to the cholesterol content of the sample. The colour produced by the esters is approximately 30 % more intense than that produced by cholesterol under identical conditions (see Table 1). Two standard

Table 1. *Colours produced by cholesterol and its esters*

(Conditions as in text, p. 873.)

| Compound | Optical density 200 μg. Cholesterol | Optical density 400 μg. Cholesterol |
|---|---|---|
| Cholesterol | 0·110 | 0·221 |
| Cholesteryl acetate | 0·141 | 0·286 |
| Cholesteryl palmitate | 0·144 | 0·283 |
| Cholesteryl stearate | 0·143 | 0·285 |
| Cholesteryl oleate | 0·139 | 0·280 |

curves are therefore required, one constructed from cholesterol and the other from any representative ester. Provided that allowance is made in this way for the enhancement of colour produced by esterification, it would appear that both cholesterol and its esters may be directly estimated by measurement of the intensity of the green colour developed when the Liebermann-Burchard reaction is allowed to proceed in the dark at 25° for 10 min.

### *The chromatographic separation of cholesterol and cholesteryl esters*

*Experiments with pure solutions.* Mixtures of known amounts of cholesteryl stearate and cholesterol were applied in light petroleum to alumina columns as above and the light-petroleum eluates were examined for chromogenic material by the Liebermann-Burchard reaction. From this experiment and from others in which cholesteryl stearate or cholesterol were examined separately, it was established that the ester was quantitatively eluted by 20 ml. light petroleum, whereas the alcohol was retained even with 150 ml. Addition of benzene to the petroleum, to 50 %, resulted in the appearance of cholesterol, spread over several fractions. When the benzene was increased to 80 % the leading edge of the eluate pattern was much sharpened, and, as was to be expected from theoretical considerations (cf. Cassidy, 1951), the use of pure benzene gave a similar pattern. In a typical experiment 506 μg. cholesteryl stearate and 275 μg. cholesterol (equivalent to a total of 575 μg. cholesterol) were dissolved in light petroleum and added to an alumina column in the usual manner. The chromatogram was developed with 30 ml. light petroleum followed by 30 ml. benzene. 5 ml. cuts were taken and the Liebermann-Burchard colour was developed. The six light petroleum eluate fractions were found to contain 154, 75, 54, 22, 0 and 0 μg. cholesterol (as the stearate) and the six benzene fractions 17, 152, 76, 23, 0 and 0 μg. cholesterol, respectively. A standard procedure has therefore been adopted in which 30–40 ml. light petroleum are used as the first eluent and followed by a similar volume of pure benzene. Under these conditions all esters tested gave similar patterns.

It was suggested by Trappe (1940) that cholesterol might be chemically altered by contact with alumina, and there is also the possibility that the cholesteryl esters may be hydrolysed. The former possibility need not be considered under the present conditions in view of recovery figures (see later, Table 2). Cholesteryl acetate and stearate, chosen for their difference in chain length, were allowed to remain in contact with alumina on the column for 12 hr. before they were eluted with light petroleum and benzene in the usual manner. In the cases of both esters 98 % of the chromogenic material was recovered in the light petroleum fraction and none in the benzene, showing that no hydrolysis of the esters had occurred.

*Experiments with plasma extracts.* It was essential to determine whether or not the elution pattern of the cholesterol in acetone-ethanol extracts of plasma was the same as that of pure solutions. Plasma extracts were therefore applied to columns and eluted successively with light petroleum and benzene. Examination of the eluates by the Liebermann-Burchard reaction showed a pattern similar to that given by mixtures of cholesteryl stearate and cholesterol, i.e. two peaks, one in the first 20 ml. of light petroleum and the other in the first 20 ml. of benzene, representing the ester and the alcohol fractions, respectively. A plasma containing 210 mg. cholesterol/100 ml. was used in a typical experiment. The extract from 0·2 ml. plasma was applied to a column which was developed with light petroleum, 30 ml., followed by an equal volume of benzene. Twelve 5 ml. cuts were collected and the Liebermann-Burchard colour was developed in each. The light petroleum eluates contained 22, 180, 67, 50, 0 and 0 μg. cholesterol, and the benzene eluates 10, 35, 26, 15, 0 and 0 μg. cholesterol respectively, thus giving a value of 159 mg./100 ml. for the ester cholesterol and 43 mg./100 ml. for the free.

There was also the danger of eluting some substance in the plasma extract along with either the ester or free cholesterol fractions which, although non-chromogenic, might interfere with the colour reaction. Samples of standard solutions of cholesteryl stearate and cholesterol were added to plasma extracts of known cholesterol content, and the whole was chromatographed as for plasma extracts. The results are shown in Table 2. Cholesteryl stearate was recovered from 96 to 98 % in the light petroleum fraction and cholesterol from 93 to 97 % in the benzene. Moreover, no anomalous peaks were detected. In a few early experiments an ether-ethanol extract of plasma was used, but this was abandoned when it was discovered that substances, probably phospholipids, which interfered with the Liebermann-Burchard reaction were eluted by benzene. Unpurified A.R. benzene also contained an impurity which gave a green colour with the Liebermann-Burchard reagents.

Table 2. *Recoveries of added cholesteryl stearate and cholesterol from plasma extracts*

| Cholesterol (mg./100 ml.) | | | | | |
|---|---|---|---|---|---|
| Amount in plasma extract | | Amount added | | Amount recovered | |
| Ester | Free | Ester | Free | Ester | Free |
| 56 | 94 | 100 | 30 | 153 | 122 |
| 56 | 94 | 150 | 60 | 200 | 151 |
| 145 | 47 | 100 | 30 | 243 | 76 |
| 145 | 47 | 150 | 60 | 292 | 105 |
| 212 | 78 | 100 | 30 | 308 | 107 |
| 212 | 78 | 150 | 60 | 357 | 136 |

Table 3. *Comparison between the Schoenheimer-Sperry and the chromatographic methods*

(In the Schoenheimer-Sperry method, total and free values are obtained by estimation, and ester by difference. In the chromatographic method, ester and free values are obtained by estimation, and total by summation.)

| Schoenheimer-Sperry. Cholesterol (mg./100 ml.) | | | Chromatographic. Cholesterol (mg./100 ml.) | | |
|---|---|---|---|---|---|
| Total | Ester | Free | Total | Ester | Free |
| 295 | 210 | 85 | 282 | 202 | 80 |
| 194 | 144 | 50 | 195 | 150 | 45 |
| 171 | 123 | 48 | 170 | 125 | 45 |
| 243 | 170 | 73 | 238 | 168 | 70 |
| 290 | 215 | 75 | 290 | 210 | 80 |
| 182 | 132 | 50 | 185 | 130 | 55 |
| 141 | 100 | 41 | 150 | 105 | 45 |
| 162 | 113 | 49 | 158 | 111 | 47 |
| 157 | 114 | 43 | 151 | 113 | 38 |
| 214 | 159 | 55 | 210 | 156 | 54 |
| 180 | 131 | 49 | 187 | 135 | 52 |
| 265 | 193 | 72 | 263 | 188 | 75 |
| 430 | 235 | 195 | 432 | 228 | 204 |
| 215 | 155 | 60 | 224 | 160 | 64 |
| 130 | 98 | 32 | 132 | 102 | 30 |

In many cases after applying the plasma extract to the column, a small residue, insoluble in light petroleum, was left behind. This was neglected as it was found to give no colour in the Liebermann-Burchard reaction. A narrow, yellow band was usually formed on the surface of the alumina, but this was not eluted by either solvent, all eluates being colourless.

In all routine experiments fractions of 20 ml., followed by two of 10 ml. each, were collected with each eluent. The final 10 ml. fraction was always a blank containing no chromogenic material. Thus even with plasma of abnormally high cholesterol content no ester can be carried into the benzene fraction and all the material is recovered.

*Reproducibility of results.* Twelve separate extracts of the same plasma were prepared, and the free and combined cholesterol determined as above. The average value for ester cholesterol was found to be 153 mg./100 ml. with a range of 143–162 mg./100 ml. and for free cholesterol 51 mg./100 ml. with a range of 45–57 mg./100 ml.

*Comparison of the present method with the Schoenheimer-Sperry method.* The applicability of this method for routine clinical estimation of plasma cholesterol was checked by comparing results obtained by the Schoenheimer-Sperry method and by chromatography using the same plasma (Table 3). The authors are grateful to Dr G. S. Boyd of this Department for allowing them to quote his results for the Schoenheimer-Sperry method and for the supply of plasma.

Although the accuracy of the present method is not as great as that of the Schoenheimer-Sperry method (1–2%), it would appear to be satisfactory for most clinical purposes.

The mean of the differences between the two methods is 4 mg./100 ml. in the case of total, ester, and free cholesterol; the ranges of the differences are 1–13, 1–8 and 1–9 mg., respectively.

## SUMMARY

1. The chromatographic separation of cholesterol from its esters using alumina and a solvent system of light petroleum followed by benzene has been achieved.

2. It has been shown that esters of cholesterol may be estimated directly by the Liebermann-Burchard reaction.

3. On the basis of the above, a method has been developed for the direct determination of free and combined cholesterol in blood plasma.

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