Spectrophotometric Determination of Cholesterol in Wool Fat Using the Zak Reaction

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In an effort to overcome some of the shortcomings of previous methods for determination of cholesterol in wool fat, the Zak reaction has been studied. A procedure is presented in which cholesterol is separated as its digitonide with subsequent development of color by the addition of ferric ammonium chloride and sulfuric acid. The method is reproducible and appears to be superior to other methods now in use.

THE spectrophotometric methods currently in use for the determination of cholesterol in wool fat are for the most part based on the Liebermann-Burchard reaction (1–3). The use of this reaction as a quantitative measure of cholesterol, however, leaves much to be desired due to the dependence of the reaction on temperature, time of color development, and stability of the reagent. The ferric chloride reaction proposed by Zlatkis, Zak, and Boyle (4) for the determination of cholesterol has been shown to be a reliable method for determination of blood cholesterol. Later publications by Zak (5–7) have set forth improvements in the original procedure.

The color produced by the Zak reagent with cholesterol has been found to be reproducible without the necessity for temperature control. The color reaches a maximum in about 10 minutes and is sufficiently stable to be read in the period of 10–50 minutes after mixing. The reagent, composed of ferric ammonium chloride ($FeCl_3 \cdot 2NH_4Cl \cdot H_2O$) in 80% acetic acid is stable for periods up to six months. This new method has, therefore, overcome most of the troublesome aspects of the procedure in which the Liebermann-Burchard reaction is used.

It was the purpose of this research to determine if the Zak reaction could be utilized in the analysis of wool fat and wool fat-containing products for cholesterol content. It appears to the authors that the reaction can be used for this purpose.

EXPERIMENTAL

Equipment.—The Beckman DK-2 recording spectrophotometer was used for determination of the various spectra. The routine colorimetric analyses were run on a Coleman Universal spectrophotometer, using square cuvettes of 1.3 cm. light path.

Materials.—Cholesterol U.S.P.,[1] after crystallization from alcohol-water and drying at 80°, had m.p. 148–149°, $[\alpha]_{25}^{D}$ ($CHCl_3$) −38.4°. The lanosterol[2] used had a m.p. of 137-138°. Wool fat U.S.P., wool fat unsaponifiable, and a liquid wool fat fraction were used in the sample analyses.[3] All other materials used were analytical reagent grade, except ethanol and potassium hydroxide, which were U.S.P., and acetone and aluminum chloride, which were N.F.

Preparation of Solutions.—Stock solutions for use in the experimental procedures were accurately prepared as shown in Table I.

TABLE I.—PREPARATION OF SOLUTIONS

Solute	Gm./100 ml.	Solvent
Ferric ammon. chlor.	2.12	80% Acetic acid
Digitonin	1.00	50% Ethanol
Cholesterol	0.20–0.30	Ethanol
Lanosterol	0.10	Ethanol
Wool fat unsaponifiable	0.20	Ethanol
Wool fat solution I[a]	0.15–0.40	20% Dioxane, 20% acetone in ethanol
Wool fat solution II[a]	0.050	Acetic acid

[a] *Wool fat or liquid wool fat fraction. Heat was necessary to bring about solution. Solution I was used for saponification and precipitation; solution II was used for direct color analysis.*

Procedure: Preliminary Saponification.—An amount of wool fat solution I (Table I) equivalent to 8–12 mg. of wool fat was placed in a 15-ml. centrifuge tube and ethanol added to make 5 ml. (if necessary). To the tube was added 0.2 ml. of 50% aqueous potassium hydroxide and one drop of phenolphthalein T.S. The tube was boiled in a water bath for 5 minutes, loosely stoppered, and heated on a sand bath in an oven at 65° for 3 hours. At the end of the heating period, 0.5 ml. of water was added and the mixture was neutralized by dropwise addition of 10% hydrochloric acid. This solution was then treated with digitonin as further described.

Digitonin Precipitation.—The digitonin precipitation was carried out on the neutralized solution resulting from the saponification contained in a 15-ml. centrifuge tube. Where saponification was not necessary, an amount of stock solution (Table I) equivalent to 2–3 mg. of cholesterol or 6–8 mg. of wool fat unsaponifiable was placed in 15-ml. centrifuge tubes and ethanol added to make 5 ml. (if needed).

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[1] Obtained from American Cholesterol Products Inc., Edison, N. J.

[2] Obtained from Wed. D. S. van Schuppen and Zoon, Holland, through the courtesy of N. I. Malmstrom Co., Brooklyn, N. Y.

[3] Obtained from the Malmstrom Chemical Corp., Newark, N. J.

Two drops of 30% aqueous aluminum chloride and 1 ml. of 1% digitonin solution were added to each tube. The tube was gently boiled in a water bath for 5 minutes and then allowed to stand at 20° for 2 hours. The mixture was centrifuged at 2,800 r.p.m. for 5 minutes and the supernatant liquid decanted. The precipitate was washed twice with 5 ml. of acetone, the precipitate being broken up each time with a fine glass rod, and then centrifuged. After the final spinning, the supernatant liquid was decanted and the precipitate allowed to dry. The residue was then dissolved in warm acetic acid and brought to volume in a 10-ml. volumetric flask.

Colorimetric Analysis.—For colorimetric analysis, 1 ml. of the acetic acid solution of the digitonide was used. The stock solutions (Table I) were diluted with acetic acid so that they contained 0.2–0.3 mg. of cholesterol or lanosterol or 0.4–0.6 mg. of wool fat unsaponifiable per ml.; 1 ml. of each was used for colorimetric analysis. Three milliliters of wool fat solution II (Table I) was used for analysis.

The specified quantity of the respective solutions was pipetted into 18 × 150-mm. tubes and acetic acid added from a buret to make 6 ml.; 0.40 ml. of ferric ammonium chloride solution was added, and the contents mixed. Four milliliters of concentrated sulfuric acid was added slowly down the side of the tube to form a layer under the acetic acid solution. The contents were then mixed thoroughly on a Vortex Jr. mixer and allowed to cool for at least 10 minutes. The absorbance of the solution at 560 mμ was taken in the period of 10–25 minutes after adding sulfuric acid. The blank consisted of 6 ml. of acetic acid, 0.4 ml. of ferric ammonium chloride, and 4 ml. of sulfuric acid, which had been mixed as before.

DISCUSSION

Ideally, it would be desirable to determine the amount of cholesterol directly, without prior treatment or separation of the cholesterol from wool fat. This would necessitate the determination of the effect of the reagent on the other constituents of wool fat which might give a color with the reagent. Lanosterol, which is the chief component of the triterpene fraction of wool fat and reported to be present in wool fat to the extent of about 15% (3), was used as a measure of this effect. On determination of the spectra of lanosterol and cholesterol (Fig. 1), it was found that a maximum of absorbance occurs for lanosterol at 490 mμ. From this plot of the absorptivities it is seen that there would be considerable interference of one constituent with the other at that wavelength. This is a situation which was found to make precise analysis of 1:1 mixtures of these components difficult to obtain.

Since in wool fat most of the cholesterol is present as fatty acid esters, the effect of this esterification would also have to be considered. Assous and Girard (8) have shown that the fatty acid esters of cholesterol do not exhibit the same rate of color development as does free cholesterol. These workers added the samples for analysis and the reagent to previously mixed and cooled acids, and allowed the reaction to take place at room temperature. Under these conditions the fatty acid esters of cholesterol did not develop any appreciable color in 1 hour, while the color developed by free cholesterol became constant after about 30 minutes.

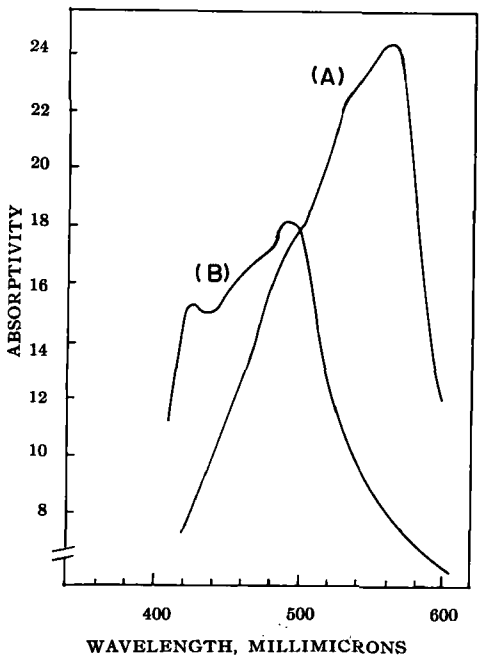

Fig. 1.—The spectra of cholesterol and lanosterol on treatment with the Zak reagent. *A*, cholesterol; *B*, lanosterol.

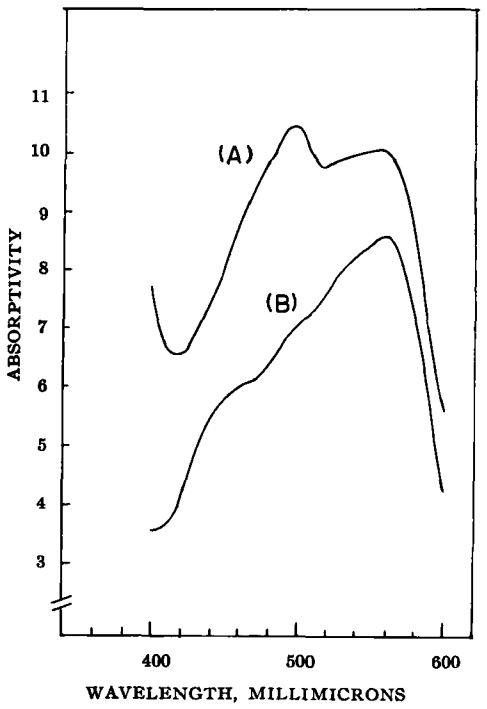

Fig. 2.—The spectra of wool fat unsaponifiable reacted directly with Zak reagent and after precipitation, with digitonin. The absorptivities were calculated using the concentration of wool fat unsaponifiable in the color solutions. *A*, reacted directly; *B*, after digitonin precipitation.

In the procedure used in the present work, elevation of temperature takes place on mixing the acetic acid solution of the cholesterol and reagent with sulfuric acid. On studying the stability of the color complex under the conditions used here, it was found that the absorbance of the color complex of untreated wool fat (containing the fatty acid esters) reached a maximum in about 60–70 minutes, while the color complex of cholesterol or cholesterol digitonide reached a maximum in about 10 minutes and remained the same or decreased slightly during the period of 1 hour after addition of the acid. This indicates that the fatty acid esters of cholesterol also react more slowly under the present conditions than does cholesterol. This is contrary to the situation with the Liebermann-Burchard reaction, in which the esters of cholesterol react more rapidly.

In sample analyses carried out on wool fat directly, the amount of cholesterol found was somewhat less than that found when wool fat was first saponified and the free cholesterol precipitated by digitonin before color development (Table II). This may be partially explained by the difference in the rate of reaction of the fatty acid esters of cholesterol, since more compatible results were obtained when the wool fat unsaponifiable fraction was analyzed by direct color analysis or by the precipitation technique (Table II).

From a practical standpoint also, it would be desirable to have a technique which would be useful in the determination of the amount of wool fat in pharmaceutical and cosmetic products. It was concluded that the best way to separate the color-producing moiety from such mixtures was by saponification and precipitation of the free cholesterol with digitonin, before color development. The saponification procedure suggested by Luddy, *et al.* (1), was used and the saponified mixture was treated directly with digitonin to precipitate the cholesterol.

It was found that lanosterol did not give a precipitate with digitonin under the conditions utilized here and so it was not necessary to calculate for lanosterol in this procedure. From an examination of the spectra of wool fat unsaponifiable, treated and untreated with digitonin (Fig. 2) it appears that there is no appreciable increase in the absorption of the treated sample at 490 mμ where a peak would be expected if there were lanosterol present.

Cholesterol digitonide was shown to have a slightly higher absorptivity than free cholesterol when treated with the iron reagent (Fig. 3). This difference can probably be accounted for by the absorbance of digitonin when treated with the reagent, since as shown by the spectrum of digitonin, there is a small amount of absorbance at the primary wavelength of cholesterol, 560 mμ.

The optimum ratio of sulfuric acid to acetic acid (2:3) in the reaction mixtures was determined by Zak (9). This ratio appears to be necessary for the proper formation of the color complex due to the amount of heat liberated on dilution of the sulfuric acid. Immediately after mixing the solutions, an amber color is observed, which changes through violet to a reddish-violet color after about 5 minutes. In time studies on the color complexes of both cholesterol and its digitonide, there was noted a decrease in absorbance of from 2–3% during the period of 10–50 minutes after mixing the solutions.

Brown and co-workers (10) have investigated the use of several agents to facilitate the flocculation of cholesterol digitonide in blood serum studies. These workers reported that aluminum chloride was very effective for this purpose. In the studies on wool fat, it was found that separation and washing of the digitonide precipitate was more easily carried out in the presence of aluminum chloride.

The viscosity of the solution resulting from the mixture of sulfuric acid and acetic acid was a source of some difficulty, particularly with respect to adequate mixing of the two acids. The use of the Vortex Jr. mixer seemed to solve this problem to a considerable extent. Occasionally in the mixing process air bubbles would become entrapped in the solution, which would usually be dissipated by the time the solution had cooled.

TABLE II.—COMPARISON OF RESULTS OBTAINED ON DETERMINATION OF CHOLESTEROL BY DIRECT TREATMENT WITH THE ZAK REAGENT AND AFTER SAPONIFICATION AND PRECIPITATION WITH DIGITONIN

Sample	Direct Method[a]	Sapon. and Pptn. Method[b]
IIIb-L	13.5[c]	19.1[c]
IVb-W	11.6	20.7
W.F. unsap.	32.5	35.4

[a] Acetic acid solutions of wool fat or wool fat unsaponifiable were analyzed colorimetrically. [b] Acetic acid solutions of the digitonide obtained from wool fat unsaponifiable or wool fat after saponification and precipitation were analyzed. [c] Gm. of cholesterol found per 100 Gm. of wool fat or wool fat unsaponifiable taken for analysis.

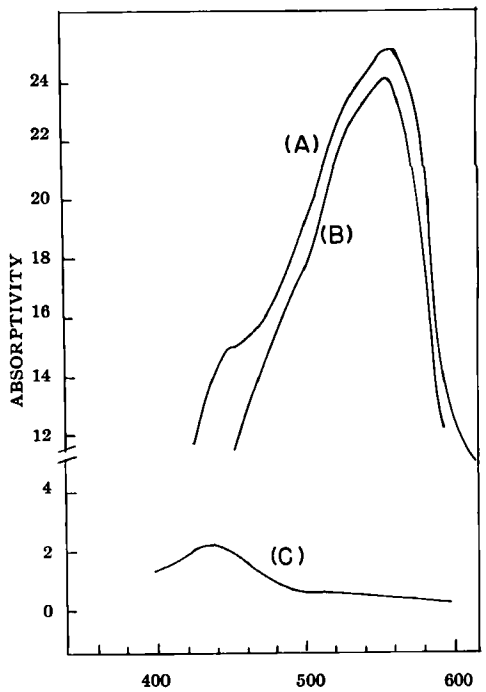

Fig. 3.—The spectra of cholesterol, cholesterol digitonide, and digitonin after treatment with the Zak reagent. The absorptivities were calculated using the concentration of the respective substances in the color solutions. *A*, cholesterol digitonide; *B*, cholesterol; *C*, digitonin.

RESULTS

The preliminary tests performed showed that the color complex formed with both cholesterol and its digitonide obeyed Beer's law (Fig. 4). This shows also the slightly greater absorbance of cholesterol digitonide than free cholesterol, as mentioned earlier.

The precision of the whole analytical procedure for determination of cholesterol in wool fat and its derivatives is shown in Table III. The difference of cholesterol content for different lots of wool fat or wool fat derivatives is also shown. However, within each lot of wool fat tested, good reproducibility was obtained with a maximum deviation from the mean of 6.8%. The average difference from the mean of all lots tested was 2.7% with a standard deviation from the mean of 3.3%. The calculations for the determination of cholesterol content were done by the use of the absorptivity of cholesterol digitonide, which had previously been determined.

The procedure as described requires some 6–7 hours to run, but does not require constant attention during the entire period. The periods of time given are for the most part minimum periods which were found to be necessary for completion of the respective reactions. The lapse of time between the addition of the sulfuric acid and the reading of the absorbance of the solution is not critical, since the readings in the analyses are taken in the period of 10–25 minutes after mixing the solutions.

TABLE III.—RESULTS OF ANALYSIS OF WOOL FAT AND WOOL FAT DERIVATIVES

Sample of Wool Fat	Chol. Found[a,b]	Mean Chol. Found[a]	% Difference from Mean
Ia-L	16.5	16.4	0.6
	16.5		0.6
	16.5		0.6
	16.2		1.2
Ib-L	16.0	16.4	2.4
	16.3		0.6
	16.8		2.4
IIa-L	19.6	18.9	3.7
	18.4		2.6
	18.7		1.0
IIb-L	18.0	17.4	3.4
	16.3		6.3
	16.9		2.9
	17.1		1.7
	18.5		6.3
IIIa-L	19.8	19.2	3.1
	18.5		3.6
IIIb-L	19.6	19.1	2.6
	20.4		6.8
	18.2		4.7
	18.2		4.7
IVa-W	21.7	20.8	4.3
	20.9		0.5
	20.2		2.9
	19.9		4.3
	21.3		2.4
	20.5		1.4
IVb-W	20.6	20.7	0.5
	21.0		1.4
	21.0		1.4
	20.3		1.9
Av. difference from mean		2.7%	
S.D. from mean (N = 31)		3.3%	

[a] Gm. cholesterol found per 100 Gm. of wool fat taken for analysis. [b] Each number represents the average obtained from color analysis of duplicate samples of acetic acid solutions of the digitonide after saponification and precipitation.

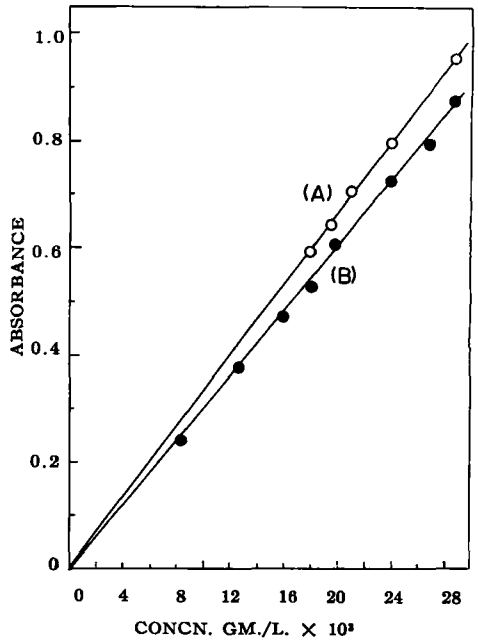

Fig. 4.—Beer's law plot of cholesterol and its digitonide using the Coleman spectrophotometer with 1.3-cm. cuvettes. *A*, cholesterol digitonide; *B*, cholesterol.

SUMMARY

A procedure is presented which is reproducible, which requires a minimum of control of conditions, and which constitutes a great improvement over previous methods in use. The reaction is quite sensitive; the analysis can be carried out using as little as 5 ml. of a mixture containing as little as 0.2% of wool fat. The reagent in use is stable for extended periods and no elaborate equipment is necessary. The method should lend itself to the analysis of many different types of pharmaceutical and cosmetic products containing wool fat or the many wool fat derivatives presently on the market.

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