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The Determination of Cholesterol and Coprosterol in Faecal Lipids

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Snog-Kjaer, Prange & Dam (1956) and Coleman & Baumann (1957) have demonstrated the microbial conversion of cholesterol into coprosterol in the mammalian large intestine. Thus there is need for the rapid and accurate simultaneous quantitative determination of cholesterol and coprosterol. This is attempted in the chromatographic procedure of Coleman, Wells & Baumann (1956), which is too time-consuming for routine determinations.

The present paper describes a procedure for such routine determinations of cholesterol and coprosterol in faeces, based on a method originally published by Brown, Zlatkis, Zak & Boyle (1954) and more recently modified by Zak, Luz & Fisher (1957), for the determination of serum cholesterol. Both the original method and that described below employ a colour reagent consisting of ferric chloride–acetic acid–concentrated sulphuric acid, which produces a coloured cholesterol derivative with an absorption peak at 560 mμ. In the modification here described the absorption is measured at 440 as well as at 560 mμ, since coprosterol forms an orange–red compound with an absorption peak at 440 mμ. The full colour development takes longer than for cholesterol and requires 35 min. to go to completion. It is stable for approximately half an hour. As faecal lipids contain interfering substances that must be removed, it was necessary to carry out the determinations on the digitonides.

For the determination of total sterols a saponification procedure becomes necessary and the method used was that described by Sperry & Webb (1950) with slight modifications.

EXPERIMENTAL AND RESULTS

Reagents and standard solutions

Solvents. Acetone (AnalaR); ethanol–water (1:1, v/v); acetic acid (Merck); aq. 30% (v/v) acetic acid; concentrated sulphuric acid (AnalaR); ethanolic 0·5N-KOH; 1% digitonin in ethanol–water (1:1, v/v) (stored in the dark); $FeCl_3,6H_2O$ (AnalaR) in acetic acid (70 mg./100 ml.; best prepared by dissolving an accurately weighed amount in the required volume of acetic acid. It is unstable in storage and must be prepared daily).

Cholesterol standard. Cholesterol, 0·100 mg./ml. (m.p. 147·5°, recrystallized from light petroleum, b.p. 50–60°), in acetone.

Coprosterol standard. Coprosterol, 0·200 mg./ml. (m.p. 101°; recrystallized from acetone at −10°), in acetone.

Procedures

Free sterols. The lipids are obtained from the minced tissues by two 8 hr. extractions with acetone (5 vol.) followed by one 24 hr. extraction with methanol–light petroleum (b.p. 50–60°) (1:10, v/v) in a Soxhlet extractor.

A portion (1 ml.) of acetone solution containing approx. 1 mg. of lipids is pipetted into a 15 ml. centrifuge tube and digitonin solution (2 ml.) is added. The mixture is stirred vigorously and warmed briefly in the water bath to aid flocculation of the precipitate. The tube is allowed to stand for 15 min. with occasional stirring. The solution is then centrifuged for 10 min. at 2500–3000 *g* and the liquid decanted. Ethanol–water (3 ml.; 1:1, v/v) is added and the precipitate dispersed thoroughly with a glass rod. It is then brought to the boil and allowed to cool for 10 min. This procedure removes occluded digitonin. After cooling, the tube is centrifuged as before, the liquid decanted and the tube drained upside down on a filter paper for 15 min. The precipitate is then dissolved in 3 ml. of $FeCl_3$ reagent at room temperature, as the coprosterol reaction is heat-sensitive. Complete solution is rapidly attained by vigorous stirring. Concentrated sulphuric acid (2 ml.) is then run down the side of the tube so as to form a bottom layer and the whole is finally mixed thoroughly and allowed to stand for 35 min. for the colour to develop fully. A blank, containing $FeCl_3$ reagent and H_2SO_4, is also prepared. The extinction (*E*) is measured, 35–45 min. after the addition of H_2SO_4, at 440 and 560 mμ in a suitable spectrophotometer (e.g. a Beckman model DU) in a 1 cm. cell. The weights of sterols are calculated as described below. It is practicable to carry out six to eight determinations simultaneously.

Total sterols. Sample (1 ml.) is pipetted into a centrifuge tube and the solvent largely removed by evaporation on a water bath. Ethanolic 0·5N-KOH (1 ml.) is added and the sample stirred with a glass rod until the lipids are completely dissolved. The tube is then immersed in a water bath at 38° for 30 min. At the end of this period the solution is neutralized to phenolphthalein with 30% acetic acid, 4–5 drops being required. The contents of the tube are then evaporated almost to dryness and taken up in 1 ml. of acetone. The solution may remain cloudy, owing to the presence of potassium salts, but these are subsequently removed when the digitonides are washed with ethanol–water (1:1, v/v). The determination is then continued as described for free sterols.

Standardization

Varying weights of cholesterol and coprosterol showed that digitonides obey Beer's law at 440 mμ and 440 and 560 mμ respectively. For the preparation of calibration curves solutions containing 0·050, 0·100, 0·150 and 0·200 mg. of cholesterol and 0·20, 0·40, 0·60, 0·80 and 1·00 mg. of coprosterol were evaporated to dryness in

15 ml. centrifuge tubes, redissolved in 1 ml. of acetone and treated as described for free sterols. The extinctions ($E_{1\,cm.}$) were measured at 440 and 560 mμ and plotted against the weights. The mean values of $E_{1\,cm.}$ for 0·10 mg. were found to be 0·3830 and 0·5980 for cholesterol and 0·1237 and 0·0867 for coprosterol at 440 and 560 mμ respectively.

Calculation. The equations used to calculate the weights of cholesterol and coprosterol are derived as follows:

x = cholesterol present (mg.);
y = coprosterol present (mg.).

Then $E_{560} = 5{\cdot}98x + 0{\cdot}867y,$
$E_{440} = 3{\cdot}83x + 1{\cdot}237y.$

Therefore $x = 0{\cdot}304\,E_{560} - 0{\cdot}2126\,E_{440},$
$y = 1{\cdot}467\,E_{440} - 0{\cdot}940\,E_{560}.$

Accuracy. The method was tested on mixtures of known composition and the recoveries were calculated. The mean recovery was 100·4% (S.D. ±2·80%) for cholesterol and 99·8% (S.D. ±3·41%) for coprosterol.

Interference by other faecal steroids

The method was also tested on faecal lipids from rats on a basal fat-free diet with a 10% corn-oil supplement. The absorption spectrum of this sample, treated as described above, is shown in Fig. 1. It is noted that in this mixture of cholesterol and coprosterol there is a peak at 550 mμ. This is due to a sharp fall in the extinction due to coprosterol above this wavelength, thus causing a decline in the combined extinctions at 560 mμ. Also shown is the spectrum of a synthetic mixture containing the calculated quantities of cholesterol and coprosterol (0·056 and 0·433 mg. respectively).

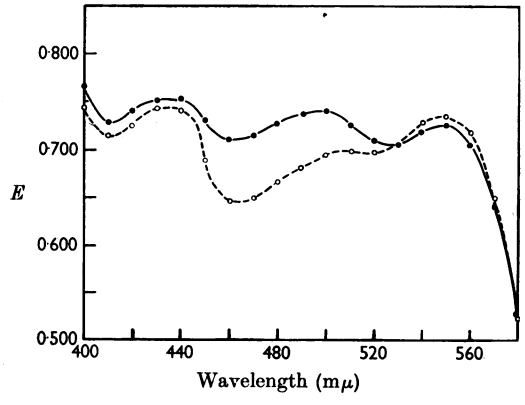

Fig. 1. Absorption spectra of faecal fat from rats on a basal fat-free diet with 10% corn-oil supplement (●) and of a synthetic mixture of the calculated amounts of cholesterol and coprosterol present (○).

Table 1. *Molecular extinction coefficient of the principal faecal sterols*

Substance	ε 440 mμ	ε 560 mμ
Cholesterol	7295	11580
Coprosterol	2386	1610
Δ^7-Cholestenol	0	0
7-Dehydrocholesterol	689	249
Cholestanol	1961	967
β-Sitosterol	5991	8064

The method was further tested for interference by other faecal sterols, namely Δ^7-cholestenol, 7-dehydrocholesterol and cholestanol as well as β-sitosterol, which may be introduced with the diet. The molecular extinctions of these sterols were determined by the above-described method (Table 1). The proportion of the total extinction was calculated for each sterol, based on the sterol composition found by Coleman *et al.* (1956). At 440 mμ it was found that cholesterol and coprosterol accounted for 96·9% of the total extinction, 7-dehydrocholesterol 0·1% and cholestanol 3·0%. At 560 mμ cholesterol and coprosterol contributed 98·3% and cholestanol 1·7% of the total extinction. The so-called 'sterol D' mentioned by these authors could not be tested.

It was found that 7-dehydrocholesterol was poorly precipitated and Δ^7-cholestenol not at all.

DISCUSSION

Although the method described above is based on that described by Zak *et al.* (1957) for the determination of serum cholesterol, a number of modifications, apart from the extension to coprosterol, have been made. Thus the determination of total as well as of free sterol is carried out via digitonide precipitation, a step necessary to eliminate interfering impurities. It appears advisable to incorporate this step into all determinations made by the method of Zak *et al.* (1957), since the present author's experience with depot fats, as well as faecal lipids, supports the conclusions reached by Rhodes (1959) that non-steroid impurities interfere with the method. In the determination of plasma cholesterol Zak *et al.* (1957) used 0·5 ml. of 1% digitonin solution. However, the weights of sterol determined in this procedure are considerably larger, and it was found necessary to increase the amount of digitonin used in order to maintain an adequate excess. The use of 2 ml. of digitonin solution produces a mixture of solvents consisting of equal parts of acetone, ethanol and water, in which the precipitation was carried out. The digitonide was brought to the boil in ethanol–water (1:1) to rid it of occluded digitonin. Digitonin gives a pale-orange compound with considerable absorption at 440 mμ when treated with ferric chloride reagent.

Since the method involves a difference of extinctions it is important to select the weight of sample so that the extinctions of neither sterol at 440 mμ is too low. The method is not considered suitable for the determination of less than 0·025 mg. of cholesterol and 0·075 mg. of coprosterol. Furthermore, it was found that optimum values for E_{440} and E_{560} are between 0·450 and 1·100. The sample quoted represents a typical determination, where 0·8765 mg. of lipid, containing 6·39 % of cholesterol and 49·40 % of coprosterol, had values of E_{440} 0·750 and E_{560} 0·707.

The difference in the spectra also reveals the presence of an unknown impurity with a peak at approx. 460 mμ, resulting in errors of 0·01 and 0·02 mg. for cholesterol and coprosterol respectively. Tests carried out on other faecal sterols (Table 1) show errors of 3·1 and 1·7 % for E_{440} and E_{560} respectively. With the faecal fat just described (fat-free diet with corn-oil supplement) the corrected extinctions should therefore be E_{440} 0·727 and E_{560} 0·695, and the recalculated proportions are therefore cholesterol 6·41 % and coprosterol 47·01 %, thus giving estimated errors of 0·31 % for cholesterol and 4·8 % for coprosterol. These seem to be almost solely attributable to cholestanol, which would appear in the determination chiefly as coprosterol.

Table 1 shows that the molecular extinction of β-sitosterol is relatively high. The effect of this on the accuracy of the method in cases where this compound is part of the diet is difficult to assess. Assuming the ingestion of 2 g. of corn oil (i.e. approx. 10 %) daily, the β-sitosterol intake would amount to 10 mg. From the work of Gould (1955), who estimated β-sitosterol absorption in the intestine to be one-tenth of that of cholesterol, and from the work of Cook & Thomson (1951) on intestinal absorption of cholesterol, it is estimated that rats could absorb 12 mg. of β-sitosterol/day. Furthermore Coleman & Baumann (1957) produced evidence that approx. 70 % of the β-sitosterol is hydrogenated in the intestine. These considerations make it appear likely that β-sitosterol will not interfere appreciably with the method unless the amounts administered are greatly in excess of those quoted above. Nevertheless, the possibility of error, particularly when faecal components other than those mentioned above are present, cannot be ruled out and the value of the method must then be reassessed.

SUMMARY

1. A method based on that of Zak *et al.* (1957) is described for the simultaneous quantitative determination of cholesterol and coprosterol, both free and total, in faecal lipids.

2. The method was tested on mixtures of known composition and it was found that the standard deviations, based on percentage recovery, were ±2·80 and ±3·41 % for cholesterol and coprosterol respectively, and the mean percentage recoveries were 100·4 and 99·8 %.

3. Tests carried out on faecal lipids indicate that other sterols normally present in faeces do not materially affect the results.

4. The method is suitable for determinations on batches of six to eight samples.

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5-Fluorouracil and Mucopeptide Biosynthesis by *Staphylococcus aureus*

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The accumulation of uridine mucopeptides has been shown to occur when some strains of *Staphylococcus aureus* are treated with penicillin (Park, 1952*a–c*; Park & Strominger, 1957; Strominger, 1957). In the strain examined, the most complicated of these compounds contained uridine diphosphate linked to one molecule each of 3-carboxyethylglucosamine (muramic acid), D-glutamic acid and L-lysine together with three molecules of alanine; two of the three alanine molecules were in