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Quantitative separation of radioactive sterols and bile acids in human faeces

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SYNOPSIS A method is described for the quantitative separation of radioactive bile acids and neutral sterols in human faeces on a potassium hydroxide-treated silicic acid column. Results obtained with faecal extracts containing metabolites derived from radioactive cholesterol, cholic acid, and chenodeoxycholic acid show that the efficiency of extraction is at least 96% and that mutual contamination between the bile-acid and sterol fractions is between 0·7 and 3·8%. The method gives better results than one based on separation of the faecal steroids into saponifiable and unsaponifiable material.

The separate determination of faecal neutral sterols and bile acids is relevant to many problems concerning the regulation of cholesterol metabolism in man. In studies in which tracers were not used (Gordon, Lewis, Eales, and Brock, 1957; Antonis and Bersohn, 1962), saponification of faecal lipid has been employed to resolve neutral and acidic components. The bile-acid fraction separated in this way may be contaminated with sterols, as the present study has shown; although this would not interfere with titrimetric measurement, it would invalidate radio-assay. Another approach has been to use three-stage counter-current distribution between 70% ethanol and petroleum ether (Rosenfeld and Hellman, 1962).

Recently, Spritz and Ahrens (1963) have used 15-stage counter-current distribution followed by column and thin-layer chromatography to isolate bile-acid and sterol fractions of purity suited to titrimetric or gravimetric determination. Hellström and Sjövall (1962) employed a cation exchange column, anion exchange chromatography on DEAE-Sephadex, and silicic acid chromatography, and then analysed bile acids by paper or gas chromatography. Bile acids have also been isolated on a Dowex 1 × 2 anion exchange column (Kuron and Tennent, 1961).

3α, 7α, 12α-Trihydroxycholanic acid (cholic acid) and 3α, 7α-dihydroxycholanic acid (chenodeoxycholic acid), the chief bile acids synthesized by the human liver, are much more polar than cholesterol. However, both acids are converted by bacterial action in the caecum and colon into numerous derivatives less polar than the parent acids, including deoxycholic, lithocholic (Rosenfeld and Hellman, 1962; Danielsson, Eneroth, Hellström, Lindstedt, and Sjövall, 1963), 3β,12α-dihydroxycholanic and isolithocholic acids (Danielsson *et al.*, 1963), several ketocholanic acids (Danielsson *et al.*, 1963), and possibly a less polar derivative of lithocholic acid (Bergström and Danielson, 1963). The presence of these derivatives of low polarity, together with mutual solubility effects in the complex lipid mixture extracted from faeces, may be responsible for the difficulty of separating bile acids from neutral sterols.

In developing the relatively simple method described in this paper, our aim was to obtain complete and quantitative separation of sterols and bile acids in a form suitable for radio-assay. We have tested the validity of the procedure in man by administering tritiated cholic and chenodeoxycholic acids and 26-^{14}C cholesterol, thus labelling the bile acids and sterols characteristic of human faeces. The method has been found satisfactory in the investigation of cholesterol metabolism in normal and hypercholesterolaemic subjects.

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MATERIALS AND METHODS

REAGENTS All solvents were of analytical grade. Diethyl ether and dioxane were purified by passage through alumina columns; alumina (Peter Spence & Sons Ltd., London) was activated at 120°C. for 15 hours. Chloroform was washed five times with 0·2 vol. of water, dried over $CaCl_2$, and distilled. The silicic acid was prepared as follows (Antonis, 1963): 100-mesh silicic acid (Mallinckrodt Chemical Works, New York City) was slurried five times with 10 vol. of distilled water, the coarse

particles being allowed to settle each time for about three minutes before the fine particles were decanted. The coarse particles, representing about 50% of the starting material, were transferred to a Buchner funnel, washed with 10 vol. of acetone, and then with 5 vol. of ether which was removed as completely as possible.

This silicic acid was treated with potassium hydroxide. In a 1-litre round-bottomed flask, 50 g. silicic acid was mixed with 200 ml. methanolic KOH 1·25% w/v, and the methanol removed in a rotary evaporator. Traces of methanol were displaced by washing the KOH-treated silicic acid in a Buchner funnel with acetone and ether. The material may be used for at least two months if stored in a stoppered container.

THIN-LAYER CHROMATOGRAPHY Two systems were used. In one, the solid phase was Kieselgel H (E. Merck AG, Darmstadt, Germany) slurried with 0·05 N-NaOH (15 g./45 ml.), and the moving phase was chloroform: methanol (77·5:22·5 v/v). The other system was that described by Eneroth (1963), in which the moving phase was diethyl oxalate: isopropanol (48:8 v/v). Cholic acid (Hopkin & Williams Ltd., Chadwell Heath, Essex), deoxycholic acid (L. Light & Co. Ltd., Colnbrook, Bucks), lithocholic acid (Steraloids Ltd., Croydon, Surrey), and cholesterol (recrystallized, Hopkin & Williams Ltd.) were used as markers.

RADIOACTIVE MATERIALS 4-^{14}C Cholesterol, specific activity 54·5 μc/mg., and 26-^{14}C cholesterol, specific activity 62·2 μc/mg., were obtained from the Radiochemical Centre, Amersham, Bucks. The cholesterol was prepared for intravenous injection by adding it to a sample of the recipient's serum, essentially by the method of Whereat and Staple (1960). Cholic acid and chenodeoxycholic acid (Steraloids Ltd.) were uniformly labelled with tritium by a modification of the Wilzbach procedure, freed from labile tritium by washing an ether solution of the acids with water, and purified by thin-layer chromatography in the two solvent systems, with pure samples of cholic and chenodeoxycholic acids as markers. After elution from the thin-layer chromatograms with acetone, both bile acids gave single titration peaks on reversed-phase partition column chromatography (Sjövall, 1953).

PROCEDURE A four-day collection of faeces is homogenized with ½ to 1 × its weight of water; an aliquot of 10 to 20 g. is weighed in a 250-ml. flask and extracted under reflux with 150 ml. acetone for four hours in a water-bath at 90°C. The solvent is filtered and the extraction repeated once with acetone and twice with 150 ml. chloroform. Fifteen g. of Dowex 50 W-X2, 50-100 mesh (Bio-Rad Laboratories, Richmond, California) is slurried in glass-distilled water and packed in a column of 1·2 cm. internal diameter. It is washed with 50 ml. N-HCl and then with distilled water until the effluent is neutral. The column is then washed with 50 ml. acetone : chloroform : water (50:50:1 v/v). The pooled extract is then passed through the column, on which part of the pigment is retained; the column is washed through with 20 ml. chloroform : acetone : water (50:50:1), and the whole eluate taken to dryness in a rotary evaporator. Two tenths of the eluate is saponified under reflux with 5 ml. N-NaOH in 80% v/v aqueous dioxane in a boiling water-bath; after 30 minutes, a further 5 ml. water is added, and saponification continued for 90 minutes. The cooled solution is acidified to *p*H 1 with N-HCl and extracted three times with 2 vol. of ether. The pooled ether extract is taken to dryness in a rotary evaporator, complete removal of water being ensured by adding 5 ml. acetone to the residue and re-evaporating.

For chromatography, 3 g. silicic acid-KOH, slurried in ether and packed in a column 8 mm. in diameter, is washed with 20 ml. acetone, then 20 ml. ether, and finally 20 ml. 70% v/v ether in hexane. The saponified faecal lipid is dissolved in 1 ml. chloroform, and 0·5 ml. is pipetted on to the column. Neutral sterols are eluted with 120 ml. 70% ether in hexane, and bile acids with 100 ml. 4% formic acid in methanol v/v. The evaporated residue of the second fraction is dissolved in 100 ml. chloroform and washed twice with 5 ml. 0·02 N-HCl. The chloroform is then taken to dryness.

The sterol and bile-acid residues are taken up in 4 ml. ethanol, and 2 ml. is transferred to counting vials for radio-assay in a Packard Tri-Carb liquid scintillation spectrometer, with 10 ml. toluene containing 0·4% diphenyloxazole and 0·01% 1,4-bis-(2-(5-phenyl-oxazolyl)-benzene. Internal standards of 4-^{14}C cholesterol or U-^{3}T chenodeoxycholic acid, containing about 50,000 c.p.m., are used to correct for quenching.

RESULTS

RECOVERY OF LABELLED BILE ACIDS AND STEROLS FROM FAECES The recovery of endogenous bile acids and sterols from faeces cannot be estimated satisfactorily by measuring the recovery of radioactive bile acids and sterols added to faeces *in vitro*, because endogenous steroids may be bound to insoluble residues in faeces. It has recently been shown, for instance, that lithocholic acid in a faecal suspension, unlike deoxycholic acid, sediments in the ultracentrifuge and may be intrabacterial (Norman and Shorb, 1962). In order to estimate the losses which occur during the extraction, we have therefore used faeces obtained from hospital in-patients, four to 30 days after intravenous injection of 4-^{14}C cholesterol, 80 μc, or after oral administration of 26-^{14}C cholesterol, 15 μc dissolved in olive oil. In the former, the endogenous neutral sterols and bile acids were labelled; in the latter, the ^{14}C was almost entirely in the neutral sterols; bile acids were not labelled because of oxidative removal of the radioactive carbon atom. Losses in the initial extraction and in the subsequent analysis of the faecal extract were measured separately.

For estimation of the efficiency of the initial extraction, the faeces were extracted with acetone and chloroform by the standard procedure and the radioactivity in the extract measured. The faecal

residue was then digested in 5-N aqueous NaOH at 100°C. for five hours, acidified, and extracted three times with chloroform. The activity in the pooled chloroform extracts were measured using an internal standard to correct for quenching due to pigment. An average of 0·4% of the total activity remained in the residue after the initial extraction (Table I).

TABLE I

RECOVERY OF RADIOACTIVE ENDOGENOUS STEROIDS BY ACETONE/CHLOROFORM EXTRACTION OF FAECES

Sterol Administered	*Activity (d.p.m.)*		*Percentage of Total Recovered in Acetone/ Chloroform*
	Residue	*Acetone/ Chloroform*	
4-^{14}C cholesterol	0	68,984	100·0
4-^{14}C cholesterol	0	43,843	100·0
4-^{14}C cholesterol	30	30,230	99·9
4-^{14}C cholesterol	55	15,790	99·6
4-^{14}C cholesterol	303	31,030	99·0
4-^{14}C cholesterol	0	21,998	100·0
4-^{14}C cholesterol	248	21,042	98·8
26-^{14}C cholesterol	215	99,167	99·8
		Mean:	99·6

Losses during the remainder of the procedure were estimated from the radioactivity recovered when samples of initial extracts, containing known amounts of activity, were submitted to the whole analytical procedure. The average loss in five experiments was 3·5% (Table II).

TABLE II

RECOVERY OF RADIOACTIVE ENDOGENOUS STEROIDS AFTER CHROMATOGRAPHY ON DOWEX AND SILICIC ACID COLUMNS

Sterol Administered	*Activity (d.p.m.)*		*Percentage Recovered*
	Faecal Extract	*Sterol Plus Bile Acid Fractions*	
4-^{14}C cholesterol	68,350	67,920	99·4
4-^{14}C cholesterol	131,170	122,290	93·2
4-^{14}C cholesterol	57,360	58,226	101·5
4-^{14}C cholesterol	16,440	14,520	88·3
26-^{14}C cholesterol	15,070	15,068	100·0
		Mean:	96·5

SEPARATION OF BILE ACIDS AND STEROLS The efficiency with which bile acids and neutral sterols are separated by this procedure was tested by analysing faeces obtained from six hospital in-patients, all without evidence of disease of the gastro-intestinal or biliary tract, given radioactive bile acids or cholesterol by mouth. Three of these patients had ^{3}H cholic acid (60 μc), two had ^{3}H chenodeoxycholic acid (60 μc) and one had 26-^{14}C cholesterol (15 μc). Faeces were collected on the fourth to sixth days and analysed as described above. Our data therefore represent recoveries on the one hand of the metabolites of the main bile acids formed by the human liver, and, on the other, of cholesterol and its C_{27} metabolites.

Table III shows the amount of activity in the non-polar and polar fractions of the faecal extracts from each of the six patients. Metabolites of cholic and chenodeoxycholic acids are eluted almost entirely in the polar fraction, contamination of the non-polar fraction being slightly greater with derivatives of chenodeoxycholic acid (2·36%) than with those of cholic acid (1·2%). Cholesterol and its C_{27} metabolites are eluted almost entirely in the non-polar fraction, 2·05% appearing in the polar fraction.

TABLE III

RADIOACTIVITY IN COLUMN ELUATES FROM FAECAL EXTRACTS OBTAINED FROM PATIENTS GIVEN LABELLED STEROIDS

Steroid Administered	*Activity in Fractions Eluted from Silicic Acid Column (d.p.m.)*		*Cross Contamination (%)*	
	70% Ether/ Hexane	*4% Formic Acid/ Methanol*		
^{3}H cholic acid	555	43,991	1·25[1]	
^{3}H cholic acid	320	43,197	0·74[1]	1·20 (mean)
^{3}H cholic acid	210	12,436	1·66[1]	
^{3}H cholic acid	2,151	188,900	1·13[1]	
^{3}H chenodeoxycholic acid	51	1,297	3·8[1]	
^{3}H chenodeoxycholic acid	473	26,260	1·77[1]	2·36 (mean)
^{3}H chenodeoxycholic acid	453	28,966	1·54[2]	
26-^{14}C cholesterol	21,510	406	1·85[2]	2·05 (mean)
26-^{14}C cholesterol	21,536	485	2·25[2]	

[1]Percentage of total count in sterol fraction
[2]Percentage of total count in bile acid fraction

THIN-LAYER CHROMATOGRAPHY OF THE BILE-ACID AND STEROL FRACTIONS In an attempt to characterize the main radioactive components of the two eluates from the silicic acid column, aliquots from the polar and non-polar fractions were analysed by thin-layer chromatography with the chloroform: methanol solvent system. After completion of the chromatography, 15-mm. bands of the dried silicic acid were scraped off the plates, eluted with ethanol and the radioactivity in the eluates assayed.

Figure 1 shows the distribution of activity in chromatograms from the faecal extracts obtained from three patients. In the polar fraction from the patient given ^{3}H cholic acid, most of the radioactivity was separated into two peaks, one close to the position of the deoxycholic acid marker and the other close to that of lithocholic acid; smaller amounts of activity were also present near the solvent front. Similarly, in the polar fraction from

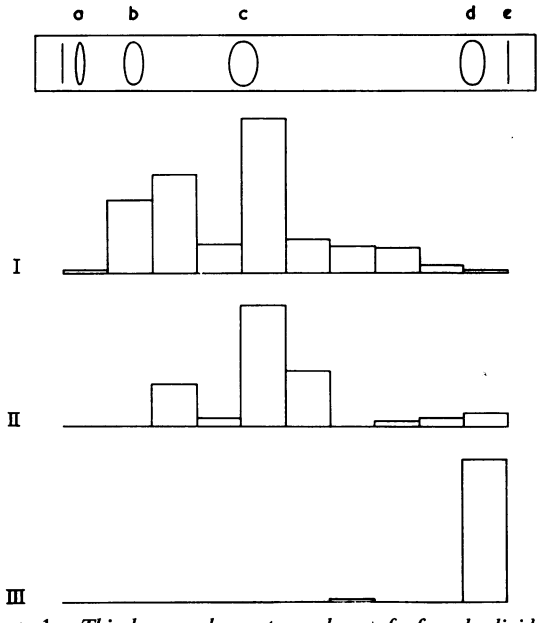

FIG. 1. *Thin-layer chromatography of faecal lipid, showing distribution of radioactivity. The solvent was chloroform : methanol (77·5 : 22·5 v/v), and Kieselgel H was used, slurried with 0·05 N-NaOH. I Polar fraction from subject given ^3H cholic acid. II Polar fraction from subject given ^3H chenodeoxycholic acid. III Non-polar fraction from subject given 26-^4C cholesterol.* a, *cholic acid;* b, *deoxycholic acid;* c, *lithocholic acid;* d, *cholesterol;* e, *solvent front.*

the patient given ^3H chenodeoxycholic acid, most of the activity was in two peaks, the smaller running slightly faster than deoxycholic acid and the larger corresponding to that of the lithocholic acid marker; a small amount of fast-running activity was again detected. In the non-polar fraction from the faeces of the patient given 26-^{14}C cholesterol, all the activity moved to a position corresponding to that of cholesterol or of material less polar than cholesterol.

SEPARATION OF BILE ACIDS AND STEROLS BY SAPONIFICATION The method was compared with one based on the separation of faecal lipids into saponifiable and unsaponifiable fractions, using the faecal extracts obtained from patients given ^3H cholic acid, ^3H chenodeoxycholic acid, and 26-^{14}C cholesterol (Table III). Aliquots of the lipid extract were saponified with N-NaOH in 80% ethanol under reflux at 90°C. for four hours. An equal volume of water was added and the unsaponifiable material extracted three times with 2 vol. hexane. The aqueous residue was acidified to *p*H 4 by adding N-HCl and extracted three times with 2 vol. ether.

The radioactivity in the unsaponifiable and saponifiable fractions from the faeces of the three patients is shown in Table IV. The saponifiable fraction contained 14·5% of cholesterol and its C_{27} derivatives, though contamination of the unsaponifiable fraction with bile-acid metabolites was not extensive.

TABLE IV

RADIOACTIVITY IN SAPONIFIABLE AND UNSAPONIFIABLE FRACTIONS FROM FAECAL EXTRACTS OBTAINED FROM PATIENTS GIVEN LABELLED STEROIDS

Steroid Given	*Activity (d.p.m.)*		*Cross Contamination (%)*
	Unsaponifiable	*Saponifiable*	
^3H cholic acid	327	17,105	1·9[1]
^3H cholic acid	219	13,751	1·6[1]
^3H chenodeoxycholic acid	334	7,703	4·2[1]
^3H chenodeoxycholic acid	322	7,604	4·1[1]
26-^{14}C cholesterol	46,508	7,903	14·5[2]

[1]Percentage of total count in unsaponifiable fraction.
[2]Percentage of total count in saponifiable fraction.

DISCUSSION

Loss of endogenous faecal steroids during the whole procedure, including extraction of the faeces and chromatographic separation of the extract, is on the average 4% (Tables I and II); this is probably a reflection of the relative simplicity of the method. Separation of bile-acid metabolites from neutral sterols is sufficiently complete for the purpose for which this method was designed, though 0·7 to 3·8% mutual contamination (Table III) might not be acceptable for other purposes. The efficiency of separation is much better than that of a method based simply on separation into saponifiable and unsaponifiable material, in which more than 10% of sterols may contaminate the saponifiable material (Table IV).

In the experiments to test the extent of contamination of one fraction by another, the faecal sterols were labelled by giving the patient 26-^{14}C cholesterol. Although no radioactivity would appear in the C_{24} bile acids, it is possible that C_{27} acids may be excreted in the faeces. It has been shown, for example, that radioactive trihydroxycoprostanic acid was excreted in the bile of a human subject given 26-^{14}C cholesterol intravenously (Staple and Rabinowitz, 1962). Traces of this substance, or of other C_{27} acids, could explain the observation that 2% of the radioactivity in the faecal extract of the patient given 26-^{14}C cholesterol was present in the polar fraction (Table III).

Thin-layer chromatography of the polar fractions from patients given radioactive bile acids shows that cholic and chenodeoxycholic acids are to some extent converted to metabolites of low polarity (Fig. 1). This is in agreement with the detailed observations of Danielsson *et al.* (1963), and such metabolites may account for the radioactivity in the non-polar fractions from patients given labelled bile acids (Table III).

It may be noted that ethanol is not used until the final stages of the procedure. It seemed possible that esterification of bile acids might otherwise occur, resulting in contamination of the neutral sterol fraction with bile-acid esters.

REFERENCES

Antonis, A. (1963). Personal communication.
——, and Bersohn, I. (1962). *Amer. J. clin. Nutr.*, **11**, 142.
Bergström, S., and Danielsson, H. (1963). *Biochem. Soc. Symp.*, **24**, 63.
Danielsson, H., Eneroth, P., Hellström, K., Lindstedt, S., and Sjövall, J. (1963). *J. biol. Chem.*, **238**, 2299.
Eneroth, P. (1963). *J. Lipid Res.*, **4**, 11.
Gordon, H., Lewis, B., Eales, L., and Brock, J. F. (1957). *Lancet*, **2**, 1299.
Hellström, K., and Sjövall, J. (1962). *J. Lipid Res.*, **3**, 397.
Kuron, G. W., and Tennent, D. M. (1961). *Fed. Proc.*, **20(1)**, 268.
Norman, A., and Shorb, M. S. (1962). *Proc. Soc. exp. Biol. (N.Y.)*, **110**, 552.
Rosenfeld, R. S., and Hellman, L. (1962). *Arch. Biochem.*, **97**, 406.
Sjövall, J. (1953). *Acta physiol. scand.*, **29**, 232.
Spritz, N., and Ahrens, E. H. Jr. (1963). In *Biochemical Problems of Lipids*, edited by A. C. Frazer, p. 66. Elsevier, Amsterdam.
Staple, E., and Rabinowitz, J. L. (1962). *Biochim. biophys. Acta (Amst.)*, **59**, 735.
Whereat, A. F., and Staple, E. (1960). *Arch. Biochem.*, **90**, 224.