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IMPROVED METHOD FOR THE DETERMINATION OF THE MAJOR NEUTRAL STEROIDS AND UNCONJUGATED BILE ACIDS IN HUMAN FAECES USING CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

An improved method has been developed for the determination of the major neutral steroids (cholesterol and 5β-cholestan-3β-ol) and unconjugated bile acids (deoxycholic acid and lithocholic acid) in human faeces, using capillary gas chromatography with flame ionization detection. The freeze-dried faecal sample was subjected to a two-stage Soxhlet extraction followed by an aqueous alkali–organic solvent partition step to separate neutral steroids from bile acids. The neutral steroids were analysed as their trimethylsilyl ether derivatives on an OV-1 capillary column. The bile acids were further purified on a Sep-Pak C_{18} cartridge and then fractionated on a Sep-Pak SIL cartridge. Unconjugated bile acids were analysed as their methyl ester-trimethylsilyl ether derivatives also on an OV-1 capillary column. Quantitation of neutral steroids and unconjugated bile acids was achieved by reference to appropriate internal standards, added to the faecal extract immediately after the Soxhlet extraction stage. The method is being used in a study of the effect of diet on the metabolic activity of human gut flora.

INTRODUCTION

The steroidal profile of human faeces may be altered in response to changes in dietary habit or the clinical manifestation of certain disease states of the large intestine, with the final contribution from different steroids representing an equilibrium between reabsorption, bacterial transformation and excretion [1].

Some of the bacterial metabolites of bile acids [2] and neutral steroids [2,3] are implicated in the aetiology of colon cancer [2–4], and the analysis of these

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components in faeces, and faecal secondary bile acids in particular, is considered to be a useful indicator of the susceptibility of individuals and populations to colon cancer [5].

As part of a study [6] on the effects of dietary supplements of plant fibre and animal fat on faecal bile acid and neutral steroid output from normal human volunteers, we have developed an improved method, reported in this paper, for the analysis of the major steroid components in faeces: cholesterol and one of its major bacterial metabolites, 5β-cholestan-3β-ol (coprostanol), and two secondary unconjugated bile acids, deoxycholic acid and lithocholic acid.

Three recently reported modifications to bile acid analysis are incorporated in our method: a mild organic solvent (Soxhlet) extraction technique for removing bile acids and neutral steroids from freeze-dried faeces [7]; the isolation and separation of unconjugated and conjugated bile acids using reversed-phase octadecylsilane bonded-silica (Sep-Pak C_{18}) and normal-phase silica (Sep-Pak SIL) cartridges [8]; and the use of capillary gas chromatography (GC) to separate and quantitate faecal neutral steroids and bile acids [9,10]. Also by adding internal standards at an early stage in the analysis, and adopting an aqueous alkali–organic solvent partition step to separate neutral steroids from bile acids, a more rapid and reliable quantitation of the major steroid components in faeces, compared with present methodology [7,11–13], is accomplished.

EXPERIMENTAL

Reference compounds

3α,12α-Dihydroxy-5β-cholanoic acid (deoxycholic acid, DCA), 3α-hydroxy-5β-cholanoic acid (lithocholic acid, LA), 3α,7β-dihydroxy-5β-cholanoic acid (ursodeoxycholic acid, UDCA), 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid, CDCA), 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid, CA), taurocholic acid (TCA), cholesterol, 5α-cholestane, coprostan-3-one and *n*-triacontane were obtained from Sigma (Poole, U.K.). 5β-Cholestan-3β-ol and 3β-hydroxy-5β-cholanoic acid were obtained from Steraloids (Croydon, U.K.) and 7α,12α-dihydroxy-5β-cholanoic acid was supplied by Cambridge Bioscience (Cambridge, U.K.). Stock solutions of the reference bile acids (1 mg/ml in methanol) and neutral steroids (1 mg/ml in *n*-hexane) were stored at 0°C. The purity of bile acids and steroids was checked by thin-layer chromatography (TLC) [7] and GC.

The following radiolabelled compounds (specific activity 50–60 mCi/mmol) were purchased from Amersham International (Amersham, U.K.): [carboxyl-^{14}C]deoxycholic acid, Na^+; [carboxyl-^{14}C]cholic acid, Na^+; [carboxyl-^{14}C]lithocholic acid; tauro[carbonyl-^{14}C]cholic acid, Na^+; [4-^{14}C]cholesterol; cholesteryl[1-^{14}C]oleate. The purity of the radiolabelled compounds was confirmed by TLC.

Reagents

Analar-grade solvents, bought from BDH or Fisons (Loughborough, U.K.), and hexamethyldisilazane and trimethylchlorosilane, supplied by Pierce & War-

riner (Chester, U.K.), were used as received. Cation-exchange resin (Dowex AG 50W-X4 H^+) was supplied by Bio-Rad Labs. (Watford, U.K.). Diazomethane, dissolved in diethyl ether–methanol (9:1), was freshly generated by the reaction of 70% aqueous potassium hydroxide with N-methyl-N-nitrosotoluenesulphonamide obtained from BDH [14].

Sep-Pak C_{18} cartridges and Sep-Pak SIL cartridges were obtained from Waters Assoc. (Cheshire, U.K.). Before use Sep-Pak C_{18} cartridges were washed with 5 ml of methanol followed by 10 ml of distilled water, and Sep-Pak SIL cartridges were washed with 10 ml of ethanol–chloroform–water (20:80:1).

All glassware was silanized with a solution of 5% dimethyldichlorosilane (BDH) in toluene to prevent surface adsorption of neutral steroids and bile acids [8].

Extraction procedure

Fresh stools were collected in the morning, from healthy male and female volunteers (aged 22–38 years) consuming a mixed free-choice diet, and stored at −20°C until analysed. A portion of the faecal sample was freeze-dried, powdered, and an aliquot (2 g) was extracted successively for 24-h periods with 350 ml of light petroleum (b.p. 40–60°C) and then with 350 ml of chloroform–methanol (1:1) in a standard Soxhlet apparatus [7,15]. After evaporation in vacuo the residue from the light petroleum extraction was redissolved in 10 ml of *n*-hexane, and the residue from the chloroform–methanol extraction was redissolved in 10 ml of methanol. Aliquots (5 ml) of the *n*-hexane and the methanolic faecal solutions, respectively, were combined, evaporated in vacuo, and the residue was redissolved in 5 ml of methanol, and stored, if necessary, at less than 5°C until the sample preparation stage.

Efficiency of the extraction procedure

Three male Sprague–Dawley rats (body weights 150–170 g) were given a single intraperitoneal (i.p.) injection of [4-^{14}C]cholesterol (2 μCi per animal) in aqueous ethanol. The animals were housed individually in all-glass metabolism cages (Jencons Scientific, Leighton Buzzard, U.K.) and allowed free access to food [R+M No. 1 (modified) diet; Special Diet Services, Whitham, U.K.] and domestic mains tap water. Faeces were collected at 48–120 h, freeze-dried, powdered, and the radioactivity determined by standard procedures. Aliquots (2 g) of pooled faeces were solvent extracted as described under *Extraction procedure* for either 6 or 24 h, and the radioactivity of the extracts and residual faeces was measured by standard procedures.

Sample preparation

A schematic flow diagram of the analytical procedure is shown in Fig. 1. Evaporations were conducted at $\leqslant 60$°C under a stream of nitrogen. Aliquots of stock solutions of the internal standards, 7α,12α-dihydroxy-5β-cholanoic acid (40 μg) and 5α-cholestane (160 μg), were added to an aliquot (0.2 ml) of the methanolic human faecal extract, and the sample was passed through Dowex 50W H^+ resin contained in a glass Pasteur pipette. After washing the resin with 3 ml of methanol, the combined eluates were evaporated, and the residue was dissolved in 1

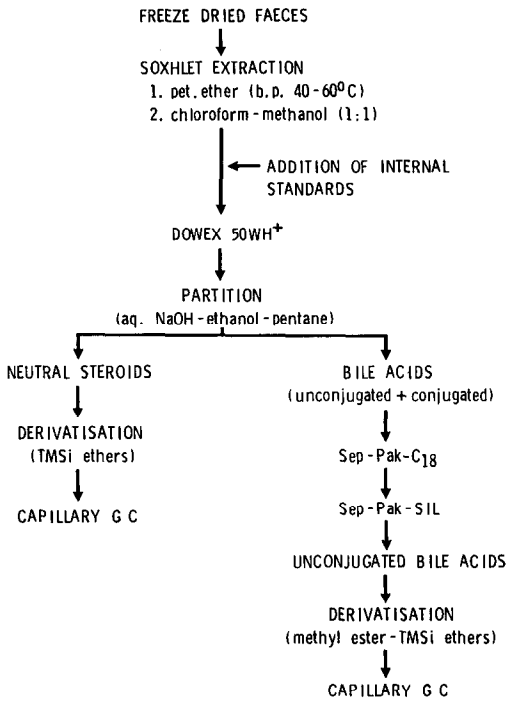

Fig. 1. Schematic flow diagram of the analytical procedure for the determination of the major neutral steroids and unconjugated bile acids in faeces.

ml of ethanol–water (7:3) and 0.2 ml of 2 *M* sodium hydroxide by sonication for 2 min.

Neutral faecal steroids and the added internal standard, 5α-cholestane, were extracted immediately from this solution into *n*-pentane (1 ml, three times) by vortex-mixing and centrifugation, and the combined pentane extracts were evaporated prior to derivatisation and GC analysis. The alkaline aqueous ethanolic extract containing unconjugated and conjugated bile acids and the added internal standard, 7α,12α-dihydroxy-5β-cholanoic acid, was neutralised with 0.2 ml of 2 *M* hydrochloric acid and evaporated. The residue was redissolved in 0.2 ml of methanol and 2 ml of 0.01 *M* hydrochloric acid by sonication for 2 min, and applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with 2 ml of 0.01 *M* hydrochloric acid, and the bile acids were eluted with 2.5 ml of methanol, twice. The methanolic eluate, collected at a flow-rate of approximately 10 ml/min, was evaporated. The residue, dissolved in three 1-ml volumes of ethanol–chloroform–water (20:80:1), was applied to a Sep-Pak SIL cartridge at 4°C. Unconjugated bile acids were removed from the cartridge by further elution with 5.5 ml of ethanol–chloroform–water–acetic acid (20:80:1:0.02) as described by Street et al. [8]. The two fractions were combined, evaporated, derivatised and analysed by GC.

Recovery

A human faecal extract, spiked with a radiolabelled neutral steroid, unconjugated or conjugated bile acid, was subjected to the solvent partition step, the Sep-

Pak C_{18} purification and the Sep-Pak SIL fractionation procedures, to check recoveries at each stage of the sample preparation (and confirm the separation of the unconjugated and conjugated bile acid fractions). Amounts of radioactivity in the eluates were determined by liquid scintillation counting.

Derivatisation

Neutral steroids were analysed as their trimethylsilyl (TMS) ether derivatives, and unconjugated bile acids as their methyl ester-TMS ether derivatives. Bile acids were esterified by treating the evaporated sample with 0.2 ml of the diazomethane reagent at 0°C for 30 min [16] followed by evaporation to dryness. TMS ether derivatives of neutral steroids and bile acid methyl esters were obtained by treating the evaporated sample with 100 μl of pyridine–hexamethyldisilazane–trimethylchlorosilane (9:3:1) for 30 min at 60°C [17]. Excess of reagents was removed at 60°C under a stream of nitrogen, and the derivatives were redissolved in a suitable volume of *n*-hexane before GC analysis.

Gas chromatography

GC was performed on a Carlo Erba HRGC 5160 Mega Series gas chromatograph, fitted with a 25 m$\times$0.32 mm OV-1 chemically bonded glass capillary column (film thickness 0.25 μm) prepared in the author's (E.B.) laboratory, and equipped with a flame ionization detector and a Packard auto-solid sampling device. Samples, dissolved in *n*-hexane, were transferred into silanized injection capsules (0.7-cm lengths of capillary tubing, closed at one end, volume capacity 3 μl), and the solvent was allowed to evaporate at room temperature. The injection capsules (up to 24) were loaded onto the carousel of the auto-solid sampling device, and GC analysis was performed with helium as carrier gas (flow-rate 3 ml/min measured at 200°C), and injection port and detector temperatures maintained at 280 and 300°C, respectively. For the analyses of the neutral steroids the column was maintained at 100°C for 1 min then programmed from 100 to 240°C at 30°C/min, from 240 to 310°C at 10°C/min and kept at 310°C for 15 min. The unconjugated bile acids were chromatographed under identical conditions except that the temperature rise from 240 to 310°C was reduced to 3°C/min. Peak-height measurements were recorded on a Spectra-Physics SP 4270 integrator. If required, unattended (overnight) GC analysis could be performed using this technique.

Quantitation

The neutral steroids cholesterol and 5β-cholestan-3β-ol, and the bile acids DCA and LA were quantitated by reference to standard calibration curves (peak-height ratio analyte/internal standard versus concentration). The curves were constructed, for each batch of faecal extracts analysed, from the GC analysis of mixtures of the two neutral steroids (80–320 μg), two bile acids (20–80 μg) and two internal standards, 5α-cholestane (160 μg) and 7α,12α-dihydroxy-5β-cholanoic acid (40 μg), which had been subjected previously to the sample preparation and derivatisation procedures.

TABLE I

PERCENTAGE RECOVERY OF RADIOLABELLED CHOLESTEROL AND BILE ACIDS ADDED TO FAECAL EXTRACTS, AFTER SOLVENT PARTITION, SEP-PAK C_{18} PURIFICATION AND SEP-PAK SIL FRACTIONATION

Results shown are the mean ± S.D. of six determinations.

Steroid	Percentage recovery (%)		
	After solvent partition*	After solvent partition and Sep-Pak C_{18} purification	After solvent partition, Sep-Pak C_{18} purification and Sep-Pak SIL fractionation
Cholesterol	106.0 ± 1.5	—	—
DCA	94.6 ± 0.4	94.4 ± 5.4	79.4 ± 6.7
LA	98.8 ± 0.3	95.6 ± 5.1	83.4 ± 1.3
CA	98.5 ± 0.2	97.8 ± 3.8	75.6 ± 2.1
TCA	99.4 ± 0.0	94.1 ± 2.6	0.6 ± 0.0**

*Cholesterol retained in the pentane phase; bile acids retained in the aqueous alkaline phase.
**After elution with 10 ml of ethanol–chloroform–water–acetic acid (20:80:3:5) followed by 6 ml of ethanol–chloroform–water–acetic acid (60:40:3:5) at 4°C [8] 83.8 ± 3.5% TCA was recovered.

Gas chromatography–mass spectrometry (GC–MS)

A VG 70-70F double-focusing mass spectrometer was interfaced to a Carlo Erba HRGC 5160 Mega Series gas chromatograph. GC column and operating conditions were as described under *Gas chromatography*. Samples were injected using a manual falling-needle type solid injector. Mass spectrometer operating conditions were: source temperature 220°C, accelerating voltage 4 kV, electron-impact energy 70 eV, trap current 200 μA. Mass spectra were recorded in the repetitive scan mode at a scan speed of 1 s/decade and processed by a VG 2035 data system.

RESULTS

Extraction efficiency

Soxhlet extractions for 24 h removed 95% of the radioactivity present in faeces collected from [4-^{14}C]cholesterol-dosed rats (three determinations) and left 5% of the original radioactivity in the faeces. Soxhlet extractions for 6 h removed 80% of the orginal faecal radioactivity.

Recovery

Table I shows the recovery of radiolabelled cholesterol and bile acids (DCA, LA, CA and TCA) at different stages of the sample preparation procedure.

Gas chromatography

Typical chromatograms for the neutral steroid (Fig. 2) and unconjugated bile acid (Fig. 3) components in a human faecal extract were obtained after subjecting each sample to the sample preparation and derivatisation procedures.

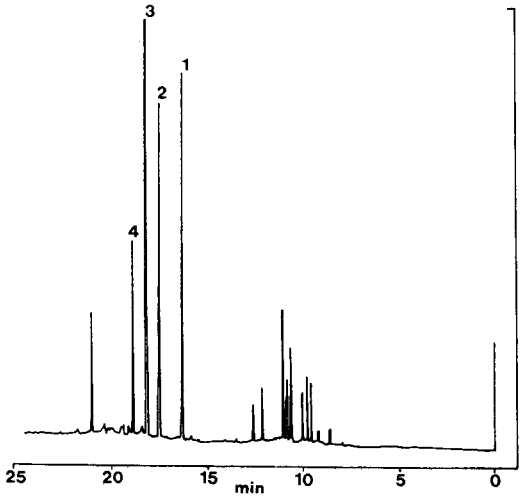

Fig. 2. Gas chromatogram of neutral steroids in a human faecal extract. For chromatographic conditions, see text. Peaks: 1 = 5α-cholestane (internal standard); 2 = triacontane (a GC marker); 3 = 5β-cholestan-3β-ol; 4 = cholesterol.

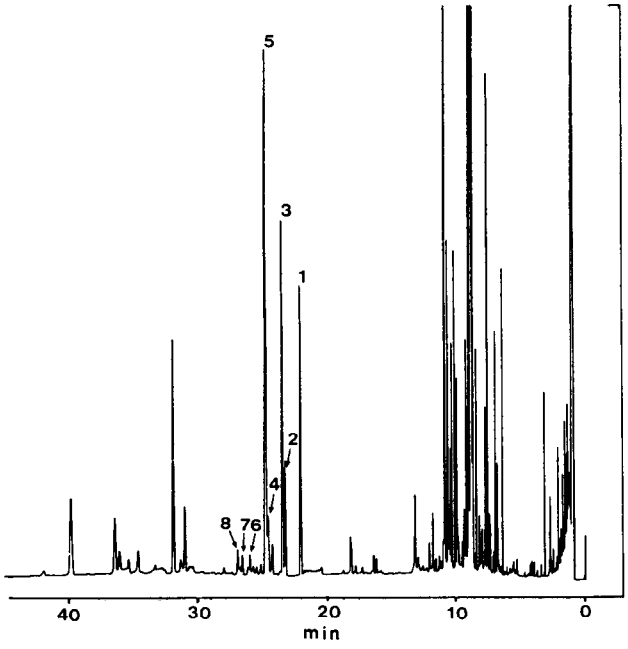

Fig. 3. Gas chromatogram of unconjugated bile acids in a human faecal extract. For chromatographic conditions, see text. Peaks: 1 = 7α,12α-dihydroxy-5β-cholanoic acid (internal standard); 2 = 3β-hydroxy-5β-cholanoic acid; 3 = lithocholic acid; 4 = 3β,12α-dihydroxy-5β-cholanoic acid; 5 = deoxycholic acid; 6 = chenodeoxycholic acid; 7 = cholic acid; 8 = ursodeoxycholic acid.

Linearity

Calibration curves were linear with minimum y-intercepts and correlation coefficients above 0.99 for the concentration ranges of the two neutral steroids

TABLE II

FAECAL CONCENTRATIONS OF THE MAJOR NEUTRAL STEROIDS AND UNCONJUGATED BILE ACIDS

Faeces were obtained in the morning from healthy subjects on a normal diet.

Subject No.	Concentration (mg/g of dried faeces)			
	Cholesterol	5β-Cholestan-3β-ol	DCA	LA
1	1.3	7.5	0.5	0.8
2	1.1	15.4	0.7	1.0
3	0.3	7.8	0.2	0.7
4	11.9	9.9	0.9	0.7
5	0.7	16.6	0.5	1.0
6	0.5	5.6	0.2	0.3
7	15.6	2.0	0.6	0.7

and two unconjugated bile acids studied. Typical linear regression equations were as follows: cholesterol, $y=3.9\cdot10^{-3}x+0.03$; 5β-cholestan-3β-ol, $y=4.8\cdot10^{-3}x+0.01$; DCA, $y=1.9\cdot10^{-2}x+0.027$; LA, $y=1.9\cdot10^{-2}x+0.004$.

Precision

Duplicate determinations of cholesterol, 5β-cholestan-3β-ol, DCA and LA in faecal extracts obtained from ten subjects were carried out using the analytical procedure (Fig. 1) and analysed by a one-way analysis of variance. Although significant variability was found for each of the four analytes between the ten subjects, the proportion of the total variation for the intra-subject determinations was less than 1%.

Coefficients of variation (calculated from the standard deviation of the intra-subject determinations and the overall mean of the twenty determinations carried out for each analyte) were 3.2% for cholesterol, 6.2% for 5β-cholestan-3β-ol, 7.7% for DCA and 2.4% for LA.

Identification and specificity

The major faecal neutral steroids (cholesterol and 5β-cholestan-3β-ol) and bile acids (DCA and LA) were identified by GC retention data and from a comparison of their complete mass spectra with those obtained from the GC–MS analysis of derivatised reference compounds. Repetitive scanning of eluted peaks was used to verify their homogeneity.

Some of the minor steroidal components in a control faecal sample were also characterised by GC–MS. Coprostan-3-one, 3β-hydroxy-5β-cholanoic acid, UDCA, CDCA and CA were identified by comparison of their mass spectra with those obtained from the GC–MS analysis of derivatised reference compounds. 3α,7β,12α-Trihydroxy-5β-cholanoic acid, 3β-12α-dihydroxy-5β-cholanoic acid and 3β (or 3α)-hydroxy-12-oxo-cholanoic acid were identified using the literature MS fragmentation patterns for these compounds [18].

Application

Using the analytical procedure (Fig. 1) the concentrations (mg/g of dried faeces) of cholesterol, 5β-cholestan-3β-ol, DCA and LA in control faeces obtained from seven human volunteers consuming their regular diet, were determined. The results are shown in Table II.

DISCUSSION

Our method for faecal steroid and unconjugated bile acid analysis employs a solvent extraction procedure [7,15] which minimises operator contact with the sample compared with the manipulations described for the extractions of wet faeces [10,19], and takes advantage of a Sep-Pak cartridge sample preparation technique [8] which is quicker than the commonly used anion-exchange (DEAP-Sephadex or DEAE-Sephadex) chromatography [7,10,12,13,18,20] for the isolation and fractionation of bile acids. By introducing the internal standards, 5α-cholestane [10] and 7α,12α-dihydroxy-5β-cholanoic acid [21], immediately after extracting the faeces, and adopting capillary [10,22] instead of packed column [7,12] GC analysis, a reliable and reproducible quantitation of the major neutral steroid and unconjugated bile acid components in faeces is achieved, without making assumptions about the efficiency of the sample preparation technique.

The effectiveness of the two-stage Soxhlet extraction step introduced by Owen and co-workers [7,15] for freeze-dried faeces was confirmed by extracting radiolabelled cholesterol and its bile acid metabolites [23] from faeces of rats dosed with [4-^{14}C]cholesterol. Extractions for two 24-h periods were required instead of the suggested two 6-h periods [7] to remove 95% of the faecal radioactivity. It was not possible to investigate alleged differences between extractions of rat and human faeces [11] using this radiochemical technique.

Soxhlet extraction of faeces with chloroform–methanol (1:1) in the presence of [carboxyl-^{14}C]lithocholic acid (or its methyl ester) for 24 h, followed by solvent partition and examination of the extracts for radioactivity, indicated that bile acid methyl esters were not produced as artefacts [10] in the neutral steroid fraction during the extraction and sample preparation stages.

The application of Sep-Pak cartridges for the isolation and fractionation of plasma bile acids [8] was extended in our work to faecal samples. Good recoveries of bile acids were obtained (Table I), and by incorporating an aqueous alkali–organic solvent partition stage neutral steroids were efficiently removed before the two Sep-Pak cartridge steps (Table I).

Although our method does not measure "saponifiable" faecal neutral steroid [10,24] and bile acid [10,20] conjugates, we checked that two representative (labelled) examples of these components, cholesteryl[1-^{14}C]oleate and ethyl[carboxyl-^{14}C]lithocholate, were not appreciably hydrolysed in brief contact with cold aqueous alkali during the partition stage (and thereby did not contribute to the final faecal neutral steroid and unconjugated bile acid concentrations).

GC–MS analysis confirmed the identity and homogeneity of the two major peaks in the chromatograms of the faecal neutral steroid (Fig. 2) and unconju-

TABLE III

FAECAL CONCENTRATIONS OF THE MAJOR NEUTRAL STEROIDS AND UNCONJUGATED BILE ACIDS DETERMINED BY VARIOUS METHODS

Faeces were obtained from healthy subjects on a normal diet.

Reference	*n*	Faecal concentration (mean ± S.D.) (mg/g of dried faeces)			
		Cholesterol	5β-Cholestan-3β-ol	DCA	LA
This method*	7	4.5 ±6.4	9.3±5.2	0.5±0.3	0.7 ±0.2
Owen et al. [7]	3	4.05±1.7	12.0±3.9	2.5±1.0	3.75±0.8
Von Breuer et al. [12]	15	—	—	1.6±0.4	1.5 ±0.4
Reddy et al. [26]	8	2.6 ±0.4	21.1±2.1	4.4±0.2	3.0 ±0.3
Moskovitz et al. [27]	24	8.9 ±2.0	16.6±2.9	3.8±0.8	3.5 ±0.5
Korpela et al. [20]	5	—	—	1.1±0.1	0.75±0.1

*The mean ± S.D. of the results shown in Table II.

gated bile acid (Fig. 3) fractions. Quantitation of these four components could therefore be performed without interference either from vegetable steroids and their bacterial metabolites [9,25] or from the epimers of DCA and LA and other minor bile acids [10,16], by using the appropriate internal standards and calibration curves.

Table III shows a comparison of our results for the major neutral steroids and unconjugated bile acids in control faeces with some recent literature values. The data quoted reflect in part the divergent analytical approaches currently practised (e.g. addition of internal standards after extraction or after sample preparation, co-elution or separation [10,13,20] of epimeric bile acids). We did not find a consistent pattern for the faecal cholesterol/5β-cholestan-3β-ol ratios between control subjects using either the method reported here or an alternative procedure [7] (unpublished data). An earlier study [28] also showed two distinct patterns of faecal cholesterol metabolism in a normal (North American) population.

We are presently attempting to broaden our analytical method to the quantitation of faecal glycine-conjugated bile acids [29], the minor faecal bile acids [16], and the epimers of DCA and LA, in order to improve the steroidal profile of human faeces reported here.

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