

QUANTITATIVE DETERMINATION OF NEUTRAL STEROIDS AND BILE ACIDS IN
HUMAN FECES BY CAPILLARY GAS-LIQUID CHROMATOGRAPHY

Jan F.C. Glatz, Frans J.M. Schouten, Geertruida den
Engelsman and Martijn B. Katan

Department of Human Nutrition, Agricultural University,
De Dreijen 12, 6703 BC Wageningen
(The Netherlands).

ABSTRACT

A method is described for the isolation and quantitative determination of human fecal neutral steroids and bile acids, to be applied especially in sterol balance studies with healthy subjects. The procedure comprises successively alkaline saponification of freeze-dried feces, extraction of neutral steroids with petroleum ether, acidification, extraction of bile acids with diethyl ether and quantification by narrow-bore capillary gas-liquid chromatography. Appropriate temperature programming allowed the identification of at least 20 different neutral steroids and 22 bile acids, among which 5 iso- and 6 oxo-bile acids.

The feasibility of the method is indicated by (i) the quantitative recovery of pure steroids added to the dry feces, (ii) a coefficient of variation for the neutral steroid and bile acid contents within and between runs of less than 5%, and (iii) the suitability for measuring amounts of individual steroids as little as 15 nmol. The neutral steroid and bile acid composition of three control pools of human feces is given.

INTRODUCTION

Studies of cholesterol balance in man require the accurate measurement of neutral steroids and bile acids in feces. Fecal steroids of cholesterol and plant sterol origin must be adequately separated. For this purpose, the combined gas-liquid chromatography (GLC) method preceded by thin-layer chromatography is usually applied (1, 2). Recently, however, Miettinen (3) established that this thin-layer chromatography step can be omitted when capillary columns are used.

Quantification of human fecal bile acids by enzymic methods, using 3-hydroxysteroid dehydrogenase is not feasible, owing to the possible presence of fecal bile acids with no 3-hydroxyl group and of substances in the acidic steroid fraction that interfere with the enzymic reaction. In this communication we describe a convenient procedure for the accurate and precise analysis of the fecal content of neutral and acidic steroids, that meets the requirements for application in sterol balance studies.

MATERIALS AND METHODS

Reference steroids

Campestanol, sitostanol, 3 α , 7 β , 12 α -trihydroxy-5 β -cholanoic acid (ursocholic acid), 3 α , 12 β -dihydroxy-5 β -cholanoic acid and 3,12-dioxo-5 β -cholanoic acid were kindly donated by Prof. D.N. Kirk, Curator of the Steroid Reference Collection, London, England. All other neutral steroids and bile acids were purchased from Steraloids, Pawling, N.Y., U.S.A., or from Serva Feinbiochemie.
Cholesterol Metabolism in Health and Disease: Studies in the Netherlands, edited by A. C. Beynen, M. J. H. Geelen, M. B. Katan, and J. A. Schouten. Ponsen & Looijen. Wageningen 1985

mica, Heidelberg, F.R.G. Purity of the steroids was checked by GLC and that of 3 α -bile acids also enzymically using 3 α -hydroxysteroid dehydrogenase (Van der Meer et al., this volume). The preparations were found to be 95-100% pure in all cases.

Reagents

Bis-silyl-trifluoroacetamide was obtained from Chrompack, Middelburg, The Netherlands; hexamethyldisilazane, trimethyl-chlorosilane and pyridine from Pierce Chemical Co., Rockford, IL, U.S.A. 2,2-Dimethoxypropane was from Merck, Darmstadt, F.R.G. and distilled prior to use. All other reagents were of analytical grade. Dimethylformamide was dried with calcium hydride and hexane with sodium sulfate.

Fecal samples

Three different control pools of freeze-dried human feces were used. Pools I and II were prepared by mixing equal quantities of freeze-dried feces obtained from 30-40 healthy subjects during a dietary trial (4). Control pool III was provided to us by Dr. G. Brydon (Wolfson Laboratories, Edinburgh, Scotland). The fecal samples used were all from subjects who consumed a mixed natural diet. For estimation of the efficiency of bile acid extraction from feces we used 4 fecal samples obtained from a volunteer after intravenous injection with [14 C]deoxycholic acid, and donated to us by Dr. F.M. Nagengast (University of Nijmegen, Nijmegen, The Netherlands). All samples were stored at -20 C.

Isolation of steroids from feces

The procedures for mild saponification and extraction of the neutral steroids and bile acids were modified from those described by Grundy et al. (1) and Miettinen et al. (2).

In a 50-ml glass-stoppered centrifuge tube 2 ml 5 α -cholestane solution (1 mM in chloroform; internal standard for neutral steroids) and 1 ml 5 β -cholanic acid/23-nordeoxycholic acid solution (both 1 mM in methanol; internal standards for bile acids) were evaporated to dryness. A 150-mg aliquot of freeze-dried feces was precisely weighed into the tube. For saponification, 2.1 ml methanol, 0.65 ml 5 M NaOH and a few boiling chips were added and the mixture was refluxed for 2 h at 80°C. We found methanol to be superior for saponification to ethanol (2) since with the latter we observed a conversion of coprostanon into epicoprostanol which did not happen with methanol. After the mixture had cooled down, neutral steroids were removed by three extractions with 5 ml petroleum ether (boiling range 60-80°C) and centrifugation for 5 min at 1000 x g. One third of the combined petroleum ether phases was transferred to a 1.5-ml vial, taken to dryness and derivatized for GLC.

Residual petroleum ether and methanol were removed from the aqueous lower phase by evaporation under N₂. The mixture was then acidified by addition of 2 ml 3 M HCl and the bile acids (and fatty acids) extracted three times with 5-ml portions of diethyl ether, vigorous shaking and centrifugation for 5 min at 1000 x g. The extracts were combined and the solvent evaporated to dryness, leaving a dark brown residue, which was derivatized as described below.

To aid in peak identification, the fecal neutral steroid mixture was further fractionated by thin-layer chromatography on 0.5 mm Florisil plates exactly as described by Miettinen et al. (2).

To measure the recovery of 14 C-bile acids from feces, 30-mg samples of the feces and the dried diethyl ether extracts obtained from these feces were

oxidized in a Liquid Scintillation Sample Oxidizer (Intertechnique, Plaisir, France). Radioactivity of $^{14}\text{CO}_2$ was measured in 10 ml Carbomax counting solution.

Derivatization and gas-liquid chromatography

The isolated neutral steroids were converted into volatile trimethylsilyl ether derivatives by addition of 0.8 ml freshly prepared silylating agent (dimethylformamide/bis-silyl-trifluoroacetamide, 2:1, v/v) and heating for 30 min at 80 °C. Excess reagents were not removed.

The bile acids were methylated with 0.5 ml 2,2-dimethoxypropane/0.5 ml methanol/50 μl 12 M HCl (5); the reaction vials were left at room temperature for 3-17 h. The methylating solution was then completely evaporated and methyl ester-TMS ether derivatives were prepared by addition of 0.5 ml freshly mixed pyridine silylating agent (pyridine/hexamethyldisilazane/-trimethyl-chlorosilane, 9:3:1, v/v) and heating for 30 min. at 80 °C. Excess reagents were evaporated and the products redissolved in 0.8 ml dry hexane. After centrifugation for 5 min at 1000 x g the clear supernatant was used for further analysis.

GLC analyses of the steroid derivatives were performed on a Packard 439 gas chromatograph, equipped with a 25 m x 0.22 mm (inner diameter) fused silica capillary column (Chrompack, Middelburg, The Netherlands) and a flame-ionization detector. A CP Sil 5 CB column was used for neutral steroids and a CP Sil 19 CB for bile acids. Hydrogen was used as carrier gas at a flow rate of 0.92 ml/min. Temperature programming was as follows: neutral steroids, 200 $\rightarrow$ 260 °C in 4 min and then isothermal at 260 °C for 20 min; bile acids, 200 $\rightarrow$ 250 °C in 2.5 min, isothermal for 12 min, 250 $\rightarrow$ 275 °C in 2.5 min, isothermal for 7.5 min and finally 275 $\rightarrow$ 300 °C in 2.5 min. Peak areas were measured with a Spectra Physics 4100 computing integrator and compared with those of standard solutions of the reference steroids.

RESULTS AND DISCUSSION

Gas-liquid chromatography

Identification of the major neutral steroids and bile acids was accomplished by matching their retention times with those of known standards of high purity. In addition, neutral steroids were identified by thin-layer chromatographic separation into three groups of steroids, following the procedure₅ described by Miettinen et al. (2, 3). This permits the distinction between Δ^5 - and ring-saturated 5α -steroids (cholesterol fraction), ring-saturated 5β -steroids (coprostanol fraction) and 3-oxo steroids (coprostanon fraction). We used the retention times of cholesterol, campesterol, stigmasterol and sitosterol and the retention indexes of the microbial cholesterol derivatives, determined with the pure compounds, to estimate the retention times of the plant steroid derivatives (Fig. 1). The results are in excellent agreement with those recently reported by Miettinen (3) and confirm his observation that fecal steroids of cholesterol and plant steroid origin are completely separated if capillary columns are used, thus permitting the omission of a preceding TLC step.

Identification of bile acids was also carried out by subjecting various representative fecal samples to GLC-high resolution mass spectrometry. Both the identity and homogeneity of the compounds were confirmed by comparing the obtained fragmentation patterns with those of known standards. Under the conditions used baseline separation of almost all bile acids was achieved (Fig. 2). The gaschromatogram is very similar to that recently observed by

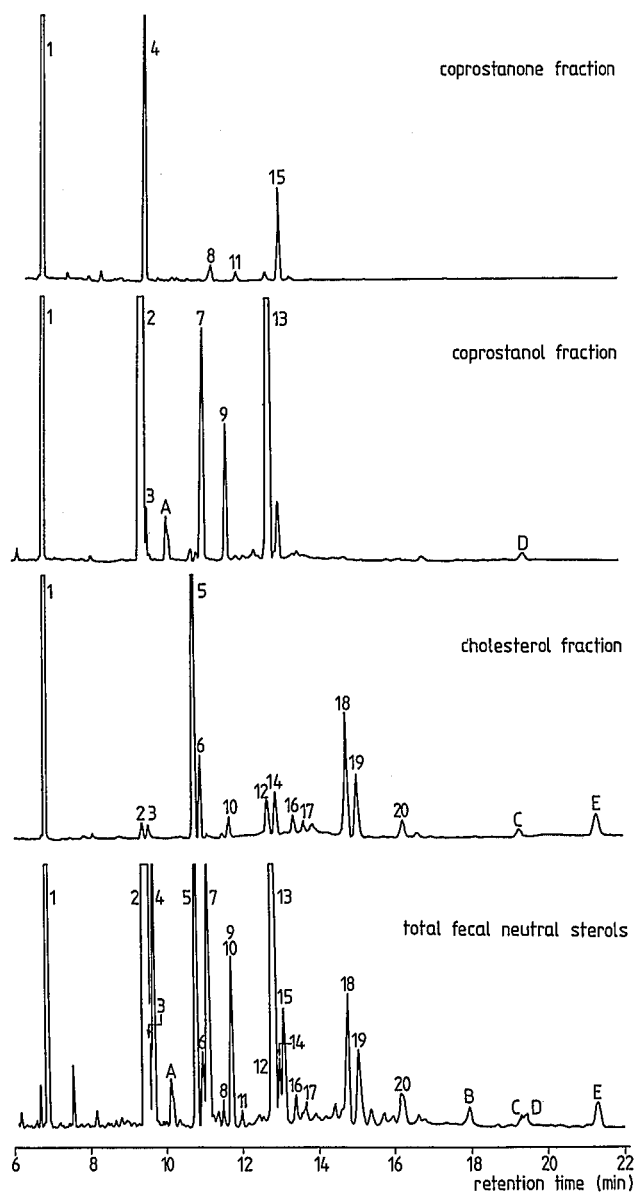


Fig. 1. Gas-liquid chromatograms on a CP Sil 5 capillary column of the TMS ether derivatives of fecal neutral steroids and of three fractions isolated with preceding TLC from this steroid mixture. Human feces pool I was used for this experiment. 1. 5α -cholestane (internal standard), 2. coprostanol, 3. epicoprostanol, 4. coprostanone, 5. cholesterol, 6. cholestanol, 7. methylcoprostanol (formed from campesterol), 8. methylcoprostanone, 9. ethylcoprostenol (formed from stigmasterol), 10. lathosterol, 11. ethylcoprostenone, 12. campesterol, 13. ethylcoprostanol (formed from sitosterol), 14. campestanol (5α), 15. ethylcoprostanone, 16. stigmasterol, 17. stigmastenol (5α), 18. sitosterol, 19. sitostanol (5α), 20. unidentified sterol, A to E, unidentified compounds.

Stellaard et al. (6).

Differences in response of the flame ionization detector for the various steroids were corrected for by specific response factors, determined in each series of analysis on the basis of the molar composition of a mixture of pure steroids. Most neutral steroids showed a response factor of 0.9-1.0, and most bile acids of 0.8-1.1, except for oxo-steroids which showed a relatively low detector response (factor 1.1-1.6). For all compounds the response was linear over a sufficiently wide concentration range.

Sample preparation

Various steps in the procedure to prepare the samples for GLC analysis were examined more closely in order to optimize and validate the method.

The efficiency with which neutral steroids and bile acids are extracted from feces by the present method was first estimated by determining the recovery of pure steroids added to feces before saponification. Both for all endogenous steroids and for the major bile acids the recovery was almost quantitative (93-104 %), except for coprostanol, which exhibited a recovery of only 88%. However, the latter steroid usually forms only 5-10 % of the total amount of excreted endogenous steroids. Extraction efficiency of bile acids was also measured from the recovery of ^{14}C -label from feces after intravenous injection of ^{14}C -labelled deoxycholic acid in a volunteer. Of the total ^{14}C -radioactivity present in the freeze-dried feces, 90-98% appeared in the bile acid extract. Thus binding of bile acids to bacteria and/or fibre residues in feces was not a problem.

A linear relationship was observed between the size of the fecal sample analyzed and the amount of neutral steroids and of bile acids recovered (Fig. 3). The methylation reaction of bile acids was complete after 3 h at roomtemperature (Fig. 4), but usually proceeded overnight.

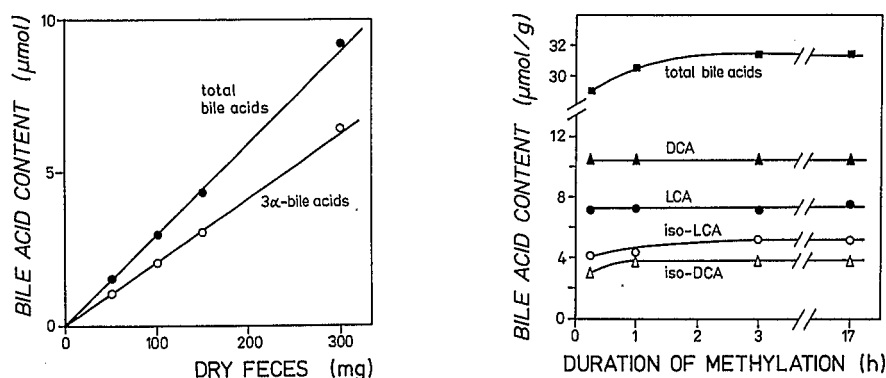


Fig. 3 (left). Effect of the amount of freeze dried human feces on the amount of 3α-bile acids (○) and total bile acids (●) recovered. The results are shown of a representative experiment with triplicate analyses using control feces pool I.

Fig. 4 (right). Time dependence methylation with 2,2-dimethoxypropane at room temperature of some individual and total amount of bile acids extracted from human feces pool I. The reaction was followed by gas-chromatographic quantitation of the methyl ester TMS ether derivatives. Results are representative for three experiments.

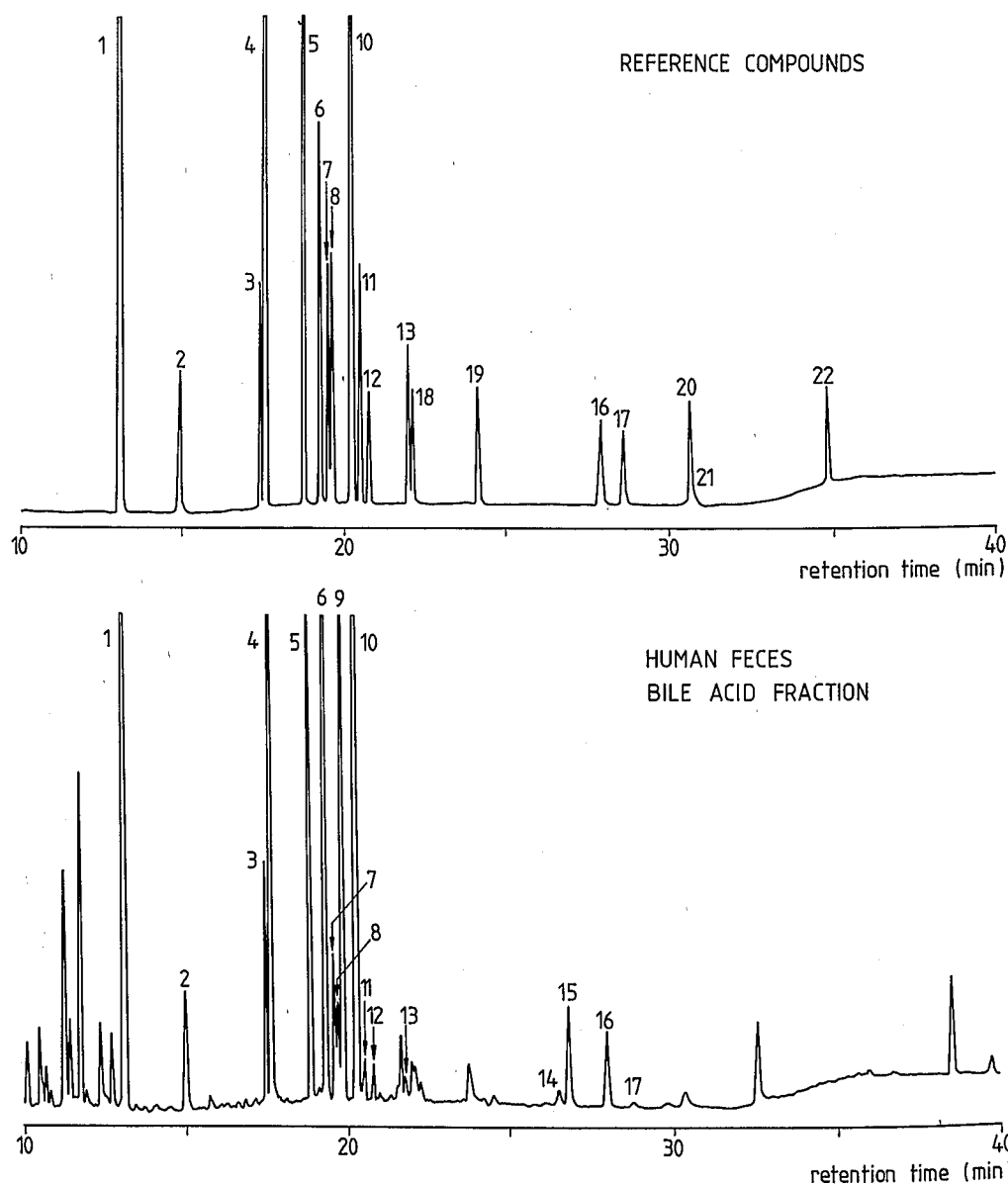


Fig. 2. Gas-liquid chromatograms on a CP Sil 19 capillary column of the TMS ether derivatives of bile acid methyl esters and other steroids as prepared from pure compounds (above) or as found in the bile acid fraction of human feces (pool I; below). 1. 5 β -cholanic acid (internal standard), 2. coprostanol, 3. cholesterol, 4. 23-nordeoxycholic acid (internal standard), 5. iso-lithocholic acid (3 β), 6. lithocholic acid, 7. iso-chenodeoxycholic acid (3 β), 8. 3 α ,12 β -dihydroxycholic acid, 9. iso-deoxycholic acid (3 β), 10. deoxycholic acid, 11. cholic acid, 12. chenodeoxycholic acid, 13. ursodeoxycholic acid, 14. unidentified dihydroxycholic acid, 15. 12-oxo iso-lithocholic acid (3 β), 16. 12-oxo lithocholic acid, 17. 7-oxo lithocholic acid, 18. ursocholic acid, 19. 3-oxo cholanic acid, 20. 7-oxo deoxycholic acid, 21. 12-oxo chenodeoxycholic acid, 22. 3,12-dioxo cholanic acid.

Both the neutral steroid and bile acid derivatives appeared to be stable when stored at 4°C, since we did not observe difference among the steroid contents when calculated from GLC analyses performed after 0, 3 and 8 days of storage (data not shown).

Steroid sulfates

The fecal samples used in this study were obtained from healthy subjects consuming a mixed natural diet. Therefore, the percentage of sulfated and/or conjugated steroids will presumably be low due to the microbial action of the colon. With control pool I we indeed found no differences between the total steroid contents determined with and without an enzymic deconjugation step and/or a solvolysis step (carried out as in Refs. 7 and 8, respectively). However, with all fecal samples examined we found that the bile acid fraction contained significant amounts of cholesterol and coprostanol (Fig. 2). It is unlikely that these steroids have entered the bile acid fraction due to incomplete extraction of neutral steroids, since with petroleum ether free steroids were quantitatively extracted from the saponified fecal mixture. We propose that the cholesterol in the bile acid fraction originates from cholesterol sulfate, which was earlier reported to be present in feces (9). When pure cholesterol sulfate was subjected to the entire extraction procedure, less than 2% of the amount was found as cholesterol in the neutral steroid fraction, but 70-80% appeared at the retention time of cholesterol in

Table I. Neutral steroid and bile acid composition of 3 control pools of human feces.

Steroid	Fecal content (μmol/g dry feces)		
	Pool I (CF 77)	Pool II (CF 84)	Pool III (CF GB)
Cholesterol	9.0	5.4	20.8
Cholesterol sulfate	1.6	1.3	2.9
Coprostanol	45.5	46.2	40.5
Coprostanon	5.5	5.1	3.6
Cholestanol	0.8	0.9	1.3
Total neutral steroids	62.3	59.0	68.9
Cholic acid	0.2	0.3	0.1
Chenodeoxycholic acid	0.2	0.2	0.2
Iso-deoxycholic acid	4.0	1.4	2.3
Deoxycholic acid	11.3	5.3	8.1
Iso-lithocholic acid	4.5	3.1	4.3
Lithocholic acid	7.7	4.1	6.7
Oxo bile acids ¹	2.7	1.6	2.8
Other bile acids	1.2	0.9	1.2
Total bile acids	31.8	16.9	25.7

Each of the feces pools was prepared by mixing equal quantities of freeze-dried feces obtained from 30-40 healthy humans consuming a mixed natural food. Values represent the means of 6-8 determinations in separate runs.

¹ Iso-chenodeoxycholic acid, 3α,12β-dihydroxy-5βcholanoic acid and an unidentified dihydroxy bile acid.

Table II. Coefficients of variation for the determination of neutral steroids and bile acids in control pools of human feces.

Steroid fraction	Coefficient of variation (%)					
	Within runs			Between runs		
	Pool I	Pool II	Pool III	Pool I	Pool II	Pool III
Cholesterol + derivatives	1.5	1.3	2.6	0.6	1.9	3.8
Plant steroids	2.3	1.1	2.6	0.7	1.5	4.0
3 α -Bile acids	2.2	3.2	1.5	2.2	4.9	4.6
Total bile acids	2.1	2.8	1.3	2.4	4.9	4.7

Values were calculated from 20 (pools I and II) or 12 (pool III) determinations distributed over 8 and 6 runs, respectively.

the bile acid fraction. Solvolysis of cholesterol sulfate will have occurred during bile acid extraction, since hydrolysis of steroid sulfates is accelerated by acids and by a low-polarity medium such as diethyl ether (8, 10). It is not excluded that sulfated bile acids, if present, were also hydrolysed by the acid-diethyl ether extraction. As to the origin of coprostanol in the bile acid fraction it is at present unclear to us whether this sterol is derived from covalently bound coprostanol or that it represents free coprostanol that is only released from the fecal matrix at a low pH.

Steroid composition of control pools of human feces

Three different pools of freeze-dried human feces were analysed for their neutral steroid and bile acid composition. Coprostanol and cholesterol represented the major neutral steroids present (Table I). About 12-19% of the fecal cholesterol was found in the bile acid fraction and is presumably derived from cholesterol sulfate. The amount of primary bile acids (cholic and chenodeoxycholic acid) was negligible compared to that of (iso)deoxycholic and (iso)lithocholic acid. In all cases the oxo-bile acids were composed of mainly 12-oxo(iso)lithocholic acid and a small amount of 7-oxo lithocholic acid. The total bile acid content of pool III was 7% higher than found when analysed as oxo-bile acids (11) by others (courtesy of Dr. G. Brydon).

Routinely, 23-nordeoxycholic acid was used as the internal standard for bile acid quantitation. For comparison, we also used 5 β -cholanolic acid as internal standard with all analyses. With the latter the steroid contents found were only -3 to +1% different from those obtained with 23-nordeoxycholic acid. We prefer the use of 23-nordeoxycholic acid because of a higher similarity with the naturally occurring bile acids.

Reproducibility

Repeated determinations on the three pools of human feces revealed coefficients of variation for the total amounts of endogenous neutral steroids, plant steroids, and 3 α - and total bile acids of 1.2-2.9% within one run and of 0.6-4.9% between runs (Table II). Differences among the fecal

samples are likely due to differences in steroid composition and in homogeneity of the freeze-dried material. On the basis of these results the coefficient of variation for the total fecal content of endogenous neutral steroids will amount to 1-4% and that for bile acids to 3-5%, when duplicate analyses are performed.

CONCLUDING REMARKS

The procedures presented in this study to determine fecal neutral steroids and bile acids were developed to meet the requirements for sterol balance studies with healthy humans consuming a mixed natural food. The recovery of pure steroids added to feces and of endogenous bile acids was found to be complete. Quantitative determination of steroids was achieved (a) by addition of the internal standards for neutral steroids and bile acids before the start of the analysis, (b) by the use of specific response factors to correct for differences in detectorresponse among the various steroids, and (c) the use of narrow-bore capillary GLC and appropriate temperature programming. The sensitivity of the method is such that in a 150-mg sample of dry feces a content of individual steroids of 0.1 $\mu\text{mol/g}$ can be analysed accurately.

ACKNOWLEDGEMENTS

The authors thank Dr. F. Stellaard (University of Munich, Munich, F.R.G.) and Dr. M.A. Posthumus (Laboratory of Organic Chemistry) for carrying out the GC-MS analyses, Dr. A. van Faassen (Central Institute for Nutrition and Food Research, CIVO-TNO, Zeist, The Netherlands) for advice with regard to the extraction procedure of fecal steroids, and Dr. R. van der Meer (Netherlands Institute for Dairy Research, Ede, The Netherlands) for stimulating discussions. This work was supported by the Netherlands Heart Foundation, grant no. 32.015 and an established investigatorship to M.B.K.

REFERENCES

1. Grundy, S.M., Ahrens, E.H. and Miettinen, T.A. Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* 6, 397 (1965).
2. Miettinen, T.A., Ahrens, E.H. and Grundy, S.M. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.* 6, 411 (1965).
3. Miettinen, T.A. Gas-liquid chromatographic determination of fecal neutral sterols using a capillary column. *Clin. Chem. Acta* 124, 245 (1982).
4. Katan, M.B., Beynen, A.C., De Vries, J.H.M. and Nobels, A. Existence of consistent hyper- and hyporesponders to dietary cholesterol in man. *Am. J. Epidemiol.* (1985), submitted.
5. Shaw, R. and Elliott, W.H. Bile acids. LV. 2,2-Dimethoxypropane: an esterifying agent preferred to diazomethane for chenodeoxycholic acid. *J. Lipid Res.* 19, 783 (1978).
6. Stellaard, F., Sackmann, M., Sauerbruch, T. and Paumgartner, G. Simultaneous determination of cholic acid and chenodeoxycholic acid pool sizes and fractional turnover rates in human serum using ^{13}C -labeled bile acids. *J. Lipid Res.* 25, 1313 (1984).
7. Roseleur, O.J. and Van Gent, C.M. A simplified method for the determination of steroids in diets and faeces. *Clin. Chem. Acta* 82, 13 (1978).
8. Galeazzi, R. and Javitt, N.B. Bile acid excretion: the alternate pathway in the hamster. *J. Clin. Invest.* 60, 693 (1977).

9. Islam, M.A., Raicht, R.F. and Cohen, B.I. Isolation and quantitation of sulfated and unsulfated steroids in human feces. *Anal. Biochem.* 112, 371 (1981).
10. Burstein, S. and Lieberman, S, Kinetics and mechanism of solvolysis of steroid hydrogen sulfates. *J. Am. Chem. Soc.* 80, 5235 (1958).
11. Evrard, E. and Janssen, G. Gas-liquid chromatographic determination of human fecal bile acids. *J. Lipid Res.* 9, 226 (1968).