Quantitative isolation and gas–liquid chromatographic analysis of total dietary and fecal neutral steroids

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SUMMARY A method for isolation and quantification of fecal neutral steroids is described which allows studies to be made of sterol balance in man or in small laboratory animals without requiring the use of radioisotopes in vivo. The critical separations of cholesterol plant sterols and their conversion products depend upon preliminary separations into three subfractions by thin-layer chromatography. Individual components in the three subfractions thus obtained are then quantitatively measured by gas–liquid chromatography of the unsubstituted 3-ketosteroids and of the trimethylsilyl ethers of the sterols.

Extractions of cholesterol-7α-H^3 added in vitro and of C^{14}-labeled neutral steroids synthesized in vivo were quantitative and highly reproducible. Several lines of evidence validate the determination of individual fecal neutral steroids by GLC.

Examples are given of the application of this technique: a sterol balance study of 27 days' duration is described in a patient whose diet included plant sterols as well as cholesterol. Representative results in man and in rats are compared to others obtained by previously described methods.

The sensitivity of the method is such that 1-g fecal aliquots containing as little as 25 μg of mixed neutral steroids can be analyzed accurately, but the procedure lends itself well to preparative scale work for more definitive study of individual neutral steroids.

KEY WORDS fecal neutral steroids · quantitative recoveries · thin-layer chromatography · gas–liquid chromatography · trimethylsilyl ethers · hydrogen flame detection · cholesterol · coprostanol · β-sitosterol · campesterol · stigmasterol · plant sterols · intestinal transformation · internal standards · man · rat

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; PE, petroleum ether (bp 60–70°); EE, ethyl ether; MeOH, methanol; TMS, trimethylsilyl; L–B, Liebermann–Burchard.

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The isolation, identification, and quantification of individual fecal neutral steroids[1] has proven to be difficult because of similarities in their molecular structures and in their physical properties. As a result, there has been no completely adequate description of the net balance (intake minus output) of cholesterol, distinct from that of the plant sterols, in intact mammalian organisms.

The present report describes a procedure by which fecal neutral steroids can be isolated and separated by TLC into groups of cholesterol and plant sterol derivatives. These groups or their individual components can then be accurately quantified by GLC. When this procedure is combined with the method for acidic steroids described in the accompanying report (1), reliable sterol balance studies can be carried out in man and in small laboratory animals. For application of these two methods there is no requirement for the in vivo administration of radioisotopes or for the feeding of sterol-free diets.

GENERAL METHODS, MATERIALS, AND APPARATUS

Solvents were glass-distilled before use, the diethyl ether over sodium, the dimethylformamide over calcium carbide as described by Eneroth, Hellström, and Ryhage (2). Solvent evaporations were carried out under nitrogen at temperatures below 40° on a rotary evaporator (Rinco Instrument Co., Greenville, Ill.) attached to a water aspirator.

[1] The term *steroids* is used in preference to *sterols* because of the significant amounts of ketonic metabolites of cholesterol which are invariably present in neutral and acidic fractions of feces.

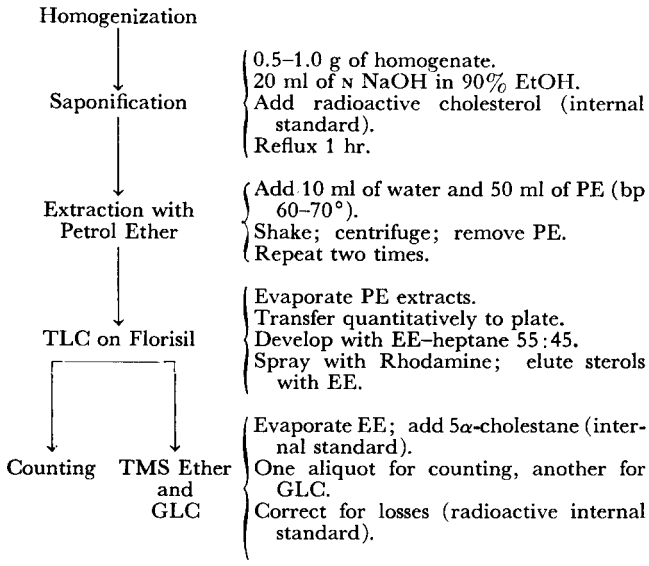

FIG. 1. Flow sheet for determination of neutral steroids in foods or feces.

Reference Standards. Cholesterol-4-C^{14} and -7α-H^3 (New England Nuclear Corp., Boston, Mass.), whether administered to patients or used as internal standards, were purified by TLC according to Mangold (3) on Silica Gel G (E. Merck, Darmstadt, Germany; distributors, Brinkmann Instruments, Inc., Great Neck, Long Island, N.Y.); plates were pre-run in MeOH–EE 1:4 and developed in acetone–benzene 1:9. 5α-Cholestane (Steraloids, Inc., Pawling, N.Y.) was used as internal standard for GLC after thorough drying but without further purification: analysis by GLC showed a single peak with an area response which was 99% of the theoretical when tested against an internal standard of repeatedly recrystallized cholesterol in the form of its TMS ether (see below). Reference samples of campesterol, stigmasterol and β-sitosterol were generously provided by M. J. Thompson, Beltsville, Md., and another sample of campesterol by P. Capella, Milan, Italy.

Thin-layer Chromatography of fecal neutral steroids was carried out on 0.5 mm layers of Florisil with binder (Research Specialties Co., Richmond, Calif.) on 20 × 20 cm plates. A smooth suspension of adsorbent was assured by brief (60 sec) mechanical blending of Florisil in water (57 g in 93 ml of water for 5 plates). The spread plates were activated at 120° for 1 hr and stored in a desiccator; a pre-run was not necessary.

Gas-Liquid Chromatography. All GLC analyses were performed on an instrument equipped with a hydrogen flame ionization detector (F and M Biomedical Gas Chromatograph, model 400, Avondale, Pa.). Columns were 6-ft glass U-tubes, 4 mm i.d., packed with silanized acid-washed Gas Chrom P (100–120 mesh) coated with 1–2% films of DC-560 (formerly called F-60), SE-30, QF-1, XE-60, or HiEff 8B (coatings and supports obtained from Applied Science Laboratories, State College, Pa.). Column temperatures were usually 240° and were accurately monitored with a precision mercury thermometer (Allihn type; Matheson Scientific Co., Philadelphia, Pa.); the temperature of the flash heater was about 300° and of the detector about 290°. Nitrogen was used as carrier gas at flows of 30–60 ml/min, inlet pressures 20–30 psi. When it was found that areas of individual peaks calculated by triangulation and by means of a mechanical integrator (Disc Instruments Co., Santa Ana, Calif.) agreed within 1%, the latter system was used for subsequent measurements; this system proved to be essential for measuring total areas of a series of unresolved peaks. All quantitative work was performed by comparison of area responses to those of known additions of 5α-cholestane as internal standard; in our hands, analyses based on volume of sample injected were grossly inaccurate.

TMS Ether Formation. TMS derivatives of neutral sterols were prepared in dimethylformamide. For formation of TMS ethers of bile acids, pyridine is a preferable solvent because silylation of the hydroxyl groups on carbons 3, 6, 7, and 12 occurs rapidly and quantitatively,[2] but this solvent promotes the formation of secondary products from 3-ketosteroids, presumably by reaction with their enol forms. This can be prevented by use of a less reactive solvent, such as dimethylformamide, which nevertheless promotes complete silylation of the hydroxyl group at carbon 3 of the neutral sterols.

The silylating reaction mixture was prepared from *N*,*N*-dimethylformamide (Matheson, Coleman and Bell, East Rutherford, N.J.), hexamethyldisilazane (Peninsular Chemical Research, Inc., Gainesville, Fla.), and trimethylchlorosilane (General Electric Co., Waterford, N.Y.) in the proportions 40:40:1. This mixture was stable for weeks if protected from water vapor.

Radioactivity Measurements were performed with a Packard Tricarb Liquid Scintillation Spectrometer (Model 3003). PPO–POPOP–toluene phosphor solution was prepared from Liquifluor (Pilot Chemicals, Inc., Watertown, Mass.). After dilution with toluene, the counting solution contained 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-[2-(5-phenyloxazolyl)-benzene per liter of toluene. Toluene-C^{14} and -H^3 (Packard Instrument Co., Inc., La Grange, Ill.) were used as internal standards to correct for quenching. Counting efficiencies were approximately 85% for C^{14} and 26% for H^3.

Clinical Aspects. In the development and validation of the present method, stools from more than 15 in-pa-

[2] Ahrens, Miettinen, and Grundy, data to be published.

tients at The Rockefeller Institute Hospital served as source material. The patients were fed solid-food diets as well as liquid formulas composed of few and well-defined ingredients (4). Diets rich in plant sterols and others devoid of them were used, and sterol analyses of formulas and of solid-food diets were performed by the same method as that described for feces. Six patients received either cholesterol-4-C^{14} or -7α-H^3, orally or intravenously, to ensure that all neutral steroids derived from cholesterol eventually became radioactive. Plasma cholesterol concentrations were measured by the method of Abell, Levy, Brodie, and Kendall (5).

QUANTITATIVE ISOLATION OF NEUTRAL STEROIDS

Figure 1, a flow sheet indicating the general aspects of the present procedure, is shown. Further details are given in the paragraphs below.

Collection and Homogenization

In clinical experiments, stools collected into clean dry weighed two-quart metal paint cans were stored at 4°. Sufficient distilled water was then added to permit the production of a homogenate that was fluid enough to be drawn up into a wide-mouthed transfer pipette; in most cases an equal dilution with water was satisfactory. After the cans had been weighed again, three 2-inch metal washers were added, and homogenization was carried out by vigorous agitation for 3 min on a $^1/_4$ hp paint mixer (Red Devil Tools, Union, N.J.). All weights were recorded to the nearest gram (Toledo Scale Co., Toledo, Ohio, model 4020). Before solids had had time to settle, aliquots of the homogenates were transferred to 125-ml glass bottles with plastic screw-tops for storage at 4°; the cans were closed and discarded.

Saponification

Dietary or stool homogenate (usually 0.5–1.0 g, so as to contain approximately 0.5–5.0 mg of sterols) was weighed to the nearest milligram into 125-ml glass-stoppered bottles. As internal recovery standard, radioactive cholesterol (either -C^{14} or -H^3) of high specific activity was added: this made possible a correction at the end of the procedure for incomplete recoveries. [If bile acids were to be determined on the same aliquot, an internal standard of radioactive bile acid was added also (1).] After addition of a few boiling chips and 20 ml of N NaOH in 90% ethanol, the contents of the bottles were refluxed for 1 hr.

Extraction of Neutral Steroids

Water (10 ml) was added to the alkaline saponification mixture, and nonsaponifiable components were extracted three times with 50 ml portions of PE. For rapid and complete phase separation, bottles were centrifuged after each extraction (1000 × *g* for 5 min), and the combined PE extracts were evaporated to dryness in a round bottom flask under nitrogen. Solutes were then transferred quantitatively to a conical centrifuge tube with 5–10 ml of PE, evaporated in a nitrogen stream to small volume (0.2–0.3 ml) and finally transferred completely to a Florisil plate with several rinses of PE. In the isolation of sterols from fat-rich samples, such as dietary fats or fatty feces, the formation of jelly-like soaps made the complete transfer of neutral steroids to the TLC plate impossible. This difficulty was overcome by equilibrating the PE phase in the centrifuge tube with 50% ethanol and discarding the lower phase. Losses of steroids in this discarded material were eventually corrected for by means of the internal recovery standard.

TLC of Neutral Steroids

The neutral steroids were then separated into groups by preparative TLC on Florisil, and at the same time freed of nonsteroidal contaminants. Two to four samples were applied per plate; after gassing the chamber with nitrogen, the plate was developed with EE–heptane 55:45 to its full length and sprayed with a half-saturated aqueous solution of Rhodamine G. The steroids were detected under UV-light. TLC of fecal neutral steroids usually produced three distinct bands which we designated Fractions I, II, and III (Fig. 2); these fractions had the mobilities of coprostanone, coprostanol, and cholesterol, respectively.

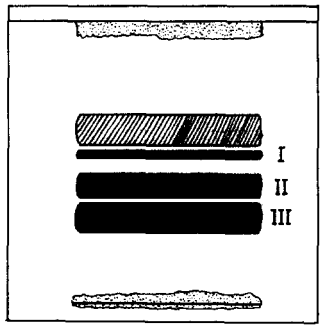

FIG. 2. Florisil TLC pattern of the fecal neutral steroids of one patient fed a diet containing corn sterols but no cholesterol.

Dietary fat (40% of total calories) consisted of residue fraction from molecular distillation of corn oil; 280 mg of corn sterols per 100 g of oil remained entirely in the form of esters. The oil was protected from autoxidation by addition of α-tocopherol (100 mg/100 g of oil).

Fraction III: cholesterol, campesterol, stigmasterol, β-sitosterol, and corresponding ring-saturated 5α-sterols. Fraction II: coprostanol and ring-saturated 5β derivatives of plant sterols. Fraction I: 3-keto coprostanone and 3-keto derivatives of plant sterols. Shaded area above I = α-tocopherol.

TABLE 1 REPRODUCIBILITY OF STEROL EXTRACTION FROM FECAL HOMOGENATE

Patient received 100 μc of cholesterol-4-C^{14} orally five days before stool collection was made.

Hydrolysis	PE Extractions*	Radioactive Steroids Extracted				
		cpm/total sample				
1. 1 N NaOH in 90% ethanol, 1 hr	First	39,520	34,770	60,785	25,880	51,040
	Second	646	586	1242	460	1000
	Third	57	63	83	36	97
2. 0.1 N HCl in 90% ethanol, 24 hr	Fourth†	114	86	172	70	158
Total cpm recovered		40,337	35,591	62,282	26,446	52,452
Amount of fecal homogenate per sample (mg)		672	594	1035	443	885
Cpm per g of homogenate		60,050	59,900	60,200	59,700	59,200

* After dilution of hydrolysis mixture to ethanol concentration of 60%.
† Made alkaline before PE extraction.

These three steroid-containing areas of the plate were collected individually in a vacuum aspirator according to Goldrick and Hirsch (6), and extracted with four 4-ml portions of EE. The combined ether eluates were evaporated under a steam of nitrogen at 40°, and the steroids dissolved in a known volume (usually 2 ml) of ethyl acetate containing a known amount of 5α-cholestane as GLC internal standard. One aliquot was taken for counting and another for GLC.

Trimethylsilylation for GLC

Since the ketonic steroids and TMS ethers of neutral sterols were found to be quantitatively determined by GLC with a hydrogen flame detector,[2] the fecal steroid mixtures were exposed to silylating reagents in order to convert free hydroxyl groups to TMS ethers. An aliquot of the steroid solution containing 5α-cholestane standard was pipetted into a disposable stoppered glass vial of appropriate size, and the solution was evaporated to dryness in a nitrogen stream with gentle warming. The silylating mixture was added (about 0.2 ml/mg of sterol), the vial closed, and the reaction mixture held at room temperature for at least 30 min; the reaction was complete by that time, provided the sample was completely free from water. When Fraction I was isolated for analysis by GLC, silylation was unnecessary since this fraction contained only 3-ketosteroids. But when Fraction I was collected with II or with II plus III, silylation was necessary for satisfactory GLC. In those instances the TMS reaction was stopped after 30 min (to prevent the formation of secondary products from the ketonic steroids), by evaporating the solvents and redissolving the steroid TMS ethers and 5α-cholestane in 0.1 ml of dry ethyl acetate; alternatively, the reaction mixture was immediately subjected to GLC.

GLC with Hydrogen Flame Detection

The ethyl acetate solution (or the TMS-reaction mixture itself) containing 5α-cholestane, ketonic steroids, and/or TMS sterols was directly injected into the GLC flash heater. All TMS ether and ketonic steroid peaks had longer retention times than 5α-cholestane on every stationary phase examined.

Quantitative GLC was performed with sterols in the form of TMS ethers because the area response of free sterols (relative to 5α-cholestane as internal standard) was always 20–40% less than theoretical on all stationary phases tested (QF-1, SE-30, DC-560, XE-60, HiEff 8B, and several others). In contrast, the area responses of 5α-cholestane, ketonic steroids, and TMS sterols were quantitative over a wide load range (0.06–120 μg) and were directly related to the absolute weights (not molecular weights) of the parent *un*substituted steroids (not their TMS derivatives); no correction factors were needed. (Detailed data will be published.)

The most effective separations were obtained on DC-560 or SE-30 columns, which were used routinely. However, even when peaks were not completely resolved, the total peak area of the neutral steroids obtained with the mechanical integrator was accurately converted to weight, because all the neutral steroids had the same ionization response.

Calculations

The total neutral steroid content of a homogenate (food, feces, or tissues) was calculated from (*a*) the total areas of the GLC peaks of all components in Fractions I, II, and III with retention times longer than that of 5α-cholestane, relative to the peak area of the internal GLC standard (5α-cholestane), and (*b*) the recovery of the radioactive cholesterol which had been added to the homogenate as internal recovery standard.

Amounts of individual components (or of groups of neutral steroids) were calculated from the GLC analyses of the three TLC fractions, each relative to the known amount of 5α-cholestane in each sample. Then, the percentage recovery of the internal recovery standard (radioactive cholesterol) was calculated by counting aliquots of Fraction III (usually 97% or more of the total radioactivity) and of Fraction II (sometimes 1–3%

of the total), and summing counts in the two fractions. Weights of neutral steroids obtained by GLC analysis in each of the three fractions were then corrected according to the total recovery of the internal recovery standard in II + III.

RESULTS

A. Isolation of Dietary and Fecal Neutral Steroids

Extraction of Neutral Steroids. In an earlier method reported from this laboratory (7) the fecal sterols were extracted after refluxing for 1–2 days, whereas in the present method the reflux period was 1 hr. To demonstrate the completeness of extraction under the latter conditions as well as the reproducibility of sampling and extraction, the experiment presented in Table 1 was designed. Various amounts of fecal homogenate (in which all the steroids had been labeled in vivo by prior administration to a patient of cholesterol-4-C^{14}) were saponified and extracted three times with PE. Each successive PE extract was subjected to TLC on Florisil, and from each the total steroid area (Fractions I + II + III) was recovered and counted. Table 1 shows that (*a*) practically all of the counts were recovered in the first three PE extractions; (*b*) a subsequent 24-hr reflux in acidic alcohol followed by PE extraction from an alkaline medium yielded only a few more counts; and (*c*) reproducibility of sampling and of extraction after saponification was excellent.

Recoveries of Fecal Steroids and of Added Radioactive Cholesterol as Functions of Ethanol Concentration. An experiment was designed to determine whether the extraction of fecal neutral steroids and of radioactive cholesterol added as internal standard was the same, and to what extent the effectiveness of extraction depended upon the concentration of ethanol in the extraction mixture. Cholesterol-7α-H^3 was added as internal recovery standard to a stool homogenate obtained from a patient given cholesterol-C^{14} orally some weeks before; in this case the fecal steroids were C^{14}-labeled, and the added recovery standard was labeled with H^3. After saponification of four aliquots of fecal homogenate, water was added to each in order to achieve the different ethanol concentrations specified in Table 2. Three PE extractions were performed on each sample, and each of the 12 extracts was counted. Finally, ethanol was made up to 63% in all four residues; the mixtures were refluxed for 24 hr and reextracted to obtain any radioactive neutral steroids not previously extracted.

Table 2 shows that (*a*) the percentage extraction of neutral steroids labeled with C^{14} in vivo and of added cholesterol-H^3 was almost the same at each extraction step, regardless of ethanol concentration and of the completeness of total sterol extraction at the step; and (*b*) steroids were quantitatively extracted by three exposures to PE only when ethanol concentrations in the lower phase reached 50% or higher: at lower concentrations, steroids were retained in the aqueous phase, possibly due to the solubilizing effects of bile salts and soaps. To determine whether the residue after the fourth extraction still contained any significant amount of C^{14}-steroids, the residue corresponding to the last column of Table 2 (63% ethanol) was acidified and extracted with chloroform, and the extract was subjected to

TABLE 2 COMPLETENESS OF EXTRACTION OF ENDOGENOUS (C^{14}-) AND EXOGENOUS (H^3-) NEUTRAL STEROIDS FROM FECAL HOMOGENATE

Feces were obtained from patient 7 days after oral administration of cholesterol-4-C^{14}; cholesterol-7α-H^3 was added to homogenate as internal standard

Hydrolysis	PE Extractions	Ethanol Concentration During PE Extraction							
		1%		25%		50%		63%	
		C^{14}-steroids	H^3-cholesterol	C^{14}-steroids	H^3-cholesterol	C^{14}-steroids	H^3-cholesterol	C^{14}-steroids	H^3-cholesterol
		*% of mean total counts**							
1. 1 N NaOH, 1 hr in various concentrations of ethanol†	First	50.2	45.5	79.9	76.5	93.1	92.3	7.1	97.1
	Second	11.0	11.0	9.4	9.4	3.9	3.5	3.7	3.7
	Third	7.8	7.8	1.3	0.7	0.5	0.2	0.4	0.3
Total		69.0	64.3	90.6	87.8	97.5	96.0	101.2	101.1
2. 1 N NaOH, 24 hr in 63% ethanol	Fourth‡	34.4	34.7	7.1	7.9	0.5	0.2	0.1	<0.1§
Grand Total		103.4	99.0	97.7	95.7	98.0	96.2	101.3	101.1

* Mean total counts for C^{14} = average of total C^{14} counts in all four samples after extraction IV. Total H^3-counts added to each homogenate = 100%.

† During reflux hydrolysis, ethanol concentrations were 2, 50, 90, and 90%, respectively, in the four experiments; each mixture was diluted with water prior to PE extraction to give the final ethanol concentrations shown.

‡ PE extraction was carried out directly from the reflux mixture.

§ Acidified with HCl and extracted with chloroform. Extract subjected to TLC; free and esterified sterol zones collected separately and counted.

TABLE 3 COMPLETENESS OF TLC SEPARATION OF FRACTION III FROM FRACTION II

	Cholesterol-C^{14} added to Stool	C^{14} Recovered	
	cpm	*cpm*	%
	17,500		
Fraction III		16,950 ± 198*	96.8 ± 1.2
Fraction II		7 ± 9	0.04

* Mean ± SD in nine replicates.

TLC on Florisil to remove all radioactive bile acids. In the solutes eluted from the free and esterified sterol zones of the plate only 0.1% of the total C^{14}-steroid counts were found.

In other similar experiments quantitative recoveries of labeled steroids were obtained from stool homogenates regardless of whether the major excretion product was cholesterol or coprostanol. Furthermore, all plant sterols or their bacterial conversion products were completely extracted from stool and food homogenates by three exposures to PE: the fourth PE extracts were invariably found by GLC to be free of these products.

Completeness of Saponification of Sterol Esters. Sterol esters were not present in the three pooled PE extracts (experiment described in Table 2), as was judged by assays of radioactivity in the sterol ester areas of thin-layer chromatograms. To show that saponification of sterol esters was complete even in the presence of a large excess of ester linkages, sterol analyses were made by the present procedure on samples of corn oil from which free sterols had been removed by molecular distillation (Distillation Products Industries, Rochester, N.Y.) so as to leave only sterol esters and triglycerides. In the distilled oil prior to saponification, analysis by TLC showed sterols only in the form of their esters; but TLC of the PE extracts after saponification under the conditions given showed sterols only in the free form. The solutes in the sterol ester zone of the latter plates were recovered, hydrolyzed, and examined by GLC: less than 0.2% of total sterols could be accounted for as unhydrolyzed sterol esters.

Cholesteryl stearate was added to fecal homogenates; after saponification under present conditions, TLC analysis of PE extracts showed no sterol esters. Similarly, cholesterol-4-C^{14} acetate added to unlabeled feces was completely saponified under present conditions, as judged by recoveries of radioactivity in the ester and free sterol areas of thin-layer chromatograms.

TLC Separations of Neutral Steroids into Groups. The separation of fecal neutral steroids into three major groups by preparative TLC on Florisil is illustrated in Fig. 2. From comparison of TLC and GLC characteristics of reference compounds with those of materials found in human, rat, and mouse feces, and from deductions[3] based on their steroid numbers, Fraction III contained Δ^5- and ring-saturated 5α-sterols, i.e., cholesterol and the three major plant sterols of corn oil (campesterol, stigmasterol, and β-sitosterol) and ring-saturated 5α-derivatives of these four sterols. Fraction II contained ring-saturated 5β-sterols such as coprostanol and the saturated 5β-homologs of the three plant sterols. Fraction I contained the ketonic steroid, coprostan-3-one, and corresponding 3-keto homologs of the three plant sterols, cholestan-3-one, if present, would be found in Fraction II.

Fractions II and III from human stools, when further subdivided by TLC on Silica Gel G, and on silver nitrate-impregnated Silica Gel G, show several minor components in addition to the major ones described above. By these successive fractionations and final analysis by GLC, human feces were found to contain several sterols believed to be precursors of cholesterol, but their quantity was small: 1–2% of all neutral steroids in Fractions I + II + III. However, in rat feces these precursors may constitute a much higher percentage of total neutral steroids.[3]

The degree of cross-contamination on preparative Florisil plates of Fraction II and Fraction III was studied by adding cholesterol-4-C^{14} as internal standard to an unlabeled fecal homogenate, and by examining the distribution of radioactivity in the TLC fractions. Table 3 shows 97% recovery of the internal standard, with negligible amounts of it in Fraction II. However, GLC of Fraction III showed that 1–3% of the coprostanol could be found in Fraction III. By the same means it was shown that no components of Fraction I were found in Fraction II, but in samples of feces from patients receiving large amounts of α-tocopherol this component (which chromatographs ahead of Fraction I) can contaminate Fraction I slightly through trailing. The TMS ether of α-tocopherol had the same retention time as TMS cholesterol on 1% DC-560 at 240°, but it never contaminated Fraction III, and its peak was clearly distinguished from that of coprostanone in GLC analysis of Fraction I.

Negligible Oxidative Changes during Isolation of Neutral Steroids. When cholesterol-4-C^{14} was subjected to repeated preparative TLC, no more than 0.5% of total radioactivity was found in the area of the plate below Fraction III. In analyses of C^{14}-neutral steroids from feces of patients given cholesterol-4-C^{14} for in vivo labeling, the labeled solutes recovered from the area of the plate below Fraction III never amounted to more than 1.5% of the counts recovered from the entire plate; this was not reduced by omission of the saponification step.

[3] Miettinen and Ahrens, data to be published.

We consider trailing to be the most likely explanation for these small losses of labeled cholesterol outside Fraction III, but the possibility of autoxidative changes (which we have not studied directly) must be borne in mind.

B. Validation of GLC Quantification

The GLC area response per unit weight of compound has been found to be different for different sterols with the usual argon ionization detectors (2, 8–12) and also with the ionization cross-section detector of Simmonds and Lovelock (13). However, with detection of TMS ethers by hydrogen flame ionization we have found strict linearity between area response and absolute weight proportions of a large number of ketonic steroids and sterols and over a wide load range.[2] These results were obtained by analyses of weighed reference samples of known purity to which defined amounts of 5α-cholestane of established purity were added as internal standard.

Validation of our procedure for measuring fecal neutral steroids by GLC was obtained through a somewhat different approach. A detailed study was made of the specific activities of the three subgroups of C^{14}-neutral steroids isolated from six fecal homogenates of three patients to whom cholesterol-4-C^{14} had been administered by mouth at least 3 weeks prior to the first stool collections; their diets were completely free of plant sterols. Under these conditions it may be assumed that each of the fecal neutral steroids would have the same specific activity after such long equilibration periods, and that any errors in GLC analysis of the three sterol fractions separated by TLC would be reflected in significant divergences of the specific activity data of the three fractions. The six fecal samples were purposely selected so as to present widely different proportions of Fractions I, II, and III in each sample, but to all 18 subsamples the same amount of 5α-cholestane was added as GLC internal standard. Thus, in Table 4 (column 2) the area response of 5α-cholestane in each case was adjusted to 100, and the areas of the three fractions were related to this. Specific activities of all fractions were similarly normalized, assigning 100 to that of cholesterol in Fraction III. Despite great differences between relative mass responses of Fractions I, II, and III in the six samples, the specific activities of the three subfractions were approximately the same (columns 6–8). The weights of the 18 different fractions varied over a 250-fold range, yet their specific activities were in close agreement. This supported the conclusion that our procedure for measurement of the various types of fecal neutral steroids by GLC was highly reliable.

Table 4 also confirmed our previous conclusion[2] that the hydrogen flame detector fails to "see" the TMS group of the TMS sterols in the effluent gas stream: although the ketonic steroids in Fraction I did not react with the silylating reagents, their specific activities were similar to those of the TMS ethers in Fractions II and III, despite the 25% heavier mass of the sterol ethers.

C. Validation of the Use of Radioactive Cholesterol as Internal Recovery Standard

Results were presented in Table 2 which demonstrated that radioactive cholesterol added to stool homogenates was quantitatively extracted from fecal homogenates. Moreover, in a large number of experiments the recovery of this internal standard at the end of the entire procedure (i.e., after subfractionation by TLC) was almost always better than 98%. However, it may be questioned whether the percentage correction for radioactive cholesterol in Fraction III applies equally to the recoveries of plant sterols in Fraction III and of steroids in Fractions I and II: application of the same correction factor for all three fractions implies that recoveries of coprostanol, coprostanone, and plant sterols after TLC are the same as those for cholesterol.

TABLE 4 VALIDATION OF GLC FOR QUANTIFICATION OF FECAL NEUTRAL STEROIDS

Analyses of six different fecal homogenates from three patients who had received cholesterol-4-C^{14} intravenously at least 3 weeks prior to stool collections; all were maintained on formula diets low in cholesterol (50 mg/day) and free of plant sterols.

Stool Sample	5α-cholestane	Relative Area Responses: Fraction III (TMS cholesterol)	Relative Area Responses: Fraction II (TMS coprostanol)	Relative Area Responses: Fraction I (coprostanone)	Relative Specific Activities: Fraction III	Relative Specific Activities: Fraction II	Relative Specific Activities: Fraction I
A-1	100	161	120	2	100	107	108
A-2	100	156	88	4	100	108	102
B-1	100	151	152	5	100	99	103
B-2	100	555	432	14	100	96	93
C-1	100	70	189	50	100	106	102
C-2	100	16	742	241	100	91	99
Mean	—	—	—	—	100	101	101

That cholesterol serves as a reliable recovery standard for all fecal steroids was supported by the following findings. When PE extracts of saponified fecal or diet homogenates contained large amounts of soaps or a precipitate at the interface, the mechanical transfer of steroids to TLC plates was seriously hindered. In these unusual cases the recoveries of radioactive internal standard might fall as low as 40–60%, and the recoveries of standard in members of duplicate analyses were often grossly different. Yet after the application of the appropriate (but different) correction factors, the quantitative data for total neutral steroids in the duplicates checked within ±5%. It was even more significant that in such hindered situations the replications between corrected duplicates were excellent whether the fecal neutral steroids were found predominantly in Fractions I, II, or III. These considerations made it seem probable that during transfer to TLC plates and during desorption after TLC, the recovery of cholesterol as internal standard was representative of that of the components in all three steroid fractions.

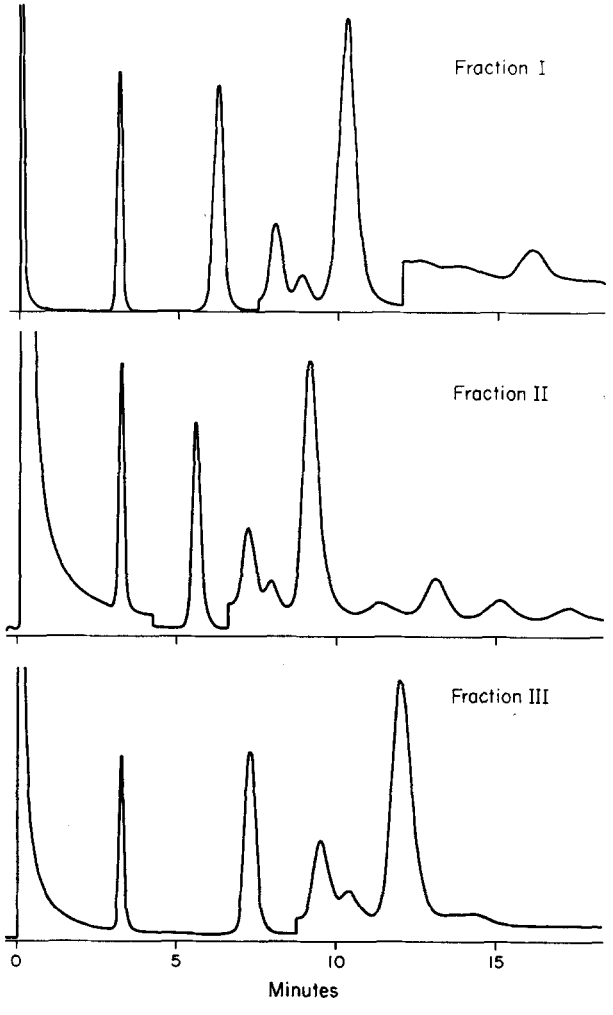

FIG. 3. GLC patterns of three TLC fractions of fecal neutral steroids when diet contains corn oil sterols.

Operating conditions held constant (1% DC-560, 240°, 30 ml/min N_2), so that internal standard (5α-cholestane, 1st component in all patterns) had same absolute retention in all runs. Successive peaks in Fraction III = cholesterol, campesterol, stigmasterol, β-sitosterol (ring-saturated, 5α-homologs not separated from Δ^5-compounds); in Fraction II = coprostanol and 5β-homologs of plant sterols in Fraction III (in same order); in Fraction I = coprostanone and 3-keto homologs of plant sterols in Fraction II (in same order).

Minor components eluting after β-sitosterol in Fraction III (or its related conversion products in Fraction II and Fraction I) have not been identified.

Radioactive cholesterol cannot be added as internal recovery standard to homogenates of feces labeled in vivo with both C^{14}- and H^3-cholesterol. But in such cases the endogenous label served as an ideal internal standard, since as shown in Table 2 the quantitative extraction of neutral steroids from fecal homogenates is a dependable baseline. By comparing the radioactivity recovered after TLC to that from an aliquot of the original extract, the losses during TLC could be readily calculated.

D. Applications of Present Method in Studies of Excretion of Neutral Steroids

Figure 3 shows the GLC patterns of fecal neutral steroids from Fractions I, II, and III from a fecal homogenate of a patient on a diet containing plant sterols from corn oil. The three GLC runs were carried out under identical operating conditions: the retention time of the internal standard, 5α-cholestane, was the same in the three runs. The need for separating Fraction III from II and I by TLC, when plant sterols are present in the diet, is made clear by comparing the GLC retention times of the various components in these three runs. Without this preliminary TLC separation, deceptive overlapping of cholesterol in Fraction III by two major plant sterol metabolites of Fraction II (5β-campestanol and 5β-stigmastenol) would make impossible the measurement of cholesterol in the feces of patients fed plant sterols, and thus prevent the separate evaluation of cholesterol and plant sterol balances. There were no serious overlaps of plant and animal steroids between Fraction I and the other fractions.

In clinical studies of sterol balance in which quantification was essential but qualitative data were not desired, it was permissible and desirable to analyze Fractions I + II + III as a single pool when patients were on diets free of plant sterols, since all the GLC peaks were produced by cholesterol and its neutral steroid conversion products. However, when the feces also contained the metabolic products of plant sterols, separate analyses were required for I + II and for III, in order to quantify separately the neutral steroids derived from cholesterol and those derived from the plant sterols. With this ap-

proach seven fecal homogenates were analyzed in duplicate. The reproducibility of the results was excellent: the standard deviation of the differences from the means of the seven pairs of duplicates was ±3.6% for cholesterol plus its metabolites, and ±2.0% for the plant sterols plus their metabolites.

A detailed description of neutral sterol balances in one patient is shown in Table 5, in which intakes and outputs of the various sterols and their metabolic products are listed. Fractions I, II, and III were separately analyzed by GLC, and in addition the ring-saturated 5α-sterols of Fraction III were isolated by TLC on silver nitrate-impregnated Silica Gel G plates. During this 27-day stool collection period, the patient ingested 71 mg of cholesterol per day and excreted 364 mg of cholesterol metabolites, and was therefore in net negative balance for cholesterol of 293 mg/day. The plant sterol data showed a net positive balance of 38 mg/day. Further, it is interesting to note that the ratio of the three plant sterols (and their conversion products) in the feces was the same as that in the diet (campesterol:stigmasterol:β-sitosterol = 18:7:75%). This similarity suggested that intestinal bacteria had attacked the three plant sterols nonselectively.

Measurements of dietary sterols were carried out in the same way as for fecal neutral steroids. In many vegetable fats most of the sterols were found to be either Δ^5- or ring-saturated 5α-derivatives, all with retention times greater than that of cholesterol. For such fats only Fraction III need be analyzed by GLC. However until further definitive studies of dietary sterols are reported, it seems necessary to verify the absence of sterols in Fractions I and II by GLC for each new dietary fat.

E. Precautions

The precision of the present procedure depends on the efficiency with which Fractions I and II are separated from III by preparative TLC. Incomplete separations are caused by poor preparation of TLC plates, by overloading the plates with steroids and/or contaminating materials in the PE extracts, by uneven application of the samples to the plates, or by such large ratios of coprostanol- to cholesterol-type compounds that solutes in Fraction II trail into Fraction III. Although a reasonable correction for these overlaps can be made by quantifying the coprostanol in Fraction III by GLC and by determining the amount of internal standard (radioactive cholesterol) which contaminates Fraction II, it is usually preferable to repeat the TLC separation in order to obtain the optimal separations of the three fractions shown in Fig. 2.

DISCUSSION

Two important features of the procedure should be stressed. (*a*) Successful separation of cholesterol from plant sterol conversion products by GLC depended upon

TABLE 5 EXAMPLE OF DAILY NEUTRAL STEROL BALANCES FOR CHOLESTEROL AND PLANT STEROLS

Analyses of a 27-day stool collection from patient J. F., 39-yr-old hypercholesteremic male, maintained at constant body weight (78 kg) by feeding liquid formula (2400 cal/day) containing 40% of calories as low-sterol corn oil (prepared by molecular distillation)

Components	Sterol Groups*				
	Cholesterol Group	Campesterol Group	Stigmasterol Group	β-Sitosterol Group	Total Plant Sterols
Δ^5-sterols†					
Intake (*mg/day*)	71.3	51.9	21.8	220.6	294.3
Distribution (%)		18	7	75	100
Output derived from Δ^5-sterols (*mg/day*)	363.9	47.4	17.3	195.0	259.7
% Δ^5, 3-OH	68	19	7	74	100
% 5β, 3-OH‡	30	19	6	75	100
% 5β, 3-keto§	2	17	6	77	100
5α-sterols‖					
Intake	0.7	5.4	1.0	16.3	22.7
Output	7.1	4.7	1.1	13.7	19.5
Balance	−6.4	+0.7	−0.1	+2.4	+3.2
Total Daily Intake					
Δ^5 + 5α	72	57.3	22.8	236.9	317.0
Total Daily Output					
Δ^5 + 5α + 5β	371.0	52.1	18.4	208.7	279.2
Net Balance (*mg/day*)	−299.0	+5.1	+4.4	+28.2	+37.8

* Each group contained Δ^5, 5α- and 5β-compounds.
† Δ^5 = cholesterol and plant sterols with double bond at C_5.
‡ Coprostanol and related 5β-compounds originating from plant sterols.
§ Coprostanone and related 3-keto compounds originating from plant sterols.
‖ Cholestanol and related 5α-compounds originating from plant sterols isolated by TLC of Fraction III on silver-nitrate impregnated Silica Gel G plates.

TABLE 6 DAILY NEUTRAL STEROID EXCRETION IN MAN AND RATS

Comparison of results obtained since 1957 in various laboratories by several methods

Authors	Method of Measurement	Number and Clinical Status	Diet and Experimental Variables	Daily Fecal Neutral Steroid Excretion
I. Human Subjects				
				mg/24 hr
Gould and Cook (1958) (15)	(various authors, methods, and subjects between 1913 and 1929)		Solid foods (undefined)	250–380
Gordon et al. (1957) (16)	Liebermann–Burchard (L–B)	4 with various illnesses	Basal = low-fat solid foods	390
			Plus coconut oil(100g)	680
			Plus sunflower seed oil (100 g)	880
Ivy et al. (1957) (17)	Digitonin pptn; L–B	11 normals	Synthetic diet (sterol- and fat-free)	
		7 males		486*
		4 females		307*
Kinsell et al. (1958) (18)	Not stated	6 with various diagnoses	Formula feeding	
			Fat-free	720–760
			Various fats (200 g)	740–1470
Engelberg (1959) (19)	Digitonin pptn.; L–B	3 normals	Ordinary solid foods (uncontrolled)	
		1 hypercholesteremic	Control period	276–662
			Plus heparin (intravenous)	319–693
Curran et al. (1959) (20)	Digitonin pptn.; L–B	5 normals	Solid foods (controlled repetitive menu: 531 mg sterols per day)	
			Control period	470–676
			Plus vanadium	236–778
Goldsmith et al. (1960) (21)	Glass paper chromatography and charring (33)	2 hypercholesteremics	Solid foods (controlled)	
			50 g of mixed fat	500–1200
			90 g of butter fat	500–1300
			90 g of corn oil	1000–1800
			90 g of butter fat plus neomycin	600–700
Powell et al. (1962) (22)	Digitonin pptn.; L–B	8 normals	Solid foods (controlled) (33% fat calories; 690 mg sterols per day)	
		5	Control	755–790
		5	Plus neomycin	1270–1699
		5	Post-treatment	765
		3	Control	640–707
		3	Plus polymixin B and bacitracin	558–1326
		3	Post-treatment	927
Aylward and Wills (1962) (23)	Digitonin pptn.; L–B	2 normals 2 hyperthyroids 1 myasthenia gravis	Solid foods (uncontrolled)	388–758
Antonis and Bersohn (1962) (24)	Digitonin pptn.; L–B	58 normals (29 white, 29 Bantu)	Solid foods (controlled)	
			40% fat calories, high fiber	
			butter	367
			sunflower seed oil	895
			40% fat calories, low fiber	
			butter	193
			sunflower seed oil	500
			15% fat calories	
			high fiber	314
			low fiber	645
Moore et al. (1962) (25)	Isotopic balance (34)	5 normals	Solid foods (controlled) (40% fat calories)	
			butter	482*
			safflower oil	584*
Haust and Beveridge (1963) (26)	Spectrophotometric	1 normal 1 diabetic	Formula feeding	
			fat-free	232–377†
			corn oil (60% of calories)	1375–1450†
Miller et al. (1962) (27)	Glass paper chromatography and charring (33)	1 hypercholesteremic	Solid foods (115 g animal fats)	
			control period	400–750
			plus nicotinic acid	600

(*Continued*)

* These values represent cholesterol (and its neutral steroid conversion products) of endogenous origin.

† These values represent cholesterol (and its neutral steroid conversion products) distinguished from plant sterols (and their conversion products).

TABLE 6 *Concluded*

Authors	Method of Measurement	Number and Clinical Status	Diet and Experiment Variables	Daily Fecal Neutral Steroid Excretion
Eneroth et al. (1964) (2)	GLC of free sterols	6 normocholesteremic 1 hypercholesteremic	Solid foods (controlled: 51% fat calories) butter corn oil	 527† 614†
Spritz et al. (1965) (7)	Florisil column, TLC and gravimetry plus isotopic balance (34)	1 normocholesteremic 2 hypercholesteremic 1 hyperglyceridemic (CHO-type)	Formula feeding (40% fat calories cholesterol-free) saturated fats unsaturated fats	 498–531*† 504–538*†
II. Rats				*mg/kg body wt per 24 hr*
Coleman and Baumann (1957) (28)	Silicic acid column; L–B	12 6 male 6 female	Synthetic diet (fat-free)	25 12
Chevallier (1960) (29)	For total: digitonide weight For endogenous: isotopic balance	7 7	Synthetic diet (20% fat calories) plus 0.025% cholesterol plus 0.52% cholesterol	 31 (endogenous plus others) 12* 130 (endogenous plus others) 22*
Gerson et al. (1961) (30)	Digitonin pptn.; $FeCl_3$ color reaction (35)	5 5	Synthetic diets fat-free 10% crude corn oil	 31 77
Bloomfield (1964) (31)	Isotopic balance (34)		Basal diet = fat-free with 0.64% cholesterol plus 20% safflower oil plus 20% butter	 28* 32*
Wilson (1964) (32)	For endogenous: isotopic equilibrium after implant of cholesterol-4-C^{14} For total: chol-H^3 feeding	6 5	Synthetic diet (16% fiber) sterol-free 0.3% cholesterol	 9* 94 (endogenous plus others) 22*
Miettinen and Ahrens[3] (1965)	For total: present method (isolation and GLC of TMS ethers) For endogenous: isotopic equilibrium after implant of cholesterol-4-C^{14} (32)	10	Synthetic (10% fat calories; sterol-free coconut oil)	13.5 (endogenous plus others) 8*

fractionating the total fecal neutral steroids by TLC into three structurally related groups, prior to the measurement of individual components by GLC. The combined use of TLC and GLC thus made it possible to carry out balance studies for cholesterol in patients fed diets which contained plant sterols. Our former dependence (7) on the feeding of sterol-free diets, or on differential in vivo isotopic labeling techniques, no longer exists. (*b*) Quantitative determination of fecal neutral steroids (as individuals or as total groups) was accomplished by GLC analysis of the ketonic steroids and of the TMS ethers of the sterols. The use of two internal standards, one for correction of losses through the multistep isolation procedure and the other for conversion of GLC peak areas into weights of neutral steroids, vastly improved the accuracy of the procedure.

Identification of free, unsubstituted neutral sterols by GLC has been reported by several laboratories. Quantification has been more difficult, however, owing to variable losses of free sterols during passage through the column, and to nonproportional detection of different sterols in the effluent gas stream in most GLC detection systems (8–13). Eneroth et al. (2), in a valuable study of fecal neutral steroids accomplished by GLC combined with mass spectrometry, stated that occasional QF-1 columns (out of the many they prepared) permitted the quantification of free sterols without corrections for losses, but to date we have not been able to carry out

satisfactory quantitative analysis of free sterols on any of the column packings studied.

On the other hand, GLC of the TMS ethers of neutral sterols, first introduced by Luukkainen, VandenHeuvel, Haahti, and Horning (14) for GLC of steroid hormones and for sterols by Wells and Makita (10), has proven in our hands to be even more satisfactory than these workers may have realized. After an extensive study of the formation of TMS derivatives with neutral sterols and bile acids and of the detection of a wide variety of TMS ethers of steroids by hydrogen flame ionization detector, we have strong evidence[2] for four conclusions: (*a*) the formation of derivatives with all hydroxyl groups at positions 3, 6, 7, and 12 was rapid and quantitative; (*b*) 3-ketosteroids were prevented from forming undesirable secondary products in the presence of silylating reagents by careful control of reaction conditions; (*c*) no losses of TMS sterols, TMS bile acid methyl esters, ketonic steroids, or 5α-cholestane during GLC could be detected, under various conditions of temperature and gas flow and with all stationary phases studied; and (*d*) with the hydrogen flame detector the ionization responses of TMS derivatives of various cholane, cholestane, and coprostane compounds—with 0, 1, 2, or 3 TMS groups—were directly proportional to the absolute weights of the parent *un*substituted steroids (and *not* of the derivatives themselves). Thus, all acidic and neutral TMS sterols and ketonic steroids, as well as 5α-cholestane, produced essentially the same ionization response per unit mass of parent unsubstituted steroid. Besides greatly simplifying the calculation of GLC results, these findings made it possible to quantify a complex mixture of neutral steroids as a group, by relating their total area to that of an appropriate internal standard. It was unnecessary to apply a separate correction factor for each component in the mixture, as would be required when other detection systems are used.

Table 6 lists the daily rate of excretion of fecal neutral steroids measured in several laboratories with a variety of methods in studies of various species, as well as representative data obtained by the present procedure in man and in rats. This summary is presented for completeness' sake, even though we have considerable reservations about the validity of some of the results obtained. For instance, in experiments in progress in six hypercholesteremic patients we have found by the present method that the daily excretion of fecal neutral steroids ranged from 328 to 746 mg; these patients were maintained at constant body weight on formula diets, with cholesterol intakes lower than 100 mg/day and with fat intakes equivalent to 40% of total calories. While the observations of some of the laboratories listed in Table 6 are in accord with these findings, others report considerably higher values. Some part of the discrepancy seems to be due to methodologic differences. It is generally recognized that colorimetric analyses based on the Liebermann–Burchard reaction, with or without digitonide formation, are inaccurate to the extent that the chromogens produced from various sterols have widely different extinction coefficients. Measurements of digitonin-precipitable sterols are also made uncertain by the different solubilities of digitonides; coprostanol, for instance, is incompletely precipitated by digitonin (36). Moreover, this approach fails to distinguish the fecal steroids derived from cholesterol and those of plant sterol origin. When large amounts of cholesterol precursors are present, as in rat feces,[3] these compounds cannot be differentiated from plant sterols or cholesterol or their neutral conversion products. The photometric procedures described by Haust and Beveridge (37, 38) are claimed to quantify cholesterol, coprostanol, and β-sitosterol in reference mixtures; nevertheless, for measurements of the complex mixtures of neutral steroid conversion products present in feces this method seems inadequate. Indeed, none of the photometric procedures measures the ketonic neutral steroids, which can form as much as one-third of the total fecal neutral steroid fraction.

Goldsmith, Hamilton, and Miller (21) developed a method for isolation of the fecal neutral sterols and ketonic steroids on silicic acid-impregnated glass paper. The lipid spots were charred with sulfuric acid and measured by densitometry. Their separations of neutral steroids on glass paper are similar to those we produce by TLC, but their method fails to distinguish the plant sterol conversion products from those derived from cholesterol, thus restricting its use to feces of patients or animals fed diets devoid of plant sterols.

The isotopic balance technique introduced in 1957 by Hellman, Rosenfeld, Insull, and Ahrens (34) has been used by Moore, Anderson, Keys, and Frantz (25) in man and by Chevallier (29) and Wilson (32) in rats. This method is based on the assumption that, after radioactive cholesterol is administered orally or parenterally, fecal steroids reach and maintain for many weeks approximately the same specific activity as plasma cholesterol. This assumption implies that all fecal neutral steroids have been derived from pools which are in rapid isotopic exchange with plasma cholesterol and thus have the same specific activity. On the basis of previous studies (7) and others more recently carried out in this laboratory[4] on patients who were maintained for many weeks on cholesterol-free diets, this assumption appears to be true in man: in four patients 46 separate determinations of fecal neutral steroid specific activity produced values which were nearly identical with those

[4] Spritz, Ahrens, Grundy, and Mietinen, unpublished data.

of plasma cholesterol sampled simultaneously. In the rat, on the other hand, this correspondence was not found:[3] specific activities of fecal neutral steroids were consistently and significantly lower than those of plasma cholesterol, whether labeling was carried out by a single dose of radioactive cholesterol or during the isotopic steady state achieved by Wilson's (32) implantation technique. Thus, in the rat the basic assumption is invalid, so that the isotopic balance technique cannot be used in this species for measuring total fecal neutral steroids.

In man, a reasonable estimate of total fecal neutral steroids of endogenous origin can be made simply by measuring the radioactivity in a fecal extract containing neutral steroids, and then translating this figure into milligrams by using the specific activity of plasma cholesterol obtained simultaneously. The method has the advantage of greater simplicity than the present purely chemical procedure, but there are four disadvantages which must be noted. Until the long-term hazards of the use of radioactive compounds in patients, especially in younger age groups, have been adequately defined, the use of the isotopic balance technique for research purposes may not seem warranted. In patients in whom the use of a radioisotope can be justified, its use for purely methodologic purposes may preclude its application in studies of an interesting physiologic problem. The isotopic balance method accounts only for steroids of endogenous origin; the effects of exogenous cholesterol and plant sterols on the excretion of endogenous steroids are usually overlooked. Finally, there may be a significant delay between the excretion of labeled cholesterol into the duodenum and its final excretion in the feces. This necessitates the use of a correction factor to compensate for the delay; the factor probably varies from patient to patient and from day to day in any one patient.

Recently Spritz, Ahrens, and Grundy (7) described the results of 5 sterol balance studies which were performed with an earlier version of the present procedure. With that method it was possible to obtain quantitative isolation and determination of all neutral steroids as a single group, but since cholesterol and plant sterol conversion products in the feces could not be measured separately, sterol balances in three studies were determined while patients were on sterol-free diets. In two other studies the diets contained plant sterols, and in these cases the isotopic balance technique was applied in addition to the chemical isolation method in order to measure total fecal neutral steroids of endogenous origin.

The present procedure has several advantages over our earlier approach: (*a*) no need for sterol-free diets, (*b*) no need for in vivo radioisotopic labeling for purely quantitative purposes, (*c*) greater simplicity at several stages in the procedure, even though the precision and reproducibility are greatly enhanced, (*d*) greater sensitivity, so that precise analyses can be carried out on smaller samples, thus permitting sterol balance studies to be performed on individual laboratory animals, and (*e*) reservation of in vivo radioisotopic labeling for application to strictly physiologic questions.

With the techniques described in this and the accompanying paper (1), it is practical for two technicians to make 12–18 measurements of neutral and acidic steroids each week. The two reports describe how the total neutral and total acidic steroids can be measured accurately in fecal samples containing as little as 50 μg of acidic steroids and 25 μg of neutral steroids per gram of feces, but in addition both procedures have been readily scaled up for isolations on the preparative scale needed for more detailed structural studies.

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