papers and notes on methodology

A simplified micro-method for quantification of fecal excretion of neutral and acidic sterols for outpatient studies in humans

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Abstract A simple and precise micro-method for measurement of daily fecal excretion of neutral and acidic sterols has been developed which utilizes sitostanol (24-ethyl-5α-cholestane-3β-ol) as fecal flow and recovery marker. Extractions of sterols were performed from 50 μl of fecal homogenate (feces–water 1:1), and analyses of neutral and acidic sterols were carried out by gas–liquid chromatography. The method is sensitive, precise, and easy to perform; the intra-assay variability yielded coefficients of variations of 1.9% and 3.5% (n=6) for neutral and acidic sterols, respectively. The results from this method were compared with those obtained with the standard fecal flow marker chromic oxide. The correlation coefficients between the two markers were compared in 16 subjects and were 0.938 and 0.998 for excretion of neutral sterols and acidic sterols, respectively. Comparison of the fecal excretion of neutral and acidic sterols in 12 subjects determined from frozen samples and aliquots (≈1 g) sent by ordinary mail to the laboratory (transport time 1 to 5 days) gave identical results using sitostanol as fecal flow marker (818 ± (SEM) 85 mg/day vs. 838 ± 89 mg/day for neutral sterols and 417 ± 59 mg/day vs. 414 ± 60 mg/day for acidic sterols). The new micro-method is ideally suited for research laboratories in need of a simple, accurate, inexpensive, and high through-put method for measuring daily fecal excretion of neutral and acidic sterols, as well as total cholesterol synthesis, and can be performed on an outpatient basis. —**Czubayko, F., B. Beumers, S. Lammsfuss, D. Lütjohann, and K. von Bergmann.** A simplified micro-method for quantification of fecal excretion of neutral and acidic sterols for outpatient studies in humans. *J. Lipid Res.* 1991. **32:** 1861–1867.

Supplementary key words sitostanol • chromic oxide • fecal balance • gas–liquid chromatography

Measurements of total cholesterol synthesis together with bile acid synthesis have been performed for over 25 years, primarily by quantifying the fecal excretion of neutral and acidic sterols (1–5). The results have yielded important information on the physiological regulation of cholesterol and bile acid metabolism (3–8) and on the effect of drugs that influence cholesterol homeostasis (9–11). However, these balance studies are time-consuming and require metabolic ward conditions; they are therefore costly and usually limited to small numbers of subjects. A more practicable method is needed to investigate more subjects, particularly on an outpatient basis, with less time-consuming laboratory handling. In the present report we describe a simplified, accurate micro-method for determining the excretion of fecal sterols. This new method introduces sitostanol (24-ethyl-5α-cholestane-3β-ol) as a fecal flow marker and is suitable for outpatient studies. This means that cholesterol and bile acid synthesis can be measured in a large numbers of subjects, which makes it especially advantageous for physiological studies in humans.

MATERIALS AND METHODS

Experimental design

In 16 healthy subjects (6 females and 10 males; age range 22 to 30 years), the fecal excretion of neutral and acidic sterols was determined by the fecal balance method (1, 2). The subjects received two different fecal markers for 7 days to correct for variations in fecal flow. The first marker was chromic oxide (60 mg t.i.d.), and the second was sitostanol (30 mg t.i.d.; purity: 91% sitostanol, 8% campestanol; Delalande Arzneimittel GmbH, Köln, Germany). Both markers were given together in stomach-soluble capsules. Two experiments were scheduled, each of 1 week duration. In the first experiment 16 subjects collected total fecal samples separately for 3 days (days 5 to 7) in plastic cans and the samples were frozen immediately at −20°C in portable deep-freeze boxes at home (Engel 10, model MHFD-010-A, Sawatuji Electric Co., Tokyo,

Abbreviations: TMS, trimethylsilyl; GLC, gas–liquid chromatography.

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Japan). The freeze boxes containing the plastic cans were returned to the laboratory. Four of the subjects received cholestyramine (Quantalan®) 4 g b.i.d. for 7 days. In the second experiment 6 of the 16 subjects, plus 6 additional volunteers who received only sitostanol (30 mg t.i.d.) as fecal marker, collected total fecal samples as described above. In addition, one aliquot (≈1 g) was placed in a plastic tube with a stopper containing a small spoon (Saarstedt Nr. 80623022, Nümbrecht, Germany) and the tubes were sent back to the laboratory by ordinary mail (transport time between 1 and 5 days). Six volunteers collected small aliquots (≈1 g) as described above over 6 consecutive days before they began fecal marker intake. Sitostanol and neutral sterols were measured and day-to-day variation in baseline fecal excretion of sitostanol was calculated. In addition, the homogenous distribution of sitostanol with the other fecal sterols was checked in 6 subjects. Neutral and acidic sterols were measured in three small fecal specimens (≈1 g) taken from three different sides of one bowel movement prior to homogenization.

During the study the subjects were on their regular, mixed solid-food diet. All subjects kept a 7-day food diary from which their cholesterol intakes were calculated by a commercial computer program (12).

The research protocol was approved by the local ethics committee and all subjects gave informed consent.

Determination of neutral and acidic sterols in total fecal samples (macro-method)

Neutral and acidic sterols were determined according to Grundy, Ahrens, and Miettinen (1), Miettinen, Ahrens, and Grundy (2), and Miettinen (13) with minor modifications. Total fecal samples from each day were thawed overnight and homogenized with distilled water (1:1, w/w). One mg 5α-cholestane (Serva Feinbiochemica, Heidelberg, Germany) and 1 mg hyodeoxycholic acid (Sigma Chemical Co., St. Louis, MO) were added to exactly 1.0 g of homogenate as internal standards for neutral and acidic sterols, respectively. After a 1-h mild alkaline hydrolysis with 10 ml 1N NaOH in 90% ethanol in a water bath at 67°C, the samples were cooled to room temperature. Five ml of water was added and the neutral sterols were extracted three times with 10 ml cyclohexane. The combined cyclohexane phases were evaporated to dryness under a stream of dry N_2 and the neutral sterols were converted to their trimethylsilyl(TMS)-ethers by adding 1.5 ml TMS-reagent (dry pyridine–hexamethyldisilazane–trichlorosilane, 9:3:1). After 30 min at room temperature, the mixture was evaporated to dryness under dry N_2. The TMS-derivatives were dissolved in 2 ml of n-decane, and after a 10-min centrifugation at 2000 U/min, 1.0 ml was transferred to vials for gas–liquid chromatographic (GLC) analysis. Two ml 10 N NaOH was added to the lower aqueous phase and the mixture was heated for 3 h at 120°C. After addition of 5 ml H_2O and cooling to room temperature, the samples were acidified to pH < 1.5 with 25% HCl, and the acidic sterols were extracted three times with 10 ml diethyl ether. The combined ether phases were evaporated to dryness under N_2. Methylation was performed by adding 2 ml dried methanol, 1.4 ml dimethoxypropane, and 20 μl concentrated HCl. The samples were mixed thoroughly and allowed to stand at room temperature for at least 1 h (14). After evaporation to dryness the bile acids were derivatized to their respective TMS-ethers as described for neutral sterols. Two ml of n-decane was added and, after centrifugation, 1.0 ml was transferred to vials for GLC analysis.

Determination of neutral and acidic sterols in aliquots (≈1 g) of fecal samples sent by mail (micro-method)

When the fecal samples arrived at the laboratory by mail they were stored at −20°C. Before analysis the samples were thawed and homogenized (Ultra-Turrax T 25, Janke & Kunkel Labortechnik, Staufen, Germany) with distilled water (1:1, w/w) in their original plastic tubes. Fifty μg 5α-cholestane and 50 μg hyodeoxycholic acid were added as internal standards to exactly 50 μl of the homogenate. All subsequent steps were similar to those described for the macro-method; however, 90% less solvent material was required.

GLC analysis of neutral and acidic sterols

GLC of fecal neutral and acidic sterols was carried out on a Hewlett Packard gas chromatograph model 5809, using an automatic on-column injection technique (automatic injector model HP 7573 A, Hewlett Packard). The chromatograph was equipped with a hydrogen flame ionization detector. Hydrogen was used as carrier gas at flow rates of 2 to 3 ml/min. Neutral and acidic sterols were separated on a 50-m fused silica capillary column (CS-FS-OV-101, inner diameter 0.32 mm; Chrompack, Middelburg, Netherlands). For optimal separation of the relevant compounds, different temperature programs were selected for neutral and acidic sterols (neutral sterols: 3 min at 150°C, 10°C min^{-1} to a final temperature of 270°C; inlet pressure 11 psi. Acidic sterols: 3 min at 150°C, 30°C min^{-1} to 240°C (15 min), then 3°C min^{-1} to a final temperature of 270°C; inlet pressure 15 psi). Representative GLC runs of both neutral and acidic sterols are shown in **Fig. 1.** Peak area integration was done by an electronic integrator (model SP 4290, Spectra Physics, San Jose, CA) equipped with the WINner software from Spectra Physics. Concentrations of different sterols were calculated from a standard curve prepared from the appropriate neutral sterols (coprostanol, coprostanone, cholestanol, cholesterol, sitosterol, and sitostanol) and acidic sterols (lithocholic acid, isolithocholic acid, deoxycholic

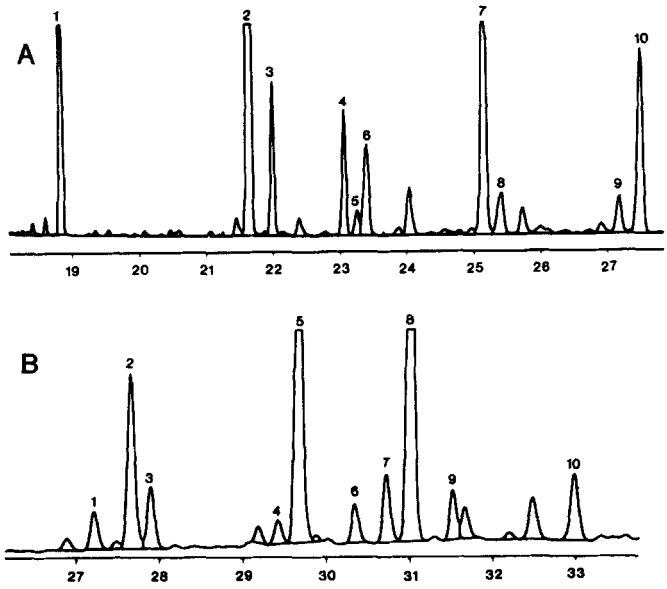

Fig. 1. A: GLC of neutral sterols and plant sterols from human feces. Identity of peaks: 1, internal standard (5α-cholestane); 2, coprostanol; 3, coprostanone; 4, cholesterol; 5, cholestanol; 6, methylcoprostanol; 7, ethylcoprostanol and campesterol; 8, ethylcoprostanone and campestanol; 9, sitosterol; 10, sitostanol. B: GLC of acidic sterols from human feces. Identity of peaks: 1, isolithocholic acid; 2, lithocholic acid; 3, isochenodeoxycholic acid; 4, isodeoxycholic acid; 5, deoxycholic acid; 6, chenodeoxycholic acid; 7, cholic acid; 8, internal standard (hyodeoxycholic acid); 9, ursodeoxycholic acid; 10, 7-ketolithocholic acid.

acid, isodeoxycholic acid, chenodeoxycholic acid, cholic acid, ursodeoxycholic acid, and 7-keto-lithocholic acid). We obtained an almost identical, 1 to 1 response for all neutral sterols using 5α-cholestane and for all acidic sterols using hyodeoxycholic acid, when the standards showed a purity greater than 98%.

Determination of chromium

Chromic oxide (Cr_2O_3) was supplied by Merck (Darmstadt, Germany) and chromium was determined according to Calvert et al. (15) with some modifications. Precisely 1.5 g of homogenized fecal samples was placed in porcelain crucibles and dried at 150°C. To ensure the complete oxidation of the organic materials, the fecal samples were completely ashed overnight at 600°C in a muffle oven (Naber, Bremen, Germany). After cooling to room temperature, the ashed residues were carefully pulverized and quantitatively transferred to 50-ml Erlenmeyer flasks. To convert the insoluble Cr (III) into soluble Cr (VI), 2 ml 85% H_3PO_4 and 3.5 ml 4.5% $KBrO_3$ were added to each sample and mixed thoroughly. The flasks were covered with watch-glasses and heated over a flame to the boiling point. The reaction was completed when the solutions had turned yellow-orange without visible bromine vapor. After cooling to room temperature the samples were transferred quantitatively to 100-ml volurimetric flasks and distilled water was added to exactly 100 ml. The solutions were allowed to stand for at least 24 h. Insoluble material was allowed to settle to the bottom of the flask. The absorbance of the solutions was read by a spectrophotometer (Syva, Darmstadt, Germany) at 340 nm; an appropriate blank was also determined and subtracted from the samples. Chromium content was determined from a standard curve of 0.25 mg to 2.5 mg Cr/dl.

Calculation of neutral and acidic sterol excretion using chromic oxide as fecal marker

Concentrations of chromium, neutral sterols, and acidic sterols in feces were calculated per gram of fecal homogenate and daily neutral and acidic sterols in feces were calculated per gram of fecal homogenate and daily neutral and acidic sterol excretion was calculated according to the following equations:

$$\text{Neutral Sterols (mg/day)} = \frac{\text{Neutral Sterols (mg/g sample)}}{\text{Cr (mg/g sample)}} \times \text{Cr (mg/day)} \qquad \textit{Eq. 1}$$

$$\text{Acidic Sterols (mg/day)} = \frac{\text{Acidic Sterols (mg/g sample)}}{\text{Cr (mg/g sample)}} \times \text{Cr (mg/day)} \qquad \textit{Eq. 2}$$

Calculation of neutral and acidic sterol excretion using sitostanol as fecal flow marker

$$\text{Neutral Sterols (mg/day)} = \frac{\text{Neutral Sterols (μg/sample)}}{\text{Sitostanol (μg/sample)}} \times \text{Sitostanol* (mg/day)} \qquad \textit{Eq. 3}$$

$$\text{Acidic Sterols (mg/day)} = \frac{\text{Acidic Sterols (μg/sample)}}{\text{Sitostanol (μg/sample)}} \times \text{Sitostanol* (mg/day)} \qquad \textit{Eq. 4}$$

*Sitostanol (mg/day) = marker sitostanol (90 mg/day) + baseline sitostanol (Eq. 5) (mg/day).

Small amounts of sitostanol are consumed with the normal Western diet (16, 17) and excreted with the feces. In order to correct for baseline fecal excretion of sitostanol, the concentration of this plant sterol was analyzed in a fecal specimen just before the first administration of the marker (day 0). Daily fecal excretion was then approximated according to the following calculation, where Neutral Sterols* (mg/day) are calculated according to Eq. 3 using the amount of marker only (90 mg/day).

$$\text{Basic Sitostanol (mg/day)} = \frac{\text{Sitostanol (μg/sample; day 0)}}{\text{Neutral Sterols (μg/sample; day 0)}} \times \text{Neutral Sterols* (mg/day)} \qquad \textit{Eq. 5}$$

Meanwhile, the basic fecal excretion of sitostanol was determined in over 200 subjects and ranged from 10 to 25 mg/day (17.5 ± 4.5 mg/day; mean ± SD).

Statistical analysis

All statistical calculations were done on an IBM computer model PS/2-80 with statistical software SSPS+. When not otherwise mentioned, the Student's *t*-test for paired observations was used. Linear regressions were calculated by the method of least squares.

RESULTS

Fecal excretion of neutral sterols, acidic sterols, and total fecal sterols using chromic oxide and sitostanol as flow marker

A graphic comparison of the two methods is presented in **Fig. 2.** Excretion rates with sitostanol as fecal flow marker were highly correlated to results calculated by chromic oxide, and linear regression analysis showed coefficients of correlation of 0.938 for neutral sterols, 0.997 for acidic sterols, and 0.984 for total fecal sterols. For the group as a whole, a small but statistically significant difference between the two markers was observed (**Table 1**): −4.6% ($P < 0.05$) for neutral sterols, −3.3% (n.s.) for acidic sterols, and −4.1% ($P < 0.05$) for total fecal sterols.

Fecal excretion of neutral sterols, acidic sterols, and cholesterol synthesis determined by macro- and micro-methods

No difference could be detected in the daily fecal excretion of neutral sterols, acidic sterols, and total fecal sterols, and cholesterol synthesis in 12 subjects, no matter whether the samples were analyzed from specimens immediately frozen (macro-method) or from small aliquots sent by ordinary mail (micro-method) using sitostanol as marker (**Table 2**). Coefficients of correlation for all 35 fecal samples analyzed by the macro- and micro-methods showed excellent agreement (0.984 for neutral sterols and 0.972 for acidic sterols, $P < 0.001$).

Baseline fecal excretion of sitostanol and distribution of sitostanol in feces

Baseline fecal excretion of sitostanol was measured in six volunteers over 6 consecutive days and the results showed a small intra-individual variation (10 ± 1 mg/day; 19 ± 3.8 mg/day; 26 ± 5.4 mg/day; 28 ± 5.4 mg/day; 30 ± 4.6 mg/day; 28 ± 5.2 mg/day; mean ± SD). When baseline sitostanol was added (90 mg/day of marker sitostanol) the coefficients of variation (CV = SD/mean × 100) ranged from 0.1 to 4.6%. Results of neutral and acidic sterol excretion measured from the three small fecal specimens (≈1.0 g) taken from three different sides of one bowel movement prior to homogenization of six subjects who received sitostanol as fecal marker (30 mg/day t.i.d.) showed a sample-to-sample variation that was very small (**Table 3**). Coefficients of variation ranged from 2.8 to 5.6% for neutral sterols and from 1.1 to 7.0% for acidic sterols, indicating a homogenous distribution of sitostanol with the other fecal sterols.

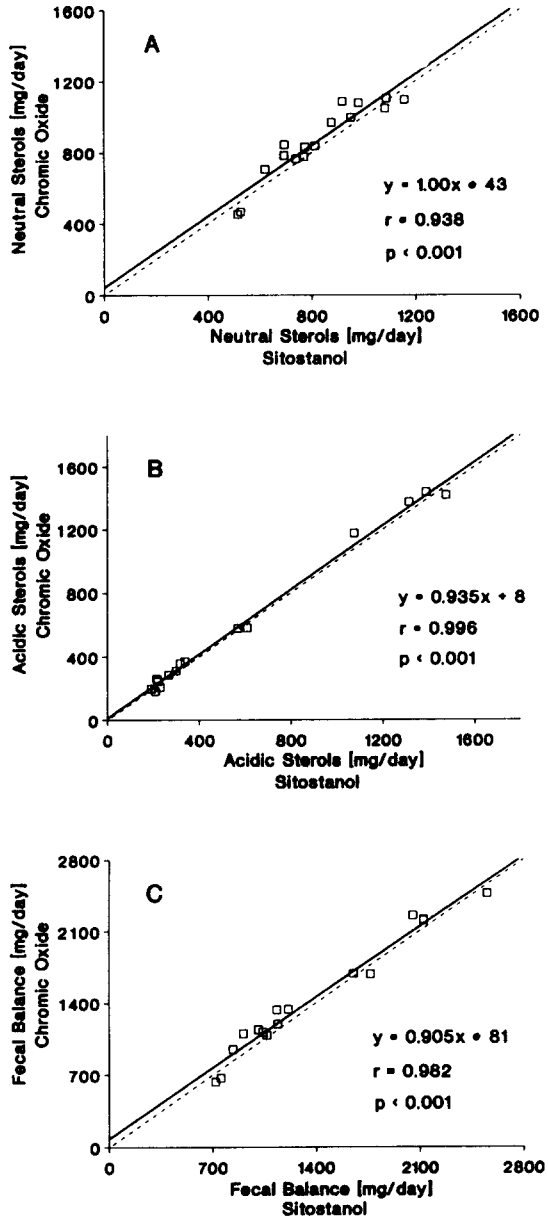

Fig. 2. Paired comparison between fecal excretion of neutral sterols (A), acidic sterols (B), and fecal balance (C, total fecal sterols) in 16 subjects, calculated with chromic oxide and sitostanol as fecal flow markers. The broken line represents identity between the two methods. The solid line demonstrates their relationship by linear regression.

TABLE 1. Fecal excretion of neutral sterols, acidic sterols, and total fecal sterols in 16 subjects using chromic oxide and sitostanol as markers

Subject	Neutral Sterols		Acidic Sterols		Total Fecal Sterols	
	C[a]	S[a]	C	S	C	S
	mg/day (mean ± SD)[b]					
1	966 ± 239	874 ± 43	371 ± 124	337 ± 71	1337 ± 332	1211 ± 41
2	996 ± 124	950 ± 54	196 ± 57	189 ± 61	1192 ± 159	1139 ± 115
3	1093 ± 31	1155 ± 119	580 ± 41	609 ± 55	1673 ± 71	1765 ± 170
4	783 ± 197	692 ± 155	358 ± 155	315 ± 128	1141 ± 171	1007 ± 104
5	1101 ± 300	1086 ± 95	578 ± 178	567 ± 70	1679 ± 478	1653 ± 164
6	450 ± 36	512 ± 10	180 ± 62	206 ± 78	630 ± 54	718 ± 72
7	1083 ± 185	917 ± 37	247 ± 35	217 ± 76	1330 ± 156	1134 ± 112
8	466 ± 14	525 ± 35	204 ± 73	227 ± 67	670 ± 74	752 ± 36
9	775 ± 237	768 ± 215	310 ± 195	297 ± 146	1084 ± 202	1065 ± 69
10	843 ± 119	693 ± 49	258 ± 29	213 ± 16	1101 ± 137	906 ± 39
11	829 ± 111	772 ± 31	284 ± 142	265 ± 128	1113 ± 220	1038 ± 143
12	706 ± 118	618 ± 77	244 ± 26	217 ± 17	950 ± 135	835 ± 82
13	762 ± 115	737 ± 66	1435 ± 205	1387 ± 269	2197 ± 91	2124 ± 204
14	835 ± 143	811 ± 152	1373 ± 80	1312 ± 32	2208 ± 168	2124 ± 131
15	1075 ± 109	978 ± 18	1175 ± 196	1074 ± 259	2250 ± 171	2052 ± 240
16	1046 ± 65	1079 ± 17	1416 ± 80	1473 ± 211	2462 ± 23	2552 ± 228
Mean ± SEM	863 ± 52	823 ± 49[c]	575 ± 120	557 ± 118[d]	1438 ± 144	1380 ± 144[c]

[a]C, calculated with chromic oxide; S, calculated with sitostanol as fecal flow marker.
[b]Values represent the mean of 3 consecutive days.
[c]Significantly different from C ($P < 0.05$).
[d]Borderline significance (P between 0.10 and 0.05).

Precision of the method

A comparison of the two methods was carried out by a sixfold analysis from the same fecal specimen from one subject who received cholestyramine (4 g b.i.d.). There was no difference in the results (chromic oxide vs. sitostanol: 687 ± 26 (SD) vs. 680 ± 13 mg/day for neutral sterols and 1328 ± 120 mg/day vs. 1317 ± 45 mg/day for acidic sterols), but the coefficients of variation were lower

TABLE 2. Results of fecal excretion of neutral sterols, acidic sterols, total fecal sterols, and cholesterol synthesis determined from specimens frozen immediately and small aliquots sent by mail

Subject	Neutral Sterols		Acidic Sterols		Total Fecal Sterols		Dietary Cholesterol	Cholesterol Synthesis	
	F[a]	M[a]	F	M	F	M		F	M
	mg/day (mean ± SD)[b]								
1	874 ± 43	917 ± 37	337 ± 71	217 ± 76	1211 ± 41	1134 ± 22	478	733 ± 41	656 ± 34
2	950 ± 54	954 ± 56	189 ± 61	293 ± 30	1139 ± 115	1247 ± 110	441	698 ± 115	806 ± 110
3	510 ± 73	525 ± 89	224 ± 50	203 ± 21	734 ± 52	728 ± 83	262	472 ± 52	466 ± 83
4	692 ± 155	727 ± 201	315 ± 128	297 ± 146	1007 ± 104	1024 ± 58	167	840 ± 104	857 ± 58
5	1086 ± 95	1155 ± 119	567 ± 70	609 ± 55	1653 ± 162	1764 ± 174	420	1233 ± 162	1344 ± 174
6	512 ± 10	525 ± 35	206 ± 78	227 ± 67	718 ± 72	752 ± 77	246	472 ± 72	506 ± 77
7	360 ± 34	367 ± 30	400 ± 79	372 ± 77	760 ± 65	739 ± 89	321	439 ± 65	418 ± 89
8	557 ± 170	563 ± 209	307 ± 177	283 ± 187	864 ± 334	846 ± 381	158	706 ± 334	689 ± 381
9	1192 ± 90	1220 ± 96	527 ± 55	520 ± 40	1719 ± 145	1740 ± 135	209	1510 ± 145	1531 ± 135
10[c]	1292	1365	916	905	2208	2270	194	2014	2076
11	939 ± 240	891 ± 238	495 ± 167	528 ± 156	1434 ± 397	1419 ± 383	220	1214 ± 397	1200 ± 383
12	848 ± 83	843 ± 39	527 ± 44	518 ± 57	1375 ± 88	1361 ± 62	286	1089 ± 88	1075 ± 62
Mean ± SEM	818 ± 85	838 ± 89 n.s.	417 ± 59	414 ± 60 n.s.	1235 ± 134	1252 ± 140 n.s.	284	951 ± 138	968 ± 144 n.s.

[a]Results from frozen (F) and mailed (M) samples.
[b]Values represent the mean of 3 consecutive days.
[c]Fecal samples from only 2 days could be obtained from subject 10.

TABLE 3. Fecal excretion of neutral and acidic sterols measured from three small fecal specimens (≈1 g) taken from three different sides of one bowel movement prior to homogenization in six subjects

Subject	Neutral Sterols	CV[a]	Acidic Sterols	CV
	mg/day (mean ± SD)			
1	353 ± 16	4.5	335 ± 16	4.8
2	537 ± 30	5.6	318 ± 22	6.9
3	1237 ± 35	2.8	569 ± 40	7.0
4	1879 ± 54	2.9	1071 ± 35	3.3
5	678 ± 40	5.8	451 ± 5	1.1
6	867 ± 26	3.0	558 ± 7	1.3

[a]CV, coefficients of variation; calculated as (SD/mean) × 100%.

with sitostanol as marker (chromic oxide vs. sitostanol: 3.8 vs. 1.9 for neutral sterols, 9.0 vs 3.4 for acidic sterols and 5.7 vs. 1.6 for total fecal sterols).

DISCUSSION

Quantification of fecal excretion of neutral and acidic sterols provides important information on the regulation of cholesterol and bile acid synthesis under various experimental conditions (3–8, 18). The standard method was developed 25 years ago by Grundy et al. (1) and Miettinen et al. (2). Complete 24-h stool collection over several weeks was necessary. Later the same group introduced markers to correct for variations in fecal flow (19) and for possible cholesterol losses by bacterial degradation during intestinal transit (20). For this purpose, the dietary intake of sitosterol needs to be known precisely, and group separation of cholesterol, plant sterols, and their bacterial conversion products by thin-layer chromatography has to precede quantification by GLC. The need for total stool collections, time-consuming procedures, huge amounts of solvents, and much technical handling is a great drawback for studies in large groups and on outpatients.

The present investigation was undertaken to develop a simple, precise, inexpensive, and high through-put method for measurement of daily fecal excretion of neutral and acidic sterols, suitable for outpatient studies. For this purpose sitostanol was introduced as a fecal flow marker. Sitostanol, a hydrogenated derivative of sitosterol, is an unabsorbable plant sterol (21–23) of negligible natural occurrence (15, 16, 24); it is structurally related to cholesterol and in the same physiological phase of the intestinal content as cholesterol (25); and it is not metabolized during intestinal transit (D. Lütjohann, C. O. Meese, J. R. Crouse, and K. von Bergmann, unpublished results). Thus sitostanol fulfills all the criteria of an ideal flow marker for cholesterol balance studies.

In the first set of experiments, fecal sterol excretion was measured using chromic oxide and sitostanol as flow markers in 16 subjects. The results showed excellent correlation (Fig. 1). These findings validate sitostanol as a fecal flow marker. Although it has been claimed in previous studies that losses of cholesterol during intestinal transit might occur under certain circumstances (20), in the present study the calculation of both neutral and acidic sterol excretion, whether with chromic oxide or sitostanol, gave almost identical results. These findings suggest that losses of cholesterol during a normal solid-food diet are negligible. This is in agreement with a recent study by Tilvis and Miettinen (26), who under similar conditions demonstrated a complete recovery of radiolabeled sitosterol as compared to chromic oxide.

Despite the excellent correlation, the calculation of sterol excretion using sitostanol revealed a small but consistent lower fecal output (≈ −4%), indicating a small methodological difference between the two markers. Several factors could be responsible for this difference. The purity of both markers was checked and can be ruled out as a source of error. Incomplete recovery of the sitostanol would result in overestimating and not in underestimating the output of fecal sterols. Variations in dietary intake of sitostanol would, of course, influence the results, but would not lead to a consistent lower output. Approximation of baseline fecal excretion of sitostanol by equation 5 could be responsible for consistent lower output; however, the day-to-day variation in baseline fecal excretion of sitostanol is so low that it is doubtful whether this small difference has any physiological significance.

From the methodological point of view, sitostanol has several advantages over chromic oxide. Extraction, derivatization, and quantification of sitostanol are performed together with neutral sterols from the same fecal specimen. Any losses of neutral sterols during the analytical procedure would be associated with equal losses of sitostanol. Sitostanol is distributed homogeneously in the feces (Table 3) and, in addition, needs no correction for sample weight. Because bile acids are extracted from the lower phase of the same fecal homogenate, there are identical conditions for calculation of acidic sterol excretion. In contrast, the calculation of sterol excretion using chromic oxide means that two fecal samples must be correctly weighed and further analytical steps are necessary. This might explain the higher precision in the analysis of neutral and acidic sterols from one fecal sample using sitostanol and the lower coefficients of variation for neutral sterol excretion in 16 subjects (9.2% vs. 15.2%; $P < 0.005$). The higher coefficient of variation of acidic sterol excretion for both markers could also be due to greater daily variations in bile acid excretion (27).

The extremely small fecal volume (50 μl homogenate) needed for quantification of neutral and acidic sterols and the simple and accurate analytical procedure prompted us to simplify this method for outpatient studies. Fecal sterol excretion determined from either total fecal homogenate frozen immediately (macro-method) or from small aliquots sent by mail (micro-method) in 12 subjects gave identical

results (Table 2). Thus, the small amount of fecal specimens randomly sampled, the environmental temperature, and the prolonged time of transport have no influence on overall results. In addition, handling the fecal specimen within its small plastic tube, from sampling to homogenization, makes this new method extremely simple, clean, and convenient for patients and technicians.

The new micro-method enables many samples to be processed with a minimum of time-consuming procedures and with smaller amounts of organic solvents. The method is accurate, precise, sensitive, inexpensive, and allows a high through-put measurement of daily fecal excretion of neutral and acidic sterols as well as of cholesterol synthesis. Results from over 200 volunteers and patients have been obtained on an outpatient basis by this method along with a measurement of their cholesterol absorption (28).

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