Fractionation of fecal neutral steroids by high performance liquid chromatography

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Summary Fecal neutral steroids were fractionated by high performance liquid chromatography (HPLC) into three major fractions: 5β-H, 3-keto steroids; 5β-H, 3β-hydroxy steroids; and 5α-H and Δ^5-3β-hydroxy steroids. This separation was achieved in about 10 minutes, with greater than 97% recovery of standards in each fraction. Gas-liquid chromatographic quantitation of fecal steroids fractionated by either HPLC or thin-layer chromatography gave nearly identical results. A method using both C18 reverse phase and silica HPLC to purify radiolabeled sterols is also described. —**Jackson, E. M., C. A. Kloss, S. T. Weintraub, and G. E. Mott.** Fractionation of fecal neutral sterols by high performace liquid chromatography. *J. Lipid Res.* 1985. **26:** 893–897.

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The separation of cholesterol and plant sterols from their fecal bacterial metabolites is necessary in sterol balance studies before quantitation by GLC. Usually silica gel TLC has been used to separate fecal neutral steroids into three fractions, 5β-H, 3-keto steroids, 5β-H, 3β-hydroxy steroids, and the parent sterols (1, 2). The TLC method is tedious, since the fractions must be visualized, scraped, and eluted from the silica. Capillary GLC can be used to quantitate cholesterol and its metabolites without prior fractionation, but cannot separate all plant sterol metabolites (3). This report describes rapid HPLC procedures for separating fecal neutral steroids into three major fractions and for purifying radiolabeled sterols.

MATERIALS AND METHODS

Chemicals

HPLC grade solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA). Cholesterol, campesterol, and β-sitosterol standards were obtained from Applied Science (Deerfield, IL), and all other steroid standards were from Steraloids, Inc. (Wilton, NH). [22,23-^{3}H(n)]β-Sitosterol and [4-^{14}C]cholesterol were purchased from Amersham Corp. (Arlington Heights, IL).

C18 and silica HPLC of radiolabeled sterols and other neutral steroids

Radiolabeled sterols were chromatographed on a 10-μm Radial-Pak C18 column, 8 mm × 10 cm, installed with a Guard-Pak in a radial compression module, RCM-100 (Waters Associates, Milford, MA). The elution solvent, acetonitrile–isopropanol–methanol 20:3:7 (v/v/v), was pumped at 2 ml/min. Unsaturated sterols were detected by UV absorption at 206 nm by a variable wavelength detector.

The efficiency of separation by the C18 column was tested by daily injection of a standard mixture of 10 μg each of cholesterol, campesterol, and β-sitosterol. Samples containing up to 2 mg of sterol, dissolved in 50–100 μl of isopropanol and diluted with an equal volume of elution solvent, were loaded into a 1-ml sample injector loop. Ideally the samples should be dissolved in elution solvent, but some steroids have limited solubility in this solvent. Since ^{14}C-labeled sterols had specific radioactivities of about 55 mCi/mmol, no more than 250 μCi was injected to avoid overloading the column.

[^{3}H]Sitosterol of high specific radioactivity (about 50 Ci/mmol) could not be detected by UV absorption unless more than 500 μCi was injected. Therefore, we added about 10 μg of the appropriate unlabeled sterol to the labeled compound before injection. The radiolabeled sterol peak corresponding to the appropriate sterol standard was collected and the recovery was calculated by counting a 5-μl portion of the eluate. Radioactive steroids were counted in Scintisol (Isolabs Inc., Akron, OH). Counting efficiencies were calculated from quenched [^{3}H]- and [^{14}C]toluene standards using an external standard method.

The sterols were prepared for further purification on silica HPLC by evaporating the solvent under nitrogen and dissolving the residue in 50–200 μl of the elution solvent, isooctane–isopropanol 975:25 (v/v). The sample was injected on a 10-μm Radial-Pak silica cartridge, 8 mm × 10 cm, protected with a silica Guard-Pak (Waters

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Abbreviations: GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl; campestanol, 24α-methyl-5α-cholestan-3β-ol; campesterol, 24α-methyl-cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; cholesterol, cholest-5-en-3β-ol; cholestanone, 5α-cholestan-3-one; coprostanol, 5β-cholestan-3β-ol; coprostanone, 5β-cholestan-3-one; ethylcoprostanol, 24α-ethyl-5β-cholestan-3β-ol; ethylcoprostanone, 24α-ethyl-5β-cholestan-3-one; methylcoprostanol, 24α-methyl-5β-cholestan-3β-ol; methylcoprostanone, 24α-methyl-5β-cholestan-3-one; sitosterol, 24α-ethyl-cholest-5-en-3β-ol; stigmasterol, 24α-ethyl-cholest-5,22-dien-3β-ol; sitostanol, 24α-ethyl-5α-cholestan-3β-ol; epicholesterol, cholest-5-en-3α-ol; epicoprostanol, 5β-cholestan-3α-ol; epicholestanol, 5α-cholestan-3α-ol; cholestenone, cholest-4-en-3-one; Δ^4-cholestenol, cholest-4-en-3β-ol.

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Associates), and eluted at 1 ml/min. The sterol peak was collected, radioactivity was determined, and the percent recovery was calculated. Amounts of [^{14}C]cholesterol greater than 250 μCi were purified on a Magnum 9, Partisil-10 column, 9.4 mm × 50 cm (Whatman Co., Clifton, NJ) by elution with isooctane–isopropanol 97:3 (v/v) at 4 ml/min.

Relative retention characteristics of steroid standards on radially compressed C18 and silica cartridges were determined with minor modifications of the above procedures. The C18 elution solvent was acetonitrile–isopropanol–methanol 20:3:7 (v/v/v), with a flow rate of 1.4 ml/min. The solvent for silica HPLC was isooctane–isopropanol 99:1 (v/v) (4), at a flow rate of 1.5 ml/min. Saturated steroids were detected with a refractive index detector.

Fractionation of fecal neutral steroids

Feces collected from baboons fed diets varying in cholesterol content and type of fat (5) were homogenized, saponified, and extracted with petroleum ether (1). Portions of the extracts expected to contain 0.5–5.0 mg of steroid were taken for fractionation by HPLC or TLC.

For HPLC fractionation, 100 μl of a chloroform solution of purified [^{3}H]β-sitosterol, containing approximately 200,000 dpm, was added to each fecal extract sample as an internal recovery standard. The samples were washed through 0.48-μm solvent-resistant nylon membrane filters (Millex-SR, Millipore Corp., Bedford, MA), and the solvent was evaporated at 40°C under nitrogen. A 10-μm silica Radial-Pak cartridge with a silica Guard-Pak (Waters Associates) was installed in the radial compression module and equilibrated with the elution solvent, isooctane–isopropanol 99:1 (v/v), at a flow rate of 2.5 ml/min. A mixture of coprostanone, coprostanol, and cholesterol standards, 50 μg each, was injected, and elution was monitored by refractive index detection. From the retention times of these standards, time intervals for collection of the fecal neutral steroid fractions were established. Samples were dissolved in 250 μl of elution solvent and loaded into a 100-μl sample injection loop. Typically, three 3-min fractions were collected by a fraction collector, beginning approximately 1.0 min after injection. The fractions were capped as they were collected, to prevent evaporation of the solvent. Of the 7.5 ml in each fraction, 3-ml portions were taken of fractions I and III, and a 1-ml portion of fraction II for GLC quantitation. Radioactivity in 1 ml of fraction III was counted and a recovery factor was calculated. The HPLC column then was washed with 7.5 ml of isooctane-isopropanol 70:30 (v/v), and was re-equilibrated with approximately 25 ml of elution solvent before the next sample was injected. After 30 or 40 samples, the column was washed with 50 ml of Reactivation Agent for silica columns (Alltech Associates, Deerfield, IL), followed by 75 ml of dichloromethane before re-equilibration with elution solvent.

Fecal extracts were also fractionated by TLC on silica gel G plates (E. Merck, Darmstadt, FRG), which were developed in diethyl ether–hexane 70:30 (v/v). Fractions separated by TLC or HPLC were quantitated by GLC using a 1.5 m × 4 mm i.d. column packed with 3% OV-17 at 240°C, with 5α-cholestane added as an internal standard (6). TMS ethers of hydroxyl-containing steroids (fractions II and III) were prepared with TriSil BSA (Pierce Chemical Co., Rockford, IL).

Components of the three fractions separated by HPLC were identified by GLC–mass spectrometry. After appropriate derivatization, a portion of each fraction was injected with a split ratio of approximately 20:1 on a 15 m × 0.32 mm i.d. BP-1 fused silica capillary column (SGE, Austin, TX) at 270°C, coupled to a Finnigan MAT 212 mass spectrometer. The mass spectra obtained from peaks in each fraction were compared with mass spectra of standards or published spectra (7).

RESULTS AND DISCUSSION

Purification of radiolabeled sterols

We used both reverse phase and silica HPLC to purify radiolabeled sterols. C18 HPLC of five different batches of [^{14}C]cholesterol resolved three or four minor peaks of radioactivity not associated with cholesterol. In four of the five batches only 1–2% of the ^{14}C was in these peaks; in the other batch, more than 5% of the radioactivity was found in the minor peaks.

Tritiated sterols were radiochemically unreliable, as others have reported for [^{3}H]cholesterol (8). We found only 85% of the tritium in the cholesterol fraction from the batch we fractionated on C18 HPLC. We purified several portions of a single lot of [^{3}H]β-sitosterol during the course of 2.5 years. The percentage of radioactivity in the β-sitosterol fraction gradually decreased from 75% to 52% during this time. For many purposes purification of radiolabeled sterols by C18 HPLC alone would be sufficient; however, we found that further purification by silica HPLC produced a reliable product for use in enzyme assays and in vivo turnover studies, and as the recovery standard for fractionation of fecal neutral steroids.

HPLC of steroid standards

Table 1 illustrates the reverse phase HPLC separation of cholesterol from many closely related steroids. Those compounds with one or more double bonds could be detected by UV absorbance at 206 nm with at least a 20-fold higher sensitivity compared to refractive index detection. Cholestanol was resolved from cholesterol by the C18 column. Also, β-sitosterol was separated from its

TABLE 1. Retention characteristics of steroid standards on C18 and silica radially compressed HPLC columns

Name	Structural Differences from Cholesterol (Cholest-5-en-3β-ol)	C18 Retention Relative to Cholesterol[a,b]	Silica Retention Relative to Cholesterol[a,c]
Coprostanone	5β-H, 3-one	0.678	0.096
Δ⁴-Cholestenone	4-en, 3-one	0.703	0.380
Epicholesterol	3α-ol	0.742	0.450
Epicoprostanol	5β-H, 3α-ol	0.763	0.757
Cholestanone	5α-H, 3-one	0.876	0.153
Δ⁴-Cholestenol	4-en	0.884	0.887
Epicholestanol	5α-H, 3α-ol	0.998	0.632
Cholesterol		1.00	1.00
Coprostanol	5β-H	1.03	0.646
Campesterol	24α-methyl	1.12	0.986
Stigmasterol	5,22-dien, 24α-ethyl	1.10	0.996
Cholestanol	5α-H	1.27	1.06
β-Sitosterol	24α-ethyl	1.27	1.01

[a]Retention relative to cholesterol is the ratio of k' of the compound to k' of cholesterol where $k' = \frac{V - V_0}{V_0}$; V = retention volume of compound; and V_0 = void volume of the column.
[b]C18 HPLC at 1.4 ml/min; elution solvent, acetonitrile–isopropanol–methanol 20:3:7; cholesterol k' = 9.10.
[c]Silica HPLC at 1.5 ml/min; elution solvent, isooctane–isopropanol 99:1; cholesterol k' = 4.99.

companion sterols, campesterol and stigmasterol, which co-eluted ahead of the β-sitosterol peak.

Table 1 also shows that relative retention factors for steroids separated by silica HPLC. Note that two compounds that did not separate from cholesterol on C18 HPLC (epicholestanol and coprostanol) were resolved by the silica column. Although cholesterol, the plant sterols, and cholestanol did not separate from each other on the silica column, those separations were accomplished by the reverse phase HPLC.

Fractionation of fecal neutral steroids

Prior to GLC analysis, fecal neutral steroids were fractionated only by silica HPLC. **Fig. 1A** shows the separation of coprostanone, coprostanol, and cholesterol into three distinct peaks that correspond to fractions I, II, and III on TLC (1). This standard mixture was used to assess the resolving power of the column and to establish the time intervals for collecting each fraction. For example, from the retention of standards shown in Fig. 1A, fraction I would be collected from 1–4 min, fraction II from 4–7 min, and fraction III from 7–10 min.

Fig. 1B shows a typical HPLC tracing of fecal neutral steroids separated into three steroid classes. The steroids were detected by refractive index rather than by UV absorbance, since the saturated steroids such as coprostanol do not absorb in the ultraviolet. Also, fecal extracts have substantial quantities of nonsteroidal components which absorb strongly in the ultraviolet, and obscure the steroid peaks. Even though there was large variability in the appearance of HPLC chromatograms from different baboons, the three classes of steroids did not overlap because the timing of fraction collection was established each day from the retention times of the three standards as described above.

We added various amounts of a mixture of coprostanol,

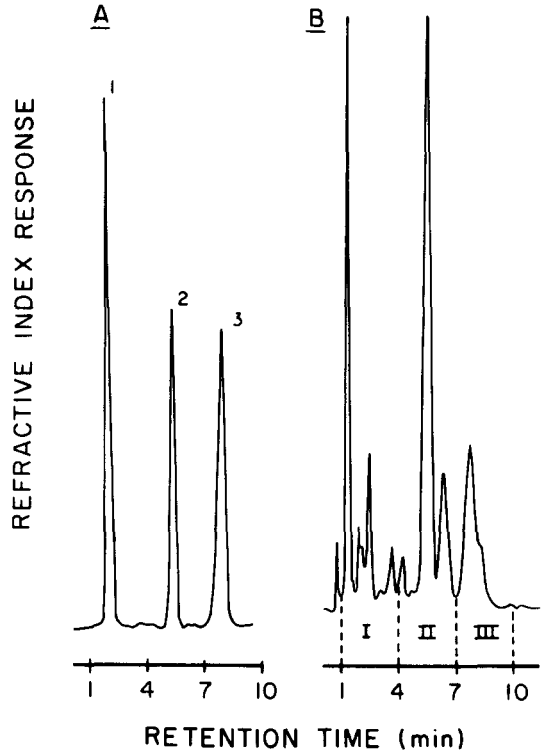

Fig. 1. Fractionation of neutral steroids by silica HPLC. A) Standards, 50 μg each; 1, coprostanone, 2, coprostanol, 3, cholesterol. B) Fecal extract showing division into three fractions: I contains 5β-H, 3-keto steroids; II contains 5β-H, 3β-hydroxy steroids; III contains Δ⁵-3β-hydroxy and 5α-H, 3β-hydroxy steroids.

cholesterol, and β-sitosterol standards (50–750 μg of each), and the internal standard, [^{3}H]β-sitosterol, to portions of a fecal extract. After separation of the three fractions by HPLC, the quantity of each standard was determined by GLC. The percent recovery was 97.7 ± 3.7 (SD) for cholesterol, 99.5 ± 2.3 for β-sitosterol, and 102.4 ± 1.7 for coprostanol. The addition of the internal standard was necessary for accurate quantitation of HPLC-fractionated neutral steroids, since the samples were dissolved in a small volume (250 μl or less) of a volatile solvent before injection, and only part of this solution was injected on HPLC. For calculation of absolute steroid recovery, the [^{3}H]β-sitosterol should be added before homogenization of the feces. We did not add it until just before fractionation so that we could accurately count low levels of ^{14}C in other portions of the highly quenched fecal extracts as part of cholesterol turnover studies.

The distribution of steroids in a baboon fecal extract among the three major fractions separated by HPLC and identified by GLC–mass spectrometry is shown in **Table 2.** As with silicic acid TLC, the 5α-stanols eluted with the corresponding Δ^5-β-ol's in fraction III. The 5α-stanols were not separated from the Δ^5-3β-ol's by GLC on a 3% OV-17 packed column, but they were resolved by the BP-1 capillary GLC column used for mass spectrometry.

Comparison of HPLC and TLC fractionation of fecal steroids

A linear regression of the recoveries of dietary β-sitosterol and its metabolites fractionated by HPLC or TLC indicated that the two methods were equivalent. For a range of values of 94–298 mg/day, the linear regression equation was y = 1.01x + 9.33, (n = 15). Comparison of TLC- and HPLC-derived values for daily excretion of cholesterol and its metabolites also showed excellent agreement (**Fig. 2**). The slightly higher values obtained by the HPLC method suggest that minor losses occurred during elution of the steroids from the TLC plate. Losses during HPLC were corrected by the recovery standard.

The coefficient of variation (C.V.) for duplicate fecal samples fractionated by HPLC was 6.7%, which was similar to that obtained by TLC, C.V. = 6.5%. The values reflect variability from all phases of the analysis, including homogenization, sampling, saponification, extraction, fractionation, and GLC quantitation. These results indicate that HPLC provides an improved alternative to TLC fractionation of fecal neutral steroids prior to GLC quantitation.

TABLE 2. Steroids identified by GLC–mass spectrometry in silica HPLC fractions of fecal extract

Name	Structural Differences from Cholesterol (Cholest-5-en-3β-ol)
Fraction I	
Coprostanone	5β-H, 3-one
Methylcoprostanone	5β-H, 24α-methyl, 3-one
Ethylcoprostanone	5β-H, 24α-ethyl, 3-one
Fraction II	
Coprostanol	5β-H
Methylcoprostanol	5β-H, 24α-methyl
Ethylcoprostanol	5β-H, 24α-ethyl
Fraction III	
Cholesterol	
Cholestanol	5α-H
Campesterol	24α-methyl
Campestanol	5α-H, 24α-methyl
Sitosterol	24α-ethyl
Sitostanol	5α-H, 24α-ethyl

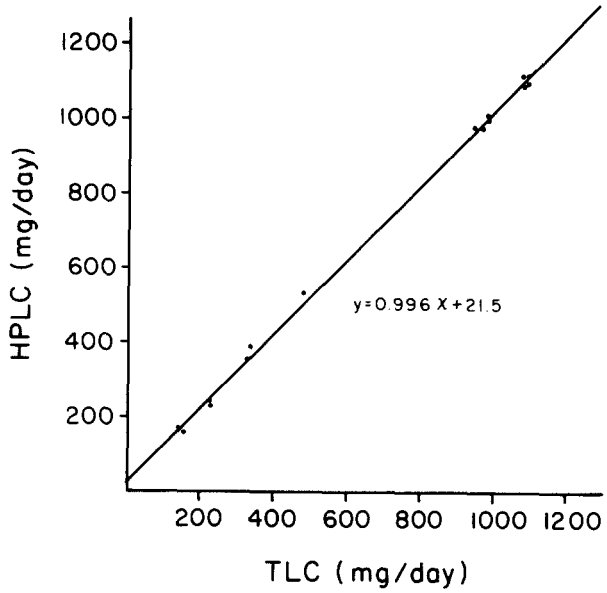

Fig. 2. Daily excretion of cholesterol and its metabolites. Comparison of values from 15 baboon fecal extracts fractionated by both HPLC and TLC before GLC analysis.

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REFERENCES

1. Miettinen, T. A., E. H. Ahrens, Jr., and S. M. Grundy. 1965. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.* **6:** 411–424.
2. McNamara, D. J., A. Proia, and T. A. Miettinen. 1981. Thin-layer and gas-liquid chromatographic identification of neutral steroids in human and rat feces. *J. Lipid Res.* **22:** 474–484.

3. Miettinen, T. A. 1982. Gas-liquid chromatographic determination of fecal neutral sterols using a capillary column. *Clin. Chim. Acta.* **124:** 245–248.
4. Ansari, G. A. S., and L. L. Smith. 1979. High-performance liquid chromatography of cholesterol autoxidation products. *J. Chromatogr.* **175:** 307–315.
5. Mott, G. E., C. A. McMahan, J. L. Kelley, C. M. Farley, and H. C. McGill, Jr. 1982. Influence of infant and juvenile diets on serum cholesterol, lipoprotein cholesterol, and apolipoprotein concentrations in juvenile baboons. *Atherosclerosis.* **45:** 191–202.
6. Mott, G. E., E. M. Jackson, and M. D. Morris. 1980. Cholesterol absorption in baboons. *J. Lipid Res.* **21:** 635–641.
7. Eneroth, P., K. Hellström, and R. Ryhage. 1964. Identification and quantification of neutral fecal steroids by gas–liquid chromatography and mass spectrometry: studies of human excretion during two dietary regimens. *J. Lipid Res.* **5:** 245–262.
8. Davidson, N. O., E. H. Ahrens, Jr., H. L. Bradlow, D. J. McNamara, T. S. Parker, and P. Samuel. 1980. Unreliability of tritiated cholesterol: studies with [1,2-^{3}H]cholesterol and [24,25-^{3}H]cholesterol in humans. *Proc. Natl. Acad. Sci. USA.* **77:** 2255–2259.