An improved gas–liquid chromatographic method for the determination of fecal neutral sterols

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Summary The analysis of fecal neutral sterols has been improved by use of a highly selective gas–liquid chromatography column packed with SP-2401. This chromatographic column allows separation of cholesterol and cholestanol and Δ^5-5α plant sterol homologs without employing silver nitrate thin-layer chromatography. Furthermore, there is no need to derivatize neutral sterols before injection. The main fecal neutral sterols are well resolved; retention times are reproducible; detector response is reproducible, linear, and sensitive to 0.2 μg. This method, successfully used for fecal samples, may be suggested as a routine method for the clinical study of cholesterol metabolism.—**Arca, M., A. Montali, S. Ciocca, F. Angelico, and A. Cantafora.** An improved gas–liquid chromatographic method for the determination of fecal neutral sterols. *J. Lipid Res.* 1983. **24:** 332–335.

Supplementary key words cholesterol • cholestanol • coprostanes • thin-layer chromatography • plant sterols • SP-2401 stationary phase

The determination of neutral sterols (NS) in feces is important in sterol balance studies, in dyslipidemias (1, 2), and in human colon cancer (3), as well as in pharmacological tests of drugs affecting intestinal cholesterol absorption (4, 5). The methods currently available include NS extraction with petroleum ether after saponification, fractionation of the extract by thin-layer chromatography (TLC), and derivatization of isolated fractions, as trimethyl silyl (TMS) ethers, before gas–liquid chromatography (GLC), for quantitative analysis (6–8).

The attempt to avoid TLC pre-separation, using temperature programmed GLC did not prove satisfactory for analytical purposes (9). The resolution of 5α from Δ^5 forms of NS is made difficult by the need of sequential TLC fractionation on silica gel and silver nitrate-impregnated silica gel plates (8). In this connection, a previous report on a hormonal steroid study described the direct resolution of cholesterol from cholestanol by GLC on SP-2401 packed column (10).

The present report describes a procedure based on GLC with the same stationary phase, which allows determination of 5α and Δ^5 forms of plant sterols and cholestane derivatives in feces without silica gel-silver nitrate TLC and NS derivatization.

MATERIALS AND METHODS

Chemicals

All solvents were analytical grade. The following reference standards were obtained from Supelco Inc., Bellefonte, PA: cholesterol, cholestanol, cholestanone, coprostanol, campesterol, stigmasterol, and 5α-cholestane. Sitosterol was purchased from Applied Science, State College, PA. The coprostanone standard solution, which was not available, was prepared through coprostanol oxidation according to a modified version of the technique of Evrard and Janssen (11). A quantitative oxidation of coprostanol into coprostanone was obtained with no evidence of by-products as judged from TLC and GLC analyses.

Thin-layer chromatography

TLC was carried out using precoated silica gel plates 0.25 mm thick (Merck, Darmstadt, FRG) developed in diethyl ether–heptane 55:45 (v/v).

Gas–liquid chromatography

A 5840A Hewlett Packard gas chromatograph was used. The glass column, 1.8 m × 2 mm ID, was packed with 3% SP-2401 on 100/120 Supelcoport. Operating conditions were as follows: oven, 220°C; injector, 250°C; flame ionization detector (FID), 270°C; nitrogen flow rate, 30 ml/min.

Analytical procedure

Stool samples were collected, homogenized, saponified, and extracted according to Miettinen, Ahrens, and Grundy (6). To determine the recovery rate 100 μg of 5α-cholestane was added to each sample before saponification. Fecal NS extracts produced three bands on silica gel plates, corresponding to Δ^5 plus 5α (fraction III), 5β (fraction II), and 3-keto compounds (fraction I) with the same mobility as cholesterol ($R_f \simeq 0.23$), coprostanol ($R_f \simeq 0.31$), and coprostanone ($R_f \simeq 0.53$), respectively. The 5α-cholestane, added as recovery stan-

Abbreviations: systematic names of the steroids referred to in the text by trivial names are as follows: 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; ethyl-coprostanol, 24α-ethyl-5β-cholestan-3β-ol; ethyl-coprostanone, 24α-ethyl-5β-cholestan-3-one; methyl-coprostanol, 24α-methyl-5β-cholestan-3β-ol; methyl-coprostanone, 24α-methyl-5β-cholestan-3-one; sitostanol, 24α-ethyl-5α-cholestan-3β-ol; sitosterol, 24α-ethyl-cholest-5-en-3β-ol; stigmasterol, 24α-ethyl-cholest-5,22-en-3β-ol. Other abbreviations are GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TMS, trimethyl silyl; NS, neutral sterols; FID, flame ionization detector; IS, internal standard; RT, retention time; RRT, relative retention time; RWR, relative weight response.

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dard, appeared as a distinct band with the highest mobility ($R_f \simeq 0.66$). The bands were scraped off the plate and eluted with chloroform (3 × 3 ml). The solvent was evaporated and the residue was dissolved in 1 ml of chloroform containing a known amount of 5α-cholestane as internal standard (IS) for GLC analysis. An aliquot of 1–2 μl of each fraction was injected into the GLC column.

Recovery

The 5α-cholestane band, treated as above described, was dissolved in 1 ml of chloroform containing a known amount of cholesterol as IS for GLC analysis.

Calculation

The peak areas obtained for each fraction, using a Hewlett Packard 5840A GC terminal, were compared with that of the IS and then corrected by a relative weight response (RWR) factor, specific for each compound and checked daily by the injection of a standard mixture of six different NS. Total and individual sterol contents, calculated by GLC analysis, were corrected with the recovery percentage obtained for the 5α-cholestane added before saponification.

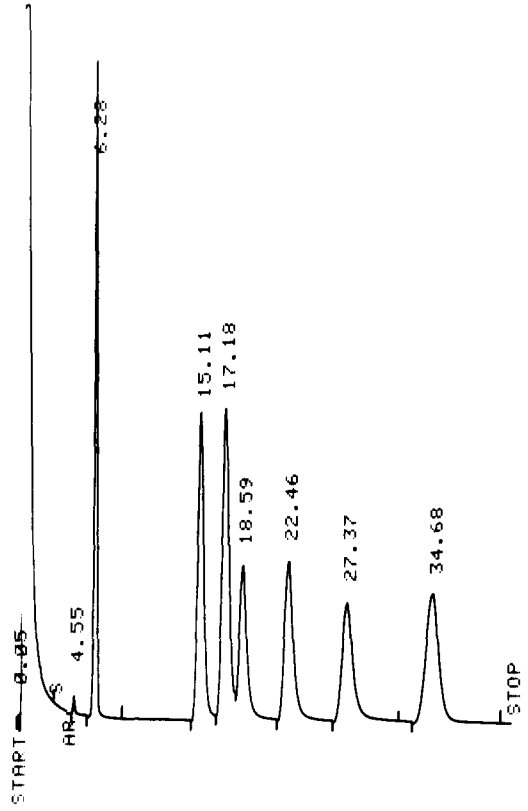

Fig. 1. GLC of standard calibration mixture of underived NS. Peak identification (RT): 5α-cholestane (6.28); coprostanol (15.11); cholesterol (17.18); cholestanol (18.59); campesterol (22.46); sitosterol (27.37); cholestanone (34.68).

TABLE 1. Relative retention times (RRT) and relative weight responses (RWR) to 5α-cholestane (IS) for free NS on SP-2401 packed column[a]

Steroid	RRT (min)[b]	RWR[c]
Coprostanol	2.434 ± 0.026	2.619 ± 0.020
Cholesterol	2.749 ± 0.026	1.567 ± 0.014
Cholestanol	2.974 ± 0.001	1.373 ± 0.017
Campesterol	3.590 ± 0.001	0.965 ± 0.014
Stigmasterol	3.710 ± 0.001	0.808 ± 0.014
Sitosterol	4.379 ± 0.001	1.249 ± 0.014
Coprostanone	5.056 ± 0.017	1.381 ± 0.023
Cholestanone	5.538 ± 0.001	0.993 ± 0.010

[a] Values are means ± SD of five to eight observations. GLC conditions as shown in Materials and Methods.
[b] Absolute retention time of 5α-cholestane (IS): 6.30 ± 0.2 min.
[c] Calculated by the formula: $\frac{\text{peak area}_{NS}/\mu\text{g injected}_{NS}}{\text{peak area}_{IS}/\mu\text{g injected}_{IS}}$.

Clinical application

This method was used on fecal samples from 14 outpatients on an ad libitum diet. Before stool collection Cr_2O_3 was administered, as a nonabsorbable marker of the 24 hr fecal flow (12), for 10–12 days.

RESULTS AND DISCUSSION

The use of the stationary phase SP-2401 has already been described for sterol analysis (10, 13), but its capability for resolving several underivatized cholesterol and plant sterol catabolites has been neglected so far.

Fig. 1 shows the chromatogram of a standard calibration mixture, under the above operating conditions, in which all NS are well resolved. It is important to stress that the resolution of cholesterol and cholestanol was proven satisfactory even if the latter represented only 1%. **Table 1** shows the RRT of the NS standards.

Fig. 2 shows the chromatograms of the three fractions isolated from a stool sample by TLC. Peak identification of 5α, 5β, and 3-keto derivatives of vegetable sterols, whose pure standards were not commercially available, were obtained by the steroid number method, as proposed by Horning et al. (14), using the following formula: steroid number = 27.00 + 5.239 × log (retention time relative to 5α-cholestane). The peak identity assignment of the 5α saturated plant sterols of fraction III was supported by $AgNO_3$-TLC; this fraction was further fractionated, as described by McNamara, Proia, and Miettinen (8), and split into two fractions with the same mobility as cholestanol (fraction IIIa) and cholesterol (fraction IIIb), respectively. The GLC analyses of these fractions, as shown in **Fig. 3,** demonstrate that peaks tentatively identified as campestanol and sitostanol appear in fraction IIIa, thus confirming their 5α-saturated structure. The comparison of GLC patterns

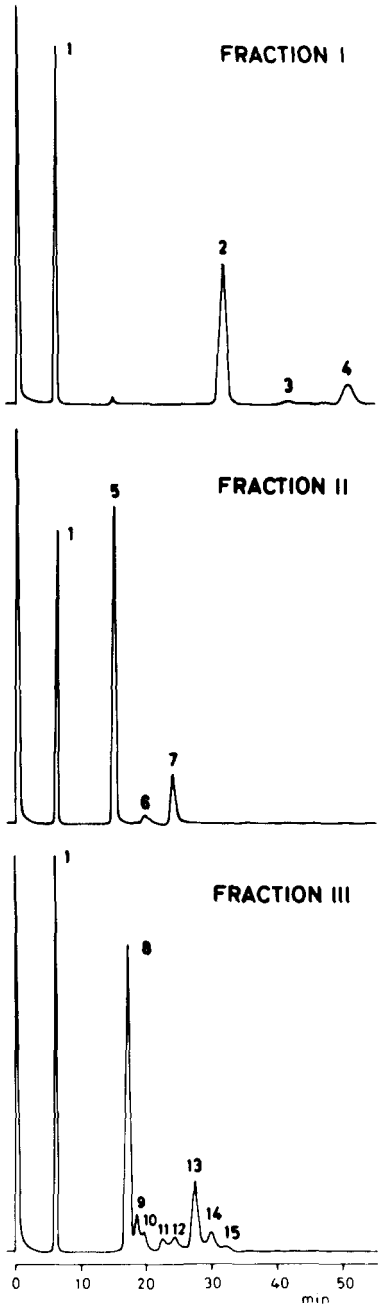

Fig. 2. GLC patterns of fractions isolated by silica gel TLC. Peak 1 = 5α-cholestane; 2 = coprostanone; 3 = methyl-coprostanone; 4 = ethyl-coprostanone; 5 = coprostanol; 6 = methyl-coprostanol; 7 = ethyl-coprostanol; 8 = cholesterol; 9 = cholestanol; 10 = unidentified peak; 11 = campesterol; 12 = campestanol; 13 = sitosterol; 14 = sitostanol; 15 = unidentified peak.

of TLC fractions shows that there is no overlapping between the components of fraction I and II, and between cholesterol and its catabolites. These results indicate that with SP-2401 packing, Δ^5 and 5α forms of NS are directly resolved so that pre-separation on $AgNO_3$-TLC can be omitted. Furthermore, fractions I and II can be analyzed together and, in case of patients on a diet free of plant sterols, the three TLC fraction can be analyzed as a pool.

The reliability of quantitative GLC analysis is evident from the linearity of the detector response for the NS standards, ranging from 0.2 to 3 μg, as shown in **Fig. 4.** Different slopes, i.e., different detector responses, for the free NS standard curves were observed, as has been reported for TMS derivatives (15). The RWRs for each NS standard are shown in Table 1; their small standard deviations demonstrate that detector responses are highly reproducible. Opposite opinions were expressed in the past about the use of underivatized sterols for GLC analysis (6, 7). Our results demonstrate that their use does not cause additional problems as compared to TMS derivatives; on the contrary, it can simplify the

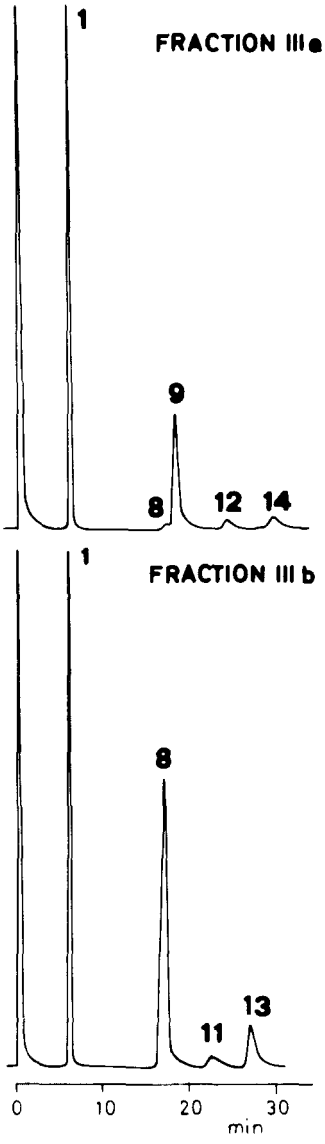

Fig. 3. GLC patterns of fraction III following separation by TLC on silica gel-silver nitrate. Peak identification as in Fig. 2.

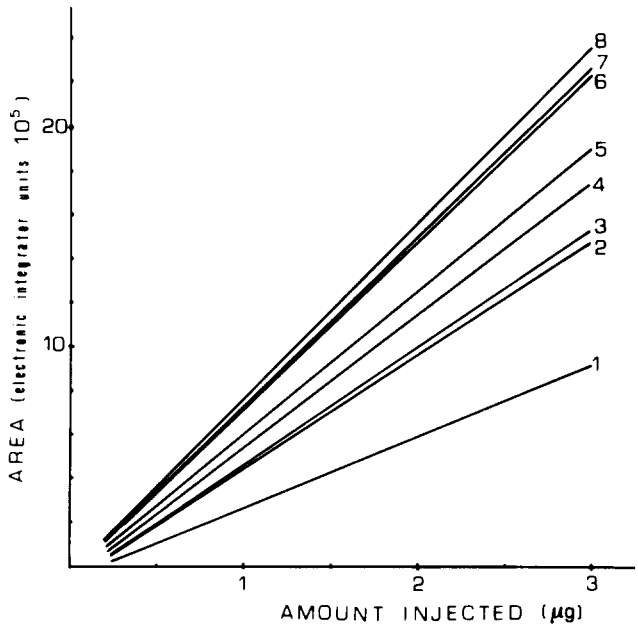

Fig. 4. Absolute detector response vs. amount injected. The equation for each curve was calculated using linear regression; correlation coefficients are reported in parentheses. Data points were obtained by eight serial dilutions of the calibration mixture. 1 = coprostanol (0.998); 2 = cholesterol (0.998); 3 = coprostanone (0.987); 4 = cholestanol (0.997); 5 = sitosterol (0.997); 6 = 5α-cholestane (0.998); 7 = cholestanone (0.998); 8 = campesterol (0.996).

routine analysis. The mean recovery of the entire procedure, obtained by 5α-cholestane addition, was 97.0 ± 2.5%.

Clinical application

According to previous findings (16, 17), approximately 97% of fecal NS daily excretion consists of cholesterol, coprostanol, and coprostanone. Cholestanol, found only in five patients, represents 2% of the daily fecal output. Cholestanone was detected only in the stools of one patient. In all the samples plant sterols and their conversion products were also found. The limited availability of standards has forced us to quantify only Δ^5 forms of plant sterols, but there is evidence that their catabolic products can also be quantified if necessary.

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