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GAS–LIQUID CHROMATOGRAPHY OF FECAL NEUTRAL STEROIDS*

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SUMMARY

A method is described for the analysis of fecal neutral steroids with a dual-column gas–liquid chromatography (GLC) system. After saponification of the fecal slurry, the neutral steroids were extracted with hexane. The GLC separation of the compounds and quantitation were achieved by simultaneous injection of the derivatized and underivatized aliquots of the extract onto dual columns under identical conditions. The neutral steroids of interest were then identified by matching the retention times with those of known standards, and identification was confirmed by use of an interfaced GLC high-resolution mass spectrometry system. The detection limit was 0.003 mg of steroid/g of fecal slurry. The precision of the method is illustrated by a relative standard deviation of 2–10% and a recovery of neutral steroids from 73–96%. The method was applied to the determination of fecal neutral steroids in a "High protein diet in colon cancer study". A considerably larger level of coprostanone than of coprostanol was observed. Data on neutral steroids in fecal samples from subjects on different diets are the subject of a separate publication.

INTRODUCTION

Along with the increased amount of research involving diet and its relationship to atherosclerosis and colon cancer, there has been considerable interest in the analysis of neutral steroids in feces, which includes cholesterol, its metabolic conversion products, and the plant steroids.

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The numerous fecal neutral steroids, due to similarity of molecular structure and physical properties, have been difficult to separate. Fifteen years ago, investigators[1–5] began using gas–liquid chromatography (GLC) for the determination of cholesterol and coprostanol. In 1964, Eneroth *et al.*[6] combined GLC with mass spectrometry (GLC–MS) for the analysis of neutral steroids in feces. Miettinen *et al.*[7] in 1965 reported on the application of GLC for the determination of fecal neutral steroids after a preliminary separation of the components by thin-layer chromatography (TLC). Ho *et al.*[8] quantified the fecal steroid components using a combination of TLC, GLC–MS, and colorimetry. These methods were time consuming and the possibility of error was increased directly with the number of steps required for the analysis.

This paper describes an analytical method for the GLC determination of fecal neutral steroids. After saponification of the slurry, the neutral steroids were extracted with hexane, then the hexane extract was divided and chromatographed underivatized and derivatized, simultaneously, on a dual-column system under identical GLC conditions.

EXPERIMENTAL

Subjects and diets

Two groups of five healthy males were fed four constant diets for one month each. The diets consumed were a control diet simulating typical North American meals, a meatless diet consisting of egg, dairy and vegetable protein, a high beef diet and a repeat of the control diet. The four diets were equivalent in all nutrients, except protein, which was doubled during the high beef diet. During the last week of the dietary regimen three fecal specimens per subject were collected[9]. Only values from control diet 1 were selected to illustrate the validity of the analytical method presented.

Materials

The neutral steroids standards, except β-sitosterol, were purchased from Analabs (New Haven, Conn., U.S.A.); β-sitosterol (>97% purity) and the silylating reagents were obtained from Applied Science Labs. (State College, Pa., U.S.A.). High-purity *n*-hexane was obtained from Phillips 66 (Borger, Texas, U.S.A.). The solid phase, Supelcoport (100–120 mesh) and the liquid phase, SP-1000, were from Supelco (Bellefonte, Pa., U.S.A.).

Gas chromatography

A Packard Model 7300 gas chromatograph with a dual-column oven and equipped with dual-flame ionization detectors was used. The two-channel electrometer of the instrument was interfaced with a Hewlett-Packard 3352B Laboratory Data System (Avondale, Pa., U.S.A.) for the identification and the quantitation of the individual neutral steroids.

The extracted samples were analyzed by injection of 1 μl of each underivatized and derivatized fecal extract into two 4 m × 2 mm I.D. double-looped U-shaped glass columns packed with 1% SP-1000 (a Carbowax modified with terephthalic acid) on 100–120 mesh Supelcoport. The column oven temperature was maintained at 245° and the linear carrier gas (nitrogen) flow-rate for both columns was kept at 5.5 cm/sec.

Mass spectrometry

A Varian Model 1520 gas chromatograph (under the conditions described above) interfaced with a high-resolution mass spectrometer CEC 21-110B was used to confirm the identity of the neutral steroids.

Sample preparation

The well-homogenized fecal slurry was precisely weighed (*ca.* 150 mg) in a 12-ml conical centrifuge tube. The internal standard, 5α-cholestane (50 μg), was then added to the weighed slurry, and the hexane solvent was evaporated using a stream of pure nitrogen gas. The homogenate was then saponified with 2 ml of 1 *M* KOH/methanol for 1 h in a dry bath at 65° with brief mixing on a Vortex-Genie every 10 min. After 1 h, the centrifuge tubes were cooled and 1 ml of deionized water was added to each tube. The neutral steroids were then extracted four times with 5-ml portions of hexane. For each extraction, the centrifuge tube was vigorously shaken for 1 min by hand and centrifuged for 10 min. The hexane layer was drawn off and the extracts were transferred into an 8-ml culture tube. After evaporating the hexane from the four combined extracts under a gentle stream of pure nitrogen, the residue was redissolved in 0.2 ml hexane, and the solution was then divided into two portions and each placed into a 1-ml conical vial. The hexane was evaporated under a stream of nitrogen in both vials. In one vial the residue was redissolved in 0.1 ml dry ethyl acetate (over CaH_2), sonicated, and 1 μl of the extract was injected into one of the two GLC columns.

To the residue in the second vial a mixture of trimethylchlorosilane and hexamethyldisilazane in dimethylformamide (1:40:40) was added. After brief sonication and standing for 30 min at room temperature, the derivatizing reagent was evaporated under a gentle stream of pure nitrogen at room temperature, and the silylated compounds were redissolved in 0.1 ml dry ethyl acetate, sonicated, and 1 μl was injected into the designated column of the dual-column system.

Calibration standard solution

The standard solution was composed of a mixture of 25 mg each of 5α-cholestane (internal standard), coprostanone, coprostanol, cholestanone, cholestanol, cholesterol, campesterol, stigmasterol, and β-sitosterol in 25 ml hexane. The retention time and the relative weight response ($RWR_{NS/IS}$) were determined for each neutral steroid *versus* an internal standard (5α-cholestane). Retention times and *RWR* values were entered into the computer and repeated injections provided the average *RWR* values.

RESULTS AND DISCUSSION

Precision and recovery study

To measure the reproducibility of the analytical-chromatographic method, a continuous precision check was performed. One fecal sample of each dietary regimen was analyzed again on a different day and the results were compared with the previous analysis. The data for the precision study are shown in Table I. The relative standard deviations for the eight neutral steroids determined ranged from 2–10%.

The amounts of coprostanol, coprostanone, and cholestanone were determined

TABLE I

REPRODUCIBILITY OF FECAL NEUTRAL STEROID ANALYSIS*

Neutral steroids	*Day***						
	1	*2*	*3*	*4*	*Average*	*Standard deviation, σ*	*Relative standard deviation, RSD (%)*
Cholesterol	0.59	0.52	0.56	0.54	0.553	0.03	5
Cholestanol	0.12	0.09	0.11	0.12	0.11	0.01	9
Cholestanone	<0.01	<0.01	<0.01	<0.01	<0.01		
Coprostanol	<0.01	<0.01	0.01	<0.01	0.003		
Coprostanone	0.29	0.35	0.31	0.35	0.330	0.03	9
Campesterol	0.10	0.09	0.10	0.10	0.098	0.01	10
Stigmasterol	0.15	0.13	0.14	0.15	0.143	0.01	7
β-Sitosterol	0.12	0.10	0.12	0.12	0.115	0.01	9

* Data calculated from chromatograms of derivatized and underivatized samples of Subject No. 3 on control diet 1.

** Independent analyses made on different days. Units are expressed as mg/g wet weight of fecal slurry.

from the derivatized extracts, while cholestanol, cholesterol, campesterol, β-sitosterol, and stigmasterol were determined from underivatized extracts.

One sample (same as chosen for the precision study) was selected for a recovery study. Thirty, 50, and 100 μg of each neutral steroid standard were added, together with 50 μg of internal standard, to 100 mg of fecal homogenate. The per cent recovery of each neutral steroid is given in Table II and ranged from 73–96% and was considered good.

The recovery study confirmed that saponification of the fecal homogenate for 1 h followed by addition of 1 ml de-ionized water and four extractions with 5-ml portions each of hexane was a reliable procedure. A critical point in the extraction step was the amount of water added before the extraction. The best recovery of the neutral steroids was obtained when the amount of water added was 30–40% of the total slurry volume (fecal homogenate, saponifying reagent, and added water). This is in agreement with the observation made in a similar study by Miettinen *et al.*[7].

TABLE II

PER CENT RECOVERY OF FECAL NEUTRAL STEROIDS

Neutral steroids	*Amount of steroid added to 100 mg of sample**		
	30 μg	*50 μg*	*100 μg*
Cholesterol	96	91	89
Cholestanol	77	82	92
Cholestanone	85	93	84
Coprostanol	81	89	76
Coprostanone	86	80	83
Campesterol	96	87	92
Stigmasterol	87	95	85
β-Sitosterol	81	73	83

* Subject No. 4 on control diet 1.

Chromatography

A comparison of the chromatograms of the underivatized and derivatized fecal extracts on an SP-1000 packing showed that a dual-column system provided the best method for the simultaneous analysis of all steroids of interest. This dual-column system made it possible to determine coprostanone, which was obscured by an unknown component when the underivatized extract was chromatographed. After trimethylsilylation of the fecal extract, coprostanone chromatographed well and was separated from background interference. When using an underivatized extract coprostanol and cholestanone eluted together, but after trimethylsilylation of the extract coprostanol eluted earlier (as the trimethylsilyl ether) and was separated from cholestanone. The GLC patterns of a representative sample are shown in Figs. 1 and 2.

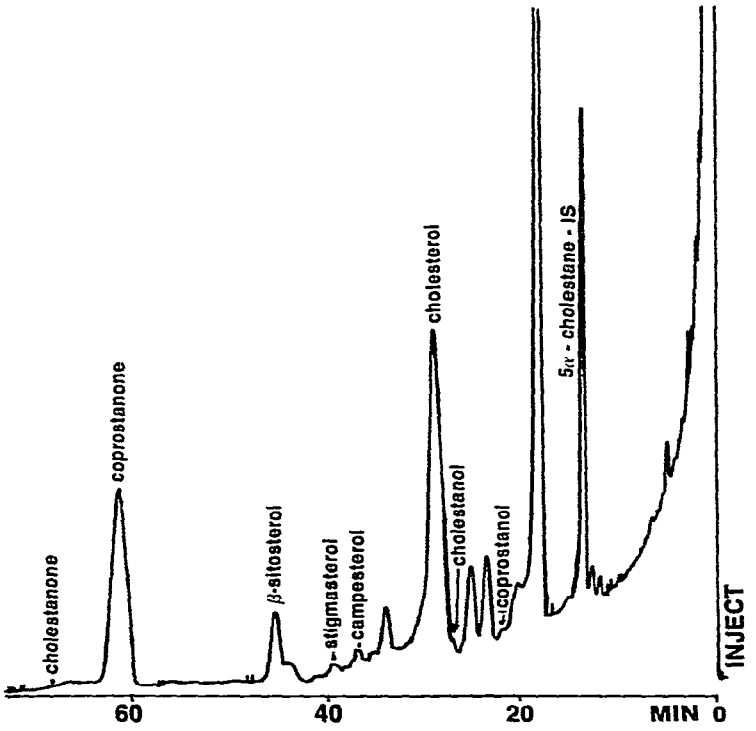

Fig. 1. GLC analysis of trimethylsilyl derivatives of neutral steroids in fecal extract. Column, packed with 1% SP-1000 on 100–120 mesh Supelcoport; glass, 4 m × 2 mm I.D., sample, 150 mg; volume injected, 1 μl; oven temperature, 245° (isothermal).

Identification of the neutral steroids of interest was accomplished by matching their retention times with the retention times of known standards of high purity (Figs. 3 and 4). The identity and homogeneity of the compounds were confirmed by subjecting a representative sample to high-resolution mass spectrometry (as described previously) and comparing the fragmentation patterns with those of the known standards.

Prior to choosing the optimal column packing, *viz.* 1% SP-1000 on Supel-

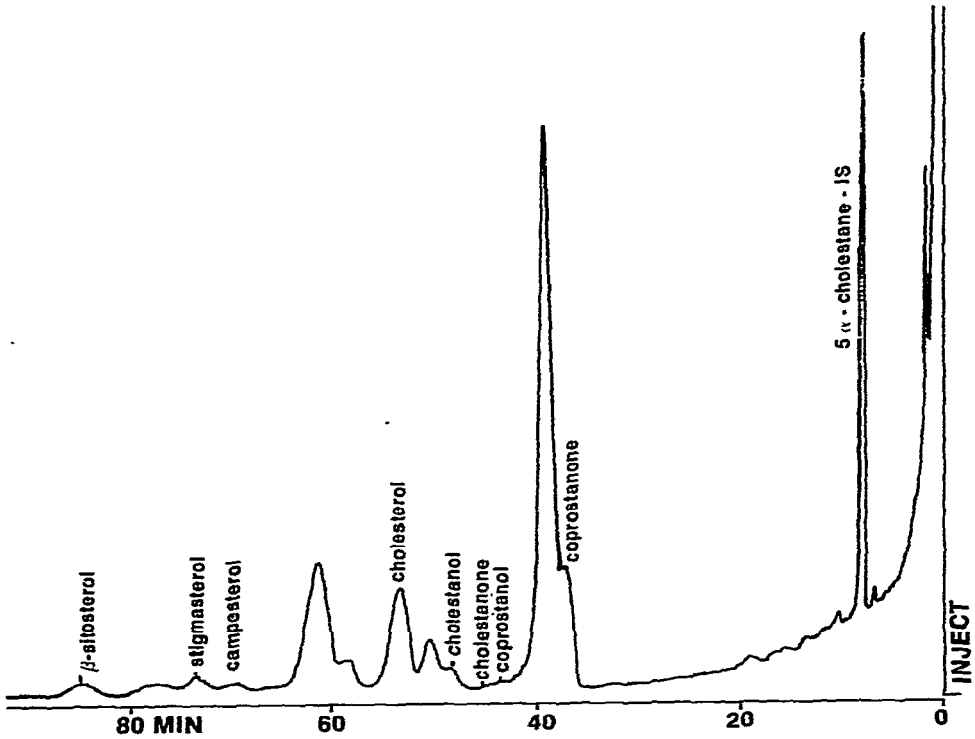

Fig. 2. GLC analysis of underivatized neutral steroids in fecal extract. For further conditions, see the legend to Fig. 1.

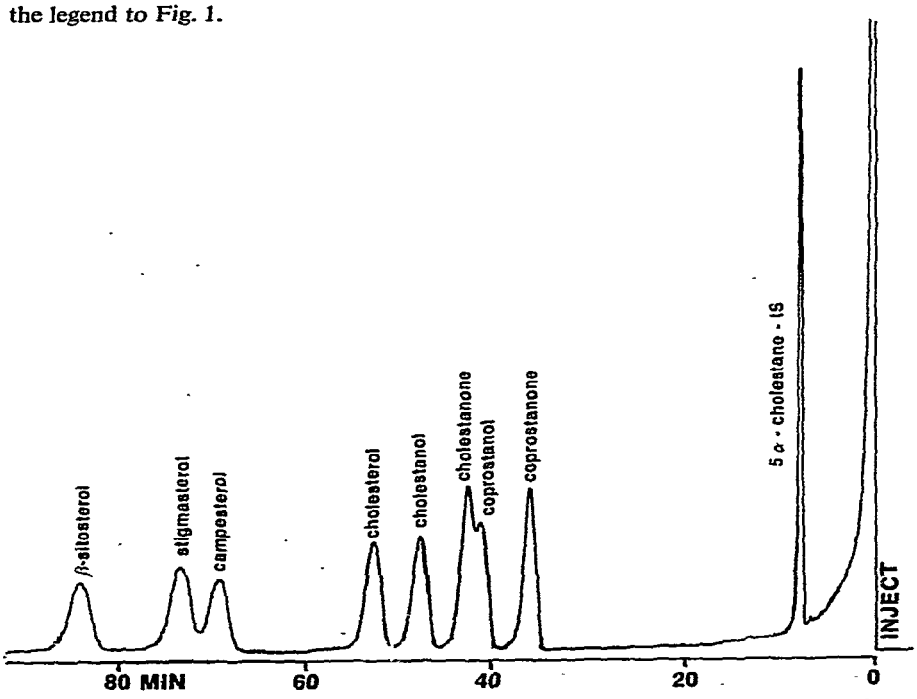

Fig. 3. GLC analysis of underivatized neutral steroids in standard mixture. Volume injected, 1 μl (400 ng of each steroid). For chromatographic conditions, see the legend to Fig. 1.

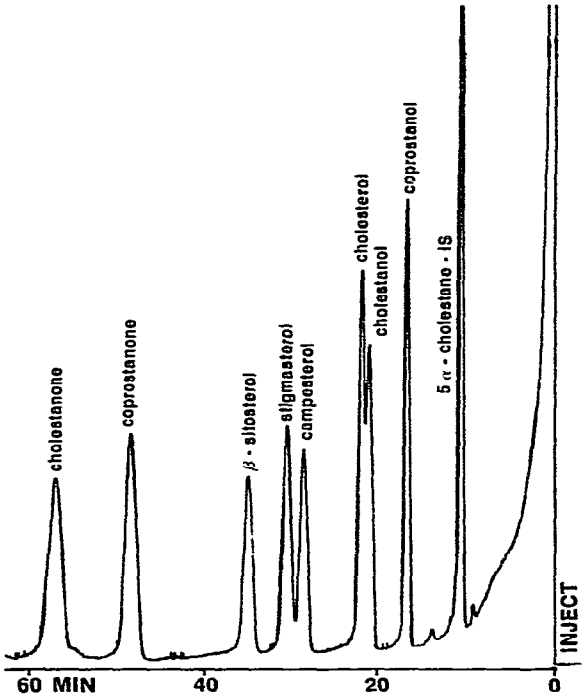

Fig. 4. GLC analysis of trimethylsilylated neutral steroids in a standard mixture. Volume injected, 1 μl (400 ng of each steroid). For chromatographic conditions, see the legend to Fig. 1.

coport 100–120 mesh, other packings, 1% OV-17, 3% SE-30, 1% SE-30, and 1% QF-1 on acid-washed Chromosorb W, and 1% QF-1 on Gas-Chrom Q had been tested under varied conditions. None of these provided a complete separation of the neutral steroids, underivatized and/or derivatized. A surface-modified Chromosorb W[10] did separate the compounds of the standard mixture well, but did not resolve them from several interfering peaks of sample background. The SP-1000 packing was most efficient and remained stable for approximately one month under the isothermal conditions used, and the large number of samples analyzed.

Although the SP-1000 substrate has not been used extensively in studies dealing with the neutral steroids, in this study the 1% SP-1000 packing showed good chromatographic efficiency (1000 theoretical plates per 1 m column length). Nordby and Nagy[11] in a study compared the chromatographic efficiency of fifteen liquid phases for the separation of sterols, and found that SP-1000 was one of the more efficient substrates among those tested.

Linearity of response

A typical calibration curve is shown in Fig. 5 for β-sitosterol demonstrating the linear detector response over a wide concentration range.

Analytical results

The results of the analyzed slurry samples from five healthy human subjects

Fig. 5. Typical standard curve for β-sitosterol. Chromatographic conditions were as given in the legend to Fig. 1.

eating a control diet are shown in Table III. These values have been converted and listed as milligram of neutral steroid per gram dry weight of fecal sample. This is an example of the results obtained in a large diet colon cancer study involving two groups of five volunteers eating four specific diets, four weeks each. The detailed design of the study and the total results are published elsewhere[12,13].

A significant observation noted in the results of this study is the comparatively large amount of coprostanone and small amount of coprostanol. Other investigators determining fecal neutral steroids[7,14,15] report the coprostanol level from 2–10 times the coprostanone level. Noteworthy is the high amount of cholesterol excreted by subject No. 1, indicating a relative low rate of conversion of cholesterol into its metabolites[16].

TABLE III

ANALYSIS OF NEUTRAL STEROIDS IN FECAL SAMPLES OF FIVE SUBJECTS ON CONTROL DIET 1*

Neutral steroids	*Subject*						
	1	*2*	*3*	*4*	*5*	*Mean*	σ**
Cholesterol	23.44	8.38	3.84	4.89	3.08	8.73	8.47
Cholestanol	0.34	0.67	0.96	0.96	0.64	0.71	0.26
Cholestanone	0.17	0.10	0.11	0.09	0.11	0.12	0.03
Coprostanol	0.17	0.10	0.11	0.09	0.11	0.12	0.03
Coprostanone	0.17	3.27	3.10	3.06	1.70	2.26	1.33
Campesterol	2.19	1.06	0.75	0.87	0.64	1.10	0.63
Stigmasterol	1.86	1.16	1.39	1.22	0.96	1.32	0.34
β-Sitosterol	7.42	2.12	0.64	1.05	0.74	2.39	2.87

* Data calculated from chromatograms of derivatized and underivatized samples. Units are expressed as mg/g dry weight of fecal sample.

** Standard deviation of a population ($N = 5$).

CONCLUSION

This chromatographic-analytical procedure, developed for the determination of fecal neutral steroids is fast, sensitive, and accurate. The method is applicable to studies in which large numbers of samples are to be analyzed quickly.

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