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GAS CHROMATOGRAPHIC ANALYSIS OF FECAL POLLUTION STEROLS ON A SINGLE COMBINED PACKED COLUMN

L. TAN, M. CLEMENCE AND J. GASS

Biochemistry Laboratories, Department of Nuclear Medicine and Radiobiology, Centre Hospitalier Universitaire, Sherbrooke, Quebec (Canada)

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SUMMARY

1. The separation of cholesterol from coprostanol and coprostanone on a single column, consisting of a combination of selective and non-selective coated supports, packed in series, is described.

2. The resolution on this combined column is better than when either phase was used alone.

3. Various functional derivatives and isomers of progesterone are also well resolved by our column, which should find general application for the gas chromatographic analysis of mixtures of steroidal compounds of similar structure.

INTRODUCTION

Recently, the possible use of coprostanol (5β-cholestane-3β-ol) as a molecular tracer for the detection of fecal water pollution has been suggested[1,2]. Since this sterol is known to be produced only by the action of the intestinal flora of higher animals by stereospecific reduction of the double bond of cholesterol[3–10], a reliable and simple analysis for its unambiguous detection in natural waters would indeed provide a desirable complement to the classical method of bacterial Coliform counting. Unfortunately, coprostanol is always excreted together with its precursor cholesterol, and because the two sterols differ in their molecular structure merely by the presence of a double bond, their successfull separation based on chromatographic mobilities would require a high degree of efficiency. Furthermore, coprostanol dispersed or dissolved for extended periods in oxygen saturated surface waters, may easily undergo oxidation to coprostanone (5β-cholestane-3-one). Thus, adequate separation of the three C_{27}-steroids coprostanol, cholesterol and coprostanone must be accomplished before coprostanol may be proposed as a suitable reference index for the chemical measurement of fecal water pollution.

We have found that in thin-layer chromatography (TLC) using different solvent systems, and including complexing of the unsaturated bond of cholesterol with silver nitrate, coprostanol consistently showed similar mobilities as its companion cholesterol

so that its positive identification by this method alone is not possible. We had expected gas–liquid chromatography (GLC) with its inherent much higher resolution capability to yield more satisfactory results. However, we discovered that on a non-polar phase such as OV-1, cholesterol was not at all separated from coprostanone, whereas on the selective phase QF-1, considerable overlap of the coprostanol and cholesterol peaks were observed.

In this paper, we report the complete gas chromatographic separation of the three fecal steroids by means of a single combined column, packed alternately with a polar and a non-polar liquid phase.

EXPERIMENTAL

Materials and methods

Reference coprostanol, coprostanone and 5α-cholestane were purchased from Sigma Chemical Company, Mo. All solvents were reagent grade and distilled before use. TLC was conducted as described previously[11]. GLC was performed on a Model 402 Hewlett Packard gas chromatograph, equipped with dual hydrogen flame ionization detectors operated in the twin-mode. Unless otherwise indicated operating conditions were: oven temperature 230°, injection port and detector temperatures 265°, nitrogen carrier gas flow 33 ml/min at 40 p.s.i.

Preparation of trimethylsilyl (TMS) derivatives

Two milligrams of steroid was dissolved in 2 drops of anhydrous pyridine, 4 drops of Trisyl reagent (Pierce Chemical Company, Rockford, Ill.) were added and the mixture stored under nitrogen for 30 min at 45°. After evaporation of the solvents with a stream of nitrogen, the residue was dissolved in hexane and the clear supernatant used immediately for the GLC analysis.

Preparation of the combination column

A 180 × 0.3 cm (I.D.) U-type glass column was packed simultaneously from either end with two layers of 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Penn.) each coated with 3% OV-1 and 3% QF-1, in such a way that each column half contained approximately equal amounts of the two types of coated support. The firmly packed column was then conditioned overnight at 250° untill a stable base line was obtained.

For preparation of standard curves, the peak areas were quantitated either by triangulation or by means of a mechanical Disc integrator and the addition of 5α-cholestane as an internal standard.

Analysis of fecal sterols from surface waters

A water sample (12 l) from a lake in the area was extracted twice with 2 l of distilled hexane in a wide mouth 20 l glass bottle by stirring vigorously with a mechanically driven stainless steel propeller for a period of 30 min. Other than glass and steel, no other material was used for the extraction apparatus, so that no contamination from hexane-soluble lipid components, other than from the water sample itself, could have been introduced. After stirring, the hexane fractions were siphoned off, pooled, dried over anhydrous sodium sulfate, and evaporated to dryness. The usually colored

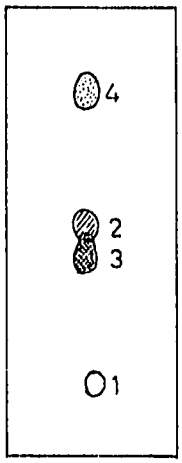

Fig. 1. Thin-layer chromatogram of 5α-cholestane (1), coprostanol (2), cholesterol (3) and coprostanone (4). Solvent system: chloroform–ether (9:1).

residue thus obtained was dissolved in dioxane and either analyzed directly, or further purified by preparative TLC on 20 × 20 cm, with 0.1 cm of Silica Gel HF_{254} (Merck, Darmstadt) coated glass plates, by means of a mechanical streaker (Rodder Instruments Corp., Los Altos, Calif.). After irrigation with a mixture of chloroform and ether (9:1), and visualization of a 1.5 cm end-strip of the chromatoplate by spraying with 50% sulfuric acid, the bands containing the suspected sterol fractions were scraped off, eluted with methanol, filtered, concentrated in vacuum, and used for the GLC analysis.

RESULTS AND DISCUSSION

On thin-layer chromatograms, coprostanol is not well separated from cholesterol, although coprostanone is (Fig. 1). Eluted with a mixture of chloroform–ether (9:1), cholesterol could not be readily defined from coprostanol. Other solvent systems such as chloroform–methanol (9:1), hexane–ethyl acetate (1:1), benzene–acetone (4:1), and the use of silver nitrate impregnated silica gel, gave similar unresolved spots. Since both compounds do not absorb in the UV, positive identification by TLC alone remains doubtful. Separation of these two sterols in the gaseous state on OV-1 and QF-1 as stationary phases, was better (Figs. 2 and 3).

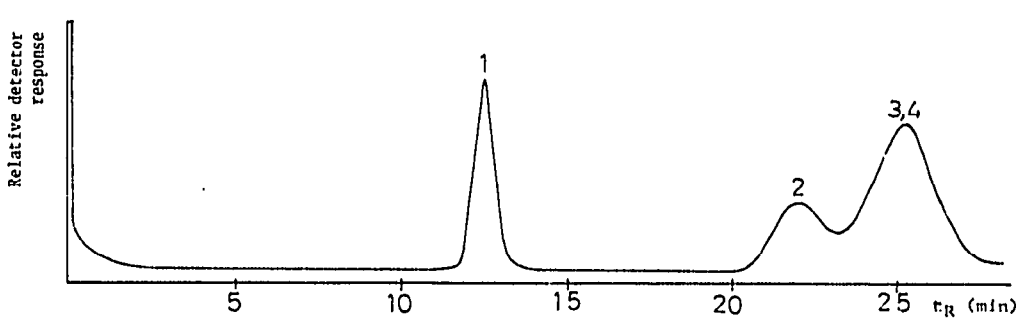

Fig. 2. Gas chromatograms of 5α-cholestane (1), coprostanol (2), cholesterol (3), and coprostanone (4) on a 3% OV-1 column. Conditions: 180 × 0.3 cm glass, oven temperature 230°, injection port and detector temperatures 265°, nitrogen carrier gas flow 33 ml/min at 40 p.s.i.

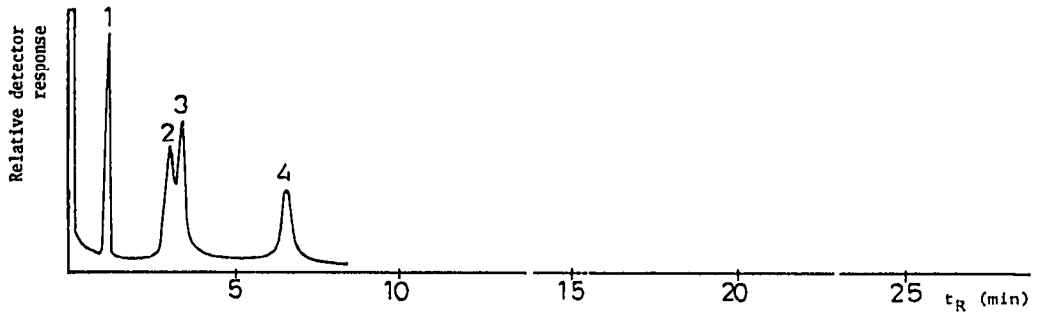

Fig. 3. Gas chromatograms of 5α-cholestane (1), coprostanol (2), cholesterol (3), and coprostanone (4) on a 3% QF-1 column. For conditions see Fig. 2.

However, although on the non-selective phase OV-1, acceptable resolution between coprostanol and cholesterol was readily achieved, cholesterol could not be distinguished from coprostanone on this column, as both compounds exhibited identical retention times. In contrast, on the selective QF-1 column, cholesterol and coprostanone were well resolved, but not cholesterol from coprostanol. Due to the high background noise of actual water sample extracts, the latter two sterols cannot therefore be readily differentiated on a QF-1 phase alone.

We have thought to combine the desirable separation properties of both phases OV-1 and QF-1 on a single column, and indeed, with our combination column coprostanone was well separated both from cholesterol and coprostanol. At the same time, almost complete resolution between coprostanol and cholesterol was achieved (Fig. 4). The practical utility of this novel column for the analysis of an actual water sample is illustrated in Fig. 5. Despite the high background noise generated by the sample, coprostanol can now easily be distinguished from cholesterol.

The identity of peaks 2, 3 and 4 of the water sample extract was established by consecutive co-injection of the same amount of sample with authentic compound and observation of area enlargement of a single, suspected peak.

That our combination column can be used for the quantitative analysis of fecal sterols is demonstrated in Fig. 6, obtained by plotting known weight ratios of coprostanol and 5α-cholestane against the ratios of their measured peak areas. As can be seen, good linearity and a zero intercept were observed.

Table I shows the properties of our combination column (C), as compared to the columns containing either only OV-1 (A) or QF-1 (B). For the compounds studied,

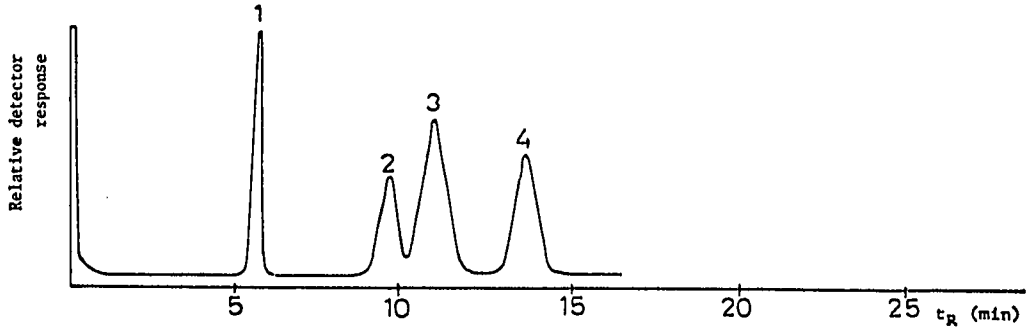

Fig. 4. Gas chromatograms of 5α-cholestane (1), coprostanol (2), cholesterol (3), and coprostanone (4) on a 3% OV-1, 3% QF-1 combination column. For conditions see Fig. 2.

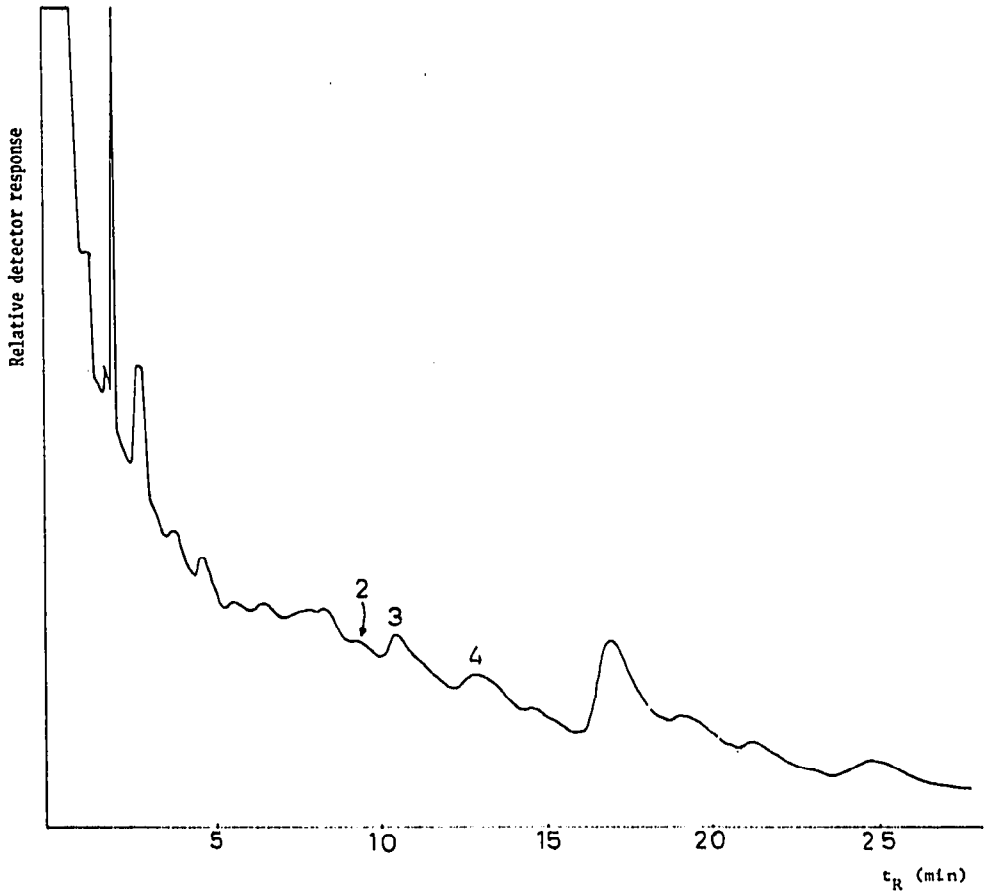

Fig. 5. Gas chromatogram of a water sample from a lake in the Sherbrooke area on the combination column, consisting of 3% OV-1 and 3% QF-1, packed in series. For conditions see Fig. 2.

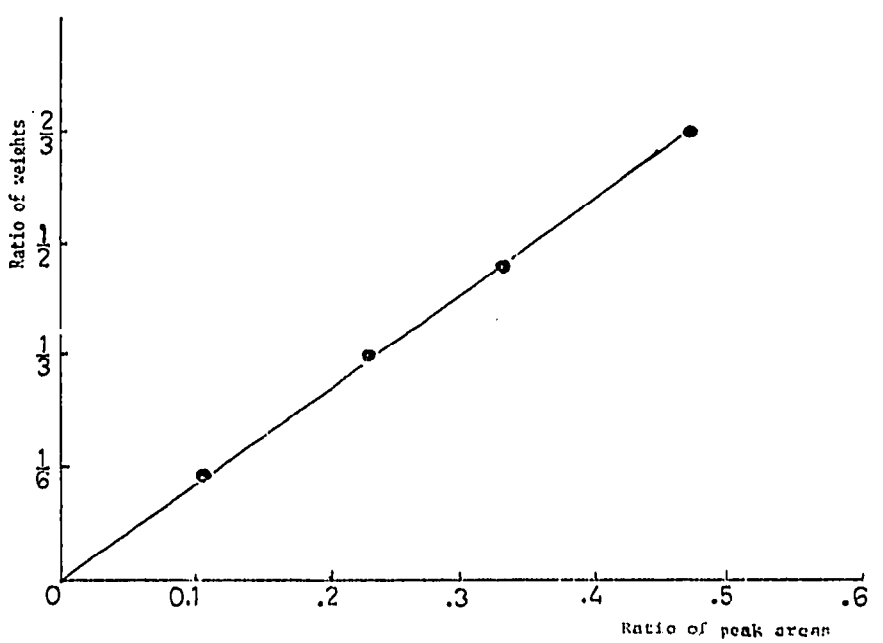

Fig. 6. Known weights of coprostanol and 5α-cholestane were co-injected on the combination column, and their ratios plotted against the ratios of the peak areas. For conditions see Fig. 2.

the optimal temperature was found to be 230°. At this temperature, our column C showed superior efficiency, as clearly indicated by the higher number of theoretical plates obtained. From these data, we may conclude that our combination column provoked retention times, in between those obtained on columns A and B, while at the same time column C combined the best separation properties of both.

The further advantages of this novel column are obvious, in particular for those chromatographers, who either have only a single column instrument, or a single detector and recorder system at their disposal. Instead of having to inject consecutively the unknown sample at least twice on a selective and non-selective column, in order to avoid identification errors due to identical retention times on one liquid

TABLE I

COMPARATIVE PROPERTIES OF COLUMNS A (3% OV1), B (3% QF1) AND C (3% OV1–QF1) TOWARDS FECAL POLLUTION STEROLS

Oven temperature	*Compound*	*3% OV-1 (A)*			*3% OF-1 (B)*			*3% OV-1/QF-1 (C)*		
		t_R[a]	*RRT*[b]	*Plates*[c]	t_R	*RRT*	*Plates*	t_R	*RRT*	*Plates*
220°	5α-Cholestane	18.8	1.00	1580	1.81	1.00	435	6.63	1.00	785
	Coprostanol	31.7	1.70	1770	4.42	2.44	380	13.0	1.97	1091
	Cholesterol	36.7	1.97	1650	5.05	2.78	545	14.4	2.17	881
	Coprostanone	36.7	1.97	1720	9.80	5.41	760	18.5	2.79	1380
	Coprostanol-TMS	41.1	2.19	1750	1.77	0.97	188	2.45	0.36	693
	Cholesterol-TMS	51.6	2.74	1680	3.46	1.91	338	18.8	2.83	762
230°	5α-Cholestane	12.9	1.00	1425	1.26	1.00	463	5.16	1.00	1610
	Coprostanol	22.6	1.75	1290	3.00	2.38	1145	9.64	1.87	1482
	Cholesterol	26.5	2.05	1570	3.44	2.73	660	10.72	2.08	1624
	Coprostanone	26.5	2.05		6.54	5.20	915	13.4	2.60	1505
	Coprostanol-TMS	24.9	1.93	1750–2040	1.89	1.50	370	11.2	2.17	1645
	Cholesterol-TMS	26.5	2.05	1480–1690	2.36	1.88	403	14.1	2.73	2003
240°	5α-Cholestane	8.50	1.00	1055	0.87	1.00	216	3.70	1.00	982
	Coprostanol	14.3	1.68	1085	2.02	2.32	495	6.90	1.86	1229
	Cholesterol	15.7	1.84	1105	2.20	2.55	385	7.56	2.04	1218
	Coprostanone	15.7	1.84	1360	4.10	4.73	435	9.71	2.62	1680
	Coprostanol-TMS	15.4	1.79	1690	1.26	1.45	880	6.29	1.70	2039
	Cholesterol-TMS	19.2	2.24	1450–1650	1.58	1.82	130	7.80	2.11	1568

[a] Absolute retention time in minutes.
[b] Retention time, relative to 5α-cholestane.
[c] Number of theoretical plates; average of at least three determinations.

phase, now a single analysis suffices for a positive identification. Indeed, our column should find general application in the analysis of mixtures of steroids with identical functional groups. For instance, the following functional isomers were also found to be easily resolved at 230°: 14α-hydroxy-4-pregnene-3,20-dione (RRT 13.34) from 21-hydroxy-4-pregnene-3,20-dione (RRT 15.15), and 3-ethoxy-2,4-pregnadien-20-one (RRT 0.61) from 3-ethoxy-3,5-pregnadien-20-one (RRT 0.71).

The gas chromatographic method using a single combination column described

in this paper, has been proven in our laboratory to be of great utility in the rapid and reliable analysis of contamination of natural waters by untreated sewage discharge, whereby as a condition, distinct resolution of the three fecal steroids coprostanol, cholesterol and coprostanone[12] had first to be achieved.

ACKNOWLEDGEMENT

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