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Paper chromatographic separation of sterol mixtures by a reversed-phase system*

Investigations of insect sterol metabolism have necessitated the separation of sterols, particularly cholesterol from 7-dehydrocholesterol, and such procedures have invariably utilized adsorption column chromatography[1,2]. This method is accurate but time-consuming (*ca.* 36 h per test).

Although there have been several reports of sterol separation by paper chromatography, especially treated paper[3-6], this laboratory has been unable to reproduce the data; of especial interest, cholesterol and 7-dehydrocholesterol would smear together and never separate when chromatographed in mixture, while individual R_F values would indicate separation possibilities. The latter phenomenon was true of mixtures of other closely related sterols.

More recently thin-layer chromatography (TLC) in a reversed-phase system was reported for the separation of mixtures of animal and vegetable fats[5], cholesterol and 7-dehydrocholesterol being two sterols separated. Although the procedure gave accurate separations on occasion, the TLC plates were difficult to prepare in an identical fashion from test to test, especially the coating procedure, and sterols must be acetylated before being chromatographed.

This paper reports on the modification of the reversed-phase paper chromatographic system for separating mixtures of certain sterols, with particular emphasis on the separation of cholesterol from 7-dehydrocholesterol.

Materials and methods

The paper chromatographic chambers used in these studies were 500-ml glass-stoppered graduate cylinders, lined with Whatman No. 1 filter paper to increase vapor saturation. The mobile phase consisted of acetic acid–acetonitrile (1:3) saturated with *n*-undecane. The stationary phase (20% *n*-undecane in chloroform) was coated onto 2.54 cm Whatman No. 1 chromatography paper by dipping the paper through the stationary phase solution and then allowing the chloroform to evaporate. The sterol(s) were spotted (10–20 μg each) on the paper and the strips placed into the chambers within 5 min from the time they were coated with the stationary phase. In this closed, ascending system it took *ca.* 5 h for a 25–26 cm length of run.

For conducting tests with a continuous ascending paper system, cork stoppers were split longitudinally and then each half lined with aluminum foil and smoothed for a tight fit. The paper strips were hung between the two halves with *ca.* 2.54 cm extending through the tip. The solvent would ascend the strip, pass through the cork stopper, and evaporate. This system was run for 16 h for best separations.

The column chromatographic system used was a 1.1 × 25 cm column of 25 g Woelm acid aluminum oxide, grade II, maintained at 29° (ref. 1). The column was eluted with *n*-hexane–benzene (95:5) and the fractions (20 ml) collected on an automatic fraction collector. The column accurately separates cholesteryl acetate from 7-dehydrocholesteryl acetate.

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The radioactive 7-dehydrocholesterol was biosynthesized by feeding [1α-^{3}H]-cholesterol to house fly larvae (*Musca domestica* L.), and the sterols extracted and purified from the adults as previously reported[2].

The solvents used in these studies were reagent grade, and the sterols and steroids were all analytical standards (>98 %). Only 7-dehydrocholesterol and ergosterol were purified further by repeated recrystallization before use[2]. All R_F and R_S values presented are means of at least five determinations, and the compounds were visualized by spraying the dry chromatograms with 10 % ethanolic phosphomolybdic acid and heating the strips for 2–4 min at 100°.

Results

The R_F values of the sterols and steroid hormones separated in 5 h on the reversed-phase paper chromatographic system are presented in Table I. Although the system adequately separated sterol esters from free sterols from the more polar steroid hormones and cholic acid, individual separations were not attained. Thus cholesterol and 7-dehydrocholesterol ran together as did all the sterols even though the R_F values for the compounds alone might indicate otherwise. Mixtures of the compounds, therefore, showed up as a large smear.

To complete separations of the sterol mixtures (the steroid hormones and cholic acid are so polar they will migrate to the front—the top of the stopper), the continuous ascending reversed-phase system was developed and the R_S values are recorded in Table II. It is shown that several useful separations can be attained, *i.e.* cholesteryl acetate from cholesterol and 7-dehydrocholesterol as well as β-sitosterol,

TABLE I

R_F VALUES OF STEROLS AND STEROID HORMONES

Development for 5 h on the reversed-phase paper chromatographic system acetic acid–acetonitrile (1:3) saturated with *n*-undecane; paper coated with *n*-undecane.

Compound	*R_F value*[a]
Cholesteryl stearate	0.01
Cholesteryl acetate	0.04
Cholestanol	0.23
β-Sitosterol	0.23
Lanosterol	0.25
Cholesterol	0.27
Campesterol	0.29
Stigmasterol	0.29
7-Dehydrocholesterol	0.40
Desmosterol	0.42
Ergosterol	0.43
Calciferol	0.44
Ecdysterone	0.84
Cholic acid	0.86
Pregnandiol	0.86
Estradiol	0.88
Androsterone	0.89
Epiandrosterone	0.89
Testosterone	0.90

[a] Spots detected with 10% ethanolic phosphomolybdic acid.

TABLE II

R_S VALUES[a] OF STEROLS

Development in a continuous ascending chamber for 16 h by the reversed-phase paper chromatographic system acetic acid–acetonitrile (1:3) saturated with *n*-undecane; paper coated with *n*-undecane.

Compounds in mixture	*Distance from origin to spot (cm)*	*R_S value*
Cholesteryl acetate	5.2	—
Cholesterol	20.5	3.94
7-Dehydrocholesterol	27.7	5.33
Cholesteryl acetate	4.5	—
Lanosterol	10.6	2.36
Cholesterol	21.6	4.80
7-Dehydrocholesterol	27.3	6.10
Cholesteryl acetate	5.6	—
Cholesterol	22.5	4.02
7-Dehydrocholesterol	28.5	5.09
Calciferol	31.6	5.64
Cholesteryl acetate	4.7	—
β-Sitosterol	15.1	3.21
Cholesterol	19.6	4.17
7-Dehydrocholesterol	25.4	5.40

[a] *S* = Cholesteryl acetate; spots were detected with 10% ethanolic phosphomolybdic acid.

TABLE III

COMPARISON OF SEPARATION EFFICIENCY OF [1α-^{3}H]CHOLESTEROL AND 7-DEHYDRO[1α-^{3}H]-CHOLESTEROL BETWEEN THE REVERSED-PHASE PAPER CHROMATOGRAPHIC SYSTEM AND AN ADSORPTION COLUMN CHROMATOGRAPHIC SYSTEM OF WOELM ACID ALUMINUM OXIDE, GRADE II

Separation procedure	*Test No.*	*Percent of radioactivity*[a]	
		Cholesterol	*7-Dehydrocholesterol*
Free sterols by reversed-phase paper system	1	95	5
	2	82	18
Acetylated sterols by column chromatography	1	93	7
	2	84	16

[a] Determinations conducted in triplicate; total radioactivity on each chromatogram averaged 21,810 c.p.m., the radiochemical purity was >98%, and the observable specific activity was 920 c.p.m./μg.

lanosterol, and calciferol. Different sterol mixtures will give slightly different R_S values for the individual sterols chromatographed; however, reproducibility within a mixture group is extremely close.

To demonstrate clean separation between cholesterol and 7-dehydrocholesterol on the continuous ascending paper system, radioisotopes were employed (Table III). When reversed-phase paper chromatography was used, 95 % of the total radioactivity behaved like cholesterol and 93 % comprised cholesterol when the sample was subse-

quently acetylated and examined by column chromatography (test No. 1). Test No. 2 showed a similar 2% difference. Thus, within 2% the samples behaved in an identical manner by two different means of chromatographic separation. The smaller quantities of radioactivity which behaved like 7-dehydrocholesterol were confirmed as that compound by ultraviolet spectroscopy (showed a typical $\Delta^{5,7}$-conjugated diene spectrum).

Discussion

Reversed-phase paper chromatographic separation of free sterols by a continuous ascending system has been shown to be very reliable, especially for separating cholesterol from 7-dehydrocholesterol. Such a separation technique is much faster than column chromatography, and although slower than TLC methods[5], it is easier to prepare and free sterols may be utilized.

It is important to point out that a standard such as cholesteryl acetate must be employed for visual recognition of the sterols, because different mixtures of sterols on identical chromatograms will give slightly different *Rs* values. Such a phenomenon is not unknown, because in gas-liquid chromatography, several liquid phases will present what appears to be good separate retention times for individual sterols, but will not separate the sterols in mixture[2].

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1 J. N. KAPLANIS, W. E. ROBBINS AND L. A. TABOR, *Ann. Entomol. Soc. Amer.*, 53 (1960) 265.
2 R. E. MONROE, T. L. HOPKINS AND S. A. VALDER, *J. Insect Physiol.*, 13 (1967) 210.
3 R. B. DAVIS, J. M. MCMAHON AND G. KALNITSKY, *J. Amer. Chem. Soc.*, 74 (1952) 4483.
4 D. KRITCHEVSKY AND M. R. KIRK, *J. Amer. Chem. Soc.*, 75 (1952) 4484.
5 J. W. COPIUS-PEEREBOOM AND H. W. BEEKES, *J. Chromatogr.*, 17 (1965) 99.
6 J. W. COPIUS-PEEREBOOM, J. B. ROOS AND H. W. BEEKES, *J. Chromatogr.*, 5 (1961) 500.

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