9 R. G. JONES, *J. Am. Chem. Soc.*, 69 (1947) 2350.
10 N. KHAN, *Org. Syn.*, 32 (1952) 104.
11 D. SWERN, G. BILLEN, T. W. FINDLEY AND J. T. SCANLAN, *J. Am. Chem. Soc.*, 67 (1945) 1786.
12 D. SWERN AND E. F. JORDAN, JR., *J. Am. Chem. Soc.*, 67 (1945) 902.
13 V. L. HANSLEY, *Ind. Eng. Chem.*, 39 (1947) 55.
14 R. F. NYSTROM AND W. G. BROWN, *J. Am. Chem. Soc.*, 69 (1947) 2548.

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Separation of vitamin D from cholesterol by thin-layer chromatography

Vitamin D* which represents the group of physiologically important 9,10-secosterols, otherwise known as open-ring B sterols, exhibits a high degree of biological activity. Since these substances occur in extremely low concentrations in animal tissues, they present a difficult problem[1], generally beyond the scope of conventional physiochemical techniques of analysis. During the course of our studies on this 'trace lipid', we developed in this laboratory highly sensitive gas–liquid chromatographic techniques for its detection in the nanogram range[2–4]. Although several purification techniques have appeared in the literature[5–8], the present paper describes a rapid and simple thin-layer chromatographic system for the prepurification of vitamin D in biological extracts prior to its determination by GLC.

Experimental

Sorbents and reagents. Silica Gel G, Silica Gel HF_{254}, and Aluminium Oxide G were obtained from E. Merck, Darmstadt, Germany. All solvents used in the study were of ACS grade, marketed by Fisher Scientific Company. The marker dye, 2,4-diaminoazobenzene, was purchased from K & K Laboratories, New York. Rhodamine 6G and antimony trichloride were obtained from the Fisher Scientific Company.

General procedure. A mixture of 30 g of sorbent and 66 ml of water was shaken vigorously for about 40 sec, while under partial vacuum created by a water pump. The slurry was immediately spread over five 20 × 20 cm plates at a thickness of 300 μ in a Shandon adjustable spreaderunoplan leveller. The plates were left at room temperature for about 30 min after which they were activated for 2 h at 110°, and stored in Brinkman aluminum carrier racks placed in metal desiccating cabinets (Arthur Thomas & Co., Philadelphia, Pa.).

The plates were generally divided into three vertical lanes, 6 cm wide, terminating on a horizontal line drawn 15 cm from the origin. Samples were spotted on a horizontal line with a 50 μl semi-automatic microsyringe (Hamilton Co., Inc., Whittier, Calif.), and developed in tanks which were previously equilibrated for 1 h with 170 ml of

* The term "vitamin D" refers to both ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3).

solvent mixture. Zones were visualized under long-wavelength ultraviolet light after spraying with Rhodamine 6G (0.4% in ethanol), or by spraying with antimony trichloride (20% in acetic acid), and heating at 200° for 3 min. R_F values were then calculated from the distances travelled by the compounds and the solvent.

Results

Table I gives the R_F values of cholesterol and vitamin D on Silica Gel G, using a variety of solvent mixtures. It can be seen that the best separations are attained either by a mixture of ethylene dichloride–benzene–acetone (90:90:20), or by a mixture of ethylene dichloride–methyl isobutyl ketone (EDC–MIK) (180:20). The latter solvent system yields zones which are more discrete than those afforded by the former. Table II gives R_F values for cholesterol, ergosterol, 7-dehydrocholesterol, vitamin D, and the marker dye, 2,4-diaminoazobenzene, on Silica Gel G, using the

TABLE I

RELATIVE MOBILITIES OF CHOLESTEROL AND VITAMIN D ON SILICA GEL G

Solvent system	R_F *values*[a]	
	Cholesterol	*Vitamin D*
Benzene–tetrahydrofuran (180:20)	0.23	0.30
Benzene–acetone (180:20)	0.31	0.38
Chloroform	0.15	0.22
Chloroform–benzene (100:100)	0.07	0.12
Chloroform–methanol (190:10)	0.59	0.63
Methylene dichloride	0.08	0.17
Ethylene dichloride	0.07	0.14
Ethylene dichloride–acetone (170–30)	0.31	0.39
Ethylene dichloride–methyl isobutyl ketone (180:20)	0.23	0.33
Ethylene dichloride–tetrahydrofuran (190:10)	0.18	0.23
Ethylene dichloride–benzene–acetone (90:90:20)	0.26	0.40

[a] Values are expressed as the mean of at least three individual determinations.

EDC–MIK solvent system. In this system vitamin D migrates faster than cholesterol, ergosterol, and 7-dehydrocholesterol, the last three compounds possessing identical R_F values. Among several dyes investigated, 2,4-diaminoazobenzene has the same R_F value as vitamin D, and can be used to locate the vitamin D zone obtained by TLC of biological extracts. In our experience replacement of Silica Gel G with aluminum oxide did not change the characteristics of the EDC–MIK solvent system. Fig. 1 illustrates the separation of cholesterol from vitamin D on Silica Gel G, using the EDC–MIK solvent system.

Purification of vitamin D from biological extracts. Biological extracts containing submicrogram amounts of vitamin D are chromatographed on silanized Silica Gel HF_{254}. Zones corresponding to vitamin D are located by reference to the position of the marker dye, 2,4-diaminoazobenzene, which is generally spotted on the lateral margins of each channel. Vitamin D is then eluted from the silica gel with ethylene dichloride, using a TLC spot extractor, and determined by GLC[4]. Recoveries have varied between 70–80% in this step.

TABLE II

SEPARATION BY THIN-LAYER CHROMATOGRAPHY OF CHOLESTEROL, ERGOSTEROL, AND 7-DEHYDROCHOLESTEROL FROM VITAMIN D ON SILICA GEL G[a]

Compounds	*R_F values*
Cholesterol	0.23
Ergosterol	0.23
7-Dehydrocholesterol	0.23
Vitamin D	0.33
2,4-Diaminoazobenzene[b]	0.33

[a] Mobile phase: ethylene dichloride–methyl isobutyl ketone (180:20).
[b] Marker dye.

Characteristics of chlorinated solvents

Since completing this work, PONCHON AND FELLERS[9] reported the separation of vitamin D from several sterols using either chloroform or a mixture of petroleum ether–benzene (50:50). Among the chlorinated solvents that are effective in separating vitamin D from cholesterol, ethylene dichloride and methylene dichloride are preferable

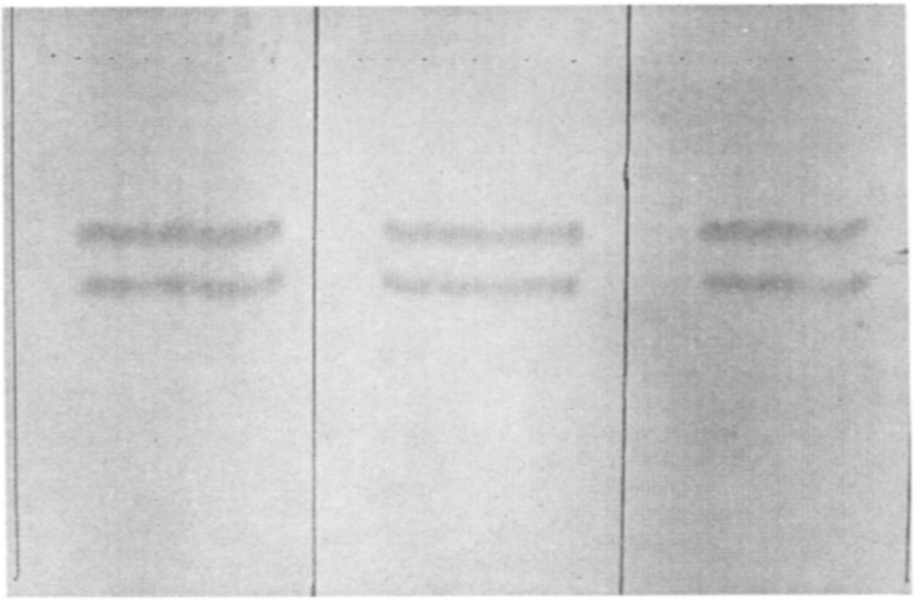

Fig. 1. TLC separation of cholesterol and vitamin D on Silica Gel G, using ethylene dichloride–methyl isobutyl ketone as the mobile phase .The upper longitudinal zone represents vitamin D and the lower one, cholesterol. Antimony trichloride was used for detecting the steroids.

to chloroform because of the sharper separations obtainable with the first two solvents. The present study has also revealed that combinations of these chlorinated solvents with ketones provided maximum resolution of the cholesterol-9,10-secosterol pair, a prerequisite for the pre-purification of biological extracts containing vitamin D prior to its determination by gas–liquid chromatography.

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1 P. P. NAIR, *Advan. Lipid Res.*, 4 (1966) 227.
2 P. P. NAIR, C. BUCANA, S. DELEON AND D. A. TURNER, *Anal. Chem.*, 37 (1965) 631.
3 P. P. NAIR AND S. DELEON, in D. KRITCHEVSKY, R. PAOLETTI AND D. STEINBERG (Editors), *Progress in Biochemical Pharmacology*, Vol. 3, Karger, Basel/New York, 1967, p. 498.
4 P. P. NAIR AND S. DELEON, *Arch. Biochem. Biophys.*, 128 (1968) 663.
5 A. MAYER, C. W. PICARD AND F. WOKES, *Pharm. Acta Helv.*, 33 (1958) 603.
6 J. W. PORTER AND D. G. ANDERSON, in E. HEFTMANN (Editor), *Chromatography*. Reinhold, New York, 1961, p. 459.
7 J. DAVIDEK AND J. BLATTNA, *J. Chromatog.*, 7 (1962) 204.
8 J. G. THEIVAGT AND D. J. CAMPBELL, *Anal. Chem.*, 31 (1959) 1375.
9 G. PONCHON AND F. X. FELLERS, *J. Chromatog.*, 35 (1968) 53.

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