[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF MEDICINE, STATE UNIVERSITY OF IOWA]

Separation of D Vitamins from Other Sterols by Paper Chromatography and the Quantitative Determination of 7-Dehydrocholesterol[1]

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The separation of vitamins D_2 and D_3 from a mixture of cholesterol, 7-dehydrocholesterol, ergosterol and sitosterol by chromatography on impregnated filter paper is described.

The separation of certain sterols by paper partition chromatography, in which the partition is effected between the organic mobile phase and the water of the hydrated cellulose of the filter paper, has been reported.[2] By using filter paper in which the surface has been altered so that partition is obtained between two organic phases, the separation of certain sterols having a low water solubility is improved. We were unable to effect satisfactory separations using acetylated filter paper[3] or benzoylated filter paper. However, the use of Quilon (stearato chromic chloride) treated paper to separate cholesterol and cholestenone has been reported.[4]

This communication reports the separation of the vitamins D_2 and D_3 (in 40 μg. quantities) from a mixture of cholesterol, 7-dehydrocholesterol, ergosterol and sitosterol by using Whatman No. 1 filter paper impregnated with Quilon.[5] In addition, a quantitative determination of 7-dehydrocholesterol (in the presence of cholesterol and ergosterol, all of which have the same R_f values) is presented by adapting Mueller's spectrophotometric method.[6]

These methods afford a way for detecting the conversion of

cholesterol ⟶ 7-dehydrocholesterol ⟶ vitamin D_3, and (a)

ergosterol ⟶ vitamin D_2 (calciferol) (b)

on a micro scale, in mammalian tissue, even in the presence of other sterols.

Experimental

Paper Chromatography.—Whatman No. 1 filter paper was prepared by immersing 1-inch strips in a 2% solution of Quilon for two minutes, running through a hand wringer and drying in an oven at 100–110° for two hours. The Quilon impregnated strips were allowed to stand at room temperature for at least 24 hours after drying in the oven. Cholesterol, 7-dehydrocholesterol and ergosterol were recrystallized before use. Sitosterol, vitamins D_2 and D_3 were used without recrystallization. A ten-microliter spot of the solvent containing the sterol was used, the concentration of cholesterol and sitosterol being 80 μg./10 μl., vitamins D_2, D_3 and 7-dehydrocholesterol 40 μg./10 μl., and ergosterol 30 μg./10 μl.

Initially, each compound was chromatographed separately; with each solvent, where R_f values for the D vitamins were different from the other sterols, the following combinations were chromatographed on separate strips: (1) vitamin D_2 and 7-dehydrocholesterol, (2) vitamin D_3 and cholesterol, (3) sitosterol and ergosterol. These combinations were chromatographed for all solvents for which a separation of the D vitamins from the other sterols is reported. In addition, with those solvents for which maximal separation is reported (Table I), the separations were confirmed by spotting vitamins D_2, D_3, cholesterol and 7-dehydrocholesterol on a single strip, as well as spotting all six of the reported sterols on a single strip and developing in the given solvent. Ascending chromatograms were run at 26 ± 2°, the solvent being allowed to migrate about 12 inches from the point of application of the compounds. The spots were developed with a 40% solution of $SbCl_5$ in chloroform, which is a somewhat more sensitive method than that previously reported.[2]

Determination of 7-Dehydrocholesterol.—To one ml. of chloroform, containing the 7-dehydrocholesterol, is added 10 ml. of antimony trichloride reagent.[7] The optical density is measured after eight minutes in the Beckman spectrophotometer, model DU, at 322 mμ, using the ultraviolet lamp and a slit width of 3.6 mm.

Discussion

Fifteen of the solvent combinations used to develop the chromatograms effected separations of the D vitamins from the other sterols (cholesterol, 7-dehydrocholesterol, ergosterol and sitosterol). These solvent combinations were: (1) methanol 95 parts by volume, H_2O 5; (2) methanol 100; (3) ethanol 95, H_2O 5; (4) methanol 40, H_2O 20, butanol 20, benzene 20; (5) methanol 85, H_2O 7.5, ether 7.5; (6) methanol 85, H_2O 4.3, skellysolve (boiling point 91–96°) 10.7; (7) methanol 60, H_2O 20, ether 20; (8) methanol 65, H_2O 10, ether 23, skellysolve 2; (9) methanol 65, H_2O 10, ethyleneglycol monomethyl ether 25; (10) methanol 65, H_2O 5, acetic acid 5, ether 25; (11) methanol 75, H_2O 10, ether 15; (12) methanol 65, H_2O 10, ether 25; (13) methanol 70, H_2O 5, ether 25; (14) methanol 60, H_2O 15, ether 25; (15) methanol 65, H_2O 15, ether 20. Certain combinations gave a maximum resolution (Table I).

TABLE I

SOLVENT COMBINATIONS GIVING MAXIMUM RESOLUTION BETWEEN THE INDICATED STEROLS

Compound		Solvent[a] MeOH 65 Water 10 Ether[b] 25	MeOH 65 Water 20 Ether 20	MeOH 95 Water 5
Vitamin D_2 (40 μg.)	R_f	0.86	0.80	0.91
Vitamin D_3 (40 μg.)	R_f	.88	.76	.91
Cholesterol (80 μg.)	R_f	.52	.49	.73
7-Dehydrocholesterol (40 μg.)	R_f	.56	.48	.67
Ergosterol (30 μg.)	R_f	.55	.43	.68
Sitosterol (80 μg.)	R_f	.55	.43	.65

[a] Parts by volume. [b] Ethyleneglycol monomethyl ether.

Mueller's[6] method for the qualitative determination of 7-dehydrocholesterol in the presence of cholesterol and ergosterol was adapted for the

(1) Aided by a grant from the Central Scientific Fund of the State University of Iowa College of Medicine.

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(4) D. Kritchevsky and M. Calvin, THIS JOURNAL, **72**, 4330 (1950).

(5) Kindly supplied by E. I. du Pont de Nemours and Company.

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(7) D. T. Ewing, V. G. Kingsley, R. A. Brown and A. D. Emmett, *Ind. Eng. Chem., Anal. Ed.*, **15**, 301 (1943).

quantitative determination of 7-dehydrocholesterol, in concentrations of 2 to 14 γ per cc., by measuring the ultraviolet absorption in the Beckman spectrophotometer for different concentrations at 322 mμ. Under these conditions, there is a linear relationship between the optical density and concentration of 7-dehydrocholesterol. Neither cholesterol, ergosterol nor calciferol interfere. By eluting 7-dehydrocholesterol from the known position on the chromatogram, the conversion from cholesterol or other precursors can be detected.

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Paper Chromatography of Steroids[1]

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The preparation and use of "Quilon" (stearato chromic chloride) impregnated paper for the reverse phase paper partition chromatography of steroids is described. The R_f values for a number of steroids in a variety of solvents are reported. Separation of cholesterol from epicholesterol, ergosterol and 7-dehydrocholesterol has been achieved. Several other separations are also reported.

Through the use of impregnated filter paper it has been possible to achieve separation of various steroid mixtures by paper chromatography. The corticosteroids have been separated using papers treated with propylene glycol,[2,3] formamide[2,3] or alumina[4,5]; the estrogens on paper treated with alumina,[4,5] glycerol,[6] ethylene glycol[6] or capryl alcohol[6]; and the androgens on papers impregnated with alumina[4,5] or a silicone.[7] Recently, Neher and Wettstein[8] have reported the separation of weakly polar steroids on paper treated with phenyl cellosolve. The successful separation of cholesterol and cholestenone on paper impregnated with "Quilon" (stearato chromic chloride) already has been reported.[9] This method also has been used for the separation of vitamins D_2 and D_3 from a mixture of sterols.[10] This report covers the results obtained by application of this method to a number of steroids. The R_f values obtained with twenty-one steroids using a variety of solvents are tabulated in Tables I, III, IV and V. Each R_f value represents the average of at least six separate chromatograms.

In the case of the weakly polar steroids, the separation of cholesterol from 7-dehydrocholesterol, ergosterol and epicholesterol has been accomplished. Stigmasterol and ergosterol have also been separated. Any two steroids whose R_f values are sufficiently far apart may be separated by this system, and in the case of a mixture of cholesterol and testosterone separation has indeed been carried out. The separations are summarized in Table II.

In the case of the corticosteroids and the androgens, the R_f values are reproducible but no resolution could be achieved with the solvent systems used. The salient feature of the data concerning these compounds is that the addition of water to the anhydrous solvent gives a higher R_f value rather than the lower one which might be expected. Thus, in methanol the corticosteroids all exhibit R_f values in the neighborhood of 0.75 and in methanol–water 9:1 the R_f values are approximately 0.80. The same variation was observed with various androgens and progestational hormones. It is possible that the systems under investigation are exceedingly sensitive to small changes in solvent composition and that the mixtures we have used have bracketed the area of greatest sensitivity. For example, the R_f values for cortisone in methanol–water 95:5, methanol–water 9:1 and methanol–water 85:15 are 0.81, 0.86 and 0.80, respectively. Other steroids exhibit similar variations. Several of the weakly polar steroids exhibit the expected lowering of R_f upon dilution of the methanol.

Addition of ammonia or formic acid to the methanol–water solvent system caused a change in the R_f values (usually higher than for methanol

TABLE I

R_f VALUES OF THE WEAKLY POLAR STEROIDS

	CH_3OH	CH_3OH-H_2O 9:1	C_2H_5OH	C_2H_5OH-H_2O 8:2
Cholesterol	0.56	0.31	0.92	0.52
Epicholesterol	.80	..	..	.97
Cholestanol	.63	..	..	.56
Stigmasterol	.52	0.27	..	.53
Sitosterol	.65	..	..	.54
Cholestenone	.82	..	0.97	.86
7-Dehydrocholesterol	.88	..	..	.94
Ergosterol	.84	0.90	..	.95

TABLE II

SEPARATIONS IN METHANOL

Compounds	R_f Values
Cholesterol/ergosterol	0.52/0.84
Cholesterol/7-dehydrocholesterol	.54/ .88
Cholesterol/epicholesterol	.56/ .81
Cholesterol/testosterone	.54/ .77
Stigmasterol/ergosterol	.47/ .85

(1) The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

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