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Removal of cholesterol from serum extracts of vitamin D by thin-layer chromatography

The main contaminant present in vitamin D* extracts from human serum is cholesterol. The most part of this can be eliminated by precipitation with a solution of digitonin[1–3]. However, further purification is required when vitamin D is to be analysed quantitatively by gas–liquid chromatography (GLC). Previous workers have used liquid chromatography[1,2,4] or thin-layer chromatography (TLC) of the isovitamin[3].

This paper compares a range of solvents, and describes a simple method, utilizing pre-coated silica gel plates, for complete elimination of cholesterol prior to GLC.

Experimental

Adsorbents and reagents. Silica Gel GF_{254} and Aluminium Oxide GF_{254} were obtained from E. Merck, Darmstadt, G.F.R. Precoated plates, Eastman Chromogram Sheet 6061, came from Eastman Kodak Ltd. Precoated plates, Baker-Flex Silica Gel IBF, were made by J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A. Crystalline vitamins D_3 and D_2 were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and DHT from Mann Research Labs. Inc., N.Y., U.S.A. All solvents and reagents were A.R. grade, the solvents being redistilled before use. Plates were sprayed with a solution of 20% phosphotungstic acid in absolute ethanol followed by warming in an oven at 100° for several minutes. Cholesterol gives a dark pink spot, vitamins D_2 and D_3 both turn orange-brown and DHT gives a pink-brown spot[5].

Preparation of plates. A slurry of 20 g of adsorbent in 40 ml of distilled water was applied to 20 × 10-cm glass plates as a uniform layer of approximately 250 nm thickness. The plates were activated at 120° for 2 h.

Solvent systems. Preliminary experiments to find a suitable solvent system for separation of vitamin D from cholesterol were carried out using silica gel precoated plates cut into strips 6 × 2 cm. These were spotted 1.5 cm from the bottom with a petroleum ether (b.p. 60–80°) solution containing 5 μg of vitamin D and 5 μg of cholesterol. They were developed in small stoppered jars containing solvent to a depth of 1 cm. Before inserting the chromatogram, the jar was shaken to saturate the air space with solvent vapour. When the solvent front had almost reached the top of the plate, the chromatogram was removed, dried in air, and sprayed with the colour reagent.

Solvent systems giving adequate separation of vitamin D and cholesterol were then applied to the prepared glass plates coated with either Aluminium Oxide GF_{254} or Silica Gel GF_{254}. Individual spots of vitamin D and cholesterol were applied at both sides as markers and mixtures were spotted in the centre of the plate. The origin was 2 cm from the bottom of the plate and development was performed in glass tanks

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

TABLE I

SOLVENT SYSTEMS TESTED

n-Hexane	*n*-Hexane–acetone (90:10)
Cyclohexane	Chloroform–methanol (99:1)
Carbon tetrachloride	Chloroform–methanol (95:5)
Chloroform	Petroleum ether (b.p. 40–60°)–benzene (90:10)
Benzene	Petroleum ether (b.p. 40–60°)–benzene (50:50)
Toluene	*n*-Hexane–chloroform (95:5)
Trichloroethane	*n*-Hexane–chloroform (90:10)
Dichloromethane	*n*-Hexane–chloroform (75:25)
Diethyl ether	*n*-Hexane–chloroform (50:50)
Ethyl acetate	*n*-Hexane–diethyl ether (80:20)
n-Butanol	Cyclohexane–heptane (50:50)
Isopropanol	Cyclohexane–ethyl acetate–water (1560:440:1)
Acetone	Isooctane–carbon tetrachloride (20:1)
Ethanol	Hexane–ethyl acetate (90:10)
Methanol	Cyclohexane–diethyl ether (50:50)
n-Hexane–acetone (95:5)	Cyclohexane–ethyl acetate (90:10)

containing solvent to a depth of 1.5 cm. Filter paper was used to line the walls of the tank, providing a vapour saturated with solvent. The developed plates were allowed to dry, sprayed with colour reagent and warmed to detect the spots.

Thirty-two solvent systems previously used by other authors[6–8] and a selection of pure solvents (Table I) were tested on the precoated strips; only five were considered suitable for further investigation (see Tables II and III) on glass plates.

Serum extracts. The extraction of vitamin D from serum gave a petroleum ether solution containing both vitamin D and cholesterol. Since only minute quantities of vitamin D were present, a good recovery was required from the TLC plate. To help prevent breakdown by UV light, the biological samples were streaked along the base-line of a precoated silica gel plate under red light. A 500-μl syringe was used for application and two extracts with marker spots were chromatographed on one plate. The plate was developed for 1 h in the dark and the zone corresponding to vitamin D was located before the plate dried. The centre of the plate was protected with a sheet of paper and the marker lanes were examined under UV light (Mineralight UVSL-25 short-wave, Ultra-Violet Products Inc., San Gabriel, Calif., U.S.A.). Vitamin D showed as a dark spot. For subsequent GLC analysis the vitamin D zone was cut from the chromatogram and eluted with hexane–ether (1:1) from the adsorbent. The recovery of vitamin D after TLC was measured by chromatographing three aliquots of [1,2-^{3}H] vitamin D_3, retaining a fourth as standard. After TLC the vitamin D was eluted from the plate, and all four samples were evaporated to dryness, dissolved in 1 ml toluene and counted in a liquid scintillation counter. The average recovery for vitamin D was 65%.

Results and discussion

Tables II and III give the R_F and R_S values of cholesterol, vitamins D_2 and D_3 and DHT obtained by the authors in the five most successful adsorbent–solvent systems. All the values are for activated glass plates. The aluminium oxide chromatograms were all rather streaky. The silica gel gave superior results in all solvent systems.

TABLE II

R_F AND R_S VALUES[a] ON SILICA GEL GF_{254}

Solvent	R_F				R_S		
	Vitamin D_3	*Vitamin* D_2	*DHT*	*Cholesterol*	*Vitamin* D_3	*Vitamin* D_2	*DHT*
Chloroform	0.31	0.32	0.46	0.27	1.14	1.17	1.67
Hexane–chloroform (50:50)	0.28	0.28	0.39	0.20	1.40	1.40	1.96
Dichloromethane	0.42	0.42	0.59	0.26	1.62	1.62	2.25
Hexane–acetone (90:10)	0.38	0.38	0.51	0.34	1.13	1.13	1.51
Cyclohexane–ethyl acetate–water (1560:440:1)	0.42	0.42	0.52	0.36	1.17	1.16	1.45

[a] R_S Cholesterol = 1.00.

TABLE III

R_F AND R_S VALUES[a] ON ALUMINIUM OXIDE GF_{254}

Solvent	R_F				R_S		
	Vitamin D_3	*Vitamin* D_2	*DHT*	*Cholesterol*	*Vitamin* D_3	*Vitamin* D_2	*DHT*
Chloroform	0.80	0.81	0.87	0.71	1.12	1.14	1.23
Hexane–chloroform (50:50)	0.53	0.52	0.68	0.43	1.23	1.21	1.57

[a] R_S cholesterol = 1.00.

Dichloromethane gave the best separation. The vitamin moved almost halfway up the plate, 2 cm in front of the cholesterol (Fig. 1). When the concentration of cholesterol in the mixture was increased to 100 × that of the vitamin, the R_F value of cholesterol decreased slightly (Table IV) while the value for vitamin D remained the same. The separation was therefore still good, despite the larger area covered by the cholesterol.

TABLE IV

VARIATION OF R_F VALUES WITH INCREASING CONCENTRATION OF CHOLESTEROL ON BAKER-FLEX PRECOATED SILICA GEL PLATE

Cholesterol: vitamin	*Cholesterol*		*Vitamin* D_3		*Vitamin* D_2	
	R_F	R_S	R_F	R_S	R_F	R_S
1:1	0.42	1.00	0.61	1.42	0.60	1.40
2:1	0.42	1.00	0.61	1.42	0.61	1.42
10:1	0.41	1.00	0.61	1.48	0.61	1.48
100:1	0.40	1.00	0.62	1.56	0.61	1.53

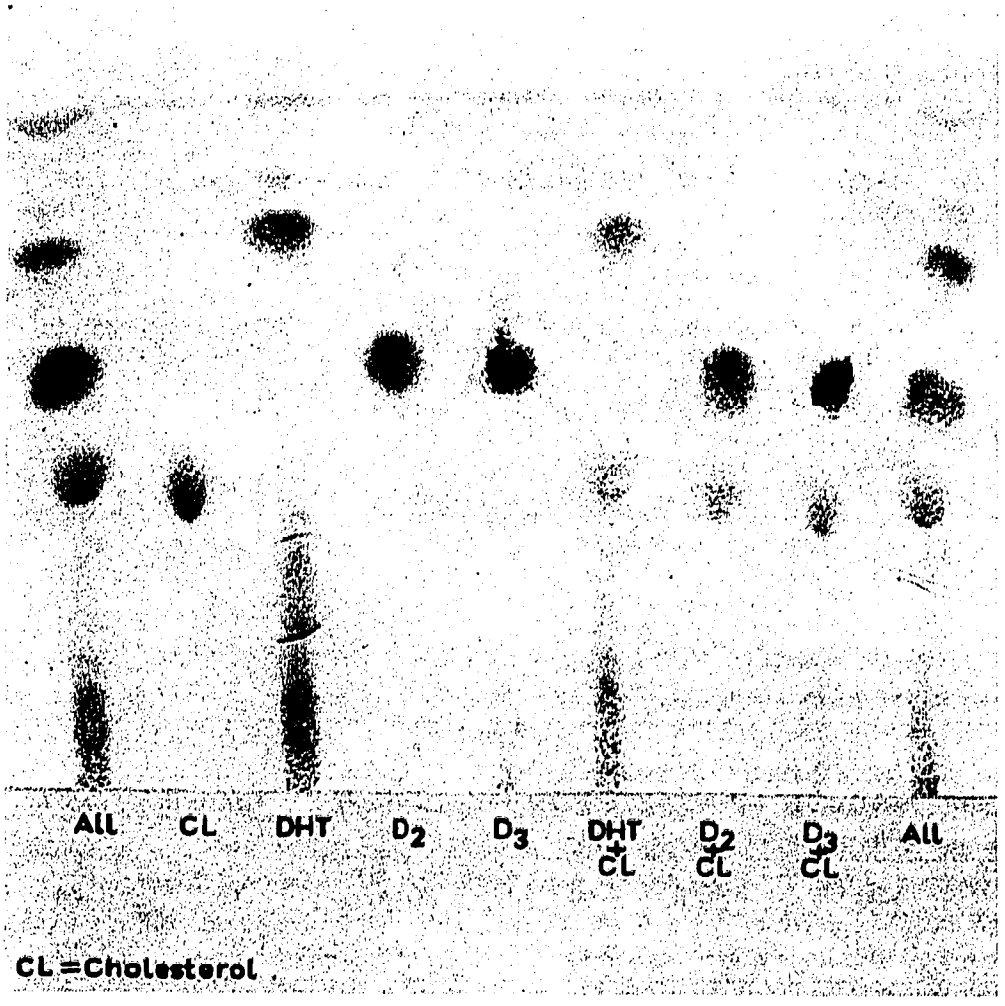

Fig. 1. TLC separation of vitamin D and cholesterol on Baker-Flex precoated silica gel plate. The chromatogram was developed for 1 h in dichloromethane and sprayed with 20% phosphotungstic acid in absolute alcohol.

The R_F values for vitamin D on precoated plates were the same as those on glass plates, but the development time was halved to 1 h. After the initial experiments to ascertain the optimum adsorbent–solvent system, precoated plates were used for routine separations.

Serum extracts chromatographed on GLC after TLC purification on Baker-Flex precoated plates gave several small unidentified peaks. By eluting a blank TLC plate

TABLE V

R_F AND R_S VALUES OF UNIDENTIFIED UV ABSORBING SPOT FROM EXTRACTS RUN ON PRECOATED SILICA GEL PLATES IN DICHLOROMETHANE

Compound	R_F	R_S
Cholesterol	0.34	1.00
Cholecalciferol	0.51	1.48
Unidentified UV absorbing spot	0.79	2.28

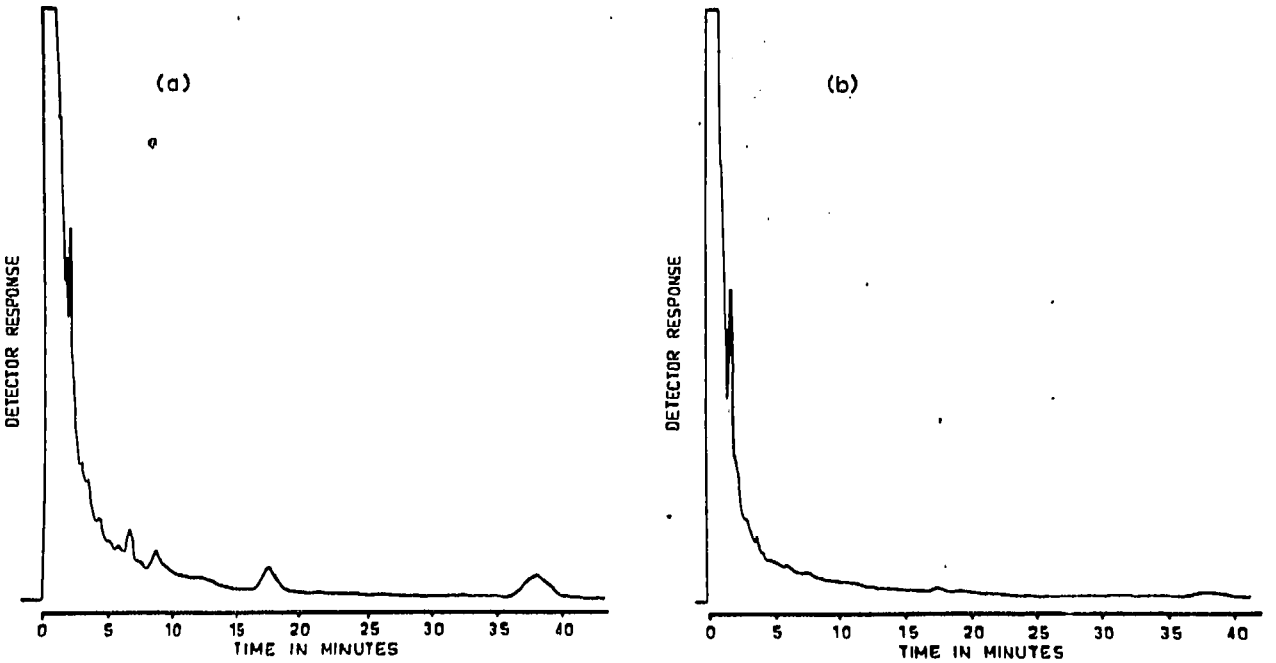

Fig. 2. Gas chromatograms of trimethylsilyl derivatives of extracts from blank Baker-Flex precoated silica gel plates: (a) from unwashed plate; (b) from plate washed once with dichloromethane. Gas chromatograph: Aerograph 2100 with flame-ionisation detector. Column 243 cm × 2 mm I.D., 4% OV-225 on high performance Chromosorb W, 100–120. Injector and detector 300°, column 230°.

it was established that these peaks were contaminants from the TLC plate (Fig. 2). These were eliminated by first developing the blank plate in dichloromethane, then drying and activating it before use. The plates were activated by heating for 60 min at 115°.

A second UV absorbing spot was detected in some chromatograms with R_F and R_S values as shown in Table V. This was possibly pre-vitamin D (ref. 5). The largest concentration, estimated from intensity of colour produced with spray reagent, was obtained in the cyclohexane–ethyl acetate–water system. This solvent system may encourage the formation of pre-vitamin D from vitamin D.

Two other reports for the separation of vitamin D from cholesterol have been published[5,9]. Some of the R_F values published by PONCHON AND FELLERS[5] are very low, so that, although the R_S values are high, the methods are impractical for separation of vitamin D from cholesterol. Aluminium oxide and chloroform apparently gave the most encouraging results but we were unable to duplicate these results.

PINELLI *et al.*[9] recommend ethylene dichloride–methyl isobutyl ketone (180:20) or ethylene dichloride–benzene–acetone (90:90:20) with Silica Gel G adsorbent. However, both R_F and R_S values are significantly lower than in our system, and the efficiency of the method in the presence of a large excess of cholesterol was not examined.

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