Separation of cholesterol from its companions, cholestanol and Δ^7-cholestenol, by thin-layer chromatography

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SUMMARY A two-step thin-layer chromatographic procedure is described for separation of cholesterol from its naturally occurring companions. Impregnation of Silica Gel G with silver nitrate retards cholesterol relative to cholestan-3β-ol and Δ^7-cholestenol, which can be separated from each other on a second plate by reversed-phase thin-layer chromatography at low temperature.

KEY WORDS cholesterol . cholestanol . Δ^7-cholestenol . separation . thin-layer chromatography . silver nitrate . interference Liebermann-Burchard estimation . ethanolic ammonia extraction

Cholestan-3β-ol (dihydrocholesterol) and Δ^7-cholestenol (lathosterol) accompany cholesterol in serum, feces, and several tissues (1, 2), and cholestanol has also been reported in gallstones and atheromatous plaques (3). Small amounts of these two sterols cannot be separated from large amounts of cholesterol by ordinary thin-layer chromatography (TLC) (4, 5) or glass paper chromatography (6, 7) because their R_F values are too close. With gas–liquid chromatography, resolution of mixtures of cholesterol and cholestanol is difficult and has been achieved only by making derivatives and using selective phases (8).

Cargill (4) and Michalec (9) separated cholestanol from cholesterol on thin-layer plates after the mixture had been subjected to bromination, which converted cholesterol to the faster-moving dibromide but left the saturated cholestanol unaltered. Unfortunately, more polar oxidation products of cholesterol were formed at the same time (4), which would make it difficult to estimate the cholesterol after developing the chromatogram. Avigan, Goodman, and Steinberg (5) were able to separate Δ^7-cholestanol from cholesterol with benzene–ethyl acetate 20:1 on Silica Gel G, but the plates had to be 40 cm long and the run took 24–36 hr. With this method, we find that cholestanol runs together with cholesterol.

We have not been able to obtain practicable separation of Δ^7-cholestenol from cholesterol with the system cyclohexane–ethyl acetate–water, 600:400:1 on Silica Gel G plates, suggested by Bennett and Heftmann (10).

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De Vries (11) introduced the use of silica gel impregnated with silver nitrate for the separation of *cis* and *trans* isomers of fatty acids by column chromatography. This principle has been extended to the TLC of lipids of several classes differing only by one double bond (5, 12–15). During a study of human fecal neutral steroids we have found that cholestanol can be completely separated from cholesterol on plates 20 cm long coated with silica gel impregnated with silver nitrate. Evidently cholesterol forms the expected silver complex at its double bond and is retarded on the plate. However, Δ^7-cholestenol is not retarded and moves ahead with cholestanol. The faster spot containing cholestanol and Δ^7-cholestenol can be resolved by a second, reversed-phase TLC at low temperature.

Materials. Silica Gel G was obtained from E. Merck AG, Darmstadt, Germany. Chloroform (which contains 2% ethanol), acetone, and petroleum ether (bp 40–60°) were supplied by British Drug Houses Ltd., Poole, Dorset, England. They were "Analar" grade and were not further purified. Benzene (British Drug Houses "Analar" grade) was redistilled. *n*-Undecane (bp 195°) came from L. Light & Co., Colnbrook, Bucks., England. The standard sterols used were cholesterol and cholestan-3β-ol from British Drug Houses; coprostan-3β-ol and Δ^7-cholestenol from Southeastern Biochemicals Inc., Morristown, Tenn. All other chemicals were from British Drug Houses, "Analar" grade except for acetonitrile (methyl cyanide).

First Separation, on Silver Nitrate-Impregnated Thin-Layer Plate. Glass plates, 20 cm long, are coated with a 250 μ layer of Silica Gel G (Merck) using a slurry (30 g/60 ml water) in the Desaga spreader, and stored in a desiccator. When required, a plate is simply sprayed with 25% aqueous $AgNO_3$ until it is evenly moistened. It is then dried in an oven at 110° for 30 min and allowed to cool to room temperature, before applying the sample. The silver nitrate layer starts to darken after standing 12–24 hr, even in the dark, so that impregnated plates should be used the day they are prepared. The best separation of cholestanol plus Δ^7-cholestenol from other sterols is obtained in chloroform–acetone 98:2. The solvent front is allowed to rise 15 cm above the origin. To make the sterols visible for light photography we have found that the best procedure is to spray with 10% ethanolic phosphomolybdic acid and then to heat at 140° for 15–20 min. This gives brown spots on silver-impregnated plates. (Other sprays for sterols such as 1% ceric ammonium sulfate in 10% sulfuric acid, and 35% ortho-phosphoric acid in water are rather unsatisfactory on silver-impregnated plates. They give less distinct spots and less characteristic colors, both in visible and UV light.)

TABLE 1 EFFECT OF SILVER NITRATE IMPREGNATION ON R_F VALUES* OF CHOLESTENOLS AND C_{27} STANOLS ON CHROMATOPLATES

	On Silica Alone	On Silica Plus Silver Nitrate	Ratio R_F on Silica + $AgNO_3$ / R_F on Silica Alone
Cholesterol	0.40	0.48	1.22
Δ^7-Cholestenol	0.39	0.57	1.46
Cholestan-3β-ol	0.39	0.59	1.52
Coprostan-3β-ol	0.51	0.72	1.40
Coprostan-3α-ol	0.51	0.74	1.46
Cholestan-3α-ol	0.58	0.80	1.40

Solvent: chloroform–acetone 98:2, run 15 cm.
* Means of four replicates on each of two pairs of plates run in the same tank simultaneously.

The effect of impregnating silica gel with silver nitrate on the R_F values of cholesterol, Δ^7-cholestenol and the C_{27} stanols is shown in Table 1. The absolute R_F values of all the sterols are increased, perhaps because the silver nitrate reduces the surface of silica available for adsorption. Cholesterol migrates 1.2 times further while Δ^7-cholestenol and the C_{27} stanols all move 1.4 to 1.5 times further. Consequently cholesterol is separated from its companions. Although cholestanol and Δ^7-cholestenol are ahead of cholesterol they run well behind coprostanol, both in a mixture of pure sterols and in a naturally occurring mixture (Fig. 1). In experiments applying this separation method for neutral lipid extracts from human feces, spots of up to 70 μg of cholesterol and 200 μg of coprostanol have been very well separated from cholestanol and Δ^7-cholestenol.

Extraction of Sterols from First Plate: Recovery Experiments. The sterols may be detected nondestructively by lightly spraying the dried plate with distilled water. If the plate is viewed against a dark background, they appear as white spots when the thin layer starts to dry. The smallest amounts that can be detected with certainty in this way are 15 μg of cholesterol and 5 μg of Δ^7-cholestenol.

For recovery experiments 50 μg of sterols were used. The spots were ringed with a fine point, allowed to dry, scraped off the plate into conical centrifuge tubes, and extracted. If methanol was used it carried over small amounts of silver ion which inhibited the Liebermann-Burchard reaction. With 3 ml of acetic anhydride, concentrated sulfuric acid, and glacial acetic acid (16), 7.9 μg of Ag^+ halved the optical density given by 50 μg of cholesterol. The following extraction procedure, which utilizes the ability of ammonia to complex with silver ion, was therefore devised.

Each scraped-off spot in a centrifuge tube was first extracted with 50% ethanolic ammonia (3 × 4 ml). The combined extracts were diluted with an equal volume of distilled water and reextracted with 3 × 10 ml redistilled benzene. The benzene extracts were washed with 20 ml of

distilled water, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness at 40–50° under reduced pressure. The residue was allowed to react with 3 ml of Liebermann-Burchard reagent, following the procedure of Abell, Levy, Brodie, and Kendall (16). Mean recoveries were 95% for cholesterol and 81% for Δ^7-cholestenol.

The same residue from the cholestanol plus Δ^7-cholestenol spot may be taken up in a small volume of chloroform for the second TLC. (If the Liebermann-Burchard reaction is not used for quantification after the second plate, spots from the first plate can be extracted simply with methanol. We have found, for instance, that the Zak et al. (17) procedure for cholesterol is not inhibited by Ag^+ if the ferric chloride is replaced by ferric nitrate to avoid cloudiness caused by silver chloride. However, this reaction seems to give less consistent results than the Liebermann-Burchard reaction.)

Second Separation, Reversed-Phase at Low Temperature. The mixture of Δ^7-cholestenol and cholestanol may be resolved conveniently by reversed-phase TLC (18) at low temperature. A silica gel plate, 20 cm long, is impregnated with stationary phase by allowing 15% *n*-undecane in petroleum ether (bp 40–60°) to rise nearly to the top of the plate in a TLC tank. Samples are applied and the plate is left on the bench 1 hr for the petroleum ether to evaporate. The chromatogram is then developed in the moving phase, acetic acid–acetonitrile 1:1, 70% saturated with undecane. Conditions: temperature −15 to −20°, height 15 cm, time 3–3$^1/_2$ hr. (The tank containing moving phase is placed in the deep freeze 15 min before the run, for temperature equilibration.) After removing undecane from the plate by heating for 1 hr at 150°, sterols may be demonstrated with ethanolic phosphomolybdic acid (Fig. 2). If the

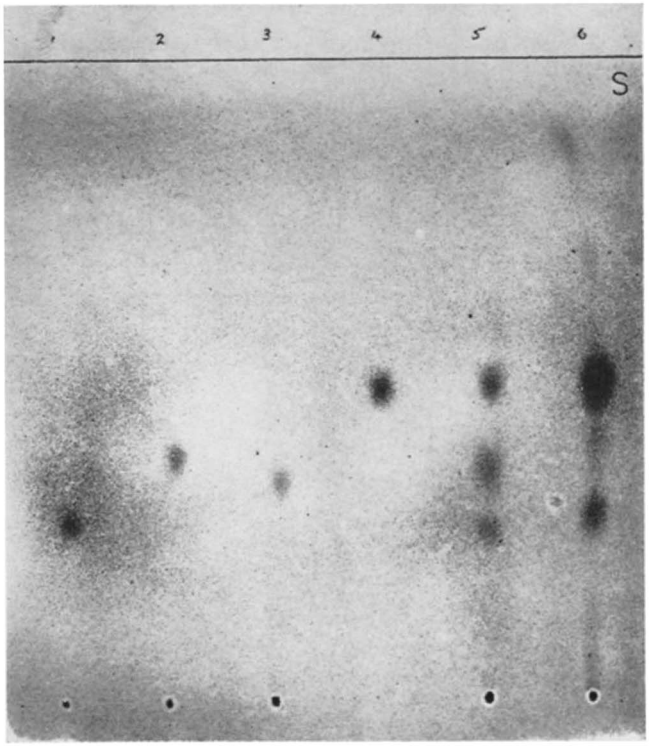

FIG. 1. Photograph of thin-layer chromatogram on Silica Gel G impregnated with silver nitrate. Sprayed with phosphomolybdic acid. *1*, 10 μg cholesterol; *2*, 10 μg cholestan-3β-ol; *3*, 10 μg Δ^7-cholestenol; *4*, 10 μg coprostan-3β-ol; *5*, mixture; and *6*, neutral lipid extract of human feces. *S*, solvent front. Origins at base of the photograph were enlarged after chromatography for easier visualization. The fast spots in the fecal extract are steroid 3-ketones and esters.

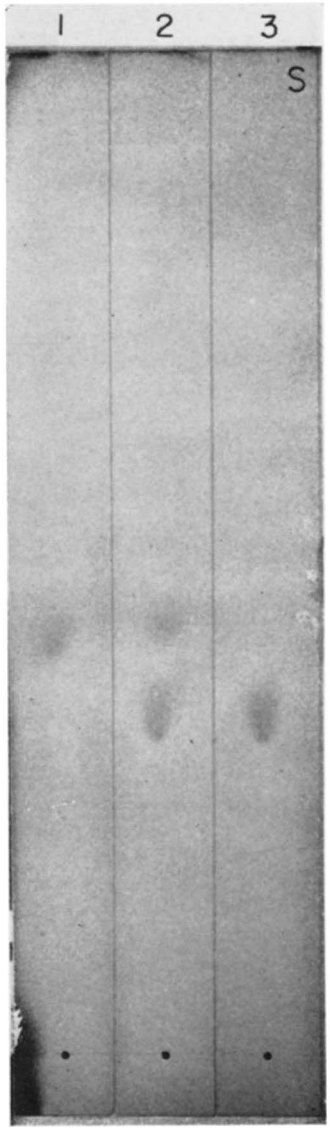

FIG. 2. Photograph of low temperature reversed-phase thin-layer chromatogram of *1*, Δ^7-cholestenol (1 μg); *2*, mixture; *3*, cholestanol (1 μg); *S*, solvent front. Origin at base of the photograph.

plate is sprayed immediately after it is taken out of the oven, it is not necessary to heat further.

The unsaturated Δ^7-cholestenol migrates faster than cholestanol, even at room temperature. But by lowering the temperature the separation factor is increased from 1.175 to 1.39. The greater separation is necessary because the edges of the spots are not as sharp as on layers of silica gel alone. The loads of sterols which can be completely separated in this system are rather small. With more than 5 μg of each sterol there is some blurring of the spots. Cholesterol migrates with cholestanol in this second system, hence the need for two steps to resolve a mixture of cholesterol, Δ^7-cholestenol, and cholestanol by TLC.

We have not been able to combine silver-nitrate impregnated separation and the reversed-phase separation in two dimensional chromatography on a single plate. Reversed-phase TLC was unsuccessful on silver nitrate-impregnated plates.

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