notes on methodology

Separation of sterols by vapor-programmed thin-layer chromatography

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SUMMARY By vapor-programmed thin-layer chromatography on silica gel, it was possible to separate cholestanol from cholesterol and stigmastanol from β-sitosterol. The method was applied for the analysis of β-sitosterol-3-^{14}C.

SUPPLEMENTARY KEY WORDS cholestanol · cholesterol · stigmastanol · β-sitosterol · β-sitosterol-3-^{14}C

By ordinary thin-layer chromatography, sterols containing one double bond are difficult to separate from the corresponding saturated sterols. Special chromatographic techniques are necessary, such as the use of silica gel or alumina impregnated with silver nitrate (1), continuous development (2), or partition chromatography with a reversed-phase system (3). We wish to report here the separation of cholestanol and cholesterol, and of stigmastanol and β-sitosterol by vapor-programmed thin-layer chromatography on silica gel.

The sterols used were the following: cholestanol (Fluka A.G., Buchs, Switzerland), cholesterol (kindly supplied by Wed. D. S. van Schuppen, Veenendaal, The Netherlands) purified by recrystallization, β-sitosterol (kindly supplied by The Upjohn Co., Kalamazoo, Mich.) of about 80% purity as shown by gas–liquid chromatography with stigmastanol and campesterol as the most probable main impurities, and stigmastanol[1] synthesized by reduction of β-sitosterol and purified by recrystallization. Solvents used were of reagent grade (E. Merck A. G., Darmstadt, Germany). The compositions of solvent mixtures are given in volume parts. Silica Gel GF 254 (Merck A. G.) was used as adsorbent, in 0.25 mm layers on glass plates, 20 × 20 cm. After spreading (Desaga apparatus), the plates were air dried for 15 min, heated for 30 min at 110°C in an oven with a fan, then cooled and stored in a dessiccator. The sterols were dissolved in benzene (0.1% w/v), and 5-μl portions were applied with a 10 μl micropipette (Desaga, Heidelberg, Germany), 2.5 cm from the bottom edge of the plate. Small bands of adsorbent, approximately 0.5 cm wide, were scraped off the side edges and the bottom edges. We used normal chambers (Desaga) and the 20 × 20 cm vapor-programming chamber (Desaga). In the latter chamber, the troughs were filled with about 5 ml each of the appropriate solvent mixtures, the plate was fixed in position, and, after allowing 10 min for equilibration, the solvent reservoir was filled with 25 ml of running solvent. Small strips, 0.5 mm thick were used as spacers to prevent the adsorbent from touching the trough walls. These strips were placed on the side walls of the chamber and fitted on to the side edges of the plate from which the adsorbent had been removed. After development the plate was sprayed with 2 N H_2SO_4 solution and heated at 110°C in an oven. Detection was carried out under UV-light, 350 nm (Camag Universal lamp), and photographs were made in daylight on Agfacolor CT 18 Diapositive film.

Fig. 1*A* shows that, with the best system in our hands, toluene–ethyl acetate (80 + 20) in an unsaturated chamber, cholestanol and cholesterol, and also stigmastanol and β-sitosterol, are not separated by ordinary thin-layer chromatography. However, by using a vapor-programming chamber, as described by de Zeeuw (4), and different mixtures of carbon tetrachloride and ether in the troughs, a separation can be achieved between the saturated and unsaturated sterols, which is shown in Fig. 1B. Vapor-programmed TLC is based on the principle that the migration of the solute spots is highly dependent on the adsorption of solvent vapors by the dry adsorbent. To obtain optimum vapor influence, a vapor-programming chamber has been designed in which the plate is developed in a horizontal position. During development the adsorbent faces a number of troughs that can be filled with liquids of different compositions, while the distance between the adsorbent and the trough walls is kept very small to prevent vapor diffusions. Thus, optimum vapor conditions can be established all over the plate. For the separation of closely related substances an accelerating vapor program should be used, i.e. the polarity of the solvent mixtures in the troughs increase from bottom to top. However, in order to prevent tailing of the spots, interspersing troughs with liquids of low polarity (retarding troughs) have to be used between the accelerating troughs. Details are described in the legend of Fig. 1B. Increased proportions of ether are used in the accelerating troughs from bottom to top, whereas carbon tetrachloride is used as retarding liquid. Benzene instead of carbon tetrachloride is used in the solvent reservoir and in the first trough to give the substances a suitable starting migration. The R_f values for cholestanol and stigmastanol are about 0.59, and those for cholesterol and β-

[1] A sample of stigmastanol was prepared by Mr. A. C. Besemer, Central Laboratory TNO, Delft, The Netherlands.

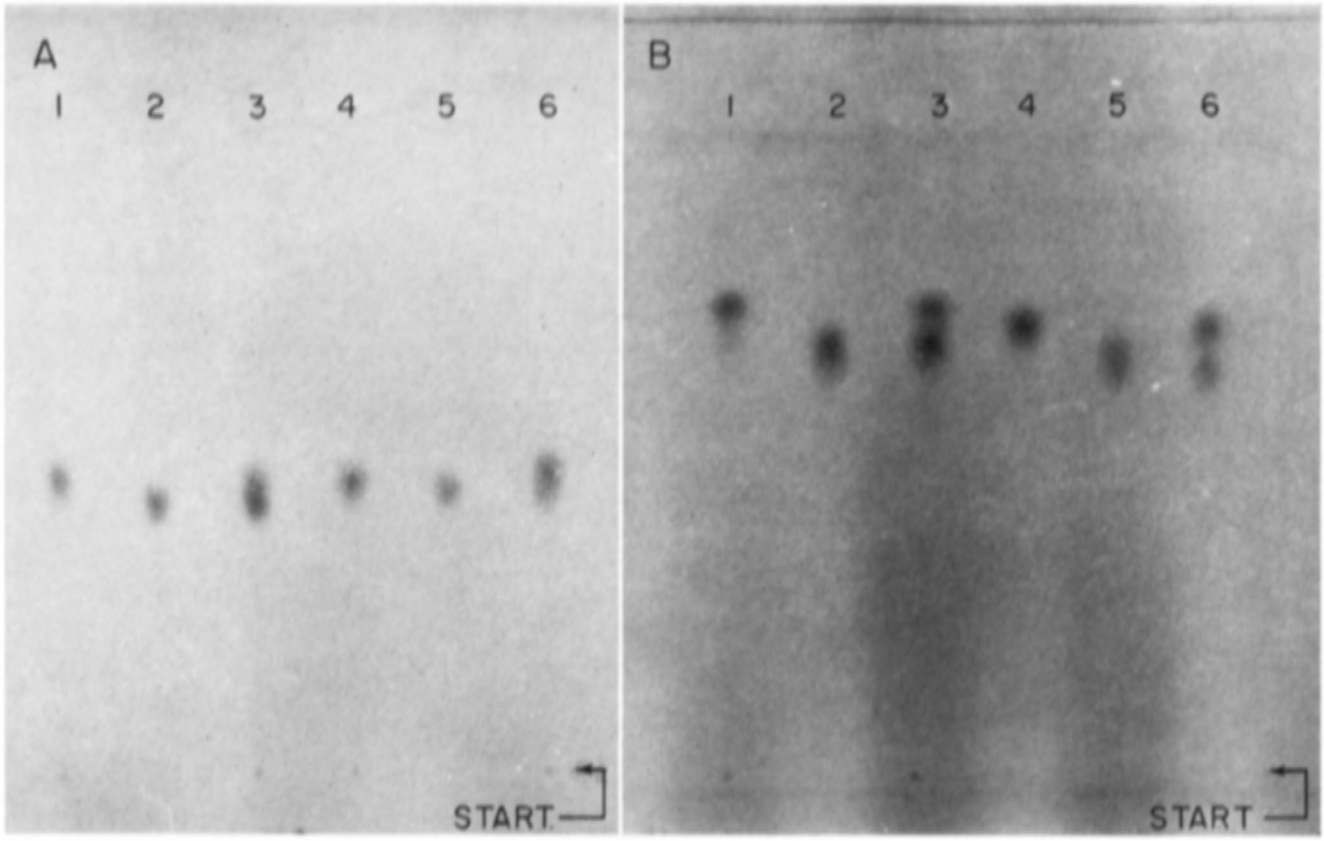

FIG. 1. Thin-layer chromatography of sterols. Temperature 20°C, relative humidity 40%. 1, β-sitosterol; 2, stigmastanol; 3, mixture of β-sitosterol and stigmastanol; 4, cholesterol; 5, cholestanol; and 6, mixture of cholesterol and cholestanol. *A:* Unsaturated chamber (Desaga), solvent system toluene–ethyl acetate (80+20), development 1 hr. *B*: Vapor-programmed chamber (Desaga), solvent system benzene–ether (90+10), saturation 10 min, development 3 hr, strips 0.5 mm thick, cooling 19°C. Troughs: 1, benzene–ether (90+10); 2, carbon tetrachloride–ether (80+20); 3 and 4, carbon tetrachloride; 5, carbon tetrachloride-ether (60+40); 6 and 7, carbon tetrachloride; 8, carbon tetrachloride–ether (60+40); 9 and 10, carbon tetrachloride; 11, carbon tetrachloride–ether (40+60); 12–21, carbon tetrachloride.

sitosterol about 0.66. A similar separation was obtained by Bennett and Heftmann (2) for cholestanol and cholesterol with continuous development, but their developing time was 6 hr, whereas ours was 3.

We have applied this method of vapor-programmed thin-layer chromatography to investigate the possible presence of stigmastanol in a sample of β-sitosterol-3-^{14}C (5). Radioautography showed that, if the β-sitosterol-3-^{14}C contains any stigmastanol, it would be less than 1%.

Manuscript received 12 November 1969; accepted 26 January 1970.

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