J. Lipid Research, January, 1963
Volume 4, Number 1

# Notes on Methodology

## Thin-layer chromatography of sterols and steroids

JOEL AVIGAN, DEWITT S. GOODMAN, and DANIEL STEINBERG

*Section on Metabolism,*
*Laboratory of Cellular Physiology and Metabolism,*
*National Heart Institute,*
*National Institutes of Health,*
*Bethesda 14, Maryland*

[Manuscript received July 25, 1962; accepted October 1, 1962.]

The chromatographic separation of mixtures of closely related nonpolar sterols has been attempted by a variety of techniques. Column chromatography of the less polar sterols is often unsatisfactory because of poor resolution, tailing, lack of reproducibility of results, slowness, and difficulty in monitoring the fractions. More recently, excellent and rapid separations of microgram quantities of sterols have been achieved by gas-liquid chromatography (1) and by glass-paper chromatography (2). Thin-layer chromatography is free of many of the difficulties posed by conventional column chromatography, and a number of authors have used this technique for separation of free and esterified cholesterol. No study has been reported, however, on fractionation by the thin-layer method of mixtures of various cholestane and lanostane derivatives, in particular of compounds related metabolically to cholesterol.

Thin-layer chromatographic fractionation of several sterols that are metabolic precursors of cholesterol was carried out by us, both for analytic and preparative purposes, in the course of studies of cholesterol biosynthesis. Plates, about 250 $\mu$ thick, prestained with Rhodamine 6G,[1] were made with the Desaga-Brinkmann applicator[2] by plating a slurry containing 55 ml of a 0.1% aqueous solution of the dye and 25 g Silica Gel G.[3] The materials to be chromatographed were applied on plates either as single spots or as bands containing up to 10 mg of material/20-cm wide plate. Sterol zones were visible, after chromatography, under UV light as pink-yellow fluorescent areas on pale backgrounds or, in the case of UV absorbing compounds, as dark areas. For recovery, the zones were scraped off with a spatula and transferred to a paper filter, and the sterols were eluted with methylene chloride or chloroform. The dye is not eluted with these solvents.

[1] National Aniline Division, Allied Chemical Corporation.
[2] Brinkmann Instruments, Inc.
[3] E. Merck, A.G.

TABLE 1. CHROMATOGRAPHY OF STEROLS ON 20-CM LONG PLATES COVERED WITH THIN LAYERS OF SILICA GEL G*

| | $R_f$ |
|---|---|
| Cholesterol | 0.49 |
| Desmosterol | " |
| $\Delta^7$-Cholestenol | " |
| $\beta$-Sitosterol | " |
| Methostenol | 0.60 |
| Lanosterol | 0.67 |
| 24,25-Dihydrolanosterol | " |
| Cholesteryl acetate | 0.93 |
| Desmosteryl acetate | 0.91 |
| Lanosteryl acetate | 0.92 |

* Running time: approximately 1 hr. Solvent: benzene–ethyl acetate 5:1.

The chromatographic system used depended on the compounds to be separated. Lanosterol, methostenol, and 27-carbon sterols as a group were separated from each other by ascending chromatography in benzene–ethyl acetate 5:1 (v/v), using 20-cm long plates covered with commercial Silica Gel G (Table 1). When a mixture of labeled lanosterol and nonlabeled cholesterol was chromatographed, the cholesterol component was usually contaminated with less than 1% of the radioactivity. The time required for this chromatography was 60–80 min.

Cholesterol, $\Delta^7$-cholestenol, and $\Delta^{5,7}$-cholestadienol were separated from each other (and from lanosterol) on 40-cm long plates also prestained with Rhodamine 6G, by chromatography in benzene–ethyl acetate 20:1 for 24–36 hr. This relatively long period was necessary to achieve maximum resolution, although the solvent did not ascend beyond about $^3/_4$ of the length of the plate because of continuous evaporation from its surface. Cholesterol and the slower moving $\Delta^7$-cholestenol were completely separated (by a distance of 4–5 cm), but $\Delta^{5,7}$-cholestadienol moved between the two, and was only partially resolved from cholesterol. Complete separation of $\Delta^{5,7}$-cholestadienol from the monounsaturated cholestenols, and from desmosterol, was, however, achieved by chromatography on 20-cm long plates impregnated with $AgNO_3$, as recommended by Mangold for the separation of cholesterol esters or of mixtures of methyl esters of fatty acids (personal communication). The plates containing the fluorescent dye were prepared as usual, and were then sprayed lightly with a saturated solution of $AgNO_3$ in 90% ethanol and dried for 10–20 min at 100°. Chromatography was done with a mixture of benzene–

ethyl acetate 5:1. Under these conditions, the $\Delta^{5,7}$-cholestadienol moved more slowly than the other compounds. In order to prevent oxidation and degradation, samples containing $\Delta^{5,7}$-cholestadienol were chromatographed in a chamber filled with $CO_2$ and protected from light. The recovery of each of the sterols after chromatography and elution was over 90%.

Sterols differing only in the degree of saturation of the side-chain (cholesterol and desmosterol, 24,25-dihydrolanosterol and lanosterol) had to be acetylated before chromatography. The separation of these acetylated compounds on silicic acid columns was investigated by Klein (3). The acetylation and extraction of micro quantities of sterols was carried out as described by Johnston, Gautschi, and Bloch (4). The sterols were dissolved in 0.2–0.3 ml anhydrous pyridine, an equal volume of acetic anhydride was added to the solution, and the samples were kept overnight at room temperature under nitrogen. The solutions were subsequently poured into ice-cold water, shaken with hexane, and the latter was washed with 0.1 N HCl, 0.1 N $KHCO_3$, and water. The acetylated esters were chromatographed with hexane–benzene 5:1 (v/v) on 40-cm long plates covered with silicic acid[4] which had been passed through a 325-mesh sieve and to which 5% of reagent grade $CaSO_4 \cdot {}^1/_2\ H_2O$ had been added. This material gave better resolution than the commercial Silica Gel G in this system. The plates were prepared from a slurry containing 50% ethanol to improve the flow properties during plating. In each pair, the steryl acetate containing the unsaturated side-chain moved more slowly than the saturated one, and the two were usually separated by a distance of about 5 cm. In a typical chromatographic run of a mixture of $H^3$-labeled 24,25-dihydrolanosteryl acetate and $C^{14}$-labeled lanosteryl acetate, the zone of lanosteryl acetate was contaminated with 2% or less of the other component and the zone of dihydrolanosteryl acetate was contaminated with even smaller amounts of lanosteryl acetate. The recovery of each of the components after acetylation, chromatography, and elution was about 70%. Rechromatography of each of the zones yielded compounds of high purity as confirmed by gas-liquid chromatographic analysis. Less satisfactory separation of cholesteryl and desmosteryl acetates has also been achieved by chromatography on 20-cm long plates of Silica Gel G, previously sprayed with $AgNO_3$ solution and dried before use. Lanosteryl acetate and 24,25-dihydrolanosteryl acetate were only partially separated under these conditions.

Mixtures of several other steroids were fractionated with benzene–ethyl acetate mixture 3:1 on the smaller size plates covered with Silica Gel G, using Rhodamine 6G as indicator. The results are shown in Table 2.

[4] Mallinckrodt Chemical Works.

TABLE 2. THIN-LAYER CHROMATOGRAPHY OF STEROIDS*

| | $R_f$ |
|---|---|
| Cholesterol | 0.57 |
| Pregnenolone | 0.40 |
| Androstane-3,17-dione | 0.64 |
| Progesterone | 0.49 |
| Androstane-3-$\alpha$-ol, 17-one, 3-acetate | 0.81 |
| Androstane-17-one | 0.89 |

* Running time: approximately 1 hr. Solvent: benzene–ethyl acetate 3:1.

Isolation of many of the sterol precursors of cholesterol was achieved by repeated chromatographic runs made consecutively in the different systems described above, and the method was applied by us in a study of cholesterol biosynthesis.[5]

## REFERENCES

1. VandenHeuvel, W. J. A., C. C. Sweeley, and E. C. Horning. *Symposium on Drugs Affecting Lipid Metabolism* Milan, Italy, 1960.
2. Swartwout, J. R., J. W. Dieckert, O. N. Miller, and J. G. Hamilton. *J. Lipid Research* **1**: 281, 1960.
3. Klein, P. D. *Anal. Chem.* **33**: 1737, 1961.
4. Johnston, J. D., F. Gautschi, and K. Bloch. *J. Biol. Chem.* **224**: 185, 1957.

[5] Goodman, DeW. S., J. Avigan, and D. Steinberg, in preparation.