THIN-LAYER CHROMATOGRAPHY AND IDENTIFICATION OF FREE STEROLS

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INTRODUCTION

A number of investigators[1-7] have applied thin-layer chromatography to the separation of free and esterified sterols. Three of these[1,6,7] have successfully separated several of the biosynthetic precursors* of cholesterol. The technique used by AVIGAN *et al.* has the disadvantage of requiring long development times for the separation of certain pairs, and others, such as cholesterol and desmosterol, could not be separated. While the reverse phase technique of COPIUS-PEEREBOOM successfully separates many of the precursors, it has the disadvantage of being difficult to prepare the plates by dipping, since the support on the plates has a tendency to crack. The procedure published by CLAUDE requires the esterification of sterols in order to effect their separation.

In April, 1964, Applied Science Laboratories, Inc. described a procedure[8] for the separation of cholesterol and desmosterol based on a principle described by DEVRIES[9] and by MORRIS[10]. We have modified and extended the use of this system to the study of a number of sterols; the results are reported herein.

EXPERIMENTAL

Preparation of plates

Thirty grams of Silica Gel H according to Stahl (Brinkmann Instruments, Inc., Long Island, New York) are slurried in 80.0 ml of aqueous 12.5 % silver nitrate. This amount will prepare approximately ten plates (10 cm × 20 cm) of about 200–300 μ in thickness. The slurry is spread on the plates with a plexiglass applicator. A standard thin-layer applicator should not be used because the silver nitrate corrodes metal unless rinsed off immediately. Allow the plates to air dry overnight; no other activation is required. Should it be desired to use plates the same day, they may be activated at 110° for one hour. It has been found that plates more than three days old tend to lose their resolving power.

* Trivial names of sterols used are as follows: cholesterol (Δ^5-cholesten-3β-ol), desmosterol ($\Delta^{5,24}$-cholestadien-3β-ol), 7-dehydrocholesterol ($\Delta^{5,7}$-cholestadien-3β-ol), cholestanol (5α-cholestan-3β-ol), lathosterol (Δ^7-cholesten-3β-ol), lanosterol (4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadien-3β-ol), dihydrolanosterol (4,4,14α-trimethyl-Δ^8-cholesten-3β-ol).

Spotting

Prior to spotting, the adsorbent on the plate should be removed according to the pattern presented by COPIUS-PEEREBOOM[4] (see Fig. 2). The adsorbent can be removed easily by placing a template on the plate and aspirating the adsorbent with a disposable pipette. All the adsorbent within the diamond shape need not be removed. It is sufficient if only the adsorbent around the edges of the pattern is aspirated. We also advise aspirating along the edges of each column thereby preventing overlap from one column to the next.

Sample size

Generally, 10 μg of sterol are spotted. However, we have been able to detect trace impurities in our sterol standards even when as little as 5 μg of sterol was used. (See impurities in Fig. 1, columns 7 and 8.)

Development

Plates of 200–300 μ in thickness are developed with chloroform–acetone (95:5 v/v). For thicker plates the same solvents are used in a 90–10 v/v ratio. Development is continued until the solvent front has moved a distance of 15–16 cm from the origin, usually one hour for shaped, and one-half hour for unshaped plates.

Identification

After removing the plates and allowing to air dry for about 15 min, spray with concentrated sulfuric acid and view under U.V. light. Sterols fluoresce. The plates are then heated for 10 min at about 110° and then examined under U.V. light again. Plates are examined before and after heating because some sterols fluoresce better under one set of conditions than under the other. In addition, this may be of some use in identification since some sterols, *e.g.* lathosterol, can be observed without heating, whereas cholestanol must be heated.

Preparation of yeast sterols

Bakers' yeast was saponified, extracted and purified according to SCHWENK *et al.*[11]. The digitonides were hydrolyzed using either pyridine or the dimethyl sulfoxide procedure of ISSIDORIDES[12]. The purified yeast sterol was then recrystallized from *n*-heptane. Solid samples of the pyridine or the dimethyl sulfoxide split sterol were analyzed by GLC and by TLC and showed no difference.

Blue tetrazolium spray

3 ml of 0.5 % blue tetrazolium in methanol; 12 ml of methanol; 2 ml of conc. ammonium hydroxide; 3 ml of 6 *N* sodium hydroxide.

RESULTS AND DISCUSSION

Fig. 1 shows a typical separation utilizing this system. It can be seen that all the standards were separated from each other. One pair of sterols, cholestanol and lathosterol, did not separate well. However, a closer examination of column eleven shows that the two compounds were beginning to resolve. These two sterols were successfully resolved as the propionic esters by CLAUDE[7].

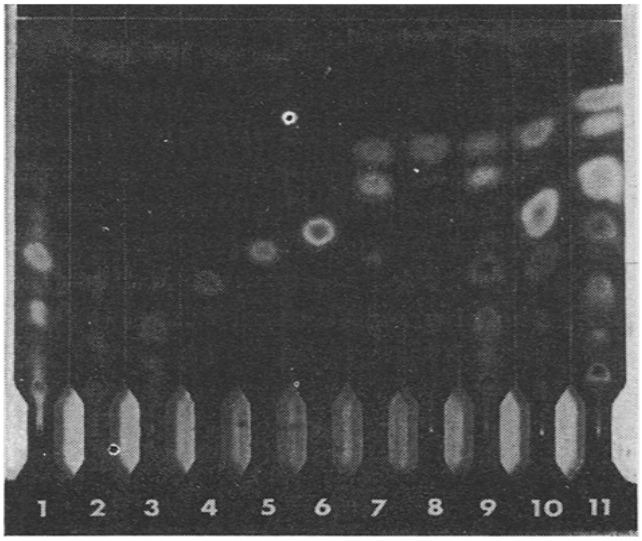

Fig. 1. Thin-layer chromatography of sterols on silver nitrate silica gel H. 1 = yeast sterols; 2 = 7-dehydrocholesterol; 3 = desmosterol; 4 = cholesterol; 5 = lathosterol; 6 = cholestanol; 7 = lanosterol; 8 = dihydrolanosterol; 9 = 7-dehydrocholesterol, desmosterol, cholesterol and lanosterol; 10 = cholesterol, lathosterol, cholestanol and dihydrolanosterol; 11 = mixture of 2 through 8. 20 μg of each sterol spotted except for 40 μg of 2 and 7 and an unknown amount of 1.

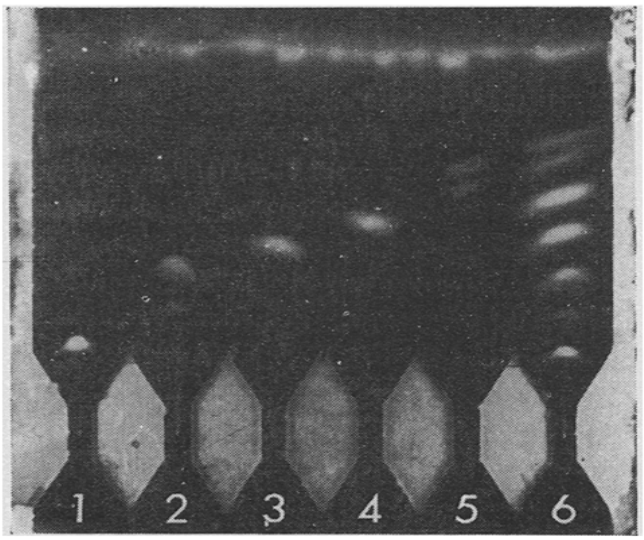

Fig. 2. Thin-layer chromatography of sterols on silver nitrate silica gel H. 1 = 7-dehydrocholesterol; 2 = desmosterol; 3 = cholesterol; 4 = cholestanol; 5 = lanosterol; 6 = mixture of 1 through 5, 20 μg of each.

Column 1 shows to the greatest degree the advantage of this system. This column was spotted with digitonin purified yeast sterols. While we do not have standards to identify each component, it can be seen that there are approximately fifteen that must be 3β-hydroxy sterols based on the specificity of the digitonin reaction.

Comparison of Figs. 1 and 2 shows the role of the diamonds in determining the shape of the sterol spot. In Fig. 2 the plate was shaped according to COPIUS-PEEREBOOM's directions, whereas in Fig. 1 the dimensions of the diamond shape were altered so as to fit more samples on the plates. Although the angles of the diamond points were the same as the angles in COPIUS-PEEREBOOM's directions, the width of the column was reduced. This had the effect of causing the sterols to chromatograph as circular spots rather than as bands, which is to be preferred when compounds have very similar R_F values.

During the course of these investigations, STECIW[13] recommended the use of the blue tetrazolium spray described by NISHIKAZE AND STAUDINGER[14] as an aid in the identification of steroids. Although it does not work on silver nitrate plates, it has proven to be advantageous with the reverse-phase system of COPIUS-PEEREBOOM. The usefulness of this spray justifies brief mention of it in this report.

Dried plates were sprayed with blue tetrazolium and then, as recommended by STECIW[13], with concentrated sulfuric acid. Each sterol gave a characteristic color ranging from red to pink for cholesterol to a green with such a closely related compound as 7-dehydrocholesterol particularly when viewed under U.V. light. The colors in most cases were sufficiently different that although two components were not completely resolved, the presence of both sterols could be ascertained on the basis of color.

NOTES ADDED IN PROOF

1. During the preparation of this manuscript COPIUS-PEEREBOOM AND BEEKES[15] and TRUSWELL AND MITCHELL[16] published chromatographic systems utilizing silica gel–silver nitrate for the separation of sterols.

2. The separation of cholestanol and lathosterol may be accomplished using this method by the following procedure: Develop the plate to a distance of 15 cm using chloroform. Remove the plate, allow to dry, then again develop the plate to a distance of 15 cm with chloroform.

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