Separation of closely related steroids by an improved technique for continuous development of thin-layer chromatograms

In recent years thin-layer chromatography (TLC) has become one of the most useful analytical methods in steroid research[1,2]. While most problems of steroid separation can be solved by appropriate combinations of solvent systems and adsorbents, certain closely related substances can only be separated by modifications of the basic method, such as reversed-phase TLC, impregnation with silver nitrate, and repeated or continuous development. The latter, although a simple and powerful method of increasing resolution, has been used only infrequently in the steroid field[3–8]. This investigation was undertaken to evaluate recent improvements in the technique[8,9] and to solve some difficult problems in steroid separations.

Cylindrical glass developing chambers*, 63 mm in diameter, were cut down to a height of 180 mm. A slit was cut in the plastic cover, 50 mm long and about 2 mm wider than the thickness of the plates. Samples (1 μg each) were spotted 23 mm from the bottom of a 50 × 200 × 0.25 mm Silica Gel G plate**. For development, the plate was placed in a chamber containing 30 ml of solvent***, so that it faced a 160 × 60 mm filter paper liner wet with solvent. The plastic cover was then fitted over the plate and onto the chamber, leaving the upper portion of the plate exposed. The open spaces in the cover were then sealed by inserting pieces of chromatographic paper, 30 × 50 × 0.6 mm, into the slit. The solvent evaporated as it reached the exposed upper portion of the layer, thus effecting continuous development.

This method is technically simpler than that previously used by us[5], and it gives equivalent or better separation. It is not recommended for solvent systems containing a low concentration of a component that is much more volatile than the major one. For example, in the hexane–ether systems we have used to separate sterol acetates[5,12], the mobilities of these compounds were not reproducible. This is probably due to the fact that the chamber is not tightly sealed and differential evaporation of the ether may occur. Substitution of ethyl acetate for ether solved this problem and gave separation equivalent to those obtained with the former systems.

Since both C-25 epimers of the steroidal sapogenins frequently, if not always, occur together[13], their separation is of some importance. Some separation of these isomers is possible by conventional TLC on Silica Gel G plates[2,12,14], but for practical purposes, such as preparative TLC, a much better resolution is required, and accordingly we investigated the use of continuous development for this purpose.

As Fig. 1 shows, our new method provides a very suitable solution to this problem. In contrast to previous methods[2,14], continuous development separates the free genins (Nos. 7–12, System B) just as well as the acetates (Nos. 1–6, System A).

We then extended the method to pairs of Δ^5- and 5α-sterols, which, being sterically similar, are inseparable by conventional TLC[1,15]. Earlier methods of resolving such pairs involved changing the polarity of one of the two components by a

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* Research Specialties, Richmond, Calif. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

** Analtech, Inc., Wilmington, Del.

*** All solvent systems were saturated with water[10,11] to prevent the formation of undesirable activity gradients on the plate.

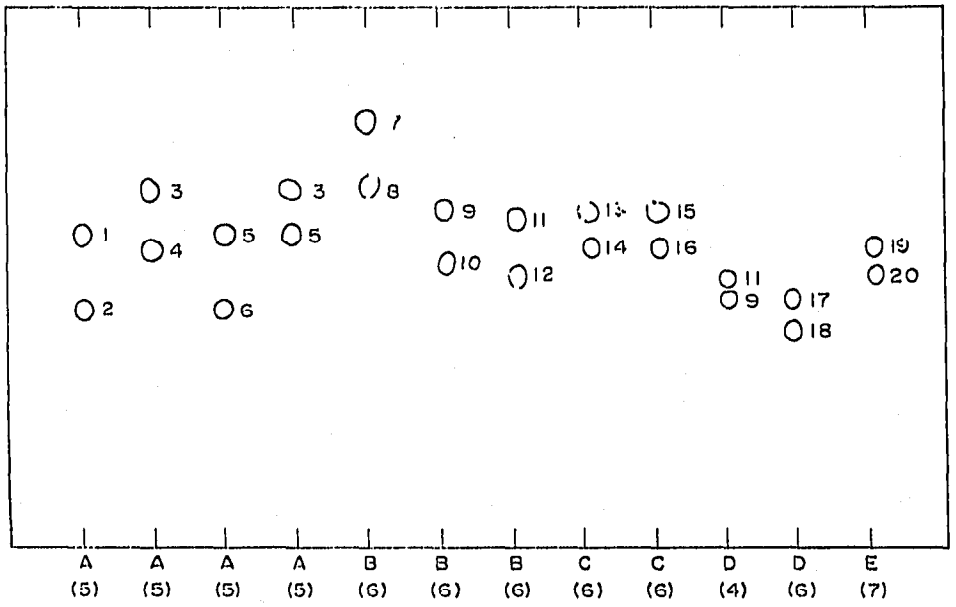

Fig. 1. Separation of steroids by continuous development on Silica Gel G. Solvent systems: A = hexane–dichloromethane (3:7); B = dichloromethane–ether (199:1); C = benzene–methanol (99:1); D = benzene–methanol (399:1); E = benzene–methanol (199:1). Development times in hours are given in parentheses. Compounds: 1 = smilagenin acetate (5β,25α-spirostan-3β-ol acetate); 2 = sarsasapogenin acetate (5β,25β-spirostan-3β-ol acetate); 3 = tigogenin acetate (5α,25α-spirostan-3β-ol acetate); 4 = neotigogenin acetate (5α,25β-spirostan-3β-ol acetate); 5 = diosgenin acetate (25α-Δ^5-spirosten-3β-ol acetate); 6 = yamogenin acetate (25β-Δ^5-spirosten-3β-ol acetate); 7 = smilagenin; 8 = sarsasapogenin; 9 = tigogenin; 10 = neotigogenin; 11 = diosgenin; 12 = yamogenin; 13 = cholesterol (Δ^5-cholesten-3β-ol); 14 = cholestanol (5α-cholestan-3β-ol); 15 = stigmasterol (24α-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol); 16 = Δ^{22}-stigmastenol (24α-ethyl-Δ^{22}-cholesten-3β-ol); 17 = gentrogenin (25α-Δ^5-spirosten-3β-ol-12-one); 18 = hecogenin (5α,25α-spirostan-3β-ol-12-one); 19 = pregnenolone (Δ^5-pregnen-3β-ol-20-one); 20 = pregnanolone (5α-pregnan-3β-ol-20-one). Spots were revealed by spraying with 50% sulfuric acid and charring on a hot plate.

selective chemical reaction prior to or during chromatography[6,16–18]. As a non-destructive expedient, COPIUS-PEEREBOOM and coworkers[19–21] used reversed-phase TLC and silver nitrate-impregnated layers to separate cholesterol and cholestanol. The latter method has also been applied to separations of this type by other workers[6,22–24].

Diosgenin acetate (No. 5) and tigogenin acetate (No. 3) are well separated in System A. However, the method was not applicable to other pairs of Δ^5- and 5α-steroid acetates, *e.g.*, cholesterol acetate and cholestanol acetate. Best results were obtained only by chromatographing the free sterols, cholestanol (No. 14) and cholesterol (No. 13), rather than their acetates, in System C (Fig. 1). An analogous pair of C_{29} sterols, Δ^{22}-stigmastenol (No. 16) and stigmasterol (No. 15) were similarly separated by this system. The method was successfully applied to three other 5α–Δ^5 pairs, tigogenin (No. 9) and diosgenin (No. 11), hecogenin (No. 18) and gentrogenin (No. 17), and 5α-pregnanolone (No. 20) and pregnenolone (No. 19) (Fig. 1).

However, mixtures of tomatidine and Δ^5-tomatidenol, 5α-pregnane-3β,20β-diol and Δ^5-pregnene-3β,20β-diol, and 5α-androstane-3β,17β-diol and Δ^5-androstene-3β,17β-diol could not be resolved by benzene–methanol systems. These compounds are more polar than the ones we have successfully separated and require a methanol concentration of 2% for adequate mobilities. This indicates that the method may be

restricted to less polar compounds, and the same limitation may also apply to the other TLC techniques for separating Δ^5–5α pairs. Reversed-phase TLC, by its nature, is only applicable to non-polar compounds, and a mixture of tomatidine and Δ^5-tomatidenol could not be resolved on silver nitrate-impregnated plates, even by continuous development[6].

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Response of steroids to sulfuric acid in thin-layer chromatography

Thin-layer chromatography is by now one of the most important analytical methods in the steroid field and a sizeable literature on this subject is now available[1]. Of the many detecting reagents in the literature sulfuric acid occupies a special place, because it reacts with all steroids to give colored and/or fluorescent zones and, after charring, permanent black-on-white chromatograms suitable for photographic or