THIN-LAYER CHROMATOGRAPHY OF STEROIDAL SAPOGENINS

RAYMOND D. BENNETT AND ERICH HEFTMANN

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Md. (U.S.A.)

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For the separation of steroidal sapogenins by paper chromatography, SANNIÉ AND LAPIN[1] used mixtures of petroleum ether, chloroform, and acetic acid, HEFTMANN AND HAYDEN[2] petroleum ether–toluene–ethanol–water systems, and MCALEER AND KOZLOWSKI[3] and WALL *et al.*[4] nonaqueous stationary phases, such as formamide, phenylcellosolve, or propylene glycol. The shortcomings of the aqueous systems are failure to attain equilibrium conditions and tailing, whereas the nonaqueous systems are technically more complicated[5] and require several hours for development. Furthermore, the separation of isomers differing in the configuration of the C-25 methyl group by paper chromatography presents difficulties. Although a good separation between smilagenin and sarsapogenin had been reported[2], the failure of OKANISHI *et al.*[6] to reproduce this result has led us to the discovery that our observation was due to an impurity in the sample of sarsapogenin*. SANNIÉ AND LAPIN[1] have also reported the separation of sarsapogenin from smilagenin, but later observations[6–8] showed little or no differences in their R_F values in the same systems.

Recently, VANDENHEUVEL AND HORNING[9] have applied gas–liquid chromatography to the separation of sapogenins. Their method is not capable of resolving C-25 isomers, and the separation of diosgenin from tigogenin is not sharp.

Thin-layer chromatography has been employed in the separation of unknown sapogenins and in the identification of neotigogenin, tigogenin, diosgenin, and gitogenin by SANDER[10–13] and in work on the structure of convallamarogenin by TSCHESCHE, SCHWARZ AND SNATZKE[14]. CARRERAS MATAS[15] impregnated thin-layer plates of silica gel with formamide and used them for partition chromatography of steroidal saponins. HEŘMÁNEK *et al.*[16,17] and ČERNY *et al.*[18] have reported R_F values of some sapogenins for spread-layer chromatography.

In the course of our work on the biosynthesis of diosgenin[19] we have found it necessary to separate small amounts of sapogenins, especially the C-25 isomers. Both partition and adsorption chromatography on thin layers were found eminently suitable to this purpose.

EXPERIMENTAL

Except as described below, chromatograms were prepared and developed as in the preceding paper[20]. Trifluoroacetates of sapogenins were prepared as follows: To 0.2

* Due to a typographical error, Table II, Ref.[2] shows "Yamogenin acetate"; this should read "Yamogenin".

ml of 0.01–0.1% solution of the sapogenins in hexane or dichloromethane, 2 μl of trifluoroacetic anhydride was added. After thorough shaking for 1 min, 1 ml of 2 N aqueous sodium carbonate was added and the mixture was again thoroughly shaken. Samples were spotted directly from the organic layer.

Preparation of plates

Unless otherwise specified, Silica Gel G was used as adsorbent. The Kieselguhr G* plates used for partition chromatography were not conditioned by development with chloroform. The silica gel–kieselguhr mixture was prepared by shaking 15 g of Silica Gel G and 15 g of Kieselguhr G in a stoppered flask for a few seconds, adding 60 ml of water, and shaking for 1 min. These plates were conditioned with chloroform.

Development

The following solvent systems were used (minutes required for development in parentheses):

A: Dichloromethane–methanol–formamide, 93:6:1 (28).
B: Toluene–ethyl acetate–formic acid, 57:40:3 (36).
C: Cyclohexane–acetone, 1:1 (26).
D: Cyclohexane–ethyl acetate–water, 600:400:1 (29).
E: Cyclohexane–ethyl acetate–water, 1000:1000:3 (28).
F: Chloroform–methanol–water, 485:15:1 (26).
G: Chloroform–methanol–water, 188:12:1 (28).
H: Chloroform–toluene, 9:1 (34).
I: Hexane–toluene–ethanol–water, 100:50:5:45 (42).
J: Hexane–ethanol–water, 40:3:7 (30).

Detection

Detection of sapogenins with 50% sulfuric acid was possible at a much lower temperature than in the case of corticosteroids[20], 120°–140° being sufficient. The lower limit of detection for diosgenin was 0.005 μg under ultraviolet light, while saturated sapogenins could be detected in 0.01 μg quantities in Systems A-H.

Partition chromatography

To ensure equilibrium conditions inside the chromatography jar, the wall of the cylindrical chamber (229 mm high × 60 mm diameter)* was lined with two strips of filter paper, 15 × 4 cm; one strip dipped into the solvent system (15 ml), the other one was impregnated with water and suspended on the opposite side without touching the solvent. This was accomplished by use of a steel paper clip held in place by an external magnet. Phase equilibrium in the chamber was attained within 6 min.

Usually 0.02 μg of each compound was applied to a Kieselguhr G plate as a 0.002% solution in hexane. Up to four samples may be spotted in the middle 3 cm of the plate, 1 cm apart. A finish line was drawn 15 cm from the starting points.

The plate was impregnated with water by supporting it, adsorbent side down, across the top of a 3-l beaker of boiling water until it was thoroughly wet. It was then placed in the hood, and, as soon as the water began to recede at the corners of the

* Brinkmann Instruments, Inc., Great Neck, N.Y.

layer, the plate was transferred to the developing chamber. It was placed in the chamber with the adsorbent layer facing the solvent-saturated liner.

RESULTS AND DISCUSSION

Preliminary experiments showed that sapogenins differing in number and/or kind of polar groups were easily separable in solvent systems commonly used in thin-layer chromatography. However, the C-25 isomers were not separated, and resolution of diosgenin–tigogenin mixtures was poor. Partition systems of the type used for paper chromatography[2] were then tried in an effort to achieve these separations. When Silica Gel G layers were used with these systems, the monohydroxysapogenins remained near or at the origin. Although impregnation of the chromatoplates with water resulted in greater mobilities, uniform impregnation of the layers could not be achieved and movement of the sapogenins was erratic. However, Kieselguhr G plates, impregnated with water, gave reproducible separations of monohydroxysapogenins. The amount of water in the layer is a critical factor; too much water causes enlargement of the spots and poor separation, while tailing occurs when the plates are too dry. The R_F values are also dependent on the water concentration and therefore exact reproducibility of mobilities is difficult. However, the pattern of separation is not affected by changes in mobilities due to variations in water content. As Fig. 1 shows, smilagenin, tigogenin, and diosgenin were separated by System I, but the C-25 isomers, smilagenin–sarsapogenin and tigogenin–neotigogenin were

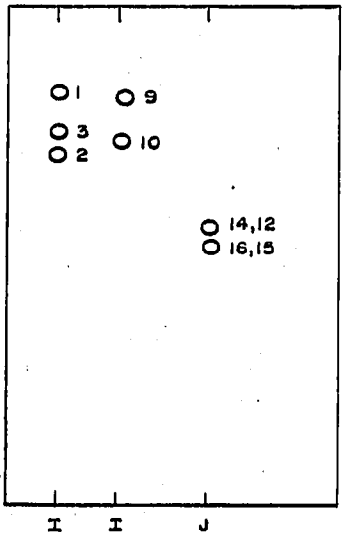

Fig. 1. Separation of sapogenins by partition chromatography on Kieselguhr G (for solvent systems A-J see text). (1) Smilagenin (5β,25α-spirostan-3β-ol); (2) Diosgenin (25α-Δ^5-spirosten-3β-ol); (3) Tigogenin (5α,25α-spirostan-3β-ol); (4) Gentrogenin (25α-Δ^5-spirosten-3β-ol-12-one); (5) Hecogenin (5α,25α-spirostan-3β-ol-12-one); (6) Isochiapagenin (25α-Δ^5-spirostene-3β,12β-diol); (7) Kryptogenin (25α-Δ^5-cholestene-3β,26-diol-16,22-dione); (8) Chlorogenin (5α,25α-spirostane-3β,6α-diol); (9) Sarsapogenin (5β,25β-spirostan-3β-ol); (10) Neotigogenin (5α,25β-spirostan-3β-ol); (11) 3-Episarsapogenin (5β,25β-spirostan-3α-ol); (12) Tigogenin acetate; (13) Diosgenin acetate; (14) Smilagenin acetate; (15) Neotigogenin acetate; (16) Sarsapogenin acetate; (17) Gentrogenin acetate; (18) Hecogenin acetate; (19) Kryptogenin acetate; (20) 3-Episarsapogenin acetate; (21) Tigogenin trifluoroacetate; (22) Diosgenin trifluoroacetate; (23) Smilagenin trifluoroacetate; (24) Sarsapogenin trifluoroacetate; (25) Neotigogenin trifluoroacetate; (26) Gentrogenin trifluoroacetate; (27) Hecogenin trifluoroacetate.

not resolved, although their relative mobilities differed slightly. In the form of their acetates, the C-25 isomers could be separated in System J, but in this system the acetates of the 5α–5β pairs tigogenin–smilagenin and neotigogenin–sarsapogenin were not separated. When the chromatoplates were impregnated with the aqueous phase of System I by the technique of BRENNER, NIEDERWIESER AND PATAKI[21], results were similar to those obtained on plates impregnated with water, except that the C-25 isomers were better separated and the resolution of diosgenin and tigogenin was poorer.

The use of System I seems to be restricted to monohydroxysapogenins, as the hydroxyketo compound gentrogenin remains at the origin, while sterols move with the solvent front. A drawback of Systems I and J is the limited concentration range; the lower limit of detection is 0.02 μg, while amounts greater than 0.2 μg give spots too large for good resolution.

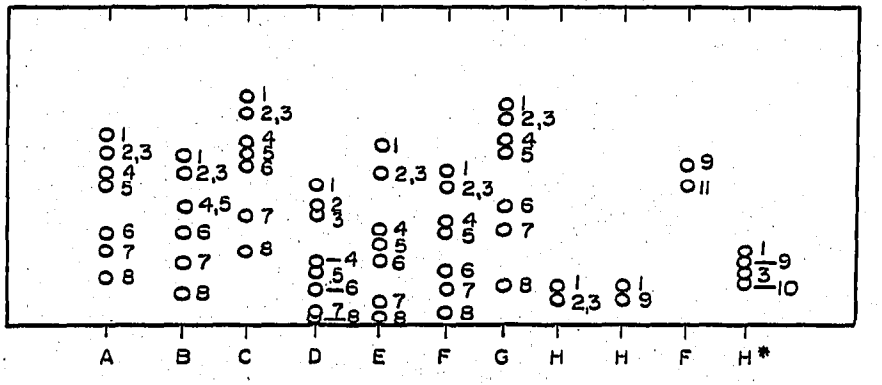

Fig. 2. Separations of sapogenins on Silica Gel G (for legend see Fig. 1). * Kieselguhr G–Silica Gel G plate.

Separations of various sapogenins on Silica Gel G are shown in Fig. 2. The A/B *cis–trans* isomers smilagenin and tigogenin were well separated in Systems A-G, and the axial–equatorial epimers sarsapogenin and 3-episarsapogenin were well resolved in System F. The wide separation of the dihydroxysapogenins isochiapagenin* (3β,12β) and chlorogenin (3β,6α) shows the sensitivity of this method in differentiating positional isomers. It is interesting that gentrogenin and hecogenin were separated by most of these systems, while diosgenin and tigogenin, having the same structural difference, were not. Only in System D was there any significant separation of diosgenin from tigogenin.

The C-25 isomers showed no differences in relative mobility in Systems A-G. In System H, however, separation between smilagenin and sarsapogenin occurred, even though they had moved only a short distance from the origin. When the mobilities were increased by the addition of as little as 1% of methanol to System H, a mixture of the isomers was no longer resolved. This left only two alternatives for increasing mobilities: decreasing the activity of the adsorbent or decreasing the polarity of the sapogenins.

When Silica Gel G was replaced by Kieselguhr G, the relative mobilities of the C-25 isomers increased and showed a considerable difference, but resolution was vitiated by tailing. However, on plates made from a 1:1 mixture of Kieselguhr G

* Prepared by reduction of gentrogenin with sodium borohydride[22].

and Silica Gel G, tailing was eliminated, while mobilities exhibited a satisfactory increase. Fig. 2 (H) shows the separation of two pairs of C-25 isomers.

The other approach to increasing mobilities was also successful. The acetates of the C-25 isomers were even better separated than the free sapogenins on Silica Gel G in System H. Corresponding 5α-, 5β-, and Δ^5-sapogenins were also separated in the form of their acetates, as were the 3α- and 3β-OH epimers. Fig. 3 shows the separation of the acetates of six monohydroxysapogenins in System H. It should be noted that the sequence of the A/B *cis–trans* isomers is reversed in the case of the

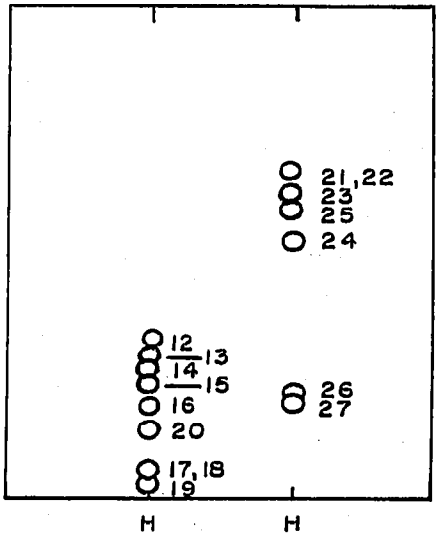

Fig. 3. Separation of sapogenin acetates and trifluoroacetates on Silica Gel G (for legend see Fig. 1).

acetates. Separation of monohydroxysapogenin acetates occurred only in System H or other solvent mixtures of low polarity, *e.g.*, chloroform–carbon tetrachloride and cyclohexane–benzene. In more polar systems, containing ethyl acetate or methanol, these acetates had equal relative mobilities.

For the rapid analysis of sapogenin mixtures it would be helpful if they could be esterified directly on the plate. This is not possible in the case of acetylation, but it was found that trifluoroacetic anhydride reacts almost instantaneously with 3-hydroxy steroids. The trifluoroacetates can be prepared by spotting the anhydride directly over compounds on the plate, but drying in the hood for several minutes is necessary to remove the trifluoroacetic acid formed as a by-product. It is therefore usually preferable to prepare this derivative as indicated in the Experimental Part. Fig. 3 shows the separation of a mixture of sapogenin trifluoroacetates in System H. The lesser polarity of these derivatives in comparison with the acetates is an added advantage.

There is a considerable difference in the reactivity of the Δ^5- and the saturated sapogenins toward the 50% sulfuric acid spray. The former quickly show a bright fluorescence, while the latter take much longer and never appear as intense. When microgram quantities are applied, the Δ^5-compounds are bright red or orange and become purple, while the saturated compounds are pale yellow and become brown.

The useful concentration range of the sapogenin method is greater than that for corticosteroids[20]. Except for the partition systems I and J, no tailing was observed with 50 μg quantities, and some separations were still possible at this level.

In the application of this method to unknown sapogenin mixtures, one of the polar Systems A-G should be used first, to give a separation into classes according to the number and kind of polar groups. This may be followed by a nonpolar system such as H to show differences at C-25, preferably after acylation. The partition system I is the most suitable for distinguishing between diosgenin and tigogenin. The combined use of several of the systems given will permit the identification of any of the monohydroxysapogenins, even if available only in microgram quantities.

SUMMARY

The application of thin-layer chromatography to the separation of mixtures of steroidal sapogenins and their esters has been investigated. Not only sapogenins differing in the number and kind of polar groups, but also those differing in A/B ring junction and in configuration of hydroxyl and C-25 methyl groups have been resolved. A rapid method for partition chromatography on thin layers of Kieselguhr G was developed. Mixed silica gel–kieselguhr layers were also found useful, as was a method for acylating hydroxyl groups directly on the thin-layer plate.

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