THIN-LAYER CHROMATOGRAPHY OF CORTICOSTEROIDS

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In recent years the technique of thin-layer chromatography on glass plates has gained recognition as a highly useful analytical method in organic and biological chemistry (for reviews *cf.* refs.[1–8]). In view of the advantages[3] of this method over paper partition chromatography, its application to the field of corticosteroids, where the latter technique has been widely used[9–11], should be explored.

STÁRKA AND MALÍKOVÁ[12], using spread-layer chromatography (in which adsorbent without binder is spread dry on a glass plate) to separate pregnanediols and pregnanetriols, also reported R_F values for cortisone, corticosterone, 17α-hydroxyprogesterone, and progesterone, and ADAMEC, MATIS AND GALVÁNEK[13] employed the same technique for the separation of 11-desoxycortisol, cortisone, cortisol, tetrahydrocortisone, and tetrahydrocortisol. METZ[14] mentioned the use of thin-layer chromatography for separation of corticosteroids without giving any examples, and other workers[15–17] have reported R_F values for various corticosteroids.

In our opinion, the spread-layer technique offers no important advantages over the conventional thin-layer method, except for preparative purposes, and has the disadvantage that the layers are easily disturbed. Accordingly, we have investigated the application of thin-layer chromatography to the identification and separation of corticosteroids and have made some observations on the relation between structure and relative mobility.

EXPERIMENTAL

The Desaga–Brinkmann apparatus for thin-layer chromatography* was used, with Silica Gel G as adsorbent. The solvents were 99 Mol % pure or Spectranalyzed grades**, if available; otherwise reagent grades were used. Chromatograms were recorded by tracing or by photographing with a Polaroid Model 800 Land Camera*** mounted on a Polaroid Model 208 Copymaker***. The procedures described by BRENNER *et al.*[18–20] were used in preparing the chromatoplates and developing the chromatograms, with the following exceptions.

Preparation of plates

Since it is impossible to prepare uniform layers on 20 × 5 cm plates by the standard technique[20], a row of 20 × 20 cm plates was placed on the template and a length of

* Brinkmann Instruments Inc., Great Neck, N.Y.
** Fisher Scientific Co.
*** Polaroid Corp., Cambridge, Mass.

glass tubing, 5 mm in diameter, was placed between the long retaining ledge and the plates. The smaller plates were then aligned in rows of four, with their longer edges parallel to the long edge of the template, on top of the larger plates, placing a few drops of water under each small plate and sliding it back and forth until it adhered well to the larger plate. It is essential that the thickness of the plates within each row be uniform. Each plate was measured with a micrometer and its thickness marked on the underside with a diamond marking pencil.

Small plates have the advantages that they will not crack when heated strongly and, of course, require much less solvent for development. In the concentration range used in our work, up to eight samples can be placed on one plate.

The plates were allowed to dry overnight in an air-conditioned room at 25° and were then developed once with chloroform[21], ten at a time, in a 30.5 × 9.9 × 27.6 cm chamber. Plates were stored in a metal rack, protected from dust by a plastic cover, and all chromatograms were developed in the same air-conditioned room.

Samples were applied as 0.01% solutions in hexane, dichloromethane, or mixtures of these solvents. Since the lower limit of detection is 0.01 μg (less for some compounds), submicrogram quantities, usually 0.1 μg of each compound, were used in this work. It is advantageous to apply quantities of less then 1 μg, because samples can be spotted closer together and separations are better, but adequate separations can still be obtained in the 5 μg range. Some tailing was observed with 10 μg or higher amounts.

Development

The following solvent systems were used:

A: Chloroform–methanol–water, 188: 12: 1.
B: Chloroform–methanol–water, 485: 15: 1.
C: Chloroform–methanol–water, 90: 10: 1.
D: Ethyl acetate–chloroform–water, 90: 10: 1.

The volume of solvent system used for development was always 15 ml; it is important that this be kept constant[20]. The point of application was marked and a finish line, 10 cm from the starting points, was drawn in the layer with a needle. When the solvent front reached this line, its flow ceased, but the plate was kept in the chamber until the solvent penetration, which is easily observed visually, appeared uniform up to the line[18]. Development took from 25 to 30 min in Systems A, B, and C and 21 min in System D. The plates were then dried in the hood under an infrared lamp*; usually 2–3 min was sufficient.

Chromatoplates prepared on microscope slides, 25 × 75 mm and 50 × 75 mm, by the method of HOFMANN[22] were very convenient for exploratory work. The solvent systems used in this study rose a 50-mm distance in 10 min or less. PEIFER[23] has recently published a different method for preparing such small plates.

Detection

For the detection of spots 50% sulfuric acid was found to be the most useful spray. When working with submicrogram quantities, it is important that the entire surface of the plate be covered by the spray, but not to the extent that it appears wet.

* A. H. Thomas Co.

The John chromatographic spray bottle* is excellent for this purpose. After the spraying, the plates were heated on a hot plate in the hood and the spots were observed under long-wave ultraviolet light with an SL-3660 Mineralight*. A surface temperature of 175°–200° is necessary to reveal all the corticosteroid spots.

Another useful spray reagent is Bromthymol Blue solution[24], which gives white spots on a dark-blue background after exposure of the plate to ammonia vapors. This method does not require heating for the detection of spots, but is less sensitive, the lower limit being about 0.1 μg.

RESULTS AND DISCUSSION

Fig. 1 summarizes the results. Compounds 1–11, all Δ^4-pregnene derivatives having keto groups at positions 3 and 20, were completely separated by System A. While progesterone and pregnane-3,20-dione were not well separated in this system due to their proximity to the solvent front, a good resolution of these compounds was achieved in the less polar System B. Of particular interest is the separation of aldosterone, cortisone, and cortisol, since paper-chromatographic methods for effecting this separation are tedious. These three compounds were even better separated in the more polar System C, as shown in Fig. 1.

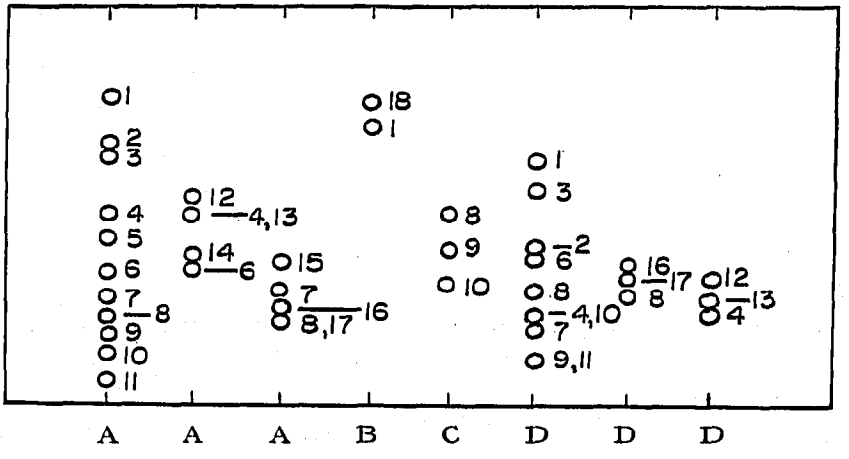

Fig. 1. Separation of corticosteroids in Systems A-D (see text). (1) Progesterone (Δ^4-pregnene-3,20-dione); (2) 17α-Hydroxyprogesterone (Δ^4-pregnen-17α-ol-3,20-dione); (3) 11-Desoxycorticosterone (Δ^4-pregnen-21-ol-3,20-dione); (4) 11-Dehydrocorticosterone (Δ^4-pregnen-21-ol-3,11,20-trione); (5) 11α-Hydroxyprogesterone (Δ^4-pregnen-11α-ol-3,20-dione); (6) 11-Desoxycortisol (Δ^4-pregnene-17α,21-diol-3,20-dione); (7) Corticosterone (Δ^4-pregnene-11β,21-diol-3,20-dione); (8) Cortisone (Δ^4-pregnene-17α,21-diol-3,11,20-trione); (9) Aldosterone (Δ^4-pregnen-18-al-11β,21-diol-3,20-dione); (10) Cortisol (Δ^4-pregnene-11β,17α,21-triol-3,20-dione); (11) 11-Epicortisol (Δ^4-pregnene-11α,17α,21-triol-3,20-dione); (12) Allopregnan-21-ol-3,11,20-trione; (13) Pregnan-21-ol-3,11,20-trione; (14) Pregnane-17α,21-diol-3,20-dione; (15) Allopregnane-11β,21-diol-3,20-dione; (16) Allopregnane-17α,21-diol-3,11,20-trione; (17) Pregnane-17α,21-diol-3,11,20-trione; (18) Pregnane-3,20-dione.

The positional isomers, compounds 2, 3, 5 and 6, 7 and the axial–equatorial 11-hydroxyl epimers 10 and 11 were separated in System A. In the two cases where analogous Δ^4, 5α, and 5β steroids were available for comparison (4, 12, 13 and 8, 16, 17), the Δ^4 and 5β steroids were not separated in System A, although the 5α com-

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pounds were separated from the other two. On the other hand, the Δ^4, 5β pairs 1, 18 and 6, 14 were separated. It is perhaps noteworthy that in the cases where separation did not occur in System A, an 11-keto group was present, while in the other two cases position 11 was unsubstituted. Separation of 4, 12, 13 and 8, 16, 17 was possible in System D; in both cases mobility was in the order $5\alpha > 5\beta > \Delta^4$.

The separation of Δ^4-pregnenes in System A was in line with the accepted concepts of "polarity"[9,11], except that compound 4, in spite of having one more keto group, moved faster than compound 5. In System D, however, the relative mobilities seemed to bear little or no relation to "polarities". Cortisol, for instance, differing from corticosterone only in having an extra OH group (17α), actually ran ahead of the latter. Cortisone and 11-dehydrocorticosterone, also differing by a 17α-OH group, followed the same pattern, but in the case of compounds 3 and 6 the 17α-hydroxy analog was more "polar".

The sequence reversals in Systems A and D should be valuable for confirming identities of compounds. Both solvent systems are suitable for the separation of the adrenocorticosteroids aldosterone, cortisone, and cortisol.

In many cases, chromatoplates on microscope slides gave adequate resolution in a short time. For instance, compounds 1, 2, 4, 5, 6, 7, 8, 10, and 11 were separated in 9 min using System A, and aldosterone was separated from cortisone and cortisol in 7 min in System D.

The time required for spots to appear after spraying with 50 % sulfuric acid may aid in their identification. Cortisol appeared almost as soon as heating began, while cortisone and 11-dehydrocorticosterone, requiring a much higher temperature, were the last to appear. When microgram quantities were used, the spots showed characteristic colors when sprayed with this reagent and heated.

Although R_F values in thin-layer chromatography are reproducible when all variables are controlled[20], they are very likely to vary from one laboratory to another. This is especially true when, as in this work, the chromatoplates have been kept in equilibrium with the atmosphere of the room. Thus, no useful purpose would be served by reporting R_F values. Correlation between results under different conditions can usually be established by including one or two reference compounds per plate.

ADAMEC, MATIS AND GALVÁNEK[13] found that their spread-layer chromatographic method for corticosteroids gave satisfactory results with urinary extracts, and they expressed the opinion that it should be applicable to quantitative analysis. MANGOLD[5] has listed the methods used for quantitative evaluation of thin-layer chromatograms and has commented on the compromise between accuracy and practicality. The speed and economy inherent in thin-layer chromatography certainly warrant further exploration of its applicability to the analysis of biologically important steroids.

SUMMARY

The separation of eighteen corticosteroids and pregnane derivatives in submicrogram quantities by thin-layer chromatography is reported. Positional isomers, axial–equatorial hydroxyl epimers, and A/B *cis–trans* isomers were successfully resolved. Rapid separation of aldosterone, cortisone, and cortisol was achieved. Reversal of some relative mobilities was observed with two solvent systems.

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