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Note

Characterization of C_{19} steroids by two-dimensional thin-layer chromatography

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Metabolic studies with steroids often produce a large number of closely related metabolites. The present paper describes a thin-layer chromatographic (TLC) system used in our laboratory for both characterization and separation of labelled steroid metabolites. The TLC plate is developed first with a "typical" and then, in the second direction, with an "atypical" solvent mixture. Such a combination results in efficient separation of a large number of unconjugated metabolites of neutral steroids on a single plate[1]. The "typical" solvent mixture is characterized by a stronger retention of hydroxyl than keto groups, whereas in the "atypical" direction the metabolites with keto groups are retained more strongly. In addition, several minor characteristics of the steroid molecule affect the mobility values in "typical" and "atypical" solvent systems in a different way[2,3].

EXPERIMENTAL

Materials and reagents

Ready-made silica gel sheets on aluminium (silica gel 60; layer thickness, 0.2 mm) were obtained from E. Merck (Darmstadt, F.R.G.). The solvents were analytical grade with the exception of hexane, which was distilled in the laboratory from a crude petroleum distillate. The reference steroids were mostly obtained as gifts from Steroid Reference Collection, Chemistry Department, Westfield College, London, U.K. The steroid acetates were prepared by dissolving the steroids overnight at room temperature in a small volume of pyridine–acetic anhydride (50:50).

Methods

Chromatographic development was carried out in an all-glass chromatography chamber. In addition, a modification of the sandwich-chamber, which considerably enhances the quality of the developments (ref. 4, p. 69), was prepared for each sheet. The chromatography sheet was placed on a thin 20 × 20 cm metal plate, and a similar plate was placed on top of it. The cover plate had small bumps, punched near the edges at intervals of 2 to 3 cm, to keep the cover plate at a distance of 2 mm from the surface of the silica gel (Fig. 1). The plates and the sheet between them were held together with paper clamps and then placed in the larger glass chamber. Several sandwich-layers can be clamped together. The solvent mixture (100 ml) was placed at the bottom of the larger glass chamber.

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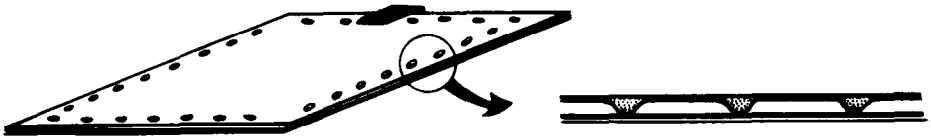

Fig. 1. The modification of the sandwich-chamber used in the present study. The width of the free space above the adsorbent is *ca.* 2 mm.

Two-dimensional TLC with steroid mixtures is best begun with a "typical" solvent mixture, here with 1,2-dichloroethane–methyl acetate (80:20). The sandwich-chamber is then opened for drying, closed again after a few minutes and run in the second direction with an "atypical" solvent mixture, here with hexane–1-hexanol (65:35). With this mixture, pure hexane front is followed at the distance of a few centrimetres by a secondary hexanolic front. The sheet is heated at 150°C for *ca.* 30 min to remove the hexanol, and the steroids are then visualized by spraying with Liebermann–Burchard reagent and heating (ref. 4, p. 855).

In the present paper the chromatographic behaviour of a number of C_{19} steroids in a "typical"–"atypical" two-dimensional system is described. The results were obtained from numerous one-dimensional and two-dimensional developments and are compiled in Fig. 2 and Table I for easy reference.

RESULTS AND DISCUSSION

A chart (Fig. 2) is more useful than numerical mobility values in the characterization of unknown metabolites. The mobilities of different steroids relative to each other are constant, whereas absolute r_F values vary on account of a number of random experimental factors. Comparison of radioactive spots in radioautography with non-labelled internal standards after staining the TLC sheet gives a good idea of the labelled metabolites of the sample. In many cases the sheet can be cut and the bits used in the quantification with scintillation counting.

The present system is naturally useless for the separation (rather than characterization) of the closely spaced polyfunctional steroids near the origin. These are better separated with a larger percentage of methyl acetate in the first solvent mixture and a larger percentage of 1-hexanol in the second direction.

The chart can also be used in the group separation of metabolites. Steroids with the same number of hydroxyl and/or carbonyl groups have approximately the same mobilities in the two-dimensional system. The steroids with two hydroxyl groups (dihydroxysteroids) move slowly with the "typical" and rapidly with the "atypical" solvent mixture. They are thus found after the two-dimensional run in one corner of the sheet (group A of Fig. 2). Similarly, monoketomonohydroxysteroids are found in another and diketosteroids in still another part of it (groups B and E). The metabolites can thus be quantificated together, *e.g.*, all monohydroxy-monoketo metabolites as a group[5].

Bidirectional chromatography with similar solvent systems for both directions leads to the result, that all chromatographed spots align on a diagonal. For many classes of compounds there exist chromatographic systems that are different enough

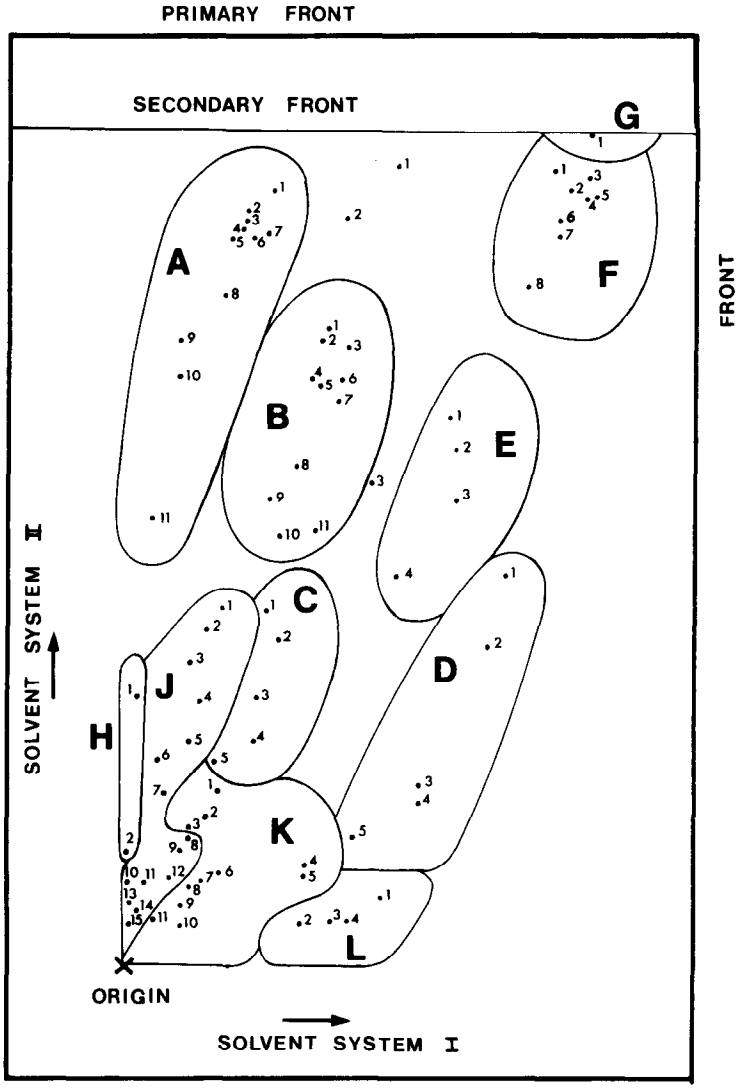

Fig. 2. A compilation of relative mobility values from a large number of one-dimensional and two-dimensional TLC developments (two to ten determinations for each steroid). The sample size was 60 nmol for each steroid. Solvent system I: 1,2-dichloroethane–methyl acetate (80:20), running distance 10 cm, development time 40 min. Solvent system II: hexane–1-hexanol (65:35), running distance 16.5 cm, development time 210 min. The points refer to the middle (densest point) of each individual steroid spot after staining. The compounds are listed in Table I.

and use the total area of the plate efficiently[6]. The systems commonly used for steroids, however, are only moderately effective in this respect[6].

"Atypicality", defined as anomalously slowly moving ketosteroids in relation to the corresponding hydroxysteroids, can obviously be due to either strongly adsorbed keto groups or weakly adsorbed hydroxyl groups. The former explanation is

TABLE I

COMPOUNDS SHOWN IN FIG. 2

Group A: Dihydroxysteroids
A1 = 3β,17β-dihydroxy-5β-androstane
A2 = 3α,17β-dihydroxy-5α-androstane
A3 = 3β,17α-dihydroxy-5-androstene
A4 = 3β,17β-dihydroxy-5α-androstane
A5 = 3β,17α-dihydroxy-5α-androstane
A6 = 3β,17β-dihydroxy-5-androstene
A7 = 3β,17β-dihydroxy-4-androstene
A8 = 3β,17α-dihydroxy-5β-androstane
A9 = 3α,17α-dihydroxy-5α-androstane
A10 = 3α,17β-dihydroxy-5β-androstane
A11 = 3α,17α-dihydroxy-5β-androstane

Group B: Monohydroxymonoketosteroids
B1 = 3α-hydroxy-5α-androstan-17-one
B2 = 3β-hydroxy-5-androsten-17-one
B3 = 3β-hydroxy-5β-androstan-17-one
B4 = 3β-hydroxy-5α-androstan-17-one
B5 = 3β-hydroxy-5α-androstan-16-one
B6 = 17α-hydroxy-5α-androstan-3-one
B7 = 17β-hydroxy-5α-androstan-3-one
B8 = 3α-hydroxy-5β-androst-9(11)-en-17-one
B9 = 3α-hydroxy-5β-androstan-17-one
B10 = 17α-hydroxy-5β-androstan-3-one
B11 = 17β-hydroxy-5β-androstan-3-one

Group C: Monohydroxymonoketosteroids with one or two conjugated double bonds
C1 = 17α-hydroxy-4-androsten-3-one
C2 = 17β-hydroxy-4-androsten-3-one
C3 = 17β-hydroxy-4,6-androstadien-3-one
C4 = 17β-hydroxy-19-nor-4-androsten-3-one
C5 = 17β-hydroxy-1,4-androstadien-3-one

Group D: Diketosteroids
D1 = 5α-androstane-3,17-dione
D2 = 5β-androstane-3,17-dione
D3 = 4-androstene-3,17-dione
D4 = 5-androstene-3,17-dione
D5 = 1,4-androstadiene-3,17-dione

Group E: Acetylated monohydroxymonoketosteroids with a conjugated double bond
E1 = 17β-acetoxy-4-androsten-3-one
E2 = 17α-acetoxy-4-androsten-3-one
E3 = 17β-acetoxy-19-nor-4-androsten-3-one
E4 = 17β-acetoxy-1,4-androstadien-3-one

Group F: Acetylated monohydroxymonoketosteroids without conjugated double bonds
F1 = 3β-acetoxy-5α-androstan-16-one
F2 = 3β-acetoxy-5-androsten-17-one
F3 = 3α-acetoxy-5α-androstan-17-one
F4 = 3β-acetoxy-5α-androstan-17-one
F5 = 3α-acetoxy-5β-androstan-17-one
F6 = 3β-acetoxy-5β-androstan-17-one
F7 = 17β-acetoxy-5α-androstan-3-one
F8 = 17β-acetoxy-5β-androstan-3-one

Group G: Acetylated dihydroxysteroids
G1 = 3α,17β-diacetoxy-5α-androstane
= 3β,17β-diacetoxy-5α-androstane
= 3α,17β-diacetoxy-5β-androstane
= 3β,17β-diacetoxy-5-androstene

Group H: Trihydroxysteroids
H1 = 3β,16α,17β-trihydroxy-5-androstene
H2 = 3β,11β,17β-trihydroxy-5α-androstane

Group J: Dihydroxymonoketosteroids
J1 = 3β,16α-dihydroxy-5-androsten-17-one
J2 = 3β,16β-dihydroxy-5-androsten-17-one
J3 = 3α,16α-dihydroxy-5α-androstan-17-one
J4 = 3α,11β-dihydroxy-5α-androstan-17-one
J5 = 3β,11β-dihydroxy-5α-androstan-17-one
J6 = 3α,16α-dihydroxy-5β-androstan-17-one
J7 = 3β,14α-dihydroxy-5α-androstan-17-one
J8 = 2α,17β-dihydroxy-4-androsten-3-one
J9 = 2β,17β-dihydroxy-4-androsten-3-one
J10 = 16α,17β-dihydroxy-4-androsten-3-one
= 6β,17β-dihydroxy-4-androsten-3-one
J11 = 11β,17β-dihydroxy-4-androsten-3-one
= 3β,7β-dihydroxy-5-androsten-17-one
= 17β,19-dihydroxy-4-androsten-3-one
J12 = 17β,18-dihydroxy-4-androsten-3-one
J13 = 11α,17β-dihydroxy-4-androsten-3-one
J14 = 14α,17β-dihydroxy-4-androsten-3-one
J15 = 3α,6α-dihydroxy-5β-androstan-17-one
= 3β,7α-dihydroxy-5-androsten-17-one
= 7α,17β-dihydroxy-4-androsten-3-one

Group K: Monohydroxydiketosteroids
K1 = 11β-hydroxy-4-androstene-3,17-dione
K2 = 3α-hydroxy-5α-androstane-11,17-dione
K3 = 3α-hydroxy-5β-androstane-11,17-dione
K4 = 2α-hydroxy-4-androstene-3,17-dione
K5 = 2β-hydroxy-4-androstene-3,17-dione
K6 = 16α-hydroxy-4-androstene-3,17-dione
K7 = 6β-hydroxy-4-androstene-3,17-dione
K8 = 14α-hydroxy-4-androstene-3,17-dione
= 3β-hydroxy-5-androstene-7,17-dione
K9 = 19-hydroxy-4-androstene-3,17-dione
K10 = 3β-hydroxy-5α-androstane-6,17-dione
K11 = 7α-hydroxy-4-androstene-3,17-dione

Group L: Triketosteroids
L1 = 4-androstene-3,6,17-trione
L2 = 1,4-androstadiene-3,11,17-trione
L3 = 4-androstene-3,11,17-trione
L4 = 5β-androstane-3,7,17-trione

TABLE I (*continued*)

Remarks
The norandrostene compounds C4 and E3 are C_{18} steroids. The three points outside the enclosed areas denote C_{21} steroids included for comparison:
1 = 3α-hydroxy-5α-pregnan-20-one
2 = 3β-hydroxy-5α-pregnan-20-one
3 = 17α-ethinyl,17β-hydroxyl-4-androsten-3-one

supported by the fact that adsorbents vary greatly in their atypicality[2]. Only very limited atypicality is found in partition chromatography in which no adsorbents are used. Moreover, the small atypicality in partition systems is obviously due to a decreased polarity of hydroxyl groups rather than an increased polarity of keto groups (ref. 7, p. 96). It is thus probable that the strongly "atypical" adsorbents, silica gel and especially magnesium silicate[2], form special strong bonds with the oxygen atoms of keto groups of steroids.

Alcohols are considered to be rather strong eluents (ref. 8, p. 20). Nevertheless, the R_F value of androstenedione, a weakly adsorbed steroid in typical systems, is only 0.17 when chromatographed with 100% 1-octanol on silica gel (ref. 2, p. 33). This too points to a strong "atypical" adsorption between the keto groups and the adsorbent. The fact that many spots tail somewhat in the "atypical" direction might be due to a relatively small capacity of the sites of the adsorbent that bond to keto groups.

On the other hand, hydroxyl groups are retained only weakly in the alcoholic "atypical" systems. Solvent hydroxyl groups presumably compete with the hydroxyl groups of the steroids for adsorption sites. In the solvent mixture the 1-hexanol (or 1-octanol) molecules probably form groups (micelles) with their hydroxyl heads close to another. Steroids with a hydroxyl group at either end of the elongated molecule could obviously replace one or two hexanol molecules from such a configuration. Adsorption would then depend mainly on the remaining functional groups.

The acetoxy group is in the "typical" direction approximately as polar as the keto group, so that steroids in group G of Fig. 2 move approximately as rapidly as steroids in group F in this direction. On the other hand, in the "atypical" direction all tested diacetoxysteroids (G) move to the secondary front and are thus not adsorbed at all. Monoacetoxymonoketosteroids (F), with their unmasked keto group, are adsorbed somewhat even in this direction.

Steroids with a functional group (hydroxyl or carbonyl) in the A-ring pointing at an angle away from the plane of the steroid skeleton (Fig. 3) are rather polar. Such configurations (either 3α-hydroxy-5β or 3-keto-5β) are found in the steroids A10, A11, B8, B9, B10, B11 and D2, all examples of steroids that are comparatively polar in both the "typical" and the "atypical" direction (Fig. 2). If such a divergent functional group is masked by acetylation, the resulting acetoxysteroid (F5) is rather apolar when compared with an acetoxysteroid with its divergent functional group intact (F8).

As the 3α-hydroxyl group of 5β-steroids is angular (equatorial), these findings do not contradict the rule known from partition chromatography, according to which

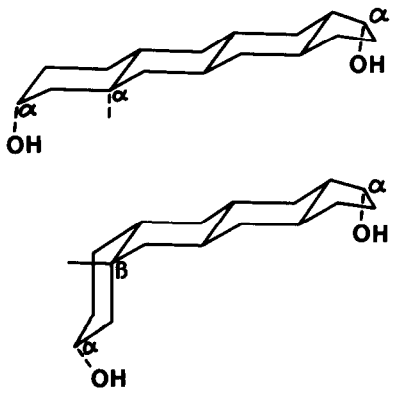

Fig. 3. The conformational structures (side projections) of 5α-androstane-3α,17α-diol (A9 in Fig. 2) and 5β-androstane-3α,17α-diol (A11 in Fig. 2).

hydroxyl groups in angular (equatorial) positions are always more polar than the corresponding axial hydroxyl groups (ref. 8, p. 5). The 3β-hydroxyl group of 5α-steroids is also angular, and A4 is indeed more polar in both directions than A2, and B9 is more polar than B3. As pointed out previously[9], the rule does not always hold for adsorption chromatography. Thus, 3α,17α-dihydroxy-5α-androstane (A9) is considerably more polar in both the "typical" and "atypical" directions than the corresponding 3β-epimer (A5). The only difference of the molecule that can explain the anomaly is the α-hydroxyl group at C-17. The configuration of the D-ring thus affects the adsorption of the A-ring.

Of the dihydroxysteroids, 3α,17α-dihydroxy-5β-androstane (A11) moves anomalously slowly with the "atypical" solvent. The total configuration of the molecule is relevant. If any of the epimeric features of the molecule is changed, the steroid is retained much less. Thus the corresponding 5α-epimer (A9), the corresponding 3β-epimer (A8), and the corresponding 17β-epimer (A10) all move more in the second direction. The 3α,17α-dihydroxy-5β-androstane molecule is almost horseshoe-shaped, with its hydroxyl groups relatively near each other (Fig. 3). Such a molecule fits very poorly in the presumed micelles, and the hydroxyl groups may affect each other's adsorption at nearby sites of the adsorbent.

The finding that minor structural differences cause different effects on the mobilities of steroids in "typical" and "atypical" systems, can be useful in difficult separations. "Typical" systems do not separate testosterone (C3) from epitestosterone (C2) at all[9]. (The only good separation, given in ref. 10, p. 88, is a misprint.) This separation is, however, relatively easy with "atypical" solvents[11]. Similarly, the separation of dehydroepiandrosterone (B2) from 5α-dihydrotesterone (B7), which is considered difficult[12], can be achieved with the "atypical" solvent system of the present paper. Even the difficult[12] pair 3α,17β-dihydroxy-5α-androstane (A2) and 3β,17β-dihydroxy-5-androstene (A6) is partially separated in the "atypical" direction.

Other separations more easy to achieve with the present "atypical" system than with the customary "typical" systems (ref. 10, p. 88) include 5α-androstanedione (D1) from 5β-androstanedione (D2), and dihydrotestosterone (B7) from etiocholan-17β-ol-3-one (B11). The pair androsterone (B1) and etiocholan-17β-ol-3-one

(B11) is hardly separable with the customary systems, whereas their R_F values differ by more than 0.2 in the present atypical system. Many more examples can be found in Fig. 2.

Tailing of the spots can be somewhat troublesome at low R_F values in the "atypical" direction. Another drawback of the present "atypical" solvent mixture is the high boiling temperature of 1-hexanol (158°C). If the separated steroids are eluted from the plate, the eluent contains hexanol, the last traces of which must be evaporated with a current of nitrogen.

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