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Note

Separation of the steroidal pairs 5α-androstenediol–5α-androstanediol and dehydroepiandrosterone–5α-dihydrotestosterone by thin-layer chromatography

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The separation of steroids which differ by only a double bond has proven difficult in many instances. Examples of such steroidal pairs are 5α-androstenediol–5α-androstanediol** and dehydroepiandrosterone–5α-dihydrotestosterone. These steroids resist separation by conventional thin-layer chromatography (TLC) and usually only differ in R_F value by approximately 0.05[1,2]. Several derivatization procedures are available which take advantage of the presence of a double bond in only one of each pair of steroids. These methods include epoxide formation[3] and hydroxylation[4]. In addition, π-complex formation can be achieved on silver nitrate impregnated silica gel plates[5] resulting in a retardation of steroids containing a double bond.

This paper reports the separation of the steroidal pairs 5α-androstenediol–5α-androstanediol and dehydroepiandrosterone–5α-dihydrotestosterone by conventional TLC after addition of bromine across the double bond whilst on the TLC plate.

MATERIALS AND METHODS

Water was glass-distilled. All organic solvents were distilled before use. Silica gel DG was from Riedel-de-Haën (Sellze, Hannover, F.R.G.). Sulphuric acid (sp. gr. 1.84 g/cm^3) was Analytical Grade. Steroids and miscellaneous chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

The preparation and running of TLC plates was as previously described[6]. Silver nitrate impregnated plates were prepared by adding 30 g of silica gel to 60 ml of a solution of silver nitrate in water (10%, w/v) to form a slurry and plates were poured as described. These plates were stored in the dark.

Epoxidation of steroids was performed using *m*-chloroperbenzoic acid as described by Azarnoff and Tucker[3]. Hydroxylation of steroids with alkaline potassium permanganate was performed by the method of Bush[4]. Bromination of steroids was

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** The following trivial names are used in this text: 5α-androstenediol = 5α-androstene-3β,17β-diol; 5α-androstanediol = 5α-androstane-3α, 17β-diol; dehydroepiandrosterone = 3β-hydroxy-5-androsten-3-one; 5α-dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one.

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performed by a modification of the method of Cargill[7] as follows. A 2% (v/v) bromine solution was prepared in absolute methanol. After application of steroids (10 μl of a 1 mg/ml ethyl acetate solution) to the TLC plates, 10 μl of the bromine solution was applied to the same origin and the solvent and excess bromine were allowed to evaporate.

Visualization of steroids was achieved by spraying the plates with a freshly prepared mixture of ethanol–sulphuric acid (1:1) and heating at 110°C for 10–20 min.

RESULTS AND DISCUSSION

The data presented in Table I is typical of the separations achievable for 5α-androstenediol–5α-androstanediol and dehydroepiandrosterone–5α-dihydrotestosterone in conventional one-dimensional TLC on silica gel. The best separation of 5α-androstenediol and 5α-androstanediol was only about 0.03 R_F values (systems IX and X, Table I). Dehydroepiandrosterone and 5α-dihydrotestosterone were separable by approximately 0.08 R_F values in solvent system II of Table I. However, it can be seen from these data and that of Heftmann[1] and Lisboa[2], that the separations achievable for these steroids are unsatisfactory for identification purposes.

Derivatization of steroids by epoxide formation or hydroxylation are lengthy procedures and require subsequent purification of steroidal derivatives. Whilst these procedures proved effective for the separation of the steroidal pairs 5α-androstenediol–5α-androstanediol and dehydroepiandrosterone–5α-dihydrotestosterone (data not shown), they are inappropriate for small quantities of steroids due to procedural loses.

Silver nitrate impregnated silica gel plates were found to be more effective for the separation of these steroidal pairs (Table II) than conventional TLC. The separation of the diols was about twice that obtainable on silica gel with a difference in

TABLE I

CONVENTIONAL TLC OF 5α-ANDROSTENEDIOL, 5α-ANDROSTANEDIOL, DEHYDROEPIANDROSTERONE AND 5α-DIHYDROTESTOSTERONE

10 μg of each steroid (prepared in ethyl acetate) was added to a separate origin. Ascending chromatograms were developed for sufficient time for the front to move approximately 12 cm. Steroids were visualized by spraying the plates with ethanol–sulphuric acid (1:1) and heating at 110°C for 10–20 min. The solvent systems were, by volume: I, benzene–ethanol (9:1); II, benzene–ethanol (14:1); III, benzene–ethyl acetate (2:1); IV, diethyl ether–benzene (2:1); V, benzene–toluene–methanol (9:1:1); VI, hexane–ethyl acetate (1:1); VII, dichloromethane–acetone (4:1); VIII, benzene–diethyl ether (2:1); IX, chloroform–methanol (98:2); X, dichloromethane–ethyl acetate–methanol (85:15:1).

Compound	R_F*									
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
5α-Androstenediol	0.37	0.26	0.38	0.61	0.23	0.57	0.67	0.23	0.34	0.26
5α-Androstanediol	0.39	0.27	0.39	0.63	0.24	0.59	0.69	0.24	0.37	0.23
Dehydroepiandrosterone	0.43	0.47	0.57	0.79	0.36	0.73	0.88	0.33	0.72	0.51
5α-Dihydrotestosterone	0.45	0.55	0.59	0.84	0.38	0.76	0.90	0.36	0.79	0.55

* Values represent the means of triplicate determinations.

TABLE II

SEPARATION OF 5α-ANDROSTENEDIOL, 5α-ANDROSTANEDIOL, DEHYDROEPIANDROSTERONE AND 5α-DIHYDROTESTOSTERONE ON SILVER NITRATE IMPREGNATED SILICA GEL PLATES

Silver nitrate impregnated silica gel plates were prepared and ascending chromatograms run as described.

Compound	R_F*			
	II	*IV*	*V*	*VIII*
5α-Androstenediol	0.25	0.26	0.47	0.57
5α-Androstanediol	0.32	0.32	0.54	0.63
Dehydroepiandrosterone	0.44	0.41	0.62	0.72
5α-Dihydrotestosterone	0.52	0.52	0.73	0.81

* The solvent systems used correspond to those described in the legend to Table I. R_F values represent the means of triplicate determinations.

R_F values of about 0.07 units. Dehydroepiandrosterone and 5α-dihydrotestosterone were separated by about 0.11 R_F values in two of the solvent systems used (systems IV and V). It was apparent that regardless of the solvent system, separation of these steroidal pairs was superior on silver nitrate impregnated silica gel plates. However, since silver nitrate quenches radioactivity[2], the usefulness of silver nitrate impreg-

TABLE III

SEPARATION OF 5α-ANDROSTENEDIOL, 5α-ANDROSTANEDIOL, DEHYDROEPIANDROSTERONE AND 5α-DIHYDROTESTOSTERONE BY TLC AFTER BROMINATION OF THE STEROIDS ON THE PLATES

10 μg of each steroid was spotted onto a separate origin. The solvent was allowed to evaporate, 10 μl of a 2% (v/v) bromine solution in methanol was added and the solvent and excess bromine were allowed to evaporate. Ascending chromatograms were run and steroids were visualized as described.

Compounds	R_F*				
	II	*III*	*IV*	*VIII*	*X*
5α-Androstenediol	0.26	0.29	0.48	0.22	0.21
5α-Androstanediol	0.29	0.29	0.50	0.24	0.21
5α-Androstenediol and bromine	0.31	0.40	0.59	0.31	0.30
Both diols and bromine	0.26	0.29	0.51	0.24	0.21
	0.30	0.40	0.59	0.31	0.30
Dehydroepiandrosterone	0.40	0.43	0.65	0.34	0.45
5α-Dihydrotestosterone	0.43	0.45	0.70	0.36	0.45
Dehydroepiandrosterone and bromine	0.55	0.63	0.81	0.56	0.64
Both sterones and bromine	0.45	0.44	0.70	0.37	0.48
	0.59	0.63	0.82	0.52	0.66

* Solvent systems were those described in the legend to Table I. R_F values represent the means of triplicate determinations.

nated plates is restricted to non-radioactive steroids or high levels of radioactivity. These criteria are commonly not achievable in investigations of steroid metabolism.

Table III shows the separations obtained using the method described for the addition of bromine across the double bond. Using this procedure 5α-androstenediol and 5α-androstanediol could be separated with an R_F difference of 0.11 (system III, Table III), superior to any of the other methods described. Similarly, dehydroepiandrosterone and 5α-dihydrotestosterone were separable by approximately 0.2 R_F values in solvent systems III and IX.

The reaction of bromine with the steroids is rapid and efficient since only one spot was visualized after derivatization. The specificity of the reaction for steroids containing a double bond allows the separation of a mixture of steroids such as those described (see Table III). In addition, this methodology has proven effective in investigations of androgen metabolism by various cell cultures[8].

In conclusion, the method described appears to be highly efficient for the separation of steroidal pairs which differ only by the presence or absence of a double bond. The method is simple and rapid and does not require lengthy manipulations as is necessary for other derivatization procedures.

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