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Note

Improved separation of some isomers and epimers of the pregnane series by thin-layer chromatography with repeated development

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Radioactively labelled steroids with high specific activity are now available for hormone research, which is performed under different experimental conditions. In the past, metabolic studies were mainly performed *in vitro* with steroid hormones of low specific activity. The amounts of the compounds applied could be chosen to be as high as necessary to recover enough material from the tissue being investigated. However, compounds with high specific activity are now available that enable one to work with smaller amounts under nearly physiological conditions. The amounts of radioactive material recovered from the tissue are often too low to be detected by methods other than a combination of thin-layer chromatography (TLC) and liquid scintillation counting.

Because small amounts of progesterone metabolites had to be separated for identification[1–3], it was necessary to develop TLC methods for the resolution of some steroid isomers and epimers.

In previous studies[4–6], R_F values for progesterone derivatives in various chromatographic systems were reported. With these systems, the separation of some isomeric and epimeric pairs was sometimes not good enough for the recovery of relatively pure compounds for further identification experiments. The TLC methods reported here give adequate separations for some pairs of closely related pregnane derivatives.

EXPERIMENTAL

Steroid compounds

3α-Hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, 5α-pregnane-3α,20α-diol, 5α-pregnane-3β,20α-diol, 5α-pregnane-3α,20β-diol and 5β-pregnane-3α,20β-diol were obtained from Ikapharm (Ramat-Gan, Israel). Mann Labs. (New York, N.Y., U.S.A.) supplied 5α-pregnane-3,20-dione and 5β-pregnane-3,20-dione, while Merck (Darmstadt, G.F.R.) supplied 5β-pregnane-3α,20α-diol. 6α-Hydroxy-4-pregnene-3,20-dione, 6β-hydroxy-4-pregnene-3,20-dione, 11α-hydroxy-4-pregnene-3,20-dione, 16α-hydroxy-4-pregnene-3,20-dione, 5α-pregnane-3β,20β-diol, 5β-pregnane-3β,20α-diol and 5β-pregnane-3β,20β-diol were purchased from Steraloids (Pawling, N.J., U.S.A.).

Materials for TLC

The sorbents aluminium oxide, neutral (Type T) without calcium sulphate binder, aluminium oxide, basic (Type T), and silica gel HF_{254}, and all organic solvents, of analytical-reagent grade, were obtained from Merck.

Preparation of TLC plates

For the purpose which was described elsewhere[1–3], commercially available pre-coated TLC plates could not be used. Glass plates were coated with silica gel or with aluminium oxide, neutral or basic, using an applicator from Desaga (Heidelberg, G.F.R.). A slurry was prepared by stirring 100 g of aluminium oxide with 170 ml or silica gel with 250 ml of distilled water. The plates were coated with the slurry to a thickness of 0.5 mm. After being coated, the plates were allowed to stand overnight at room temperature and then heated at 110–120° for 1 h.

The spots of the steroids were stained with iodine vapour after development. The solvent systems used for the separation of the steroids are given in Tables I and II.

TABLE I

MOBILITIES OF CLOSELY RELATED COMPOUNDS OF THE PREGNANE SERIES

The separations were performed on layers of aluminium oxide, basic or neutral, without calcium sulphate binder, 0.5 mm thick. Abbreviations: B = benzene, C = cyclohexane, E = diethyl ether, EtAc = ethyl acetate, EtOH = ethanol, P = light petroleum, W = water. *n* = Number of experiments.

Compound	*Aluminium oxide*					
	Neutral				*Basic*	
	*B–EtOH (98:2)**		*P–E (60:40)***		*C–EtAc–W (125:125:0.4)*	
	n	R_F	*n*	R_F	*n*	R_F
5α-Pregnane-3,20-dione			8	0.49		
5β-Pregnane-3,20-dione			8	0.38		
3α-Hydroxy-5α-pregnan-20-one	7	0.67				
3β-Hydroxy-5α-pregnan-20-one	7	0.52				
20α-Hydroxy-4-pregnen-3-one	7	0.53				
20β-Hydroxy-4-pregnen-3-one	7	0.61				
6α-Hydroxy-4-pregnene-3,20-dione	8	0.24			3	0.27
6β-Hydroxy-4-pregnene-3,20-dione	8	0.36			3	0.42
11α-Hydroxy-4-pregnene-3,20-dione	8	0.30			3	0.31
16α-Hydroxy-4-pregnene-3,20-dione	8	0.24			3	0.22
5α-Pregnane-3α,20α-diol	6	0.32			4	0.51
5α-Pregnane-3α,20β-diol	6	0.40			4	0.65
5α-Pregnane-3β,20α-diol	6	0.35			4	0.56
5α-Pregnane-3β,20β-diol	6	0.37			4	0.60
5β-Pregnane-3α-20α-diol	6	0.21			4	0.44
5β-Pregnane-3α,20β-diol	6	0.25			4	0.50
5β-Pregnane-3β,20α-diol	6	0.45			4	0.63
5β-Pregnane-3β,20β-diol	6	0.48			4	0.67

* Developed twice.

** Developed five times.

RESULTS AND DISCUSSION

The separation of the closely related steroids used in this study is demonstrated by the R_F values given in Tables I and II. As reported[4–6], TLC of the pregnane series had been performed on silica gel with and without added calcined calcium sulphate. In this work, aluminium oxide, neutral or basic, was used additionally, often giving a better separation than silica gel.

TABLE II

MOBILITIES OF CLOSELY RELATED COMPOUNDS OF THE PREGNANE SERIES

The separations were performed on layers of silica gel HF_{254}, 0.5 mm thick. Abbreviations: E = diethyl ether, EtAc = ethyl acetate, P = light petroleum, T = toluene. n = Number of experiments.

Compound	*Silica gel HF_{254}*					
	*P–E (60:40)**		*P–E (40:60)**		*T–EtAc (90:10)***	
	n	R_F	n	R_F	n	R_F
5α-Pregnane-3,20-dione	12	0.75			8	0.67
5β-Pregnane-3,20-dione	12	0.63			8	0.59
3α-Hydroxy-5α-pregnan-20-one					4	0.47
3β-Hydroxy-5α-pregnan-20-one					4	0.38
6α-Hydroxy-4-pregnene-3,20-dione			6	0.28		
6β-Hydroxy-4-pregnene-3,20-dione			6	0.44		
5α-Pregnane-3α,20α-diol	4	0.27	3	0.59		
5α-Pregnane-3α,20β-diol	4	0.39	3	0.68		
5α-Pregnane-3β,20α-diol	4	0.32	3	0.62		
5α-Pregnane-3β,20β-diol	4	0.37	3	0.64		
5β-Pregnane-3α,20α-diol	4	0.16	5	0.43		
5β-Pregnane-3α,20β-diol	4	0.21	5	0.51		
5β-Pregnane-3β,20α-diol	4	0.39	5	0.70		
5β-Pregnane-3β,20β-diol	4	0.43	5	0.75		

* Developed ten times.
** Developed five times.

Pairs of isomers or epimers, such as 5α- and 5β-pregnane-3,20-dione, 3α- and 3β-hydroxy-5α-pregnan-20-one, 20α- and 20β-hydroxy-4-pregnen-3-one, the hydroxy-4-pregnene-3,20-diones and the pregnanediols, can easily be separated by TLC on silica gel HF_{254} with solvent systems such as chloroform–ethanol (90:10) and benzene–ethanol (95:5, after developing twice). The results achieved with these systems are described elsewhere[3].

Because of the last-mentioned separations of the steroid pairs, it is acceptable that compounds with different functional groups show the same R_F values in one of the systems (Tables I and II). If steroid pairs gave a poor separation in one of the chromatographic systems, their R_F values are not shown in Table I or II.

The R_F values were determined with a solvent front 100 mm from the origin. Because the solvent front usually migrates 150 mm on a 200 × 200 mm plate, the separation of epimers such as 5α-pregnane-3β,20α-diol from 5α-pregnane-3β,20β-diol, with R_F values of 0.32 and 0.37, respectively, on silica gel with light petroleum

(b.p. 60–80°)–diethyl ether (60:40, developed 10 times) may be considered to be complete (Table II).

The separation of 5α-pregnane-3,20-dione from 5β-pregnane-3,20-dione was more effective with the systems applied (Tables I and II) than that obtained by Lisboa[6], and was similar to that obtained by Wright[4].

3α-Hydroxy-5α-pregnan-20-one could easily be separated from its 3β-isomer on aluminium oxide with benzene–ethanol (98:2, after developing twice), and the same was true for the separation of 20α-hydroxy-4-pregnen-3-one from its 20β-epimer (Table I). Both separations were more complete than those reported by Lisboa[5,6]. Additionally, the hydroxy-4-pregnene-3,20-dione isomers could be separated better on aluminium oxide (basic or neutral) (Table I) than on silica gel G[4,7]. Berthou *et al.*[8] reported that the separation of the 5α- and the 5β-pregnanediols could be achieved only by a combination of thin-layer and gas–liquid chromatography. However, with the solvent system benzene–ethanol (98:2) on aluminium oxide, neutral, the 5α-pregnanediols could be separated from the 5β-epimers (Table I). Each series could then be separated into the four isomers on silica gel HF_{254} with one of the solvent systems containing light petroleum and diethyl ether after 10 developments (Table II).

CONCLUSION

TLC with the chromatographic systems reported here and using repeated development is a simple and effective method for the separation of closely related compounds of the pregnane series, especially when small amounts of substances are present, as in metabolic studies using radioactive steroids. These radioactive compounds can then be detected on thin-layer chromatograms by comparing them with reference steroids after analyzing the TLC fractions by liquid scintillation counting. It is obvious that the chromatographic systems reported here can also be applied to similar separation problems in hormone research.

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