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SEPARATION OF ANDROSTERONE FROM EPIANDROSTERONE, AND DEHYDROEPIANDROSTERONE FROM ITS 3-HYDROXY EPIMER BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The application of thin-layer chromatography to the separation of 13 steroids, including androstanes, 4-androstenes and 5-androstenes, using silica gel and 1,2-propanediol-impregnated cellulose is described. After group-wise separation of various C_{19} steroids on silica gel, the 3-hydroxy epimers of 5α-androstanes and 5-androstenes can be separated by thin-layer chromatography on impregnated cellulose plates. The chromatographic procedure is rapid and makes the prior formation of steroid derivatives unnecessary.

INTRODUCTION

The analytical separation of derivatives of androstane and androstene has beeen achieved by paper partition chromatography using Zaffaroni-type or Bush-type solvent systems[1,2]. However, these methods have some drawbacks, such as the long development time and, often, poor reproducibility. For the separation of C_{19} steroids, adsorption chromatography on silica gel is simpler and quicker than partition chromatography on paper. But, unfortunately, separation of the four 3α-(or 3β-)hydroxy-5α-(or 5β-)androstan-17-ones is not possible in a number of different solvent systems, as described by Lisboa[3,4]. These steroids were separately identified by formation of steroid acetates or by chromatography on aluminium oxide F_{204} and application of specific colour reactions[5].

Recently, our attention has been focused on the enzymatic conversion of C_{19} steroids by *Streptomyces hydrogenans*[6,7]. Notably, a small activity of 4-ene-3-oxo-steroid-5α-reductase could be detected in the cytosol fraction of the cells[8], leading to numerous androstane metabolites. Therefore, we have searched for a sensitive, reproducible and rapid method for the separation and identification of minute amounts of these testosterone metabolites. We combined the advantages of partition chromatography and thin-layer chromatography (TLC) by using TLC plates pre-coated with cellulose and impregnated with 1,2-propanediol.

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EXPERIMENTAL

Materials

All reagents used were of analytical grade (Merck, Darmstadt, G.F.R.), with the exception of 1,2-propanediol, which was of technical grade (Merck). 5β-Androstane-3,17-dione was obtained from Steraloids (Pawling, N.Y., U.S.A.); 4-androstene-3α,17β-diol from Schering (Berlin, G.F.R.); all other steroids were obtained from Merck. Table I lists the 13 C_{19} steroids used.

TABLE I

STEROID NUMBER, SYSTEMATIC NAMES, TRIVIAL NAMES AND ABBREVIATIONS FOR THE 13 STEROIDS STUDIED

*Number**	*Systematic name*	*Trivial name*	*Abbreviation*
1	3α-Hydroxy-5α-androstan-17-one	Androsterone	3α ol 5α A 17 one
2	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	3β ol 5α A 17 one
3	17β-Hydroxy-5α-androstan-3-one	5α-Dihydrotestosterone	17β ol 5α A 3 one
4	17β-Hydroxy-5β-androstan-3-one	5β-Dihydrotestosterone	17β ol 5β A 3 one
5	5α-Androstane-3, 17-dione	Androstanedione	5α A 3, 17 one
6	5β-Androstane-3, 17-dione	Etiocholanedione	5β A 3, 17 one
7	Androst-4-ene-3, 17-dione	Androstenedione	Δ^4 A 3, 17 one
8	3β-Hydroxyandrost-5-en-17-one	Dehydroepiandrosterone	3β ol Δ^5 A 17 one
9	3α-Hydroxyandrost-5-en-17-one	—	3α ol Δ^5 A 17 one
10	Androst-4-ene-3α, 17β-diol	—	3α, 17β ol Δ^4 A
11	Androst-5-ene-3β, 17β-diol	Androstenediol	3β, 17β ol Δ^5 A
12	5α-Androstane-3β, 17β-diol	Androstanediol	3β, 17β ol 5α A
13	17β-Hydroxyandrost-4-en-3-one	Testosterone	17β ol Δ^4 A 3 one

* See also Fig. 1.

TLC plates were purchased from Merck, with the following specifications: pre-coated silica gel plates 60 F_{254}, 20 × 20 cm, 250 μm layer thickness; pre-coated cellulose plates without fluorescent indicator, 20 × 20 cm, 100 μm layer thickness.

Methods

One-dimensional chromatography was performed on either pre-coated silica gel (60 F_{254}) or cellulose plates impregnated with 1,2-propanediol. Every chromatogram was run in completely saturated tanks[9]. Steroid samples of 30 μg were dissolved in 20 μl of either dichloromethane or diethyl ether. Samples were spotted 1.5 cm from the lower edge of the plate and at least 3 cm from the lateral border.

TLC on silica gel

Steroid samples were spotted on the start line and the chromatograms developed by the ascending technique with chloroform–ethyl acetate–light petroleum (b.p. 60–80°) (50:45:5, v/v/v; system A) as mobile phase[10]. The plates were removed when the solvent had ascended to a distance of 1 cm from the upper edge of the plate. The development time was 60 min.

Impregnation of cellulose plates and chromatography

Prior to impregnation of the cellulose thin-layer plates, 1.5 cm wide cellulose

strips were removed from two opposite edges of the plate. The origin line was labelled with a pencil, and small spacer plates (1 × 1 cm, 1 mm thickness) were placed at the four corners of the plate. A second cellulose plate was placed over the first, with cellulose layers facing one another. The pair of plates was held together by four clamps and immersed in a 35% solution of 1,2-propanediol in methanol. After 10 min, two clamps positioned opposite the start line were removed. The pair of plates, still held by two clamps, was dried under vacuum for 15 min. Following drying, the other two clamps were removed, and the back sides of the plates and the 1.5-cm-wide cellulose-free strips at the two edges were thoroughly cleaned with a methanol-soaked cloth and again dried under vacuum for 30 min. The plates were developed for 90 min with benzene–cyclohexane (50:50, v/v; system B).

Detection of steroids

After development the plates were allowed to dry in air. UV-absorbing steroids were marked under UV light (low-pressure mercury resonance arc with filter, Mineralight Model SL 2537). Most steroids were detectable by iodine vapour, which yielded reversibly coloured spots without destroying the marked steroid[11]. The following identification reactions were also employed[12]: spray of a solution of 0.2% of 2,4-dinitrophenylhydrazine in 2 *M* HCl (yellow-olive to yellow-brown colour); spray of 3.5% phosphotungstomolybdic acid in ethanol, followed by heating for 10 min at 120° (green-blue colour); spray of 1 g of vanillin in 100 ml of concentrated sulphuric acid, followed by heating for 10 min at 120° (red-violet colour)[13].

RESULTS

The chromatographic results obtained by application of the two different systems to 13 C_{19} steroids are summarized in Fig. 1 and Table II. A primary separation of the steroids or steroids groups was achieved by TLC on silica gel using solvent system A. Androsterone, epiandrosterone and 5α-dihydrotestosterone (steroids 1–3), as well as dehydroepiandrosterone and its 3α-epimer, 3α-hydroxy-5-androstene-17-one (steroids 8 and 9), migrate as a group of spots. Similarly, 4-androstene-3α,17β-diol, androstenediol, androstanediol and testosterone (steroids 10–13) form a group of spots with very close R_S values. (R_F values relative to S = testosterone). Similar results were obtained by Verhoeven *et al.*[14] by TLC separation of the steroids on silica gel, developed in methylene chloride–diethyl ether (85:15, v/v). We confirmed their findings with regard to the R_S values 1.30, 1.34 and 1.56 for steroids 1–3, and 0.82 and 0.84 for steroids 11 and 12, respectively, in that solvent system.

As shown in Table II, steroids 1–3 and 10–13, which form two groups of spots on silica gel in solvent system A, can be adequately separated after development on impregnated cellulose plates. This also applies to steroids 8 and 9. Only androstenediol and androstanediol, both carrying a 3β-hydroxy group (steroids 11 and 12) are unresolved in all chromatographic systems mentioned in this paper.

Although steroids are usually more sensitive to colour reactions after chromatography on silica gel, the steroids could be adequately detected after separation on cellulose plates. Because of the chemical reactivity of cellulose, only colour reagents that do not severely damage the cellulose layer can be used. Therefore, the vanillin–sulphuric acid reaction can be applied only to silica gel plates. On the other hand, phosphotungstomolybdic acid is useful on cellulose plates (Table III).

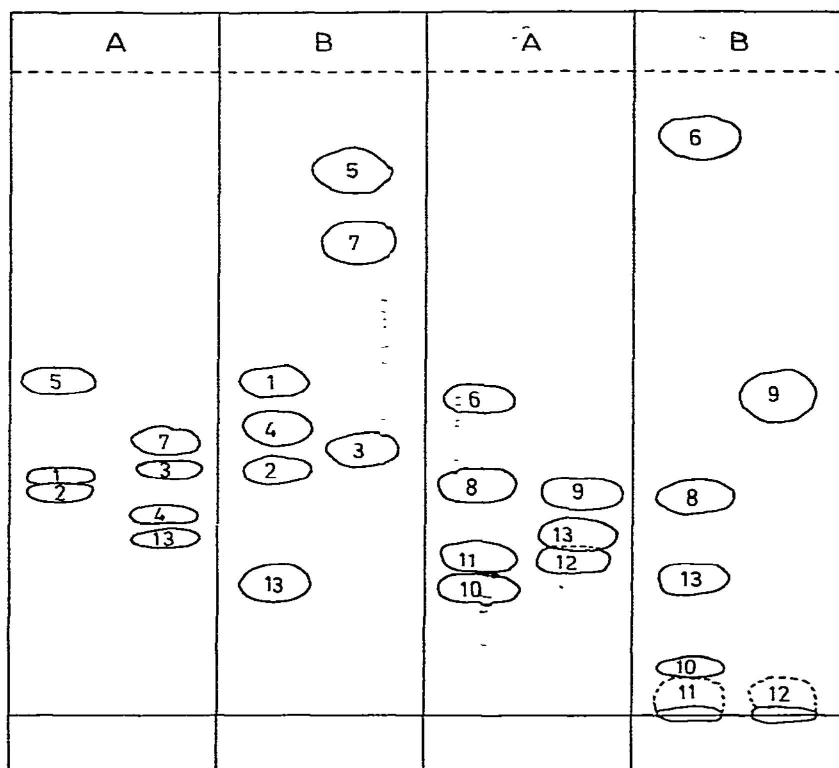

Fig. 1. Diagram of TLC of 13 steroids on Silica Gel F_{254} (solvent system A), or 1,2-propanediol-impregnated cellulose (system B). For explanation of the numbers, see Table I. System A: chloroform–ethyl acetate–light petroleum (b.p. 60–80°) (50:45:5, v/v/v). System B: benzene–cyclohexane (50:50, v/v).

TABLE II

R_S-VALUES (S = TESTOSTERONE) OF 13 C_{19} STEROIDS AFTER CHROMATOGRAPHY ON SILICA GEL (SYSTEM A) OR 1,2-PROPANEDIOL-IMPREGNATED CELLULOSE (SYSTEM B)

System A: chloroform–ethyl acetate–light petroleum (b.p. 60–80°) (50:45:5, v/v/v). System B: benzene–cyclohexane (50:50, v/v).

Number	*Abbreviation*	*Silica gel (system A)*	*Impregnated cellulose (system B)*
1	3α ol 5α A 17 one	1.35	2.58
2	3β ol 5α A 17 one	1.25	1.88
3	17β ol 5α A 3 one	1.37	2.03
4	17β ol 5β A 3 one	1.11	2.22
5	5α A 3, 17 one	1.89	4.22
6	5β A 3, 17 one	1.76	4.43
7	Δ^4 A 3, 17 one	1.54	3.66
8	3β ol Δ^5 A 17 one	1.27	1.67
9	3α ol Δ^5 A 17 one	1.23	2.43
10	3α, 17β ol Δ^4 A	0.69	0.33
11	3β, 17β ol Δ^5 A	0.88	0.0
12	3β, 17β ol 5α A	0.87	0.0
13	17β ol Δ^4 A 3 one	1.00	1.00

TABLE III

SENSITIVITY OF COLOUR REACTIONS FOR 30 μg OF 13 STEROIDS SUBJECTED TO TLC ON SILICA GEL OR 1,2-PROPANEDIOL-IMPREGNATED CELLULOSE

++, Strong colour; +, weak colour; —, no reaction.

Number	*Abbreviation*	*Iodine vapour*		*2,4-Dinitrophenylhydrazine*		*Vanillin–sulphuric acid*		*Phosphotungstomolybdic acid*	
		*A**	*B***	*A*	*B*	*A*	*B*	*A*	*B*
1	3α ol 5α A 17 one	++	++	++	++	++			+
2	3β ol 5α A 17 one	++	++	++	++	++			+
3	17β ol 5α A 3 one	++	++	++	++	++			+
4	17β ol 5β A 3 one	++	++	++	++	++			+
5	5α A 3, 17 one	++	++	++	++	++			+
6	5β A 3, 17 one	++	+	++	++	++			—
7	Δ^{4} A 3, 17 one	++	++	++	++	++			++
8	3β ol Δ^{5} A 17 one	++	+	++	++	++			—
9	3α ol Δ^{5} A 17 one	+	—	+	—	++			++
10	3α, 17β ol Δ^{4} A	—	—	—	—	++			++
11	3β, 17β ol Δ^{5} A	+	—	—	—	++			++
12	3β, 17β ol 5α A	+	—	—	—	++			++
13	17β ol Δ^{4} A 3 one	++	+	++	++	++			++

* After developing on silica gel plates in solvent system A.

** After developing on 1,2-propanediol-impregnated cellulose plates in solvent system B.

DISCUSSION

This paper describes a method for the separation of several C_{19} steroids by ascending TLC without prior derivatization of the samples, which is often accompanied by a considerable loss of material. In contrast to other chromatographic methods, TLC separation on impregnated cellulose is a rapid procedure and is recommended especially for the separation of various 3-hydroxy epimers of C_{19} steroids, such as androsterone and epiandrosterone. Moreover, minute amounts of different C_{19} metabolites that are usually inseparable in TLC systems on silica gel, can be identified; for example, androstene-3,17-dione (steroid 7) often overlaps other minor C_{19}-metabolites, such as 5α-dihydrostestosterone (steroid 3).

In a comparative study, Berthou *et al.*[15] determined the separation factor (ratio of relative R_F values of two steroids to be separated) for four pairs of androstanes. They reported no separation (ratio = 1) for steroids 1 and 2, or only very slight separation (maximum ratio = 1.15), when chromatography was performed on silica gel, using six different solvent systems.

To our knowledge, this is the first report of the separation of androsterone and epiandrosterone, as well as of other 3-hydroxy epimers of C_{19} steroids, by TLC on impregnated cellulose plates. On the other hand, androstenediol and androstanediol (steroids 11 and 12) are not separable by the chromatographic systems used in these experiments. We did not observe any dependence of migration of steroids on their position on the plate (the so-called "edge effect"), provided the tanks were thoroughly saturated with the vapour of the mobile phase. A similar system of 1,3-propanediol-impregnated cellulose plates has been described by Voelcker *et al.*[16] for the separation of highly labile metabolites of cyclophosphamide.

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