Notes

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Separation of some 17-ketosteroids by means of horizontal thin-layer chromatography

Horizontal thin-layer chromatography has already gained recognition as a valuable technique for the separation, purification and identification of several hormonal steroids. In our laboratory, this technique has been used extensively for pure substances[1] and for urinary oestrogens[2,3] plasma progesterone[4] and testosterone in biological media[5,6]. So far, however, the horizontal method has found only limited application in the separation of neutral 17-ketosteroids[7].

This communication describes a simple and reproducible horizontal thin-layer chromatographic technique, in which the separation and characterization of neutral 17-ketosteroids is effected on a single silica gel layer.

Materials and methods

The pure 17-ketosteroids studied are listed in Table I.

TABLE I

17-KETOSTEROIDS INVESTIGATED

Trivial name	*Systematic name*
Androstenedione	Androst-4-ene-3,17-dione
Androsterone	3α-Hydroxy-5α-androstan-17-one
Dehydroepiandrosterone	3β-Hydroxy-androst-5-en-17-one
Epiandrosterone	3β-Hydroxy-5α-androstan-17-one
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one
11-Ketoandrosterone	3α-Hydroxy-5α-androstane-11,17-dione
11-Hydroxyandrosterone	3α,11β-Dihydroxy-5α-androstan-17-one
11-Ketoetiocholanolone	3α-Hydroxy-5β-androstane-11,17-dione
11-Hydroxyetiocholanolone	3α,11β-Dihydroxy-5β-androstan-17-one

These steroids, obtained from Mann Research Laboratories (New York, N.Y.), were prepared as standard solutions in absolute ethanol.

All solvents were redistilled before use. Absolute ethanol and acetone, both analytical reagent grade, were obtained from British Drug Houses (B.D.H., London). Benzene (thiophene free), ethyl acetate and other solvents used were R.P. grade, and were obtained from Prolabò (distributor: Biraghi, Naples).

Silica Gel G (7731; Merck, Darmstadt) was sieved and then washed before use with absolute ethanol.

The Desaga outfit and the "B.N.-Kammer" for horizontal thin-layer chromatography (Desaga, Heidelberg) were purchased from Pabisch (Milan).

Thin-layer chromatography

Glass plates (20 × 20 cm) were coated with a micro-layer (0.25 mm thickness) of Silica Gel G, a slurry of which was prepared by vigorously shaking 20 g of the adsorbent with 50 ml of twice-distilled water for 2 min in a glass stoppered 250 ml Erlenmeyer flask.

The plates were then left on the bench for 15 min; dried in an oven at 60° for 12 h, pre-washed with ethanol or the same solvent system employed for the chromatographic separation, activated at 110° for 1 h and, finally, stored in a glass desiccator over dry silica gel. Samples (1–10 μg) of neutral 17-ketosteroids in absolute ethanol (singly and as mixtures), were spotted in successive small portions (to prevent undue spreading of the spots) by means of micropipettes approximately 1.5 cm from the lower edge and 2.0–2.5 cm from the sides of the plates. This procedure was found necessary in order to obtain reproducible R_F and R_A values.

The plates were developed, at room temperature, using horizontal migration in a B.N.-Kammer chromatographic apparatus according to LUISI *et al.*[1]

The plates were then removed from the chamber, dried (first at room temperature and then in an oven at 50° for 10–15 min) and sprayed with a 50:50 v/v solution of sulphuric acid in absolute ethanol (Engel's reagent) followed by heating in an oven at 80° for 15 min.

Results and discussion

Following an eluotropic solvent series we examined the different solvents separately in order to find the best solvent. Non-polar solvents did not cause any appreciable displacement of the 17-ketosteroids and polar ones caused total migration without separation. Therefore it was concluded that systems constituted of two solvents of different polarity (and in various proportions) would be the most useful for our purpose, especially the solvent system benzene–ethyl acetate (50:50, v/v). Different proportions of this solvent system (and others investigated) were found useful for the separation of 17-ketosteroids in groups.

Table II shows both R_F and R_A (R_F value of a single 17-ketosteroid relative to androstenedione) values of several 17-ketosteroids investigated.

The results obtained provide strong evidence that pure samples of 17-keto-

TABLE II

AVERAGE R_F AND R_A VALUES OF STANDARD 17-KETOSTEROIDS ON HORIZONTAL THIN-LAYER CHROMATOGRAPHY IN A BENZENE–ETHYL ACETATE (50:50 V/V) SYSTEM

S.D. is the standard deviation of a single estimate and N is the number of replicate analyses.

Compounds	N	$R_F \pm$ S.D.	$R_A \pm$ S.D.
Androstenedione	30	0.80 ± 0.04	— —
Androsterone	30	0.75 ± 0.04	0.93 ± 0.03
Dehydroepiandrosterone	30	0.72 ± 0.04	0.90 ± 0.03
Epiandrosterone	30	0.69 ± 0.04	0.86 ± 0.03
Etiocholanolone	30	0.66 ± 0.03	0.83 ± 0.02
11-Ketoandrosterone	25	0.52 ± 0.03	0.65 ± 0.02
11-Hydroxyandrosterone	25	0.50 ± 0.03	0.62 ± 0.01
11-Ketoetiocholanolone	25	0.44 ± 0.02	0.55 ± 0.01
11-Hydroxyetiocholanolone	25	0.41 ± 0.02	0.51 ± 0.01

TABLE III

R_F VALUES OF STANDARD 17-KETOSTEROIDS ON ASCENDING THIN-LAYER CHROMATOGRAPHY IN A BENZENE–ETHYL ACETATE (50:50 v/v) SYSTEM

S.D. is the standard deviation of a single estimate and N is the number of replicate analyses.

Compounds	*N*	$R_F \pm S.D.$	
Androstenedione	20	0.69	0.05
Androsterone	20	0.66	0.06
Dehydroepiandrosterone	20	0.64	0.05
Epiandrosterone	20	0.64	0.05
Etiocholanolone	20	0.54	0.05
11-Ketoandrosterone	10	0.30	0.04
11-Hydroxyandrosterone	10	0.29	0.04
11-Ketoetiocholanolone	10	0.22	0.04
11-Hydroxyetiocholanolone	10	0.21	0.03

steroids can be separated by horizontal thin-layer chromatography on Silica Gel G in a suitable solvent system. However, the same degree of separation was not achieved using an ascending thin-layer technique with the same solvent system (Table III).

In particular, it was not possible (by ascending migration) to separate dehydroepiandrosterone from epiandrosterone or the 11-keto and 11-hydroxy-derivatives of androsterone and etiocholanolone, respectively.

With quantitative application of the method described in mind, recovery experiments were carried out. For this purpose different adsorbents were employed (Silica Gel G, H and HF_{254}, from Merck, Darmstadt).

After chromatographic migration and evaporation of the solvents from the plates, the steroids were located; the silica gel was then loosened with a microspatula, aspirated into a glass elution apparatus and the steroids were eluted five times, under positive pressure, with 0.3 ml redistilled absolute ethanol. The ethanol was evaporated off in a water bath at 50° under nitrogen. The Zimmermann reaction was carried out according to DREKTER *et al.*[8]. The results obtained are reported in Table IV.

TABLE IV

MEAN % RECOVERY (± STANDARD DEVIATION) OF 17-KETOSTEROIDS FROM DIFFERENT SILICA GEL ADSORBENTS AFTER MIGRATION ON A THIN-LAYER IN BENZENE–ETHYL ACETATE (50:50 v/v) SYSTEM

Compounds	*N*	*Silica Gel*		
		G	*H*	HF_{254}
Androstenedione	10	95.4 ± 5.3	98.2 ± 3.1	99.0 ± 2.0
Androsterone	10	96.6 ± 5.7	96.5 ± 3.3	96.3 ± 3.0
Dehydroepiandrosterone	10	96.5 ± 3.2	97.1 ± 2.1	99.2 ± 1.1
Epiandrosterone	10	97.3 ± 3.1	97.8 ± 3.0	98.3 ± 2.2
Etiocholanolone	10	96.6 ± 4.1	95.8 ± 4.1	97.2 ± 3.0
11-Ketoandrosterone	5	94.4 ± 4.6	95.2 ± 3.2	95.3 ± 2.7
11-Hydroxyandrosterone	5	90.1 ± 3.1	91.6 ± 3.5	90.7 ± 4.0
11-Ketoetiocholanolone	5	91.6 ± 5.2	92.2 ± 4.1	92.8 ± 5.1
11-Hydroxyetiocholanolone	5	89.5 ± 6.6	93.3 ± 5.7	92.7 ± 4.3

These results indicate that the method described is useful for quantitative analysis. It should be emphasized that for quantitative analysis, it is necessary to pre-purify the silica gel (by extracting the powder, for example, with ethanol) or, and this is, in our opinion, more convenient, to pre-wash the plates (with ethanol and the same solvent system employed for the chromatographic separation) in order to prevent background discoloration, especially when the plates are sprayed with sulphuric acid and heated, and to reduce the silica gel blanks.

The technique is now being applied to the separation and quantitation of neutral urinary 17-ketosteroids.

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1 M. LUISI, C. SAVI AND V. MARESCOTTI, *J. Chromatog.*, 15 (1964) 428.
2 M. LUISI, A. CARNICELLI, G. GAMBASSI AND G. F. MENCHINI, *Biochim. Biol. Sper.*, 5 (1966) 173.
3 M. LUISI AND V. MARESCOTTI, in C. CASSANO (Editor), *Research on Steroids*, Il Pensiero Scientifico (Rome), II (1965) 171.
4 M. LUISI, G. GAMBASSI, V. MARESCOTTI, C. SAVI AND F. POLVANI, *J. Chromatog.*, 18 (1965) 278.
5 M. SURACE, M. LUISI, E. MONETA, V. MARESCOTTI AND F. POLVANI, *Excerpta Med. Found., Int. Cong. Ser. No. 101*, (1965, publ. 1966), p. 16.
6 A. CARNICELLI, M. GRIECO, G. F. MENCHINI, P. SABA AND M. LUISI, *Boll. Soc. Ital. Biol. Sper.*, 43 (1967) 36.
7 P. M. REISERT AND D. SCHUMACHER, *Experientia*, 19 (1962) 84.
8 I. J. DREKTER, S. PEARSON, E. BARTOZAC AND T. H. MCGAVACK, *J. Clin. Endocrinol. Metab.*, 7 (1947) 795.

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