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Note

A solvent system for the separation of steroids with estrogenic and progestational activity by two-dimensional thin-layer chromatography

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Many applications of one-dimensional thin-layer chromatography (TLC) for the separation of hormonal steroids have been described[1]. Minor applications have been reported for the two-dimensional technique[2–7], which is particularly useful when the resolution is difficult or impossible with the former type of chromatography. In our studies concerning the separation of hormonal steroids belonging to the same

TABLE I

R_F VALUES OBTAINED BY TWO-DIMENSIONAL TLC WITH SOLVENTS I AND II

Colours after spraying with sulphuric acid and UV absorption.

Steroids	*R_F values*		*Colours after H_2SO_4 + heating*		*UV absorption (254 nm)**
	Solvent I	*Solvent II*	*Visible light*	*UV light (350 nm)*	
Progestogens					
Progesterone	0.40	0.39	Colourless	Colourless	+
17α-hydroxyprogesterone hexanoate	0.44	0.48	Blue-violet	Pink	+
Medroxyprogesterone acetate	0.36	0.35	Green-blue	Lemon yellow	+
Megestrol acetate	0.36	0.34	Yellow-green	Lemon yellow	+
Ethinyltestosterone	0.24	0.42	Violet	Pink	+
Norethindrone	0.23	0.37	Violet	Deep pink	+
Norethindrone acetate	0.40	0.45	Violet	Pink	+w
Norethynodrel	0.30	0.59	Weak violet	Weak pink	+
Lynestrenol	0.43	0.76	Weak brown	Violet	—
Ethynodiol diacetate	0.57	0.77	Weak brown	Violet	—
Estrogens					
Estradiol	0.15	0.47	Orange-yellow	Lemon yellow	+v.w.
Estradiol 3-benzoate	0.28	0.51	Pink-orange	Weak yellow	+
Estradiol 17β-valerate	0.31	0.76	Yellow	Weak green-yellow	+v.w.
Estradiol cyclopentylpropionate	0.33	0.76	Yellow	Weak green-yellow	+v.w.
Estradiol dipropionate	0.63	0.83	Yellow-orange	Green-yellow	+v.w.
Ethinylestradiol	0.17	0.63	Red-violet	Yellow-orange	+v.w.
Mestranol	0.35	0.71	Red-violet	Yellow-orange	+v.w.
Estrone	0.24	0.62	Yellow	Green-yellow	+v.w.

* w. = weak; v.w. = very weak.

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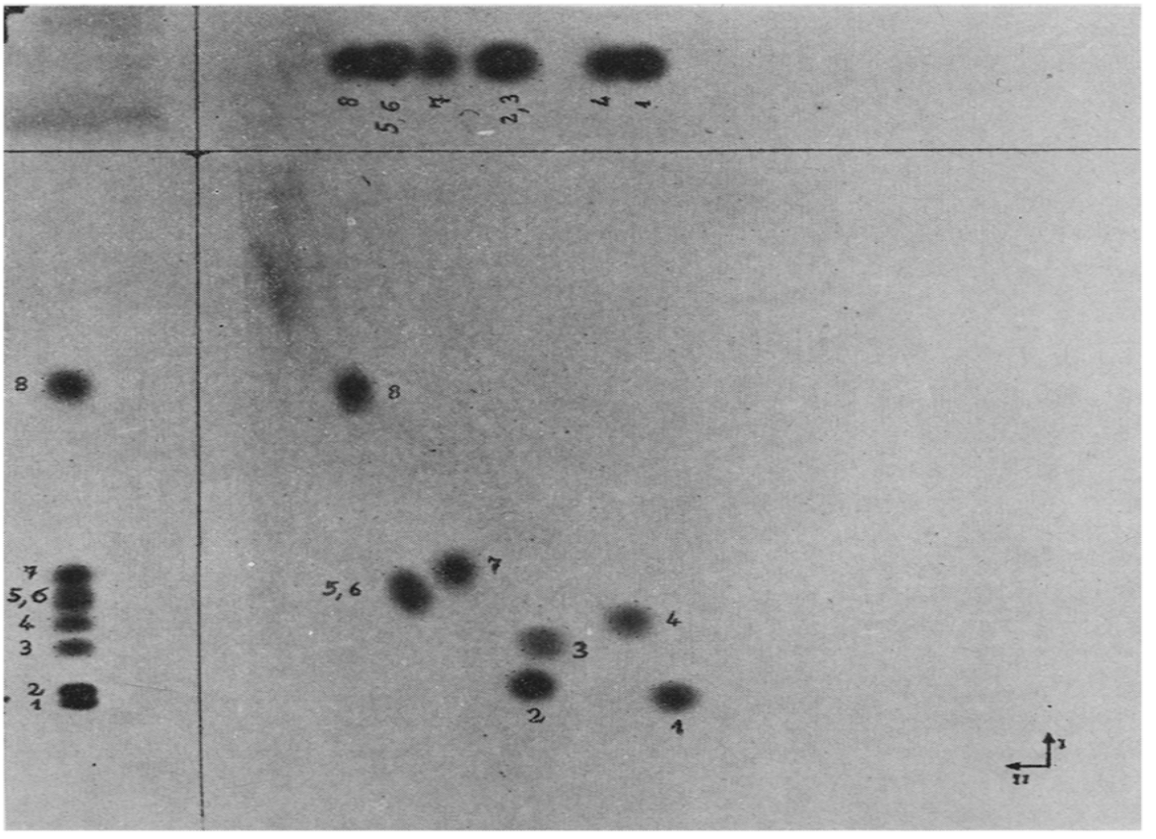

Fig. 1. Two-dimensional TLC of estrogens with solvents I (toluene–95% ethanol, 90:10) and II (butyl acetate–light petroleum –acetic acid, 70:30:1). Detection: 50% sulphuric acid and heating. 1 = Estradiol; 2 = ethinylestradiol; 3 = estrone; 4 = estradiol 3-benzoate; 5 = estradiol 17β-valerate; 6 = estradiol 17β-cyclopentylpropionate; 7 = mestranol; 8 = estradiol dipropionate.

class or different classes[8–11], we examined the usefulness of the solvent system for TLC which is proposed here. It is particularly suitable when the number of components to be analyzed is high. This system is very suitable for the separation and identification of the most important estrogens and progestogens which are frequently present in pharmaceutical preparations and are included in the principal Pharmacopoeias.

EXPERIMENTAL

Reagents

The solvents used were of analytical-reagent grade.

Solutions for spotting

The steroids were of analytical-reagent grade or for pharmaceutical use; their mixtures were prepared in chloroform–methanol (1:1) at a concentration of 0.1% for each compound.

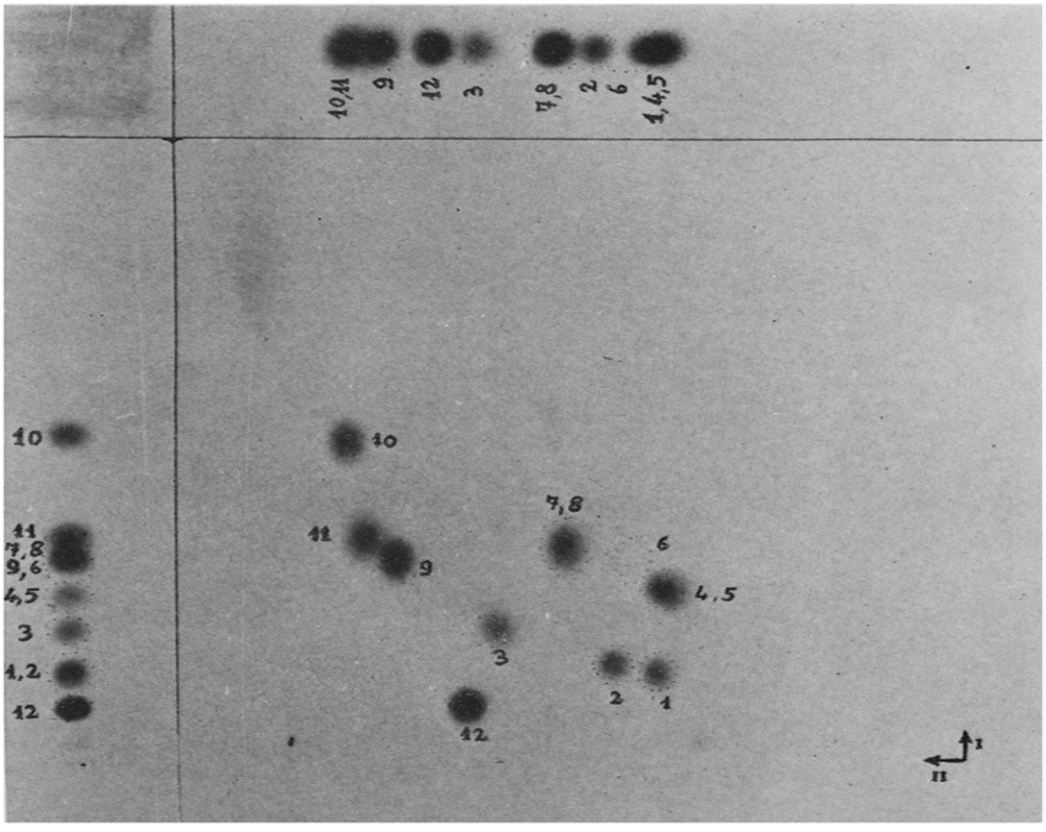

Fig. 2. Two-dimensional TLC of progestogens plus mestranol and ethinylestradiol with solvents I and II. Detection: 50% sulphuric acid and heating. 1 = Norethindrone; 2 = ethinyltestosterone; 3 = norethynodrel; 4 = medroxyprogesterone acetate; 5 = megestrol acetate; 6 = progesterone; 7 = norethindrone acetate; 8 = 17α-hydroxyprogesterone hexanoate; 9 = lynestrenol; 10 = ethynodiol diacetate; 11 = mestranol; 12 = ethinylestradiol.

Thin-layer chromatography

Pre-coated silica gel 60 F_{254} plates (20 × 20 cm, 0.25 mm thickness; Merck, Darmstadt, G.F.R.) were used as purchased. The solvent systems used were: (I) toluene–95% ethanol (90:10), (II) butyl acetate–light petroleum (b.p. 40–70°)–acetic acid (70:30:1). Each solvent was placed in a chromatographic chamber lined with filter-paper. The chamber was saturated with solvent vapour for 2 h prior to use. The ambient temperature was about 20°. For sample application, 5 μl of solution, corresponding to 5 μg of each steroid, were applied to the plate in the right-hand corner, by means of a Hamilton micro-syringe, at a point 2-cm distant from the right-hand edge and at the same distance from the bottom edge. On the same plate, in order to verify the movement of steroids in the two solvents, the same sample was spotted on two lanes, each 3 cm wide, tracing the first parallel to the left-hand edge and the other parallel to the upper edge of the plate. After the first run with solvent I, when the solvent front had arrived at the transverse trace, the plate was removed and dried in air for 20 min in the dark. The second run was then performed with solvent II, after rotating

the plate through 90° in a clockwise direction, until the solvent front arrived at the second transverse trace. The plate was examined under UV light (254 nm), sprayed with sulphuric acid–water (1:1) and placed in an oven at 110° until the characteristic colours appeared (about 10 min). The colours were examined under visible and UV light (350 nm).

RESULTS AND DISCUSSION

The R_F values in the two solvents and the characteristic colours for the compounds studied are reported in Table I. The chromatograms of the separations obtained with mixtures of the estrogens and of progestogens are shown in Figs. 1 and 2, respectively. In the progestogen mixture, ethinylestradiol and mestranol, which are the two estrogens more commonly used with progestogens in contraceptive formulations, were also added (Fig. 2). The selected solvents were obtained by testing different mixtures, considering the rule of isoeluotropic solvents of Neher[1].

Of the eight estrogens examined, only two esters of estradiol, the valerate and the cyclopentylpropionate, were not resolved.

Of the progestogens, two pairs were not separated: norethindrone acetate–17α-hydroxyprogesterone hexanoate and medroxyprogesterone acetate–megestrol acetate. In the other cases, the separations were good and reproducible and identification was easy and clear.

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