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The separation of C_{19}-16-unsaturated steroids from C_{21} and other C_{19}-steroids by two-dimensional thin-layer chromatography

Two-dimensional thin-layer chromatography (TLC) has been successfully applied in the purification of testosterone semi-carbazone from plasma extracts[1] and in the separation of groups of steroids of similar polarity[2]. In our work on the biosynthesis of the very non-polar C_{19}-16-unsaturated steroids from pregnenolone and progesterone, it has become increasingly important to study this group of compounds in relation to the rather more polar metabolites, such as testosterone, also formed in testicular incubations. Using the system benzene–ether (9:1) and running twice to separate the 16-unsaturated steroids[3] other metabolites were inadequately separated. These have hitherto been eluted "en bloc" and re-run on another TLC plate using a system such as benzene–acetone (4:1)[4]. In order to save time it was therefore necessary to find a method of separating some sixteen compounds, formed from pregnenolone or progesterone in testis incubations, with polarities ranging from very non-polar (*e.g.* 4,16-androstadien-3-one) to more polar (*e.g.* 17α-hydroxypregnenolone). It seemed worthwhile to investigate whether two-dimensional TLC could be utilised to achieve such a separation on one plate.

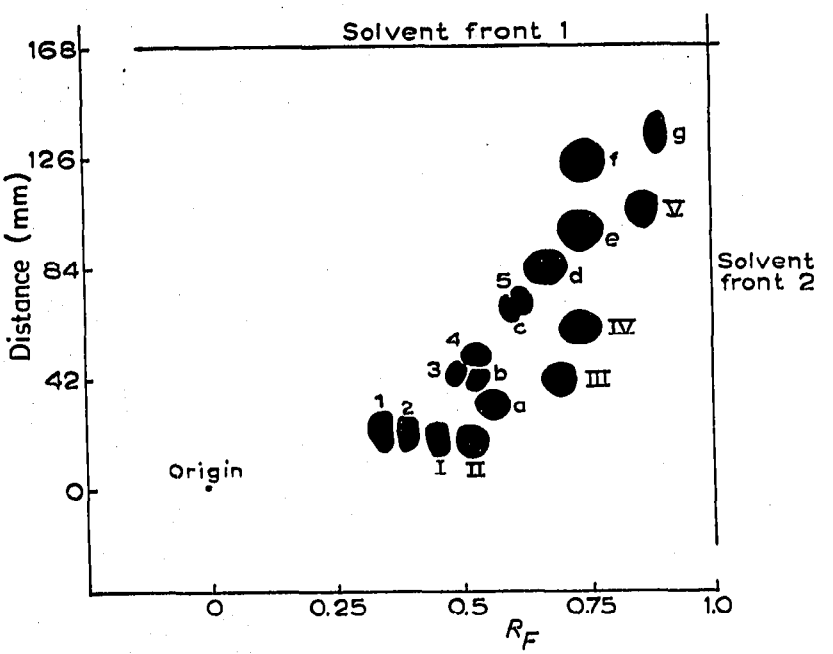

Fig. 1. Two-dimensional TLC of a mixture of steroids using benzene–ether (9:1), run twice, as the first system and benzene–methanol (9:1) as the second. Δ^4-3-Oxosteroids: I = testosterone; II = 17α-hydroxyprogesterone; III = 4-androstenedione; IV = progesterone; V = 4,16-androstadien-3-one. Δ^5-3β-Hydroxysteroids: 1 = 17α-hydroxypregnenolone; 2 = 5-androstene-diol; 3 = dehydroepiandrosterone; 4 = pregnenolone; 5 = 5,16-androstadien-3β-ol. Other steroids: (a) androsterone; (b) aetiocholanolone; (c) 5α-androst-16-en-3β-ol; (d) 5β-androst-16-en-3α-ol; (e) 5α-androst-16-en-3α-ol; (f) 1,3,5(10),16-oestratetraen-3-ol; (g) 5α-androst-16-en-3-one. Steroids were located by heating with ALLEN[5] reagent and the intensity of spots improved by then spraying with iodine in light petroleum[3]. A copy of the plate was made using Kodagraph Document Copying paper C13 (Kodak Ltd., London) from which a photographic print was subsequently obtained.

TLC plates (20 × 20 cm) were spread with a layer (0.5 mm thick) of Kieselgel G (Merck, Darmstadt). In some experiments pre-coated plates (Anderman & Co. Ltd., London) were used. Authentic steroid mixtures or radioactive tissue extracts, dissolved in chloroform (25 μl), were spotted at one corner of the plate at a point 2 cm from each edge. Two washings of 25 μl of chloroform were likewise applied to the origin. When dry, the plate was placed in benzene–ether (9:1) and the solvent allowed to run up twice, the plate being dried between each run. Tanks were used with and without filter paper wicks on separate occasions but it was consistently found that, although the development without wicks took longer, a slightly better separation of steroids was achieved by this method. After drying, the plate was turned through 90° and developed once in the second solvent system; benzene–methanol (9:1) gave a better separation than benzene–acetone (4:1). Δ^4-3-Oxosteroids were detected in UV light (254 nm) and Δ^5-3β-hydroxysteroids by heating with ALLEN[5] reagent.

Fig. 1 shows a typical separation of sixteen steroids, many of which are likely to be encountered in incubations of testis tissue. Two curves can be drawn through the positions of the various steroids; one relating Δ^4-3-oxo steroids (I–V) and the other relating Δ^5-3β-hydroxysteroids (1–5). Moreover, radioactive metabolites have been successfully separated. Testis tissue from human testicular feminization cases and from normal rats has been incubated separately with [4-^{14}C]pregnenolone, proge-

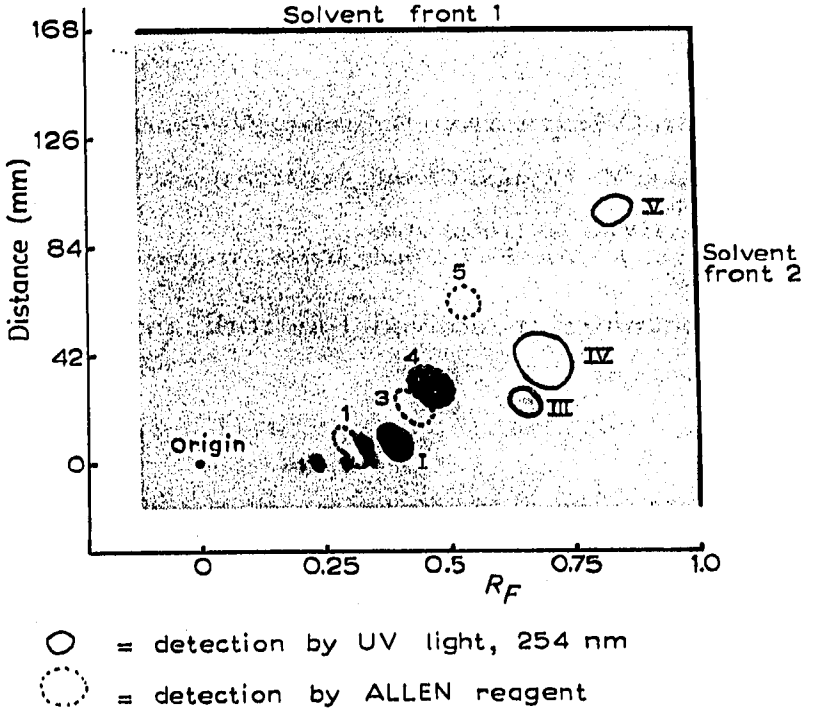

Fig. 2. Radioautograph of a two-dimensional thin-layer chromatogram of ^{14}C-steroids from an incubation of rat testis homogenate with [4-^{14}C]pregnenolone. Carrier steroids were added to the homogenate before extraction and were located after chromatography with UV light, 254 nm, or with ALLEN[5] reagent. Δ^4-3-Oxosteroids: I = testosterone; III = 4-androstenedione; IV = progesterone; V = 4,16-androstadien-3-one; Δ^5-3β-hydroxysteroids: 1 = 5-androstenediol; 3 = dehydroepiandrosterone; 4 = pregnenolone; 5 = 5,16-androstadien-3β-ol. Solvent systems were as in Fig. 1.

sterone and testosterone. After the addition of carrier steroids, extraction was performed with ethyl acetate, the extract applied to a plate and two-dimensional TLC carried out as described above. Radioautography (Fig. 2), usually for 42 h, revealed the location of radioactive metabolites and carrier Δ^4-3-oxosteroids were readily seen with UV light (254 nm). Radioactive Δ^5-3β-hydroxy steroids, such as pregnenolone and dehydroepiandrosterone, were located on the inner curve and tentatively identified by comparison with the separation of a mixture of authentic steroids, performed in the same tank and under identical conditions as the radioactive steroid separation. The final positions of these compounds were found to be reproduced reasonably well on different plates. Further identification of metabolites was achieved using one-dimensional TLC in a number of solvent systems.

Thus, by this method, a separation of very non-polar 16-unsaturated steroids from other C_{19}- and C_{21}-steroids can be quickly achieved on one plate. It is hoped that a similar technique will be developed to separate 16-unsaturated steroids and corticosteroids so that the biosynthesis of the former group of compounds may be investigated in adrenocortical preparations.

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