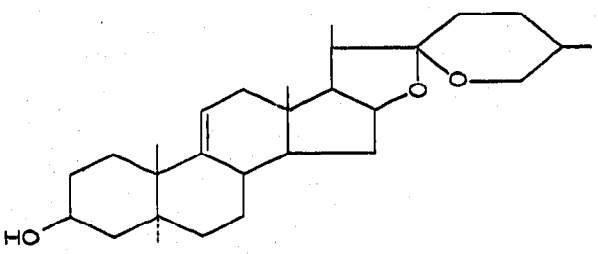
CHROM. 3570

Thin layer chromatography of synthetic steroids on silica gel impregnated with silver nitrate

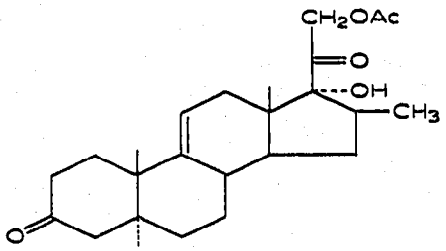
Thin layer chromatography (TLC) on silica gel impregnated with silver nitrate was first used by BARRETT *et al.*[1] and by MORRIS[2] to distinguish between saturated and unsaturated compounds. Subsequent papers on the application of this technique to steroids have been predominantly concerned with the separation of sterols, either free[4–8] or as their acetates[4,8,9]. The adsorbent generally employed is silica gel, with or without plaster of Paris. There appears to be no advantage in using neutral alumina[7], and impregnated alumina plates rapidly darken on exposure to light. Silver nitrate complexing has also been used to isolate ethynyl steroids from nonsteroidal materials in biological fluids[3].

An investigation into the synthesis of corticosteroids from sapogenins produced a number of intermediates containing a 9(11)-double bond in ring C. These were contaminated with the corresponding ring C-saturated analogue, and it was necessary to estimate the amount of saturated material present. Silica gel impregnated with silver nitrate proved an effective means of separation and enabled impurities to be estimated by visual comparison with standards of known concentration.

The starting point in the investigation was $\Delta^{9(11)}$-tigogenin(I). This was converted to ring A-saturated corticosteroids such as 21-acetoxy-17α-hydroxy-16β-methyl-5α-pregn-9(11)-ene-3,20-dione(II), without any reaction on the 9(11)-bond.



(I)



(II)

At each step in the conversion the C-saturated and 9(11)-derivatives could be separated only by the silver nitrate technique. Two other groups of unsaturated derivatives were available for comparison at certain stages of the synthesis. Δ^5-compounds were synthesised from diosgenin (Δ^5-tigogenin) and a few Δ^{11}-compounds were produced from Δ^{11}-tigogenin, which was obtained by the treatment of hecogenin 12-tosylhydrazone with sodium methoxide in dimethylformamide.

None of these groups of derivatives could be separated from one another on ordinary silica gel, but the majority were separated by complexing the unsaturated compounds with silver nitrate (Tables I and II). The unsaturated complexes move more slowly than the saturated material. The Δ^{11}-derivatives were easiest to separate from the C-saturated compounds and the Δ^5-derivatives the most difficult *i.e.* the order of R_F values is C-saturated $> \Delta^5 > \Delta^9 > \Delta^{11}$.

Two of the intermediates had a double bond elsewhere, at the C_{16} position as

TABLE I

R_F VALUES ON NORMAL SILICA GEL

Steroid	*Ring C saturated*	*Unsaturated derivatives* Δ^9	Δ^5	Δ^{11}	*System**
Sapogenins					
(1) 3β-Hydroxy-5α,20α,25*R*-spirostane (Tigogenin)	0.40	0.43	0.43	0.44	C_9EA_1
3-Acetates					
(2) 3β-Acetoxy-5α-pregn-16-en-20-one	0.39	0.35	0.37	0.36	C_4T_1
(3) 3β-Acetoxy-16-methyl-5α-pregn-16-en-20-one	0.35	0.36	0.36	—	C_4T_1
(4) 3β-Acetoxy-16ξ-(5-acetoxy-4-methyl-valeroxy)-5α-pregnan-20-one	0.24	0.22	—	0.23	C_4T_1
(5) 3β,26-Diacetoxy-5α-furost-20(22)-ene	0.48	0.46	—	—	C_4T_1
3-Alcohols					
(6) 3β,17α-Dihydroxy-16-methylene-5α-pregnan-20-one	0.44	0.41(e)**	0.48	—	C_1EA_1
(7) 3β-Hydroxy-16,17-oxido-16β-methyl-5α-pregnan-20-one	0.39	0.38	0.39	—	C_9EA_1
(8) 21-Bromo-3β,17α-dihydroxy-16-methylene-5α-pregnan-20-one	0.50	0.50	—	—	C_1EA_1
Corticosteroids					
(9) 21-Acetoxy-3β,17α-dihydroxy-16β-methyl-5α-pregnan-20-one	0.45	0.46	—	—	C_1EA_1
(10) 21-Acetoxy-3β,17α-dihydroxy-16-methylene 5α-pregnan-20-one	0.44	0.44	—	—	C_1EA_1
(11) 21-Acetoxy-17α-hydroxy-16β-methyl-5α-pregnane-3,20-dione	0.43	0.48	—	—	C_9EA_1
Related compounds					
(12) 3β-Acetoxy-5α-pregnan-20-one	0.44	0.39	0.42	—	C_4T_1
(13) 3β-Acetoxy-16β-methyl-5α-pregnan-20-one	0.40	0.42	0.42	—	C_4T_1

* See *Experimental*.
** (e) = Elongated spot.

part of the ring D system. In addition to running the Δ^5-, Δ^9-, and Δ^{11}-diene derivatives these two intermediates (Tables I and II, entries 2 and 3) were compared with the fully saturated and with the Δ^5- and Δ^9-monoene derivatives (Tables I and II, entries 12 and 13).

During the past three years, several other kinds of steroids have been examined on silver nitrate impregnated silica gel. Examples of these classes of steroids are included in Tables III and IV. As a general guide we have found that when there is a 3-ketone present in ring A, it is frequently possible to separate saturated compounds from conjugated monoenes and dienes without reverting to the use of silver nitrate complexing. There is thus no necessity to use silver nitrate to separate the progesterones listed, and no advantage for the sterols. The androgens, containing a 3-hydroxy group, are separated however, and the corticosteroids are completely separated whereas only a partial separation is achieved on normal silica gel.

There are some limitations on the type of location reagent that can be used when silver nitrate is present. Iodine is not satisfactory[10] nor can tetrazolium salts be

used to locate sterols[6] or reducing corticosteroids. Sterol workers have produced on silica gel spots that fluoresced in ultra violet light, either by spraying with dibromofluorescein[8] or concentrated sulphuric acid[6], or by incorporating Rhodamine 6G in the plates[4]. Sterols have also been located by charring the spots, with 50 % sulphuric acid[5] on silica gel or with phosphoric acid[7] on alumina.

We have employed methanolic zinc chloride[11] as location reagent throughout, to obtain spots fluorescing under ultra violet light at 366 mμ. The colours produced are less vivid than those seen on untreated silica gel, but remain useful for distinguishing between close-running compounds. When silver nitrate is present, zinc chloride has proved at least as sensitive as sulphuric acid on silica gel. One disadvantage is that the sprayed plates darken on exposure to visible or ultra violet light, so that it is desirable to cover them with a cloth when they are not being examined.

When heating sprayed plates, we have found it necessary to use a well vented oven, preferably equipped with an extractor fan. If the fumes produced are not

TABLE II

R_F VALUES ON SILVER NITRATE SILICA GEL

Steroid	*Ring C saturated*	*Unsaturated derivatives* Δ^0	Δ^6	Δ^{11}	*System**
Sapogenins					
(1) 3β-Hydroxy-5α,20α,25*R*-spirostane (Tigogenin)	0.44	0.21	0.32	0.17	C_9EA_1
3-Acetates					
(2) 3β-Acetoxy-5α-pregn-16-en-20-one	0.58	0.43	0.57	0.30	C_4T_1
(3) 3β-Acetoxy-16-methyl-5α-pregn-16-en-20-one	0.55	0.44	0.55	—	C_4T_1
(4) 3β-Acetoxy-16ξ-(5-acetoxy-4-methyl-valeroxy)-5α-pregnan-20-one	0.46	0.32	—	0.26	C_4T_1
(5) 3β,26-Diacetoxy-5α-furost-20(22)-ene	0.67	0.54	—	—	C_4T_1
3-Alcohols					
(6) 3β,17α-Dihydroxy-16-methylene-5α-pregnan-20-one	0.26	0.16	0.18	—	C_1EA_1
(7) 3β-Hydroxy-16,17-oxido-16β-methyl-5α-pregnan-20-one	0.35	0.21	0.29	—	C_9EA_1
(8) 21-Bromo-3β,17α-dihydroxy-16-methylene-5α-pregnan-20-one	0.39	0.28	—	—	C_1EA_1
Corticosteroids					
(9) 21-Acetoxy-3β,17α-dihydroxy-16β-methyl-5α-pregnan-20-one	0.38	0.22	—	—	C_1EA_1
(10) 21-Acetoxy-3β,17α-dihydroxy-16-methylene-5α-pregnan-20-one	0.33	0.21	—	—	C_1EA_1
(11) 21-Acetoxy-17α-hydroxy-16β-methyl-5α-pregnane-3,20-dione	0.38	0.22	—	—	C_9EA_1
Related compounds					
(12) 3β-Acetoxy-5α-pregnan-20-one	0.54	0.43	—	—	C_4T_1
(13) 3β-Acetoxy-16β-methyl-5α-pregnan-20-one	0.58	0.44	0.56	—	C_4T_1

* See *Experimental*

TABLE III

R_F VALUES ON NORMAL SILICA GEL

Class	*Parent compound*	R_F	*Derivatives*				*System**
			Δ^1	Δ^4	Δ^5	$\Delta^{1,4}$	
Androgens	3β-Hydroxy-5α-androstan-17-one (Epiandrosterone)	0.33	—	—	0.33	—	C_9EA_1
Progesterones	5α-Pregnane-3,20-dione	0.57	—	0.48	—	—	C_9EA_1
Sterols	5α-Cholestan-3-one	0.51	0.47	0.37	0.55	—	C_1T_1
Corticosteroids	11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione (Dihydrocortisol)	0.45	0.43	0.38	—	0.35	MA_4MC_1

* See *Experimental.*

TABLE IV

R_F VALUES ON SILVER NITRATE SILICA GEL

Class	*Parent compound*	R_F	*Derivatives*				*System**
			Δ^1	Δ^4	Δ^5	$\Delta^{1,4}$	
Androgens	Epiandrosterone	0.32	—	—	0.26	—	C_9EA_1
Progesterones	Pregnanedione	0.68	—	0.56	—	—	C_9EA_1
Sterols	Cholestanone	0.59	0.52	0.40	0.58	—	C_1T_1
Corticosteroids	Dihydrocortisol	0.44	0.39	0.32	—	0.26	MA_4MC_1

* See *Experimental.*

removed there is a decrease in sensitivity of detection, at least with zinc chloride sprayed plates.

A further advantage of zinc chloride on ordinary silica gel layers is that after location with this reagent the plates can be oversprayed with sulphuric acid to produce different colours and occasionally to locate additional spots.

Sterol workers used silver nitrate in aqueous solution of 12.5 % w/v[6] and 30 % w/v[7], which was slurried with the silica gel during preparation of the plates. Alternatively, plates were prepared with the usual aqueous slurry and silver nitrate solution, 5 %[5] or saturated[4], then sprayed on the dried plates.

Spraying gives the advantage that only part of the plates need be treated so that separations can be compared directly on the sprayed and unsprayed sections. It is however less likely to produce a uniformly impregnated plate. MORRIS has reported[12] that for a series of simple lipids there is no improvement in separations between the minimum level of 2 % w/w and a level of 30 % w/w of silver nitrate (*i.e.* 1 % to 15 % w/v) in Silica Gel G.

We found that the incorporation of about 10 % w/v silver nitrate when preparing the plates was satisfactory, and did not investigate the effect of varying this amount. Because of the corrosive attack of silver nitrate on the Desaga chromium-plated brass applicator that was originally used to prepare TLC plates, we changed to an anodised aluminium applicator which has proved satisfactory.

The solvents used to obtain sterol separations were mainly two component systems such as chloroform-acetone (19:1)[6,7] or benzene–ethyl acetate (5:1)[4], although single solvents such as chloroform[5] were also employed. The advantage of a single solvent system is that the composition does not change over a period of time. In practice, however, this is frequently outweighed by the higher resolution that is obtainable with a two component system. We used *n*-butyl acetate for our initial studies but now prefer chloroform–ethyl acetate (1:1 or 9:1) for sapogenins and synthetic 3-alcohols, and chloroform–toluene (4:1) for 3-acetates.

R_F values have been quoted in this report since they are the most convenient method of denoting the relative running position of the steroids. In particular they indicate clearly whether or not a separation is achieved, which usually requires a difference in R_F value of at least 0.05. However, no precautions were taken to control humidity, for example, so that the R_F values should be regarded as indications of the relative running positions only. We have found that by using lined tanks enclosed in polythene bags and by storing plates in containers (but *not* desiccators) R_F values with fresh solvent systems are reasonably reproducible. It is important that plates should be reactivated if they have been prepared before the day on which they are used.

Experimental

Preparation of plates. 40 g of Silica Gel G (Macherey, Nagel) were slurried with 75 ml of water containing 8 g of silver nitrate (B.D.H.). The suspension was used to coat five 20 cm × 20 cm plates (or their equivalent) using a Quickfit and Quartz anodised aluminium applicator to give a nominal 0.25 mm layer. The plates were left 10 min in air and then activated by heating for 1 h at 120°. Stored plates were reactivated before use.

Steroids were applied as 1 % solutions in chloroform–methanol (1:1), using a 2λ micro pipette (Drummond microcap) to give a loading of 20 μg. Tanks were lined with Whatman 3 MM paper on the side facing the coated plate and No. 14 paper on the opposite side. They were also enclosed in polythene bags.

The plate was developed for 30 min and was then dried with a hair-drier and sprayed with 30 % methanolic zinc chloride solution. It was heated for 30 min at 130° in a well ventilated oven. On removal from the oven it was covered with a second plate, and protected from light with a cloth, until it was examined under U.V. light of 366 mμ wavelength.

Solvent systems:

C_1EA_1	chloroform–ethyl acetate (1:1)
C_9EA_1	chloroform–ethyl acetate (9:1)
C_4T_1	chloroform–toluene (4:1)
C_1T_1	chloroform–toluene (1:1)
MA_4MC_1	methyl acetate–methylene chloride (4:1)

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Thin-layer chromatographic separation of flavonoids in *Medicago* (Papilionaceae)

Biochemical systematics which has developed very rapidly within the last decade, has revealed the significance of flavonoids as favored taxonomic markers among many secondary constituents of plants. Significant correlations between flavonoid distribution patterns and morphological features of various plant species, have been reported in literature[1] and therefore the present study on the distribution of flavonoids in different species of *Medicago* (Papilionaceae), was taken up. Although paper chromatography has been successfully used for the separation of flavonoids, thin layer chromatography (TLC), due to its greater sensitivity and shorter developing time, has recently been widely used in the separation of anthocyanins[2], flavonoids[3] and secondary phenolic compounds[4,5]. In the present communication, a simple, rapid and a very sensitive technique for separation of the flavonoids of *Medicago* species, by means of commercially produced Silica Gel and Cellulose Eastman Chromagram Sheets, is briefly discussed.

Materials and methods

Petals from fifty flowers of perennial and annual species of *Medicago*, weighing approximately 0.05 g, were dried at room temperature (70–80°F). The dried petals were ground in 1 ml petroleum ether (boiling range 37.8°–58.2°) and thus the carote-