THIN-LAYER CHROMATOGRAPHY OF STEROIDS ON STARCH-BOUND SILICA GEL CHROMATOPLATES

LELAND L. SMITH AND THEODORE FOELL
Wyeth Laboratories, Inc., Radnor, Pa. (U.S.A.)

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Early work associated with the thin-layer chromatographic technique was done on chromatoplates prepared using various starches as the binder for the adsorbent[1–4]. Little attention[5–7] has been given to this means of operation since the reports of STAHL[8,9], who employed gypsum as binder in his technique. To our knowledge only one instance has been reported where steroids have been chromatographed on starch-bound thin-layers[6]. We wish to report our experience of the past two years using thin-layer silica gel chromatoplates prepared with rice starch for the analysis of several classes of steroids.

The gypsum-bound thin-layer chromatoplates prepared according to STAHL[8] are fragile and do not withstand handling, transportation, storage, display, etc. without flaking. They cannot be marked with pencil satisfactorily[9]. These disadvantages offset the advantages of use of corrosive reagents and destructive methods of visualization. We find that for routine analysis the durable thin-layers obtained using starch as binder are the preparation of choice. Starch-bound thin-layers resist flaking, may be stored and transported freely, and can be marked with pencil both before and after chromatographic irrigation.

The methods for preparation of the thin-layers using rice starch (other starches may also be used) as binder are modifications of the procedures of REITSEMA[4], and commercially available spreading equipment is used. The technique is otherwise that of STAHL. The solvent systems useful with gypsum-bound plates are of use on starch-bound plates also. In our experience, with a given solvent system the mobility of a given steroid on starch-bound plates may be greater than, less than, or equal to its mobility on gypsum-bound plates (using "Kieselgel G" of E. Merck, Darmstadt). Relative mobility data for several steroids are presented in Table I.

The silica gel–water–rice starch mixture before spreading has a pH of 2.7, and the thin-layers so produced (Procedure A) have an acid reaction to indicator paper. These acid thin-layers have been acceptable for most routine purposes.

Thin-layer chromatography of sensitive steroids is not without artifact formation, however. Sensitive steroid 16β-esters are altered on alumina chromatoplates[10], and we have observed cases where acid-sensitive ethylene ketals have been hydrolyzed on the acidic rice starch-bound silica gel plates.

Neutralization of the silica gel–water–rice starch mixture to pH 6.4 (range 6.1–6.7) affords a neutral chromatoplate (Procedure B) with the durability and chromatographic properties of the acidic plates. The neutral thin-layers offer a more suitable means of analysis where sensitivity to acid is encountered. Thin-layers prepared with

TABLE I

RELATIVE MOBILITY DATA OF SEVERAL CLASSES OF STEROIDS

Steroid	R_F			
	Hexane–ethyl acetate (4 : 1)	*Hexane–ethyl acetate (1 : 1)*	*Ethyl acetate*	*Benzene–2-propanol (4 : 1)*
C_{18}-steroids				
Estrone	0.07	0.57	—	—
Estrone 3-acetate	0.06	0.55	—	—
Estrone 3-methyl ether	0.24	0.68	—	—
Estradiol-17α	0.03	0.42	—	—
Estradiol-17α 3-methyl ether	—	0.52	—	—
Estradiol-17β	0.02	0.37	—	—
Estradiol-17β 3-methyl ether	0.07	0.47	—	—
Ethinylestradiol-17β	0.04	0.56	—	—
Estriol	0.00	0.02	—	—
Equilin	0.04	0.50	—	—
Equilenin	0.04	0.47	—	—
C_{19}-steroids				
Testosterone	0.01	0.15	0.47	—
19-Nortestosterone	0.01	0.12	0.42	—
17α-Methyltestosterone	0.01	0.17	0.48	—
4,5α-Dihydrotestosterone	0.04	0.32	0.63	—
17α-Methyl-4,5α-dihydrotestosterone	0.03	0.32	0.63	—
17α-Ethinyltestosterone	0.03	0.28	0.70	—
4-Androstene-3,17-dione	0.02	0.20	0.55	—
5α-1-Androstene-3,17-dione	—	0.32	0.69	—
5α-Androstane-3,17-dione	—	0.42	0.72	—
1,4-Androstadiene-3,17-dione	0.00	0.14	0.44	—
Dehydroisoandrosterone	0.02	0.25	0.59	—
C_{21}-steroids				
Progesterone	0.05	0.36	—	—
3β-Hydroxy-5-pregnen-20-one	0.06	0.38	—	—
3β-Hydroxy-5,16-pregnadien-20-one	0.05	0.37	—	—
3β-Acetoxy-5,16-pregnadien-20-one	0.34	0.75	—	—
3β-Hydroxy-5α-16-pregnen-20-one	0.09	0.44	—	—
Cortisone	—	0.07	0.38	0.75
Cortisone 21-acetate	—	0.18	0.64	—
Hydrocortisone	—	0.06	0.38	0.55
Hydrocortisone 21-acetate	—	0.17	0.62	—
Prednisone	—	0.06	0.38	0.69
Prednisone 21-acetate	—	0.17	0.61	—
Prednisolone	—	0.05	0.35	0.46
Prednisolone 21-acetate	—	0.13	0.57	—
Cortexone	—	0.22	0.56	0.96
Cortexone 21-acetate	—	0.37	0.71	—
Reichstein's Substance S	—	0.15	0.51	—
11-*epi*-Hydrocortisone	—	0.02	0.20	0.33

(continued on p. 341)

TABLE I *(continued)*

Steroid	R_F			
	Hexane–ethyl acetate (4 : 1)	*Hexane–ethyl acetate (1 : 1)*	*Ethyl acetate*	*Benzene–2-propanol (4 : 1)*
C_{27}-steroids				
Diosgenin	0.18	0.67	—	—
Diosgenin 3-acetate	0.73	—	—	—
Tigogenin	0.18	0.67	—	—
Tigogenin 3-acetate	0.74	—	—	—
Smilagenin	0.26	0.72	—	—
Smilagenin 3-acetate	0.73	—	—	—
Hecogenin	0.02	0.31	0.58	—
Hecogenin 3-acetate	0.26	0.89	—	—
Gentrogenin 3-acetate	0.25	0.89	—	—
Sarsapogenin 3-acetate	0.68	—	—	—
Chlorogenin	0.00	0.04	0.18	—
Kryptogenin	0.00	0.22	0.25	—
Pennogenin	0.04	0.48	0.68	—
Tomatidine	0.00	0.02	0.03	—
Cholesterol	0.36	0.73	—	—
5α-Cholestan-3-one	0.77	0.91	—	—

buffers or at high pH did not have the durability properties of favor, and we have not studied these preparations further. STAHL has reported on such thin-layer chromatoplates, however[11,12].

Detection of steroids on the rice starch-bound plates is accomplished by the same means as would normally be used on paper chromatograms or on gypsum-bound thin-layers. Although the starch-bound plates contain organic matter and thus cannot be subjected to the extremes of heat and chemical exposure possible for gypsum-bound plates, we have encountered relatively few interferences from the rice starch. The greater durability of the plates in day-to-day operations more than accommodates for these limitations.

We have applied many visualization procedures successfully without modification. Thus antimony trichloride, phosphoric acid, trichloracetic acid, 2,4-dinitrophenylhydrazine, the Zimmermann reagent, etc. work well, as does isonicotinic acid hydrazide for detection of Δ^4-3-ketones and $\Delta^{1,4}$-3-ketones[13]. Quenching of ultraviolet light fluorescence permits ready detection of Δ^4-3-ketones, $\Delta^{1,4}$-3-ketones, and other unsaturated steroids. The soda fluorescence procedure of BUSH[14] specific for Δ^4-3-ketones cannot be applied.

Phosphomolybdic acid (10% in ethanol)[15] is the most widely useful visualization technique at our disposal. Whereas phosphomolybdic acid frequently does not give good sensitivity on paper chromatograms and severe background coloration occurs, on the rice starch-bound thin-layer chromatograms (Procedure A) excellent sensitivity and contrast has been achieved with a number of different unsaturated steroids. By heating the sprayed chromatoplate until the solvent front appears as an intense blue line (usually not more than ten minutes at 100°) the steroid zones appear as well

contrasted blue spots against a lemon yellow background which does not deteriorate even after several days.

Sensitivity measurements indicate that less than 0.06 μg of estrone, 0.025 μg of 3β-hydroxy-5-pregnen-20-one, 0.125 μg of progesterone or testosterone can be detected on chromatographically irrigated plates (prepared by Procedure A). The sensitivity of phosphomolybdic acid for progesterone on chromatoplates of different composition is: Procedure A, 0.125 μg; Procedure B, 0.25 μg; Silica gel G according to STAHL, 1 μg.

The sensitivity of the phosphomolybdic acid reagent is definitely a function of acidity, and on neutral plates (Procedure B) the sensitivity is less. A modified reagent incorporating hydrochloric acid must be used in order to obtain comparable sensitivities. Very little color formation occurs on alkaline plates. Neutral or acid plates previously visualized with phosphomolybdic acid lose the lemon yellow background color on spraying with 10% ethanolic alkali, and blue spots on a colorless background result.

With many phenolic steroids, their methyl ethers and acetate esters, phosphomolybdic acid gives a red color which turns blue on heating. This intermediate red color appears immediately after spraying for 1,3,5(10),16-estratetraen-3-ol methyl ether and for certain other unsaturated phenolic steroids. The phenolic steroids may also be detected on thin-layers with the Turnbull blue reagent (1% Fe Cl_3–1% $K_3Fe(CN)_6$)[16].

Reducing steroids cannot be detected as such on rice starch-bound plates using alkaline tetrazolium salts or with alkaline silver nitrate for the background coloration is too intense. Tetrazolium salts can be used for this purpose on gypsum-bound plates, however[17], and where such reducing steroids must be detected, the gypsum-bound plates must be used.

Concentrated sulfuric acid can be used on the rice starch plates, this finding being reported quite early in the use of such thin-layers for chromatography[2,18]. We have detected 5α-cholestan-3-one (which does not respond to phosphomolybdic acid) as a rose violet coloration using concentrated sulfuric acid spread over the irrigated chromatoplate. Deterioration will take place in a few hours. The Liebermann-Burchard reagent (acetic anhydride–concentrated sulfuric acid, 4:1) can be used satisfactorily for unsaturated sterols on the rice starch-bound chromatoplates.

Reproducible mobility data of the same quality as obtained on gypsum-bound thin-layers can be obtained with the starch-bound plates. Relative mobility of steroids in the several solvent systems studied is influenced by the amount of steroid applied to the plate. For routine analysis a 1–5 μg sample is used. For 10 μg samples the resolved spots are still only about 1.2–1.5 cm^2 in size. Heavily loaded plates (25–50 μg) can be run; however, comparison of R_F values on such analyses with R_F data obtained with less sample is not appropriate.

Preparative work can be done with the rice starch-bound chromatoplates without interference from the starch. A 10 mg sample of testosterone was applied to an acid plate, run, located under ultraviolet light, and eluted with methanol, yielding 8.8 mg of crystalline testosterone identified by infrared spectra.

EXPERIMENTAL

The apparatus available commercially from C. Desaga GmbH, Heidelberg, was used throughout. The 20 × 20 cm plates were prepared five at a time, with a 275 μm thin-layer of the silica gel preparation.

Silica gel preparation

Procedure A. Thirty grams of finely divided silica gel (Fisher No. S-158, No. 1 impalpable powder) and 1.5 g of powdered rice starch (Matheson, Coleman, and Bell) were placed in a 250 ml erlenmeyer flask and 50 ml of water was added. The mixture was stirred gently to wet all the material, then heated on a steam bath for twenty minutes, until the preparation thickened. An additional amount of water was then added (usually 20 ml but possibly as little as 15 ml depending on the batch of silica gel), the mixture was stirred well to break up any lumps, and the preparation was again heated on a steam bath for twenty minutes. The mixture was cooled to room temperature on the desk, mixed well, and spread with the Desaga apparatus. The amounts used are sufficient to fill the apparatus and will coat five 20 × 20 cm glass plates.

Procedure B. The exact same proportions of silica gel, water, and rice starch were used, except that the 50 ml of water initially added is composed of 34 ml of water plus 16 ml of 0.1 *N* sodium hydroxide solution. The procedure is otherwise the same as Procedure A.

The thin-layers are made by spreading either preparation with a uniform motion. After air drying in place for 10–20 min (loss of appearance of moisture) the plates are stacked in a metal frame and dried in an oven at 100° for three hours. Whereas the plates are ready for use after drying for as little as two hours, they are conveniently left in the oven at 100° until just prior to use, at which time they are removed, cooled, and spotted with the samples in the usual way. Samples of 1 mg/ml concentration in methanol are applied with suitable microliter pipettes on the start line, etc., and developed with the selected solvent. Desaga rectangular glass chambers, 10 × 22 × 21 cm, were used, with a filter paper liner to assure saturation of the chamber with the solvent. Solvent rise of about 15 cm in 60 min is usual with the solvent systems described.

Detection

After air drying for a few minutes the chromatoplates (Procedure A) were sprayed in a horizontal position with a 10% solution of reagent phosphomolybdic acid in 95% ethanol. The sprayed plate was dried with a hand-held electric hot air drier until the odor of ethanol was lost, then dried at 100° in an electric oven until the solvent front appeared as a clearly visible blue line or zone (not over ten minutes). Overheating past this point will darken the lemon yellow background and faint spots will not be observed. After cooling the blue spots are outlined in pencil and their positions recorded with the Haloid Xerox 914 copy machine[19].

For neutral chromatoplates prepared according to Procedure B, 4 ml of concentrated hydrochloric acid was added to each 100 ml of the 10% phosphomolybdic acid reagent solution.

SUMMARY

A thin-layer chromatographic procedure for steroids is described wherein the thin-layer is prepared from silica gel with rice starch as binder.

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