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The use of silica gel modified with ammonium bisulfate in thin-layer chromatography*, **

Direct spectrodensitometry of substances separated by thin-layer chromatography (TLC) has proven to be an attractive means for quantitation[1]. However, there are some limitations to the methodology since spraying techniques for visualization are generally not reproducible and the background is not always uniform. It would be of advantage if the detection reagent could be incorporated into the adsorbent matrix. We have previously described the use of silica gel impregnated with phosphomolybdic acid for TLC[2].

The use of silica gel containing ethanolic ammonium bisulfate in TLC of a number of steroids and lipids is described herein. Different visible colors and fluorescence were obtained when the plates were heated following development of the chromatogram. The method should have wide applicability since sulfuric acid reacts with many compounds on heating.

Materials and methods

The glass plates coated with Silica Gel G containing no organic binder (250 μ thick) were dipped into a saturated solution of ammonium bisulfate in absolute ethanol. This solution was prepared by adding excess salt to the ethanol and heating it in a hot water bath (70°) for 1 h to form a saturated solution. This is allowed to stand one day before preparing the plates. If fresh solutions are used the reactions on the plates are weak and not complete. Crystalline ammonium bisulfate should always be present in the bottom of the storage bottle. The solution is poured off the crystals for plate preparation. After use the excess solution is filtered and returned to the storage bottle for use in later plate preparation.

The plates are allowed to lie flat, gel side up, in the ethanolic solution poured into a shallow tray. After 30 sec, the plates are removed and allowed to dry at room temperature.

For quantitative work the plates were scored into lanes 10 mm wide to fit the beam of the Schoeffel Model 3000 spectrodensitometer. The steroids used were dissolved in acetone to give 0.1 mg/ml concentrations. These solutions were applied on the plates to give a streak across the lane rather than a spot in the middle of the lane. After chromatography using the appropriate solvent systems (for most steroids, benzene–ethanol (85:15)) the plates are allowed to dry at room temperature to permit evaporation of the solvent.

For steroids the plates are then heated in an oven at 140° for 15 min. For the fatty acids a temperature of 200° was necessary. Since the exploratory experiments described in this report concern only the listed steroids and lipids, for other substances different developing solvents and different heating conditions may be required.

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Results and discussion

Table I lists the spectral characteristics of the steroids and lipids so far studied. The C_{21} compounds appear to have similar absorption and maximal activation near 365 nm. In contrast the estrogens showed maximal activation near 340 nm. The lipids showed a brown color and little fluorescence after heating. No color was produced by heating the lipids at 140°; the higher temperature was required. The background has been consistently low.

TABLE I

SPECTRAL PROPERTIES OF STEROIDS AND LIPIDS SEPARATED BY NH_4HSO_4 TLC

Compound	*Absorption (nm)*	*Emission (nm)*	*Visible color*	*Visbile fluorescence[a] under 360 nm lamp*
Cortisol	365	490	tan	green
Corticosterone	365	495	tan	green
Desoxycorticosterone	365	585	purple	red
17α,21-Dihydroxy-pregn-4-en-3, 20-dione	365	495	red	red
Testosterone	365	495	tan	orange
Estriol	340	520	purple	lavender
Estradiol-17β	370	530	pink	yellow
Estrone	340	510	grey	yellow
3β-Hydroxy-5α-pregnane-20-one	365	485	grey	green
β-Cortol	370	450	grey	blue
Cholesterol	380	490	grey	yellow
Squalene	340–360	—[b]	brown	—[b]
Oleic acid	340–360	—	brown	—
Methyl nervonate	340–360	—	brown	—
Cholesteryl oleate	340–360	—	brown	—
Diolein	340–360	—	brown	—

[a] Viewed under the UV lamp in a dark room.
[b] Weak fluorescence was seen.

Preliminary experiments indicate that some materials separated with the modified silica gel can be recovered. If elution is carried out before heating 89 % of [6,7-^{3}H] 16-epiestriol was obtained. Elution of testosterone and cortisol by shaking the silica gel with ethanol followed by centrifugation and aspiration of the solvent resulted in recoveries of 80 and 85 %, respectively. Thus the method should be useful for separation and recovery of compounds which do not absorb light provided they show a reaction in the plate. Heating of the section of the TLC containing the reference substance will serve to visualize the location of the substances separated. The reference lanes can be cut away from the plate.

The addition of ammonium bisulfate to the silica gel had little effect on the separation of the steroids with some exceptions. Table II shows how epimers of estriol were separated on the modified silica gel but not on the normal silica gel plate. The two 16,17-*cis* epimers of estriol separated well. The two *trans* epimers were not separated although they were separated from the *cis* epimers.

The steroids can be detected at the nanogram level by their fluorescence on the plates. For the lipids 0.1 μg is readily detected. Linear calibration curves are obtained after spectrodensitometry of colored zones.

To assess the reproducibility of color development, eight replicate spottings were made on one 20 × 20 cm TLC plate of silica gel containing ammonium bisulfate. Using an estradiol-17β solution, 0.5 μg was put in each of the zones. A standard deviation (S.D.) of 0.06 cm^2 was found in an average peak area of 5.09 cm^2 ($\sim$1.2 %) after scanning with a Schoeffel Model 3000 spectrodensitometer. Reproducibility of

TABLE II

SEPARATION OF EPIMERIC ESTRIOLS BY TLC

Estriol	R_F *values*	
	Plain Silica Gel G	*Silica gel* + NH_4HSO_4
16α, 17β	0.35	0.09
16α, 17β	0.47	0.44
16α, 17β	0.47	0.18
16β, 17α	0.35	0.09

the scanning was assessed by scanning the same spot fifteen times. This gave an S.D. of 0.027 cm for the peak heights having an average of 10.0 cm. The reproducibility from plate to plate was determined by densitometry of 0.3, 0.4, 0.5 and 0.6 μg spots on four different plates. The S.D. here was 0.33 cm^2 for the replicate 0.3 μg spots having an average area of 5.55 cm^2 · ($\sim$6 %). For the 0.6 μg spots with an average area of 9.37 cm^2 the S.D. was 0.35 cm^2 · ($\sim$3.7 %).

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