graph III gas flow detector as well as visually, and it was determined that none of the streaks exceeded 3/16 in. width, front to back.

Materials

The model and the reservoirs depicted were fabricated largely of 1/4 and 1/8 in. plexiglas. Because the reservoirs are of this material, only lower alcohols, methanol and ethanol and aqueous solutions have been successfully tested in them. Investigators desiring to use solvents reactive with plexiglas would have to fabricate the reservoirs from higher polymer plastics such as polypropylene, or from glass or stainless steel. In order to insure proper contact between the wicks and the TLC coating, the reservoirs and their track area must be perfectly parallel to that of the plate carrier frame. The wicks, also, must be of uniform height, shape and composition if the relative rates of application are to be similar between wicks.

It is most advantageous to use either a variable speed motor or a rheostat-controlled 2 r.p.m. motor, so as to permit fine adjustment of the period required for drying of the spots between applications. A more detailed discussion of these considerations is contained in ref. 1. A U.S. patent is pending on this device.

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Thin-layer chromatographic separation of steroids and their localization by diazo dyes

Steroid hormones generally occur in low concentrations in biological fluids and tissues as a result of which they present a difficult analytical problem. Several chromatographic purification techniques in combination with gas–liquid chromatography (GLC) have been described in the literature for the determination of these substances[1–7].

Recently we have developed a highly reproducible and sensitive GLC column, for the detection of steroids in the nanogram range[8], for which it was desirable to have a simple prepurification procedure. The present paper describes a suitable method using marker azo dyes for the specific localization of each class of steroids on thin-layer chromatograms. A similar technique has been described earlier[9].

Materials

Reference steroids were obtained from commercial sources (Steraloids, Rawling, N.Y.; Mann Research Laboratories, New York and Sigma Chemical Company, St. Louis, Mo.). Azo dyes were purchased from K and K Laboratories, Plainview, N.Y.; National Aniline Division, New York, N.Y. and Eastman Organic Chemicals, Rochester, N.Y. All solvents used in this study were ACS grade, marketed by Fisher Scientific Company, Pittsburgh, Pa. Adsorbosil 4 was purchased from Applied Science Laboratories, State College, Pa. Regisil (bistrimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane) was a generous gift from Regis Chemical Company, Chicago, Ill.

Methods

A mixture of 30 g of Adsorbosil 4 and 45 ml of water was shaken vigorously for 1 min, while applying partial vacuum created by a water pump. The slurry was immediately spread over five 20 × 20 cm plates at a thickness of 300 μ using a Shandon adjustable spreader Unoplan leveller (Shandon Scientific Company, Sewickley, Pa). The plates were left at room temperature for about 30 min before activation at 110° C for 2 h. These plates were stored in metal desiccating cabinets (Arthur Thomas and Company, Philadelphia, Pa). The plates were divided into three vertical lanes, 6 cm wide terminating at a line drawn 15 cm from the origin to indicate the distance the solvent had to travel. Samples were spotted on a horizontal line with a 50 μl semiautomatic microsyringe (Hamilton, Whittier, Calif.) and developed in Brinkman tanks at an ambient temperature of 75 $\pm$3°F. The chambers were previously saturated for 1 h with 200 ml of the developing solvent system benzene–methanol (175:25). The inside walls of the jar were lined by fiberglas paper (Reeves Angel, Clifton, N.J.) 20 cm high, the lower end dipping in the solvent.

The plates are then sprayed with 5% phosphomolybdic acid in ethanol and heated at 200°C for 3 min. The migration of each of the steroids were calculated relative to that of Sudan III and expressed as R_{dye} values. The zones corresponding to steroids and dyes were eluted from the plates and allowed to react with 0.1 ml Regisil in 1.0 ml of acetonitrile at 100°C for 30 min in a screw capped tube. After evaporation, the silyl ethers were redissolved in acetonitrile and subjected to GLC on an SE-30–TMCBA (tetramethylcyclobutandiol adipate) column as described earlier[8].

Results

Table I summarizes the R_{dye} values for a large number of steroids, illustrating good separations among various groups. While monohydroxy steroids have R_{dye} values between 0.82 and 0.81, the addition of ketonic functional groups shifts their R_{dye} values to between 0.70 and 0.62. Similarly dihydroxy steroids and their analogs possessing ketonic functional groups show R_{dye} values between 0.57 and 0.35. R_{dye} values between 0.34 and 0.31 are generally representative of trihydroxy steroids and their keto analogs. The R_{dye} values given in the table are expressed as the mean of four individual determinations, and are consistently reproducible.

Table II presents the R_{dye} values corresponding to those of the steroids examined, obtainable by using various azo dyes. Fig. 1 illustrates the use of this technique, by showing the localization of the 17 ketosteroids between the zones of *p*-aminoazobenzene and 2,4-diaminoazobenzene.

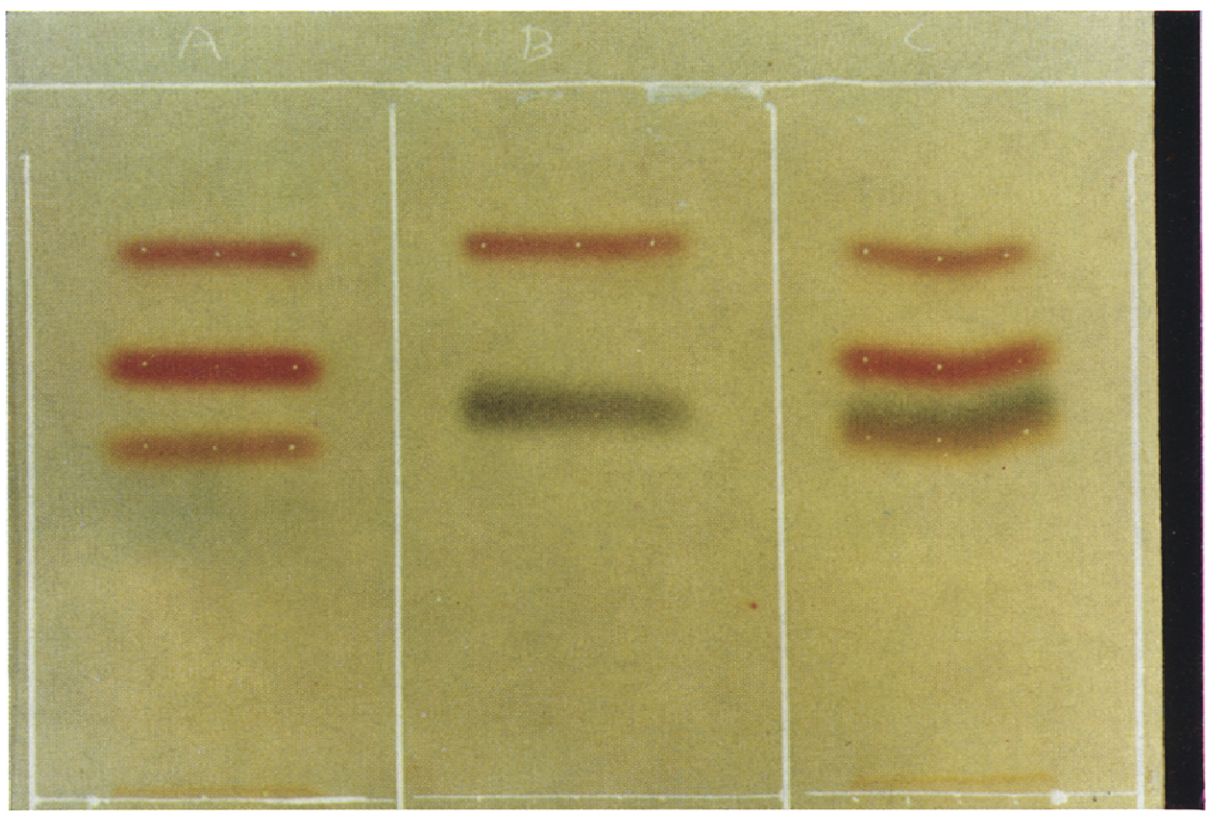

Fig. 1. Thin-layer chromatography of 17-ketosteroids and reference dyes on "Adsorbosil 4" using benzene–methanol (175:25) as the mobile phase. The left channel from top to bottom shows zones corresponding to Sudan III, *p*-amino-azobenzene, and 2,4-diamino azobenzene. The middle channel represents Sudan III and a mixture of androsterone, epiandrosterone, dehydroepiandrosterone and etiocholanolone. On the right channel the dyes and steroids have been spotted together from a mixture. Phosphomolybdic acid was used as the spray reagent.

TABLE I

R_{dye} VALUES OF STEROIDS ON ADSORBOSIL 4 THIN-LAYER PLATES

Results are expressed as R_{dye} values obtained by dividing the absolute migration of the steroid by that of Sudan III. The values represent means of four individual determinations. Mobile phase: benzene–methanol (175–25).

Chemical name	*Trivial name*	R_{dye}
Monohydroxy compounds		
5α-Cholestan-3β-ol	Cholestanol	0.82
5-Cholesten-3β-ol	Cholesterol	0.82
5,7-Cholestadien-3β-ol	7-Dehydrocholesterol	0.82
5,7,11-Cholestatriene-24β-methyl-3β-ol	Ergosterol	0.82
Monohydroxymonoketone compounds		
5α-Androstan-3α-ol-17-one	Androsterone	0.67
5α-Androstan-3β-ol-17-one	Epiandrosterone	0.63
5α-Androstan-17β-ol-3-one	Androstanolone	0.69
5β-Androstan-3α-ol-17-one	Etiocholanolone	0.66
5-Androsten-3β-ol-17-one	Dehydroepiandrosterone	0.69
4-Androsten-17α-ol-3-one	Epitestosterone	0.62
4-Androsten-17β-ol-3-one	Testosterone	0.59
5α-Pregnan-3β-ol-20-one	Allopregnanolone	0.67
5β-Pregnan-3α-ol-20-one	Epipregnanolone	0.68
4-Pregnen-20α-ol-3-one		0.65
4-Pregnen-20β-ol-3-one		0.65
5-Pregnen-3β-ol-20-one		0.67
1,3,5(10)-Estratrien-3-ol-17-one	Estrone	0.70
Monohydroxydiketone compounds		
4-Pregnen-21-ol-3,20-dione	Desoxycorticosterone	0.70
4-Pregnen-17α-ol-3,20-dione	17-Hydroxyprogesterone	0.61
Dihydroxy compounds		
5α-Androstan-3α,17β-diol	Dihydroandrosterone	0.51
5-Androsten-3β,17α-diol		0.42
5α-Pregnan-3β-20β-diol		0.48
5β-Pregnan-3α,20α-diol	Pregnandiol	0.44
1,3,5(10)-Estratriene-3,17β-diol	17β-Estradiol	0.48
Dihydroxymonoketone compounds		
5-Pregnene-3β,17α-diol-20-one		0.47
1,3,5(10)-Estratriene-3,17β-diol-16-one		0.45
Dihydroxydiketone compounds		
4-Pregnene-17α,21-diol-3,20-dione	11-Desoxycortisol	0.57
4-Pregnene-11β,21-diol-3,20-dione	Corticosterone	0.45
Dihydroxytriketone compounds		
4-Pregnene-17α,21-diol-3,11,20-trione	Cortisone	0.36
Trihydroxy compounds		
5β-Pregnane-3α,17α,20α-triol	Pregnantriol	0.35
1,3,5(10)-Estratriene-3,16α,17β-triol	Estriol	0.31
Trihydroxydiketone compounds		
4-Pregnene-11β,17α,21-triol-3,20-dione	Cortisol	0.35
1,4-Pregnadiene-11β,17α,21-triol-3,20-dione	Prednisolone	0.31
4-Pregnene-11β,21-diol-17-al-3,20-dione	Aldosterone	0.45

TABLE II

R_{dye} VALUES OF SEVERAL DYES ON ADSORBOSIL 4 THIN-LAYER PLATES

Results are expressed as R_{dye} values obtained by dividing the absolute migration of the dye by that of Sudan III. Values represent mean of 3 individual determinations.

Sudan III	1.00
Sudan Black B	0.83
p-Aminoazobenzene	0.79
p-Hydroxyazobenzene	0.69
2-4-Diaminoazobenzene	0.62
4-4'-Dihydroxyazobenzene	0.38
Bismarck Brown B	0.37
Bismarck Brown Y	0.34
p-Nitrophenylazoresorcinol	0.27
Diamond Olive Chrome G	0.21

Discussion

The solvent system described in this paper effectively separates several classes of steroids on the basis of the number of hydroxyl and ketonic functional groups on the molecule. Besides being highly reproducible, the method is simple and allows the non-destructive localization of these compounds by comparison with known reference azo dyes. The presence of the azo dyes does not affect the retention time of the steroids on GLC[8] following their elution from thin-layer plates.

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