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THIN-LAYER PARTITION CHROMATOGRAPHY OF STEROIDS USING VOLATILE STATIONARY PHASES

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SUMMARY

A simple technique for using Bush-type (volatile stationary phase) chromatographic systems on thin-layer plates composed of Silica Gel GF is described. The R_F values of several steroids of biological interest are presented for both aqueous methanolic and aqueous acetic acid systems. Suggestions for the modification of paper partition chromatography systems for use with silica gel plates are also presented.

INTRODUCTION

Progress in the development of solvent systems suitable for the separation, by adsorption thin-layer chromatography, has been extensive. Many such systems for separating steroids of the estrane[1,2], androstane[3-5] and pregnane[6-9] series have been published. Nevertheless, there is a practical need for partition chromatography, in that certain steroid pairs commonly found in biological materials can be separated only with difficulty by adsorption chromatography. Common examples include progesterone and androstenedione, pregnenolone and dehydroepiandrosterone, and 20α- and 20β-dihydroprogesterone.

Although many workers have used reversed-phase partition chromatography on thin-layer plates for the resolution of lipid and sterol mixtures[10], VAEDTKE AND GAJEWSKA[11] were the first to report the use of thin-layer partition chromatography for the separation of steroids. These workers used Zaffaroni-type systems in which impregnation of the plate with the liquid stationary phase is required. To our knowledge, the use of thin-layer partition chromatography using Bush-type solvent systems has not been achieved. We should therefore like to present a simple, reproducible technique for using volatile stationary phase partition chromatography systems on Silica Gel GF plates and to present examples of its usefulness.

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MATERIALS AND METHODS

Preparation of tank

A Brinkman rectangular TLC tank (Cat. No. 25-10-22) is lined with Whatman filter paper (two thicknesses of No. 1 or one thickness of No. 3MM). A trough is placed in the bottom of the tank which should be wide enough to hold a 20 cm plate. We have used a trough and support obtained for paper chromatography tanks (Will Scientific Co., Cat. Nos. 8430 and R 8429). The lid of the tank should have a hole, approximately ½ in. in diameter, fitted with a cork. The lid is sealed on with a film of starch–glycerine paste[12] and held firmly with two five-pound lead weights, in order to minimize evaporation of solvent from the atmosphere of the tank. The tank is prepared by saturating the paper liner with 200 ml of stationary phase, which forms a layer in the bottom, and 75 ml of mobile phase which overlays the stationary phase. The atmosphere should be allowed to equilibrate for at least 1 h.

Preparation of plates

Thin-layer plates are prepared with Mallinckrodt Silica Gel TLC-7 GF, 30 g in 60 ml of water, and are spread in the customary way to make five plates, 20 cm × 20 cm × 250 μ. The plates are left to dry at room temperature for about 30 min, and then dried in an oven at 100° for 2 h. We have confirmed the report of HEFTMANN[13] that plates which have been left to dry for 24 h at room temperature perform as well as those dried at 100°. This eliminates the need for taking special precautions for storage of plates. If Merck (Brinkman) Silica Gel GF is used, the R_F values are about half of those obtained with Mallinckrodt silica gel. The radical changes in solvent composition thus required make it desirable to use the Mallinckrodt product. The reason for this difference is not apparent, but since it is also found when comparing customary adsorption TLC solvent systems for the two products, there is reason to suspect it arises from differences in the preparation of the two silica gels[14].

Preparation of solvent systems

Biphasic solvent systems are prepared in the manner for paper partition chromatography, by mixing the appropriate solvents in the indicated ratios in a separatory funnel and allowing the two layers to separate. The two phases thus formed are stored in glass bottles with glass stoppers.

Conditions of chromatography

The samples and standards are spotted on the plates exactly as for adsorption TLC. The plate is then placed in the trough, and left to equilibrate for 2 h at room temperature. A funnel with a plastic tube attached is then used to add 40 ml of the mobile phase directly into the trough, through the hole in the lid. When removing the funnel, one should avoid dripping solvent onto the plate. Development requires 30–60 min, depending on the solvent system used. After the solvent has risen to the desired height (usually 15 cm from the origin) the plate is removed and dried in the fume hood. The trough can be removed and the tank re-used at least two more times.

Chromatographic systems used

Abbreviations used in solvent system nomenclature are taken from BUSH[12].

L/80: Cyclohexane–methanol–water (100:80:20)

LT81/80: Cyclohexane–toluene–methanol–water (89:11:80:20)
LT51/80: Cyclohexane–toluene–methanol–water (83:17:80:20)
LT41/80: Cyclohexane–toluene–methanol–water (80:20:80:20)
LT21/80: Cyclohexane–toluene–methanol–water (67:33:80:20)
L/A85: Cyclohexane–acetic acid–water (100:85:15)
LT21/A80: Cyclohexane–toluene–acetic acid–water (67:33:80:20)
LT41/A85: Cyclohexane–toluene–acetic acid–water (80:20:85:15)
LT41/A80: Cyclohexane–toluene–acetic acid–water (80:20:80:20)
LT31/F80: Cyclohexane–toluene–90% formic acid–water (80:20:89:11)

The use of cyclohexane rather than *n*-hexane, light petroleum etc. is recommended for the reason given by BUSH[12], namely, that ΔR_M values are sometimes increased when using a cyclic hydrocarbon as a major component of the mobile phase.

Similarly we recommend the use of toluene rather than benzene because $\Delta R_{M(r)}$ values are frequently slightly greater with toluene. The toxicity of toluene is also appreciably less than benzene.

EXPERIMENTAL

The R_F and R_M values for a number of common steroids of biological interest are presented in Tables I and II, for methanolic and acetic or formic acid systems, respectively.

The R_F values presented in Tables I and II are, in all cases, slightly less than those reported for similar biphasic solvent systems used for paper partition chromatography (PPC)[12,15,16]. Some of the ΔR_M values due to modification of structure ($\Delta R_{M(r)}$ values) obtained with thin-layer partition chromatography (TLPC) are presented in Table III. These values are quite similar to those reported for PPC[12,15,16], suggesting that partition rather than adsorption effects predominate.

TABLE I

R_F AND R_M VALUES FOR SOME COMMON STEROIDS IN AQUEOUS METHANOLIC SYSTEMS

	L/80		*LT81/80*		*LT41/80*		*LT21/80*	
	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
Progesterone	0.49	0.017	0.62	−0.213	0.71	−0.388	0.76	−0.501
Androstenedione	0.22	0.550	0.32	0.327	0.47	0.052	0.57	−0.122
Pregnenolone	0.32	0.327	0.40	0.176	0.53	−0.052	0.60	−0.176
Dehydroepiandrosterone	0.15	0.753	0.20	0.602	0.31	0.348	0.37	0.231
20β-Dihydroprogesterone	0.22	0.550	0.31	0.348	0.43	0.122	0.52	−0.035
20α-Dihydroprogesterone	0.14	0.788	0.22	0.550	0.32	0.327	0.40	0.176
7-Oxo-cholesterol	0.89	−0.908	0.95	—	0.94	—	0.94	—
Cholesterol	S.F.	—	S.F.	—	S.F.	—	S.F.	—

Systematic names, abbreviations and trivial names used refer to the following chemical substances: progesterone (P^4) = pregn-4-ene-3,20-dione; androstenedione (A^4) = androst-4-ene-3,17-dione; pregnenolone (P^5) = pregn-5-en-3β-ol-20-one; dehydroepiandrosterone (DHEA) = androst-5-en-3β-ol-17-one; 20β-dihydroprogesterone (20β-P^4) = pregn-4-en-20β-ol-3-one; 20α-dihydroprogesterone (20α-P^4) = pregn-4-en-20α-ol-3-one; cholesterol (chol.) = cholest-5-en-3β-ol; 7-oxo-cholesterol (7-oxo-chol.) = cholest-5-en-3β-ol-7-one.

TABLE II

R_F AND R_M VALUES FOR SOME COMMON STEROIDS IN AQUEOUS ACETIC AND FORMIC ACID SYSTEMS

	L/A85		*LT41/A85*		*LT21/A80*		*LT31/F80*	
	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
Progesterone	0.13	0.825	0.41	0.158	0.50	0.00	0.25	0.477
Androstenedione	0.05	1.131	0.21	0.575	0.31	0.347	0.08	1.061
Pregnenolone	0.19	0.629	0.49	0.017	0.55	−0.087	0.44	0.104
Dehydroepiandrosterone	0.09	1.033	0.31	0.347	0.37	0.231	0.17	0.688
20β-Dihydroprogesterone	0.08	1.058	0.32	0.327	0.41	0.158	0.15	0.753
20α-Dihydroprogesterone	0.05	1.210	0.24	0.501	0.35	0.268	0.11	0.908
7-Oxo-cholesterol	0.35	0.268	0.61	−0.194	nd[a]	—	0.53	−0.052
Cholesterol	0.73	−0.432	0.84	−0.720	nd	—	0.83	−0.689

[a] No detection.

It is of special interest to note that the $\Delta R_{M(r)}$ (Δ^4-3-one→Δ^5-3β-ol), whether for the C_{21} steroids or the C_{19} steroids, changes from a positive value for the aqueous methanolic systems, to a negative value for the aqueous acidic systems. This observation has also been noted for PC systems[12] and is of particular analytical value. For example, for practical purposes, pregnenolone has a slower mobility than progesterone in aqueous methanolic systems, but a faster mobility in aqueous acidic systems.

TABLE III

$\Delta R_{M(r)}$ FOR THREE STRUCTURAL CHANGES

	20α-OH → 20β-OH	*Δ^4-3-one → Δ^5-3β-ol*	*C_{19} → C_{21}*
L/80	−0.23	+0.25	−0.50
LT81/80	−0.20	+0.34	−0.49
LT51/80	−0.20	+0.34	−0.49
LT41/80	−0.20	+0.35	−0.44
LT31/80	−0.21	+0.36	−0.38
LT21/80	−0.22	+0.34	−0.39
L/A85	−0.15	−0.15	−0.36
LT41/A85	−0.17	−0.18	−0.37
LT21/A80	−0.11	−0.10	−0.33
LT31/F80	−0.16	−0.37	−0.58

DISCUSSION

The advantages of TLC as compared to PC are multiple and need not be belabored. The establishment of a simple technique for using Bush-type solvents in thin-layer partition chromatography (TLPC) enables the investigator to utilize the advantage of partition chromatography without sacrificing the ease of TLC.

A theoretical problem of practical concern in adsorption chromatography is that compounds differing in the number of carbon atoms cannot be easily separated.

One approach has been to use low-polar solvents with repeated development[17], and STÁRKA AND HAMPL[18] have described mathematical formulae by which the number of developments for optimal separation could be calculated. Earlier workers have used the Zaffaroni type systems in which the stationary phase is impregnated onto the supporting silica gel. A disadvantage of such systems is the difficulty of removing the stationary phase after development of the chromatogram. This difficulty is eliminated by using the Bush-type, volatile, stationary phase as described in this report.

The aqueous methanolic and the aqueous acetic acid systems for which data are presented in Tables I and II, respectively, represent a small part of the total range of systems reported for use with PC. The polarity of these systems is suitable for the polarity of the steroids presented as examples. The separation of more polar steroids would necessitate the use of more polar solvent systems.

As a first approximation, paper partition chromatography (PPC) systems may be used with this technique (TLPC). However, we have noted that a slight decrease in the methanol concentration of the stationary phase (*e.g.*, L/85→L/80) is usually necessary in order to get similar R_F values in PPC and TLPC, respectively. Alternatively, the polarity of the mobile phase may be decreased (*e.g.*, LT21/80→LT41/80).

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