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Note

Studies on the reversed-phase thin-layer chromatography of ecdysteroids on C_{12} bonded and paraffin-coated silica

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The ecdysteroids are polar, polyhydroxysteroids, the best known among them are the moulting hormones of insects and crustaceans. In a previous report[1] we described the use of reversed-phase thin-layer chromatography (RP-TLC) on C_{18}, C_8 and C_2 bonded silicas for the separation and purification of ecdysteroids. As well as offering another chromatographic system for the identification of these substances. RP-TLC gives recoveries of ecdysteroids from the plate much higher than recoveries from silica (*ca.* 90% as compared with *ca.* 50% for silica). This has obvious implications for the isolation of trace quantities of ecdysteroids (or similar polar substances) from biological samples. However, problems associated with RP-TLC include expense, the limited range of plate sizes available, and the need to apply samples in an organic solvent, because of the hydrophobic properties of the plate. Recent reports[2,3], suggested that this last problem has been overcome, and we were encouraged to re-examine RP-TLC for ecdysteroids.

Furthermore, we were reminded by the editor of the earlier use of paraffin-coated silica, now eclipsed by the bonded type. We were stimulated by our correspondence to re-examine the simple paraffin-coated plates and compare them with the modern chemically bonded reversed-phase silica. Paraffin-coated plates have recently been the subject of a review[4]. The relatively cheap paraffin-coated plates appear to be a neglected area of chromatography worthy of greater use, now that reversed-phase systems have come into widespread use.

EXPERIMENTAL

Ecdysteroids used in this study were gifts from a number of sources.

Preparation and use of RP-TLC plates

Paraffin-coated plates were prepared according to Mangold[5]. Whatman HP-K 10 × 10 HP-TLC plates (Uniscience, Cambridge, Great Britain), were developed in a solvent system consisting of dichloromethane containing the appropriate percentage of refined heavy paraffin oil, "Nujol" (2.5, 5, 7.5 and 10%, v/v). For the majority of

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the experiments reported here a 7.5% loading was used. When the solvent front reached the top of the plates they were removed from the tank and allowed to dry in a fume cupboard. For some experiments these plates were washed in dichloromethane before coating with paraffin.

Preparative TLC plates (0.6 mm thick, 10 × 20 cm plates) were prepared in the laboratory from silica gel PF_{254} (Merck, Darmstadt, G.F.R.) as described by Bielby *et al.*[6]. These plates were converted to reversed phase as described above using 7.5% paraffin in dichloromethane.

OPTI-UP C-12 RP-TLC plates (Fluka, Buchs, Switzerland) were used as supplied without pre-treatment.

All plates were equilibrated over the solvent used for chromatography for at least 30 min before being developed. Solutions of ecdysteroids, either in methanol or water, were applied to the plates by syringe either as spots or as a streak. Plates were then developed in solvent systems consisting of water–methanol mixtures. After chromatography, ecdysteroids were visualised on the plate by fluorescence quenching at 254 nm.

To determine the efficiency of recovery, the appropriate R_F zone was removed from the plate. Ecdysteroids were then eluted from the silica by repeated washing the methanol (3 ml, followed by 1 ml). The combined methanolic extracts were then evaporated to dryness in a 1-ml Reacti-vial (Pierce and Warriner, Chester, Great Britain). The recovery of ecdysteroids was then estimated by gas chromatography. Gas chromatographic procedures used in this study are described in detail by Bielby *et al.*[6].

RESULTS AND DISCUSSION

Chromatographic properties of coated and bonded RP-TLC plates

The chromatographic properties of the OPTI-UP C-12 RP-TLC plates were determined by chromatographing a mixture of ecdysone, 20-hydroxyecdysone, and makisterone A in solvent systems composed of water to which varying amounts of methanol (0–100%) had been added. Fig. 1A shows that the R_F values varied between 0 and 0.55 for these compounds. Not only was it possible to develop these plates in entirely aqueous solvent systems, it was also possible to apply the sample as an aqueous solution, although this had to be done with a syringe, as capillary action was insufficient to overcome the hydrophobic property of the plate. Applying samples in water has the advantage that they remain as tight spots on the hydrophobic silica. The chromatographic properties of the OPTI-UP plates were found to be very reproducible. The R_F values of eleven representative ecdysteroids on these plates are given in Table I.

The chromatographic properties of paraffin-coated plates were assessed in a similar way (Fig. 1B), Makisterone A was not resolved from ecdysone on these plates, so it was omitted from the mixture. Aqueous samples could also be applied to paraffin coated plates in the same way as for the bonded plates. These "homemade" plates were rather less reproducible than the bonded plates, but this may reflect our inexperience in their preparation rather than inherent irreproducibility. Compared with preparing chemically bonded RP-TLC plates in the laboratory[1], the preparation of coated plates is simpler, less time consuming and less expensive (both in reagents and solvents).

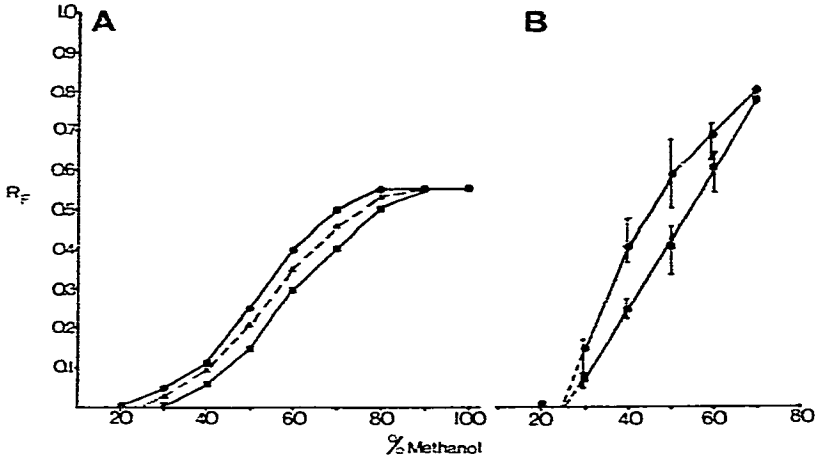

Fig. 1. A Effect of solvent composition on the chromatography of ecdysone (■), 20-hydroxyecdysone (●), and makisterone A (▲) on OPTI-UP C-12 reversed-phase thin-layer plates. B Effect of solvent composition on the chromatography of ecdysone (■), and 20-hydroxyecdysone (●) on 7.5% paraffin-coated silica RP-TLC plates. Error bars show the range of values obtained in four replicate determinations.

The effect of varying the degree of coating between 5 and 10% paraffin is relatively small. With 40% methanol in water as mobile phase the R_F value of ecdysone was 0.34 (5% paraffin), 0.29 (7.5% paraffin) and 0.29 (10% paraffin), and 7.5% was chosen for all further work. The time required for development was strongly dependent on the particle size of the silica and the quantity of organic

TABLE I

R_F VALUES OF ECDYSTEROIDS ON REVERSED-PHASE THIN-LAYER PLATES USING BONDED AND COATED PHASES

Compound	*Solvent composition: methanol–water*					
	40:60		*50:50*			
	OPTI-UP C-12		*Paraffin-treated Whatman plates*		*Paraffin-treated preparative plates*	
	R_F	R_M^*	R_F	R_M^*	R_F	R_M^*
Ecdysone	0.35	1.0	0.37	1.0	0.39	1.0
20-Hydroxyecdysone	0.44	1.26	0.52	1.41	0.52	1.33
Poststerone	0.38	1.09	0.52	1.41	—	—
Ponasterone A	0.29	0.83	0.20	0.54	0.15	0.38
Inokosterone	0.40	1.14	0.50	1.35	—	—
Cyasterone	0.43	1.23	0.57	1.54	—	—
Muristerone A	0.39	1.11	0.46	1.24	0.43	1.1
Polypodine B	0.44	1.26	0.55	1.49	—	—
Makisterone A	0.39	1.11	0.43	1.16	0.42	1.07
Ajugasterone C	0.41	1.17	0.46	1.24	—	—
2-Deoxyecdysone	0.25	0.71	0.24	0.65	0.18	0.46

* R_M is the R_F relative to ecdysone.

TABLE II

EFFICIENCY OF RECOVERY OF ECDYSTEROIDS FROM RP-TLC PLATES

Quantity applied (ng)	*Mean recovery (%)*		
	OPTI-UP	*Paraffin-coated Whatman plates*	*Paraffin-coated preparative plates*
500	–	–	95.6
250	83.0	–	106.6
200	85.3	81.8	–
100	100.0	74.3	87.6
50	92.0	69.0	68.3
25	85.0	–	–
10	69.0	–	–

modifier in the solvent, and independant of the degree of coating. The preparative paraffin-coated RP-TLC plates with much coarser particles developed much faster than the paraffin-coated Whatman RP-TLC plates (with 50% methanol, preparative RP-TLC 0.16 cm min^{-1}, Whatmans RP-TLC 0.026 cm min^{-1}, and OPTI-UP RP-TLC 0.082 cm min^{-1}). With 20% methanol on the Whatman plate the solvent migrated at 0.017 cm min^{-1} whilst at 80% this increased to 0.13 cm min^{-1}. Identical values were obtained for all degrees of coating from 2.5 to 10%. The R_F values of ecdysteroids on both paraffin-treated Whatman and preparative RP-TLC plates were very similar (Table I). When R_F values relative to ecdysone are compared the paraffin-coated plates appear to give slightly superior resolution to the bonded plates. Differences in selectivity are also apparent between the plates particularly for poststerone and 2-deoxyecdysone.

Recovery of ecdysone applied to RP-TLC plates

As we noted in our previous report[1] recovery of ecdysteroids from RP-TLC

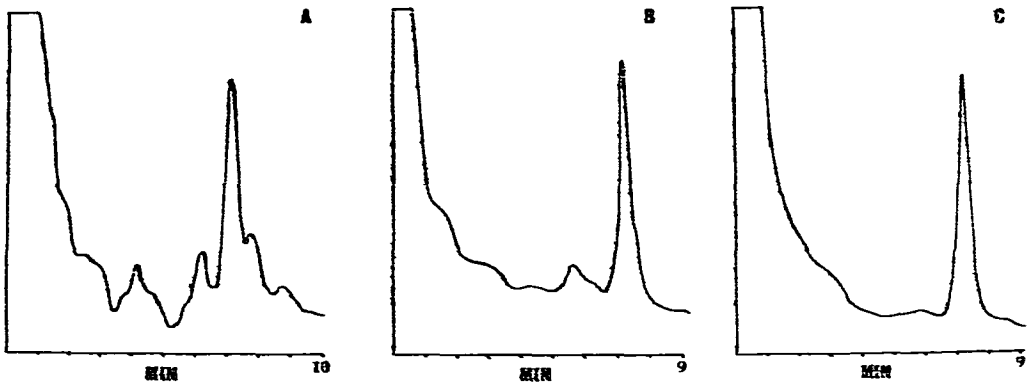

Fig. 2. Gas chromatographic traces, with electron-capture detector, of ecdysone (100 ng) recovered from Whatman HP-K silica plates which had been impregnated with paraffin oil. Trace A shows the result with no pre-treatment. B shows effect of washing the plate with dichloromethane before impregnation and use. Trace C shows a sample which had been recovered from a laboratory-prepared preparative TLC plate impregnated with paraffin.

plates is more efficient than from silica. The plates examined in this study also gave good recoveries even down to 10–50 ng per plate. Recovery of ecdysone from all three types of RP-TLC plates are given in Table II. The Whatman HP-TLC plates had to be washed in dichloromethane before conversion to RP-TLC for quantiative work. This was due to the co-elution of compounds which interfered with the detection of ecdysone using the electron capture detector. These contaminants, which were removed by washing, prevented quantification of ecdysone at less than 200 ng/plate (Fig. 2). This problem was not observed with the OPTI-UP RP-TLC plates or the paraffin-treated preparative RP-TLC plates (where the silica had been extensively washed before spreading). For quantities of material of the order of 200 ng per plate, all three types of plate gave similar results, however below 100 ng per plate the OPTI-UP plates gave better results (of the order of 70% recovery of 10 ng).

Reversed-phase chromatography was introduced first with a bonded phase, when Howard and Martin[7] treated silica with dimethyldichlorosilane. This was quickly followed by a number of systems using coated solids. The very first isolation of an insect pheromone, bombykol, was achieved with the help of chromatography on paraffin impregnated silica gel[8], but subsequently interest in reversed-phase systems was eclipsed by other developments. High-performance liquid chromatography has brought bonded reversed-phase systems back into fashion, and RP-TLC has come back with it. Coated TLC plates remain neglected and deserve re-examination.

Both coated and bonded RP-TLC plates are well suited to the chromatography of ecdysteroids.

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