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EFFECT OF THE MOBILE PHASE COMPOSITION ON THE RETENTION BEHAVIOUR OF DIPHENYLSILICA PRE-COATED PLATES*

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SUMMARY

The retention of some rifamycins and steroids on diphenyl bonded pre-coated silica gel plates, in relation to the mobile phase used, was examined by thin-layer chromatography. Neat organic solvents, non-aqueous and aqueous binary mixtures were tested as eluents. By comparison of retention data for rifamycins and steroids, respectively, under non-aqueous and aqueous conditions, a dual retention mechanism on this diphenyl phase was found. Interactions with the residual silanol groups seemed to prevail when employing as mobile phase the more lipophilic solvents tested, such as chloroform or dichloromethane, whereas interactions with the aryl groups of the bonded phase prevailed when using high polarity alcohols or aqueous mixtures. As a consequence, by changing the mobile phase, a large variation in selectivity with a concomitant change in retention order of the test compounds was observed.

INTRODUCTION

In recent years many new chemically modified silica gel thin-layer chromatographic (TLC) plates, pre-coated with the same materials as used in high-performance liquid chromatography (HPLC), have led to an increasing practical and theoretical interest in reversed-phase TLC[1]. Aqueous mixtures of methanol and acetonitrile were generally employed as the mobile phases, and there have been few systematic studies of the use of other solvents[2–5]. This is unexpected: one of the advantages of TLC over column liquid chromatography is the possibility of a rapid screening of solvents for use as the mobile phase. Moreover, the information obtained by the screening of solvents may be useful in two-dimensional TLC, where a suitable choice of the eluents may allow the separation of very complex mixtures of compounds by using two different types of interaction in two directions[6].

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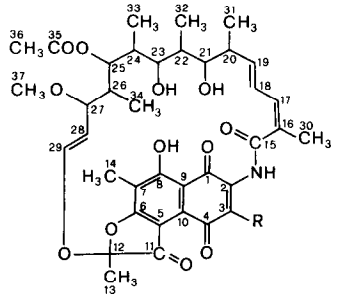
In order to investigate the role of the mobile phase composition and as an extension of previous studies[3,5,7], this paper reports the results of a systematic study on diphenyl bonded pre-coated silica gel plates (d-PH); a silica bonded phase with two phenyl groups), which have recently become commercially available. There is a growing interest in the study of these new arylsilica phases in HPLC[8–12] and TLC[2,13–15] as their selectivity may differ significantly from that of alkylsilicas such as octyl- or octadecyl-bonded phases.

The present work was accomplished by employing a wide variety of eluents, ranging from methanol–water (50:50, v/v) to neat *n*-hexane. Two classes of compounds, differing in skeletal structure, size and polarity, were chosen as test solutes: steroids and rifamycins. In the hormone class, compounds with different degrees of unsaturation and chemical functions, such as two pregnane, two androstane and three estrane derivatives, were chosen. In the antibiotic class, rifamycins in their quinone (S) or hydroquinone (SV) forms, the last with different substituent groups at C(3), were tested. For comparison, the retentions of these compounds on RP-18 plates were also studied.

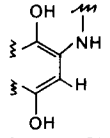
EXPERIMENTAL

Materials

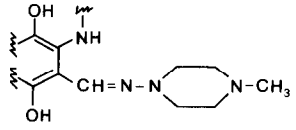
The model compounds used were the rifamycins listed in Fig. 1 and the following steroids: progesterone (1), pregnenolone (2) (pregnane derivatives); testosterone (3), androstendione (4) (androstane derivatives); estrone (5), estradiol (6) and estriol (7) (estrane derivatives). All solvents were of analytical reagent grade. Approximately



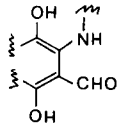
1) Rifamycin S



2) Rifamycin SV



3) Rifampicin



4) 3-formyl-rifamycin

Fig. 1. Structural formulae of rifamycin S (quinone form) and of some 3-substituted derivatives of rifamycin SV (hydroquinone form).

TABLE I

hR_F VALUES OF RIFAMYCINS ON DIPHENYL (a) AND OCTADECYL (b) SILICA GEL PRE-COATED PLATES IN VARIOUS ORGANIC SOLVENTS AS MOBILE PHASES

Eluent	*Rifamycin 1**		*Rifamycin 2*		*Rifamycin 3*		*Rifamycin 4*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
n-Hexane	0	0	0	0	0	0	0	0
Cyclohexane	0	0	0	0	0	0	0	0
Chloroform	91	91	3	13	31	36	13	34
Dichloromethane	23	71	0	43	0	0	0	40
Tetrahydrofuran	99	96	99	96	98	97	97	98
Acetone	83	86	98	98	86	83	99	97
Acetonitrile	–	–	–	–	–	–	–	–
1-Butanol	72	99	98	99	65	60	97	98
1-Propanol	74	88	96	89	71	67	96	90
2-Propanol	42	82	92	86	56	66	94	85
Ethanol	80	88	94	93	86	86	94	93
Methanol	95	94	98	96	95	95	98	96

* Numbering as in Fig. 1.

1% sample solutions in chloroform for the rifamycins, in methanol or methanol–chloroform for the steroids, were used for TLC.

Single- and two component solvent systems were employed as mobile phases. Table I lists the neat organic solvents investigated. The binary non-aqueous solvent systems tested were *n*-hexane–chloroform and *n*-hexane–ethanol, at various ratios. The binary aqueous solvent systems examined were mixtures containing 10–90% (v/v) of an organic modifier and water (or aqueous solutions). The organic modifiers were methanol, 1-propanol, 2-propanol, acetonitrile and tetrahydrofuran. The aqueous solutions were 0.025–1 *M* lithium chloride, 0.025–1 *M* ammonium sulphate, 0.1 *M* hydrochloric acid and 0.1 *M* sodium hydroxide.

Thin-layer chromatography

TLC was routinely carried out on diphenyl-F_{254} RP (Whatman, Clifton, NJ, U.S.A.) and Stratocrom SiF_{254}-$C_{18}W$ (made by Whatman for Farmitalia-Carlo Erba, Milan, Italy) pre-coated plates.

The following parameters were kept constant: size of the plates (5 cm × 5 cm); solvent volume (12 ml) in the developing tank (10.5 cm high × 6.5 cm × 6.5 cm); time prior to the insertion of the plates (20 min); distance of starting line from the bottom (1 cm) and distance of development (3.5 cm). After the application of the spots (0.2–0.3 μl), ascending development was carried out at room temperature.

Rifamycins are coloured compounds which do not require special detection, while the steroid spots were located under UV light by quenching of the fluorescence at 254 nm. A Perkin-Elmer Model 650-10s fluorimeter equipped for densitometric measurements, in the reflectance mode, was used to measure directly the spots of steroids after their TLC separation. The plates were scanned in a direction parallel to that of the solvent flow.

TABLE II

hR_F VALUES OF STEROIDS ON DIPHENYL (a) AND OCTADECYL (b) BONDED SILICA GEL PRE-COATED PLATES IN VARIOUS ORGANIC SOLVENTS AS MOBILE PHASES

Eluent	*Steroid* 1*		*Steroid 2*		*Steroid 3*		*Steroid 4*		*Steroid 5*		*Steroid 6*		*Steroid 7*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
n-Hexane	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyclohexane	8	0	8	0	0	0	0	0	0	0	0	0	0	0
Chloroform	99	91	97	82	96	82	99	92	91	85	81	74	14	0
Dichloromethane	92	68	88	62	82	47	90	74	76	60	70	52	7	0
Tetrahydrofuran	99	97	98	98	98	99	99	98	97	96	99	98	98	96
Acetone	93	71	81	74	81	74	76	71	93	86	90	80	78	84
Acetonitrile	92	47	85**	41**	90**	46**	85	57	85**	65**	99	69	98**	71**
1-Butanol	86	87	87	94	90	94	86	92	86	97	92	97	91	98
1-Propanol	86	86	88	93	86	90	81	89	81	94	89	95	89	98
2-Propanol	81	71	85	84	87	76	80	71	86	81	91	83	91	84
Ethanol	81	81	85	87	83	81	80	81	84	86	89	95	86	90
Methanol	98	96	98	98	98	97	99	99	97	99	97	89	99	97

* Numbering as in Experimental.

** Elongated spot.

RESULTS AND DISCUSSION

Single- and two-component solvent systems, *e.g.*, non-aqueous and aqueous–organic mixtures, with or without addition of an inorganic salt, were tested as eluents. The solvents chosen had increasing polarities and differed in the proton-donor solubility parameter[16].

Mobile phase effects

Non-aqueous eluents. In Tables I and II the R_F values of four rifamycins and seven steroids on d-PH and C_{18} bonded pre-coated silica gel plates, respectively, are reported, using twelve organic solvents. The R_F values of rifamycins in acetonitrile are not reported, as in this eluent the spots showed long tails. From the data listed in Table I it appears that the d-PH plates are quite selective for the rifamycins in many eluents, *e.g.*, chloroform, 1-butanol, 2-propanol. When the R_F values in the twelve eluents were compared, on d-PH plates, the sequences of retention of the rifamycins and of the estrane derivatives did not increase according to the strength of the eluents[16]. The above compounds showed higher R_F values in tetrahydrofuran than in other more polar solvents, and were more strongly retained in 2-propanol than in 1-propanol or in 1-butanol.

On d-PH plates, changes in the organic solvent employed as mobile phase affected the retention of rifamycins to different extents. An understanding of the rôle of the mobile phase in retention and selectivity is not easy to achieve, as different and competitive interactions between the mobile phase, solutes and stationary phase may occur. However, in our case, when the solvents are lipophilic such as *n*-hexane, cyclohexane, chloroform and dichloromethane, interactions between the rifamycins and the residual silanol groups at the surface of the stationary phase seem to prevail. The quinone form (rifamycin S) is much less strongly retained than the hydroquinone forms of rifamycins 2–4 due to their polarity and possibility of forming hydrogen bonds to the silanol groups. When the solvents are polar, the retention of the rifamycins is due, principally, to interactions with aryl groups of the bonded phase.

This possibility of two different retention mechanisms depending on the mobile phase is in agreement with a study of Halpaap *et al.*[2] on the chromatographic behaviour of polycyclic aromatic hydrocarbons on C_{18}, C_8 and d-PH modified silica gel plates. An adsorption mechanism was postulated when a lipophilic eluent (*n*-heptane or *n*-hexane) was employed as mobile phase, whereas a reversed-phase partition mechanism, even in the absence of water, was assumed when using highly polar alcohols, acetone or acetonitrile.

In order to test further the hypothesis of a dual retention mechanism on the diphenyl phase, eluents consisting of binary mixtures of a non-polar solvent (*n*-hexane) and co-solvents with different polarities such as chloroform and ethanol were examined. Table III shows the R_F values obtained with *n*-hexane–chloroform and *n*-hexane–ethanol with increasing concentration of the more lipophilic solvent. A plot of the retention, R_M, *vs.* percentage chloroform or ethanol for the four rifamycins, Fig. 2a, was linear only for the first system where silanophilic interactions were believed to prevail. The non-linear retention behaviour observed for the *n*-hexane–ethanol system (Fig. 2b) is consistent with the hypothesis of a dual retention mechanism[17]. Indeed the retention cannot occur by silanophilic or solvophobic in-

TABLE III

hR_F VALUES OF RIFAMYCINS IN BINARY NON-AQUEOUS MIXTURES ON DIPHENYL PRE-COATED PLATES

Eluent		*Rifamycin**			
		1	*2*	*3*	*4*
n-Hexane–chloroform	(5:5)	65	0	0	0
	(4:6)	71	0	14	0
	(3:7)	74	0	22	5
	(2:8)	80	1	25	7
	(1:9)	90	3	31	11
n-Hexane–ethanol	(9:1)	31	0	0	0
	(8:2)	42	0	5	0
	(7:3)	58	3	10	9
	(6:4)	71	72	34	73
	(5:5)	73	81	43	86
	(4:6)	79	92	58	93
	(3:7)	80	94	79	95

* Numbering as in Fig. 1.

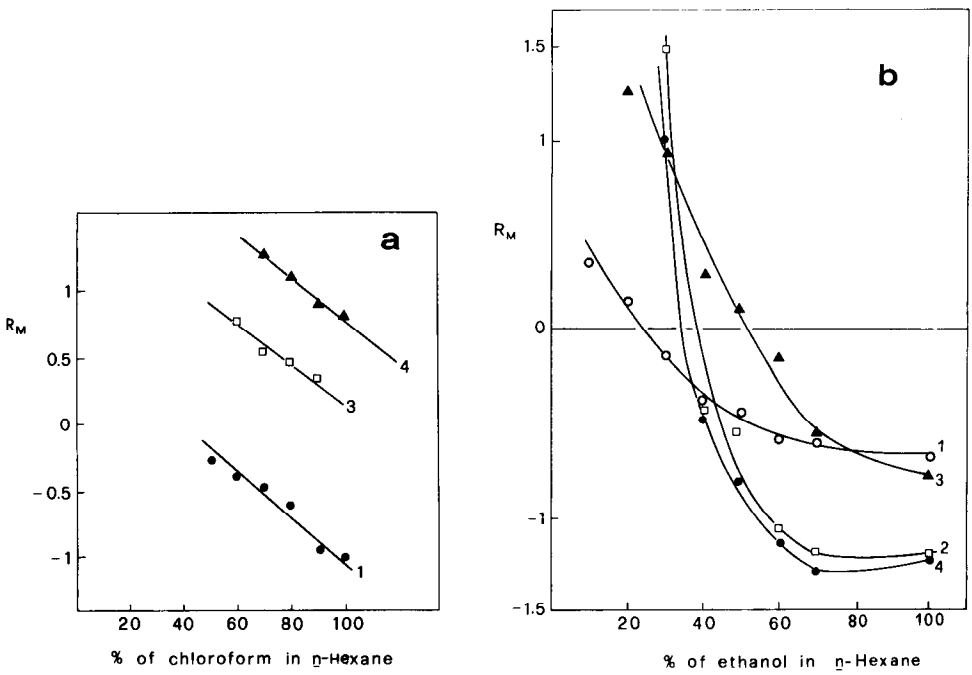


Fig. 2. Plots of retention factor, R_M, for rifamycins on d-PH plates against the composition of the binary organic mixtures used as eluents. (a) *n*-Hexane–chloroform; (b) *n*-hexane–ethanol. Numbering of rifamycins as in Fig. 1.

teractions alone, because each mechanism would predict a monotonic decrease in retention with decreasing concentration of one of the solvent components[18].

As regards the steroids, the results in Table II indicate that their retention on d-PH pre-coated plates was generally rather weak except for estriol (compound 7) in the more lipophilic eluents, such as dichloromethane and chloroform. Estriol, a compound with a phenolic A ring and two alcoholic OH groups, was also the most strongly retained on amino-modified and silica gel pre-coated plates, employing the same two eluents[6]. This fact is in agreement with our hypothesis of an adsorption mechanism for estriol on d-PH plates when using lipophilic eluents.

Aqueous–organic eluents. The effect of these eluents on the retention of the rifamycins and steroids on d-PH plates was investigated with various organic modifier–water mixtures. Tables IV and V report the R_F values for both series of test compounds, obtained by employing methanol, 1-propanol, 2-propanol, acetonitrile and tetrahydrofuran as organic modifiers. The addition of water to these solvents increased the retention of the two classes of compounds, except that in the case of 1- and 2-propanol with rifamycins no difference or higher R_F values were obtained. A similar unusual behaviour was reported earlier for some anthraquinones: on C_{18} plates, the same (1-propanol, 2-propanol) or higher (1-butanol) R_F values were obtained when water was added to these organic modifiers[19].

Further evidence for a dual retention mechanism on the d-PH pre-coated plates is found by comparison of the retention data of rifamycins and steroids with non-aqueous and aqueous mixtures as mobile phases. In Figs. 3 and 4 the retention sequences of rifamycins and steroids, respectively, using different mobile phases are compared: aqueous–organic solvents, neat alcohols, chloroform and dichloromethane. In Fig. 3, the sequence of retention for the rifamycins is virtually the same in

TABLE IV

RETENTION DATA, hR_F, FOR RIFAMYCINS ON DIPHENYL PRE-COATED PLATES IN AQUEOUS–ORGANIC ELUENTS

Eluent	*Rifamycin**			
	1	*2*	*3*	*4*
Methanol–water (8:2)	97	97	96	97
(7:3)	87	89	74	90
(6:4)	70	70	49	63
(5:5)	42	61	29	47
1-Propanol–water (7:3)	88	84	70	84
(5:5)	91	86	87	90
2-Propanol–water (8:2)	85	89	88	91
(5:5)	85	90	86	91
Acetonitrile–water (5:5)	74	87	79	84
(4:6)	57	74	63	74
Tetrahydrofuran–water (8:2)	94	91	88	88
(5:5)	87	85	86	88

* Numbering as in Fig. 1.

TABLE V

RETENTION DATA, hR_F, FOR STEROIDS ON DIPHENYL PRE-COATED PLATES IN AQUEOUS–ORGANIC ELUENTS

Eluent	*Steroid**						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Methanol–water (8:2)	59	59	69	67	66	70	83
(6:4)	16	5	27	27	18	20	57
(5:5)	5	0	16	16	14	18	43
2-Propanol–water (5:5)	48	48	66	60	51	66	80
Acetonitrile–water (6:4)	56	49	71	66	43	53	71
(5:5)	29	29	51	42	40	51	72
Tetrahydrofuran–water (5:5)	40	38	56	55	38	44	61
(4:6)	16	16	31	31	17	20	40

* Numbering as in Experimental.

the aqueous–organic eluents and in the alcohols, but differs significantly from that observed in the more lipophilic eluents. One reason for this may be that the same retention mechanism occurs with the first two groups of mobile phases. The retention sequence of rifamycins in alcohols or aqueous–organic solvents is in good agreement with their polarities, apart from a slight discrepancy in some eluents for rifamycin S and rifampicin. This may be explained as a particular affinity of the substituent group at C(3) of rifampicin for the diphenyl stationary phase.

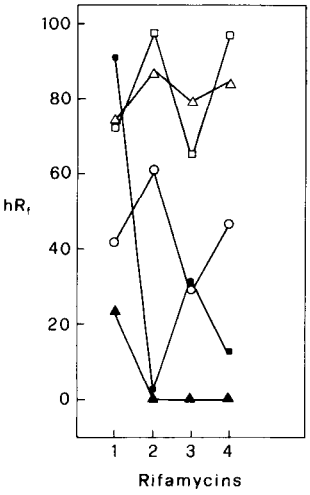


Fig. 3. Graph illustrating the dependence, on d-PH plates, of the retention order of rifamycins on the mobile phase composition: ○—○, methanol–water (1:1); △—△, acetonitrile–water (1:1); □—□, 1-propanol; ■—■, chloroform and ▲—▲, dichloromethane. Numbering of rifamycins as in Fig. 1.

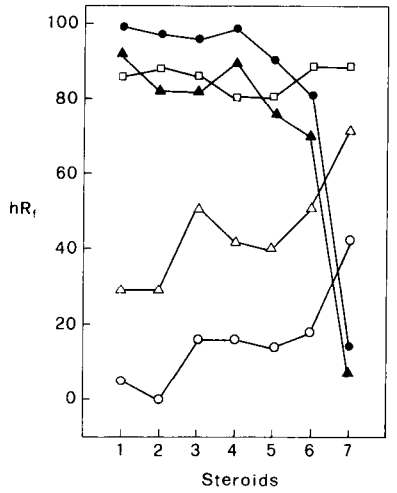


Fig. 4. Graph illustrating the dependence, on d-PH plates, of the retention order of steroids on the mobile phase composition: ○—○, methanol–water (1:1); △—△, acetonitrile–water (1:1); □—□, 1-propanol; ●—●, chloroform and ▲—▲, dichloromethane. Numbering of steroids as in Experimental.

Comparison between the retention sequences of the steroids in the aqueous–organic and the more lipophilic eluents suggests, in this case too, a dual retention mechanism on the diphenyl phase. The retention order of the three estrane derivatives (compounds 5–7) was 5 > 6 > 7 in the aqueous–organic mobile phases, in accord with the increasing polarity of the compounds, whereas it was the opposite in chloroform or dichloromethane. Moreover, pregnane derivatives (compounds 1 and 2), the least polar of the steroids tested, showed the highest R_F values in dichloromethane and the lowest in the other two mobile phases. Fig. 4 shows that, when employing the alcohols as mobile phases, the difference in the retention between the various steroids was too small to assume that, as in the case of rifamycins, the same retention mechanism was occurring in both the aqueous-organic eluents and in the alcohols.

By combining the two retention mechanisms, the seven steroids may be separated by two-dimensional TLC. A good separation was achieved by employing *n*-hexane–chloroform (1:1) in the first direction, followed by water–tetrahydrofuran (1:1) in the second direction.

Effect of the ionic strength. Lithium chloride was preferred to the sodium chloride generally used with RP pre-coated plates[4,14,15], in view of its good solubility in the aqueous–organic mixtures tested as mobile phases. Problems arose only with tetrahydrofuran: demixing was observed above 40% water. However, in the presence of lithium chloride no demixing was noted. In the case of acetonitrile–aqueous lithium chloride (1:1), the limiting concentration of lithium chloride was 0.5 *M*. Furthermore the R_F values of rifamycins on C_{18} pre-coated plates with acetonitrile–aqueous lithium chloride had to be discarded as spots of irregular shape were obtained, whereas in the absence of the salt the chromatographic development was not possible[3]. Table VI lists the R_F values of rifamycins obtained by employing various mixtures of water–organic modifiers (methanol, 2-propanol, acetonitrile and tetrahydrofuran) at increasing concentration of lithium chloride and at a constant water–organic modifier ratio (1:1). In the case of methanol the ratio 8:2 was also tested. Comparison of the R_F values of rifamycins in water–organic modifiers with the corresponding values in aqueous lithium chloride–organic modifier as mobile phases showed that the addition of lithium chloride significantly increased the retention on d-PH layers, but to different extents depending on the compound and on the eluent. Furthermore it is noteworthy that a more distinct increase in the retention was evident at low salt concentration (0.025–0.1 *M*) and that only small differences in the R_F values were obtained by increasing the salt concentration from 0.1 to 1 *M*. Similar results were obtained by substituting lithium chloride with ammonium sulphate.

As regards the steroids, the addition of a lithium chloride aqueous solution to methanol, 2-propanol and acetonitrile has a negligible effect on their R_F values.

The data suggest that on d-PH pre-coated plates, upon addition of inorganic salts to the eluent the retention of the test solute may vary or not depend principally on the solutes under examination. However the results obtained with the rifamycins on the d-PH pre-coated plates is of particular interest: the addition of an inorganic salt to the mobile phase may improve the separation of the spots to a considerable extent as reported for the first complete TLC separation of three underivatized catecholamines[14].

TABLE VI

RETENTION DATA, hR_F, FOR RIFAMYCINS ON DIPHENYL (a) AND OCTADECYL (b) PRE-COATED PLATES IN AQUEOUS–ORGANIC ELUENTS AT VARIOUS CONCENTRATIONS OF LITHIUM CHLORIDE

Eluent	*Rifamycin* 1*		*Rifamycin 2*		*Rifamycin 3*		*Rifamycin 4*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Methanol–water (8:2)	97	50	97	70	96	54	97	68
–0.05 *M* LiCl (8:2)	74	40	87	68	74	54	85	68
–0.1 *M* LiCl (8:2)	71	37	85	68	65	54	81	67
–1 *M* LiCl (8:2)	60	31	75	58	57	50	71	54
Methanol–water (5:5)	42	6	61	24	29	6	47	16
–0.05 *M* LiCl (5:5)	10	0	28	0	10	0	14	0
–0.1 *M* LiCl (5:5)	5	0	22	0	8	0	11	0
–1 *M* LiCl (5:5)	3	0	14	0	10	0	6	0
2-Propanol–water (5:5)	85	62	90	74	86	74	91	71
–01. *M* LiCl (5:5)	55	41	74	74	70	74	70	71
–0.5 *M* LiCl (5:5)	50	40	70	68	68	74	67	65
–1 *M* LiCl (5:5)	47	37	65	69	66	73	60	64
–2.5 *M* LiCl (5:5)	44	34	64	60	67	74	61	57
–5 *M* LiCl (5:5)	46	33	61	57	65	76	57	59
Acetonitrile–water (5:5)	74	–	87	–	79	–	84	–
–0.05 *M* LiCl (5:5)	28	–	82	–	65	–	65	–
–0.1 *M* LiCl (5:5)	20	–	60	–	68	–	50	–
–0.5 *M* LiCl (5:5)	20	–	54	–	67	–	50	–
Tetrahydrofuran–water (5:5)	87	30	85	80	86	70	88	72
–0.025 *M* LiCl (5:5)	40	19	56	57	58	55	52	54
–0.1 *M* LiCl (5:5)	35	20	54	57	60	57	54	50
–0.5 *M* LiCl (5:5)	26	21	45	55	56	53	40	50
–1 *M* LiCl (5:5)	18	14	38	43	54	52	36	40

* Numbering as in Fig. 1.

Characteristics of diphenyl plates

As for C_{18} pre-coated plates from the same source[3,4], the d-PH phase became detached from the glass support when eluents with high water contents were employed. However, a higher percentage of water (about 60%) than previously reported for C_{18} (35–40%) may be employed. The addition of lithium chloride to the mobile phase, successfully employed with the C_{18} plates[3], suffices to extend the application range at 90% of water in methanol, acetonitrile, tetrahydrofuran and 2-propanol mixtures, with a drastic and very convenient decrease in the development time (from 20–25 min to a few minutes). Nevertheless, in some cases, the addition of the inorganic salt may affect the retention of the test solutes, as was pointed out above. No damage to d-PH pre-coated plates was observed on employing strongly acidic or basic eluent mixtures, *e.g.*, methanol–0.1 *M* hydrochloric acid (6:4) or methanol–0.1 *M* sodium hydroxide (6:4); it is important to note that with respect to column HPLC, the range of eluent pH was widened, mainly to basic values.

From a comparison of the R_F values reported in Tables I and II it appears that when neat organic solvents are used as eluents, rifamycins and steroids, respectively,

may be more strongly retained on d-PH then on C_{18} plates, depending on the mobile phase employed. However, there are few differences in the selectivity patterns of steroids between these two types of plates, so that the retention order among these compounds changed little. As regards the rifamycins, the same retention order on both types of plates was also observed, except in the case of rifamycins, the only one in the quinone form, when higher chain alcohols were employed as mobile phases.

As shown in Table VI, the addition of lithium chloride to the mobile phase, studied with methanol and 2-propanol as the organic modifiers, has a larger effect on the R_F values of the rifamycins on d-PH than on the C_{18} layers. In the case of tetrahydrofuran as eluent, the effects of salt addition were comparable.

CONCLUSIONS

The data suggest that, on the d-PH TLC plates, two different retention mechanisms may operate, depending on the mobile phase used: interactions between the solutes and the surface silanol groups or the aryl groups of the stationary phase. Indeed, the first retention mechanism seems to be prevalent when employing as mobile phase the more lipophilic solvents, like chloroform and dichloromethane, and the second when using some alcohols and aqueous–organic mixtures.

As regards the use of d-PH bonded silica gel pre-coated plates, they may be employed with all twelve organic solvents tested and with binary mixtures containing up to 60% of water. Replacing the water in the eluent with lithium chloride solutions, mobile phases containing up to 90% of water may be employed and the development time decreased drastically. The addition of lithium chloride to the mobile phase increased the rifamycin retention, but did not significantly affect the steroid retention. It appeared that, depending on the solutes under examination, the addition of an inorganic salt to the eluent may affect spot resolution and improve the separation.

These dual retention mechanisms related to the choice of mobile phase and the possibility of varying the retention by adding an inorganic salt may be useful for the separation of mixtures of varying complexity by means of two dimensional TLC, an analytical technique that is becoming increasingly important[20]. Indeed in the present work the seven steroids examined were separated by two-dimensional chromatography.

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