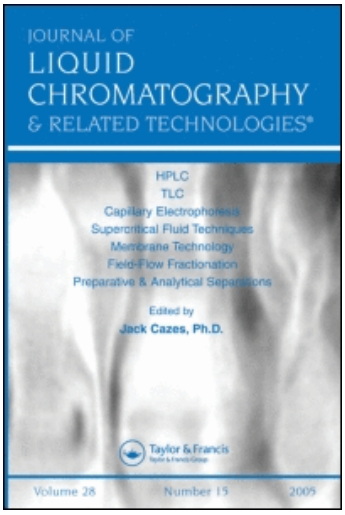

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APPLICATIONS OF A MODIFIED "ISOHYDRIC SOLVENT SYSTEM" IN HPLC ON SILICA GEL FOR THE ANALYSIS OF THE MACROLIDE ANTIBIOTICS TURIMYCINS AND SPIRAMYCINS

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ABSTRACT

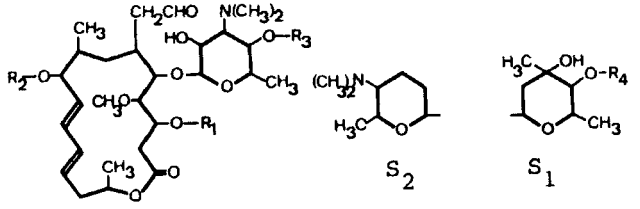
The behaviour of some macrolide antibiotics in high-performance liquid chromatography on silica gel using an isohydric eluent system and its modifications are described. These modifications involve water content and diethylamine content variations in the eluent, and also column temperature variations, using column packing material previously described. Extention to other types of silica gel packing materials was also performed. The experiments showed excellent results for the separations of the macrolide antibiotic complexes Turimycins and Spiramycins and proved that the chromatographic process is not only an adsorption one but that partitioning effects also play an important role.

INTRODUCTION

The problem of separation of various macrolide antibiotics is frequently encountered. In several instances, the efficiencies of the separations by high-performance thin-layer chromatography (H.P.T.L.C.) and high-performance liquid chromatography (H.P.L.C.) are not satisfactory due to the similar structures and large molecular weights of these components.

Table I

Structure of Spiramycin and Turimycin Macrolide Antibiotics



Name	Abbreviated name	R_1	R_2	R_3	R_4
Spiramycin 1	Sp 1	H	S_2	S_1	H
2	Sp 2	$COCH_3$	S_2	S_1	H
3	Sp 3	$COCH_2CH_3$	S_2	S_1	H
Turimycin H_2	H_2	H	H	S_1	$COCH_3$
H_3	H_3	H	H	S_1	$COCH_2CH_3$
H_4	H_4	H	H	S_1	$COCH_2CH_2CH_3$
H_5	H_5	H	H	S_1	$COCH_2CH(CH_3)_2$

The introduction of the isohydric solvent system theory by Thomas et al. (1-4) as an all-round eluent system on silica gel, prompted us to investigate the application of this theory to the separation of two macrolide antibiotic complexes; Turimycins and Spiramycins (5-10) (Table I).

During these investigations it became clear that several chromatographic parameters could be improved to a great extent by modifying the original isohydric solvent system.

EXPERIMENTAL

High-Performance Liquid Chromatography

A Varian 4100 liquid chromatograph or a Spectra Physics SP 8000 liquid chromatograph was used, equipped with a variable wavelength detector (Varichrom, Varian, Palo Alto, Ca, USA), set at 232 nm.

The temperature of the column was controlled using a waterbath (Varian) or a heated air oven (Spectra-Physics). Samples, dissolved in the eluent, were injected using a six way Valco valve with a sample loop of 20 mm^3.

Reagents and Materials

- Di-isopropylether, methanol and iso-octane were of analytical grade (Merck, Darmstadt, G.F.R.)
- Diethylamine (U.C.B., Brussels, Belgium) and water were destilled in an all-glass apparatus prior to use
- Column packing materials :
  - Spherosil XOA-600 (5 µm)(Prolabo, Paris, France), spherical porous silica gel, specific surface area, 580 m^2/g, mean pore diameter 90 Å
  - Lichrosorb Si 60 (5 µm)(Merck, Darmstadt, G.F.R.) irregular silica gel, specific surface area, 500 m^2/g, mean pore diameter 60 Å
  - Lichrospher Si 100 (5 µm)(Merck, Darmstadt, G.F.R.) spherical porous silica gel, specific surface area 250 m^2/g, mean pore diameter 100 Å
  - Spherisorb S 5W (5 µm)(Phase Separations, Queensferry, U.K.) spherical porous silica gel, specific surface area 220 m^2/g, mean pore diameter 80 Å
- Columns (150 mm x 4.6 mm I.D., stainless-steel tubing with 2 µm porosity frits) were filled by means of a slurry technique (slurry : 2 g silica gel in 15 ml carbontetrachloride:methanol (80:20), methanol as pressuri-

zingsolvent, upward filling).

- Spiramycins : bulk powders and pure components were obtained from S.P.E.C.I.A. (Paris, France)
- Turimycins : bulk powders and pure components were obtained from Dr. Fricke, Forschungszentrum für Molekularbiologie und Medizin, Jena,(G.D.R.)

RESULTS AND DISCUSSION

A normal isohydric eluent system is a combination of two isohydric solvent mixtures, one apolar and the other polar in nature (1,3). The original isohydric eluent used in our experiments consisted of the apolar mixture A[Di-isopropylether:iso-octane (500:500)] and the polar mixture B [Di-isopropylether:methanol:water (500:474:26)]. To suppress the ionization of the basic functions in the macrolide molecules, 0.2 % diethylamine was added to each combination of A and B.

A first trial for the separation of the Turimycin and the Spiramycin group was made by plotting the capacity factors of the various antibiotics as a function of the inverse of the molar water fraction, for different concentrations of mixture B in the eluent. As pointed out by Thomas et al., there was a linear relationship between the capacity factors and the inverse of the molar water fraction in the eluent. A representation of this phenomenon for the Spiramycins is given in figure I . A mixture of about 70 to 80 per cent A and 30 to 20 per cent B showed the best results for the separation of Spiramycins and Turimycins. Figure II gives as an example, the separation of Turimycins on 80A-20B . To improve the resolution between Turimycins H_4 and H_5 and the Spiramycins 2 and 3, the effects of water content and diethylamine concentration of the eluent were studied and also the effect of column temperature variation, as these showed to be important to improve the column efficiency.

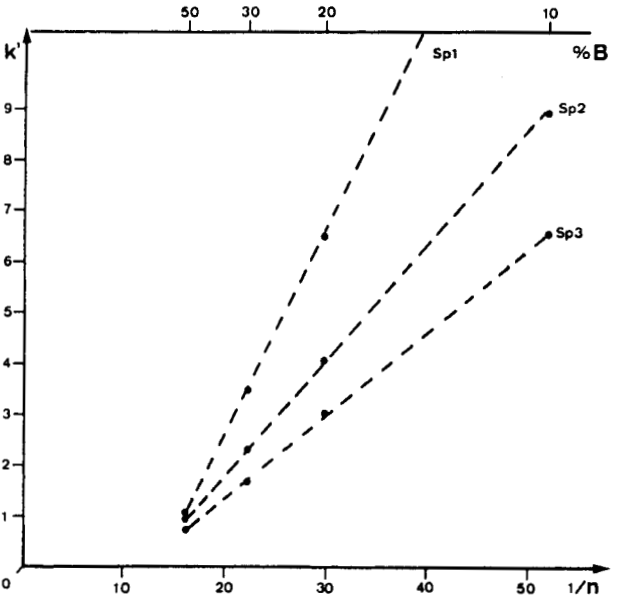


FIGURE I

Capacity factors of Spiramycins as a function of the inverse of the molar water fraction in the eluent (mixture B with 2.6% H_2O).

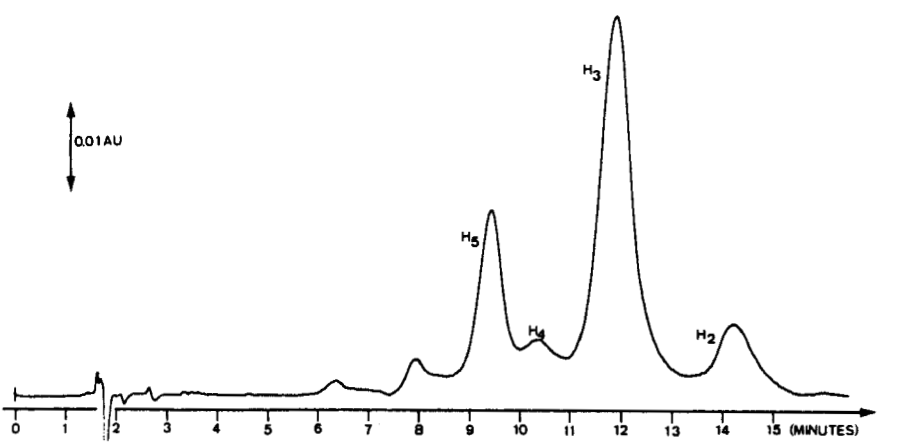


FIGURE II

Separation of components of a Turimycin bulk powder (H_5, H_4, H_3 and H_2, X : unknowns)

Column : Spherosil XOA 600, 5 µm, 150 mm x 4.6 I.D.
Eluent : 80A-20B with 0.2 % diethylamine (B with 2.6 % H_2O)
Flow : 1.0 $ml.min^{-1}$
Detection : 232 nm
Temperature : ambient

Variation of the water content of mixture B

When using an isohydric eluent system as described (1), the activity of the adsorbent is equal to one (α_a = 1) by definition. Normally, to be isohydric, mixture B should contain 2.604 % of water and mixture A a negligable amount of 0.004 % of water. By altering the amount of water in mixture B, the activity of the adsorbent is also changed. To study this effect on the different macrolide antibiotics, the water content of mixture B was changed in following steps : 0 %-0.65 %-1.3 %-2.6 % (α_a = 1)-3.9 % and 4.55 % water in mixture B. At 5.2. % water in mixture B, demixing was noticed when adding A and B together at a ratio of 70 A and 30 B.

In contradiction with the adsorption theory of Thomas et al., the capacity factors of the Turimycins and Spiramycins did not decrease, but they showed an increase with increasing water content of mixture B. This effect can be seen in figure III, where capacity factors of

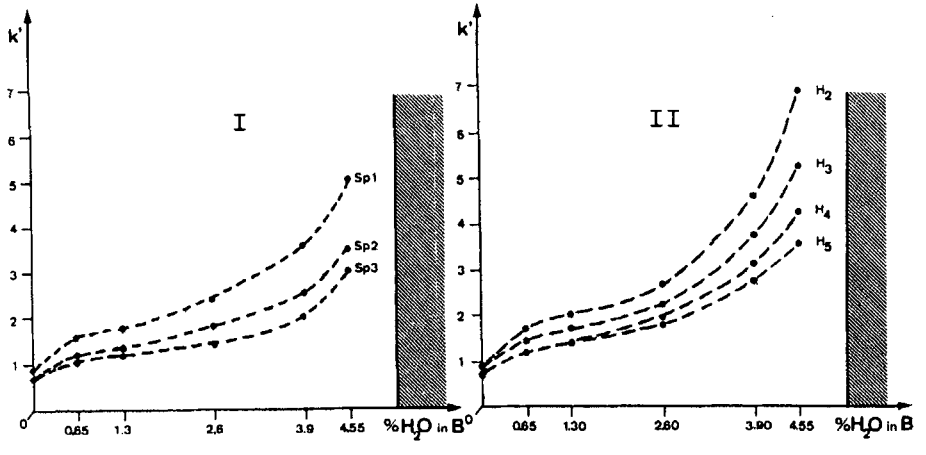


FIGURE III

Capacity factors (k') of Spiramycins (I) and Turimycins (II) as a function of the water content of mixture B (Eluent: 70A-30B, with 0.2% diethylamine)

TABLE II

Column-efficiencies (N) and Resolutions (R_S) for Spiramycins and Turimycins as a Function of the Percentage of Water in Mixture B

% Water in mixture B	N Sp 1	R_S Sp 2 - Sp 3	N H_3	R_S H_4 - H_5
0 %	-	-	-	-
0.65 %	1496	0.77	1491	-
1.30 %	2561	1.25	2567	-
2.60 %	2127	1.38	3570	0.56
3.90 %	1702	1.53	2976	1.27
4.55 %	1364	1.71	2376	1.58

Turimycins and Spiramycins are given as a function of the water content in mixture B at the same proportion (70:30) of mixtures A and B and with the same amount of organic base (0.2 % diethylamine) in the eluent. From table II one may also notice that the efficiency of the column was maximal for 2.6 % water in mixture B for the Turimycins (calculated on Turimycin H_3) and for 1.3 % water in mixture B for the Spiramycins (calculated on Spiramycin 1). Nevertheless, the resolutions between Turimycins H_4 and H_5 and Spiramycins 2 and 3 were optimal for 4.55 % water in mixture B. This improvement in resolution with less efficiency is the result of the important contribution of the capacity factor and selectivity terms in the equation of the resolution :

$$R_S = \frac{1}{4} \sqrt{N} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k'}{k' + 1}\right)$$

An example of this effect is shown in figure IV where chromatograms are given of the separations of Turimycins at 2.6 % water and 4.55 % water in mixture B.

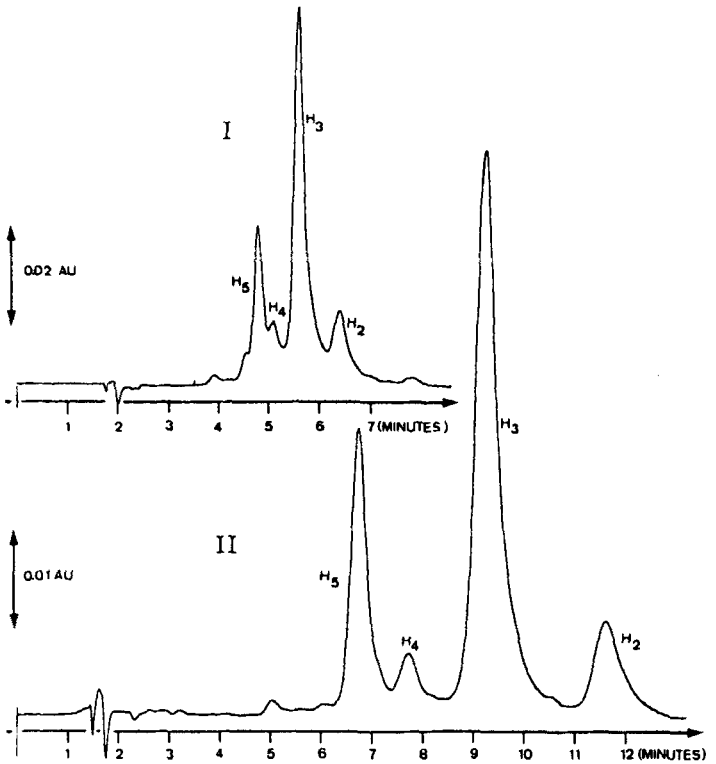


FIGURE IV

Influence of the water content of mixture B on the separation of Turimycins.
Column : Spherosil XOA 600, 5 µm, 150 mm x 4.6 mm I.D.
Eluent : 70A-30B (B with 2.60 % H_2O (I) and with 4.55 % H_2O (II)), with 0.2 % diethylamine
Flow : 1.0 $ml.min^{-1}$
Detection : 232 nm
Temperature : ambient

Variation of the diethylamine concentration in the eluent

The influence of the concentration of organic base in the eluent on capacity factors, efficiency and resolution was studied, by adding different amounts of diethylamine to the original isohydric mixture (70A-30B; 2.6 % water in B). The effect on the capacity factors of Turi-

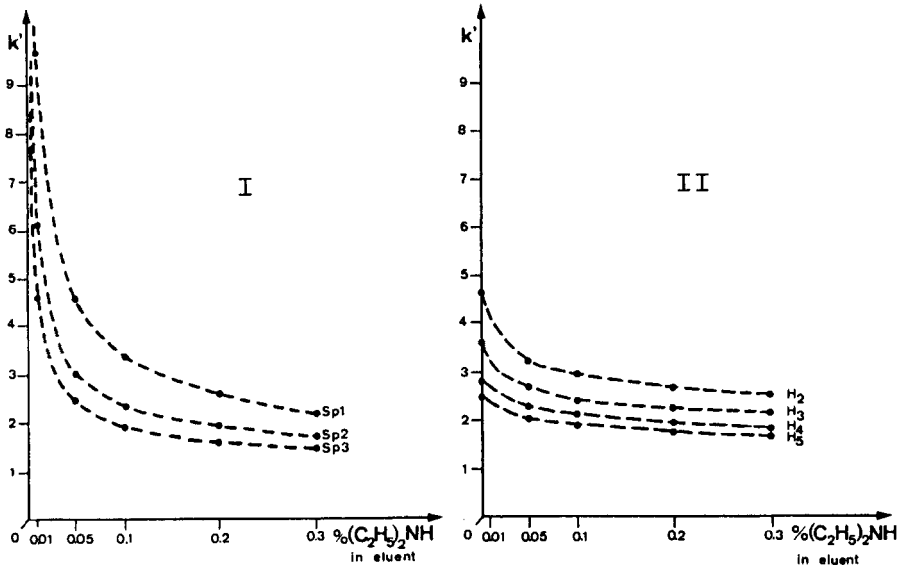


FIGURE V

Influence of diethylamine concentration in the eluent on the capacity factors (k') of Spiramycins (I) and Turimycins (II) (Eluent : 70A:30B, with 2.6 % H_2O in B)

mycins and Spiramycins are given in figure V. From this figure one can see that the Spiramycins do not elute from the column when there is no diethylamine in the eluent, due to their supplementary basic function in the forosamine sugar moiety (S_2; see table I), while Turimycins elute within a reasonable time. In table III it can be seen that the resolution is not maximal for concentrations of diethylamine for which column efficiency is maximal (0.05 % diethylamine in the eluent), due to a great contribution of the capacity factors in the equation of the resolution. Figure VI represents the separations of Spiramycins at 0.3 % and 0.05 % diethylamine in the eluent, showing extreme differences in efficiencies for slight differences in base concentrations.

TABLE III

Column-efficiencies (N) and Resolutions (R_S) for Spiramycins and Turimycins as a Function of the Percentage of Diethylamine in the Eluent

% Diethylamine in the eluent	N Sp 1	R_S Sp 2 - Sp 3	N H_3	R_S H_4 - H_5
0 %	-	-	3414	-
0.01 %	2920	2.75	3677	1.35
0.05 %	3830	2.54	4820	1.29
0.1 %	3292	2.05	3910	1.06
0.2 %	2328	1.46	3011	0.83
0.3 %	1072	0.95	2228	0.57

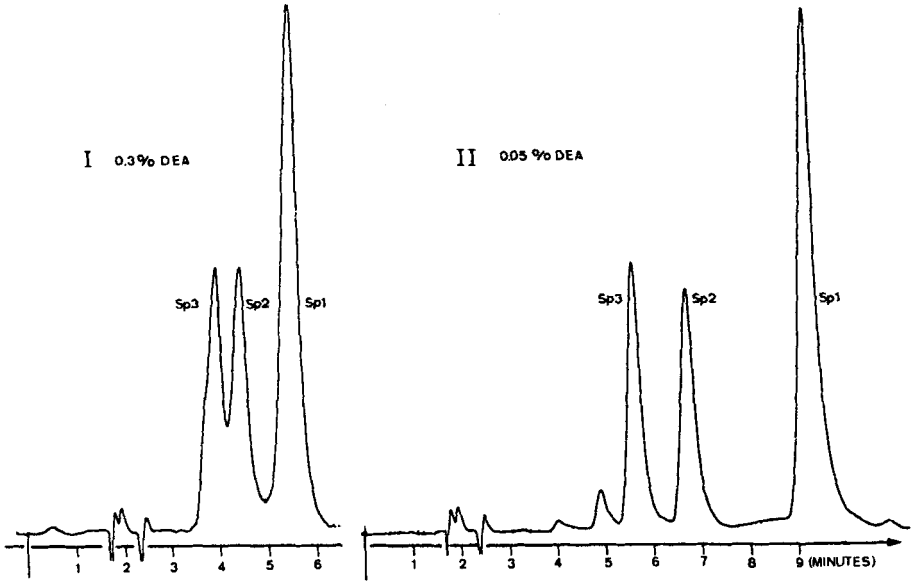


FIGURE VI

Influence of the diethylamine concentration of the eluent on the separation of Spiramycin antibiotics (Eluent 70A:30B; B with 2.6 % H_2O)

I : 0.3 % D.E.A.
II : 0.05 % D.E.A.
Flow : 1.0 ml.min^{-1}
Detection : 232 nm
Temperature : ambient

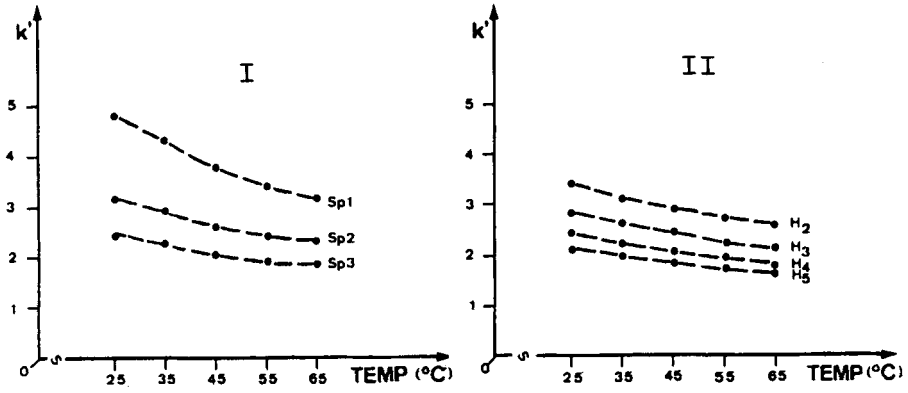


FIGURE VII

Influence of column temperature on the capacity factors (k') of Spiramycins (I) and Turimycins (II). Eluent : 70A:30B, with 0.05 % diethylamine (B with 2.6 % H_2O)

Variation of column temperature

The effect of column temperature variation on the separation of the macrolide antibiotic complexes was investigated using the original isohydric eluent system, i.e. the combination 70 A - 30 B, whereas B contained 2.6 % water, but with 0.05 % diethylamine. As this is already a partially optimized system (0.05 % diethylamine instead of 0.2 % originally), the results of temperature variation were not as spectacular as for water- and diethylamine content variation (Figure VII). Capacity factors slightly decreased with increasing column temperatures but the selectivities for the pairs :

$$\text{Sp 2- Sp 3} \left(\alpha^{\text{Sp 2}}_{\text{Sp3}} = \frac{k' \text{ Sp 2}}{k' \text{ Sp 3}}\right) \text{ and}$$

$$\text{H}_5\text{-H}_4 \left(\alpha^{\text{H5}}_{\text{H4}} = \frac{k' \text{ H5}}{k' \text{ H4}}\right) \text{ remained practically unchanged.}$$

In table IV it can be seen that there is an optimum at 35°C for Turimycins and for Spiramycins at higher tem-

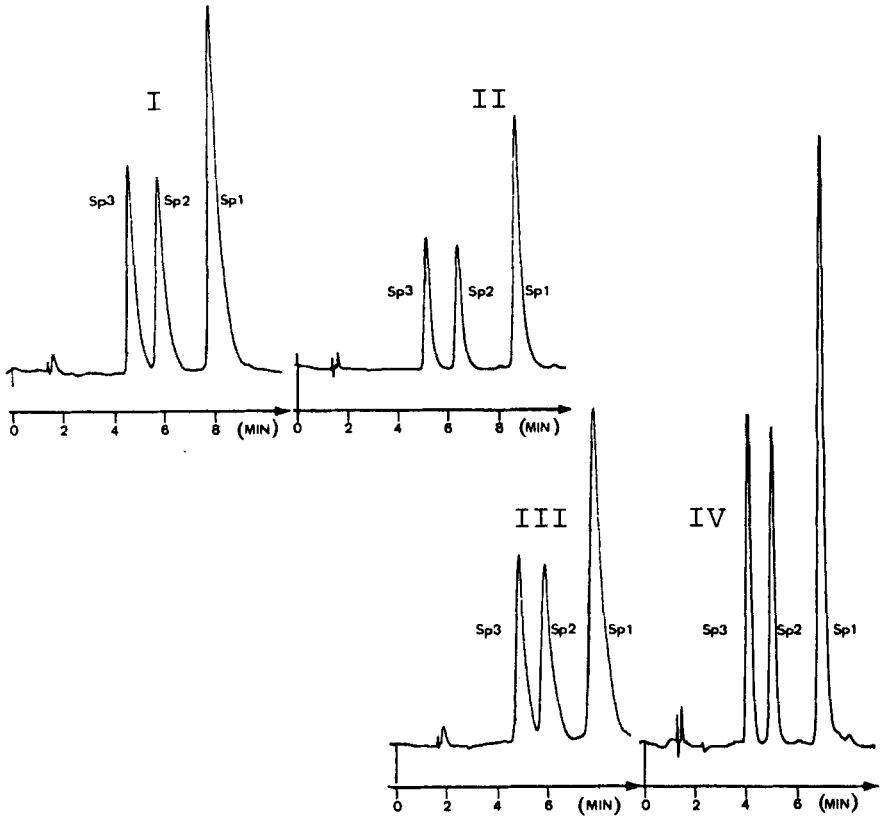


FIGURE VIII

Separation of Spiramycins on four types of silica gel columns.
Eluent : 70A-30B with 0.05 % diethylamine (B with 4.55 % H_2O)
Flow : 1.0 ml.min^{-1}
Detection : 232 nm
Temperature : 45°C
Columns : 150 mm x 4.6 mm I.D.

I : Lichrosorb Si60
II : Spherosil XOA 600
III : Lichrospher Si100
IV : Spherisorb S-5-W

TABLE IV

Column-efficiencies (N) and Resolutions (R_s) for Spiramycins and Turimycins as a Function of the Column Temperature

Column temperature	N Sp 1	R_s Sp 2 - Sp 3	N H_3	R_s H_4-H_5
25°C	3127	2.39	3761	1.07
35°C	3930	2.49	4815	1.19
45°C	4900	2.85	4715	1.15
55°C	5841	2.88	4769	1.13
65°C	6776	2.88	4615	1.09

peratures, but the resolution was satisfactory even at lower temperatures.

Effect of the silica gel adsorbent

With an optimized eluent system, consisting of 70A - 30B (with 4.55 % water in mixture B and 0.05 % diethylamine in the total) and a column temperature of 45°C, four types of silica gel were compared to investigate the contribution of their specific surface area or their pore diameter on the separation of the group of Spiramycins. Excellent separations were obtained with the different types, but a relationship between the capacity factors and pore diameters or specific surfaces could not be fount (Figure VIII).

CONCLUSION

Using a modified isohydric eluent system, Turimycin and Spiramycin macrolide antibiotics are well separated and both adsorption- and partition effects are

observed. When plotting k'-values againtst the percentage of water in mixture B, one may notice that the curvature obtained has the same slope as the water adsorption isotherm of silica gel (11) (Figure III).

Two hypotheses can be put forward. The first one is that capacity factors (or relative retentions on the column) of Spiramycins and Turimycins are increasing with increasing water content of mixture B, due to a partitioning process. In the second hypothesis, one may believe that the increased water content of mixture B (and hence increased mass of water on the silica gel surface), decreases the mass of diethylamine adsorbed on the silica gel surface. As a result of this decreased diethylamine concentration, the retention times of Spiramycins and Turimycins increase as evidenced by the data obtained when the diethylamine concentration was varied. (see : Variation of the diethylamine concentration in the eluent).

The first hypothesis was stated by following experiments : as only Turimycins did elute from the column at 0 % diethylamine in the eluent (see : Variation of the diethylamine concentration in the eluent), Turimycins H_5 and H_3 were used to check their behaviour with different water concentrations in the eluent at 0 % diethylamine. As can be seen from table V, where capacity factors of Turimycins H_5 and H_3 are given as a funtion of the percentage of water in mixture B at 0 % diethylamine, the capacity factors increased with increasing water content of the eluent, indicating a partitioning process.

Nevertheless, this may not exclude the fact that diethylamine may be washed out at higher water levels in the eluent and produce higher retentions of the macrolides.

TABLE V

Capacity factors of Turimycins H_5 and H_3 as a Funtion of the Percentage of Water in Mixture B at 0 % Diethylamine in the Eluent

% Water in mixture B	k' H_5	k' H_3
0 %	1.39	2.28
0.65 %	2.02	3.53
1.30 %	2.15	3.97
2.60 %	2.49	4.67
3.90 %	2.97	5.93
4.55 %	4.06	8.33

A water layer is build up on the silica gel surface and instead of adsorption chromatography on a highly deactivated adsorbent of which the activity is kept constant, mixed mechanisms of adsortion and partition take place.

Temperature effects are of minor influence, when using very low amounts (0.05 %) of organic base in the eluent, but they may contribute to a great extent to the efficiency of the column when using up to 0.3 % of organic base in the eluent. This can be explained by a lower uptake of base by the silica gel at higher temperatures as low organic base concentrations give very high plate numbers for the column.

Although the isohydric solent theory was based on the use of one particular silica gel (Spherosil XOA 600) other silica gels may be used, resulting in simular separation possibilities.

For the moment, the same experiments are performed in our laboratory on a great number of basis drugs and

the use of these isohydric systems in high-performance thin-layer chromatography on silica gel is also under investigation. Results will be presented in a next paper.

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