CHROM. 3992

PARTITION DATA OF PENICILLINS DETERMINED BY MEANS OF REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

G. L. BIAGI, A. M. BARBARO, M. F. GAMBA AND M. C. GUERRA
Istituto di Farmacologia e Farmacognosia dell'Università di Bologna (Italy)
(First received December 10th, 1968; revised manuscript received January 11th, 1969)

SUMMARY

The R_M values of 11 penicillins were measured by means of a reversed-phase thin-layer chromatographic method with various concentrations of acetone in the mobile phase.

R_M values were calculated by interpolation or extrapolation from the ranges of linearity between R_M values and composition of the mobile phase. The influence of substituent groups on the R_M values of the penicillins and disagreement with $\Sigma\pi$ values are pointed out.

INTRODUCTION

The partition coefficient is one of the most important factors in controlling the interaction of drugs with biological systems[1].

HANSCH *et al.*[2-6] found very good structure–activity correlations by using a substituent constant π. This was defined as log (K_B/K_A) where K_A and K_B are the octanol–water partition coefficients of a parent compound A and its derivative B, differing by a substituent group X. Thus the constant π indicates the change in the logarithm of the partition coefficient resulting from the introduction of the substituent group X. The π values for many substituent groups were directly measured by HANSCH *et al.*[4,5,7] for a variety of drugs and used in order to calculate the partition coefficient function $\Sigma\pi$ of other compounds. However, as pointed out by HANSCH *et al.*[6], the calculated $\Sigma\pi$ values cannot be a complete substitute for the experimental determination of the partition coefficient because of possibile group interactions. BIRD AND MARSHALL[8] found some anomalies in the $\Sigma\pi$ values of penicillins when they were compared with their experimental partition coefficients. However, in order to avoid the practical difficulties often presented by the direct determination of the partition coefficient, BOYCE AND MILBORROW[9] suggested the use of the chromatographic R_M value ($R_M = \log(1/R_F - 1)$). This was shown to be related to the logarithm of the partition coefficient between the mobile and stationary phase of a chromatographic system[10-12].

In a previous paper we showed that it was possible to find, by means of a reversed phase thin-layer chromatographic method, very good correlations between the R_M values and the biological activity of bis-dichloroacetamides and vitamin K

analogues[13]. Preliminary results obtained in structure–activity studies with antibiotics have already been reported[14].

The purpose of the present work is to show that reversed-phase thin-layer chromatography is a suitable technique for the determination of partition data for penicillins.

MATERIALS AND METHODS

Glass plates measuring 20 × 20 cm were coated with Silica Gel G in the usual manner[15]. A stationary non-polar phase was obtained by impregnating the Silica Gel G layer with Silicone DC 200 (350 cS) from Applied Sciences Laboratories. The impregnation was carried out by developing the plates in a 5 % silicone solution in ether. Eight plates could be impregnated, in a single chromatographic chamber, containing 200 ml of the silicone solution. The plates were left in the chamber for 12 h, that is for several hours after the silicone solution had reached the top of the plates. This method of impregnating Silica Gel G layers has also been used by ROOMI *et al.*[16] with silicone oil, by BOYCE AND MILBORROW[9] with liquid paraffin and by several other investigators as reported by KIRCHNER[17]; furthermore it has been recommended by ANKER *et al.*[18] for impregnating Silica Gel G layers.

Previous to this, immersion was tried, but it was concluded that immersion is much more apt to cause damage to the thin layer, which was also noted by KIRCHNER[17]. In a few experiments the glass plates were coated with Kieselguhr G (Merck). This is particularly indicated for partition chromatography because it should act as an inert adsorbent[19,20]. The sequence of the penicillins from the most hydrophilic to the most lipophilic did not change. This was considered evidence that there is no interaction between adsorbent and compounds. However when the Kieselguhr G layers were impregnated in the same way as those of Silica Gel G all the compounds migrated too close to the solvent front, even if the mobile phase was only water. When the Kieselguhr G layers were impregnated with higher amounts of silicone oil the R_F values of the penicillins were lower. It was therefore evident that by adjusting the degree of impregnation it would be possible to obtain R_F values similar to those measured on Silica Gel G layers.

In order to avoid the "edge effect" and uneven migration of the solvent front, the silicone impregnated layer was cut 2 cm from each lateral edge and the chromatographic chamber was saturated with the mobile phase vapor as indicated by STAHL[15].

A migration of 10 cm was obtained on all the plates by cutting the layer at 12 cm and spotting the compounds on a line 2 cm from the lower edge of the plate. The mobile phase saturated with silicone was an aqueous buffer (sodium acetate–Veronal buffer at pH 7.4) alone or mixed with various quantities of acetone. Two plates were developed simultaneously in a chromatographic chamber containing 200 ml of mobile phase. The following penicillins were studied: benzylpenicillin, phenoxymethylpenicillin, methicillin, nafcillin, oxacillin, chloxacillin, dichloxacillin, phenethicillin, ampicillin, methylenampicillin, carboxybenzylpenicillin.

The experiments were carried out with the sodium or potassium salts and/or the free acids. The compounds were dissolved in distilled water or acetone (3 mg/ml) and 1 μl of solution was spotted on the plates in randomized allocations in order to avoid any systematic error. The developed plates were dried and sprayed with an alkaline

solution of potassium permanganate. After a few minutes at 120°, yellow spots appeared on an intensely pink background. The penicillins could also be identified by spraying with the iodine azide solution[15]. On a yellow background white spots appeared. The R_M values were calculated by means of the formula:

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

RESULTS

The spraying of the developed plates with potassium permanganate or iodine–azide solutions resulted in the appearance of round spots at different distances from the starting line (Fig. 1). Tailing was only detectable in the case of methylenampicillin.

The data obtained in the present work are partially reported in Table I. Negative and positive R_M values derive from R_F values respectively greater and smaller than 0.5. Higher and/or positive R_M values indicate compounds more lipophilic than those represented by a lower and/or negative R_M value.

The means of the experimental R_F values are plotted in Fig. 2 against the composition of the mobile phase. It can be seen that the R_F values increase with the acetone concentration in the mobile phase for each compound. However above

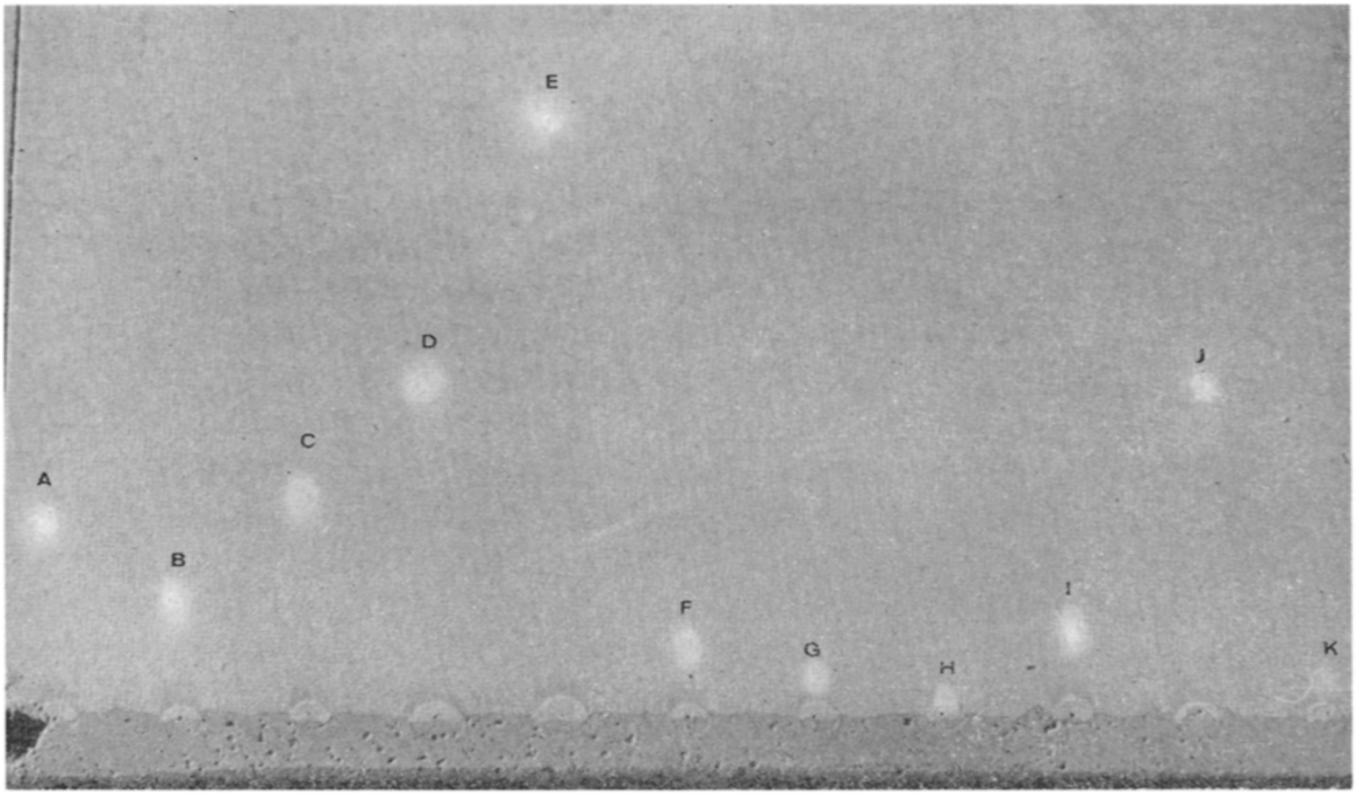


Fig. 1. Reversed-phase TLC of penicillins. From the left to the right: (A) Benzylpenicillin; (B) Phenoxymethyl penicillin; (C) Methicillin; (D) Ampicillin; (E) Carboxybenzylpenicillin; (F) Oxacillin; (G) Chloxacillin; (H) Dichloxacillin; (I) Phenethicillin; (J) Methylenampicillin; (K) Nafcillin. Stationary phase: silicone oil on Silica Gel G layer; mobile phase: acetone–buffer 2%. Detection: iodine–azide solution. Amounts: 3 μg of each compound.

TABLE I

THE MEANS OF THE R_M VALUES TOGETHER WITH THEIR STANDARD ERROR

The R_M values calculated from the R_F values obtained with 30, 40 and 50% acetone in the mobile phase are not included because they are clearly out of the range of linearity.

Acetone concentration in the mobile phase (%)	*Compounds*				
	Benzyl-penicillin	*Methicillin*	*Nafcillin*	*Phenoxymethyl-penicillin*	*Methylen-ampicillin*
0	0.59 ± 0.01	0.55 ± 0.01	1.47 ± 0.03	0.93 ± 0.02	−0.24 ± 0.01
2	0.38 ± 0.03	0.31 ± 0.01	1.26 ± 0.05	0.71 ± 0.04	−0.53 ± 0.02
4	0.28 ± 0.02	0.13 ± 0.02	1.06 ± 0.03	0.60 ± 0.03	−0.61 ± 0.03
6	0.11 ± 0.01	−0.07 ± 0.01	0.81 ± 0.02	0.42 ± 0.02	−0.80 ± 0.03
8	0.06 ± 0.01	−0.15 ± 0.02	0.75 ± 0.02	0.34 ± 0.01	−0.78 ± 0.02
10	−0.07 ± 0.01	−0.27 ± 0.01	0.60 ± 0.01	0.21 ± 0.01	−0.85 ± 0.02
12	−0.24 ± 0.02	−0.47 ± 0.02	0.42 ± 0.03	0.03 ± 0.03	−0.86 ± 0.01
14	−0.28 ± 0.02	−0.53 ± 0.02	0.35 ± 0.01	0.01 ± 0.02	−0.89 ± 0.01
16	−0.33 ± 0.01	−0.59 ± 0.02	0.22 ± 0.00	−0.10 ± 0.01	−0.91 ± 0.01
18	−0.40 ± 0.02	−0.63 ± 0.02	0.14 ± 0.02	−0.19 ± 0.02	−0.87 ± 0.06
20	−0.51 ± 0.02	−0.73 ± 0.01	−0.00 ± 0.03	−0.29 ± 0.02	−0.84 ± 0.06
22	−0.44 ± 0.05	−0.73 ± 0.04	−0.05 ± 0.01	−0.33 ± 0.01	−0.81 ± 0.03
24	−0.61 ± 0.01	−0.82 ± 0.02	−0.15 ± 0.01	−0.42 ± 0.01	−0.83 ± 0.03

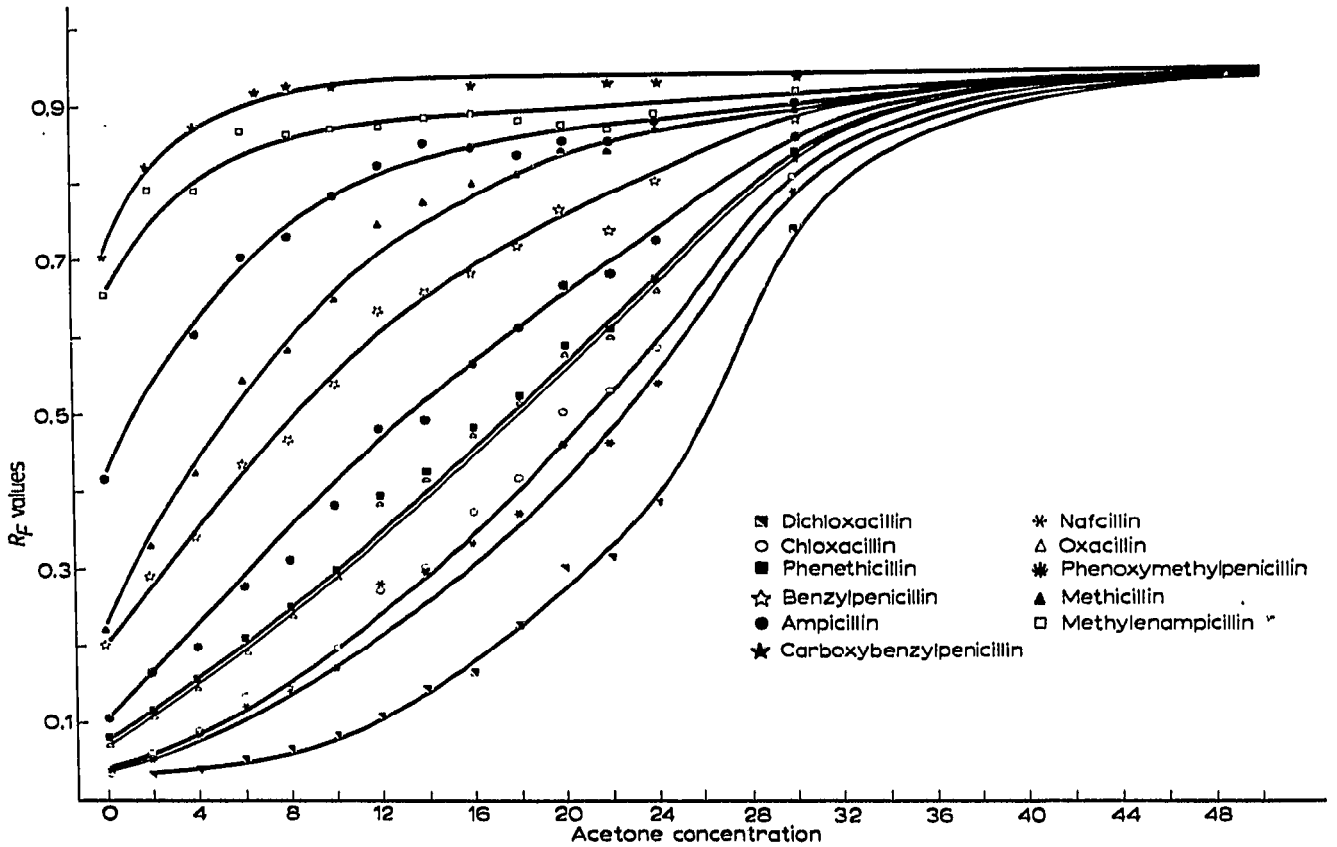


Fig. 2. The R_F values of the penicillins tested are plotted against the composition of the mobile phase. Each point represents the mean of 8–12 determinations. Some experiments carried out with the penicillin free acids instead of the sodium or potassium salts gave identical results. In some other experiments mobile phases at pH 2.6 and 5.0 instead of 7.4 were used. The migration of the compounds did not show any significant difference. The R_F values corresponding to 40 and 50% acetone in the mobile phase were not reported because of the overlapping of the curves.

picillin	*Chloxacillin*	*Dichloxacillin*	*Phenethicillin*	*Oxacillin*	*Carboxybenzyl-penicillin*
,14 ± 0.01	1.36 ± 0.02		1.11 ± 0.02	1.10 ± 0.02	−0.37 ± 0.03
,05 ± 0.01	1.18 ± 0.05	1.51 ± 0.06	0.88 ± 0.03	0.91 ± 0.04	−0.66 ± 0.06
,18 ± 0.02	1.02 ± 0.03	1.39 ± 0.03	0.75 ± 0.03	0.77 ± 0.03	−0.83 ± 0.04
,37 ± 0.04	0.86 ± 0.01	1.25 ± 0.02	0.57 ± 0.01	0.61 ± 0.01	−1.03 ± 0.07
,43 ± 0.04	0.78 ± 0.02	1.16 ± 0.03	0.49 ± 0.02	0.50 ± 0.02	−1.08 ± 0.05
,56 ± 0.06	0.69 ± 0.01	1.04 ± 0.02	0.37 ± 0.02	0.40 ± 0.01	−1.09 ± 0.02
,66 ± 0.03	0.41 ± 0.02	0.90 ± 0.04	0.18 ± 0.02	0.20 ± 0.03	−1.10 ± 0.03
,75 ± 0.04	0.39 ± 0.02	0.76 ± 0.02	0.13 ± 0.02	0.14 ± 0.01	−1.09 ± 0.01
,73 ± 0.02	0.30 ± 0.02	0.70 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	−1.09 ± 0.02
,69 ± 0.03	0.23 ± 0.01	0.53 ± 0.01	−0.03 ± 0.01	−0.03 ± 0.02	−1.10 ± 0.03
,75 ± 0.03	0.07 ± 0.01	0.36 ± 0.02	−0.13 ± 0.02	−0.16 ± 0.02	−1.11 ± 0.04
,73 ± 0.04	0.06 ± 0.05	0.33 ± 0.01	−0.17 ± 0.01	−0.19 ± 0.02	−1.14 ± 0.03
,86 ± 0.02	−0.06 ± 0.02	0.20 ± 0.01	−0.30 ± 0.02	−0.28 ± 0.01	−1.14 ± 0.02

a certain acetone concentration they tend to migrate with the solvent front. The most hydrophilic compounds, carboxybenzylpenicillin and methylenampicillin, are the first to reach a maximum R_F value. On the other hand, at 0 % acetone in the mobile phase the most lipophilic compounds remained close to the origin; dichloxacillin did not move at all. The transformation of the R_F values into R_M values allowed us to obtain the plots shown in Fig. 3. These show that for each compound there is a linear relationship between the R_M values and the composition of the mobile phase over a particular range of acetone concentration. The straight lines in Fig. 3 were calculated by means of the least squares method from the R_M values in the linearity range. Accordingly, the straight lines were fitted with the R_M values obtained with mobile phases containing up to a maximum of 12–16 % acetone. In the case of methylenampicillin and carboxybenzylpenicillin only 4 and 5 data points, corresponding up to a maximum respectively of 6 and 8 % acetone in the mobile phase, were used. Higher acetone concentrations gave R_M values outside the linearity range, as it can be seen in Fig. 3 especially for the lower compounds.

According to BOYCE AND MILBORROW[9], the R_M values in the linearity range were considered to be satisfactory; over this range there are maximum increments of R_M for each compound and among different compounds.

In order to obtain a R_M value for each compound in a standard system, where all of them could be compared, the theoretical R_M values at 0 % acetone in the mobile phase were calculated by interpolation or extrapolation from the linear part of the curve.

The calculations were carried out by means of the equations of the straight lines of Fig. 3. In this way it was possible to calculate the R_M value for dichloxacillin in a silicone oil–water system as well.

The calculated R_M values are reported in Table II. These show the influence of

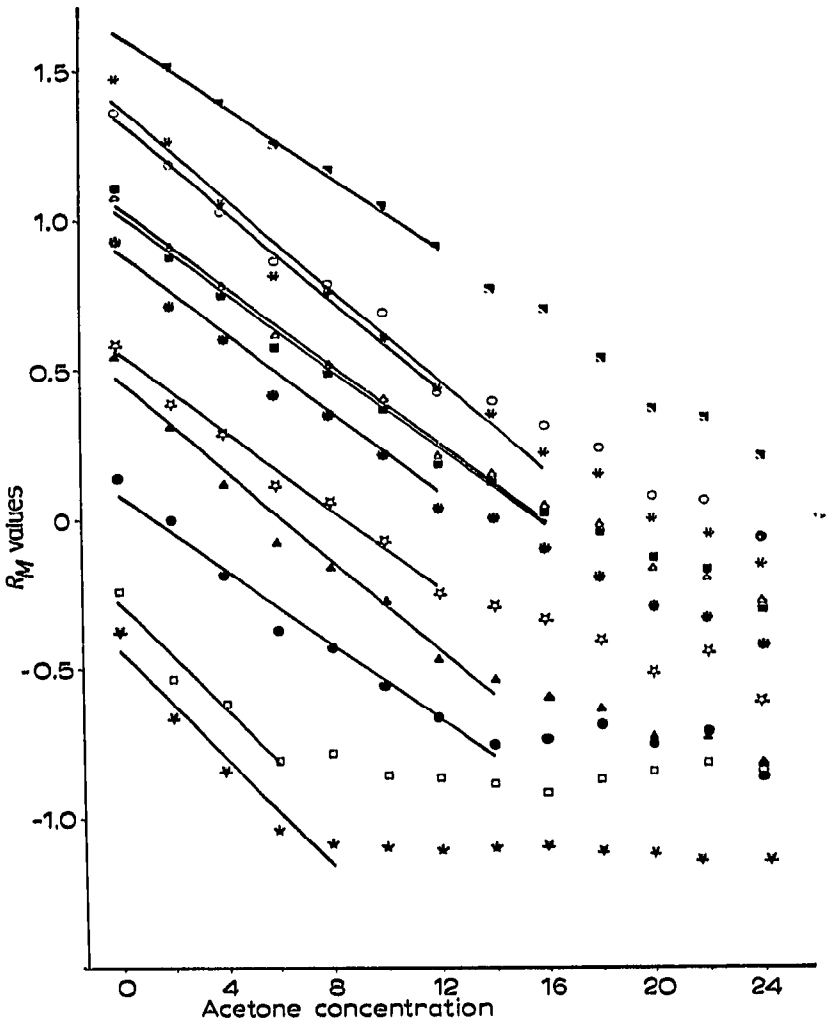


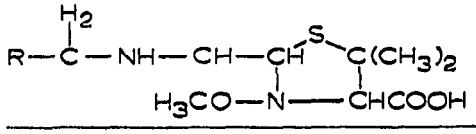
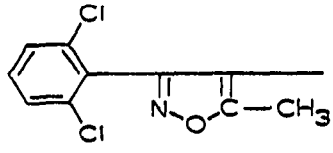
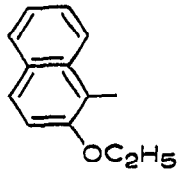
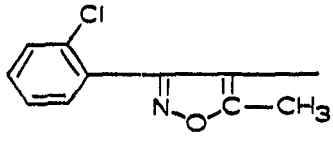
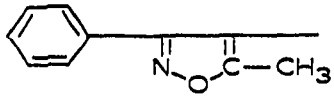
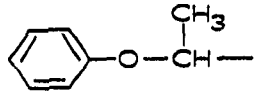
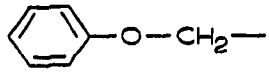
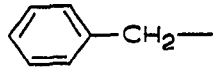
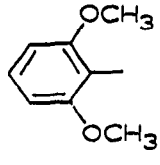
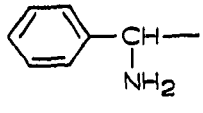
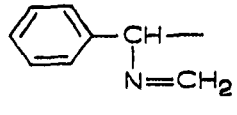
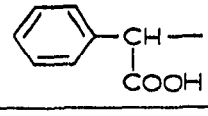
Fig. 3. The R_M values are plotted against the composition of the mobile phase. The straight lines were calculated by means of the least squares method. Each point represents the mean of 8–12 values. The R_F values corresponding to 30, 40, 50% acetone in the mobile phase are not reported. The penicillins are marked as in Fig. 1.

the substituent groups on the partition coefficients of the penicillins. The introduction of Cl atoms into the aromatic ring of oxacillin causes an increase in lipid-solubility. This is manifested by the increased R_M values of chloxacillin and dichloxacillin. The introduction of a CH_3 group into the side chain of phenoxymethylpenicillin explains the increased R_M value of phenethicillin. On the other hand methicillin becomes more hydrophilic than benzylpenicillin as a result of the absence of the benzylmethylene group and the introduction of OCH_3 groups in the 2- and 6-positions. An increase in water-solubility also results from the introduction of NH_2, $N = CH_2$ and COOH groups in the side chain of benzylpenicillin. This also holds for ampicillin, methylenampicillin and carboxybenzylpenicillin.

The $\Sigma\pi$ values calculated by BIRD AND MARSHALL[8] for six of the penicillins tested in the present work are also reported in Table II. It can be seen that the calculated $\Sigma\pi$ values for benzylpenicillin indicate a relative lipid-solubility greater than that expressed by the R_M value.

TABLE II

LIST OF THE PENICILLINS ACCORDING TO THE DECREASING LIPOPHILIC CHARACTER OF THEIR MOLECULES AS EXPRESSED BY THEIR (EXTRAPOLATED) R_M VALUES AND SOME $\Sigma\pi$ VALUES

Compound	 R	R_M	$\Sigma\pi$
Dichloxacillin		1.62	
Nafcillin		1.39	3.54
Chloxacillin		1.34	
Oxacillin		1.05	
Phenethicillin		1.03	2.61
Phenoxymethylpenicillin		0.89	2.11
Benzylpenicillin		0.55	2.69
Methicillin		0.47	1.47
Ampicillin		0.07	0.84
Methylenampicillin		−0.28	
Carboxybenzylpenicillin		−0.46	

DISCUSSION

The data of the present work show in the first place that there is a range of linear relationship between the R_M values of penicillins and composition of the mobile phase. SOCZEWINSKI AND WACHTMEISTER[21] were the first to demonstrate this relationship by using phenolic compounds and a mobile phase consisting of various concentrations of dimethylsulfoxide in water. They also pointed out that this relationship is valid only provided that the solvent mixtures do not deviate markedly from ideal solutions. A similar relationship was also found by BOYCE AND MILBORROW[9] with N-*n*-alkyltritylamines and aqueous acetone as mobile phase. The practical importance of this relationship is that it allows the calculation, in the range of maximum accuracy, of theoretical R_M values for each tested compound in a chosen standard system. The effect of substituent groups on the partition coefficients of the penicillins are in agreement with the results obtained by HANSCH *et al.*[22,23], and BIRD AND MARSHALL[8]. They used the partition coefficient function $\Sigma\pi$ and were able to show the hydrophobic character of substituents such as the CH_3 group in the side-chain of phenoxymethylpenicillin or Cl atoms in the *para* positions of the aromatic ring. They also pointed out the increase in hydrophilic properties resulting from the introduction of OCH_3 and NH_2 groups in the aromatic ring or side chain, respectively, and from the subtraction of a benzylmethylene group. As regards the disagreement between the R_M and $\Sigma\pi$ value of benzylpenicillin, it is important to note that BIRD AND MARSHALL[8] also found a discrepancy between the $\Sigma\pi$ values of benzylpenicillin and the logarithm of its experimental octanol–water partition coefficient. This suggests that it is possible to obtain, by reversed phase TLC, results which agree with those achieved by the direct measurement of the partition coefficient between two immiscible phases.

BOYCE AND MILBORROW (9) indicated several practical advantages of the measurement of the R_M values over the direct determination of the partition coefficients. It is also pointed out that the use of silicone as stationary phase in the method described offers the possibility of detecting the compounds with unspecific reagents such as alkaline potassium permanganate or chromic–sulfuric acid.

Finally as regards the suitability of the silicone oil–aqueous acetone system for measuring R_M values for correlation with biological activity, some comment is necessary.

COLLANDER[24–26] pointed out that the nature of the phases should not affect the results in a qualitative sense. He supported this with thermodynamic considerations and found that ether–water and olive oil–water partition coefficients are equally correlated with penetration into Nitella cells. In addition, IWASA *et al.*[27] showed that there is a very good correlation between the π values and the ΔR_M values of phenols as calculated by means of HANSCH's method and reversed-phase chromatography, respectively. In that particular case the two phases of the chromatographic system were triethylene glycol and diisopropyl ether. This supports COLLANDER's[26] finding that the logarithms of the partition coefficients of a compound in two different sets of solvents are linearly related. BOYCE AND MILBORROW found a parabolic relationship between the biological activity of N-*n*-alkyltritylamines and the R_M values obtained with a stationary phase represented by liquid paraffin and a mobile phase constituted of acetone–water 70%. In preliminary work with the present silicone oil–aqueous acetone system, a good correlation between R_M values and biological activity[13,14] was

found. On the other hand it should be pointed out that the calculated R_M values in Table II correspond to a mobile phase without acetone.

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