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REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY AND PARTITION COEFFICIENTS OF PENICILLINS

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SUMMARY

R_M values for eight penicillins have been measured by a reversed-phase thin-layer chromatography system using *n*-octanol as the stationary phase. There is a near perfect linear relation between the R_M values measured at pH 3 or pH 4 and the logarithm of the partition coefficients of the penicillin free acids between *n*-octanol and water.

The change of R_M with change in pH of the developing buffer is close to a theoretical value for measurements at pH 3 and 4, but it is too low for measurements at pH 4 and 5.

Attention is drawn to an unusually low HANSCH π value for the methyl group of phenethicillin.

INTRODUCTION

Several biological properties of penicillins have been shown[1-3] to be quantitatively or qualitatively related to their partition coefficients. BIAGI *et al.*[1] have used R_M values from a thin-layer chromatographic (TLC) system for correlation of antibiotic properties. BIRD AND MARSHALL[2] used calculated HANSCH π values[4] and, to a lesser extent, measured partition coefficients, for correlation of the extent of serum binding of many penicillins.

BIAGI *et al.*[5] have recently presented R_M results from their system developed with buffers of different pH and suggested that such studies might provide a model system for investigating penetration of penicillins through biological membranes. We show in this paper that BIAGI's results are not in accord with theory and we present an alternative reversed-phase TLC system for penicillins and compare results from it with measured partition coefficients for the same set of compounds.

EXPERIMENTAL

Thin-layer chromatography

Glass plates, 20 × 20 cm, were coated with a 0.5 mm layer of Camag microcrystalline cellulose (67.5 g to 330 ml water) using a motorised spreader. The plates were dried at room temperature overnight, heated in an oven at 110° for 1.5 h, cooled

on the bench for 10 min and then impregnated with *n*-octanol by developing in a 10% solution of *n*-octanol in acetone for 1 h in a chromatographic chamber previously equilibrated with the octanol solution. The *n*-octanol was purified by successive washing with dilute HCl, water, dilute NaOH and water before distillation. Acetone was allowed to evaporate from the plates on the bench for 15 min after removing them from the chamber.

Spots (1 μl) of the penicillin solutions (2 mg/ml in water) were then applied and the plates were placed in the developing buffer 30 min after removal of the plates from the octanol solution. The buffer was a 0.5 *M* solution of 6-aminohexoic acid adjusted to the chosen pH with HCl and saturated with *n*-octanol. The chromatographic chamber was equilibrated with buffer solution in the normal way. Development was continued until the solvent front reached the 10-cm mark (about 2 h). The wet plates were placed in a tank saturated with ammonia vapour for 15 min, dried, sprayed with 10% acetic acid in acetone and then with starch iodine solution. The penicillins appear as white spots on a blue background.

Partition coefficients

The partition coefficients of the penicillin free acids between *n*-octanol and water were determined by a modification of BRÄNDSTRÖM's method[8]. This is a rapid titrimetric method in which a neutralised aqueous solution of the sample is adjusted to a chosen pH to give a reasonable concentration of unionised sample, the organic solvent is added and the vigorously-stirred mixture is kept at the chosen pH by titration, while equilibration of sample between the phases occurs. The partition coefficient is calculated from the volumes of the phases and titrant used and the weight of sample taken. BRÄNDSTRÖM used the method only for amines, but we have applied it to acids using HCl as the titrant. Eqn. 1 has been derived for acids in the same way as BRÄNDSTRÖM's eqn. 10 for bases.

$$P = \frac{WV_0V_2}{SV_1((V_0 - V_1 - V_2))} \qquad (1)$$

where:

P is the partition coefficient of the organic acid;

W is the volume of aqueous phase at equilibrium;

V_0 is the volume of HCl (molarity m) equivalent to the weight of sample acid used;

V_1 is the volume of HCl added to the neutral sample solution to obtain the working pH;

V_2 is the volume of HCl added to maintain the pH during equilibration. This volume is corrected by deduction of the volume found in a blank experiment, without sample;

S is the volume of *n*-octanol used.

If the working pH is below 5, volumes V_1 and V_2 must be corrected by deduction of the volume of acid required to adjust the relevant volume of water alone to that pH.

The corrected volumes are:

$$V_1' = V_1 - \frac{10^{-pH}((W'' + V_1))}{m}$$

$$V_2' = V_2\left(1 - \frac{10^{-pH}}{m}\right)$$

where W' is the initial volume of the aqueous phase.

Trial experiments with the method indicated that accurate neutralisation of the initial sample solution was critical for obtaining reliable results and caused considerable difficulty. This step can be eliminated by including the ionisation constant (K_a) of the sample acid in the equation, as follows. The expression for K_a after addition of V_1 ml HCl is

$$K_a = \frac{[H^+]\,(V_0 - V_1)}{V_1}$$

Thus

$$V_1 = \frac{V_0}{1 + \frac{K_a}{[H^+]}} = \frac{V_0}{\alpha}$$

Substituting for V_1 in eqn. 1 gives

$$P = \frac{WV_2\alpha}{S\left[V_0\left(\frac{\alpha - 1}{\alpha}\right) - V_2\right]} \qquad (2)$$

Eqn. 2 can be used in two ways. If K_a is known P can be calculated direct. If K_a is not known then both K_a and P can be determined by a graphical method using measurements at two or more pH values. Several values of P are calculated from the measurements at any one pH, using various trial values of K_a. A plot of P against K_a gives a curve the shape of which varies with the pH at which the measurements were made. The point of intersection of these curves gives the P and K_a values for the sample. Measurements are needed at only two pH values provided these are chosen so that the lines intersect approximately at right angles. The partition coefficients for penicillins reported here were determined using eqn. 4, either graphically or by direct calculation with a pK_a of 2.72.

RESULTS AND DISCUSSION

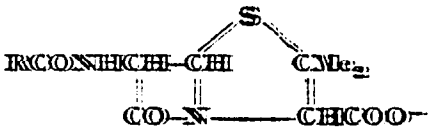
The mean log P values and R_M values for measurements at pH's 3, 4 and 5 are given in Table I. The TLC system gave clearly defined round spots with no tailing. Regression analysis by the method of least squares gives the following equations:

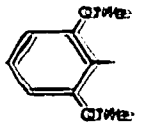
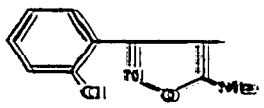
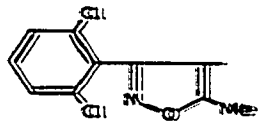
		r	
pH 3	$R_M = 1.035 \log P - 1.892$	0.997	(3)
pH 4	$R_M = 1.036 \log P - 2.628$	0.998	(4)
pH 5	$R_M = 0.861 \log P - 2.719$	0.973	(5)

where r is the correlation coefficient.

TABLE II

THE LOG P, R_M AND ΔR_M VALUES FOR EIGHT PENICILLINS



Penicillin	R	log P[a]	R_M[b]			ΔR_M	
			pH 3	pH 4	pH 5	pH 3–4	pH 4–5
Methicillin		1.13	—0.60	—1.34	[c]	0.74	—
Benzyl	$PhCH_2$—	1.76	0.09	—0.66	—1.21	0.75	0.55
Phenoxymethhyl	$PhOCH_2$—	2.01	0.34	—0.37	—0.87	0.71	0.50
Phenethicillin	PhOCH(Me)—	2.19	0.50	—0.23	—0.95	0.73	0.72
Propicillin	PhOCH(Et)—	2.58	0.97	0.21	—0.52	0.76	0.73
Oxacillin		2.34	0.57	—0.15	—0.71	0.72	0.56
Cloxacillin		2.44	0.71	0.07	—0.64	0.70	0.65
Dicloxacillin		2.83	1.18	0.44	—0.23	0.74	0.67

[a] P was determined using eqn. 2 and the graphical method with measurements at pH's 3 and 4, except for oxacillin and dicloxacillin where measurements were at pH 4 only and a pK_a of 2.72 was used in eqn. 4. The graphical method gave pK_a values of 2.70–2.72 for all six penicillins to which it was applied. This is in good agreement with literature values[8] from potentiometric titration. The log P values for the first five penicillins listed differ from those reported previously[2]. The weight, rather than the volume, of *n*-octanol was used in calculation of the latter results[2] and we subsequently realised that most literature values of P are calculated with w/v concentrations.

[b] Each R_M is the mean of between 5 and 10 spots on 3 to 5 plates. Each plate had at least one spot of each penicillin.

[c] At this pH methicillin ran very close to the solvent front so that an accurate R_M value could not be obtained.

Both R_M and P have been measured with *n*-octanol as the organic phase so that in an ideal system the slope of these correlation lines would be exactly 1.000. The results at pH 3 and 4 show a slope quite close to one and a near perfect correlation. We have no explanation of the slight deviation of the slope from one. Possibly it is related to the different concentrations of salts in the aqueous phases used for the R_M and P measurements or to some interaction of the penicillins with the cellulose in the TLC system. The results at pH 5 show considerably more scatter than those

at the lower pH values. This is probably due to the difficulty of making accurate measurements of the more closely-spaced spots at this pH (all seven penicillins had R_F values between 0.6 and 0.95). Such inaccuracies are probably at least a partial cause of the considerably lower slope at pH 5. However, some of the scatter is not due to experimental inaccuracies. Thus at pH 5 phenethicillin has a lower R_M than phenoxymethylpenicillin. This is contrary to what is expected, because introduction of a methyl group should increase hydrophobic character, and it is the opposite of the results at pH 3 and 4. The result at pH 5 was checked many times with different samples of the two penicillins. We can think of no explanation of this anomaly.

The ΔR_M values (*i.e.* the difference between R_M values for the same penicillin at two pH values) in Table I show a near constant value for pH 3–4 with a mean of 0.73, and a much more variable value for pH 4–5 with a mean of 0.63. A theoretical value of ΔR_M can be calculated as follows. For an acid of ionisation constant K_a,

$$R_M = R'_M - \log\left(1 + \frac{K_a}{[H^+]}\right) \qquad (6)$$

where R_M is the value observed on a reversed-phase thin-layer chromatogram developed with a buffer of hydrogen ion concentration $[H^+]$; and R_M' is the pH independent value of R_M for the unionised form of the acid.

Eqn. 6 is derived in the same way as an analogous equation for bases given by BÜCHI AND FRESEN[7], assuming that the ionised acid is insoluble in the organic phase.

ΔR_M values can be calculated from eqn. 6 as follows:

$$\Delta R_M = (R_M \text{ at } pH_1) - (R_M \text{ at } pH_2) = -\log\left(1 + \frac{K_a}{[H^+]_1}\right) + \log\left(1 + \frac{K_a}{[H^+]_2}\right) \qquad (7)$$

To a first approximation the ionisation constants of all penicillins without an ionising group in the side-chain are the same, so ΔR_M should be a constant for measurements at any pair of pH values. An average value for K_a of penicillins of $1.9 \cdot 10^{-3}$ (pK_a of 2.72) has been found in this and other[8] work. With this value for K_a, eqn. 7 gives a ΔR_M of 0.84 for pH 3–4 and 0.98 for pH 4–5. Thus the observed ΔR_M values are too low. There are two obvious possible explanations of these discrepancies. Either the effective pH of the TLC system is not the same as the bulk pH of the developing buffer or the assumption in the derivation of eqn. 6 is not valid. We do not have enough evidence to choose between these possibilities.

Derivation of eqn. 6 assumes that only the unionised form of the penicillin partitions into the octanol phase. There may be some solution of ionised penicillin in the octanol as an ion pair, either with the buffer cation or with a metal cation. It seems likely that the effect of such behaviour would become more obvious as the pH increases because a small amount of penicillin dissolved as an ion pair would have a greater relative effect at higher pH values where the concentration of penicillin in the octanol is lower. Thus this effect would probably be in the correct direction to account for the greater discrepancy between observed and theoretical ΔR_M values as the pH increases.

The alternative explanation of the discrepancies concerns the effective pH of

the TLC system during development. Throughout our work we have checked some developed plates for the presence of pH variations by spraying with universal indicator or with other indicators chosen to show a pH change in the region of interest. With the cellulose–aminohexoic acid system the indicators gave a very uniform colour over the entire plate, apart from a very small zone near the solvent front. This was the main reason for our choice of the aminohexoic acid buffer system. When *n*-octanol-coated cellulose plates were developed with citrate or phosphate buffers, definite pH gradients were observed. Nevertheless, this check does not provide absolute confirmation that the pH during development is exactly that of the developing buffer. Consequently an effect of the thin layer on the buffer pH cannot be eliminated as a possible cause of the ΔR_M discrepancies. This effect would not need to be very large, at least for the pH 3 and 4 measurements where an actual pH difference of 0.9 unit would give a calculated ΔR_M of 0.73, in agreement with the observed value.

BIAGI *et al.*[5,9] give R_M values for ten penicillins on a silicone oil-coated Silica Gel G layer developed with a sodium acetate–veronal buffer at pH 2.6, 7.4, and 9.4. The mean ΔR_M values are 0.22 for pH 2.6–7.4 and 0.31 for pH 2.6–9.4. The corresponding theoretical values from eqn. 7 are 4.43 and 6.43 respectively. Thus this system exhibits behaviour which is very far from ideal. This is very probably due to the use of Silica Gel as the solid support. BÜCHI AND FRESEN[7] state that sodium ions can interchange with hydrogen ions on silica gel plates and they recommended cellulose as a more inert support. The very low ΔR_M values found by BIAGI *et al.* suggest that the effective pH during development has not changed anywhere near so much as the bulk pH of the buffer; *i.e.* the silica gel exerts a buffering effect on the system. By spraying with indicators we have observed pH gradients, or fronts where the pH changes, on silica gel plates coated with *n*-octanol or silicone oil and developed in various buffers at various pH values. These results, and the low ΔR_M values of BIAGI *et al.*, indicate that silica gel is not a suitable support for TLC studies where development at a known pH is important.

A desirable feature of a TLC system for measurement of R_M values for use in structure–activity correlations is that it should be capable of covering a wide range of hydrophobic character in the samples. This can be achieved in the system presented here by development at various pH values to give convenient R_F values. Results for the set of compounds under investigation can then be made comparable by calculating the R_M values appropriate to a single pH by the use of measured ΔR_M values. However, this TLC system does not give meaningful results from the very lipophobic penicillins, ampicillin and carbenicillin (α-amino- and α-carboxy-benzylpenicillins, respectively). These compounds ran very close to the solvent front at all pH values studied. This is a realistic result because the presence of an ionised group in the side-chain makes it very unlikely that any significant amount of these penicillins would partition in to the octanol phase. BIAGI *et al.*[5,9] obtained measurable R_M values from these two penicillins. We feel that there must be some doubt as to whether this indicates a genuine partition in to the organic phase in view of our results and the presence of an ionised group in the side-chain.

The π values which can be deduced from the measurements reported here require some comment. The HANSCH substituent constant π is defined[4] as $\log P_x - \log P_H$, where P_x and P_H are the partition coefficients between *n*-octanol and water of the substituted and parent compounds, respectively.

Thus π for the methyl group is 0.18 from phenethicillin and phenoxymethylpenicillin and 0.39 from propicillin and phenethicillin. These values, especially the former, are low compared with the value of 0.5 which has been found[10] for the methyl or methylene group in very many compounds. Possibly the geometry of the penicillin molecule is such that the methyl group is partly shielded from the solvent and so cannot exert its full hydrophobic effect. The only other methyl group π values below 0.4 that we know of were reported[11] for some benzothiadiazine derivatives. Cloxacillin gives a π value for the ortho chlorine of 0.10 while the second ortho chlorine in dicloxacillin, has a π of 0.39. These values are low compared with those reported[4,12] for chlorine in *o*-chlorophenol, anilline and phenoxyacetic acid. However, we have found that π for chlorine in *o*-chloronitrobenzene is 0.40. This is a more meaningful standard for comparison because both the nitro and 3-isoxazolyl groups are electron withdrawing.

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