Interactions between Amines and Phospholipids: A Chromatographic Study on Immobilized Artificial Membrane (IAM) Stationary Phases at Various pH Values

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The chromatographic capacity factors (log k') for 23 amines were measured by High Performance Liquid Chromatography (HPLC) on a stationary phase composed of phospholipids, the so-called 'Immobilized Artificial Membrane' (IAM). The chromatographic behaviour of the compounds, which consist of primary, secondary, and tertiary amines, and compounds with endocyclic amino functions, was studied with eluents at various pH values (7.0, 5.5, and 3.0). The results were compared both to the octanol/buffer partition values of neutral forms (log P) and to those of mixtures of neutral and ionised forms, existing at the three pH values above mentioned (log $D^{7.0}$, log $D^{5.5}$, and log $D^{3.0}$). At pH 7.0, the log k' of all secondary and tertiary amines overlapped with those previously observed for neutral isolipophilic compounds. This behaviour was also observed for primary amines, but only for compounds fully ionised at this pH. In contrast, the partially ionised primary amines at pH 7.0 and the compounds with an endocyclic amino function both showed stronger interactions with phospholipids than expected on the basis of log P. The changes in retention observed with eluents at pH 5.5 indicated that retention varies with the ionisation degree of the analytes. At pH 3.0, the interaction between phospholipids and the ionised forms of the amines considered was impaired probably by a change in the charges on the IAM surface. The present study indicates that phospholipids are a partitioning phase that better accommodates the neutral forms of primary amines than does octanol. Moreover, the phospholipid phase is much less sensitive to the ionisation of analytes than octanol, but only at pH 7.0 and 5.5; indeed, the ionised forms of all the amines considered are retained to the same extent as expected for hypothetical neutral isolipophilic compounds. We can thus conclude that, for amines, the partition scale in phospholipids is distinct from the one in octanol.

Introduction. – The ability to cross biological membranes strongly affects the pharmacokinetic behaviour of drugs and their capability to access the receptor site. At present, the reference parameter for the prediction of passive diffusion through these biological barriers is the measure of lipophilicity, expressed as the logarithm of the partition coefficient between octanol and an aqueous phase [1]. When the partition of neutral forms only is considered, it is indicated as 'log P'. For ionisable compounds, the term 'log D' can be used to indicate the lipophilicity values actually displayed by the mixture of the ionised and neutral forms existing at the experimental pH.

Although both log P and log D have been recognised in Quantitative Structure-Activity Relationship (QSAR) studies to effectively correlate with various biological phenomena, these parameters have often proven inadequate to predict the partition of ionised compounds (both acids and bases) in biological membranes [2][3]. This could be due to the inadequacy of the octanol partition system to mimic the complex

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interactions between biomembranes and the ionised forms of the compounds. Therefore, since the main constituents of biomembranes are phospholipids, the use of the latter as the partitioning phase can be expected to yield *in vitro* data that better mimic the *in vivo* interactions between drugs and biomembranes.

Recently, the partition of a solute between phospholipids and an aqueous phase can also be measured by the HPLC determination of the chromatographic capacity factor, k', on the so-called Immobilized Artificial Membrane (IAM) stationary phases [4]. These phases are composed of lecithin monolayers (phosphatidylcholine), wherein each lipid molecule is covalently linked to propylamine/silica; unreacted propylamine moieties can be end-capped with methyl glycolate (IAM.PC.MG).

The chromatographic capacity factor, k', is linearly related to K, the equilibrium partition coefficient of a solute that partitions between the stationary phase and the mobile phase; thus:

$$k' = (V_s/V_m)\ K$$

where V_m is the total volume of solvent within the HPLC column, and V_s is the volume of the IAM interphase created by immobilised phospholipids [5]. The phase ratio V_s/V_m in this equation is constant for a given IAM column, making the difficult experimental measurement of V_s unnecessary.

Data pertaining to the partition on phospholipids obtained by the IAM-HPLC method have been reported to be distinctive from log P measures [6]. In our previous work [7–9], we determined the log k' values on IAM for 42 compounds (including both neutral and ionised molecules) measured with (or extrapolated to) a completely aqueous eluent at pH 7.0 ($\log k_{7.0}$). While, for neutral compounds, we found that the scale of log $k_{7.0}$ values on IAM correlated well with the scale of log P, a quite surprising chromatographic behaviour on IAM was observed for ionised compounds. In fact, in most cases, their retention was found to depend on log P values instead of log $D^{7.0}$. In other words, the log $k_{7.0}$ values for ionised compounds overlapped with those of hypothetical neutral compounds with the same log P (isolipophilic compounds). Moreover, among the amines considered in our previous work, amlodipine, tocainide (the two primary amines), and W36017 (a scarcely lipophilic tertiary amine), although ionised, showed even higher retention times at pH 7.0 than expected for neutral isolipophilic compounds [7][9].

The particular behaviour of bases may seem to arise from two artefacts of the chromatographic technique, namely the occurrence of ion-pair mechanisms and secondary retention effects from either alcoholic functional groups (end-capping of silica amine) [10] or free silanols. However, the retention of ionised compounds was demonstrated not to derive from ion-pair mechanisms [7], and the occurrence of secondary retention effects from alcoholic functional groups of methyl-glycolate moieties has recently been excluded [11]. As to free silanols, no direct evidence to exclude their occurrence can be gained, because all the experimental conditions quenching silanol electrostatic interactions (*e.g.*, ionic-strength variations) could simultaneously affect the possible polar interactions that occur at the level of phospholipid charged heads. However, it is important to remember that the behaviour of amines on IAM parallels that observed in phospholipid vesicles (liposomes), where secondary retention mechanisms cannot take place. On this last partition phase, the

positively charged forms of bases were also found to have a relatively high affinity for phospholipids compared with the respective lipophilicity values in octanol [12–14]. Moreover, a recent study comparing partition measures on liposomal and IAM phases has revealed that an involvement of silanols in the IAM chromatography was evident for acids only (although to an insufficient extent to fully account for the possible high affinity of ionised forms). In contrast, for bases, the partition data from IAM at various pH values substantially overlapped with those from liposomes [15]. Therefore, it is reasonable to infer that the particular behaviour observed on IAM for bases really arose from their interactions with phospholipids. However, to date it is not clear which structural motifs of analytes govern the occurrence of stronger retention on IAM than expected on the basis of log *P*.

The aim of the present work was to elucidate the behaviour of basic compounds on the IAM.PC.MG phase in terms of the influence of different kinds of amino functions (primary, secondary, and tertiary), the value of lipophilicity, and the ionisation degree of analytes. We have investigated the behaviour of 17 basic model compounds on IAM compared with six amines previously studied at pH 7.0 (amlodipine, tocainide, nicardipine, prilocaine, trimecaine, and tetracaine) [7][9]. Therefore, the whole set considered included 23 compounds (primary, secondary, and tertiary amines) with both log *P* and pK_a spanning over a wide range of values (0.56–4.96 and 1.10–10.15, resp.). The measurements were performed with eluents at three different pH values (7.0, 5.5, and 3.0) in order to investigate the effect on retention of both the ionisation degree of the analytes and the possible modifications of phospholipid interaction capability occurring at different pH values.

Experimental. – All samples were obtained from commercial sources. The chemicals were of HPLC grade and used without further purification.

Chromatographic System. A *600E* liquid chromatograph (*Waters-Millipore*, Milford, MA, USA) equipped with a *7125 Rheodyne* injection valve (fitted with a 20-µl loop) and a *486* UV detector (*Waters*) set at λ of maximum absorbance for each compound was used. The stainless-steel column was an IAM.PC.MG (4.6 × 150 mm; *Regis Chemical Company*, Morton Grove, IL). The chromatograms were recorded by a *746 Data Module* (*Millipore*).

Chromatographic Conditions. The eluents were mixtures of different percentages of MeCN and phosphate-buffered saline (PBS) at pH 7.0, 5.5, and 3.0; the buffer at pH 3.0 was prepared by dropwise addition of orthophosphoric acid (65% (*w*/*w*) aq. soln.) to a 0.10M soln. of NaH_2PO_4; the buffers at pH 5.5 and 7.0 were prepared by dropwise addition of a 0.10M soln. of Na_2HPO_4 to a 0.10M soln. of NaH_2PO_4. Chromatography was carried out at r.t. with a flow rate of 0.9 ml/min. All samples were dissolved in MeOH (*ca.* 10^{-3} M); 20-µl samples were injected in the chromatograph. Chromatographic retention data are expressed by the logarithm of the capacity factor, log k', defined as $\log k' = \log[(t_r - t_o)/t_o]$, where t_r and t_o are the retention times of the drug and a non-retained compound (citric acid), respectively. Log k' values were determined with completely aq. eluents for all compounds eluting within 20 min (corresponding to $\log k' = 1.0$). Other compounds were eluted with mobile phases containing MeCN fractions (ϕ) ranging from 10 to 30% (*v*/*v*). Linear relationships between log k' and ϕ values were found for all compounds over the range of the eluent composition examined ($r^2 \geq 0.990$).

Lipophilicity Parameters from Octanol/H_2O System. Partition coefficients of the neutral species, log *P*, were taken from the literature [16][17] or determined according to the 'shake-flask' procedure [1]; partition coefficients of the mixtures of ionised and neutral forms existing at pH 7.0, 5.5, and 3.0 (log $D^{7.0}$, log $D^{5.5}$, and log $D^{3.0}$, resp.) were measured by the 'shake flask' procedure. Octanol was used as the lipophilic phase. For log *P* measurements, the hydrophilic phase was an aq. soln. at pH 12.5 (2.85 g of KCl and 0.53 g of NaOH in 1 l of H_2O). Since the pK_a values for the amines considered never exceed 10.15, at pH 12.5 they are present, in practice, completely in their neutral form; therefore, the partition measurements performed with aq. phases at this pH value can be assumed to represent the log *P* values of the neutral forms.

For log $D^{7.0}$, log $D^{5.5}$, and log $D^{3.0}$ measurements, the hydrophilic phases were buffer solns. at the corresponding pH values prepared as described above (*Chromatographic conditions*).

Partitions were performed at r.t. Quantification after partition was performed spectrophotometrically by comparison against calibration graphs of concentration *vs.* the absorbance previously obtained in the UV region at λ corresponding to the maximum absorbance for each compound.

The pK_a values were taken from the literature [18][19].

All values of log k', log P, and log D reported are the average of at least three measurements; the 95% confidence interval never exceeded 0.04 for each log k' value and 0.06 for each log P and log D value.

Statistical Analysis. A commercially available statistical package for personal computers was used for linear regression analysis. Requirements of significant regression analysis were observed.

Results and Discussion. – Aside from two compounds bearing an endocyclic amino function (compounds 1 and 2), the set of analytes selected for the present study (*Table 1*) included primary (compounds 3–14), secondary (compounds 15–18), and tertiary (compounds 19–23) amines. *Table 1* summarises the respective pK_a, log P, log $D^{7.0}$, log $D^{5.5}$, and log $D^{3.0}$ values.

Table 1. *p*K$_a$ *and Lipophilicity Parameters at Different pH Values for the Amines Considered*

No.	Compound	pK_a [a])	log P [b])	log $D^{7.0}$ [c])	log $D^{5.5}$ [c])	log $D^{3.0}$ [c])
1	Pyridine [d])	5.23	0.65	0.70	0.44	– 1.56
2	Acridine [d])	5.58	3.40	3.17	2.57	1.00
3	Aniline [d])	4.63	0.90	0.91	0.87	– 0.61
4	*p*-Toluidine [d])	5.08	1.39	1.43	1.24	– 0.41
5	*p*-Nitroaniline [d])	1.10	1.39	1.40	1.38	1.28
6	2-Chloroaniline [d])	2.57	1.90	1.84	1.91	1.73
7	1-Naphthylamine [d])	3.93	2.25	2.38	2.22	1.30
8	2-Amino-1,1-biphenyl [d])	3.82	2.84	2.60	2.64	1.86
9	Benzylamine	9.31	1.09	– 1.10	– 1.49	– 2.35
10	2-Phenylethylamine	9.82	1.41	– 1.06	– 1.16	– 2.30
11	4-Methylbenzylamine	9.41	1.58 [c])	– 0.67	– 1.10	– 2.01
12	*N*-Methylnaphthalen-1-amine	9.3 [e])	2.88 [c])	0.13	– 0.79	– 1.37
13	Tocainide [d])	7.8	0.56	– 0.33	– 0.82	– 1.17
14	Amlodipine [d])	9.1	3.30	1.30	– 0.50	0.35
15	*N*-Ethylaniline	5.11	2.16	2.04	1.78	0.14
16	*N*-Methylbenzylamine	9.58	1.52	– 0.10	– 0.18	– 0.18
17	*N*-Methylphenethylamine	10.15	1.68 [c])	– 0.02	– 0.01	– 0.06
18	Prilocaine	7.8	2.21	1.05	– 0.27	– 1.04
19	*N*,*N*-Dimethylaniline	5.15	2.31	2.21	1.99	0.65
20	*N*,*N*-Dimethyl-*p*-toluidine	5.33	2.81	2.59	2.27	0.74
21	Trimecaine	7.4	2.73	2.11	0.50	– 0.59
22	Tetracaine	8.5	3.55	1.96	0.56	– 1.48
23	Nicardipine	6.5	4.96	2.88	2.49	0.82

[a]) Values from [18–19]. [b]) Values from [16–17]. [c]) Experimentally determined data. [d]) Outliers. [e]) Value estimated on the basis of structural analogy.

The logarithms of the capacity factors on IAM columns extrapolated to, or measured at, 100% aqueous phase at the different pH values considered are indicated as log $k_{7.0}$, log $k_{5.5}$, and log $k_{3.0}$; these are reported in *Table 2*, together with the percentages of the ionised forms calculated according to the *Henderson-Hasselbach* equation at each pH value considered.

Table 2. *Log* k′ *Values on IAM.PC.MG and Percentages of Ionised Forms at Different pH Values*

No.	Compound	log k_{calc} [a])	log $k_{7.0}$	log $k_{5.5}$	log $k_{3.0}$	Ionized forms [%] [b])		
						pH 7.0	pH 5.5	pH 3.0
1	Pyridine [c])	−0.525	−0.007	−0.171	–	2	35	99
2	Acridine [c])	1.719	2.027	1.918	0.681	4	55	T
3	Aniline [c])	−0.321	0.124	0.079	−0.694	N	12	98
4	*p*-Toluidine [c])	0.079	0.525	0.383	−0.284	1	27	99
5	*p*-Nitroaniline [c])	0.079	0.930	0.958	0.964	N	N	1
6	2-Chloroaniline [c])	0.495	0.835	0.851	0.723	N	N	27
7	1-Naphthylamine [c])	0.781	1.462	1.454	0.708	N	3	89
8	2-Aminobiphenyl [c])	1.262	1.862	1.808	1.012	N	2	87
9	Benzylamine	−0.165	0.012	−0.306	−0.580	T	T	T
10	2-Phenylethylamine	0.095	0.227	−0.022	−0.260	T	T	T
11	4-Methylbenzylamine	0.234	0.312	0.117	−0.152	T	T	T
12	*N*-Methylnaphthalen-1-amine	1.295	1.092	0.811	0.542	T	T	T
13	Tocainide [c])	−0.601	0.532	0.175	−0.082	86	T	T
14	Amlodipine [c])	1.645	2.593	2.470	2.163	T	T	T
15	*N*-Ethylaniline	0.707	0.782	0.589	−0.426	1	29	99
16	*N*-Methylbenzylamine	0.185	0.134	−0.227	−0.547	T	T	T
17	*N*-Methylphenethylamine	0.316	0.329	0.048	−0.282	T	T	T
18	Prilocaine	0.751	0.621	0.422	−0.006	86	T	T
19	*N,N*-Dimethylaniline	0.829	0.926	0.724	−0.565	1	31	99
20	*N,N*-Dimethyl-*p*-toluidine	1.238	1.203	0.873	−0.183	2	40	T
21	Trimecaine	1.175	1.210	0.760	0.310	71	T	T
22	Tetracaine	1.843	1.754	1.554	1.188	97	T	T
23	Nicardipine	2.990	3.142	2.385	2.003	24	91	T

[a]) Values calculated from log *P* by *Eqn. 3*. [b]) N = Negligible degree of ionisation (less than 1% ionised); T = Totally ionised (more than 99% ionised). [c]) Outliers.

Relationships between Lipophilicity and log $k_{7.0}$ *Values.* The log $k_{7.0}$ values for the whole set of 23 amines correlated to the lipophilicity values according to the following equations:

$$\log k_{7.0} = 0.719\ (\pm 0.072)\ \log P - 0.564\ (\pm 0.172) \quad (1)$$
$$n = 23 \qquad r = 0.909 \qquad s = 0.358$$

$$\log k_{7.0} = 0.459\ (\pm 0.101)\ \log D^{7.0} + 0.436\ (\pm 0.175) \quad (2)$$
$$n = 23 \qquad r = 0.705 \qquad s = 0.609$$

In these equations and those that follow, 95% confidence limits are given in parentheses, *n* is the number of compounds, *r* is the correlation coefficient, and *s* is the standard deviation.

These equations clearly show that the ranking order of elution on IAM for amines at different ionisation degrees correlates better with log *P* (lipophilicity values of the neutral forms) than with log $D^{7.0}$ (lipophilicity values actually displayed by the mixture of neutral and ionised forms at pH 7.0). However, a closer look at *Eqn. 1* shows a high standard deviation, indicating a quite scattered log $k_{7.0}$ population. Therefore, we decided to compare the log $k_{7.0}$ values observed for the amines with the values expected for neutral isolipophilic compounds. These were calculated by considering a relation

between $\log k_{7.0}$ and $\log P$ for neutral compounds, which we had found in a previous study [8] (*Eqn. 3*):

$$\log k_{7.0} = 0.816\ (\pm 0.035)\ \log P - 1.055\ (\pm 0.140) \qquad (3)$$
$$n = 10 \qquad r = 0.993 \qquad s = 0.111$$

This equation was relative to three hydrocarbons (benzene, toluene, and naphthalene) and seven non-ionisable dihydropyridine calcium-channel blockers (nifedipine, nitrendipine, isradipine, nimodipine, nisoldipine, felodipine, and lacidipine) and had already been used to generate the $\log k_{7.0}$ values expected for neutral compounds on the basis of their $\log P$. Noteworthy is that, in our previous work [9], the experimental $\log k_{7.0}$ values for eleven bases (local anaesthetics) were in excellent agreement with the $\log k_{7.0}$ values calculated from $\log P$ by *Eqn. 3*. It is important to remember that the local anaesthetics were ionised at the experimental pH value, and the soundness of their $\log k_{7.0}$ values to describe partition in phospholipids had been verified by comparison with partition data in phospholipid vesicles (liposomes).

Among the amines considered in this study, the $\log k_{7.0}$ values for ten compounds (No. 1–8 and No. 13–14 in *Table 2*) were significantly higher than those calculated by *Eqn. 3*. In contrast, this equation predicted, within the limits of its standard deviation, the experimental $\log k_{7.0}$ values for 13 compounds (No. 9–12 and No. 15–23 in *Table 2*). Therefore, we split the compounds into two subclasses: a first one including compounds 1–8 and 13–14, here defined as 'outliers', and a second one including compounds 9–12 and 15–23, here defined as 'non-outliers'. Hence, we recalculated the relationship between $\log k_{7.0}$ and $\log P$ by considering these two subsets separately. The regression equations $\log k_{7.0}$ *vs.* $\log P$ were:

for the outliers:

$$\log k_{7.0} = 0.776\ (\pm 0.090)\ \log P - 0.354\ (\pm 0.191) \qquad (4)$$
$$n = 10 \qquad r = 0.950 \qquad s = 0.287$$

for the non-outliers:

$$\log k_{7.0} = 0.800\ (\pm 0.033)\ \log P - 0.999\ (\pm 0.084) \qquad (5)$$
$$n = 13 \qquad r = 0.991 \qquad s = 0.118$$

As can be seen in the *Figure*, these equations produced two quite parallel straight lines on the relative plot.

Poorer relation equations were found between $\log k_{7.0}$ and $\log D^{7.0}$ values for both subsets (for the first subset: $r = 0.618$; for the second subset: $r = 0.766$).

The subset of outliers is made up of compounds containing either an endocyclic or a primary amino function, provided that the latter is not totally ionised at pH 7.0. An exception is represented by amlodipine, which belongs to this subset despite its being a totally ionised primary amine. Therefore, the presence of a primary or endocyclic amino function appears essential for stronger interactions with phospholipids to occur than expected on the basis of $\log P$.

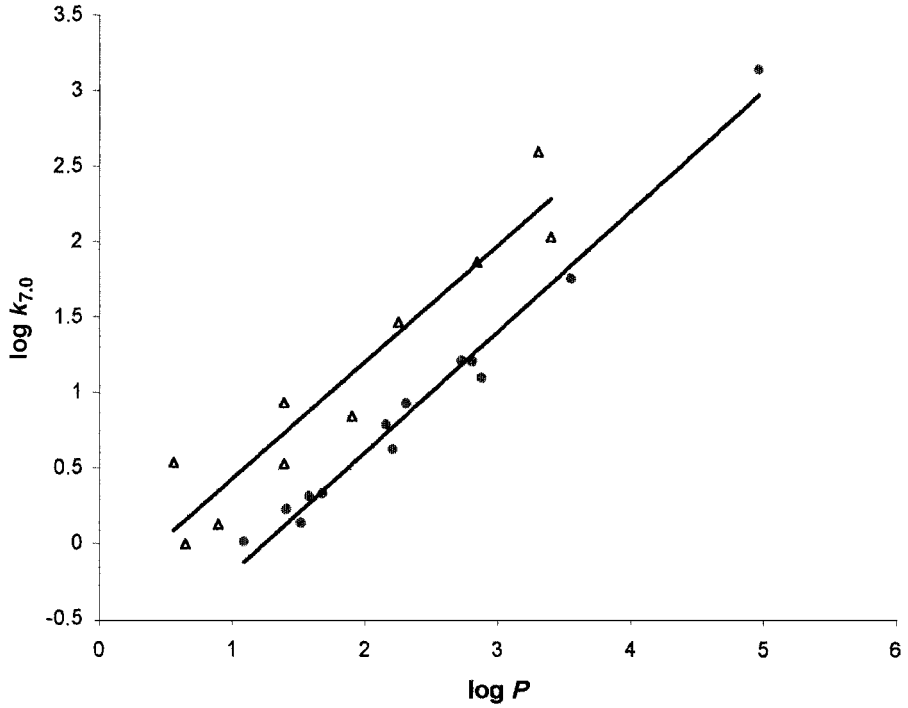

Figure. *Plots of log* $k_{7.0}$ vs. *log* P *values for outliers* (△) *and non-outliers* (●)

The subset of non-outliers included all the secondary and tertiary amines, regardless of their degree of ionisation, as well as the primary amines totally ionised at pH 7.0, except for amlodipine. The presence of six fully ionised amines in this subset (compounds No. 9–12, 16, and 17) indicates that, at pH 7.0, phospholipids are able to mask the effect of an electric charge on the amino function and to interact with fully protonated amines to the same extent as with neutral isolipophilic compounds. This effect is consistent with the relatively high affinity of positively charged bases for phospholipids on liposomal partition phases observed by other authors [12–14].

Although high retention values for ionised compounds have already been observed on IAM [7–9], we investigated the variations of IAM parameters in relation to the different ionic strengths of the eluent at pH 7.0, this to further exclude that ion-pair elution mechanisms were involved in the retention of ionised forms. Two test compounds, an outlier (*p*-nitroaniline) and a non-outlier (2-phenylethylamine), were eluted with phosphate buffers at concentrations of 0.10, 0.05, and 0.01M. As shown in *Table 3*, the outlier, which was non-ionised at pH 7.0, did not appreciably change its retention time in relation to the different ionic strengths. In contrast, the non-outlier, which was totally ionised at pH 7.0, increased its retention time on the IAM phase when the ionic strength was lowered. This behaviour is in contrast to that expected for an ion-pair elution mechanism. It confirms that the interactions between phospholipids and the non-outliers fully ionised at pH 7.0 include an electrostatic component capable of counteracting the negative influence of an electric charge on the analyte.

Table 3. *Log $k_{7.0}$ Values for* p-*Nitroaniline and 2-Phenylethylamine with Eluents at pH 7.0 at Different Ionic Strengths*

PBS Molarity	log $k_{7.0}$	
	p-Nitroaniline	2-Phenylethylamine
0.10M	0.930	0.227
0.05M	0.937	0.453
0.01M	0.922	1.040

Relationships between Lipophilicity and log $k_{5.5}$ *Values.* For the whole set of 23 amines, the lipophilicity parameter best describing the retention on IAM at pH 5.5 was still the lipophilicity value relative to the non-ionised forms, log P ($r = 0.852$), while the equation obtained considering log $D^{5.5}$ was less well-correlated ($r = 0.639$).

However, analogously to what occurred at pH 7.0, much better relation equations between lipophilicity and chromatographic parameters on IAM were observed only when the two subsets – outliers and non-outliers – were considered separately.

For the subset of the outliers, the relation equation between log $k_{5.5}$ and log $D^{5.5}$ values was very poor ($r = 0.524$) while a reasonable relation equation was found to correlate log $k_{5.5}$ values with log P:

$$\log k_{5.5} = 0.811\ (\pm 0.081)\ \log P - 0.515\ (\pm 0.172) \qquad (6)$$
$$n = 10 \qquad r = 0.962 \qquad s = 0.258$$

This equation shows a slope value practically equal to that of *Eqn. 3* relative to neutral compounds, while its intercept value is higher than in *Eqn. 3* (but lower than in *Eqn. 4*). This indicates that, at pH 5.5, the interactions between outliers and phospholipids are still higher than expected for isolipophilic neutral compounds and slightly lower than at pH 7.0.

Similarly, the log $k_{5.5}$ values for the subset of non-outliers correlated significantly better with log P than with log $D^{5.5}$ values ($r = 0.690$):

$$\log k_{5.5} = 0.706\ (\pm 0.034)\ \log P - 1.084\ (\pm 0.087) \qquad (7)$$
$$n = 13 \qquad r = 0.988 \qquad s = 0.122$$

We observed that the amines negligibly ionised at both pH 7.0 and 5.5 (*e.g.*, No. 5 – 8) did not appreciably change their retention times at these different pH values. In contrast, amines totally ionised at both these pH values (*e.g.*, No. 9 – 12, and No. 16 and 17) showed shorter retention times at pH 5.5 than at pH 7.0. These results indicate that, at pH 5.5, the retention mechanism on IAM for the neutral forms of the amines considered is substantially similar to that observed at pH 7.0, but it is reduced for ionised forms. Therefore, the slight differences between the equations at pH 5.5 and those at pH 7.0 can be referred not only to the more extensive degree of ionisation of some analytes but also to a reduction of the retention capability of the IAM phase at pH 5.5 toward the ionised forms.

Relationships between Lipophilicity and log $k_{3.0}$ *Values.* Since, at pH 3.0, pyridine was not retained on the IAM phase, the log $k_{3.0}$ values were determined for 22 compounds only. At pH 3.0, most of the amines considered were extensively or totally ionised, and the relationships observed for the whole set of compounds between log $k_{3.0}$, and both log P and log $D^{3.0}$ values were much poorer that at the other pH values ($r = 0.732$ and $r = 0.382$, resp.).

Considering the two subsets separately, none of the lipophilicity parameters correlated with log $k_{3.0}$ values for the subset of outliers.

For the subset of non-outliers, the lipophilicity parameter that best correlated with the chromatographic indexes on IAM was log P. However, the relation equation observed at pH 3.0 was poorer that at the other pH values:

$$\log k_{3.0} = 0.669\ (\pm 0.091)\ \log P - 1.509\ (\pm 0.234) \quad (8)$$
$$n = 13 \qquad r = 0.912 \qquad s = 0.329$$

No relationship was found between log $k_{3.0}$ and log $D^{3.0}$ values.

Eqn. 8 confirms that log P is the lipophilicity parameter that best describes the retention on IAM for the subset of non-outliers at every pH value considered. A comparison between *Eqn. 8*, and both *Eqns. 5* and *7*, relative to the relationships between the same parameters at pH 7.0 and 5.5, indicates that the retention of these amines on the IAM phase further decreases at pH 3.0. As can be seen in *Table 2*, all amines of this subset are quite totally ionised at pH 3.0. However, this does not fully account for the loss of retention observed on the IAM. In fact, the compounds of the subset fully ionised at both pH 7.0 and 3.0 are retained on the IAM phase at pH 7.0 to the same extent as expected for neutral isolipophilic compounds (*Eqn. 5*); however, they show a dramatic decrease in retention time at pH 3.0, despite a practically constant degree of ionisation. This suggests that the retention capability of the IAM phase for protonated forms changes as a function of the pH and is impaired at the lowest pH value.

Differently from non-outliers, outliers include molecules with a different ionisation degree (*Table 2*). The loss of any relationship between log $k_{3.0}$ and log P for the subset of outliers is consistent with the loss of IAM interaction capability toward the protonated forms observed at pH 3.0. Actually, at pH 3.0, poorly protonated compounds, *e.g.*, *p*-nitroaniline, still show high retention values, while extensively ionised compounds show a noticeable decrease in their retention times. Assuming that only the neutral forms are still appreciably retained on IAM at this pH, retention should correlate with the lipophilicity parameters accounting for the ionisation degree of the analytes (log $D^{3.0}$). However, the validation of this hypothesis necessarily required the exclusion of amlodipine, since this amine interacts with phospholipids also in its protonated form.

Indeed, a moderate relationship was found between log $k_{3.0}$ and log $D^{3.0}$ values.

$$\log k_{3.0} = 0.492\ (\pm 0.097)\ \log D^{3.0} + 0.072\ (\pm 0.122) \quad (9)$$
$$n = 8 \qquad r = 0.900 \qquad s = 0.300$$

The lack of correlation with log P ($r = 0.669$) values confirms that the description of retention on IAM at pH 3.0 must take into account the ionisation degree of the analytes, in contrast to that observed at both pH 7.0 and 5.5.

As to the exclusion of amlodipine, it is the only fully protonated amine that still undergoes a strong additive extra-interaction on the IAM stationary phase at pH 3.0. Our previous study on IAM phases [7] demonstrated the occurrence of a mechanism involving the protonated form. It consists of a lipophilic interaction component that acts as the driving force for the partition of the analyte into the lipophilic core of phospholipids, which in turn allows a co-operative polar interaction between the protonated primary amino function and the polar head group of phospholipids, hence referred to as 'extra-interaction'. Differently from that proposed for the amines considered in this study, this mechanism implies an IAM behaviour with a low sensitivity of amlodipine to pH changes (see *Table 2*) and a high sensitivity to changes in the ionic strength of eluent [7]. The simultaneous occurrence of polar and lipophilic interactions for this compound seems to depend also on specific structural features (*e.g.*, distance between primary amino function and lipophilic moiety of the analyte) that make the structure of amlodipine complementary to that of phospholipids [6].

Conclusion. – The present study indicates that the retention behaviour of amines on the IAM.PC.MG stationary phase cannot be predicted *a priori* on the basis of octanol/H_2O lipophilicity parameters.

When the measurements are performed with eluents at pH 7.0, the retention of the protonated forms of amines on this phospholipid phase is stronger than expected on the basis of their lipophilicity. This peculiar capability is progressively lost as the pH of the eluent decreases, and completely disappears with eluents at pH 3.0. This is probably a consequence of conformational changes and/or variations in charge distribution occurring for phospholipids as a function of the pH, according to their pK_a value of *ca.* 2 [20]. Indeed, in buffer at pH below 3.5, a drop of partition values was also observed for phosphatidylcholine liposome-buffer systems [14].

Moreover, the neutral forms of all primary amines and the compounds bearing an endocyclic amino function undergo stronger interactions with phospholipids than would be expected based on the respective log P values. In this subset, amlodipine behaves differently from other primary amines when either pH or ionic strength of the eluent are varied. This has allowed us to hypothesise that at least two different mechanisms can produce stronger interactions between amines and phospholipids than expected for neutral isolipophilic compounds on the basis of their partition data in the octanol/H_2O system.

The first kind of mechanism hypothesised refers to amlodipine, a highly lipophilic molecule. It probably consists of an additive electrostatic interaction between the polar head groups of phospholipids and the protonated amino function of the analyte, supported by a lipophilicity-driven force. The second kind of mechanism hypothesised applies to scarcely lipophilic compounds, such as the outliers considered in this work, and refers to the non-ionised fraction of analytes. It should arise from a more favourable accommodation of the hydrophilic primary (or endocyclic) amino function in phospholipids than in octanol due to the different chemical nature of the two phases. This mechanism of interaction does not include any electrostatic component, because it

is insensitive to the ionic-strength variations of the eluent and disappears on the ionisation of the amine. It is important to note that *p*-nitroaniline, which was not ionised at every pH value considered, always behaved as an outlier (*Table 2*). This indicates that the particular behaviour observed on IAM phase for neutral forms of primary amines is substantially generated by the interactions occurring at level of phospholipids. Indeed, this study does not allow us to exclude the occurrence of secondary retention effects of free silanols. However, it is reasonable to assume that their possible occurrence plays only a minor role in retention, since these effects are known to be pH-dependent and to take place only in a narrow range of pH values [15].

These results emphasise the different interaction capabilities of amphiphilic and ordered phases, such as IAM, as opposed to bulk lipophilic phases, such as octanol.

The observation that the interaction capability of phospholipids toward protonated forms of amines changes with variations in the pH of the aqueous phase should be taken into account when considering partition data obtained at acidic pH values from both IAM and liposomal systems. Actually, pH changes in the *in vitro* systems can be assumed to affect the ionisation degree of analytes in a way parallel to that occurring *in vivo* in different biological compartments. In contrast, it seems highly unlikely that biomembrane phospholipids can directly contact acidic fluids *in vivo* and, consequently, undergo the same modifications of their interaction capability as observed *in vitro*.

Finally, this study indicates that some hydrophilic amines can interact with phospholipids to such a surprisingly strong extent as to imply a better capability to cross biomembranes than expected on the basis of their log *P* values. The implications of these findings for pharmacokinetics should be considered in the design of new drug candidates.

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REFERENCES

[1] A. J. Leo, C. Hansch, D. Elkins, *Chem. Rev.* **1972**, *71*, 525.
[2] L. G. Herbette, G. Gaviraghi, T. Tulenko, R. Preston Mason, *J. Hypert.* **1993**, *11*, *Suppl 1*, 13.
[3] R. Kaliszan, A. Nasal, A. Bucinski, *Eur. J. Med. Chem.* **1994**, *29*, 163.
[4] C. Pidgeon, S. Ong, H. Liu, X. Qiu, M. Pidgeon, A. H. Dantzig, J. Munroe, W. J. Hornback, J. S. Kasher, L. Glunz, T. Szczerba, *J. Med. Chem.* **1995**, *38*, 590.
[5] L. R. Snyder, J. J. Kirkland, in 'Introduction to Modern Liquid Chromatography', John Wiley & Sons, New York, 1979, p. 15.
[6] S. Ong, H. Liu, X. Qiu, G. Bhat, C. Pidgeon, *Anal. Chem.* **1995**, *67*, 755.
[7] F. Barbato, M. I. La Rotonda, F. Quaglia, *Eur. J. Med. Chem.* **1996**, *31*, 311.
[8] F. Barbato, M. I. La Rotonda, F. Quaglia, *J. Pharm. Sci.* **1997**, *86*, 225.
[9] F. Barbato, M. I. La Rotonda, F. Quaglia, *Pharm. Res.* **1997**, *14*, 1699.
[10] R. J. Markovich, X. Qiu, D. E. Nichols, C. Pidgeon, B. Invergo, F. M. Alvarez, *Anal. Chem.* **1991**, *63*, 1851.
[11] F. Barbato, A. Bertschinger, M. T. Quercia, P.-A. Carrupt, M. Reist, M. I. La Rotonda, B. Testa, Abstracts of Symposium 'Log P 2000 – Lipophilicity in Drug Disposition', Lausanne, March 5 – 9, 2000, P-A13.
[12] R. P. Austin, A. M. Davis, C. N. Manners, *J. Pharm. Sci.* **1995**, *84*, 1180.
[13] P. Barton, A. M. Davis, D. J. McCarthy, P. J. H. Webborn, *J. Pharm. Sci.* **1997**, *86*, 1034.
[14] C. Ottiger, H. Wunderli-Allenspach, *Eur. J. Pharm. Sci.* **1997**, *5*, 223.
[15] C. Ottiger, H. Wunderli-Allenspach, *Pharm. Res.* **1999**, *16*, 643.
[16] R. F. Rekker in 'The Hydrophobic Fragmental Constant', Eds. W. T. Nauta, R. F. Rekker, Elsevier Scientific Publishing Company, Amsterdam-Oxford-New York, 1977, Vol. 1, p. 39.
[17] A. Pagliara, E. Khamis, A. Trinh, P. A. Carrupt, R. S. Tsai, B. Testa, *J. Liq. Chrom.* **1995**, *18(9)*, 1721.

[18] 'Handbook of Chemistry and Physics', Ed. R. C. Weast, The Chemical Rubber Co., Cleveland Ohio, 1972–1973, 53rd edition, D117–D119.
[19] P. N. Craig, in 'Comprehensive Medicinal Chemistry', Eds. C. Hansch, P. G. Sammes, J. B. Taylor, Pergamon, New York, 1990, Vol. 6, p. 237.
[20] A. D. Bangham, *Prog. Biophys. Mol. Biol*. **1968**, *18*, 29.

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