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Correlation between in situ absorption and in vitro dialysis data found in the presence of surfactants

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Summary

Dialysis rate constants of 4-alkylanilines in free solution (k_d) are virtually the same as those found in the presence of polysorbate 80 at its critical micelle concentration (k_c). In both cases, a dependence of molecular weight has been observed although the differences are very small. Such in vitro behaviour clearly differs from that found in in situ absorption experiments, where the influence of surfactant on the biological membrane and its environment leads to very dissimilar rate constant values in the absence and in the presence of surfactant at this concentration. A different picture is found when surfactant is present in the dialyzing solution at supramicellar concentration; dialysis rate constants (k_s) decrease progressively as molecular weight and lipophilicity increase, due to micellar solubilization of the solutes. Fitting equations are analogous to those found for absorption studies. Free and micelle-solubilized amine fractions in situ are correlated with those found in vitro, so that the latter could be utilized for predictive purposes, thus helping to correctly design further in situ absorption studies.

Introduction

In former papers some attempts to explain the effects of the surfactants on intestinal absorption of drugs and other xenobiotics were made (Plá-Delfina et al., 1987; Collado et al., 1988). Apart from the main conclusions, these studies revealed the possibility of calculating the free (F_f) and the micelle-solubilized (F_m) solute fractions remaining in the intestinal luminal fluid on the basis of the apparent absorption rate constant experimentally determined from perfusion solutions by means of an in situ rat gut technique, in the presence of a surfactant at its critical micelle concentration (CMC) or at supramicellar surfactant concentration (SMC), namely k_c and k_s, respectively:

$$F_f = \frac{k_s}{k_c} \quad (1)$$

$$F_m = 1 - \frac{k_s}{k_c} \quad (2)$$

The present work attempts to determine the behaviour of the compounds used in previous animal absorption studies (*p*-alkyl-substituted anilines) when subjected to in vitro dialysis tests, in order to attain two main objectives.

(1) Analysis of the dialysis experiments as compared with absorption tests. More specifically,

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the possible extrapolation of F_f and F_m in situ values from those found in vitro. Since development of animal studies is time-consuming and requires some deal of specialization, the objective could be of interest in obtaining information for correct planning and development of the in situ absorption studies.

(2) Validation of some of the conclusions arising from the in situ experiments on the mechanisms governing the effects of surfactants in the intestinal absorption of xenobiotics, mainly in the presence of supramicellar concentrations of the amphiphile.

Theoretical

Dialysis of substances follows first-order kinetics (Flynn et al., 1974; Bottari et al., 1975; Tsuji et al., 1982), so that

$$C = C_o \cdot e^{-k_d \cdot t}$$

where C is the solute concentration in the dialyzing solution at any time, t, C_o is the starting solute concentration, and k_d represents a dialysis rate constant, in reciprocal time. When the dialyzing solution contains a surfactant, this equation remains perfectly functional, regardless of the amphiphile concentration (Bottari et al., 1975; Tsuji et al., 1982).

In the presence of a surfactant at any supramicellar concentration, SMC, an equilibrium of the solute is established between surfactant micelles and free solution which follows the general partition laws (Collett and Koo, 1975; Tomida et al., 1978). Since only the free form of the solute is available for dialysis, the resulting global equilibrium can be described as:

$$\frac{dF_m}{dt} = k_1 \cdot F_f - k_2 \cdot F_m$$

$$\frac{dF_f}{dt} = -k_1 \cdot F_f + k_2 \cdot F_m - k_d \cdot F_f$$

where k_1 and k_2 are constants governing the equilibrium between micelles and free solution. Material balance shows that:

$$\frac{dF_m}{dt} + \frac{dF_f}{dt} = \frac{dF_t}{dt} = -k_d \cdot F_f \quad (3)$$

where F_t is the total solute content, both free and solubilized in micelles. Since the apparent dialysis rate constant for total solute, k_s, has also been shown to be first-order, we have:

$$\frac{dF_t}{dt} = -k_s \cdot F_t \quad (4)$$

and since Eqns. 3 and 4 describe the same process, it can be writen:

$$k_d \cdot F_f = k_s \cdot F_t \quad (5)$$

One can assume that F_t is unity. Therefore, the free and solubilized fractions of the solute remaining in the dialyzing fluid can be calculated as follows:

$$F_f = \frac{k_s}{k_d} \quad (6)$$

$$F_m = 1 - \frac{k_s}{k_d} \quad (7)$$

Consequently, if dialysis constants in the absence and in the presence of surfactant at SMC (k_d and k_s, respectively) are experimentally determined, the free and solubilized fractions of the solute in the dialyzing fluid can be calculated.

Let us point out here that the dialysis constant of the solute in free solution (k_d) should, theoretically, be the same as that obtained in the presence of surfactant at or below its CMC, i.e. in the absence of micelles (k_c), since no substantial interactions can be expected between surfactant and solutes or the porous hydrophillic materials constituting the dialyzing membrane. Therefore, it can be also assumed that Eqns. 1 and 2 are fulfilled in this case, and should be identical to Eqns. 6 and 7, respectively. In order to confirm this point, however, dialysis experiments in the presence of surfactant at CMC were developed in

this work, and the results were compared with those found in the absence of surfactant. In so doing, unwanted effects that can arise from interactions surfactant/solutes or surfactant/membrane will be avoided.

As indicated above, Eqns. 1 and 2 perform well for in situ absorption tests. The absolute values of the in situ and the in vitro rate constants, however, were presumed to be very dissimilar because – contrary to that which would occur in dialysis tests – the surfactant elicited important effects on the absorbing lipophilic membrane and its microenvironment. This means that absorption rate constants in the absence and presence of surfactant at CMC are completely different (Plá-Delfina et al., 1987; Collado et al., 1988). Despite these features, it was thought that, on the basis of kinetic similarities, a correlation could be obtained between in situ and in vitro F_f or F_m values. According to the Collander equation (Collander, 1951), which is fully confirmed by more recent work (Leo et al., 1971), when partition phases are of similar character, and especially, when one is dealing with true homologous compounds, it can be assumed that:

$$\log P = a \cdot \log P' + b$$

where P and P' are partition coefficients found in two related systems. Solubilization conditions in vitro – where no significant interactions can be expected – may be very different from those prevailing in situ – where surfactant adsorption on the biological membranes and the presence of endogenous substances are undoubtedly a definite source of such interactions – and therefore, it can be also assumed that the internal partition coefficients of the solutes between micelles and free solution in situ and in vitro (P_a' and P_a, respectively) are different for a given solute. But, since the partitioning phases are substantially the same, P_a' and P_a could be still related through the above equation, so that:

$$\log P_a' = a \cdot \log P_a + b$$

since, as has been pointed out above, internal partition phenomena are governed by the general partition equations (Collett and Koo, 1975; Tomida et al., 1978).

As previously demonstrated and from reported data (Plá-Delfina et al., 1987; Collado et al., 1988):

$$P_a' = \frac{F_m'}{F_f'} \tag{8}$$

and

$$P_a = \frac{F_m}{F_f} \tag{9}$$

where F_f' and F_f are the free fractions of the solute in situ and in vitro, respectively, while F_m' and F_m represent the corresponding solubilized fractions. Therefore:

$$\log \frac{F_m'}{F_f'} = a \cdot \log \frac{F_m}{F_f} + b \tag{10}$$

Eqn. 10 and its mediated transforms such as:

$$\log\left(\frac{1}{F_f'} - 1\right) = a \cdot \log\left(\frac{1}{F_f} - 1\right) + b \tag{11}$$

could be used for in situ predictions from in vitro data, provided that some in situ experiments for a limited number of homologs are available. This may help to better delineate the optimal range and conditions for further animal tests and perhaps also to make some predictions about surfactant/xenobiotic interactions. Contrarily, the prediction of in situ k_c or k_s constants from those found in vitro was considered to be impossible unless lipophilic membranes resembling biological barriers were utilized for the in vitro experiments. Work is in progress in our laboratory on this subject and we expect to report more on it in the near future.

Materials and Methods

Test compounds

Five homologous anilines were utilized as dialyzing solutes (aniline and 4-methyl-, 4-ethyl-, 4-propyl- and 4-butyl-derivatives). Solutes were handled as bases at 0.05% (w/v) in the dialysis

fluid, except 4-butylaniline, which was used at 0.02% due to its lower solubility. As model surfactant, polysorbate 80 was selected in order to avoid interactions with solutes and to compare results with those found in the absorption in situ experiments (Plá-Delfina et al., 1987). Surfactant was added to amine solutions at suitable concentrations, identical to those employed in situ, and the solutions were buffered to a final pH of 7.5, as in colonic absorption tests.

Dialysis apparatus and technique

A dissolution test-type apparatus, in conformity with U.S.P. requirements (M-1635 Turu-Grau Dissolution Tester, type DT-6, 6.88 HP, 200 v [Turu-Grau S.A., Tarrasa, Spain]) was made suitable for dialysis tests. It consists basically of a water bath with 6 openings into which the same number of round-bottom glass vessels with 1000-ml capacity can be placed. Each vessel is furnished with a plastic cover with openings for stirring blades (stainless steel, 3 mm thick, perfectly centered in the vessels) and for sample withdrawals. The joint is supplied with a 1500 W resistance, a thermostat, and a 4.5 litres/m-capacity driving pump. Stirring speed is regulated by a high-precision tachometer. For dialysis experiments, the lower end of the dialysis membrane (Dialysis tubing-wisking, size 2-18/32 inches, pore size 24 Arms [Medicell International, London N1 1LX]) was closed with a tight double-knot, and the upper end was adjusted with a perforated rubber stopper; the whole was introduced in one of the vessels, which was filled with the outer dialysis solution through the opening for samplings.

The dynamic dialysis method was used. The outer dialysis medium was a physiological saline solution buffered to pH 7.5 with Sörensen phosphates. In experiments carried out with surfactant, polysorbate 80 at its CMC – estimated to be 0.0022% w/v (Plá-Delfina et al., 1987) – was added to this fluid in order to prevent permeation of free surfactant molecules (Patel and Kostenbauder, 1958; Blanchard, 1980). Inner dialysis fluids were constituted by the same solution containing variable amounts of amines or amines and surfactant, buffered to a final pH of 7.5. Three series of experiments were carried out: (1) in the absence of surfactant; (2) in the presence of polysorbate at CMC; and (3) in the presence of the surfactant at a clearly SMC, which was 5%, w/v.

Dissolution vessels were filled with the outer dialysis medium so that the fluid height would be the same as that contained in the dialysis bag (about 900 ml); this volume will assure sink conditions throughout the experiments for all the amines tested. The stirring blades and the thermostat were then switched on, and when a temperature of 37 °C was attained, the dialysis bag (of about 11 cm height) was filled with 10 ml of the working amine solution and placed in the vessel (zero time). Samples were taken at 15, 30, 45, 60, 90 and 120 min through the opening in the rubber stopper (which remained closed during the intervals between samplings), and placed in gauged 10-ml flasks for amine content analysis.

The apparent dialysis rate constants were calculated, for each condition, by least-squares linear regression analysis of the remaining amine concentrations, expressed as percentage of the initial amine concentration (100%) versus time (in reciprocal hours). In order to prevent potential amine adsorption effects on the dialysis membrane, the initial sample was not used for regression. Dialysis rate constants employed for correlation were the means of 5 experiments. Since at the end of the experiments no significant volume variation was observed in the dialyzing solutions, no correction factor was applied to the experimental data.

Analysis of samples

The diazotization and coupling technique, modified as previously described (Plá-Delfina et al., 1987; Collado et al., 1988), was used for amine evaluation in samples. Absorbance values, measured at 540–570 nm, in a Perkin-Elmer Lambda 3 automatic spectrophotometer, were fully in the range of linearity previously established for each compound.

Fitting equations to data and statistical methods

The best fit for dialysis constants found for the tested compounds in the absence of micelles (i.e. k_d and k_c values) versus lipophilicity, as assessed

by chloroform/pH 7.5 aqueous buffer partition coefficient, P, previously determined (Plá-Delfina et al., 1987) was found to be:

$$\log k_{d,c} = a \cdot \log P + b \qquad (12)$$

The reliability of this type of correlation will be discussed later.

Dialysis rate constants determined in the presence of surfactant at SMC (i.e. in the presence of micelles, k_s values) were fitted against P through a previously established equation deduced from absorption experiments (Plá-Delfina et al., 1987):

$$\log k_s = a \cdot \log P - \log(B' \cdot P^{a'} + 1) + b \qquad (13)$$

where a and b have the same meaning as in Eqn 12, and a' and B' are parameters describing the correlation between internal partition coefficients (micelles/solution), P_a, and bulk-phase partition coefficients, P:

$$\log P_a = a' \cdot \log P + \log B'$$

Since log P is linearly related to molecular weight, M, for true homologous compounds, Eqns. 12 and 13 can be written as follows:

$$\log k_{d,c} = a \cdot M + b \qquad (14)$$

and

$$\log k_s = a \cdot M - \log(B' \cdot 10^{a' \cdot M} + 1) + b \qquad (15)$$

In order to fit free or solubilized fractions of the solutes in vitro and in situ, Eqn. 11 and its non-logarithmic transforms were utilized.

As statistical criteria to test the goodness of fits, the correlation coefficients between experimentally found and model-predicted values was used, as well as, in most cases, the AIC values (Akaike, 1976); the smallest AIC value is indicative of the best fit. In order to compare means, the classical t-test was utilized.

Results

In Table 1, dialysis rate constants found in the absence of surfactant (k_d), in the presence of it at CMC in the dialyzing fluid (k_c), and in the presence of the surfactant at SMC (k_s), are shown. Statistical comparison based on a t-test shows that k_d and k_c values are not significantly different for each compound, thus confirming the above assumption that no appreciable interaction arises between dialyzing membrane or solutes and the surfactant. It was decided, therefore, that k_d and k_c values, as members of the same statistical population, can be considered globally in order to obtain a mean $k_{d,c}$ value, which is shown in the third column of Table 1 under the paragraph of "mixed constant"; these values have been used in the present study for correlation purposes whenever dialysis rate constants in the absence of micelles in the working fluid have been employed.

TABLE 1

Dialysis rate constants found in the absence and in the presence of surfactant at different concentrations

Tested anilines	Dialysis rate constant ± S.D. (h^{-1})			
	Free solution (k_d)	Polysorbate CMC (k_c)	Mixed constant ($k_{d,c}$)	Polysorbate SMC (k_s)
Aniline	1.159 ± 0.073	1.179 ± 0.047	1.169 ± 0.059	1.094 ± 0.140
4-Methyl-	1.030 ± 0.071	1.029 ± 0.022	1.029 ± 0.049	0.751 ± 0.029
4-Ethyl-	1.010 ± 0.112	0.888 ± 0.133	0.953 ± 0.136	0.440 ± 0.049
4-Propyl-	0.912 ± 0.040	0.876 ± 0.019	0.894 ± 0.035	0.210 ± 0.023
4-Butyl-	0.880 ± 0.065	0.864 ± 0.104	0.872 ± 0.082	0.092 ± 0.025
Number of tests	5	5	10	5

TABLE 2

Structural and physicochemical features of the tested compounds which have been used for correlations

Tested anilines	Molecular weight (M)	Chloroform partition coefficient (P) [a]	Internal partition coefficient (P_a)	In vitro fraction free amine (F_f)	In situ fraction free amine (F_f') [a]
Aniline	93.12	23.96	0.068	0.936	0.667
4-Methyl-	107.15	83.70	0.370	0.730	0.464
4-Ethyl-	121.18	191.08	1.165	0.462	0.337
4-Propyl-	135.21	981.40	3.255	0.235	0.221
4-Butyl-	149.24	2350.74	8.524	0.105	0.142

[a] From Plá-Delfina et al. (1987).

Some structural and physicochemical features of the tested compounds which have been used for correlations are shown in Table 2. In Table 3, equation parameters describing correlations according to Eqns. 12–15 are shown.

Figs. 1 and 2 give the correlations between dialysis rate constants found in different conditions, and bulk-phase partition coefficients, P, or molecular weights, M, according to Eqns. 12–15. In both Figs. 1 and 2, $k_{d,c}$ values have been used for correlations in the absence of micelles, although individual k_d and k_c mean values have been drawn on the plots as independent points. In Fig. 3, the correlation between free in vitro and in

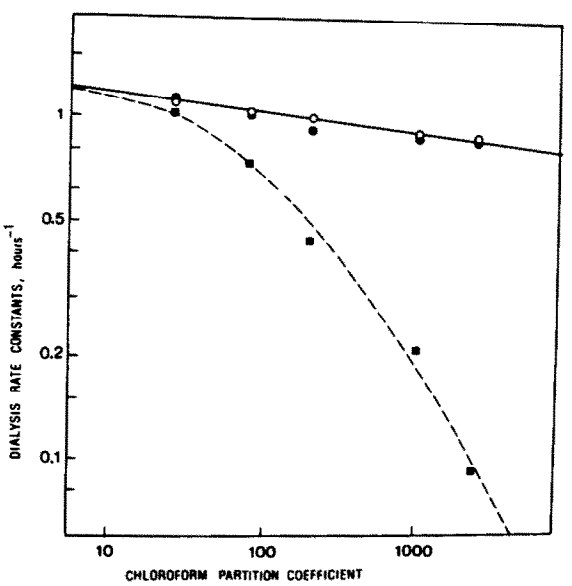

Fig. 1. Double logarithmic plots showing the correlations between dialysis rate constants and bulk-phase partition coefficients. The former were determined in the absence of micelles (continuous line, Eqn. 12) or in the presence of a supramicellar concentration of surfactant (dotted lines, Eqn. 13). Open circles are mean k_d, black circles mean k_c, and black squares mean k_s values. See also Tables 1 and 3.

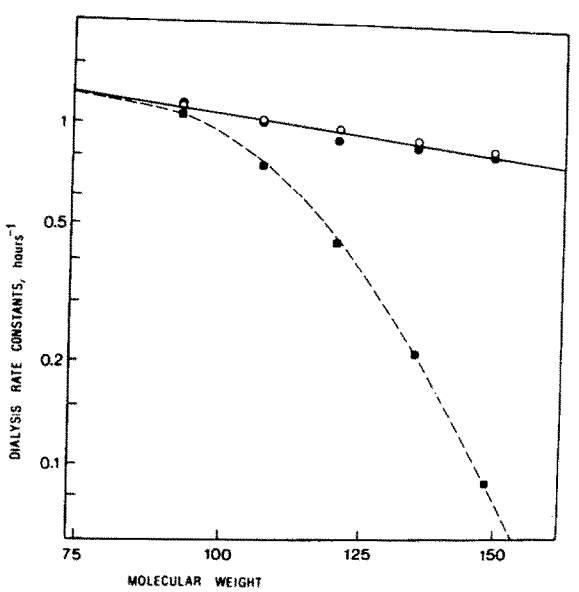

Fig. 2. Semilogarithmic plots representative of the correlations between dialysis rate constants and molecular weight. Symbols are the same as in Fig. 1, but the correlation equations are 14 and 15, respectively. Note the better correlation found for k_s values relative to that obtained with partition coefficients.

TABLE 3

Equation parameters representative of the correlations relating diffusion and partition constants or molecular weights

Correlated variables (Eqns. 12–15)	a	b	B	r
log k_d and log P	−0.0581	0.1361	1.368	0.987
log k_c and log P	−0.0658	0.1394	1.378	0.902
log $k_{d,c}$ and log P	−0.0620	0.1384	1.375	0.966
log $k_{d,c}$ and M	−0.0022	0.2629	1.832	0.970

Correlated variables (Eqns. 13 and 15)	a'	b'	B'	r
log P_a and log P	1.0067	−2.4229	3.78×10^{-3}	0.988
log P_a and M	0.0366	−4.4536	3.52×10^{-5}	0.993

situ solute fractions (F_f and F_f', respectively) is represented according to the non-logarithmic form of Eqn. 11.

Discussion

Dialysis rate constants found in the absence of micelles

As pointed out above, dialysis rate constants found in free solution and in the presence of the surfactant at CMC are not statistically different and can be fitted together versus lipophilicity parameters (Table 1, $k_{d,c}$ values). This means that the dialysis process is not appreciably affected by the surfactant in the absence of micelles in the dialyzing fluid. This would also indicate that diffusion layer phenomena and membrane polarity modifications, two factors of decisive importance for in situ absorption tests (Plá-Delfina et al., 1987; Collado et al., 1988), are virtually lacking throughout the dialysis process. This clearly differentiates in vitro and in situ behaviour, and will prevent any comparison and/or extrapolation for the corresponding rate constants.

As the nature of correlations is concerned, Eqns. 12 and 14 give the best fit; the particular correlation equations are given in Table 3. This could be in accordance with some observations showing that diffusion through porous membranes is, at least for some molecular weight ranges, exponentially related to molecular weight, M (Renkin, 1954; Beck and Schultz, 1970; Loehry et al., 1970). Since M has been shown to be related in a linear fashion to log P for true homologous series of compounds (Plá-Delfina and Moreno, 1981), a physicochemical basis for Eqn. 12 could be found. On the other hand, it is convenient to point out here that on the basis of the linear character of the log $k_{d,c}$/log P or M plots, the fitting of the k_s values becomes feasible in light of the criteria outlined from in situ experiences, as will be shown immediately.

Dialysis rate constants found in the presence of micelles

As can be observed in Figs. 1 and 2, the k_s values decrease progressively and significantly as lipophilicity or molecular weight increase, as a result of solubilization of amines in the micelles. Eqn. 13 is fully functional for data fitting, which means that our previous conclusions derived from in situ experiments are correct and that micellar solubilization does indeed acts as the limiting step

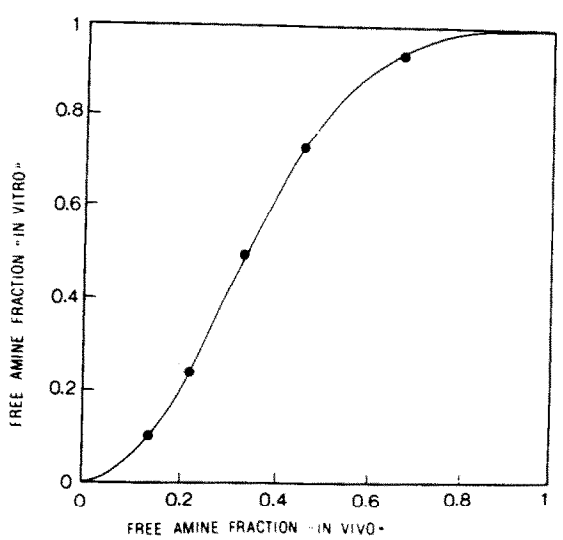

Fig. 3. Sigmoidal plot showing the tentative correlation between the calculated free amine fractions remaining in the dialyzing fluid, in vitro (F_f), and those remaining in the intestinal luminal fluid, in situ (F_f'), in accordance with Eqn. 19, a non-linear transform of Eqn. 11. See also Table 2.

for diffusion. The mathematical expression of the correlation between k_s and P is, in this case:

$$\log k_s = -0.0620 \cdot \log P - \log(0.00378 \cdot P^{1.0067} + 1) + 0.1384 \quad (16)$$

The theoretical line in Fig. 1 has been drawn according to this equation, which produces a good fit ($r = 0.985$; AIC = -15.05. Deviations from the theoretical line seem to be due to small experimental errors in P determination rather than to any other cause because when molecular weights are considered for correlation instead of log P (Eqn. 15), we have:

$$\log k_s = -0.00225 \cdot M - \log(0.0000352 \times 10^{0.0366 \cdot M} + 1) + 0.2629 \quad (17)$$

an equation for which the statistical parameters become considerably better ($r = 0.992$; AIC = -18.47), as shown in Fig. 2. Thus the versatility of Eqns. 13 and 15 (as applied to both in situ and in vitro data) as well as their functionality (for lipophilicity as well as for molecular weight when true homologous compounds are utilized) is assessed.

Correlation between parameters found in vitro and in situ

From the outlined criteria, it is easy to see that any extrapolation concerning diffusion rate constants is impossible. However, on the basis of the experimental studies developed in the presence of the surfactant at SMC, some correlation can be expected between internal partition coefficients found in situ and in vitro: i.e. between the free and solubilized fractions of amine remaining in the working solutions along the entire range of lipophilicity of the tested compounds. The logits of the free amine fractions (F' and F, respectively), as shown in Table 2, were consequently fitted to Eqn. 11 with the following result:

$$\log\left(\frac{1}{F_f'} - 1\right) = 0.5142 \cdot \log\left(\frac{1}{F_f} - 1\right) + 0.2856 \quad (18)$$

From this equation, the value of the free amine fractions prevailing in situ for each compound can be calculated from the F_f value found in dialysis tests by applying the non-logarithmic form of the latter equation:

$$F_f' = \frac{1}{1 + 1.9303\left(\frac{1}{F_f} - 1\right)^{0.5142}} \quad (19)$$

This expression is sigmoidal in nature and has excellent statistical parameters ($r = 0.999$; AIC = -37.86), as shown in Fig. 3. Although this type of correlation is apparently complex, it could easily be used for predictions from F_f dialysis data if in situ determinations for a very limited number of homologs were carried out.

Concluding remarks

Although, as has been pointed out above, the differences are very small, it is somewhat surprising that dialysis constants in the absence of micelles are molecular weight dependent within the narrow range of daltons considered here (90–150). This may be related to the pore diameter of the dialyzing membranes, and it is quite possible that the use of other membrane types would not discriminate dialysis rate constants for the tested compounds and would yield identical $k_{d,c}$ values for all amines. As far as correlation equations are concerned, however, this point is perhaps irrelevant since, in this case, Eqns. 12 and 14 would collapse to an asymptotic rate constant value (i.e. k_o), and Eqns. 13 and 15 would be simplified to the following:

$$\log k_s = \log k_o - \log(B' \cdot P^{a'} + 1) \quad (13a)$$

and:

$$\log k_s = \log k_o - \log\left(B' \cdot P^{a' \cdot M} + 1\right) \qquad (15a)$$

This would give rise to bilinear-type curves with an asymptotic value, k_o, as partition coefficient or molecular weight decrease:

$$\lim_{P \to 0} k_s \frac{k_o}{B' \cdot P^{a'} + 1} = k_o$$

$$\lim_{M \to 0} k_s \frac{k_o}{B' \cdot 10^{a' \cdot M} + 1} = \frac{k_o}{B' + 1} = k_o$$

since in the latter expressions, B' is near zero, as can be seen in Table 3.

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