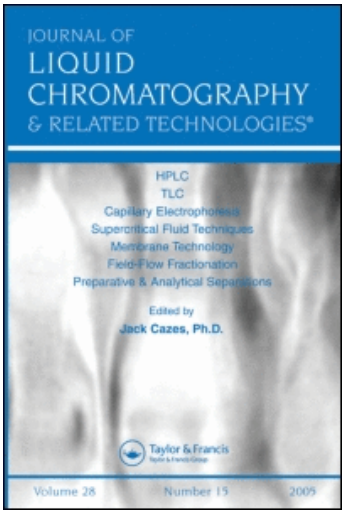

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RELATIONSHIP STUDY BETWEEN REVERSED PHASE HPLC RETENTION AND OCTANOL/WATER PARTITION AMONG AMPHOTERIC COMPOUNDS

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ABSTRACT

The retention of eight non-congeneric amphoteric compounds was followed in a reversed phase (RP HPLC) system (C_{18}/methanol-water vol. 50:50) in the pH range 4 - 9. The chromatographic behavior of the amphoterics is explained by means of their species distribution diagram (pH profile of the protonation macro- and microspecies) in the same pH interval.

Maximum retention was observed at the isoelectric point of the compounds even in cases when the zwitterionic species was in a great excess over the neutral (uncharged) one. This finding reveals, that the *retention at the* isoelectric point (ie.p.) must be generated by the retention of the less polar, neutral form. The great similarity of pH dependent partition (octanol-water) and retention (C_{18}/methanol-water) pattern provides further proof to the concept, that in case of amphoteric compounds the neutral species is transferred into the octanol phase during octanol/water partition.

Linear correlation analysis showed that $logP_{oct/w}$ and $logk'_{C18}$ are analogue lipophilicity parameters i.e.: the physico-chemical content of $logk'_{C18}$ corresponds to that of the octanol/water true partition coefficient.

INTRODUCTION

The biological, therapeutical importance of amphoteria relies on the amphoteric character of the human body proteins and polypeptides, further, on the relatively large number of amphoteric drug compounds. The unambiguous mathematical definition of lipohilicity and protolytic dissociation for amphoterics is a much more complicated task than in case of monofunctional acids or bases, where the true partition coefficient (logP, the concentration rate of the neutral, uncharged species in the organic and the aqueous layer) can be obtained by Eqns. 1 and 2[1]:

$$\text{acids:} \quad logP = logP_{app} + \log (1 + 10^{pH-pKa}) \qquad (1)$$

$$\text{bases:} \quad logP = logP_{app} + \log (1 + 10^{pKa-pH}) \qquad (2)$$

Due to the similarity of their physico-chemical content, a very close relationship exists between the RP HPLC retention (t_R ; logk') and $logP_{oct/w}$, which was recognized earlier and could be utilized in a rather broad spectrum. This correlation proved particularly significant in RP HPLC systems where C_{18} as stationary phase was applied[2]. For this, numerous of examples could be referred from the circle of non-dissociating compounds[3], acids[4] and bases[5].

Relationship between $logP_{app}$ and logP was derived[6] also for amphoteric compounds including the stepwise dissociation constants (pK_{a1}, pK_{a2}):

$$\log P = \log P_{app} + \log (1 + 10^{pH-pKa1} + 10^{pKa2-pH}) \quad (3)$$

Eqn.3 is valid only for "ordinary" amphoterics (in general: $\Delta pK_a \rangle 4$), where beside the cation and anion forms the uncharged (but none of the the zwitterionic) species is present in the equilibrium mixture.

A more complicated relationship must be valid between logP and $\log P_{app}$ if $\Delta pK_a \ll 4$, i.e. overlapping protonation exists thus the solution of such compounds contains also the zwitterionic form ("zwitterionic amphoterics").

The relative concentration of protonation microspecies at a given pH value can be expressed by the protonation microconstants[7] ($k_1^{\pm}$ $k_2^{\pm}$ k_1^o k_2^o in Figure 1). The latters may be calculated if pK_{a1} and pK_{a2} macroconstants are known and one of the microconstants is experimentally available.[7,8] On this basis the diagram of pH dependent distribution of microspecies for a given amphoteric can be prepared (microspeciation). This diagram may supply valuable information about the actual protonation state and lipophilicity of the compounds. Takács-Novák et al.[9,10,11] basing on certain experimental data suggest, that only the neutral species partitions between the octanol and aqueous phase. The authors derived a relationship between logP and $\log P_{app}$ for zwitterionic amphoterics[10]:

$$\log P = \log P_{app} + \log (1 + 1/ k_1^o [H^+] + k_2^o / k_2^{\pm} + k_2^o [H^+]) \quad (4)$$

It seems probable, that the values of microconstants and data of species distribution as well as the true partition coefficients may provide valuable ideas to define the mechanism of effect for amphoteric drug compounds.

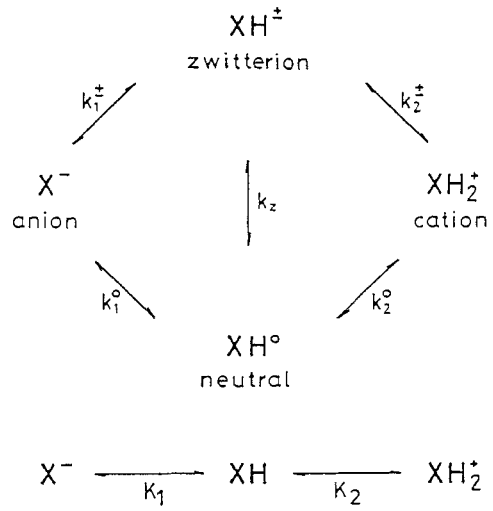


FIGURE 1 Protonation scheme of amphoterics

In the course of the present work the pH profile of reversed phase (RP HPLC) retention was followed in pH range 4 - 9. Since the retention on C_{18} stationary phase showed an evident analogy with octanol/water partition, it seemed reasonable anticipation, that the pH dependent RP HPLC retention should allow some new insight to the equilibrium partitioning state of different amphoteric compounds.

The C_{18} retention, although with some reserves,[2,13] is accepted as a lipophilicity parameter, moreover, in some cases, it proved suitable in QSAR research[12,14]. The RP_{C18} HPLC provided particularly good correlations with the octanol/water partition, when the mobile phase, beside water (aqueous buffer solution) contained methanol as organic modifier.[15,16] The correlation between $\log k'_{C18}$ and $\log P_{oct/w}$ was also close in case of ionizable compounds[17] although certain authors[18,19] suggested the use of correction. Our results unanimously reveal the

predominant contribution of the neutral (less polar) species in developing the final retention of the amphoteric solute. This behavior can be met regardless of that the uncharged form is the major or the minor component of the equilibrium solution.

EXPERIMENTAL

Model substances

Pyridoxine (HCl), Morphine ($HCl.3H_2O$), Nitrazepam, Sulfadimidine were a quality of Hungarian Pharmacopoeia.[20] Perfloxacin, Norfloxacin were synthesized at Chinoin Pharmaceutical Works (Budapest), Niflumic acid was also generously supplied by its manufacturer, Gedeon Richter Chemical Works (Budapest). All these substances were used without further purification. 11-amino undecanoic acid (11-AA) 99% (Aldrich).

Materials

Buffer solutions (for Chromatography) in pH range 3 - 8 were prepared by mixing the proper volumes of 0.067 M aqueous solutions of potassium dihydrogenphosphate and disodium hydrogenphosphate (KH_2PO_4, $Na_2HPO_4.2H_2O$ anal. grade, Reanal, Budapest). The pH of these solutions was tested by pH-metry. As mobile phase the 50:50 vol. mixture of the buffer solutions (20°C) and methanol (20°C) was used. After mixing of the solutions a final pH control at the ready for use methanolic mobile phase was performed.

For pH measurement combined glass electrode (Radiometer GK2320 C) and a reference pH meter (Radiometer PHM93) were used. The solutions were thermostatted at 20°C and stirred by magnetic stirrer.

TABLE 1

The pH shifting effect of methanol in aqueous buffer solutions

pH (± 0.02)		Δ
aqueous buffer solution	after methanolic dilution (1 :1)	(shifting)
3.12	4.03	0.91
5.08	6.10	1.02
6.09	7.27	1.18
7.47	8.74	1.27
8.40	9.24	0.87

Electrode calibration was performed using standard buffer solutions (Aldrich, pH 2 - 11). The accepted pH values are the average of three subsequent readings, the standard deviation was less than ±0.02 pH unit.

Table 1 shows the pH values of the aqueous and the corresponding methanolic buffer solutions.

Methanol, HPLC grade (Chemolab,Budapest).

Chromatography

The HPLC apparatus was comprised in Waters (Millipore,USA) Model 501 solvent delivery system, Labor MIM (Budapest,Hungary) Model QE 308 variable wavelength UV photometer as detector, Yokogava (Tokyo,Japan) Type 3051 recorder. For the detection of 11-AA refractometry was applied (Waters Differential Refractometer, Model R 401). The packing was filled into steel columns 250 x 4.6 mm. I.D.) the adsorbent, Hypersil 5 ODS was purchased from Bioseparation Technique Ltd., (Budapest, Hungary) in a particle size 5 μm.

As eluent the 50:50 mixture of methanol-aqueous buffer solutions was used. The eluents, after pH control, were filtered and degassed prior to chromatography. The flow rate was 1.0ml/min. A Model 7125 sampling valve (Rheodyne, Berkley, USA) was applied.

The column temperature was controlled by recirculating water through an isolated stainless jacket from thermostat (Ultrathermostate, MLW Type U_2C, Freital, Germany).

The model substances were solved in the eluent. The chromatograms were recorded and the retention data were collected by a Hewlett-Packard integrator Model 3396 Ser.2.

Each retention data was calculated as an average of three parallel runs. The mobile phase hold up time was signalled by the solvent peak of methanol.

Determination of the octanol/water pH-partition profile

The traditional shake-flask method was used for logP measurements. The apparent partition coefficients ($logP_{app}$) were determined in wide pH range at 7 different pH values including the ie. point pH. The experimental details and the obtained $logP_{app}$ values were published elsewhere.[9,10] Here only the pH-partition profile of the molecules is presented in Figure 3.

Determination of the protonation macro- and microconstants

The protonation macroconstants were determined by standard methods (potentiometry or UV spectroscopy) at $25 \pm 0.1^{\circ}C$, $I = 0.2M$ ionic strength. Combined pH-metry and UV spectroscopy was applied to determine the protonation microconstants.[8,11] The pH-dependent relative concentrations (%) of microspecies were calculated using

protonation microconstants. The distribution diagrams are shown in Figure 4.

RESULTS, DISCUSSION

In Table 2, the RP HPLC retention values of the model compounds and also the pK values as well as the isoelectric points are included. Figure 2 shows the structural formulas of the compounds.

In assigning the pH interval to be studied (4 - 9) the stability of the chromatographic column and the reproducibility (comparability) of the results were considered. This limitation occasionally diminished the possibilities to compare the RP HPLC retention and octanol/water partitioning behavior in a wider pH interval. The pH profile of octanol/water partition is shown by Figure 3, while pH dependence of species distribution (macro- and microspeciation) can be seen on Figure 4.

As the species distribution in the equilibrium mixtures at the ie.p. concerns, the model compounds may be divided into three groups. 11-AA is a typical zwitterionic amphoteric that is at the pH of ie.p. practically only the zwitterionic species exists; on the contrary, nitrazepam and sulfadimidine are true ordinary amphoterics. In their solution, beside the cation and the anion form, as third component the neutral (uncharged) species is present. The other five (compds No. 2-6 in Table 2) substances represent a transition: in their solution at the pH interval, near to the ie.p., the zwitterionic and neutral species co-exist in commensurable amount. The relative concentration of the neutral form increases from niflumic acid (compd. No. 2) through the morphine (compd. No. 6).

TABLE 2

RP-HPLC retention times (min) of model substances

N°	substance	pK_{a1} pK_{a2} (ie.p.)	pH of the eluent				
			4.03	6.10	7.27	8.74	9.24
1.	11-AA	10.74 4.56 (7.65)	5.40	4.65	4.80	5.10	5.55
2.	niflumic acid	4.44 2.26 (3.35)	57.10	29.70	17.10	13.80	12.90
3.	pyridoxine	9.16 5.05 (7.10)	3.50	5.47	6.86	5.67	5.53
4.	norfloxacin	8.51 6.22 (7.37)	3.62	5.24	8.90	7.60	5.62
5.	pefloxacin	7.80 6.02 (6.91)	4.03	13.31	33.00	14.80	8.42
6.	morphine	9.54 8.34 (8.94)	2.77	2.95	4.93	8.60	10.70
7.	nitrazepam	10.66 2.94 (6.80)	12.60	12.20	12.30	12.50	12.50
8.	sulfadimidine	7.38 2.36 (4.87)	3.35	3.75	3.52	3.39	3.48

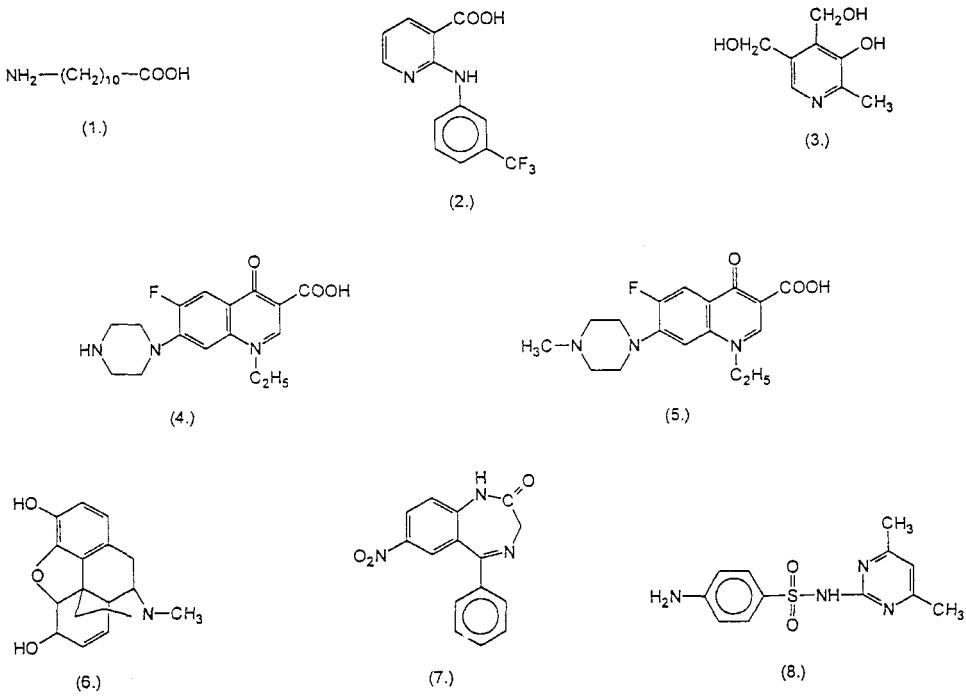


FIGURE 2 Chemical structure of the model compounds

Figure 5 shows the pH profile of RP HPLC retention of model compounds expressed by logk' values. The shape of curves are very characteristic.

In case of **11-AA** mild retention depression my be observed in the region of ie.p. This is a behaviour which must be typical of pure zwitterionic amphoterics where the charged poles are isolated.

The retention of **niflumic acid** shows a definite increase, approaching to the ie.p. though, the relative concentration of the zwitterion at this region is higher than 90%. This fact unambiguously indicates the adsorbance of the neutral form since from the two

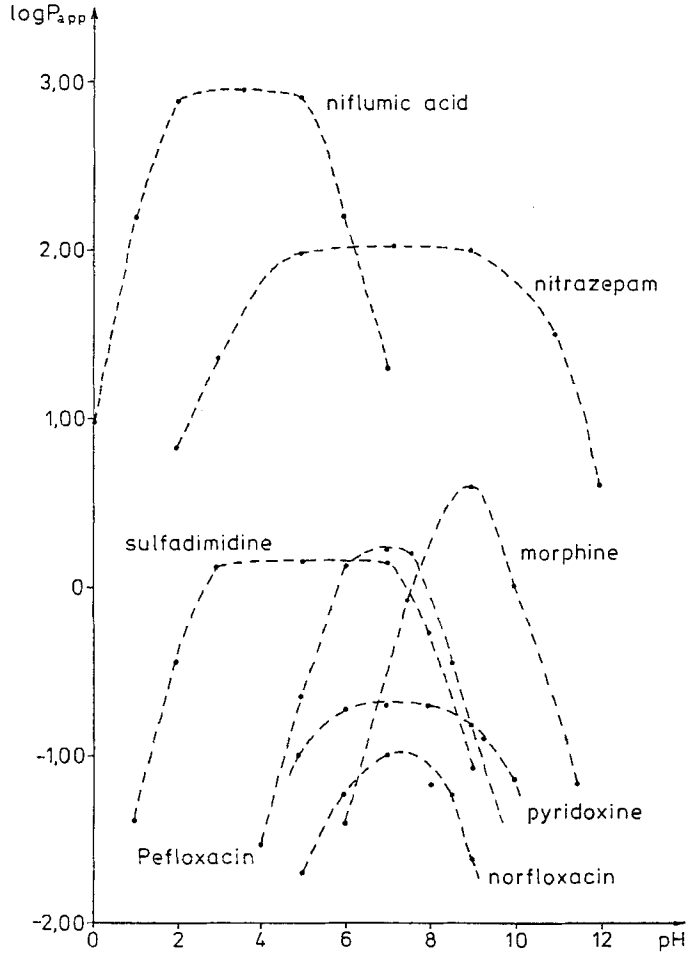
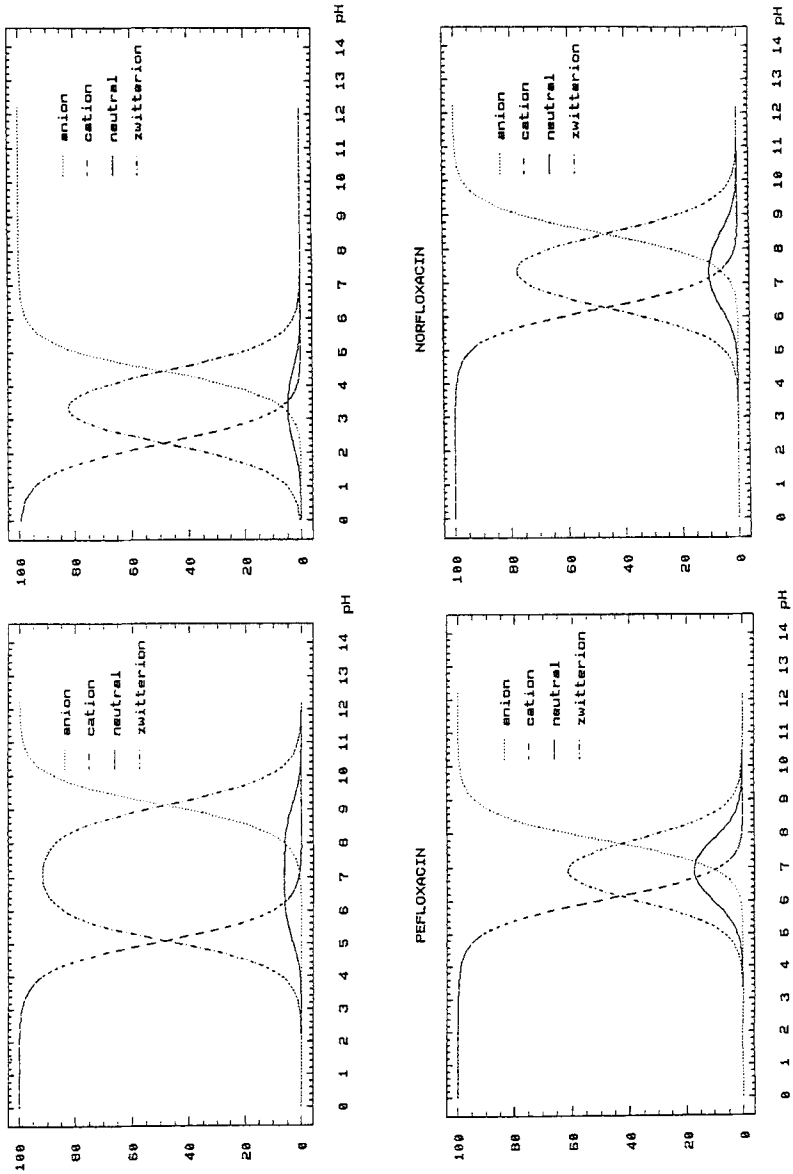


FIGURE 3 pH profile of octanol/water partition



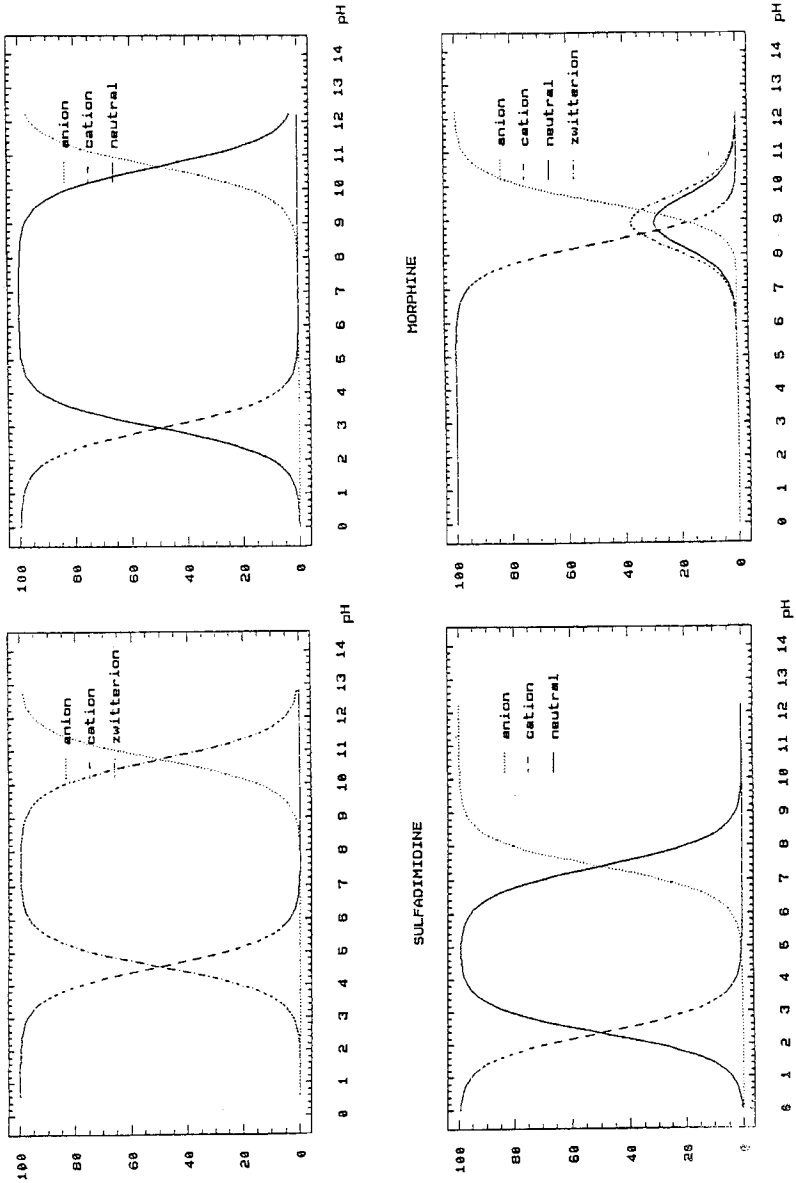


FIGURE 4 Species distribution diagram of the model substances

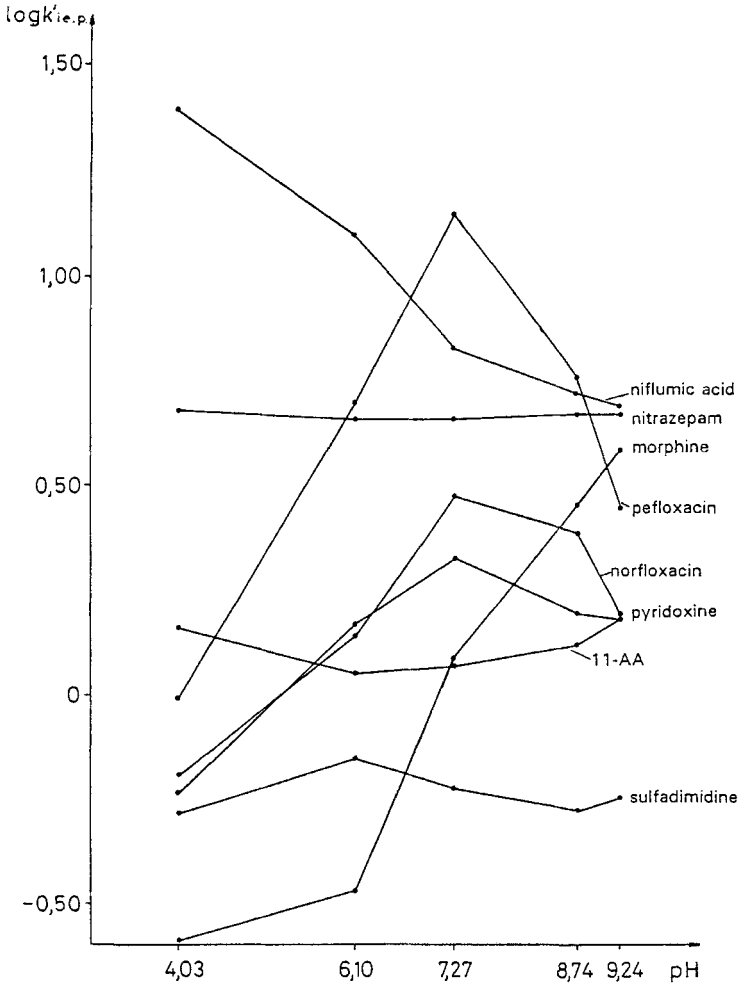


FIGURE 5 pH profile of RP-HPLC retention

protonation isomers $XH^{\pm}$ and XH^{o}, the neutral form is the less polar i.e. more lipophilic. The very steep rise of the retention by pH depression may be a consequence of the great difference in acid-base strength (ie.: lipophilicity) of the niflumic acid cation and anion species (7-ie.p. = 3.65). The plausibility of this explanation is supported by the species distribution diagram (Figure 4). Near to the ie.p. the amount (contribution to the retention) of the more polar anionic form rapidly decreases followed by the appearance of the cationic form. Quite reasonable to assume, that a maximum retention could be achieved at the ie.p. (pH 3.35). In addition, the ion pairing between niflumic acid and phosphate ions[11] as a weak retention modifying factor must not be excluded.

Norfloxacin and **pefloxacin** show a definite retention maximum at the ie.p. region, evidently caused by the adsorbance of the neutral form existing in gradually increasing concentration. The significantly higher retentions in the alkaline region (cf. retentions at pH 4.03 and 9.24) may arise from differences in the neutral form concentrations:

pH	z %	n %	a %	c %
4.03	0.73	0.21	-	99.06
9.24	2.72	0.77	96.51	-

z: zwitterionic, n: neutral, a: anionic c: cationic form

or may be indicators of different lipophilicity of cationic and anionic species. However, this latter assumption is less probable since the acid--base strength of pefloxacin's carboxyl and amine groups is rather equal (7-ie.p. = 0.09).

The retentions of **morphine** are increasing towards the alkaline zone and must decrease above pH ⟩ 9.24 (which interval not tested).

The correspondent species distribution diagram clearly shows, that the retention increase is related to the adsorbance of the neutral from.

Pyridoxine has a definite maximum curve. The maximal retention appears at the ie.p., indicating again the adsorbance of the neutral form.

The retentions of **nitrazepam** are located along a straight line; by means of the species distribution diagram (Figure 4) it can be seen that through the pH interval (4.03 - 9.24) studied the uncharged is the practically existing form. The monoions appearing at the peripheral alkaline and acidic pHs, due to their almost equal and small polarity (7 - ie.p. = 0.20) have hardly influence on the overall retention; the retention curve describes an almost straight line. The **sulfadimidine** curve runs similar to that of nitrazepam, though, the low retention values may reduce the reliability of evaluation.

The results above unambiguously confirm the conception about the dominant contribution of the neutral species adsorbance to the retention of amphoterics. This effect seems to work quite independently from the actual ratio of the zwitterionic and uncharged form, appearing not only by the ordinary amphoteric compounds but also by those representing a transition between the pure ordinary and zwitterionic amphoterics. This experience, in addition to the similar feature of lipophilicity pH profile and retention pH profile (cf. Figures 3 and 5) proves by a series of non-congeneric compounds, that partitioning properties of C_{18}/methanol-water and octanol/water systems are very similar.

By linear regression analysis between the true partition coefficients (data in Table 3) and $\log k'_{ie.p.}$ values a correlation coefficient r = 0.768 has been found including all molecules. If the two fluoroquinolones (compds. 4 and 5) had been omitted from the analysis the r value

TABLE 3

The true partition coefficients (logP) and the capacity factors ($logk'_{ie.p.}$) of model compounds

substance	logP	$logk'_{ie.p.}$ [c]
niflumic acid	4.43[a]	1.379
pyridoxine	0.33[a]	0.326
norfloxacin	-0.02[a]	0.484
pefloxacin	1.07[a]	1.146
morphine	1.22[a]	0.587
nitrazepam	1.96[b]	0.670
sulfadimidine	0.19[b]	-0.152

a.) logP value calculated by Eqn. 4
b.) logP value calcutated by Eqn. 3
c.) logk' value calculated from retention times (Table 2); t_o = 2.20 (min).

has improved to 0.948, which can be considered a significant linear relationship. (The outlier behavior of fluoroquinolones points out to some specific interactions and requires further investigations.)
Based on the above findings, $logk'_{ie.p.}$ retention and logP (true partition coefficient) as physico-chemical content concerns, should be regarded analogue lipophilicity parameters.

REFERENCES

1. Leo, A., Hansch, C., Etkins, D.: Chem. Rev. 71. 525 (1971)

2. Haggerty, W.J., Murrill, E.A.: Res. Dev. 25. 30 (1974)

3. Koopmans, R.E., Rekker, R.F.: J. Chromatogr. 285. 267 (1984)

4. Hafkenscheid, T.L., Tomlinson, E.: Int. J. Pharm. 16. 225 (1983)

5. ibid.: J. Chromatogr. 292. 305 (1984)

6. Asuero, A.G.,: Int. J. Pharm. 45. 157 (1988)

7. Noszál, B.: Acid-base properties of bioligands. p. 18-55. in: Burger, K. (Ed.) Biocoordination chemistry, Coordination equilibria in biologically active systems. Ellis-Horwood, Chichester, 1989.

8. Takács-Novák, K., Noszál, B., Hermecz, I., Keresztúri, G., Podányi, B., Szász, Gy.: J. Pharm. Sci. 79. 1023 (1990)

9. Takács-Novák, K., Józan, M., Hermecz, I., Szász, Gy.: Int. J. Pharm. 79. 89 (1992)

10. Takács-Novák, K., Józan, M., Szász, Gy.: Int. J. Pharm. (1994) (in press)

11. Takács-Novák, K., Avdeef, A., Box, K.J., Podányi, B., Szász Gy.: J. Pharmaceut. Biomed. Anal. (1994) (accepted for publication).

12. Braumann, Th.: J. Chromatogr. 373. 191 (1986)

13. Antle, P.E., Goldberg, A.P., Snyder, L.R.: J. Chromatogr. 321. 1 (1985)

14. Shalaby, A., Budvári-Bárány, Zs.: Hankó-Novák, K., Szász, Gy.: J. Liquid Chromatogr. 7. 2493 (1984)

15. Haky, J.E., Young, A.M.: J. Liquid Chromatogr. 7. 675 (1984)

16. El Tayar, N., Van de Waterbeemd, H., Testa, B.: Quant. Struct. Act. Relat. 4. 69 (1985)

17. ibid: J. Chromatogr. 320. 293 (1985)

18. Horváth, Cs., Melander, W., Molnár, I.: Anal. Chem. 49. 142 (1977)

19. Fong. M.H., Aarons, L., Moso, R., Caccia, S.: J. Chromatogr. 333. 191 (1985)

20. Pharmacopoeia Hungarica Ed. VII., Vol. 2., Medicina, Budapest, 1986.

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