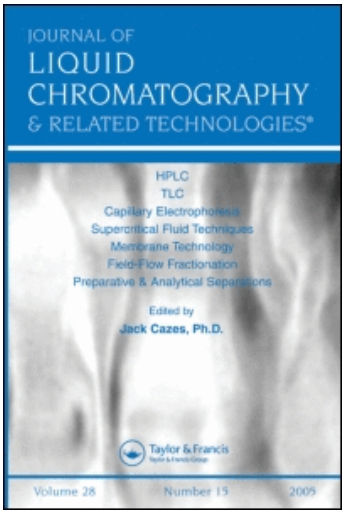

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Zs. Budvári-bárány[a]; Gy. Szász[a]; K. Takács-novák[a]; I. Hermecz[b]; A. Lóre[a]
[a] Institute of Pharmaceutical Chemistry Semmelweis University of Medicine, Budapest, Hungary [b] Chinoin Pharmaceutical Works, Budapest, Hungary

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THE pH INFLUENCE ON THE HPLC-RETENTION OF CHEMOTHERAPEUTIC FLUOROQUINOLONE DERIVATIVES

ZS. BUDVÁRI-BÁRÁNY[1], GY. SZÁSZ[1], K. TAKÁCS-NOVÁK[1], I. HERMECZ[2], AND A. LÓRE[1]

[1]*Institute of Pharmaceutical Chemistry*
Semmelweis University of Medicine
Budapest, Hungary
[2]*Chinoin Pharmaceutical Works*
Budapest, Hungary

ABSTRACT

The gyrase inhibitor fluoroquinolone derivatives were investigated by RP-HPLC methods in C_{18}/methanol-phosphate buffer system. Considering the amphoteric feature of the substances it was reasonable to study the relationship between their chromatographic behaviour vs. pH of the mobil phase. The formation of k' values was characterized by a maximum curve, and the retention maxima could be found close to or at the pH values of isoelectric point of the compounds. This may be explained by the presence of the zwitter ionic or the unionized form of the derivatives at this pH value.

By addition of ion-pairing (IP) agent (cetrimide or hexanesulfonic acid) in different concentration to the eluent a definite k' value increase (formation of a characteristic saturation type curve) was observed indicating an ion-pairing mechanism.

In case of IPHPLC significant correlation was found between the octanol-water partition coefficients measured at the isoelectric point or at pH 7.4, and the retention values of the compounds.

INTRODUCTION

The gyrase inhibitor fluoroquinolone derivatives, due to their therapeutic importance, attract permanent interest of numerous fields of pharmaceutical research. In the investigation of metabolism, pharmakokinetics, analytical control of these substances HPLC is the most often used method. On the latter field in a large number of papers a great variety of experimental conditions may be met concerning the composition of the mobile phase (pH, ionic strength and pH adjusting-, ion-pairing-, peak symmetry improving agents).

In the great majority of the publications* dealing with the analysis of fluoroquinolones reversed phase HPLC (RPHPLC) method (ODS stationary phase) and very rarely normal phase HPLC was used. In RPHPLC phosphate buffer with different pH as aqueous component of the mobile phase, methanol and/or acetonitrile as organic modifiers were applied. The ionic strength of the eluent was adjusted by the addition of sodium chloride, -perchlorate or phosphate salts. As ion-pairing agents tetrabutilammonium salts, Na dodecylsulfate, trichloroacetic acid were used. Worth to note, that ion pairing activity on behalf of phosphate or acetate ions also was observed (1). For peak shape improvement high acidity of the eluent (low pH adjusted by phosphoric acid) increased ionic strength and different additives e.g. citric acid, perchloric acid or tertiary amine salts, served (2).

The pK_a values of the fluoroquinolones containing piperazinyl substituent (Fig. 1) range in the interval 7.5-8.5

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*Authors are grateful to colleague T. Gábor (Institute of Pharmaceutical Chemistry, Budapest) who gave us free run of his compilation of fluoroquinolones' HPLC literature.

for the ammonium form and 5.5-6.0 for the carboxylic group (3). Consequently, the substances begin to be completely ionized in acidic region at pH 5.5-6.5 and in the alkaline zone 7.5-8. The same compounds are in non-ionized form at their isoelectric points which are between pH 6.5-7.5. Our investigations aimed to get data about RPHPLC retention values of fluoroquinolone through a wide pH interval (3-8) with or without the use of an ion-pairing agent, and to provide systhematic principles to the HPLC behaviour of fluoroquinolones.

EXPERIMENTAL

Materials

Samples of fluoroquinolones, derivatives of nalidixic acid, oxolinic acid (Fig. 1) were generously supplied by Chinoin Pharmaceutical Works and were used without further purification .

Methanol, LiChrosolv^{R} for chromatography (Merck).

Phosphate buffer solutions were made by mixing in different ratio of 0.025 M or 0.067 M solutions of potassium dihydrogenphosphate, (KH_2PO_4) and dibasic sodium hydrogenphosphate ($Na_2HPO_4.7H_2O$), Aldrich.

Cetrimide, Reanal (Budapest)

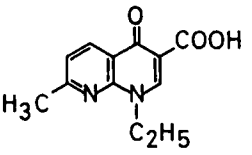
Hexanesulfonate sodium, Aldrich.

Sodium chloride, Reanal (Budapest).

Chromatography

The HPLC apparatus was comprised in an ISCO pump, Model 2350 (USA) and an ISCO variable wavelength absorbance detector. The column effluent was monitored at 254 nm. Chromatographic system included as reversed phase LiChrosorb C_{18} 5 μm (Merck)

1. Nalidixic acid



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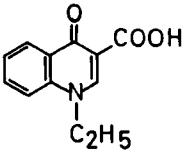

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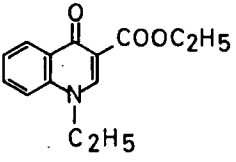
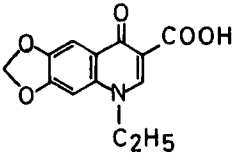
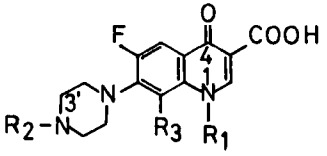


4. Oxolinic acid

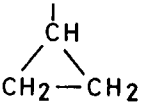




	R_1	R_2	R_3
5. Norfloxacin	C_2H_5	H-	H
6. Norfloxacin--3-ethylester			

Fig. 1.

Structure of model compounds

7. N-acetyl-norfloxacin	C_2H_5	CH_3CO-	H
8. 8-F-Norfloxacin	C_2H_5	H-	F
9. Pefloxacin	C_2H_5	CH_3-	H
10. 8-F-Pefloxacin	C_2H_5	CH_3-	F
11. Lomefloxacin	C_2H_5	H- (3'-CH_3)	F
12. 8-des-F-Lomefloxacin	C_2H_5	H- (3'-CH_3)	H
13. Amifloxacin	$NHCH_3$	CH_3-	H
14. Ciprofloxacin		H-	H

15. Ofloxacin

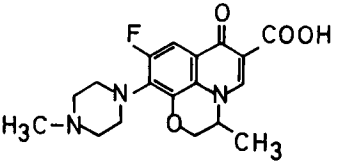


Fig. 1 (continued)

in a column measured 250x4.6 mm i.d. The mobile phase contained methanol-phosphate buffer 50:50 per cent (a series of buffer solutions was used in the interval of pH 3 through 8 in increasing order with 0.5 pH units). The pH of the buffer solutions was adjusted to the declared value with an accuracy 0.05 unit with occasional addition of phosphoric acid 85 per cent or sodium hydroxyde solution 5 M, before the mixing with the methanol. The pH values were measured by a Digital pH-meter (Radelkis OP-211/1, Budapest).

RESULTS AND DISCUSSION

The capacity factors of the compounds vs. pH of the eluent are listed in Table 1. It can be seen, that the relation between pH of eluent and the k' value of the compounds may be characterized, with few exception, by a maximum curve. Considering the amphoteric feature of the substances, it seems evident, that the k' maxima are generated by the zwitter ionic or the unionized form of the fluoroquinolone derivatives (Fig. 2). This interpretation is supported by the fact, that the retention maxima can be found at pH values close to the isoelectric point of the compounds. A pH-measuring after the addition of methanol i.e. in a methanol-buffer 50:50 mixture showed pH value increase with about one pH unit (Table 1). This is in good accordance with the earlier finding that buffer acidity was suppressed by the addition of ethanol (4). At a pH more acidic or alkaline than that of the isoelectric point the protonated or the deprotonated form of the compounds are present which move with lower k' values (see Table 1). It can be seen that N-methylation of piperazine causes very significant k' increase (compare k's of norfloxacine and pefloxacine). It is also worth to note that fluor--substitution at position C_8 significantly decreases the retention values (compare k's of pefloxacine and 8-F-pefloxacine, des-8-F-lomefloxacine, and lomefloxacine). Similar tendency was observed in the relation of lipophylicity (5). The difference between the k'_{max} values of nalidixic and oxolinic acids reflects the difference in their pK_a values (6.13 and 6.91 resply).

Table 1

pH dependence of the retention (k')

compound (isoelectric point)[3]	pH							
	3* 3.83**	5* 6.02**	5.5* 6.50**	6.0* 7.03**	6.5* 7.50**	7.0* 8.09**	7.5* 8.60**	8* 9.07**
nalidixic acid	3.11	3.42	3.42	3.04	1.68	1.09	0.57	
H-4437	1.76	1.83	1.93	1.70	1.26	0.64	0.32	0.35
H-4380	1.82	1.93	2.02	2.07	2.0	2.01	2.08	
oxolinic acid	1.86	2.10	2.13	2.2	1.68	1.23	0.51	0.43
norfloxacin (7.37)	0.43	0.72	1.17	1.63	2.04	1.98	1.11	0.51
norfloxacin-3-ethyl ester	0.42	0.64	1.05	1.64	3.22	5.46	7.63	7.90
n-acetyl-norfloxacin	3.40	3.58	3.40	3.07	2.04	1.04	0.41	0.29
8-F-norfloxacin (7.44)	0.38	0.58	0.79	0.90	0.98	0.92	0.86	0.49
pefloxacin (6.91)	0.45	2.35	4.93	8.69	8.0	5.54	2.38	1.42
8-F-pefloxacin (6.73)	0.50	0.71	0.94	1.09	1.17	1.16	1.03	0.76
lomefloxacin (7.14)	0.52	0.71	0.93	1.08	1.18	1.11	1.02	0.76
8-des-F-lomefloxacin (7.19)	0.57	0.87	1.41	1.96	2.72	2.49	1.65	0.88
amifloxacin (6.5)	0.40	1.65	3.48	5.41	5.17	3.18	1.44	1.14
ciprofloxacin (7.5)	0.48	0.79	1.21	1.66	1.87	1.67	0.98	0.52
ofloxacin (7.0)	0.40	1.03	1.94	3.40	4.19	3.32	1.72	1.07

* Measured in aqueous buffer solution ** Measured in methanol-buffer solution mixture 50:50

Mobile phases: methanol 50% + phosphate buffer (pH = 3-8) 50% I = 0.1 adjusted by NaCl.

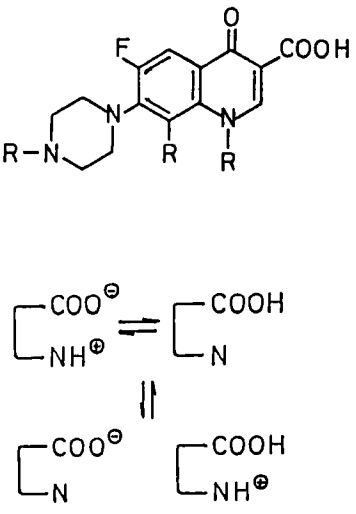


Fig.2.

Equilibrial species in a fluoroquinolone solution

In RPHPLC of fluoroquinolones the addition of an ion pairing agent to the mobile phase to keep a good peak symmetry as well as to assure a higher selectivity is a quite often used solution. Also in this respect, depending upon the quality of the analytical task, a good number of mobile phase variety can be found in the fluoroquinolone-literature. In this work the chromatographic behaviour of substances under the simplest but reasonable conditions for ion-pair formation was investigated. The results are summarized in Tables 2-4.

In Table 2 the k' values of the compounds are shown when eluents with two different acidic pH values (5.5; 3) were used. The ionic strength of the eluent was adjusted to 0.1 in every cases. The ion-pairing effect of hexanesulfonic acid may be

Table 2

Ion pair formation with hexanesulfonate

Compound	k'			
	pH 5.5*		pH 3.0*	
	(1)	(2)	(3)	(4)
nalidixic acid	5.14	4.38	3.79	3.42
H-4437	1.78	1.90	1.70	1.66
H-4380	1.93	1.93	1.67	1.60
oxolinic acid	2.30	2.29	1.94	1.78
norfloxacin	2.70	3.48	0.76	1.46
norfloxacin-3-ethylester	1.80	2.51	0.52	1.07
n-acetyl-norfloxacin	3.66	4.38	3.76	3.67
8-F-norfloxacin	1.08	1.90	0.52	1.15
pefloxacin	8.83	10.31	1.17	1.85
8-F-pefloxacin	4.37	5.57	0.69	1.39
lomefloxacin	1.42	2.04	0.61	1.31
8-des-F-lomefloxacin (7.19)	2.12	3.66	0.72	1.58
amifloxacin	7.14	7.43	0.83	1.38
ciprofloxacin	2.60	3.52	0.82	1.54
ofloxacin	4.35	4.90	0.81	1.44

Mobile phase:

(1) and (3) Methanol 50% + 0.067 M phosphate buffer 50%

(2) and (4) 0.01 M hexanesulfonate in methanol 50% + 0.067 M phosphate buffer 50%

(1-4) I = 0.1 (adjusted by NaCl)

* pH values measured in aqueous solution

Table 3

The effect of phosphate and hexanesulfonate on the retention of fluoroquinolones

	k'		
	(1)	(2)	(3)
pefloxacin	0.46	1.17	1.85
ciprofloxacin	0.49	0.82	1.54
norfloxacin	0.43	0.76	1.46
8-F-norfloxacin	0.38	0,52	1.15

Mobile phase:

(1) Methanol 50% - 0.025 M phosphate buffer (pH = 3) 50%

(2) Methanol 50% - 0.067 M phosphate buffer (pH = 3) 50%

(3) 0.01 M hexanesulfonate in methanol 50% - 0.067 M phosphate buffer (pH = 3) 50%

I = 0.1 (adjusted by NaCl).

obeserved in columns 2 and 4. The retention increase on the addition of the ion-pairing agent is significant at all of the piperazine derivatives with the exception of amifloxacine. This may be explained by the relatively low isoelectric point (6.5) of this compound. (When a series of eluents with hexanesulfonate concentration from 0.005 M through 0.03 M was applied the hexanesulfonate concentration vs. k' relationship gave a characteristic saturation type curve. This phenomenon supports the fact of ion-pair formation.) The

Table 4

Ion pair formation with cetrimide

Compounds	k'			
	pH 7.5		pH 8.0	
	(1)	(2)	(3)	(4)
nalidixic acid	0.92	3.16	0.77	6.21
H-4437	0.42	1.33	0.38	2.10
H-4380	1.21	0.48	1.88	0.59
oxolinic acid	0.54	1.19	0.43	1.76
norfloxacin	1.27	1.14	0.51	1.07
norfloxacin-3-ethylester	4.77	0.72	7.85	0.61
n-acetyl-norfloxacin	0.50	0.76	0.29	0.97
8-F-norfloxacin	0.82	0.96	0.49	1.25
pefloxacin	2.36	2.42	1.42	3.23
8-F-pefloxacin	2.01	3.25	0.76	2.00
lomefloxacin	1.00	1.25	0.76	1.93
8-des-F-lomefloxacin	1.51	1.38	0.80	1.45
amifloxacin	1.45	2.41	1.14	3.79
ciprofloxacin	1.23	1.21	0.52	1.17
ofloxacin	1.72	2,03	1,07	3.07

Mobile phase:

(1) and (3) Methanol 50% + phosphate buffer (0.067 M) 50%

(2) and (4) 0.01 M cetrimide in methanol 50% + phosphate buffer (0.067 M) 50%

(1-4) I = 0.1 adjusted by NaCl

Table 5

Apparent log P values of fluoroquinolones at the isoelectric point and the blood pH-s

Compound	log P_{app} at ie. point[5]	log P_{app} pH 7.4[5]
norfloxacin	-1.03 +/- 0.04	-1.03 +/- 0.04
pefloxacin	0.27 +/- 0.04	0.18 +/- 0.05
lomefloxacin	-0.80 +/- 0.10	-1.03 +/- 0.09
8-F-norfloxacin	-1.13 +/- 0.09	-1.13 +/- 0.09
8-F-pefloxacin	-0.09 +/- 0.03	-0.21 +/- 0.05
8-des-F-lomefloxacin	-0.75 +/- 0.07	-0.80 +/- 0.10
amifloxacin	0.23 +/- 0.03	0.05 +/- 0.02
ciprofloxacin	-1.08 +/- 0.05	-1.11 +/- 0.04
ofloxacin	-0.39 +/- 0.05	-0.44 +/- 0.02

* $\log k'_c = 0.33 \log P_{pH\ 7.4} + 0.42$

$r = 0.918 \qquad n = 9$

** $\log k'_{hs} = 0.43 \log P_{pH\ 7.4} + 0.88$

$r = 0.924 \qquad n = 9$

** $\log k'_{hs} = 0.385 \log P_{ie.} + 0.82$

$r = 0.888 \qquad n = 9$

* k' values and mobile phase see in Table 4, column (2).

** k' values and mobile phase see in Table 2, column (2).

c = cetrimide

hs = hexanesulfonate

relatively small k' increasing effect of hexanesulfonic acid may be a consequence of the complexing ability of phosphate itself i.e. a competition between hexanesulfonate and phosphate anions for fluoroquinolonium cations. This tendency is clearly reflected by the data of Table 3.

In Table 4 the ionpairing effect of cetrimide is shown. (Since the relationship cetrimide concn. vs. retention manifested itself in the form of a saturation-like curve and cetrimide in a 0.01 M concentration caused maximum retention increase, the latter was chosen for the illustration.)

A rather close correlation was found between the octanol-water partition coefficients measured at the isoelectric point or at pH 7.4 (log P see in Table 5) and the retention values (log k') of the compounds when an ion-pairing agent was added to the mobile phase.

As a theoretical basis for this latter correlation could serve that partition coefficient at the isoelectric point is generated by an interaction between n-octanol (water) and the less polar form (zwitter ion or uncharged species) of the substances. In the other hand, retention value in the presence of an ion-pairing agent represents an interaction between C_{18} stationary phase and the less polar ion-pairs formed with cetrimide or hexanesulfonate respectively.

This experience provides further proof for adequacy of RP(C_{18})HPLC to the traditional shake flask method of partition coefficient determination, and showes the suitability of the RPHPLC method for the QSAR research.

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