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Investigation of the retention/pH profile of zwitterionic fluoroquinolones in reversed-phase and ion-interaction high performance liquid chromatography

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Abstract

The retention/pH profiles of three fluoroquinolones, ofloxacin, norfloxacin and ciprofloxacin, was investigated by means of reversed-phase high performance liquid chromatography (RP-HPLC) and reversed-phase ion-interaction chromatography (RP-IIC), using an octadecylsilane stationary phase and acetonitrile as organic modifier. Sodium hexanesulphonate and tetrabutylammonium hydroxide were used as sources of counter ions in ion-interaction chromatography. The retention/pH profiles under in RP-HPLC were compared to the corresponding lipophilicity/pH profiles. Despite the rather hydrophilic nature of the three fluoroquinolones positive retention factors were obtained while there was a shift of the retention maximum towards more acidic pH values. This behavior was attributed mainly to non-hydrophobic silanophilic interactions with the silanized silica gel material of the stationary phase. In ion-interaction chromatography the effect of counter ions over a broad pH range was found to be ruled rather by the ion pair formation in the mobile phase which led to a drastic decrease in retention as a consequence of the disruption of the zwitterionic structure and thereupon the deliberation of a net charge in the molecules. At pH values at which zwitterionic structure was not favored both the ion-exchange and ion pair formation mechanisms were assumed to contribute to the retention.

Keywords: Fluoroquinolones; Retention/pH profile; Ion-interaction chromatography; Zwitterions; Ion pairs; Ion-exchange

1. Introduction

The presence of charged centers in many drugs may be essential for their biological activity and their passage into cells and across other membranes [1,2]. According to a study using Oxford Molecular's Chem-X software, about 15% of the ionizable drugs compounds listed in World Drug Index 1999 can be classified as ampholytes, many of which exhibit zwitterionic characteristics [3]. Zwitterions represent a particular type of solutes with intra- and inter-molecular interactions, which influence their physicochemical characteristics. Their partitioning behavior, expressed as octanol–water $\log D$, changes as a function of pH in the internal environment and the dissociation constants resulting in most cases in a bell shaped profile. A U-shaped $\log D$/pH curve may also occur in the case of absence of intramolecular compensation of the ionizable groups [3–5]. The partitioning profile of solutes can be studied through the determination of retention factors by reversed-phase high performance liquid chromatography (RP-HPLC) [6,7]. Retention factors, in their logarithmic form ($\log k'$), are related with $\log D$ via Collander type equations and their change as a function of pH may be considered to resemble the $\log D$/pH profile, since similar ionization corrections can be applied to retention [8]. In the case of acidic compounds however this parallelism has been disputed [9]. Moreover, no systematic studies have been conducted to relate the retention behavior of zwitterionic species with their direct partitioning in the isotropic octanol–water system.

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Hydrophobic ion pairing is also a fascinating topic recently reviewed by Meyer and Manning [10]. Hydrophobic ion pairs result in a considerable increase of partition coefficients. They have been applied in many fields, as in the extraction procedures of charged molecules, in the lipophilization of peptides, in enhancing the solubility of enzymes in organic solvents, as well as to improve the absorption of ions [11–14]. Endogenous substances like prostaglandins, bile acids and eventually phospholipids, may form hydrophobic ion pairs influencing the biological properties of drugs [15]. The analogy of ion pair solvent extraction to ion pair chromatographic retention was recognized some time ago [16]. In reversed-phase ion pair chromatography, known also as ion-interaction chromatography, the presence of hydrophobic ions in the mobile phase generally enhances the retention of oppositely charged molecules [17]. The physicochemical phenomena underlying the retention mechanism are still not fully understood. One hypothesis suggests the formation of neutral ion pairs in the mobile phase, which are retained on the non-polar stationary phase [18]. In contrast, the ion-exchange model assumes that the ion interacting reagent is first adsorbed on the stationary phase giving it an ion-exchange character [19]. These hypothesis represent limiting cases and most probably both mechanisms contribute to retention. A deeper insight into the composite mechanistical processes is obscured due to the lack of physicochemical data and the difficulties associated with the accurate determination of the relevant equilibrium constants. Nevertheless, the measured retention factors are considered to depend on hydrophobicity, as well as on the nature and concentration of the ion interacting reagent [20]. In the special case of zwitterionic compounds the additional tautomeric equilibrium of the inner salt formation should be taken into account. Thus, two opposite trends in retention may be expected: increase due to ion pair formation between the opposite charged centres and decrease due to the disruption of the zwitterionic structure and the deliberation of a net charge. If the ion-exchange model is considered, the retention of zwitterions should be the outcome of attractive and repulsive forces. According to the dipole approach the zwitterionic analyte is regarded as a dipole, which is aligned in such a way as to minimize the electrostatic repulsion and hence the attractive force is always higher than the repulsive force and retention factors increase [21]. The dipole approach has been applied to analyze mainly retention data of amino acids and peptides. In contrast, in the case of of zwitterionic cephalosporins, previously investigated by our group, a drastic decrease in the retention was observed in presence of ion interacting reagents in agreement rather with the ion pair hypothesis [22].

In the present study, we extended our research on the behavior of zwitterionic drugs applying RP-HPLC and RP-IIC to three zwitterionic fluoroquinolones, ofloxacin, norfloxacin and ciprofloxacin. Their chemical structures can be found in USP 28 [23]. They contain two proton acceptor groups, a carboxylate and a piperazine moiety, capable to form zwitterionic inner salt. Their acid–base equilibria have been well established [24]. Their log D/pH profiles have been reported to follow bell shaped curves reflecting the intramolecular charge compensation within the zwitterionic species. A maximum is reached around the physiological pH with log D values −0.39, −1.00 and −1.03 for ofloxacin, norfloxacin and ciprofloxacin, respectively [25]. The two-fold aim of the present study was to explore the compatibility between reversed-phase retention/pH and the lipophilicity profiles as well as the effect of ion interacting reagents upon the retention/pH profiles.

2. Materials and methods

Ofloxacin, norfloxacin and ciprofloxacin of pharmaceutical purity grade were kindly provided by Hoechst Marion Roussel (France) Vianex A. E. Athens, Greece and Bayer Hellas A.B.E.E. Athens, Greece, respectively.

Solvents of HPLC grade, tetrabutylammonium hydroxide (TBA) and sodium hexanesulphonate (Hex) were purchased from Lab-Scan Analytical Sciences Ltd., Ireland. Phosphoric acid, boric acid, and acetic acid (analytical reagent grade) were purchased from Fluka. Water was de-ionised and further purified by means of a Milli-Q Plus water purification system (Millipore Co, USA).

The HPLC system consisted of a Waters Model 501 solvent-delivery system with a Waters Model 486 variable-wavelength UV–vis detector (flow cell 8 μl). The injection system was Rheodyne Model 7125 equipped with a 5-μl loop and the syringe used was a 100-μl Hamilton–Bonaduz–Schweiz. The integrator–recorder used was a Hewlett-Packard Model HP 3394A and the effluent was monitored at 300 nm.

Two ODS columns (A) and (B) of the same type (250 × 4 mm i.d.), packed with LiChrosorb RP-18 (particle size 10 μm), served as stationary phases: column (A) served for the investigation of the effect of TBA on retention, column (B) for the investigation of the effect of Hex. Prior to add any counter ion in the mobile phase, both columns were used in order to assess the reversed-phase retention profile. Acetonitrile/universal buffer 25:75 was used as the mobile phase. Universal buffer was prepared by mixing equal volumes of 0.1 M phosphoric acid, 0.1 M acetic acid and 0.1 M boric acid. The pH of the mobile phase was appropriately adjusted by NaOH 10 M, to values ranging from 2.5 to 7.5. Experiments were also performed using acetonitrile/water 25:75 as the mobile phase or methanol/water 25:75. The pH was appropriately adjusted by phosphoric acid 25% or NaOH 1 M to the same range.

Tetrabutylammonium hydroxide was added in the universal buffer at concentrations 2.5, 50, 100 and 200 mM and the pH of the mobile phase was appropriately adjusted by NaOH 10 M to values ranging from 2.5 to 7.5.

Sodium hexanesulphonate was added in water at low (2.5 and 7.5 mM) and high (0.1 and 0.2 M) concentrations. The

pH was appropriately adjusted to values ranging from 2.5 to 7.5 by phosphoric acid 25% or NaOH 1 M.

The mobile phase was always degassed by filtering through a nylon membrane filter (0.45 μm, Millipore) under vacuum and delivered at a flow rate of 1.0 ml/min.

All measurements were performed at room temperature (25 ± 2 °C). Retention times t_r were measured at least in duplicate, and they were converted into the logarithm of the retention factor log k via the equation:

$$\log k = \log\left(t_r - \frac{t_0}{t_0}\right),$$

t_0 being the retention time needed for the eluent to pass through the column measured as a perturbation peak of the baseline. t_0 ranged from 1.98 to 2.35 min (flow rate 1.0 ml/min), but was constant within the same day and for the same mobile phase composition.

3. Results and discussion

3.1. Reversed-phase retention behavior

The HPLC retention of ofloxacin, norfloxacin and ciprofloxacin, expressed as log k, was monitored as a function of (apparent) pH using acetonitrile/universal buffer 25/75 and is depicted in Fig. 1. Considering the corresponding lipophilicity profiles reported in literature [24] the following remarks have to be made. (1) The maximum in retention was observed at pH 4, considerably shifted towards more acidic pH compared to the reported log D/pH profiles [24,25]. (2) Although isocratic retention factors are expected to be lower than octanol–water distribution coefficients, positive log k values were obtained in contrast to the hydrophilic nature of the three fluoroquinolones as reflected in their negative log D values at the isoelectric points. The use of phosphoric acid or NaOH instead of the universal buffer to control the pH of the mobile phase led to a small systematic further increase in retention with no considerable influence in the shape of the log k/pH profiles. The shift in the retention maximum may only partly be explained by the fact that the pH of the mobile phase corresponds to the apparent pH due to the presence of

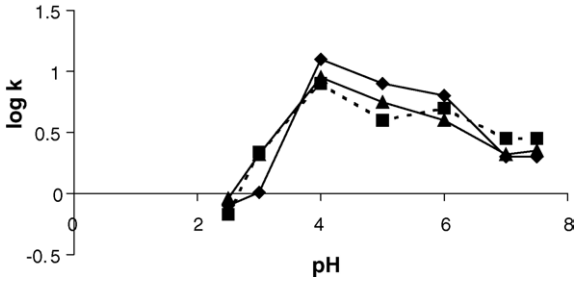

Fig. 1. Log k/pH profile of the three fluoroquinolones using acetonitrile/universal buffer 25:75 as mobile phase: (♦) ofloxacin, (■) norfloxacin and (▲) ciprofloxacin.

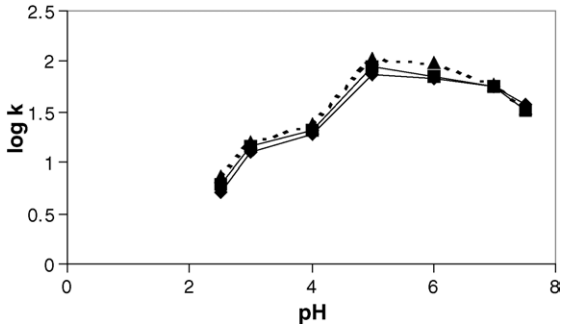

Fig. 2. Log k/pH profile of the three fluoroquinolones using methanol/water 25:75 as mobile phase: (♦) ofloxacin, (■) norfloxacin, (▲) ciprofloxacin.

acetonitrile. The use of methanol as organic modifier led to further increase in the retention while the log k/pH profiles, presented in Fig. 2, showed the maximum at pH 5–6, still lower than the physiological pH. The active role of the stationary phase, demonstrated already by Carr et al. [26], may be critical for solutes, which bear functional groups possible to participate in non-hydrophobic interactions [27]. The free silanol groups of the silanized silica gel material in their ionized state may lead to electrostatic interactions with positively charged analytes, a phenomenon extensively studied by many authors [18,28,29]. Less investigated is the hydrogen bond formation of the protonated silanols with hydrogen bond acceptor sites [30] like carbonyl oxygen. In both circumstances prolonged retention is observed. In the special case of zwitterionic compounds, such interactions may as well interfere with the ionization state and the tautomeric equilibrium. The effect of the organic modifier in the log k/pH profile may also be associated with its hydrogen bond donor capability to compete with that of the silanol groups. This assumption could explain why in presence of methanol the shift in the retention maximum was smaller. The active role of the stationary phase in the retention/pH profile of quinolones has been commented also by Barbosa et al. [31] concerning a polystyrene–divinylbenzene copolymer column packing. The deviations reported by these authors are considerably smaller than the shifts observed in the present study and this provides further evidence of the more intensive influence of the silanized silica gel packing material. The solute–stationary phase non-hydrophobic interactions described above may further be responsible for the generally enhanced retention compared to the hydrophilic nature of the three fluoroquinolones. According to this viewpoint the reversed-phase retention of the zwitterionic fluoroquinolones cannot be considered to reflect pure partitioning characteristics.

3.2. The effect of counter cation in retention

Column A was used to investigate the effect of TBA on the retention of the three fluoroquinolones. A strong decrease in retention was observed particularly at pH 3 and 4. At these pH values the fluoroquinolones eluted practically with

the hold-up volume ($t_r \sim t_0$). This behavior is illustrated in Fig. 3 together with the log k/pH profiles obtained under the same conditions (column A, 25/75 ACN/universal buffer) in absence of TBA for direct comparison. As shown in Fig. 3, increase in TBA produced further decrease in retention. At pH 2.5 the decrease in retention could be explained by repulsive forces between the TBA cation adsorbed on the stationary phase and the protonated piperazine group in agreement with the ion-exchange model. However, according to this model at higher pH, at which both the acidic and basic centers are ionized, an increase in retention should rather be expected since the dipole approach, formulated to explain the retention of zwitterionic analytes, implies that the attractive forces are always higher than the repulsive [21]. In contrast, ion pair formation in the mobile phase between the carboxylate anion and the TBA counter ion may be expected to cause disruption of the zwitterionic structure, leading to the deliberation of the positive charge and thereupon to rapid elution. In this aspect, the ion pair formation hypothesis provides a reasonable explanation for the decrease in the retention of the fluoroquinolones in presence of TBA.

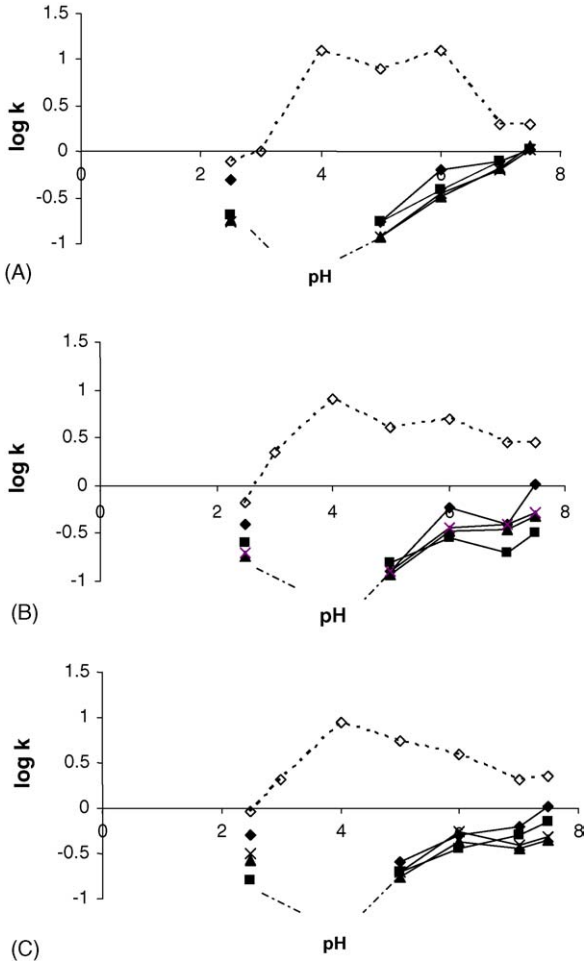

Fig. 3. Log k–pH profile of (A) ofloxacin (B) norfloxacin and (C) ciprofloxacin, using acetonitrile/universal buffer 25:75 as mobile phase in absence (◊) and after addition of various concentrations of TBA (–): (♦) 2.5 mM, (■) 50 mM, (▲) 100 mM, (×) 200 mM. Further decrease in retention at pH 3–4 is represented by dashed lines.

3.3. Effect of counter anion on retention

Column B was used to investigate the effect of sodium hexanesulphonate (Hex) on retention. Water appropriately adjusted by phosphoric acid or NaOH to the desired pH was used as the aqueous component of the mobile phase in order to avoid possible competition with the anions present in the universal buffer. Fig. 4 illustrates the log k/pH profiles obtained upon addition of 0.1 M of Hex in the mobile phase. The profiles obtained under the same conditions (column B/25:75 ACN/H_2O) in absence of the counter anion, are also depicted in Fig. 4 for direct comparison. At pH 2.5 and 3.0, considerable increase in retention was observed which could be considered as the outcome of both the ion-exchange and the ion pair formation mechanism. At pH 4.0–6.0, however, the decrease in retention supports the assumption that ion pair formation in the mobile phase is the predominant mechanism. Thus, as postulated also in the preceding section the ion pair formation between the protonated piperazine group and the hexanesulphonate anion may cause disruption

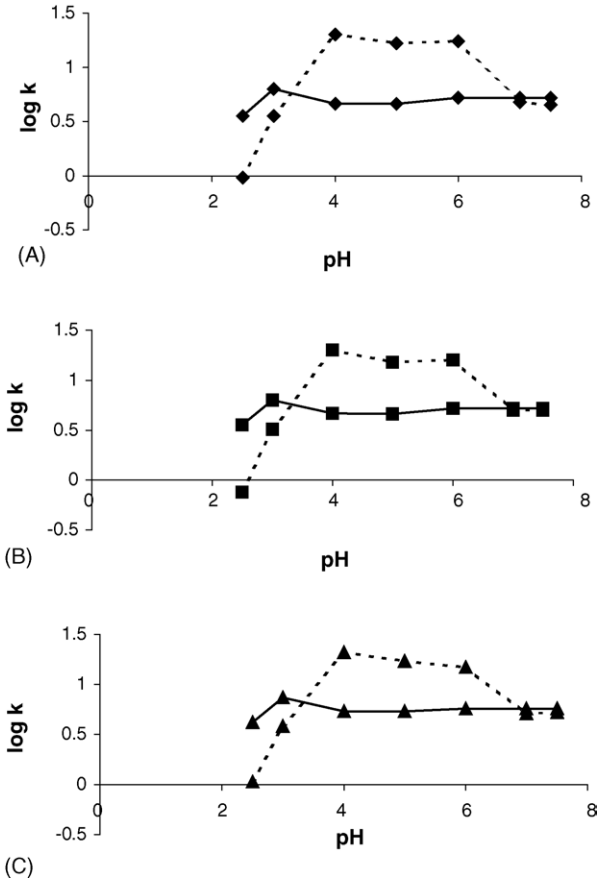

Fig. 4. Log k–pH profile using acetonitrile/water 25:75 as mobile phase in absence (- - -) and after addition of 0.1 M sodium hexanesulphonate (–): (A) (♦) ofloxacin (B) (■) norfloxacin and (C) (▲) ciprofloxacin.

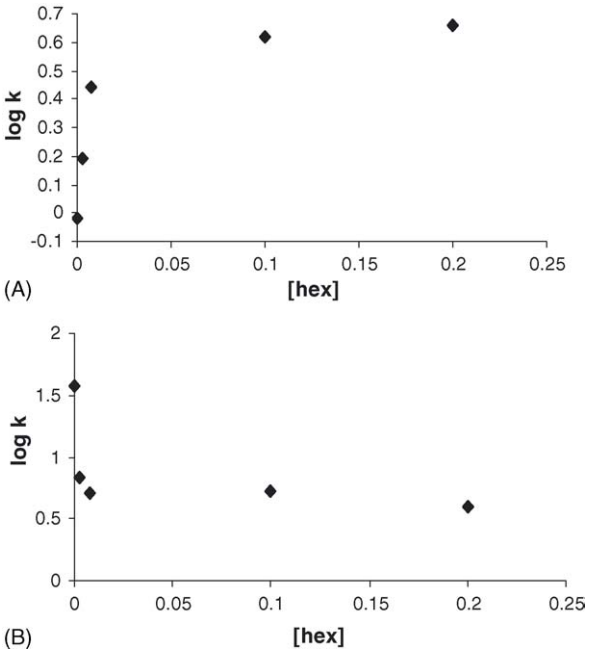

Fig. 5. Log k vs. the concentration of sodium hexanesulphonate [Hex] for ofloxacin: (A) pH 2.0 and (B) pH 6.0.

of the intramolecular charge compensation, deliberation of the negative charge and thereupon rapid elution. At higher pH, the reduced protonation of the piperazine group may cause limited ion pair formation in the mobile phase and retention should be influenced also by the attractive and repulsive forces predicated by the ion-exchange model. As a result of the counter balance between the two mechanisms practically no effect in retention was observed.

The opposite trends in retention were also reflected in the log k/[Hex] profiles obtained at different pH values. At pH 2.5 with increasing counter anion concentration the retention followed a hyperbolic relationship, approaching a maximum plateau at 0.2 M, as expected by either the ion-exchange or the ion pair formation model. At pH 3.0, no clear relationship between retention and counter anion concentration was obtained. At higher pH values (4.0–6.0) a low concentration of hexanesulphonate proved to be sufficient for the disruption of the intramolecular zwitterionic structure. Thus, rapid elution was observed already upon addition of 2.5 mM hexanesulphonate. Increase of the counter anion concentration had no further effect on retention. Examples of the log k/[Hex] profiles are shown in Fig. 5.

4. Conclusions

The retention of zwitterionic compounds in RP-HPLC may be considerably influenced by non-hydrophobic interactions. In the case of ofloxacin, norfloxacin and ciprofloxacin silanophilic interactions were considered to be responsible for the relatively high retention in respect to their log D/pH profiles [25] as well as for the shift of the retention maximum towards more acidic pH values. In the light of the above observations, the use of chromatographic data as substitutes of log D in the case of zwitterionic compounds should be faced with some skepticism.

In ion-interaction chromatography at a pH range at which both acidic and basic centers are ionized, the effect of the counter ions was considered to be ruled rather by ion pair formation which led to a drastic decrease in retention as a consequence of the disruption of the zwitterionic structure and thereupon the deliberation of a net charge in the molecules. These results are in agreement with our previous findings concerning the behavior of zwitterionic cephalosporins in ion-interaction chromatography [22]. The predominance of the ion pair formation mechanism in retention may be related to the molecular mass and bulk of the zwitterionic fluoroquinolones which, in contrast to the amino acids and peptides so far investigated [21] may not have easy access as dipoles on the stationary phase. In this aspect ion-interaction chromatography is comparable to ion pair solvent extraction and may provide insight on the putative behavior of zwitterionic drugs in presence of endogenous hydrophobic ions within living organisms.

References

[1] Y. Ishihama, Y. Oda, N. Asakawa, Microscale J. Pharm. Sci. 83 (1994) 1500–1507.
[2] K. Palm, K. Luthman, J. Ros, J. Casjo, P. Artursson, J. Pharmacol. Exp. Ther. 291 (1999) 435–443.
[3] J. Comer, K. Tam, Lipophilicity profiles: theory and measurement, in: B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy (Eds.), Pharmacokinetic Optimization in Drug Research, Wiley-VCH, Zurich, 2001, pp. 276–304.
[4] A. Pagliara, P.-A. Carrupt, G. Caron, P. Gaillard, B. Testa, Chem. Rev. 97 (1997) 3385–3400.
[5] R. Scherrer, Biolipid pKa values and the lipophilicity of ampholytes and ion pairs, in: B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy (Eds.), Pharmacokinetic Optimization in Drug Research, Wiley-VCH, Zurich, 2001, pp. 351–381.
[6] J.G. Dorsey, M.G. Khaledi, J. Chromatogr. A 656 (1993) 485–499.
[7] A. Tsantili-Kakoulidou, A. Varvaresou, Th. Siatra-Papastaikoudi, O. Raevsky, Quant. Struct. Act. Relat. 18 (1999) 482–489.
[8] H. van de Waterbeemd, M. Kansy, B. Wagner, H. Fischer, Lipophilicity measurement by reversed-phase high performance liquid chromatography (RP-HPLC), in: V. Pliška, B. Testa, H. van de, Waterbeemd (Eds.), Lipophilicity in Drug Action and Toxicology, VCH, Weinheim, 1996, pp. 73–87.
[9] F. Lombardo, M.Y. Shalaeva, K.A. Tupper, F. Gao, J. Med. Chem. 44 (2001) 2490–2497.
[10] J.D. Meyer, M.C. Manning, Pharm. Res. 15 (1998) 188–193.
[11] K.M. Akamatsu, Y. Yoshida, H. Nakamura, M. Asao, H. Iwamura, T. Fujita, Quant. Struct. Act. Relat. 8 (1989) 195–203.
[12] D. Quintanar-Guerrero, E. Alleman, H. Fessi, E. Doelker, Pharm. Res. 14 (1997) 119–127.
[13] J. van Gelder, M. Witvrouw, C. Pannecouque, G. Henson, G. Bridger, L. Naesens, E. de Clercq, P. Annaert, M. Shafiee, G. van de Mooter, R. Kinget, P. Augustijns, Int. J. Pharm. 186 (1999) 127–136.
[14] R. Neubert, Pharm. Res. 6 (1989) 743–747.
[15] K. Tacáks-Novák, G. Szász, Pharm. Res. 16 (1999) 1633–1638.
[16] I.M. Johansson, K.-G. Wahlund, G. Schill, J. Chromatogr. 149 (1978) 281–296.

[17] H. Zou, Y. Zhang, M. Hong, P. Lu, J. Chromatogr. 625 (1992) 169–175.
[18] C. Horvath, W. Melander, I. Molnar, P. Molnar, Anal. Chem. 49 (1977) 2295–2305.
[19] N.E. Hoffman, J.C. Liao, Anal. Chem. 49 (1977) 2231–2234.
[20] E. Tomlinson, T.M. Jeffries, C.M. Riley, J. Chromatogr. 159 (1978) 315–358.
[21] T. Cecchi, F. Pucciarelli, P. Passamonti, P. Cecchi, Chromatographia 54 (2001) 38–44.
[22] C. Pistos, A. Tsantili-Kakoulidou, M. Koupparis, J. Liq. Chromatogr. 26 (6) (2003) 937–952.
[23] USP 28, NF-23, The United States Pharmacopeial Convention Inc. (2005).
[24] K. Takács-Novák, M. Jósan, I. Hermecz, G. Szász, Int. J. Pharm. 19 (1992) 89–96.
[25] K. Takács-Novák, B. Noszál, I. Hermecz, G. Kerestúri, B. Podányi, G. Szász, J. Pharm. Sci. 79 (1990) 1023–1028.
[26] P.W. Carr, J.J. Li, A.J. Dallas, D.I. Elkens, L.C. Tan, J. Chromatogr. A 656 (1993) 113–133.
[27] D. Sýkora, E. Terasová, M. Popl, J. Chromatogr. A 758 (1997) 37–51.
[28] P. Jandera, S. Buncekova, M. Halama, K. Novotna, M. Nepras, J. Chromatogr. A 1059 (2004) 61–72.
[29] U.D. Neue, K.V. Tran, A. Méndez, P.W. Carr, J. Chromatogr. A 1063 (2005) 35–45.
[30] N.El. Tayar, A. Tsantili-Kakoulidou, T. Roethlisberger, B. Testa, G. Gal, J. Chromatogr. 439 (1988) 237–244.
[31] J. Barbosa, R. Bergers, V. Sanz-Nebot, J. Chromatogr. 823 (1998) 411–422.