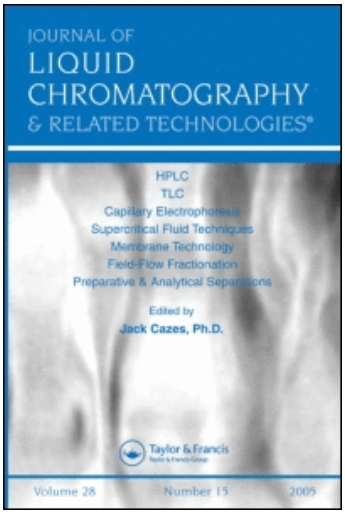

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The Effect of Ion Pairing Reagents in the Retention Profile of Zwitterionic Cephalosporins

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ABSTRACT

The retention of three zwitterionic cephalosporins, Cefepime, Cefpirome, and Ceftazidime, in a broad pH range was studied by means of reversed phase high performance liquid chromatography (RP-HPLC) and reversed phase ion-pair liquid chromatography (RP-IPC), using an octadecylsilane stationary phase and acetonitrile as organic modifier. Sodium hexane-, heptane-, and octane-sulphonate and tetrabutylammonium hydroxide were used as sources of counter ions in ion pair chromatography. The presence of the permanently charged quaternary nitrogen in the cephalosporin molecules leads to zwitterionic species over a broad pH range, masking

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the carboxylic acid function and stabilizing the retention. The effect of the counter ion depends on the ionization state of the compounds. Formation of ion pairs with the opposite charged center of the molecules led to significant increase in retention. At pH values favoring the zwitterionic species a decrease in retention was observed as a result of the disruption of the intramolecular interactions and the deliberation of the free charge. The extent of ion pair formation and the competition with the zwitterionic species, depended on the counter ion concentration in the mobile phase and was reflected in retention. The effect of the size of the counter anion alkyl chain on retention was investigated at different pH as well.

Key Words: Cefepime; Cefpirome; Ceftazidime; Zwitterions; High performance liquid chromatography; Ion-pair liquid chromatography.

INTRODUCTION

Zwitterions represent a particular type of solute with intra- and intermolecular interactions, which influence their partitioning behavior in a manner as yet insufficiently, explored. The two charged groups interact strongly, resulting in partial neutralization and a consequent increase in lipophilicity. In most cases the lipophilicity of the zwitterion, expressed, as the octanol–water partition coefficient log *P*, is higher than that of the cationic or anionic species but lower than the lipophilicity of the neutral form. The intercharge distance has been found to be important, exerting an inverse proportional influence on lipophilicity and affecting the log *P* difference between the neutral and the zwitterionic species.[1–4]

Ion pair formation in the case of charged drug molecules also leads to partial neutralization and increase in lipophilicity, facilitating the partitioning in non-polar media and penetration through membranes.[5,6] In partition experiments for ionized substances, the nature and concentration of the counter ion should always be taken into account.[7] Ion pairing is reflected in extraction constants, the magnitude of which depends on the size of the ions involved.[8] The ion-pairing concept has been applied in extraction procedures of charged molecules in their chromatographic analysis, in the lipophilization of peptides, as well as, to improve the absorption of ions.[9–11]

Ion pairing may affect zwitterions, disrupting the intra-molecular interaction between the charged centers. The potential competition between ion pairing and zwitterionic species may influence the partitioning behavior of such compounds and should be taken into account in their isolation and in the analytical procedures. To our knowledge, however, no relevant systematic investigations are reported in literature.

The partitioning characteristics, as well as the dissociation equilibria of solutes, can be studied through their retention properties, as determined by reversed phase high performance liquid chromatography (RP-HPLC). The use of HPLC capacity factors as alternatives of *n*-octanol water distribution coefficients, has been well established.[12,13] For ionizable compounds, the effect of pH on log k' parallels the log D–pH profile and relevant studies can be used for the assessment of dissociation constants.[14] Likewise, the presence of zwitterionic species is expected to have an analogous impact in the retention as in direct partitioning.

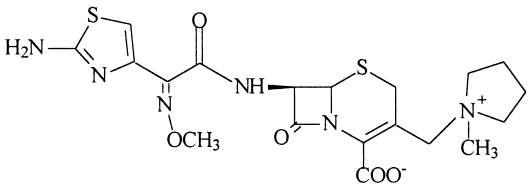
Reversed phase ion pair liquid chromatography (RP-IPC) has been used for the measurement of the relative lipophilicity of ionic organic compounds, which are weakly, or not at all retained, in HPLC.[15] In RP-IPC, the retention mechanism is based on the ion-pairing concept and the capacity factors are related to the extraction constant of the ion pair and the concentration of the pairing ion. Thus, besides the hydrophobicity, the nature and concentration of the ion pair reagent regulate retention.[16]

In the present study, both RP-HPLC and RP-IPC were used in order to investigate the retention profile of zwitterionic cephalosporins, and to monitor the potential competition between ion pairing and zwitterionic species. Three cephalosporins were chosen: Cefepime, Cefpirome, and Ceftazidime. Their structures are presented in Fig. 1. They contain a strong acidic carboxylic group directly attached on the cephem nucleus, a weak basic aminothiazole center in the side chain, and a permanently charged quaternary nitrogen atom at position 3 of the cephem nucleus. Over a broad pH range, they exist as zwitterions, formed between the carboxylate anion and the positively charged quaternary nitrogen atom. To a lesser extent, zwitterionic species formation is possible between the carboxylate anion and the protonated aminothiazole moiety in a limited pH range. Ceftazidime bears a second carboxylic group in the side chain, which further contributes to the complicated dissociation equilibria. In Table 1, the dissociation constants reported in literature[17–20] are presented.

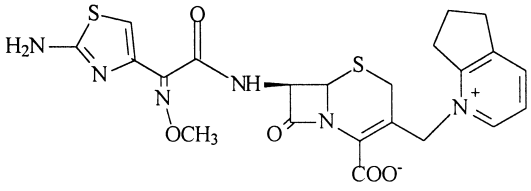
EXPERIMENTAL

Cefepime dihydrochloride, monohydrate, Cefpirome sulphate, and Ceftazidime dihydrochloride of pharmaceutical purity grade were kindly provided by Bristol-Myers Squibb (Italy) SPA, Hoechst Marion Roussel (France) and Pharmaserv-Lilly A.E. Athens, Greece, respectively.

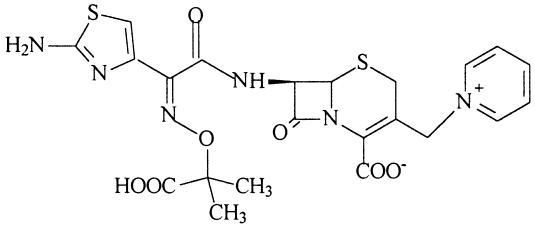
Solvents of HPLC grade and hexane-, heptane-, and octane-sulphonate sodium salts were purchased from Lab-Scan Analytical Sciences Ltd., Ireland. Phosphoric acid, boric acid, and acetic acid (analytical reagent grade) were purchased from Fluka. Water was deionised and further purified by means of a Milli-Q Plus water purification system (Millipore Co, USA).



Cefepime.2HCl.H2O



Cefpirome.H_2SO_4



Ceftazidime.2HCl

Figure 1. Chemical structures of Cefepime, Cefpirome, and Ceftazidime.

The HPLC system consisted of a Waters model 501 solvent-delivery system with a Waters Model 486 variable-wavelength UV–Vis detector (8 μL flow cell). The integrator-recorder used was a Hewlett Packard model HP 3394A. 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 effluent was monitored at 300 nm.

Two ODS columns (A) and (B) of the same type (25 cm × 4 mm I.D.), prepacked with LiChrosorb RP-18 (particle size 10 μm), were used as stationary phases; column (A) was used for the investigation of the effect of

Table 1. Dissociation constants of the three cephalosporins under study.

	pKa		
Ionization center	Cefepime	Cefpirome	Ceftazidime
$COOH \rightarrow COO^-$ (Cephem nucleus)	1.3[16] 1.5[17]		1.9[19]
$NH^+ \rightarrow N$ (Aminothiazole)	3.2[16] 3.1[17] 3.36, 3.03[18]	3.10, 3.04[18]	4.1[19]
$COOH \rightarrow COO^-$ (Side chain)			2.7[19]

Note: Numbers in superscript square brackets correspond to literature references.

counter anions on retention, column (B) for the investigation of the effect of a counter cation. With column (A), the mobile phase consisted of acetonitrile/water mixture, 25 : 75. Sodium hexane-sulphonate, heptane-sulphonate, and octane-sulphonate were added in the mobile phase at low (0.0025–0.0075 M) and high (0.1–0.2 M) concentrations. In each case, the pH was appropriately adjusted by phosphoric acid to values ranging from 2 to 6. Experiments were performed under the same chromatographic conditions in absence of the counter anion. With column (B), the mobile phase consisted of acetonitrile/universal buffer mixtures 10 : 90. Universal buffer was prepared using equal volumes of 0.1 M phosphoric acid, 0.1 M acetic acid, and 0.1 M boric acid. Tetrabutylammonium hydroxide was added in concentrations of 0.0025, 0.005, 0.1, and 0.2 M. The pH was appropriately adjusted by phosphoric acid to values ranging from 2.5 to 7.5. The same experiments were performed also in absence of the counter cation. In that case, the pH was adjusted either by phosphoric acid or NaOH.

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 ($25 \pm 2^\circ C$). Retention times t_r were measured in duplicate, and they were converted into the logarithm of the capacity factor $\log k'$ via the equation:

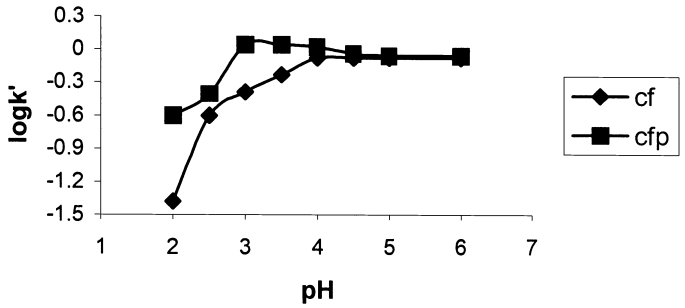
$$\log k' = \log\left(\frac{t_r - t_0}{t_0}\right), \quad t_0 \text{ being the retention time of acetonitrile.}$$

RESULTS AND DISCUSSION

Stationary Phase: Column A, Mobile Phase: Acetonitrile/Water Mixtures 25 : 75, pH Range: 2–6

Reversed Phase Retention Behavior

The $\log k'$-pH profiles obtained using column A and acetonitrile/water mixtures 25 : 75 as mobile phase in a pH range 2–6, reflected the dissociation equilibria of the three cepahalosporins and are presented in Fig. 2. For



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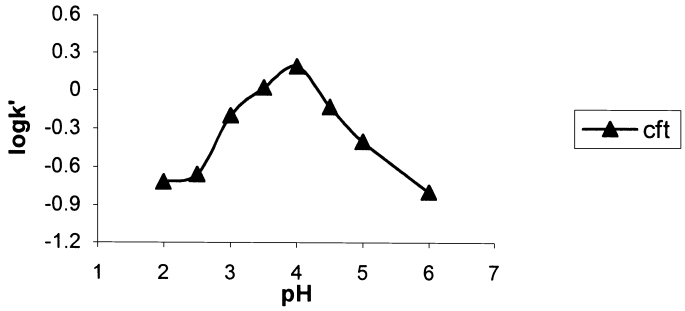


B.

Figure 2. $\log k'$–pH profiles of the three cephalosporins using acetonitrile/water 25/75 as mobile phase. (A) Cefepime (cf) and Cefpirome (cfp); (B) Ceftazidime (cft).

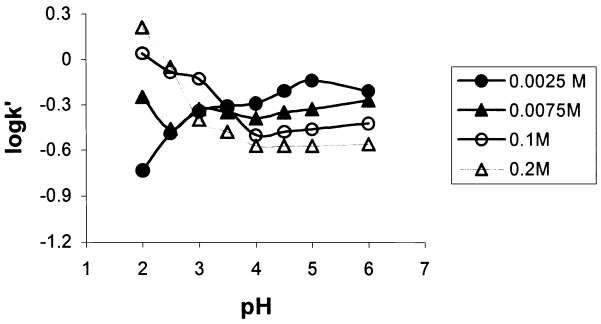
cefepime and cefpirome [Fig. 2(A)], the $\log k'$ values increased with increasing pH, reaching a plateau. This behavior should be attributed to the presence of the quaternary nitrogen atom, which interacts with the carboxylic anion to zwitterionic species, thus, bestowing invariable partitioning characteristics at pH > 4, a profile similar to that of a monoprotic weak base.[21] An analogous behavior of these two cephalosporins in capillary zone electrophoresis has been reported in literature. In that study, both cefepime and cefpirome behaved as monoprotic bases showing a zero electrophoretic mobility at pH above 4.5, whereas, at lower pH they presented positive electrophoretic mobilities.[19]

For ceftazidime a maximum is observed in the $\log k'$/pH profile [Fig. 2(B)], due to the dissociation of the second carboxylic group. This profile is analogous to that of an ampholyte with one basic and one acidic center.[21]

Effect of Counter Anions on Retention

The effect of counter anions on the retention of the three cephalosporins was investigated upon addition of different concentrations of alkylsulphonates in the mobile phase. In Fig. 3, the $\log k'$/pH profile obtained in presence of different concentrations of sodium hexanesulphonate is presented. At pH 2 and 2.5 a considerable increase in retention was observed, reflecting the ion pair formation of the counter anion with the protonated aminothiazole ring. On the contrary, at higher pH values, a decrease in retention was noticed as a result of the disruption of the intramolecular interaction between the carboxylic anion and the quaternary nitrogen atom, and the deliberation of the negative charge. To some lesser extent, deprotonation of the aminothiazole ring may also contribute to the decrease of the retention, since it affects the degree of ion pair formation with the hexanesulphonate anion. These effects led to an inverse $\log k'$–pH profile at higher concentrations of hexanesoulphonate. At full ionization of the carboxylic group and full deprotonation of the aminothiazole group a low level plateau was reached for cefepime and cefpirome [Fig. 3(A), (B)]. In the case of ceftazidime $\log k'$ values kept decreasing with increase of pH, due to the ionization of the second carboxylic group [Fig. 3(C)].

The increase in retention at lower pH values followed a hyperbolic relationship with increasing counter anion concentration, approaching a maximum value in all cases. This behavior is comparable to the influence of ion pair formation in the distribution coefficients of ionized compounds.[7] The decrease in retention at higher pH values was found to approach a minimum value with increasing counter anion concentration in the case of cefepime and cefpirome. In the case of ceftazidime, retention decreased dramatically, even at very low concentrations of the counter anion; Thus,



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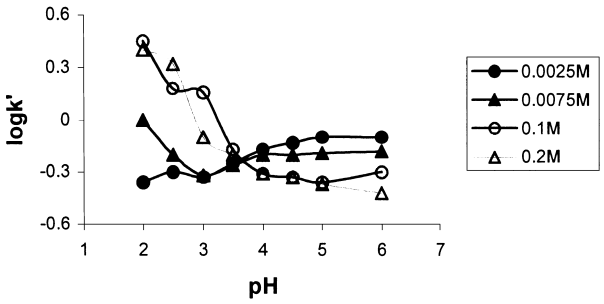

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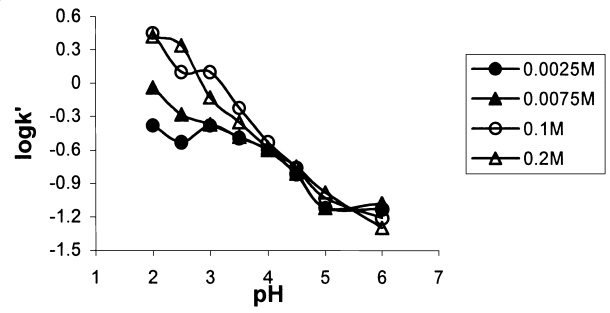


C.

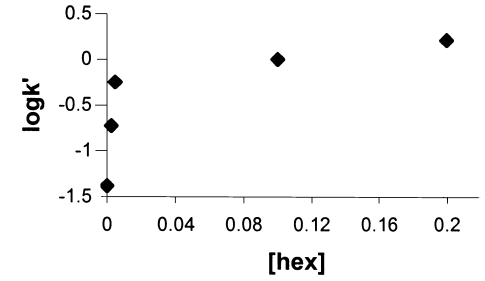
Figure 3. log k'–pH profiles using acetonitrile/water 25/75 with various concentrations of sodium hexanesulphonate, as mobile phase. (A) Cefepime; (B) Cefpirome; (C) Ceftazidime.

any further increase in counter anion concentration did not affect the retention. Representative examples for each of the above described behaviors are illustrated in Fig. 4(A)–(C). Less rationale was the effect of the counter anion concentration at the crucial cutoff pH values, reflecting more complex equilibria. At pH 3, a clear parabolic relationship was found for cefepime [Fig. 5(A)]. For cefpirome and ceftazidime, a significant decrease in retention was manifested upon addition of low sodium hexanesulphonate concentrations. At the concentration of 0.1 M, $\log k$ values were higher than those in absence of the counter anion, followed by a decrease in retention at 0.2 M hexanesulphonate [Fig. 5(B)].

Analogous results were obtained by the addition of heptane- and octanesulphonate in the mobile phase (profiles not shown). The effect of the number of carbon atoms of the alkyl chain of the counter anion depended on the pH and is presented in Fig. 6. At pH 3 [Fig. 6(A)], at which the partially protonated aminothiazole ring still contributes to ion pair formation, replacement of sodium hexanesoulphonate with heptane- and octane-sulphonate led to a drastic enhancement of retention. At pH 4 and 5, at which retention is influenced by the presence of a negative charge in the molecule, the increase of the alkyl chain did not affect the $\log k'$ values. Moreover, at pH 5, the $\log k'$ values of ceftazidime decreased upon addition of the higher alkyl sulphonate homologs [Fig. 6(C)].

Stationary Phase: Column B, Mobile Phase: Acetonitrile/Universal Buffer Mixtures 10 : 90, pH Range: 2.5–7.5

Column B was used to investigate the effect of tetrabutylammonium hydroxide on the retention of the three cephalosporins. The selection of universal buffer was predicated by the reduced reproducibility of the retention times in presence of tetrabutylammonium hydroxide, when water was used as the aqueous component of the mobile phase. The use of universal buffer did not affect the shape of the $\log k'$/pH profiles in RP-HPLC, it led, however, to a systematically reduced retention. Therefore, a lower percentage of acetonitrile in the mobile phase was used throughout. In Fig. 7, the $\log k'$–pH profiles of the three cephalosporins, obtained at 10% acetonitrile, are illustrated in the presence of different concentrations of tetrabutylammonium hydroxide, as well as, in absence of the counter cation. In the case of cefepime and cefpirome, tetrabutylammonium interacted with the carboxylate anion and competed with its engagement in the zwitterionic species. Thus, the molecules developed a positive charge and were eluted more rapidly. The effect was already evident at pH 2.5, indicating adequate dissociation of the carboxylic group at that low pH. Upon addition of different concentrations of tetrabutylammonium



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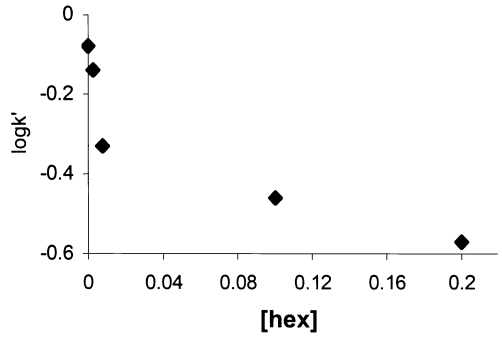

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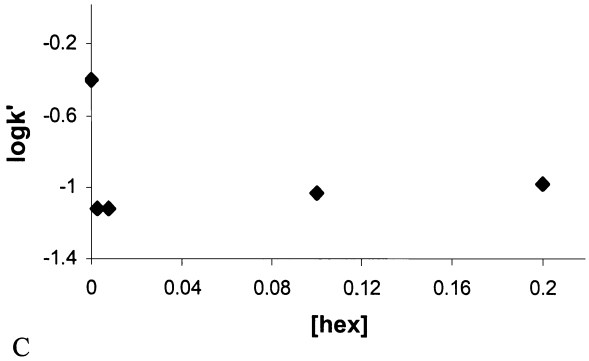
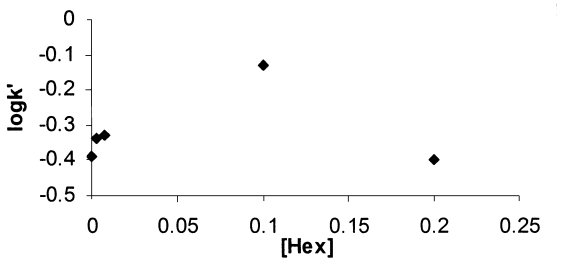


C

Figure 4. log k vs. the concentration of sodium hexanesulphonate. (A) Cefepime, pH: 2.0; (B) Cefepime, pH: 5.0; (C) Ceftazidime, pH: 5.0.



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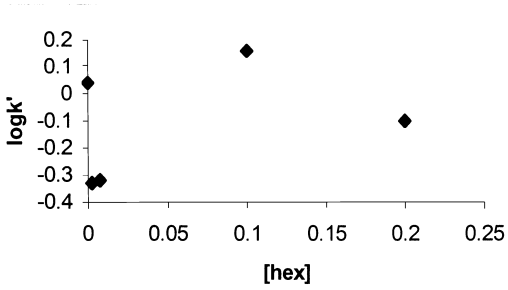

B

Figure 5. $\log k$ vs. the concentration of sodium hexanesulphonate at pH 3.0 (A) Cefepime; (B) Cefpirome, the same profile is observed for ceftazidime.

hydroxide, a hyperbolic relatioship with $\log k'$ was observed reaching a minimum value throughout the pH range studied, and the profiles (not shown) were analogous with that illustrated in Fig. 4(B). The effect of tetrabutylammonium on the retention profile of ceftazidime reflected more complex equilibria. At pH 2.5, a considerable decrease in retention was observed, as a result of the disruption of the zwitterionic structure. With increasing pH, retention increased, reaching $\log k'$ values much higher than those obtained in the absence of the counter cation. This behavior should be attributed to the dissociation of the second carboxylic group, which may form ion pairs with tetrabutylammonium, the zwitterionic species thus, remaining intact. However, after reaching a maximum, retention declined with further increase of pH, indicating that next to ion pair formation, some competition with the zwitterionic species persists. The decrease or increase in retention

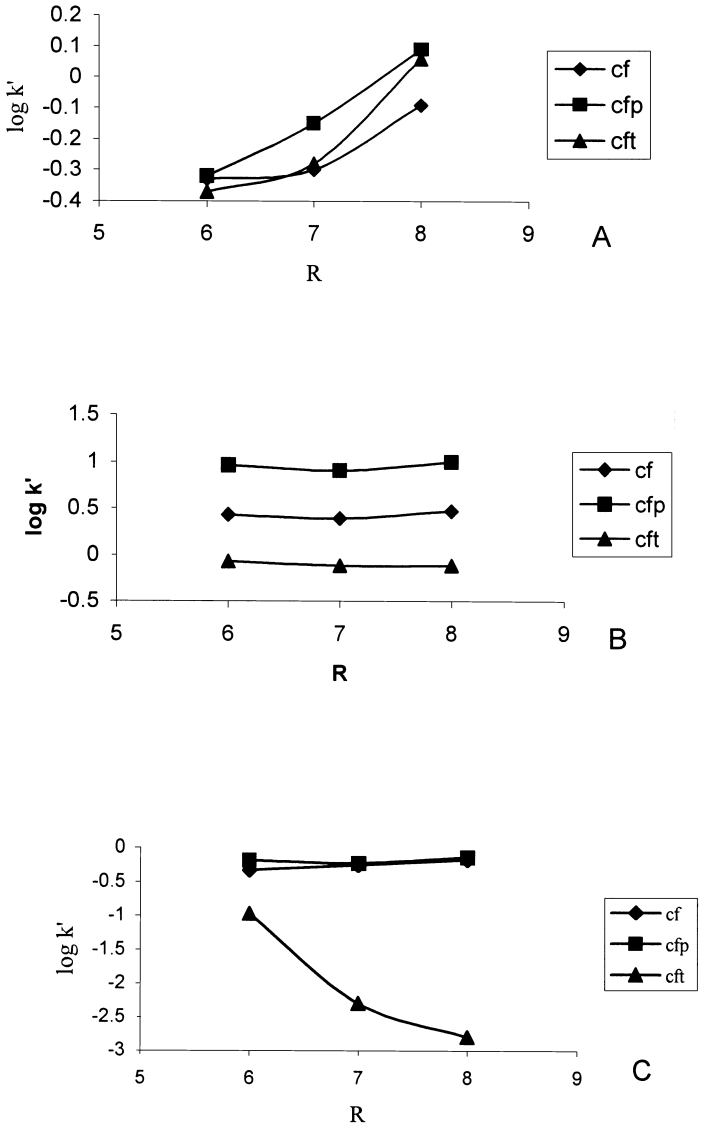

Figure 6. Effect of the number of carbon atoms of the counter anion alkyl chain (R) on retention. (A) at pH 3; (B) at pH 4; (C) at pH 5.

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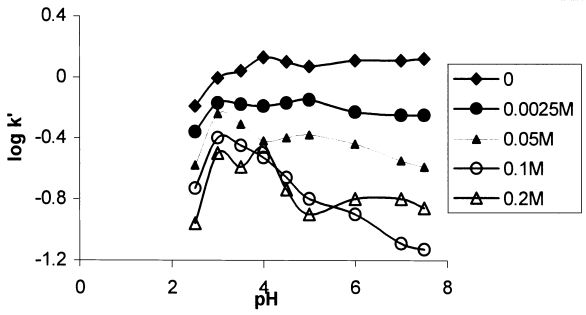

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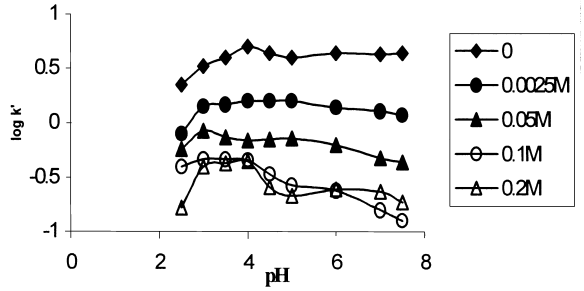

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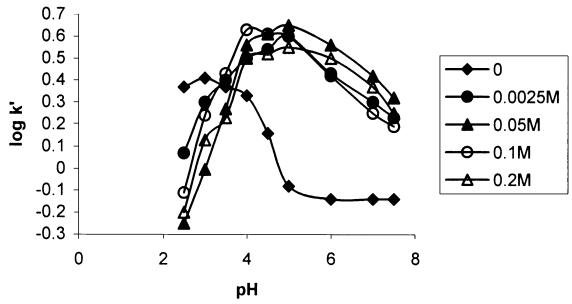

C

Figure 7. log k'–pH profiles of (A) Cefepime; (B) Cefpirome; (C) Ceftazidime at various concentrations of tetrabutylammonium hydroxide and 10% acetonitrile in the mobile phase.

were very pronounced, even at 0.0025 M concentration of tetrabutylammonium, and further increase in the counter cation concentration did not affect the retention significantly.

CONCLUSIONS

Reversed phase ion pair chromatography, in combination with RP-HPLC, offers a useful technique in the investigation of ion pair and zwitterionic equilibria. The interaction between the charged centers in the zwitterionic species resulted in a retention profile analogous to that of monoprotic bases for cefepime and cefpirome, while ceftazidime, due to the second carboxylic group, exhibited the behavior of an ampholyte. The presence of a hydrophobic counter anion, led to a considerable increase in retention at low pH values as a result of ion-pair formation with the protonated aminothiazole ring. Upon deprotonation at higher pH, the counter anion interacted with the quaternary charged nitrogen atom, disrupting the zwitterionic structure. With tetrabutylammonium, disruption of the zwitterionic structure occurred as a result of its interaction with the carboxylate anion in the whole pH range studied, leading to reduced retention. In the case of ceftazidime more complex equilibria occurred, due to the dissociation of the second carboxylic group. The effect of the counter ions in retention depended on their concentrations in the mobile phase.

These findings reveal the competition between ion pairing and zwitterionic species and the relevant consequences in the partitioning behavior. They contribute to the understanding of the physicochemical characteristics of the cephalosporins under study, and may serve as a support in the selection of the suitable conditions to solve problems related to their analysis or pharmacokinetic profiles.

REFERENCES

1. Tacáks-Novák, K.; Kökési, J.; Podányi, B.; Noszál, B.; Tsai, R.S.; Carrupt, P.-A.; Testa, B. Microscopic protonation/diprotonation equilibria of the anti-inflammatory agent piroxicam. Helv. Chim. Acta **1995**, *78*, 553–562.
2. Pagliara, A.; Carrupt, P.-A.; Caron, G.; Gaillard, P.; Testa, B. Lipophilicity profiles of ampholytes. Chem. Rev. **1997**, *97*, 3385–3400.
3. Tacáks-Novák, K.; Szász, G. Ion-pairing of quaternary ammonium drugs: the influence of counter ions of different lipophilicity, size and flexibility. Pharm. Res. **1999**, *16*, 1633–1638.

4. Bouchard, G.; Carrupt, P.-A.; Testa, B.; Gobry, V.; Girault, H.H. The apparent lipophilicity of quaternary ammonium ions is influenced by galvani potential difference, not ion-pairing: cyclic voltammetry study. Pharm. Res. **2001**, *18*, 702–708.
5. Hansch, C.; Leo, A. *Exploring QSAR Fundamentals and Applications in Chemistry and Biology*; ACS: Washington, DC, 1995; 158–161.
6. Neubert, R. Ion-pair transport across membranes. Pharm. Res. **1989**, *6*, 743–747.
7. Tsantili-Kakoulidou, A.; Piperaki, S.; Panderi, I.; Csizmadia, F.; Darvas, F. Prediction of distribution coefficients from structure. The influence of ion pair formation as reflected in experimental and calculated values. Quant. Struct.–Act. Relat. **1997**, *16*, 315–316.
8. Shim, C.K.; Nishigaki, R.; Iga, T.; Hanako, M. Determination of extraction constant, true partition coefficient and formation constant of ion-pair complexes of quaternary ammonium salts, tetrabutylammonium bromide and isopropamide iodide, with some organic ions by a solvent extraction technique. Int. J. Pharm. **1981**, *8*, 143–151.
9. Akamatsu, K.M.; Yoshida, Y.; Nakamura, H.; Asao, M.; Iwamura, H.; Fujita, T. Hydrophobicity of di and tripeptides having ionizable side chains and correlation with substituent and structural parameters. Quant. Struct.–Act. Relat. **1989**, *8*, 195–203.
10. Quintanar-Guerrero, D.; Alleman, E.; Fessi, H.; Doelker, E. Application of the ion-pair concept to hydrophilic substances with special emphasis on peptides. Pharm. Res. **1997**, *14*, 119–127.
11. van Gelder, J.; Witvrouw, M.; Pannecouque, C.; Henson, G.; Bridger, G.; Naesens, L.; de Clercq, E.; Annaert, P.; Shafiee, M.; van de Mooter, G.; Kinget, R.; Augustijns, P. Evaluation of the potential of ion-pair formation to improve the oral absorption of two potent antiviral compounds. Int. J. Pharm. **1999**, *186*, 127–136.
12. Kaliszan, R. Ion-pair high performance liquid chromatography. Quant. Struct.-Act. Relat. **1990**, *9*, 83–87.
13. Bechalany, A.; Tsantili-Kakoulidou, A.; El Tayar, N.; Testa, B. Measurement of lipophilicity indices by reversed-phase high-performance liquid chromatography: comparison of two stationary phases and various eluents. J. Chromatogr. **1991**, *541*, 221–229.
14. Hardcastle, J.E.; Jano, I. Stability study of cefepime in different infusion solutions. J. Chromatogr. A **1998**, *717*, 39–56.
15. Zou, H.; Zhang, Y.; Hong, M.; Lu, P. Measurement of partition coefficients by reversed phase ion-pair liquid chromatography. J. Chromatogr. **1992**, *625*, 169–175.
16. Tomlinson, E.; Jeffries, T.M.; Riley, C.M. Ion-pair high performance liquid chromatography. J. Chromatogr. **1978**, *159*, 315–358.

17. Fubara, J.O.; Notari, R.E. Influence of pH, temperature and buffers on cefepime degradation kinetics and stability predictions in aqueous solutions. J. Pharm. Sci. **1998**, *87*, 1572–1576.
18. Rabouan-Guyon, S.M.; Guet, A.F.; Courtois, P.Y.; Barthes, D.M.C. Stability study of cefepime in different infusion solutions. Int. J. Pharm. **1997**, *154*, 185–190.
19. Mrestani, Y.; Neubert, R.; Munk, A.; Wiese, M. Determination of dissociation constants of cephalosporins by capillary zone electrophoresis. J. Chromatogr. A **1998**, *803*, 273–278.
20. *AHFS Drug Information 94*; McEvoy, G.K., Ed.; American Society of Hospital Pharmacists Inc. Am. Soc. Hosp. Pharm., Inc.: Bethesda, MD, 1994; 130 pp.
21. van de Waterbeemd, H.; Testa, B. The parametrization of lipophilicity and other structural properties in Drug Design. In *Advances in Drug Research*; Testa, B., Ed.; Academic Press: New York, 1987; Vol. 16, 85–227.

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