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Reversed-phase high-performance liquid chromatography of amphoteric β-lactam antibiotics: effects of columns, ion-pairing reagents and mobile phase pH on their retention times

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ABSTRACT

The separation of five amino β-lactam antibiotics by reversed-phase high-performance liquid chromatography was studied as an insight into their retention behaviour. These five amphoteric compounds are cephradine, cephalexine, cefaclor, ampicillin and amoxicillin. Both octadecylsilane-bonded silica (C_{18}) columns and phenyl-bonded silica (phenyl) columns were used, with mobile phase pH values between 2.5 and 7.4. In the absence of ion-pairing reagents the retention times for all the five compounds were the shortest at pH 4–6. The phenyl column was found to improve the separation between cephradine and ampicillin at pH values lower than 3, when these two compounds appeared as fused peaks on the C_{18} column. Only C_{18} columns were used to study the ion-pair effect at various pH values. The retention times on C_{18} columns, with mobile phases both with and without ion-pairing reagents, were compared. The addition of 0.005 or 0.02 *M* tetraethylammonium acetate to the mobile phase did not result in significant ion-pair formation, except at pH values higher than 5.5. A strong ion-pairing effect was obtained at pH values higher than 6 with 0.005 or 0.02 *M* tetrabutylammonium phosphate, and the retention was decreased at pH values lower than 4. On the other hand, 0.005 *M* heptanesulphonic acid exhibited an ion-pair retention effect at pH values lower than 5. The molecular structures and pK_a values were used to account for the retention behaviour of these antibiotics in the various mobile phases.

INTRODUCTION

β-Lactam antibiotics are widely used for the treatment of infections. Many new β-lactams with higher potency and/or broader spectra are still being approved annually as a result of intensive synthesis efforts as well as growing market demand. The methods for the analysis of β-lactams include microbial assay [1,2], hydroxylamine assay [1], high-performance liquid chromatography (HPLC) [1,3], iodometric assay [1] and immunoassay [4]. Non-aqueous titration is also used for the acidic and basic functional groups to assay some amino β-lactams [1]. Of all

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these methods, HPLC is superior to the others when specificity and simultaneous analysis are considered.

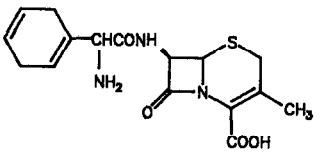
Salto *et al.* [5] conducted a systematic study of the ionization effect of pencillins and cephalosporins on their interaction with a bonded C_{18} stationary phase. Three amino β-lactams, cephalexin, cephradine and ampicillin, were among the compounds they studied. These three compounds showed V-shaped plots of capacity factors *versus* pH in mobile phases containing 10, 20 or 30% methanol. Between pH 5 and 6, their retention was the shortest. The authors suggested that in this pH range the compounds existed in a dipolar form, containing a positive charge and a negative charge, which decreased the hydrophobic interaction with the reversed-phase packing. Rouan *et al.* [6] found that at a constant ionic strength of 0.15, when the pH was increased from 2 to 8, the retention also had a minimum around the isoelectric point (pH 4–6) for the two amphoteric cephalosporins that they studied. When the ionic strength was low (0.003) and the pH was increased from 2 to 8, the retention of these amphoteric cephalosporins decreased and tended to become constant. It was proposed that the retention occurred essentially by way of solvophobic interactions at high ionic strength (0.15), and by way of both solvophobic and silanophilic interactions at low ionic strength (0.003).

In 1986, Moats [7] studied three amphoteric β-lactams by HPLC using gradient elution with 0.01 *M* orthophosphoric acid–acetonitrile (pH 1.6). He found that the addition of 0.01 *M* tetramethylammonium (TMA) chloride to the mobile phase not only decreased the retention but also sharpened the peaks. The blocking effect of TMA on the residual silanol groups of the silica support was suggested to be the cause. Later, in 1987, Moats and Leskinen [8] described the HPLC of penicillin G, ampicillin and amoxicillin with mobile phases of pH 1.6 and 4.6 with various acetonitrile contents. Their data showed that, on bonded reversed-phase (RP) columns, penicillin G was retained longer then the two aminopenicillins when the mobile phase had an acetonitrile content of less than *ca.* 50%; however, the retention sequence was reversed when the acetonitrile content was greater then *ca.* 50%.

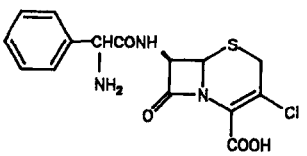
The fact that amphoteric β-lactams behave as a combination of acidic and basic compounds makes them suitable for study as model β-lactams in HPLC. Furthermore, the use of ion-pairing reagents in RP-HPLC can serve as a powerful tool in the search for the retention mechanism. The articles concerning HPLC of β-lactams [5–8] so far have dealt with only a few amphoteric compounds. A systematic study of the simultaneous RP-HPLC of more amphoteric β-lactams, with mobile phases of various pH and containing an ion-pairing reagent, remains to be carried out.

With this in mind, we have investigated the RP-HPLC behaviour of β-lactam antibiotics. Three cephalosporins (cephradine, cephalexin and cefaclor) and two penicillins (ampicillin and amoxicillin) were studied. These compounds, have an amino β-lactam structure in common, but differ in part from each other as shown

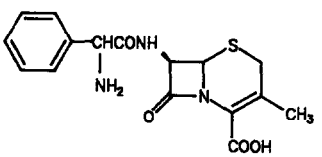
in Fig. 1. This makes them a good set of compounds from which the correlation between their structural moieties and column retention can be studied. Both cationic and anionic ion-pairing reagents were used in the mobile phase.



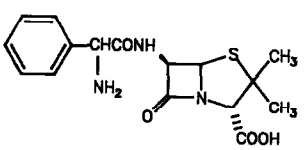
Cephradine



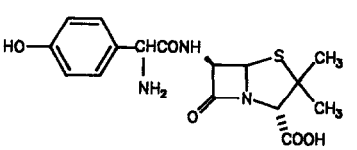
Cefaclor



Cephalexin



Ampicillin



Amoxicillin

Fig. 1. Structures of the five β-lactams studied.

EXPERIMENTAL

Chemicals and reagents

Ampicillin, amoxicillin, cephradine and cephalexin were from Sigma (St. Louis, MO, U.S.A.). Cefaclor was obtained from the water extract of 250-mg Keflor capsules (Lilly, Taipei, Taiwan). Methanol was L.C. reagent grade from Alps (Taipei, Taiwan). Phosphoric acid and monobasic and dibasic sodium phosphate were reagent grade from Wako (Osaka, Japan). Heptanesulphonic acid (HSA) and tetrabutylammonium phosphate (TBA) were from Waters (Milford, MA, U.S.A.). Tetraethylammonium acetate (TEA) was from Sigma.

Distilled water purified by Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.) was used.

Apparatus

The Waters HPLC system consisted of an M-6000 pump, a photodiode array detector, a temperature control system and an NEC CP6 printer. Two types of column, Waters μBondapak C_{18} and μBondapak Phenyl (both 300 mm × 3.9 mm I.D., particle size 10 μm) were used.

Chromatographic technique

The columns were maintained at 27 ± 1°C. The following HPLC conditions were used: (a) C_{18} column with 0.01 *M* phosphate buffer–methanol (73:27 or 88:12, v/v); (b) phenyl column with 0.01 *M* phosphate buffer–methanol (73:27, v/v); (c) C_{18} column with 0.005 *M* TEA and 0.01 *M* phosphate buffer–methanol (73:27, v/v); (d) C_{18} column with 0.02 *M* TEA and 0.01 phosphate buffer–methanol (88:12, v/v); (e) C_{18} column with 0.005 *M* TBA and 0.01 *M* phosphate buffer–methanol (73:27, v/v); (f) C_{18} column with 0.02 *M* TBA and 0.01 *M* phosphate buffer–methanol (88:12, v/v); (g) C_{18} column with 0.005 *M* HSA and 0.01 *M* phosphate buffer–methanol (73:27, v/v); (h) C_{18} column with 0.005 *M* HSA and various concentrations of phosphate buffer–methanol (73:27, v/v), pH 4.4.

For each set of conditions a new column was used. Their plate numbers were measured to be between 2500 and 3500 per column.

The detection wavelength was set at 254 nm. An additional wavelength at 280 nm and a photodiode array detector were used to identify individual peaks of the β-lactam mixture. Solutions of individual compounds were injected to confirm the identity of the peaks in the mixture.

RESULTS AND DISCUSSION

For amoxicillin, ampicillin, cephradine and cephalexin, the pK_a value of the carboxylic acid group on the β-lactam ring is between 2.4 and 2.7 [4,9,10]. For cefaclor, this value is reduced to less than 2.4 by the *ortho*-substitution of an electron-withdrawing chlorine atom. All five compounds have amino functions

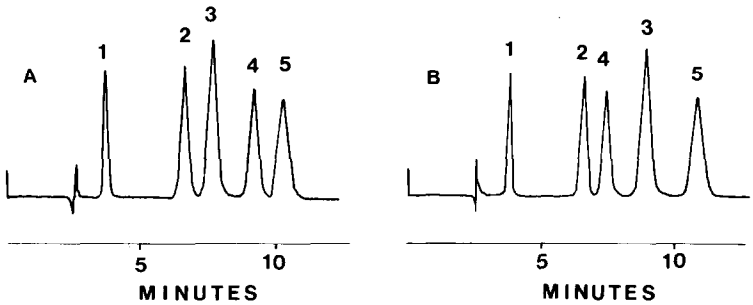


Fig. 2. Chromatographic separations of the five β-lactams. (A) C_{18} column; (B) phenyl column. Both columns were 300 mm × 3.9 mm I.D. Mobile phase, 0.01 *M* phosphate buffer–methanol (73:27, v/v), pH 3.6; flow-rate, 1 ml/min.

with pK_a values between 7.2 and 7.4 [4,9,10]; only amoxicillin has a phenolic substitution, leading to a third pK_a of 9.6 as well as a higher polarity. Cephradine is unique in having a cyclohexanediene group, which is more lipophilic than the phenyl group of the other four compounds.

Fig. 2 shows the difference of the two chromatograms using the same mobile phase but different types of column.

Figs. 3–7 were obtained by plotting the capacity factor (*k*) *versus* pH. Fig. 3 shows the plot using a C_{18} column in the absence of ion-pairing reagents. In this plot, *k* values decreased in the order cephradine, ampicillin, cephalexin, cefaclor and amoxicillin, obviously in accordance with decreasing lipophilicity. Being totally ionized between pH 4 and 6, the five compounds show their shortest retention times in this pH range. With a pH approaching 2.5, the carboxylic acids were only half-ionized, so their retention significantly increased. Similarly, as the pH approached 7.3, amino groups were only half-ionized, leading to increased retention times. Cefaclor and caphalexin have similar lipophilicities, so their peaks

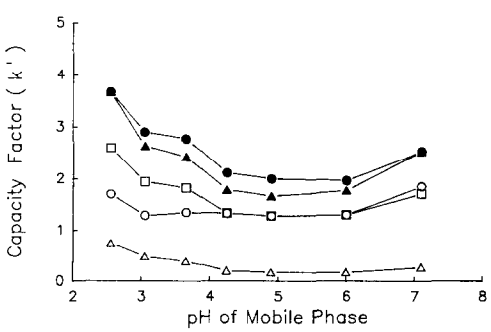


Fig.3. Effect of pH on the retention of five β-lactams on a C_{18} column. Mobile phase, 0.01 *M* phosphate buffer–methanol (73:27, v/v); flow-rate, 1 ml/min; detection wavelength, 254 nm. Analytes: (●) cephradine; (▲) ampicillin; (□) cephalexin; (○) cefaclor; (△) amoxicillin.

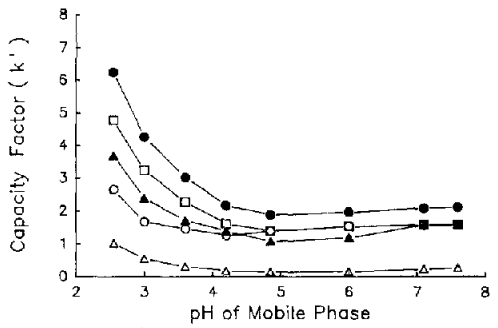


Fig. 4. Effect of pH on the retention of five β-lactams on a phenyl column. Conditions and symbols as in Fig. 3.

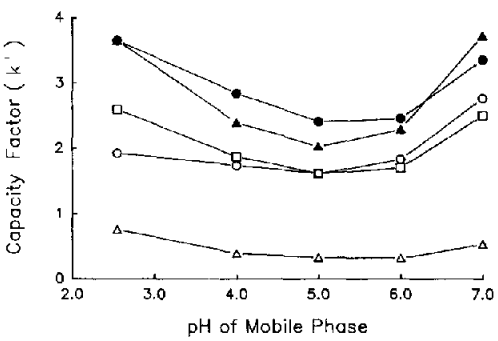


Fig. 5. Effect of pH on the retention of five β-lactams on a C_{18} column using ion-pairing reagent TEA in the mobile phases. Mobile phases, 0.005 *M* TEA in 0.01 *M* phosphate–methanol (73:27, v/v). Other conditions and symbols as in Fig. 3.

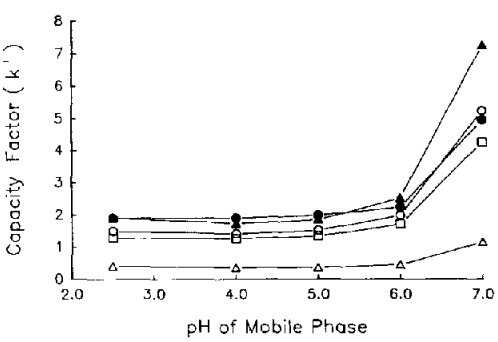


Fig. 6. Effect of pH on the retention of five β-lactams on a C_{18} column using ion-pairing reagent TBA in the mobile phase. Mobile phase, 0.005 *M* TBA in 0.01 *M* phosphate buffer–methanol (73:27, v/v). Other conditions and symbols as in Fig. 3.

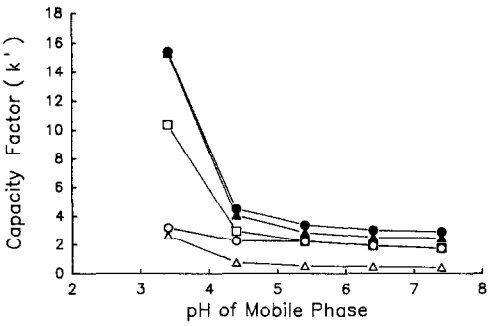


Fig. 7. Effect of pH on the retention of five β-lactams on a C_{18} column using ion-pairing reagent HSA in the mobile phase. Mobile phase, 0.005 *M* HSA in 0.01 *M* phosphate buffer–methanol (72:27, v/v). Other conditions and symbols as in Fig. 3.

were fused between pH 4.2 and 6.0. However, the retention of cephalexin increased more than that of cefaclor below pH 4.2. This is because the carboxylic acid of cefaclor has a pK_a value lower than that of cephalexin, leaving its carboxylic acid mostly ionized at a pH as low as 2.5. Fig. 3 also shows that cephradine and ampicillin had fused peaks at both pH 2.5 and 7.1, which are close to their pK_a values. Even when the methanol content was decreased from 23 to 12%, these two peaks remained fused. This suggests the use of a mobile phase with a pH different from that of the pK_a values of the test compounds in order to improve their separation.

Fig. 4 shows a separation of the five β-lactams on a phenyl column using the same mobile phase as in Fig. 3. The main difference between these two types of column lies in the change of *K* values for cephradine, ampicillin and cephalexin. The phenyl column more clearly resolved cephradine and ampicillin, even when the pH of the mobile phase was similar to their pK_a values (2.5 and 7.3). The elution order of cephalexin and ampicillin was also reversed. The only structural difference between cephalexin and ampicillin is their β-lactam ring, the former containing a six-membered dihydrothiazine ring; the latter a five-membered thiazolidine ring. The phenyl column has a higher affinity for the dihydrothiazine ring than for the thiazolidine ring, therefore the retention time of cephalexin was higher on the phenyl than on the C_{18} column. This may also explain the good separation for cephradine and ampicillin on the phenyl column. π–π interactions between the solutes and the stationary phase may explain the longer retention on the phenyl column, as proposed in a study by Okamoto and Jinno [11] on the retention behaviour of three antiepileptic drugs on phenyl columns. In the present study, the dihydrothiazine ring not only is six-membered, but also has a double bond in the ring, thus enhancing its retention on a phenyl column by π–π interaction.

A comparison of Figs. 3 and 5 indicates that the addition of 0.005 *M* TEA to the mobile phase with 27% methanol, gave a better separation at a pH higher

than 5.5. However, better separation was not obtained at pH values higher than 5.5 by using 0.02 *M* TEA in the mobile phase containing 12% methanol. This indicates that increasing the TEA concentration, along with decreasing the methanol content, does not necessarily permit a better separation, despite the fact that a longer retention time is obtained.

Fig. 6 shows the separation of β-lactams on a C_{18} column in the presence of 0.005 *M* TBA. Above pH 6, ion-pair formation was obvious, the most spectacular change being the longer retention of ampicillin relative to that of cephradine and cephalexin. The fact that ampicillin was more methyl groups next to its ion-pair-forming carboxyl group accounted for its longer retention. In a study of ion-pair HPLC conducted in 1987 with aminoglycosides, Inchauspe *et al.* [12] suggested that when the methyl group of the aminoglycoside is *ortho* to the ion-pair-forming group, the retention time was enhanced more than when the methyl group was located at other positions. The reason is that, during elution, the ion-pair-forming group was interacting with the ion-pairing reagent on the column surface and the *ortho*-substituted methyl group was close enough to interact with the column, resulting in an enhanced retention. This can be applied to β-lactams. When the pH was lower than 4, the β-lactams, in the form of ammonium ions, had shorter retention in the presence of TBA. "Electrostatic repulsion" as suggested by Bidlingmeyer *et al.* [13] can be used to explain the above phenomena. Being adsorbed on the column in the form of lipophilic ammonium ions, TBA exerted electrostatic repulsion against the retention of less lipophilic ammonium ions, in this case the β-lactams. Similar results were observed in a study of a few amino β-lactams on an RP column when 0.01 *M* TMA was added to a mobile phase of pH 1.6 [7]. The addition of TMA decreased the retention time and markedly sharpened the peaks. Here TMA was proposed to behave as a silanol-blocking agent instead of an ion-pairing agent. For the investigated β-lactams, since TBA is much more lipophilic than TMA, it might not have a significant silanol-blocking effect.

Fig. 7 shows the separation on a C_{18} column in the presence of 0.005 *M* HSA. The retention of β-lactams was not increased at a pH of 7.4 when most of the amino groups were not protonated. When the pH was lower than 5, ion-pair formation began to emerge as shown by the comparison between Figs. 3 and 7.

Fig. 8 shows that, in the presence of 0.005 *M* HSA, an increase in the phosphate concentration was followed by decreased retention, especially for the more lipophilic compounds: cephradine, ampicillin and cephalexin. It has been observed by Horvath *et al.* [14] that the addition of salts would tend to reduce the retention because they would compete with the ion-pairing reagent in forming ion pairs with analytes. However, the retention slightly increased at high phosphate concentrations (Fig. 8). An increase in the surface tension of the mobile phase caused by a high salt concentration was suggested by the same authors to account for the effect of increasing retention.

In conclusion, amino β-lactams showed V-shaped plots of capacity factors

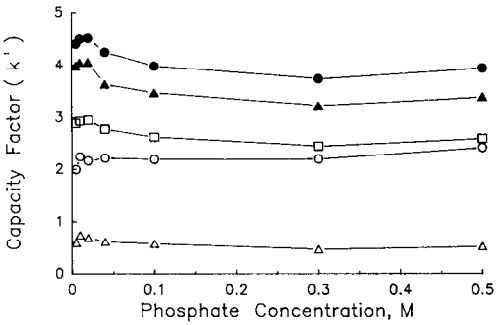


Fig. 8. Effect of phosphate buffer concentration on the retention of five β-lactams containing HSA in the mobile phase of pH 4.4. Mobile phase, 0.005 *M* HSA in various concentrations of phosphate buffer–methanol (73:27, v/v). Other conditions and symbols as in Fig. 3.

versus pH on a C_{18} column. Between pH 5 and 6 their retentions were the shortest. When both a cephalosporin and a penicillin have similar retentions on a C_{18} column, the use of a phenyl column may result in a better separation as the cephalosporin will have a longer retention than the penicillin.

For all β-lactams the RP-HPLC retention times should increase after the addition of 0.005 *M* TEA of TBA to the mobile phase above pH 6. For β-lactams containing basic group(s), the use of 0.005 *M* TBA in a mobile phase below pH 4 will result in decreased retention times compared with the same mobile phase without TBA. When the mobile phase has a pH lower than 5, the addition of 0.005 *M* HSA will increase the retention of the β-lactams that contain basic groups.

REFERENCES

1 USP Committee of Revision, *USP XXI*, USP Convention, Rockville, MD, 1990.
2 J. de Louvois, *J. Antimicrob. Chemother.*, 9 (1982) 253.
3 M. C. Roun, *J. Chromatogr.*, 340 (1985) 361.
4 W. Sadee and G. C. M. Boolen, *Drug Level Monitoring: Analytical Technique, Metabolism, and Pharmaceutics*, Wiley-Interscience, New York, 1980, pp. 364–365 and 370–371.
5 F. Salto, J. G. Prieto and M. T. Alemany, *J. Chromatogr.*, 69 (1980) 501.
6 M. C. Rouan, F. Abadie, A. Leclerc and F. Juge, *J. Chromatogr.*, 275 (1983) 133.
7 W. A. Moats, *J. Chromatogr.*, 366 (1986) 69.
8 W. A. Moats and L. Leskinen, *J. Chromatogr.*, 386 (1987) 79.
9 A. Wade (Editor), *Pharmaceutical Handbook*, The Pharmaceutical Society of Great Britain, The Pharmaceutical Press, London, 18th ed., 1980, p. 233.
10 J. P. Hou and J. W. Poole, *J. Pharm. Sci.*, 58 (1969) 1510.
11 M. Okamoto and K. Jinno, *J. Chromatogr.*, 395 (1987) 171.
12 G. Inchauspe, P. Delrieu, P. Dupin, M. Laurent and D. Samain, *J. Chromatogr.*, 404 (1987) 53.
13 B. A. Bidlingmeyer, S. N. Diming, W. P. Price Jr., B. Sachok and M. Petrusek, *J. Chromatogr.*, 186 (1979) 419.
14 C. Horvath, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.