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Optimization of separation and migration behavior of cephalosporins in capillary zone electrophoresis

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Abstract

The influences of buffer pH, buffer concentration and buffer electrolyte on the migration behavior and separation of 12 cephalosporin antibiotics in capillary zone electrophoresis using three different types of buffer electrolyte, including phosphate, citrate, and 2-(*N*-morpholino)ethanesulfonate (MES), were investigated. The results indicate that, although buffer pH is a crucial parameter, buffer concentration also plays an important role in the separation of cephalosporins, particularly when cefuroxime and cefazolin, cephalexin and cefaclor, or cefotaxime and cephapirin are present as analytes at the same time. The electrophoretic mobility of cephalosporins and electroosmotic mobility measured in citrate and MES buffers are remarkably different from those measured in phosphate buffer. With citrate buffer, optimum buffer concentration is confined to a small range (35–40 m*M*), whereas buffer concentrations up to 300 m*M* can be used with MES buffer. Complete separations of 12 cephalosporins could be satisfactorily achieved with these three buffers under various optimum conditions. However, the separability of 12 cephalosporins with citrate or MES buffer is better than that with phosphate buffer. As a consequence of a greater electrophoretic mobility of cephalosporins than the electroosmotic mobility with citrate buffer at pH below about 5, some cephalosporins are not detectable. The cloudiness of the peak identification and of the magnitudes of the electrophoretic mobility of cefotaxime and cefuroxime reported previously are clarified. In addition, the pK_a values of cephradine, cephalexin, cefaclor, and cephapirin attributed to the deprotonation of either an amino group or a pyridinium group are reported, and the migration behavior of these cephalosporins in the pH range studied is quantitatively described.

Keywords: Buffer composition; Cephalosporins; Antibiotics

1. Introduction

Cephalosporins are antibiotics for the treatment of Gram-positive and Gram-negative infections. These antibiotics derived from the 7-aminocephalosporanic acid composed of a β-lactam ring fused with a dihydrothiazine, but differ in the nature of the substituents attached at the 3- and/or 7-positions of the cephem ring. These substituents affect either the pharmacokinetic properties or antibacterial spectrum. For the past two decades, these antibiotics are generally separated and determined by high-performance liquid chromatography (HPLC). Numerous HPLC procedures have been proposed for the separation and/or quantification of these antibiotics [1–5].

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In recent years, capillary electrophoresis (CE) has become an important separation technique owing to its advantageous features, such as extremely high column efficiency, small sample volumes and rapid analysis, in comparison with HPLC [6–11]. The applications of this technique to the separation and/or determination of cephalosporins have previously been demonstrated using either micellar electrokinetic chromatography (MEKC) [12–18] or capillary zone electrophoresis (CZE) [18–21].

The separation of nine cephalosporins was performed by MEKC using sodium dodecyl sulfate (SDS) and sodium *N*-lauroyl-*N*-methyltaurate as anionic surfactants with phosphate buffer at pH 9.0 [12]. Twelve cephalosporins were separated under similar electrophoretic conditions, but at pH 6.0 [13]. Five cephalosporins were analyzed with a borate buffer containing SDS at an alkaline pH [15]. The determinations of cefuroxime in human serum [16] and of cefotaxime in plasma [17,18] by MEKC were demonstrated.

On the other hand, the determinations of cefotaxime in plasma with phosphate buffer or with borate buffer were first illustrated by CZE [18,19]. Recently, the separation and determination of nine cephalosporins in urine and bile sample solutions [20] and in plasma sample solution [21] were performed by CZE using either a citrate buffer at pH 6.0 or a phosphate buffer at pH 7.2. Dissociation constants of nine cephalosporins were determined using citrate, acetate, and phosphate buffers in three different pH regions ranging from 2 to 9 [22]. However, the influence of the buffer pH on the migration behavior and separation of the cephalosporins by CZE were less rigorously investigated and the effects of buffer concentration and buffer type were incompletely examined or even lacking.

It should be pointed out that some of the results reported previously by Mrestani et al. [20–22] seem to be not very consistent. For instance, the pH-dependence of the electrophoretic mobility curves of cefotaxime and cefuroxime level off at pHs greater than about 4.2 [22], but the curves of the migration time versus buffer pH level off at pHs greater than about 7.5 [21]; the electrophoretic mobility of cefotaxime and cefuroxime reported previously [22] are two orders of magnitude smaller than that of cefamandole, but cefamandole migrates in between cefotaxime and cefuroxime [20]. The difference in pK_a values between these two cephalosporins may be less than 0.2, as was determined from the potentiometric measurements [22] because the mobility curves of cephalexin and cefaclor shown in [22] are very close to each other. Moreover, the peak identification of cefotaxime and cefuroxime [20–22] may be questionable because their peak identification was not confirmed by our preliminary results. Furthermore, the cause of the disappearance of the signals of some cephalosporins with a citrate buffer at a pH below about 5 is speculated, but not clearly understood [22].

In order to clarify all this cloudiness, a more thorough investigation with regard to the effects of buffer pH and buffer concentration on the migration behavior and separation of cephalosporins is certainly needed. Furthermore, in view of the modification on the capillary surface with the use of a zwitterionic buffer such as sodium 2-(*N*-morpholino)ethanesulfonate (MES), an investigation on the migration behavior and separation of cephalosporins using such a buffer electrolyte is desirable. A comparative study on this matter using these three different types of buffer electrolyte is also worthy for investigation. In this work, 12 cephalosporins, which have a similar basic structure but with different substituents attached to the cephem ring, are selected. These cephalosporins are prescribed antibiotics and are commercially available. Here, we present the results of our investigations.

2. Experimental

2.1. Apparatus

All CE experiments were performed on a Beckman P/ACE System 5500 equipped with a UV detector for absorbance measurements at 214 nm. The dimensional spectral scan of the CE separation was performed on a Beckman P/ACE System MDQ with a photodiode array detector (Beckman Coulter, Fullerton, CA, USA). Uncoated fused-silica capillaries, purchased from Polymicro Technologies (Phoenix, AZ, USA), were used. The dimensions of the capillary were 57 cm×50 μm I.D. for the Beckman P/ACE System 5500 instrument and 60.2

cm×50 μm I.D. for the Beckman P/ACE System MDQ. The effective length of the capillary is 50 cm from the injection end of the capillary. The CE system was interfaced with a microcomputer and a laser printer. The System Gold software of Beckman was used for data acquisition. For pH measurements, a pH meter (Suntex Model SP-701, Taipei, Taiwan) was employed with a precision of ±0.01 pH unit.

2.2. Chemicals and reagents

Twelve cephalosporins were obtained from Sigma (St. Louis, MO, USA). MES and mesityl oxide (used as a neutral marker) were purchased from Tokyo Kasei Kogyo (TCI, Tokyo, Japan). All other chemicals were of analytical-grade. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

Standard solutions of cephalosporins at various concentrations ranging from 10 to 50 μg/ml were prepared by dissolving analytes in an aqueous solution. The pH of a phosphate buffer was adjusted to the desired pH value by mixing various proportions of a certain concentration of sodium dihydrogenphosphate solution with the same concentration of disodium hydrogenphosphate solution. Similar procedures were followed to adjust the pH of a citrate buffer by mixing various portions of a certain concentration of trisodium citrate solution with the same concentration of citric acid solution, and to adjust the pH of a MES buffer solutions by mixing salt solutions with the corresponding acid solutions. All buffer solutions, freshly prepared weekly and stored in a refrigerator before use, were filtered through a membrane filter (0.22 μm).

2.3. Electrophoretic procedure

When a new capillary was used, the capillary was washed 30 min with 1.0 *M* NaOH solution, followed by 20 min with deionized water at 25°C. Before each injection, the capillary was prewashed for 5 min with running buffer and postwashed for 2 min with deionized water, 5 min with 1.0 *M* NaOH and 5 min with deionized water to maintain proper reproducibility of run-to-run injections. Sample injections were done in a hydrodynamic mode over 4 s under a pressure of 0.4 p.s.i. The measurements were run at least in triplicate to ensure reproducibility. Applied voltages of 20 kV for the phosphate buffer and 30 kV for the citrate and MES buffers were selected to keep the total current less than 100 μA in order to avoid experimental complications resulting from Joule heating. For instance, the current measured for a phosphate buffer at 150 m*M* with an applied voltage of 20 kV is 78.7 μA. The detection wavelength was set at 214 nm. The relative standard deviation of migration time is less than 0.6% ($N=5$).

For peak identification, on-column UV spectra (200–300 nm with a 2-nm wavelength increment) of cephalosporins were recorded simultaneously during the electrophoretic separation. Spiking with the analyte to be identified was also employed.

2.4. Mobility calculations

The electrophoretic mobility of analytes was calculated from the observed migration times with the equation:

$$\mu_{ep} = \mu - \mu_{eo} = \frac{L_d L_t}{V} \cdot \left(\frac{1}{t_m} - \frac{1}{t_{e_o}}\right)$$

where μ_{ep} is the electrophoretic mobility of the analyte tested, μ is the apparent mobility, μ_{eo} is the electroosmotic mobility, t_m is the migration time measured directly from the electropherogram, t_{eo} is the migration time for an unchanged solute, L_t is the total length of capillary, L_d is the length of capillary between injection and detection and V is the applied voltage.

3. Results and discussion

The optimization of the separation of cephalosporins in CZE can be achieved by manipulation of separation parameters, such as buffer pH and buffer concentration, of a selected buffer electrolyte. In this work, the combined effects of buffer pH and buffer concentration are taken into consideration to obtain the optimized separation of 12 cephalosporins. Three different buffer electrolytes, including phosphate, citrate and MES are selected to examine the effects of buffer electrolytes on the migration behavior and separation of cephalosporins. The structures of the

12 cephalosporins selected are shown in Fig. 1. According to the nature of the carboxyl group of cephalosporins, these analytes are categorized into two classes. The cephalosporins belonging to class I, possessing their carboxyl groups in the acid-form, consist of cephradine (**1**), cephalexin (**2**) and cefaclor (**3**), whereas cephalosporins belonging to class II, used as sodium salt, include the rest of the nine cephalosporins.

3.1. Influence of buffer pH

The pH of the buffer plays an important role in the separation of ionizable analytes since it determines the extent of the ionization of the analytes [23,24]. Moreover, the charge of the capillary wall surface and the zeta potential are influenced by buffer pH. Thus, manipulation of buffer pH becomes a key strategy to optimize the separation in CZE. For minimizing any possible degradation due to β-lactam hydrolysis of cephalosporins, the separations were performed in a weak acidic, neutral or a weak basic medium.

Fig. 2 shows the influence of buffer pH on the electrophoretic mobility of 12 cephalosporins with a phosphate buffer (100 m*M*) in the pH range of 5.5–7.8. The electrophoretic mobility (migrating in the opposite direction to the electroosmotic flow) of cephalosporins belonging to class I increases sigmoidally with the pH of the buffer, whereas the electrophoretic mobility of cephalosporins belonging to class II, except cephapirin (**7**), remains essentially constant with increasing the pH of the buffer.

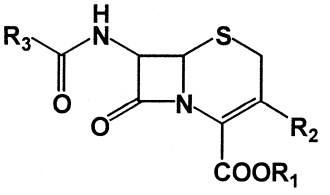
As shown in Table 1, the class I cephalosporins possess a carboxylic acid group with pK_{a1} values in the range 1.5–3.1 and an amino group with pK_{a2} values in the range 6.8–7.4 [22,25,26]. Thus, these cephalosporins exist as zwitterionic species in the pH range studied. As the carboxylic acid group is fully dissociated at pH>5, the variation in the electrophoretic mobility of these three cephalosporins in the pH range from 5 to 9 is a result of the increase in the degree of deprotonation of an amino group at the 3-position of the cephem ring. The migration behavior of these cephalosporins is predictable, once the pK_{a2} value and the limiting electrophoretic mobility at $pH \geq pK_{a2}+2$ (or adequate mobility data) are available. In fact, the pK_{a2} values of cephradine (**1**), cephalexin (**2**) and cefaclor (**3**), which will be described later in Section 3.4, are determined to be 7.27, 6.96 and 6.92, respectively.

The electrophoretic mobility of cephapirin (**7**) increases with increasing buffer pH from pH 5.5 to 7.8. The variation of the electrophoretic mobility of cephapirin (**7**) in this pH range is believed due to the deprotonation of its pyridinium group at the 7-position of the cephem ring. The pK_{a2} value of this pyridinium group, which will be described later in Section 3.4, is determined to be 4.72. Thus, the results that cephapirin (**7**) appears before cefsulodin (**5**) at a pH below 5.9 and it elutes together with cefazolin (**10**) at a pH above 7.0 is predictable.

As shown in Fig. 2, the electrophoretic mobility of cefotaxime (**6**) and cefuroxime (**9**) is essentially pH-independent because these two cephalosporins do not possess any acidic or basic functional groups with pK_a values lying in the pH range studied. With the phosphate buffer (100 m*M*) at pH 6.2, the observed magnitudes of the electrophoretic mobility of cefotaxime (**6**) and cefuroxime (**9**) are $-9.03 \cdot 10^{-5}$ and $-9.65 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$, respectively, which are greater than those reported previously [22] by a factor of about 45. In addition, the observed electrophoretic mobility of cefamandole (**8**) is $-9.34 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$, which is in between the values of cefotaxime (**6**) and cefuroxime (**9**). Evidently, the results indicate that the correctness of mobility data of these two cephalosporins reported previously [22] are questionable.

As the primary amino group of class I cephalosporins contribute to their migration behavior in the pH range studied, all members of class I cephalosporins migrate at a slower velocity toward the anode than those of class II cephalosporins at pH below 7.0. Thus in the pH range 5.5–7.0, excluding cephapirin (**7**), the migration of the 11 cephalosporins selected follows the order: cephradine (**1**)< cephalexin (**2**)<cefaclor (**3**)<cefoperazone (**4**)< cefsulodin (**5**)<cefotaxime (**6**)<cefamandole (**8**)< cefuroxime (**9**)<cefazolin (**10**)<cephalothin (**11**)< ceftriaxone (**12**). Compared with other members of class II cephalosporins, it is expected that ceftriaxone migrates with the greatest mobility towards the anode because it carries an extra negative charge in the R_2 substituent.

It is noted that, with phosphate buffer at a



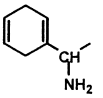
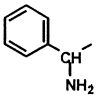
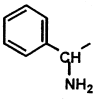
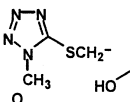
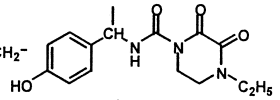
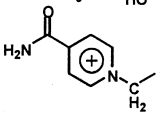
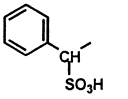
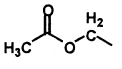
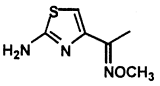
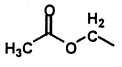
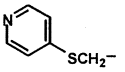
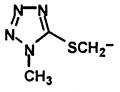
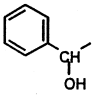
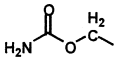
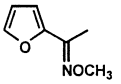
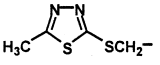
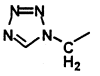
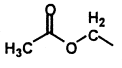
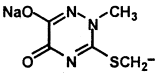
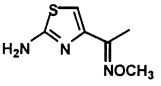
No.	Solutes	R_1	R_2	R_3
1	Cephradine	H	CH_3	
2	Cephalexin	H	CH_3	
3	Cefaclor	H	Cl	
4	Cefoperazone	Na		
5	Cefsulodin	Na		
6	Cefotaxime	Na		
7	Cephapirin	Na		
8	Cefamandole	Na		
9	Cefuroxime	Na		
10	Cefazolin	Na		
11	Cephalothin	Na		
12	Ceftriaxone	Na		

Fig. 1. Structures of cephalosporins selected.

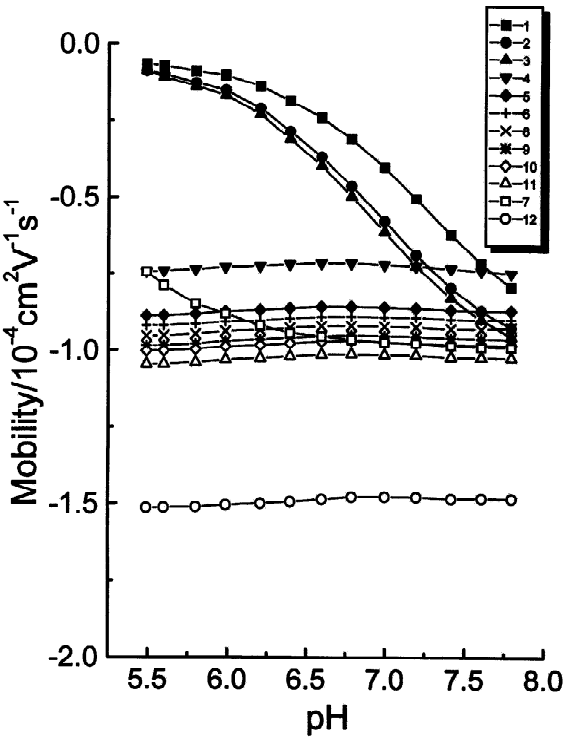


Fig. 2. Electrophoretic mobility of 12 cephalosporins obtained with phosphate buffer (100 m*M*) at varied pH in the range 5.5–7.8. Capillary: 57 cm×50 μm I.D.; the length between column inlet and detector, 50 cm. Injection method: hydrodynamic mode; pressure, 0.4 p.s.i., 1 p.s.i.=6894.76 Pa; injection time, 4 s. Other operating conditions: 20 kV, 25°C and current, 47~91 μA. Peak identification: 1=cephradine, 2=cephaloxin, 3=cefaclor, 4=cefoperazone, 5=cefsulodin, 6=cefotaxime, 7=cephapirin, 8=cefamandole, 9=cefuroxime, 10=cefazolin, 11=cephalothin, 12=ceftriaxone.

concentration of 100 m*M*, the resolution of peaks between cephalexin (**2**) and cefaclor (**3**) improves as the pH of the buffer increases from 5.8 to 7.0. In fact, the peaks of these two cephalosporins are barely resolved at pH 5.8, but they are well resolved at pHs above 6.2. Since all of the cephalosporins, except cephapirin (**7**), are well separated, complete separations of the 12 cephalosporins can be achieved by careful manipulation of buffer pH at a pH above 6.2. Fig. 3 shows the electropherograms of cephalosporins obtained with a phosphate buffer (100 m*M*) at pHs 5.8, 6.2, 6.4 and 7.0. As shown in Fig. 3B and C, complete separations of these cephalosporins were achieved with phosphate buffer (100 m*M*) at pHs 6.2 and 6.4. As ceftriaxone (**12**) elutes relatively far behind cephalothin (**11**), the peak of ceftriaxone which appears at about 20–23 min, depending on the pH of the buffer, is not shown in Fig. 3.

3.2. Influences of buffer concentration

It is well known that, for a given type of buffer electrolyte, the magnitude of the electroosmotic flow (μ_{eo}) depends mainly on the zeta potential which decreases with decreasing buffer pH and/or increasing ionic strength (or buffer concentration) of the buffer solution. Hence, at a given buffer pH, it is expected that an increase in the ionic strength (or buffer concentration) results in a decrease in the zeta potential, thus leading to a decrease in the value of $\mu_{eo.}$ Similar arguments can be applied to account for the variation in the electrophoretic mobility (μ_{ep}) of analytes.

At a particular buffer pH, buffer concentration plays a significant role in the separation of cephalosporins. This is particularly true when cefuroxime (**9**) and cefazolin (**10**) are simultaneously present as analytes because the resolution of the peaks between these two cephalosporins is concentration-dependent. They are hardly resolved with the phosphate buffer at a concentration of 20 m*M*, partially resolved at 50 m*M* and are well separated at a concentration above 100 m*M*. On the other hand, the separation of cephalexin (**2**) and cefaclor (**3**) and that of cefotaxime (**6**) and cephapirin (**7**) depend also on the concentration of a buffer electrolyte. At pH 6.0, the peaks of cefaclor (**3**) and cephalexin (**2**) are barely resolved with phosphate buffer at 50 m*M*. These two peaks are incompletely resolved at 100 m*M* and are baseline separated at 150 m*M* with an applied voltage of 20 kV. Similarly, the peak of cephapirin (**7**) almost merges with cefotaxime (**6**) at 50 m*M* and is merely resolved at 100 m*M*. Fig. 4 shows such electropherograms of cephalosporins obtained with a phosphate buffer at pH 6.0. Thus, to achieve optimum separation of these 12 cephalosporins, a phosphate buffer at a concentration of at least 100 m*M* at pH 6.2 or at a concentration above 150 m*M* at pH 6.0 is required.

3.3. Influences of buffer electrolytes

It was found that the migration behavior of

Table 1
The pK_a values and mobility data of cephalosporins

Peak	Analytes	pK_{a1}	pK_{a2}			Limiting mobility[a]: this work
		Literature values	Literature values	This work		
				Curve-fitting	Inflection point	
1	Cephradine	2.63[c]	7.30[c]	7.27	7.27	0.95
2	Cephalexin	3.11[b](2.34)[b] 2.56[c]	6.79[b](7.08)[b] 6.88[c]	6.96	6.96	0.99
3	Cefaclor	2.69[b] 1.5[d]	7.38[b](7.19)[b] 7.2[d]	6.92	6.92	0.01
4	Cefoperazone	2.6[d]				
5	Cefsulodin					
6	Cefotaxime	2.09[b](2.9)[b] 3.4[d]				
7	Cephapirin			4.65	4.72	1.13
8	Cefamandole	2.46[b](2.60)[b] 2.7[d]				
9	Cefuroxime	2.04[b](2.17)[b]				
10	Cefazolin	2.1[d]				
11	Cephalothin	2.5[d]				
12	Ceftriaxone	3.2[d]	3.2[d]			

[a] Mobility in units of 10^{-4} cm^2 V^{-1} s^{-1}.
[b] Ref. [22], values in parathesis determined from potentiometric measurements.
[c] Ref. [25].
[d] Ref. [26].

cephalosporins in citrate buffer was significantly different from that of cephalosporins in phosphate buffer at pH 6.0 [20,21], and the separation of cephalosporins was remarkably improved on addition of tetraalkylammonium salts to the SDS solution [13]. We thought that the surface modification of the capillary wall with the use of a zwitterionic buffer, such as MES, and that the electrostatic forces of the attraction and repulsion between cephalosporins and zwitterionic buffer electrolytes, would influence significantly the migration behavior and separation of cephalosporins. Thus, it would be of interest to examine the effects of separation parameters using these two types of buffer electrolyte.

3.3.1. Citrate buffer

Fig. 5 shows the effect of the buffer pH on the migration and separation of cephalosporins in the pH range 5.3–6.7 using citrate buffer at a concentration of 40 m*M*. As can be seen, the trends in the variation of the electrophoretic mobility of these 12 cephalosporins as a function of buffer pH with citrate buffer are similar to those observed with the phosphate buffer shown in Fig. 2. The electrophoretic mobility of class I cephalosporins, as well as cephapirin (**7**), increases considerably, but the electrophoretic mobility of class II cephalosporins decreases about 10% of their magnitudes with increasing buffer pH from 5.3 to 6.7. The electrophoretic mobility of cephalosporins measured in citrate buffer has a 1.2–1.3 fold increase, compared with the corresponding mobility measured in a phosphate buffer, whereas the electroosmotic mobility has a 1.1–1.3 fold increase with increasing buffer pH from 5.5 to 7.0. With the citrate buffer at a pH below 5.3, the absolute magnitudes of the electrophoretic mobility of class II cephalosporins, expect cephapirin (**7**) and cefoperazone (**4**), exceed that of the electroosmotic mobility, thus leading to the disappearance of the signals of the cephalosporins that elute later than cefoperazone (**4**). Similar phenomena were also observed with a citrate buffer at a lower concentration, but at a lower buffer pH. The disappearance of the signals of these cephalosporins occurred at pH below 5.0 with citrate buffer at a concentration of 35 m*M*.

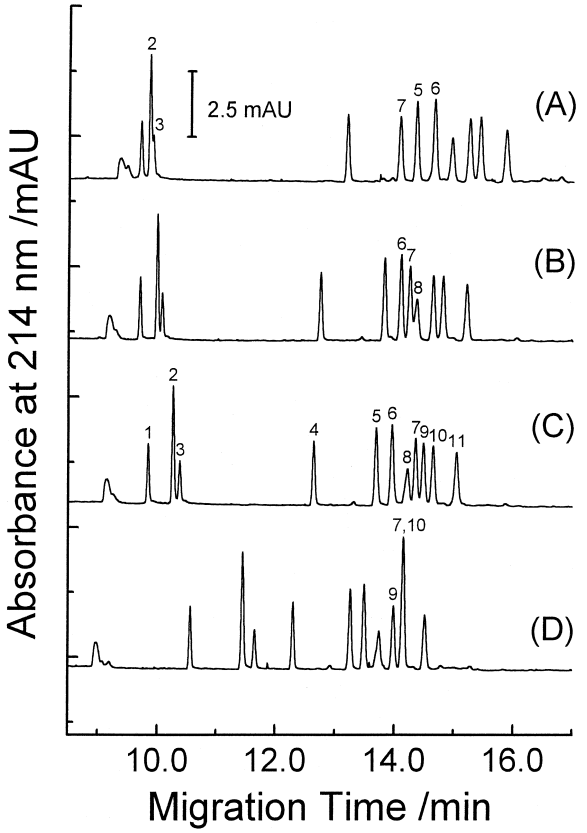


Fig. 3. Electropherograms of cephalosporins obtained with phosphate buffer (100 m*M*) at varied pH: (A) 5.80, (B) 6.21, (C) 6.4, (D) 7.00. Operating conditions and peak identification as for Fig. 2.

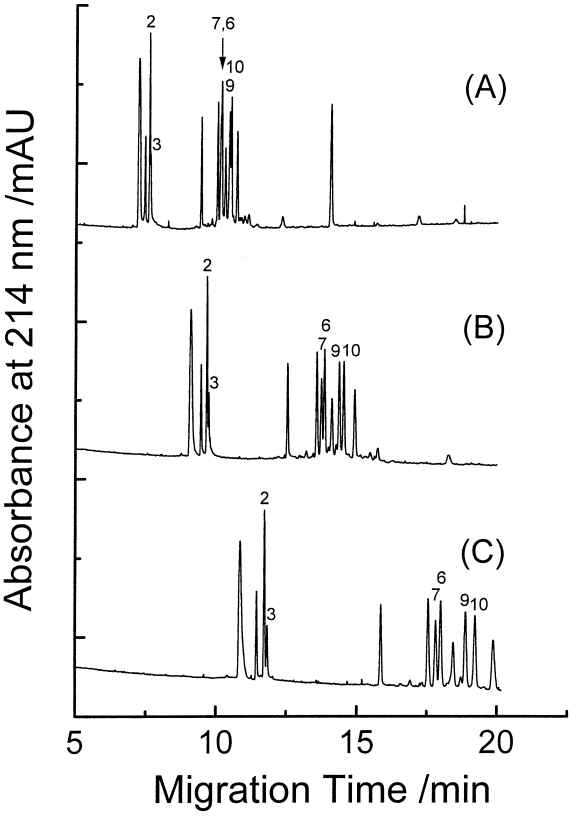


Fig. 4. Electropherograms of cephalosporins obtained with phosphate buffer at varied concentrations at pH 6.0: (A) 50 m*M*, (B) 100 m*M*, and (C) 150 m*M*. Operating conditions and peak identification as for Fig. 2.

As indicated in Fig. 5, with the citrate buffer at a concentration of 40 m*M*, the peaks of cephalexin (**2**) and cefaclor (**3**) are well separated in the pH range studied. A similar situation is encountered to the peaks of cefuroxime (**9**) and cefazolin (**10**). Hence, by manipulating the buffer pH in the range 5.5–6.2, the overlapping of the peak of cephapirin (**7**) with the others may be avoided and complete separations of the 12 cephalosporins selected can be easily achieved. As a matter of fact, complete separations of 12 cephalosporins can be achieved at pHs 5.5, 5.8, 6.0 and 6.2. Fig. 6 shows the electropherograms of the 12 cephalosporins obtained with the citrate buffer at these pH values.

The optimum concentration of the citrate buffer is confined in a small range. With the citrate buffer at a concentration of 50 m*M*, the current of the electrophoretic system exceeds 100 μA at pHs above 6.0. Hence, in order to avoid experimental difficulties due to excessive Joule heating, the use of the citrate buffer at concentrations greater than 50 m*M* is not recommended. On the other hand, at buffer concentrations below 35 m*M*, the peaks of cephalexin (**2**) and cefaclor (**3**) are baseline separated only at a pH above 6.2. Unfortunately, the peaks of cefuroxime (**9**) and cefazolin (**10**) can not be well resolved at pHs above 5.6. Thus complete separation of the 12 cephalosporins is impossible to achieve with citrate buffer at concentrations below 35 m*M*.

As the extent of the increase in the magnitude of the electroosmotic mobility (μ_{eo}) and the extents of the increase in the electrophoretic mobility of class I

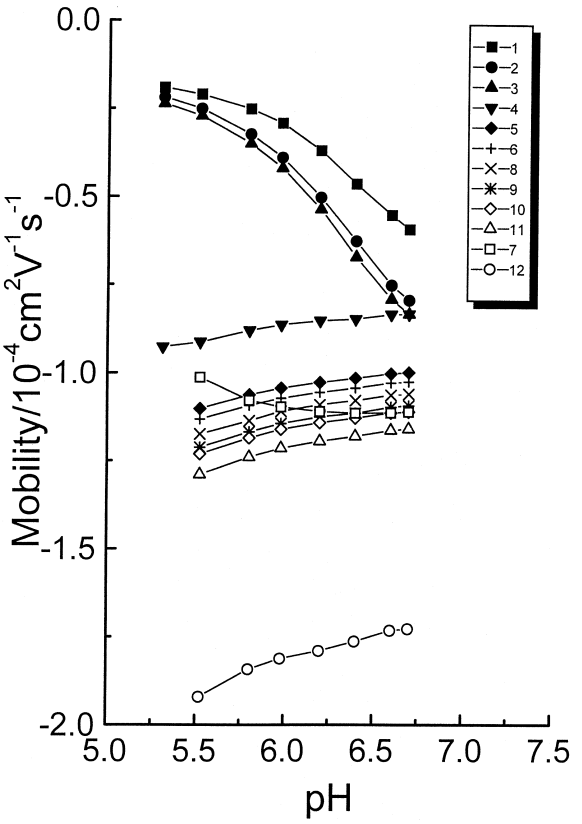


Fig. 5. Electrophoretic mobility of cephalosporins obtained with citrate buffer (40 m*M*) at varied pH in the range 5.5–6.7. Operating conditions and curve identification as for Fig. 2, except that the applied voltage is 30 kV.

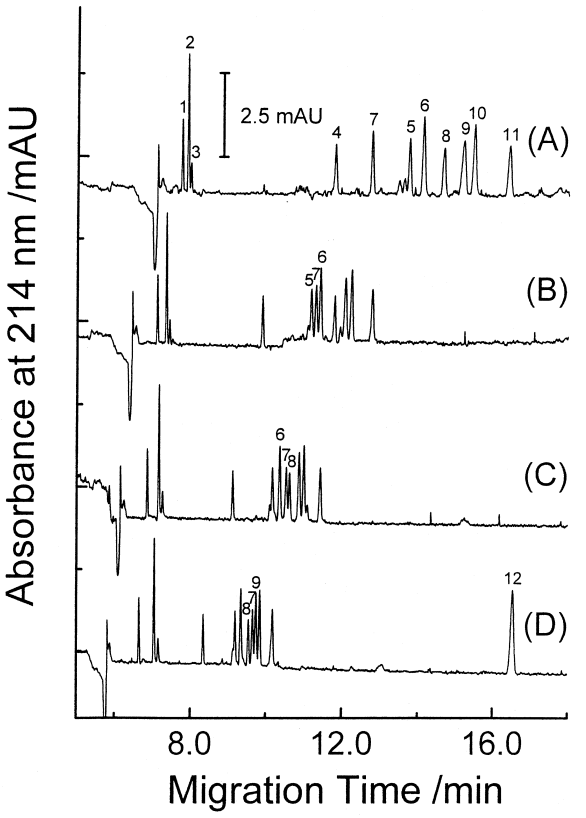


Fig. 6. Electropherograms of cephalosporins obtained with citrate buffer (40 m*M*) at varied pH: (A) 5.5, (B) 5.8, (C) 6.0, (D) 6.2. Operation conditions and peak identification as for Fig. 5.

cephalosporins and the decrease in the electrophoretic mobility of class II cephalosporins with the citrate buffer are much greater than those of the corresponding mobility with the phosphate buffer when the pH of the buffer increases from 5.3 to 6.7, the difference between the electrophoretic mobility of each analyte and electroosmotic mobility at a particular buffer pH is greater with the citrate buffer than with the phosphate buffer. Comparing Fig. 6 with Fig. 3, better separability and shorter analysis time is clearly illustrated when using the citrate solutions as a buffer electrolyte.

3.3.2. MES buffer

Fig. 7 shows the variation of electrophoretic mobility of cephalosporins as a function of buffer pH in the range 5.5–7.3 using MES buffer at a concentration of 260 m*M*. In general, the trends in the variation of the electrophoretic mobility of these 12 cephalosporins as a function of buffer pH with MES buffer are similar to those observed with the phosphate and citrate buffers (shown in Figs. 2 and 5, respectively). However, the electrophoretic mobility of each individual cephalosporin increases about 5% of its magnitude when the buffer pH increases from 5.5 to 7.3. The electrophoretic mobility of cephalosporins measured in MES buffer has a slight increase, compared with the corresponding mobility measured in the phosphate buffer. Since the magnitude of μ_{eo} decreases quite rapidly from $2.85 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ at pH 5.5 to $2.25 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ at pH 6.2, and then gradually decreases further to $2.18 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ at pH 7.0, the peaks of

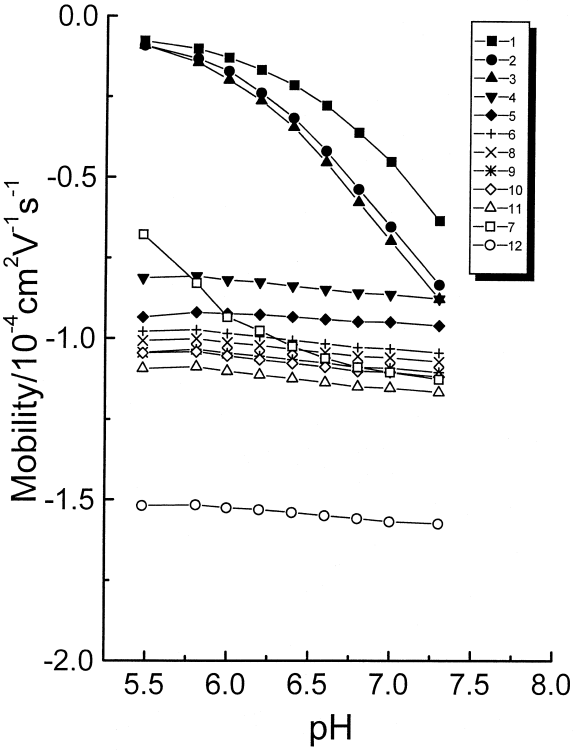


Fig. 7. Electrophoretic mobility of cephalosporins obtained with MES buffer (260 m*M*) at varied pH in the range 5.5–7.3. Operating conditions and curve identification as for Fig. 5.

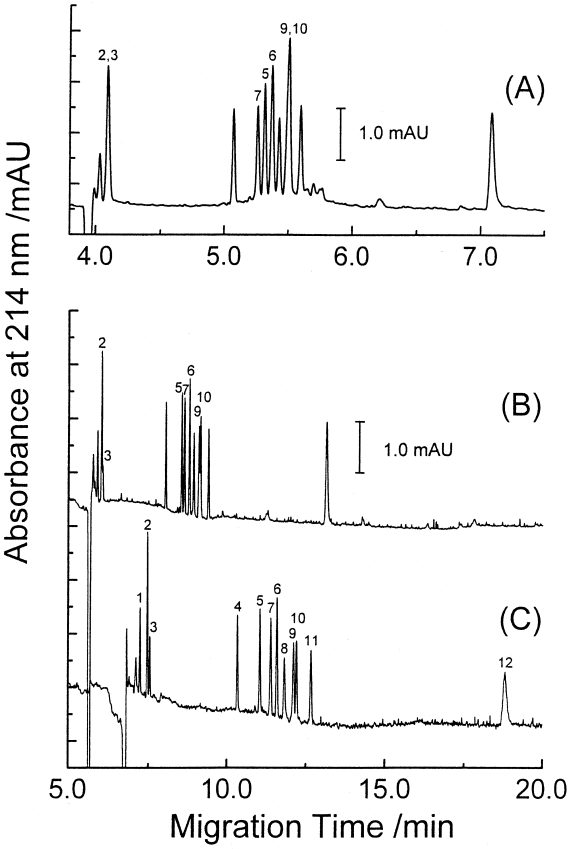


Fig. 8. Electropherograms of cephalosporins obtained at pH 6.21 with MES buffer at varied concentrations: (A) 40 m*M*, (B) 150 m*M*, (C) 260 m*M*. Operating conditions and peak identification as for Fig. 5.

cephalosporins move towards the position of neutral marker as the buffer pH decreases. To achieve complete separation of these 12 cephalosporins, the buffer pH at 6.2 should be selected when using the MES buffer at the concentration of 260 m*M*. With this buffer concentration, the current measured was about 70 μA.

The electrophoretic mobility of cephalosporins increases with increasing buffer concentration. Fig. 8 shows the electropherograms of cephalosporins obtained with the MES buffer at concentrations of 40, 150 and 260 m*M* at pH 6.21. The migration of cephapirin (**7**) is markedly influenced by the concentration of the MES buffer. The peak of cephapirin (**7**) appears before cefsulodin (**5**) with the buffer concentration at 40 m*M*. However, cephapirin (**7**) comigrates with cefsulodin (**5**) at 150 m*M*, but elutes later than cefsulodin (**5**) at or above 260 m*M*. Moreover, the resolution of the peaks between cephalexin (**2**) and cefaclor (**3**) and that of the peaks between cefuroxime (**9**) and cefazolin (**10**) are significantly affected by varying the concentration of the MES buffer. In fact, these two pairs of peaks are well resolved at a concentration greater than 260 m*M* with the MES buffer at pH 6.2.

It should be noted that complete separations are much easier to achieve when cephalexin (**2**) and cefaclor (**3**) or cefuroxime (**9**) and cefazolin (**10**) are not simultaneously present as analytes. As shown in Fig. 8A, most of the cephalosporins studied can be

easily and effectively separated even with the MES buffer at a concentration as low as 40 m*M* at pH 6.21.

3.4. Determination of pK_{a2} value and prediction of migration behavior

The pK_{a2} values of class I cephalosporins in the phosphate buffer (100 m*M*) and that of cephapirin (**7**) in the citrate buffer (35 m*M*) can be determined from the inflection points of the electrophoretic mobility curves which are the plots of electrophoretic mobility versus buffer pH. To determine the pK_a value of an analyte, a simulated mobility curve which is best-fitted to the experimental mobility curve is first obtained through the utilization of the Sigmoidal Fit of Microcal Origin software (version 5.0) so that small differences in the electrophoretic mobility ($\Delta\mu$) for every small increment of buffer pH (ΔpH), say 0.005, in the pH range studied are calculated. The first or second derivative of $\Delta\mu$ with respect to ΔpH is then plotted against buffer pH for each mobility curve and the pH value of the inflection point corresponding to the pK_a value is determined. Fig. 9 shows such plots for cephradine (**1**), cephalexin (**2**), cefaclor (**3**) and cephapirin (**7**). The arrows shown in Fig. 9 indicate the positions of the inflection points of these mobility curves. The pK_a values of cephradine (**1**), cephalexin (**2**), cefaclor (**3**) and cephapirin (**7**) determined are 7.27, 6.96, 6.92 and 4.72, respectively. As the literature values of the pK_{a2} of nicotinic acid attributed to the pyridinium group is 4.81 [27], the determined pK_{a2} value of cephapirin (**7**) would be a reasonable value.

As mentioned earlier, the carboxylic acid group of cephalosporins is fully dissociated at pH>5 and the variation of the electrophoretic mobility of cephradine (**1**), cephalexin (**2**), cefaclor (**3**) and

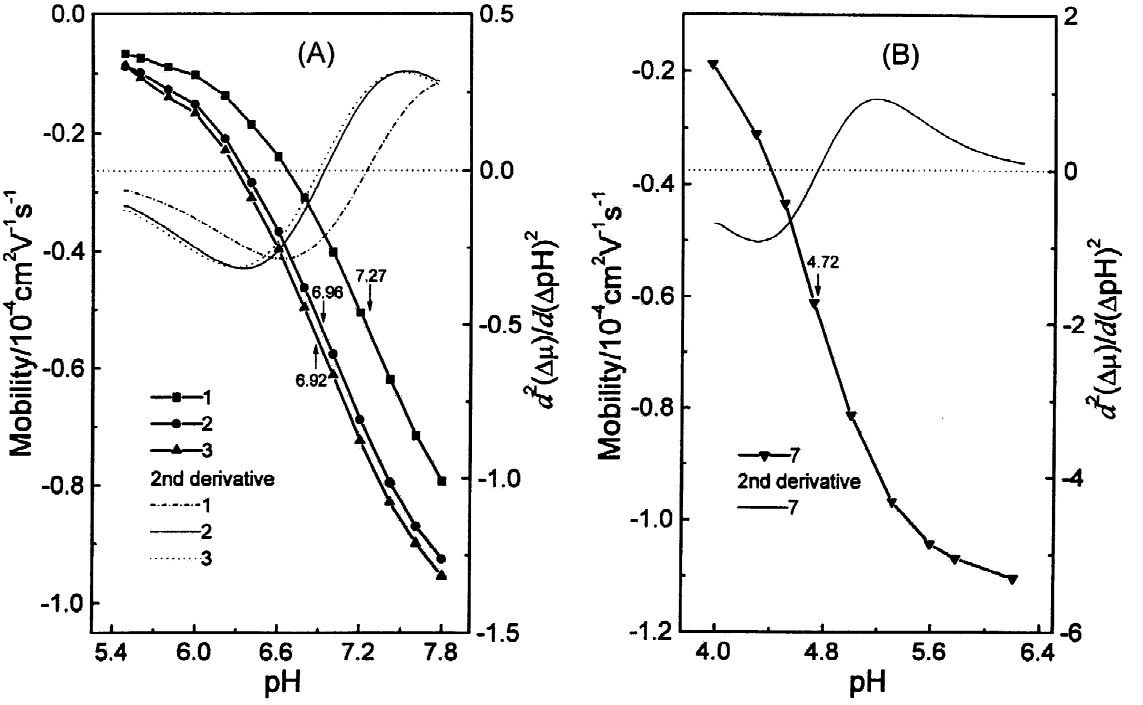


Fig. 9. The pK_{a2} values of cephalosporins determined from the inflection points of pH-dependent mobility curves: (A) cephradine (**1**), cephalexin (**2**) and cefaclor (**3**) in phosphate buffer (100 m*M*), (B) cephapirin (**7**) in citrate buffer (35 m*M*). Other operating conditions in the cases of (A) and (B) are the same as for Fig. 2 and Fig. 5, respectively. Arrows indicate the inflection points of mobility curves.

cephapirin (**7**) in the pH range studied is the result of the increase in the degree of deprotonation of either an amino group or a pyridinium group of the specific cephalosporin. Thus, the effective electrophoretic mobility (μ_{eff}) of these cephalosporins as a function of buffer pH can be described by the following equation modified from the one used for cationic species [28,29]:

$$\mu_{eff} = \frac{[H_3O^+]\mu_{BH^+}}{[H_3O^+] + K_{a2}} - \mu_{BH^+} = -\frac{K_{a2}\mu_{BH^+}}{[H_3O^+] + K_{a2}} \quad (1)$$

where $\mu_{BH^+}$is the electrophoretic mobility of the protonated form of a zwitterionic solute. Accordingly, the migration behavior of these cephalosporins in the pH between p$K_{a2}-2$ and p$K_{a2}+2$ can be predicted using Eq. (1), provided that the pK_{a2} values and the necessary mobility data are available.

To determine the pK_{a2} values of cephalosporins properly from the curve-fitting method, the trial values of pK_{a2} and limiting mobility of each individual cephalosporin are first estimated from the mobility curve. The best-fitted pK_{a2} value and limiting mobility are then determined by varying the trial values of these two parameters until the predicted mobility curve calculated by using Eq. (1) is best fitted to the experimental mobility curve through the utilization of Microcal Origin software. Fig. 10 shows the mobility curves obtained for these four cephalosporins. As can be seen, the agreement between the predicted mobility curves (represented by solid or dash lines) and the observed mobility curves (shown by data points) is very good. Table 1 lists the pK_{a2} values and the mobility data evaluated, together with the pK_a values reported in the literature [22,25,26]. The pK_{a2} values determined from the inflection-point and curve-fitting methods agree very well. It is noted that a small correction to the effective mobility is sometimes necessary for the best fit of mobility curves for class I cephalosporins. For instance, for the mobility curves shown in Fig. 9A, a small correction of -0.04 was made. This is perhaps due to a small contribution to the electro-

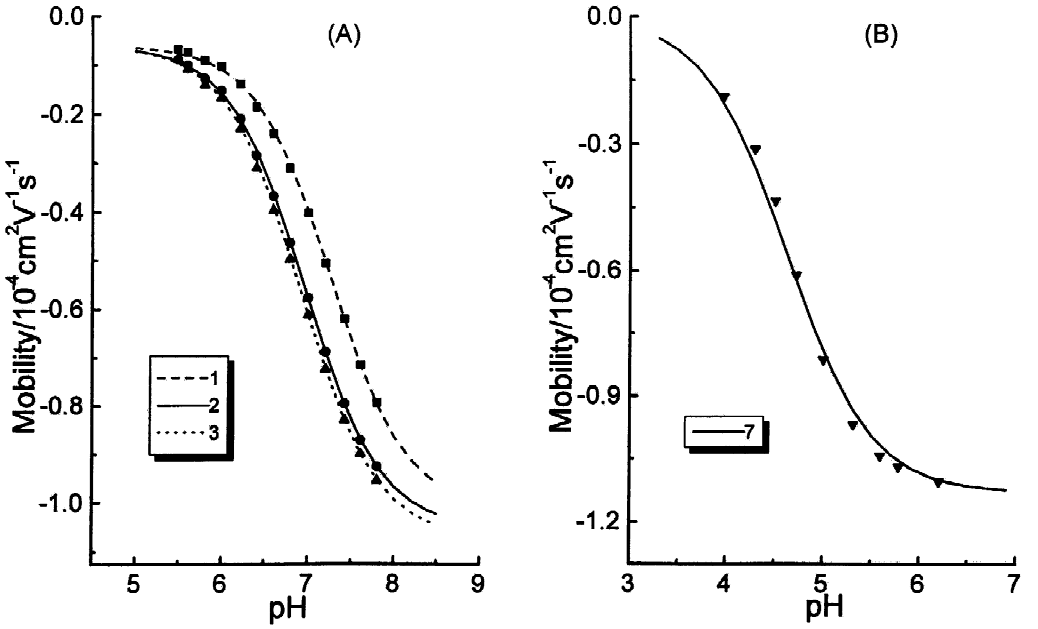


Fig. 10. The agreement between the predicted mobility curves (represented by solid and dash lines) and observed mobility curves (shown by data points) for cephalosporins: (A) cephradine (**1**), cephalexin (**2**) and cefaclor (**3**), (B) cephapirin (**7**).

phoretic mobility from other structural moieties of cephalosporins.

It should be pointed out that the pK_{a2} value of cefaclor (**3**) reported previously by Mrestani et al. [22] was not accurate enough because the mobility curves of cephalexin (**2**) and cefaclor (**3**) shown in Fig. 3 from [22] are very close to each other and the difference in the pK_{a2} values of these two cephalosporins determined by the CE method may be less than 0.2, as was determined from the potentiometric measurements [22]. The results shown in Figs. 9A or 10A clearly demonstrate that the difference in pK_{a2} values between cephalexin (**2**) and cefaclor (**3**) is only 0.04, with the pK_a value of cefaclor (**3**) being smaller than that of cephalexin (**2**). Therefore, a more accurate pK_{a2} value of cefaclor (**3**) than that reported previously [22] is obtained in this work.

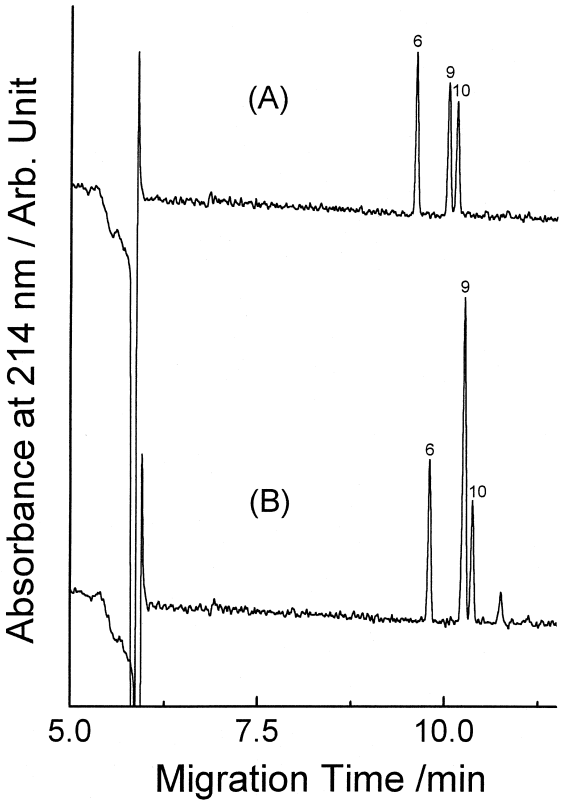


Fig. 11. Electropherograms of cefotaxime, cefuroxime and cefazolin obtained with citrate buffer (50 m*M*) at pH 6.0: (A) a mixture of standards (50 μg/ml each), (B) a standard solution of (A) spiking with Lifurox (50 μg/ml). Operation conditions and peak identification as for Fig. 5.

3.5. Peak confirmation

In the course of our investigation, the migration order of cephalosporins between cefotaxime (**6**) and cefuroxime (**9**) is contradictory to that reported by Mrestani et al. [20]. In order to confirm the correctness of our assignments for these two peaks, a real sample of cefuroxime (Lifurox) obtained from Lilly (Taiwan) was injected into the CE system. Fig. 11A shows the electropherogram obtained for a standard solution containing cefotaxime (**6**), cefuroxime (**9**) and cefazolin (**10**) (50 μg/ml each dissolved in an injection fluid). A amount of 20 μg/ml of urea was added to the sample solutions in order to examine whether urea contained in urine samples can affect the migration order of cefotaxime (**6**) and cefuroxime (**9**) using citrate buffer (50 m*M*) at pH 6.0. Fig. 11B shows the electropherogram of these three analytes obtained after spiking with Lifurox (50 μg/ml). As can be seen, the relative migration time of the cefuroxime standard and that of Lifurox to cefazolin (**10**) or to cefotaxime (**6**) is agreeable. Evidently, the result confirms that our assignments for these peaks should be beyond doubt. On the contrary, the assignments made by Mrestani et al. [20] are questionable.

4. Conclusions

Although manipulation of buffer pH is a key strategy to optimize the separation of cephalosporins in CZE, combined effects of buffer pH and buffer concentration should be taken into consideration to optimize the separation of cephalosporins, particularly when cefotaxime and cephapirin, cephalexin and cefaclor, or cefuroxime and cefazolin, are simultaneously present. Complete separations of 12 cephalosporins are achievable with citrate, MES and phosphate buffers under various optimum conditions. However, citrate and MES buffers are superior to the phosphate buffer in the CZE separation of the cephalosporins. The migration behavior of these cephalosporins in the pH range studied is described.

Acknowledgements

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