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Migration behavior and separation of sulfonamides in capillary zone electrophoresis
II. Positively charged species at low pH

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Abstract

The migration behavior and separation of sixteen sulfonamides as positively charged species at low pH were systematically investigated by capillary zone electrophoresis using citrate buffer as a background electrolyte. Optimized separation parameters were determined. The results indicate that buffer pH and buffer concentration are two important separation parameters. However, buffer pH has a larger effect on the selectivity and resolution of sulfonamides than buffer concentration. In particular, the resolution of sulfamethizole and sulfamonomethoxine is improved with increasing buffer concentration, whereas that of sulfamerazine and sulfathiazole improves with increasing buffer pH, and that of sulfameter, sulfadimethoxine and sulfaquinoxaline improves with increasing buffer concentration and buffer pH. Complete separation of sixteen sulfonamides was achieved using citrate buffer (500 m*M*) at pH 2.1 and an applied voltage of 30 kV. Moreover, the migration order of sulfonamides is primarily determined by their pK_{a_1} values.

Keywords: Food analysis; Buffer composition; Sulfonamides

1. Introduction

Sulfonamides are anti-bacterial and anti-infective compounds used widely in veterinary practice. A major concern with the use of these compounds is that residues may be present in animal food products and may pose a health threat to consumers [1,2]. In addition, sulfamethazine was reported to be a possible carcinogenic substance [3]. Thus, interest in the development of new and sensitive analytical methods to separate and to analyze these compounds continues unabated.

Capillary electrophoresis (CE) has been proven to be a powerful separation technique and it is widely applied to diverse analytical samples [4–8]. The advantageous features of CE techniques are extremely high efficiency, high resolution, rapid analysis and the requirement for a small sample volume.

The separation of sulfonamides by CE was usually conducted in the mode of capillary zone electrophoresis (CZE) [9–16] or micellar electrokinetic chromatography (MEKC) [13,17]. These compounds were separated as negatively charged species by CZE at an optimum pH in the range of 6.0–7.5, using various types of buffer, with or without the addition of electrolyte modifier. Effective mobilities of fifteen sulfonamides were determined over the pH range of 3.2–8.2 and pK_a values were determined for most of

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the analytes by Ackermans et al. [10]. However, over this wide pH range, electrophoretic mobilities of these analytes were measured only at four different pH values (i.e., 3.2, 4.0, 7.0 and 8.2) using different buffer solutions. Several selected sulfonamides were separated with a phosphate (50 m*M*)–borate (50 m*M*) buffer containing either β-cyclodextrin (2 m*M*) at pH 6.4 or β-cyclodextrin (3 m*M*) at pH 6.0 by Ng et al. [11–13]. Ricci and Cross [14] reported that eighteen sulfonamides were resolved within 22 min using 50 m*M* phosphate buffer at pH 7.5, but the peaks between sulfathiazole and sulfamethoxypyridazine and those between sulfadiazine and sulfadimethoxine were not resolved. In our previous report [16], the precise optimization of buffer pH was found to be crucial for further improving the separation of the thirteen sulfonamides that were selected. With the addition of either an appropriate amount of an organic modifier or a low concentration of β-cyclodextrin (0.5 m*M*) to the phosphate (50 m*M*)–borate (50 m*M*) buffer at pH 6.85 and with an applied voltage of 20 kV, effective separations of these thirteen sulfonamides were successfully achieved within a short time. This approach is particularly useful in enhancing the separation of sulfamethoxypyridazine and sulfathiazole.

For sulfonamides tested in this study, two dissociation equilibria exist. As shown in Fig. 1, pK_{a_1} is the dissociation constant of the equilibrium between the positively charged, protonated amino group of sulfonamide and its electrically neutral conjugate base, whereas pK_{a_2} refers to the equilibrium involving the loss of the sulfonamide proton to yield its negative charged conjugate. Therefore, depending on the pH of the buffer employed, sulfonamides can be separated by CZE either as negatively charged, deprotonated species or as positively charged, protonated species.

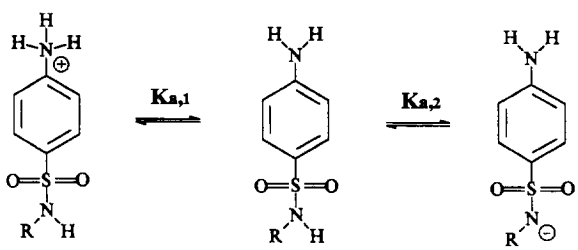

Fig. 1. Dissociation equilibria of sulfonamide involving K_{a_1} and K_{a_2}.

According to the pK_{a_1} values reported previously for some sulfonamides [14], sulfonamides are expected to separate as positively charged species at a pH of around 2.0. As citrate buffer is an excellent background electrolyte for use in the separation of positively charged β-blockers at low pH [18], the use of this buffer electrolyte to separate positively charged sulfonamides was also attempted. Since the separation of sulfonamides as positively charged species has never been reported in the literature, we present the results of a systematic investigation into the effects of buffer concentration and buffer pH of the migration behavior and separation of sixteen sulfonamides by CZE.

2. Experimental

2.1. Chemicals and reagents

Sixteen sulfonamides, originally purchased from Sigma (St. Louis, MO, USA), were supplied as a gift from the Taiwan Meat Development Foundation. The structures of these sulfonamides are shown in Fig. 2. Citric acid (Shimakyu, Osaka, Japan) and trisodium citrate dihydrate (Showa, Kyoto, Japan) were obtained from the indicated suppliers. Methanol was of HPLC grade (Mallinckrodt, Pris, KY, USA) and was used without further purification. Mesityl oxide (MO; TCI, Tokyo, Japan) was used as a neutral marker. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

Standard solutions of sulfonamides were prepared at a concentration of about 0.25 m*M* in a methanol solution. The pH of the citrate buffer was adjusted by mixing various proportions of a certain concentration of a citric acid solution with the same concentration of a trisodium citrate solution or a 1 *M* HCl solution to reach the desired pH. All solutions were filtered through a membrane filter (0.22 μm) before use.

2.2. Apparatus

Separations were made using a CE system that was described previously [16]. The capillary dimen-

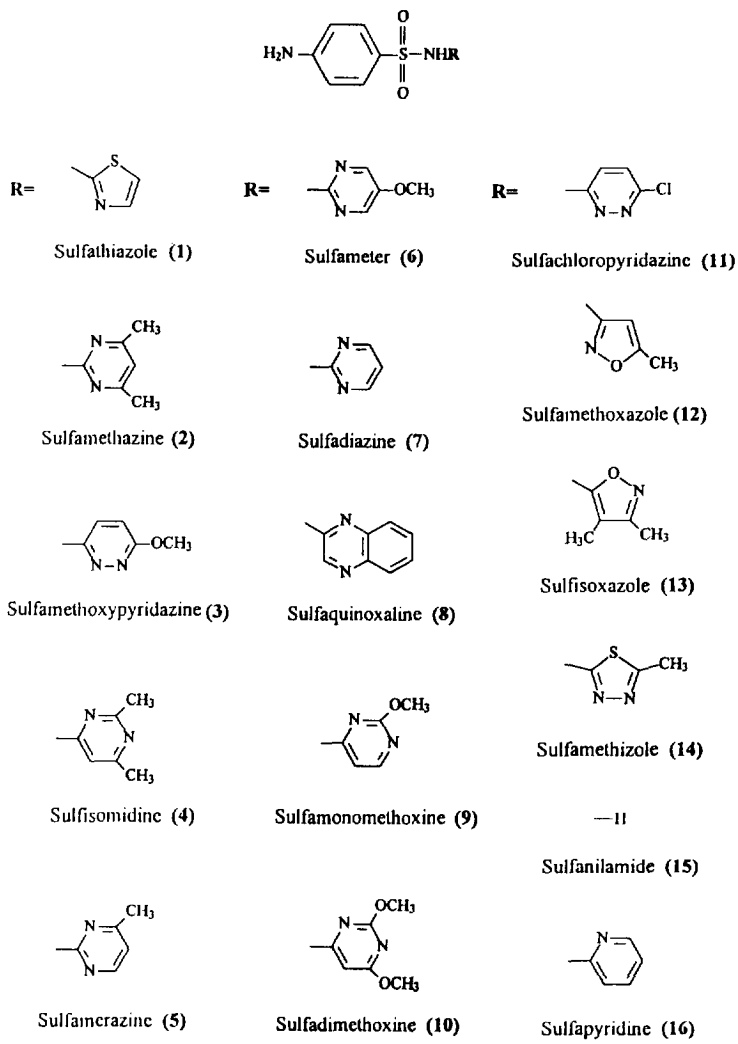

Fig. 2. Structures of the sixteen sulfonamides that were tested.

sions were 67 cm×50 μm I.D. The UV position is 7.0 cm from the cathodic end. Sample injection was done in a hydrodynamic mode over 2 s. The CE system was interfaced with a microcomputer and printer with software CE 500 1.05 A. For pH measurements, a pH meter (Suntex Model SP-701, Taipei, Taiwan) was employed with a precision of ±0.01 pH unit.

2.3. Electrophoretic procedure

When a new capillary was used, the capillary was washed using a standard sequence described previously [19]. To ensure reproducibility, all experiments were performed at 25°C and measurements were performed at least in triplicate. The capillary was prewashed for 6 min with running buffer before each injection to maintain proper reproducibility for run-to-run injections. The detection wavelength was set at 254 nm.

2.4. Mobility calculation

The electrophoretic mobility of analytes was

calculated from the observed migration time using the equation:

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

where μ_{ep} is the electrophoretic mobility of the solute 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 (mesityl oxide as the neutral marker), L_t is the total length of the capillary, L_d is the length of the capillary between the injection end and the detector, and V is the applied voltage.

3. Results and discussion

3.1. Effect of buffer concentration

It has been reported that increasing buffer concentration has a favorable effect on the separation and resolution of analytes [20,21]. The resolution of sulfonamides as negatively charged species was found to be improved by increasing the buffer concentration [14]. This is particularly true for the pair of peaks belonging to sulfamethoxypyridazine **3** and sulfathiazole **1**.

The electrophoretic mobility of sulfonamides was found to decrease with increasing buffer concentration. In general, the electrophoretic mobility of most of the sulfonamides selected in this study decreases at about the same rate when the buffer concentration varies from 150 to 500 m*M*. Thus, the selectivity of most of the sulfonamides is affected very little by an increase in buffer concentration. However, the electrophoretic mobility of sulfanilamide **15**, which possesses a primary amino group, is relatively less affected by buffer concentration than that of sulfamethazine **2**. This leads to the reversal of the migration order of these two sulfonamides when the buffer concentration is varied from 150 to 500 m*M* at pH 2.1 and an applied voltage of 30 kV.

The resolution of some co-migrating sulfonamides improves considerably by increasing the buffer concentration. Compounds **14** and **9**, as well as compounds **6**, **10** and **8**, migrate together when using citrate buffer at 150 m*M*. However, complete separation of the sixteen sulfonamides is achieved using a buffer concentration at 500 m*M*. Fig. 3 shows the electropherograms of sixteen sulfonamides obtained with citrate buffer (150 and 500 m*M*) at pH 2.1 and an applied voltage of 30 kV.

It should be noted that, in the separation of sulfonamides with a high concentration of citrate buffer, no serious experimental difficulties were encountered as a result of Joule heating. The current generated in the capillary is 84 μA with 500 m*M* citrate buffer.

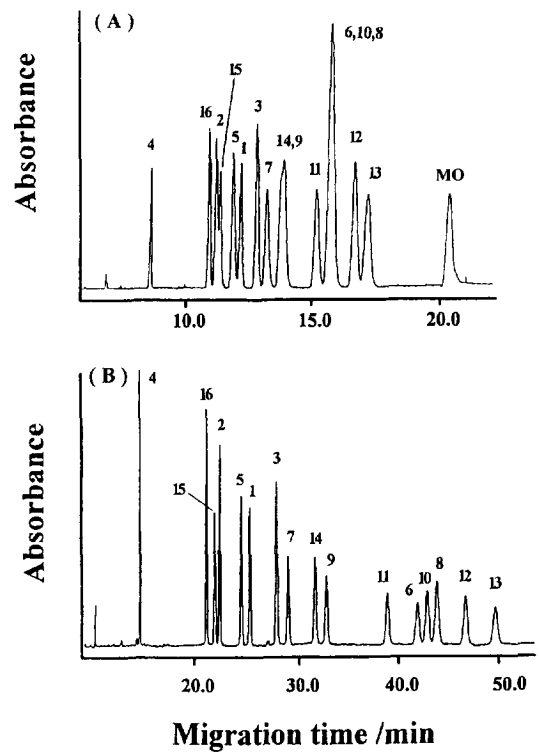

Fig. 3. Electropherograms of sulfonamides obtained with citrate buffer at concentrations of (A) 150 m*M* (with a current at 37 μA) and (B) 500 m*M* (with a current of 84 μA) at pH 2.1 and with an applied voltage of 30 kV. Capillary: 67 cm×50 μm I.D. fused-silica. Other operating conditions: 30 kV, 25°C. Peak identification: 1 = sulfathiazole; 2 = sulfamethazine; 3 = sulfamethoxypyridazine; 4 = sulfisomidine; 5 = sulfamerazine; 6 = sulfameter; 7 = sulfadiazine; 8 = sulfaquinoxaline; 9 = sulfamonomethoxine; 10 = sulfadimethoxine; 11 = sulfachloropyridazine; 12 = sulfamethoxazole; 13 = sulfisoxazole; 14 = sulfamethizole; 15 = sulfanilamide; 16 = sulfapyridine.

3.2. Effect of buffer pH

Since buffer pH is a very important separation parameter in CZE, manipulation of buffer pH often becomes a key strategy in optimizing a separation [19,21]. For separating positively charged sulfonamides with citrate buffer, the pH of the buffer is restricted in a small range of low pH (1.4–2.6). The effect of buffer pH on the migration behavior of sulfonamides, as positively charged species, can be approximately described by the following equation [24]

$$\mu_{ep} = \left(\frac{[H^+]}{K_a + [H^+]} \right) \mu_{BH}^+$$

where μ_{ep} is the electrophoretic mobility of a sulfonamide at a given pH, μ_{BH}^+ is the limiting mobility of the corresponding sulfonamide as a positively charged species and K_a is the dissociation constant. Accordingly, a sigmoidal curved for the migration behavior of each individual sulfonamide is predictable when electrophoretic mobilities are plotted against buffer pH.

Fig. 4 shows the electrophoretic mobility of sulfonamides obtained at various pH values in the range 1.6–2.2 using citrate buffer (500 m*M*) at 30 kV. As expected, the electrophoretic mobility of each individual sulfonamide decreases sigmoidally with increasing buffer pH. It should be noted that the electrophoretic mobility of sulfanilamide **15**, which possesses a primary amino group, is relatively more affected by buffer pH than the electrophoretic mobility of other sulfonamides. Therefore, a reversal of the migration order between sulfanilamide **15** and sulfapyridine **16** may occur when the buffer pH is varied from 1.6 to 2.2. Moreover, the resolution of some sulfonamides that co-migrate at pH 1.6 can be considerably improved by increasing the pH of the buffer. Compounds **5** and **1**, as well as compounds **6**, **10** and **8**, are resolved completely at a pH above 2.0. On the other hand, the resolution of sulfamethiazole (**14**) and sulfamonomethoxine (**9**) decreases gradually with increasing buffer pH. Consequently, these protonated sulfonamides are best separated at pH 2.1. Fig. 5 shows the electropherograms of sixteen sulfonamides obtained at pH values of 1.6 and 2.0 to illustrate the effect of the variation of buffer pH on

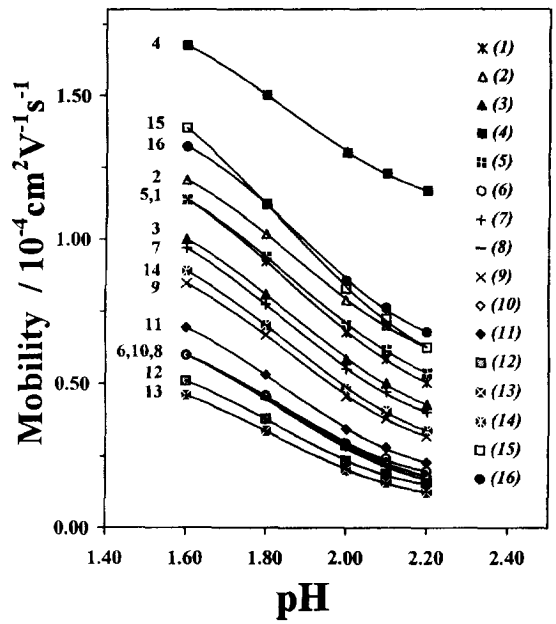

Fig. 4. Electrophoretic mobility of sulfonamides obtained using a 500 m*M* buffer at varying pH values in the range 1.6–2.2. Other operating conditions and peak numbering are as for Fig. 3.

the selectivity and the resolution of these sulfonamides at low pH.

It is interesting to note that the migration order of positively charged sulfonamides is primarily determined by pK_{a_1}. Data on the electrophoretic mobility of sixteen sulfonamides measured with 500 m*M* citrate buffer at pH 2.1 is presented in Table 1 and the pK_{a_1} values were determined [22]. By plotting electrophoretic mobility as a function of pK_{a_1}, a linear correlation with $r^2 = 0.956$ can be obtained. This is similar to the phenomenum observed for sulfonamides as negatively charged species, in which the electrophoretic mobility correlates linearly with the pK_{a_2} values [23].

4. Conclusion

Complete separation of sixteen sulfonamides as positively charged species was successfully achieved for the first time by capillary zone electrophoresis using citric acid as a background electrolyte at low

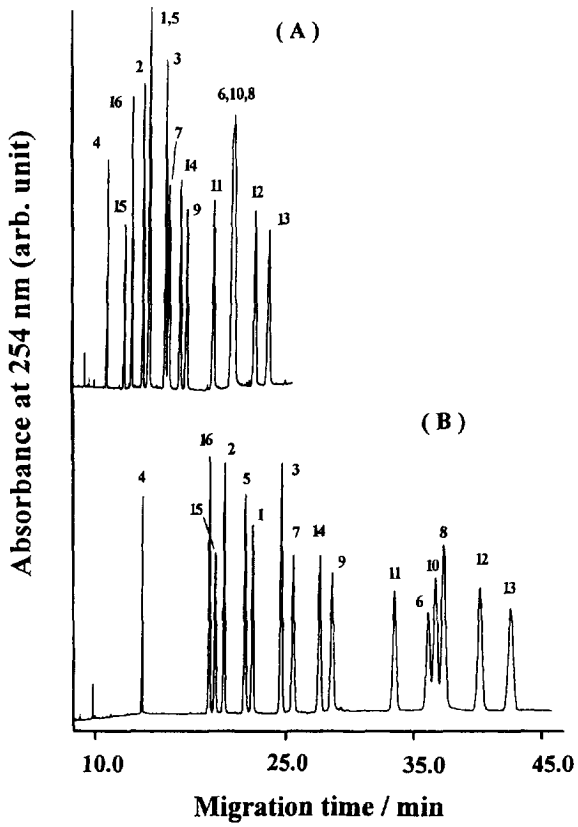

Fig. 5. Electropherograms of sulfonamides obtained with a buffer pH of (A) 1.6 and (B) 2.0. Other operating conditions and peak numbering are as for Fig. 3

Table 1
Electrophoretic mobility (μ_{ep}) of sixteen sulfonamides under optimum separation conditions and their pK_{a_1} values

Sulfonamides	μ^{a}_{ep}	$pK^{a}_{a,1}$
(4) Sulfisomidine	1.23	2.64±0.03
(16) Sulfapyridine	0.76	2.28±0.05
(15) Sulfanilamide	0.72	2.15±0.04
(2) Sulfamethazine	0.70	2.26±0.04
(5) Sulfamerazine	0.62	2.17±0.04
(1) Sulfathiazole	0.59	2.08±0.04
(3) Sulfamethoxypyridazine	0.50	2.08±0.03
(7) Sulfadiazine	0.47	2.02±0.04
(14) Sulfamethizole	0.41	1.98±0.03
(9) Sulfamonomethoxine	0.38	1.97±0.04
(11) Sulfachloropyridazine	0.28	1.88±0.04
(6) Sulfameter	0.24	1.84±0.05
(10) Sulfadimethoxine	0.23	1.84±0.04
(8) Sulfaquinoxaline	0.22	1.83±0.05
(12) Sulfamethoxazole	0.19	1.74±0.05
(13) Sulfisoxazole	0.16	1.60±0.05

[a] Mobility in units of 10^{-4} cm^2 V^{-1} s^{-1}.
[b] From Ref. [22].

pH. The migration order of these sulfonamides is primarily determined by their pK_{a_1} values. Although longer migration times are required, this approach provides an alternative method for separating sulfonamides.

Acknowledgments

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