Separation and quantitation of azimilide and its putative metabolites by capillary electrophoresis

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Abstract

Reliable methods based on capillary electrophoresis (CE) have been developed for the separation and quantitation of azimilide, an antiarrhythmic drug under development at Procter & Gamble Pharmaceuticals (P&GP). Both capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) were employed in the separation of azimilide from its impurities, degradants and/or metabolites. Separation of azimilide from NE-11178, F-410, F-1054 and F-1292 was obtained by MECC at pH 9 with 50 mM sodium dodecyl sulfate (SDS). The separation of azimilide and NE-10171, a key metabolite of azimilide, was difficult because their structures differ by only a single methyl group. The best separation was achieved under acidic pH conditions with cetyltriethyl ammonium chloride (CTAC) additive in the buffer. All of the CE separations were completed within a substantially shorter time and with better resolution than the corresponding high-performance liquid chromatography (HPLC) separations. Quantitation was done with azimilide and NE-10171. Calibration curves ranging from 10 to 1000 µg/ml were obtained with R^2 greater than 0.997 for both azimilide and NE-10171. The back-calculated concentrations of the calibration standards and the recoveries of the quality control (QC) samples were within the acceptance range currently used for HPLC methods. These results demonstrated the viability of CE as an alternative technique for drug metabolism studies in support of pharmaceutical development. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Azimilide

1. Introduction

Capillary electrophoresis (CE) has progressed from the stage of a research curiosity to a maturing separation technology with widespread applicability in the past decade [1,2]. The commercialization of CE instruments has enabled this technique to solve various industrial problems. At the same time, publications related to various CE applications have grown exponentially [3–6]. CE has proven to be a powerful technique for the rapid separation of large biological molecules, such as proteins and DNA, with high efficiency and high resolution [7,8]. CE is also a very powerful technique for the separation of small inorganic and organic compounds [4]. Actually, relative to large biomolecules, CE separation of small molecules presents fewer issues. The charge

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interaction between small molecules and the capillary wall is less of an issue compared with that between the wall and large molecules, especially proteins. In addition, there is no need to be concerned about loss of biological activity. For the CE separation of neutral compounds, a secondary mechanism is required. The most common way of separating neutral molecules is to add surfactants to the separation buffer. When the concentration of the surfactant reaches its critical micellar concentration, a pseudo-stationary phase is formed within the buffer system. Neutral compounds will partition between the micelle and the aqueous buffer. This is known as micellar electrokinetic capillary chromatography (MECC). Thus, there is a great potential for the application of CE in pharmaceutical research and development.

Azimilide is being developed as a Class III antiarrhythmic and antifibrillatory drug for the treatment of arrhythmias [9–12]. Azimilide selectively prolongs the action potential duration in calf cardiac fibers in a concentration-dependent manner [13]. The prolongation of the action potential duration is thought to result from the blockade or slowing of potassium current [14]. Both clinical and non-clinical studies have shown promising results that azimilide can be developed as a highly potent and safe drug. However, azimilide forms multiple degradants when subjected to extreme light and/or heat conditions. When administered to humans and animals, azimilide undergoes complicated metabolic processes resulting in several metabolites. The metabolic profile is different in different species, i.e. human vs. animals. The structure of azimilide along with some of its degradants and metabolites are listed in Table 1. The pharmacological functions of these metabolites are currently under investigation.

In order to support the development of azimilide, a series of analytical methods are in place to quantitate these metabolites and degradants. Most of the current analytical methods are reversed-phase high-performance liquid chromatography (HPLC) methods with UV detection. Some of the methods require a run time as long as 50 min under isocratic conditions. Most of these HPLC methods were validated for the separation of azimilide from only one or two potential metabolites or degradants under specific conditions. Any alteration, such as adding one more

analyte to the method, would require lengthy and costly new method development and validation. Hence, it is desirable to have a general method, which can rapidly separate most, if not all, of the potential analytes in a single method with high speed and high resolution. This is difficult, if not impossible, using HPLC because of the diverse physical and chemical properties of those metabolites or degradants (Table 1). On the other hand, CE appears more able to achieve this goal. A strength of CE is its ability to separate small ions with high efficiency and resolution. However, the relatively low reliability of CE for quantitation has hampered its utility in pharmaceutical analysis. The goal of this work was to explore the utility of CE as a quantitative technique, similar to HPLC, in developing viable separations and reliable assays for azimilide and critical impurities, degradants and metabolites.

This paper presents separations of azimilide from its degradants and metabolites under different conditions. CE appeared to be a good analytical method candidate for the analysis of all of these compounds. CE quantitation was demonstrated using the separation of azimilide and desmethyl azimilide, NE-10171, as an example. The results demonstrated that CE, with simple UV detection, can quantitate azimilide and NE-10171 with reliable precision and accuracy in the concentration range of 10–1000 μg/ml.

2. Experimental

2.1. Buffers and reagents

All buffers were prepared in 0.2 μm filtered deionized water (Milli Q water, Millipore, Bedford, MA, USA). Sodium phosphate (Fisher Scientific, Fair Lawn, NJ, USA) buffer was adjusted to pH 9 with NaOH (Aldrich, Milwaukee, WI, USA). Boric acid (99.5%, Aldrich, Milwaukee, WI, USA) buffer was made and adjusted to pH 9 with NaOH. The sodium acetate (Sigma, St Louis, MO, USA) buffers were adjusted to pH values of 4 and 5 with glacial acetic acid (J. T. Baker, Philipsburg, NJ, USA). Sodium dodecyl sulfate (SDS, Polyscience, Warrington, PA, USA) and polyoxyethylene (23) lauryl ether, (Brij® 35, polyoxyethylene (23) lauryl ether,

Table 1
Structures of azimilide and its metabolites/degradates

Name	Structure
Azimilide (NE-10235)	
NE-10171 Free Base	
NE-11178	
F-410	
F-1054	
F-1292	

$C_{12}H_{25}(OCH_2CH_2)_{23}OH$; Aldrich) were used as anionic and neutral additives, respectively. Cetyl triethyl ammonium chloride (CTAC; Aldrich) was used as a cationic additive. Methanol and acetonitrile were from J. T. Baker. All azimilide-related compounds, such as azimilide dihydrochloride (NE-10064), NE-10171, NE-10133, F-1292, NE-11178, F1054 and F-410, were supplied in-house at P&GP. Milli-Q deionized (DI) water was prepared in-house as well.

2.2. Preparation of sample solutions

Stock solutions (1.0 mg/ml) of azimilide, NE-10171 and NE-10133 were prepared by dissolving each compound in 10% methanol, 10% acetonitrile and 80% DI water. Standard solutions were prepared from these stock solutions by subsequent dilutions with DI water. Stock solutions (0.1 mg/ml) of F-1292, NE-11178, F-1054 and F-410 were prepared by dissolving each compound in acetonitrile. DI water was used in the following sequential dilutions for these stock solutions. A 10% organic is needed to keep F-1054 and F-410 in aqueous solution.

2.3. Capillaries and coating

Bare silica capillaries (50 and/or 75 μm I.D. and 360 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Silica capillaries were treated with 1 M potassium hydroxide and rinsed with water before use. The polyacrylamide-coated capillaries were supplied by Bio-Rad (Hercules, CA, USA). The polyacrylamide coating was prepared following the procedures developed by

Hjertén et al. [15]. A Celect™ amine-coated capillary was purchased from Supelco (Bellefonte, PA, USA). Capillaries with an epoxy coating were prepared in-house following the procedures described elsewhere [16]. In general, the epoxy coating was prepared in a two-step procedure. First, γ-glycidoxypropyl trimethoxysilane (GOX) was coated onto the capillary surface after it was activated with KOH. Then, ethyleneglycol diglycidylether (EGDE) was co-polymerized with the epoxy ring on the GOX to form a cross-linked and stable coating.

2.4. Instrumentation

Most of the separations were performed on a P/ACE 2100 system (Beckman Instruments, Fullerton, CA, USA). Some of the experiments were done on the same instrument after it was upgraded to the 5000 series. Both normal (anode at the capillary inlet) and reversed polarity (cathode at the capillary inlet) modes were used in this work. The selection of either polarity was based on the characteristics of several parameters, such as the capillary coating, pH, buffer additives, etc. Most of the detection was done by UV at 340 nm. Azimilide and most of its related compounds have their maximum absorption at 340 nm. The capillaries were thermostated using the liquid cooling system provided with the instrument. Data acquisition was done on several computers including a 386/33 MHz IBM (Armonk, NY, USA) computer and Pentium 200 MHz Compaq (Austin, TX, USA) computers. The software used includes PACE/2000 software (V 2.1), System Gold (V 6.0) and P/ACE Station for Windows 95.

3. Results and discussion

The structures of azimilide along with its degradants and metabolites are listed in Table 1. The structure includes phenylfuryl hydantoin with an amine side chain containing two tertiary amines. Based on these structures, it is expected that the separation of azimilide from NE-11178 and F-1292 by CE at basic pH (e.g. above pH 9) should be straightforward. Azimilide ($pK_a \sim 9$) is almost neutral, while NE-11178 and F-1292 are negatively charged at this pH. Thus, capillary zone electro-

phoresis (CZE) can be used to separate these compounds. If neutral compounds, such as F-1054 and F-410, are involved in the separation, it is necessary to introduce a secondary separation mechanism, such as chromatographic partition. MECC is a good fit for this purpose because it should allow the simultaneous separation of both neutral and ionic compounds. However, the separation of azimilide from NE-10171 may be challenging due to their structural similarities. Our experimental results confirmed all of the above predictions.

CE has been recognized as a powerful separation technique with high efficiency. However, concerns remain as to whether CE can deliver the same reliability as HPLC for quantitation. To be reliable for quantitation, a rugged CE method should address the following issues: sensitivity, specificity, linearity, precision, accuracy and reproducibility. Sensitivity of a specific CE method depends on the specific requirements of the assay. In UV-based methods, CE is less sensitive compared to HPLC because CE has a much shorter light pass length (100–400-fold less). Significant effort and progress have been made in improving the sensitivity of CE by either using a longer path length, such as the bubble cell [17] or the Z-cell [18]. However, CE usually demonstrates good specificity and linearity. Key issues remain in precision, accuracy and reproducibility. We report in this paper our efforts to address these issues for the separation of azimilide and its metabolites. Specificity, linearity, precision and accuracy were determined by evaluating the calibration standards and QC samples.

3.1. Separation of azimilide at basic pH

3.1.1. Separation of azimilide and NE-11178 and F-1292 by CZE

In the separation of seven tricyclic amine antidepressant drugs by CZE, Solomon et al. [19] demonstrated that the best result was achieved at a pH close to the pK_a values of the secondary or tertiary amine present in these compounds. No separation was achieved at pH values above the pK_a values of the bases. However, decreasing the pH affects not only the analytes but also the silanol groups on the capillary surface. When the pH is lower than the pK_a of the analytes, but higher than that of the silanol

groups, the analytes are positively charged and the capillary surface is negatively charged. The charge interactions between the positively charged analytes and the negatively charged capillary surface will affect the separation results under these pH conditions. Therefore, the optimal pH for the separation of amine compounds is near their pK_a values. At this pH, the amine compounds demonstrated no significant wall interactions [19].

Azimilide and its degradates and metabolites contain amine groups. Since the pK_a of these amine groups is near nine, it was expected that these compounds could be separated at or near pH 9. Preliminary studies indicated that there was peak tailing when these compounds were separated at a pH far below 9 [20]. Thus, we started the separation of azimilide from its degradants and metabolites at pH 9 by both CZE and MECC. As Fig. 1 shows, the separation of azimilide, NE-11178 and F-1292 was achieved within 4 min by CZE using bare silica capillaries in borate buffer at pH 9 (Fig. 1). This separation was relatively straightforward because all of the analytes had different charge/mass ratios. Azimilide was almost neutral while both NE-11178 and F-1292 were negatively charged at this pH. Being near neutral, azimilide was eluted primarily by the electro-osmotic flow (EOF). Both NE-11178 and F-1292 migrated against the EOF, but were overpow-

ered by the EOF and thus were carried to the cathodic end. The overall result was that both NE-11178 and F-1292 moved more slowly than azimilide. Since F-1292 had the same charge as NE-11178 but a lower molecular mass, it had a higher charge/mass ratio and thus a higher electrophoretic mobility to counter the EOF. Therefore, F-1292 stayed in the capillary for longer and was detected last. Compound identities were confirmed by spiking individual compounds.

3.1.2. Separation of azimilide from F-410 and F-1054 by MECC

NE-10171, F-410 and F-1054 were also neutral at pH 9 and could not be separated from azimilide by CZE. However, F-410 and F-1054, along with the charged metabolites NE-11178 and F-1292, could be separated from azimilide by MECC within 6.5 min in a borate buffer at pH 9 with 50 mM SDS additive (Fig. 2). Surfactant SDS formed micelles, which served as a pseudo-stationary phase for the separation of these neutral compounds. In this case, both charge and hydrophobic interactions played a role in the separation process. First, the migration order of azimilide, NE-11178 and F-1292 was exactly reversed in this case, compared to the earlier case of
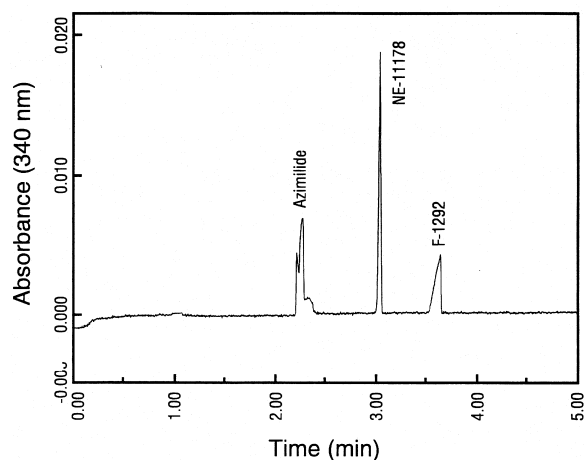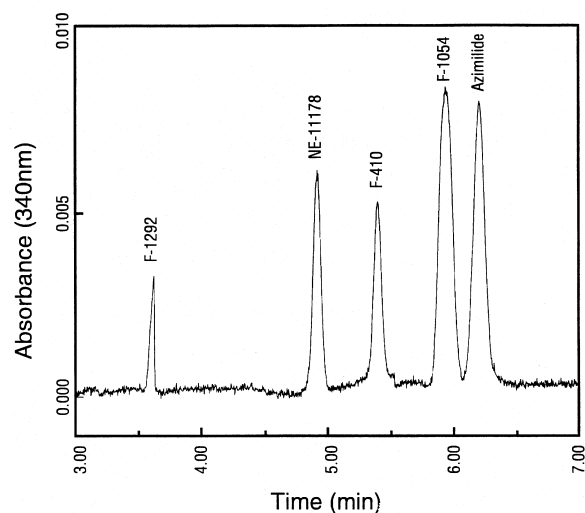

Fig. 1. Separation of azimilide, NE-11178 and F-1292 by CZE at pH 9.0. Experimental conditions: 50 cm separation length bare silica capillary (75 μm I.D. 363 μm O.D.), 20 mM sodium borate at pH 9, 30 kV.

Fig. 2. MECC separation of azimilide, F-1054, F-410, F-1292 and NE-11178. Experimental conditions: 50 cm separation length capillary (75 μm I.D., 363 μm O.D.), 50 mM SDS in 20 mM sodium borate buffer at pH 9.

CZE (Fig. 1). This change in migration order of F-1292, NE-11178 and azimilide resulted from charge interaction and hydrophobic interaction. In MECC, analytes partition between the hydrophobic interior of the micelles and the running buffer, and migration times represent the total time analytes spend in both the micelles and the buffer before they reach the detection window. Since SDS micelles are negatively charged and migrate against the EOF, analytes that interact more with the micelles would stay for a longer time in the column. Since azimilide had less negative charge than F-1292 and NE-11178, it had less charge repulsion to the micelles and tended to partition into the micelles easier. At the same time, the amount of organic moiety in structures and, thus, the hydrophobic interaction between micelles and analytes increased from F-1292 to NE-11178 to azimilide. Thus, both charge and hydrophobic interactions dictated the migration sequence.

Second, the migration order of three neutral analytes, F-410, F-1054 and azimilide, can be explained by hydrophobic interaction between the micelles and the analytes. Azimilide was the most hydrophobic species and, thus, had the strongest interaction with the micelles. F-1054, though smaller in size, had more interaction with the micelles than F-410, because it was totally neutral and exhibited a higher tendency to interact with the micelles.

Therefore, azimilide can be separated from all but one (NE-10171) related compound at pH 9 by MECC. The separation of azimilide from NE-10171 was not successful at this pH by either CZE or MECC.

3.2. Effects of MECC conditions on the separation of azimilide

3.2.1. Effect of pH
The effect of buffer pH on azimilide separation in MECC was significant. The migration times of azimilide, F-410 and F-1054 decreased first and then increased slightly between pH 8 and pH 10, with the minimum being around pH 9. Two factors were affected by the pH change. First, the ionization of the silanol groups on the surface of the capillary increased and resulted in shorter migration times at higher pH. Second, the charges on the analytes

became more negative and resulted in longer migration times. When the pH is below nine, silanol ionization was the dominant factor while the net charge on the analytes became the dominant factor at pH values above nine.

3.2.2. Effect of potential and buffer ionic strength
According to CE theory [3], a linear relationship exists between the inverse of the migration time $(1/t)$ and the applied voltage (V). This linear relationship was confirmed and the coefficients of determination (R^2) were 0.997 or better for the separations of azimilide, F-1054 and F-410.

In general, higher electrical potential should be selected whenever the other conditions, such as heat dissipation and the current, permit. Increasing the electrical potential has an additional advantage in the increased separation efficiency. Since Joule heating was adequately controlled through efficient heat dissipation by the instrument's thermostated temperature control system, the highest potential (30 kV) available on the instrument was used in most of our studies. However, we chose to limit the current below 50 µA for most of the separations, and never exceeded 100 µA of current for any of the separations.

Actually, it was found that moderate borate concentrations showed optimal separations. When the buffer concentration was low (< 5 mM), resolution between azimilide and F-1054 diminished. Increasing the buffer concentration increases separation efficiency, resolution and migration time. For azimilide separations at basic pH, a 20-mM borate buffer with 30 kV represented the best compromise between resolution and migration times for these analytes.

3.2.3. Effect of surfactants and modifiers
Two surfactants, SDS and Brij® 35, were tested for the separation of azimilide, NE-11178, F-1292, F-1054 and F-410 by MECC at pH 9. All five analytes were baseline separated at pH 9 when 50 mM of the negatively charged surfactant, SDS, was used (Fig. 2). The neutral surfactant, Brij® 35, could improve the resolution when used in combination with SDS (Fig. 3). However, these analytes were not resolved when Brij® 35 was used alone.
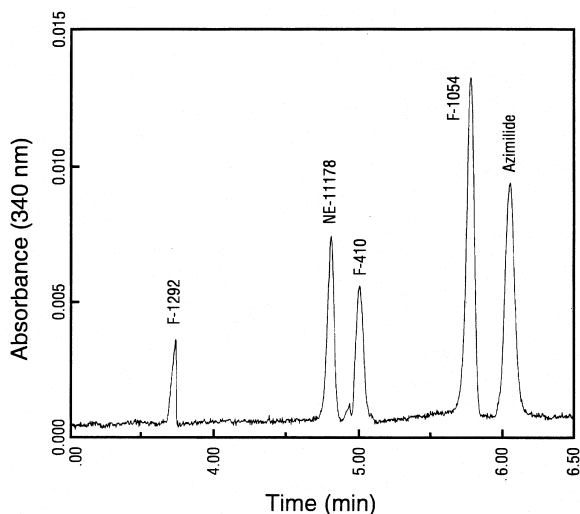
Fig. 3. Separation of azimilide, F-1054, F-410, F-1292 and NE-11178 by MECC at pH 9 with mixed surfactants. Experimental conditions: similar to those given in Fig. 2 except that 2.5 m*M* Brij® 35 was added in addition to the 50 m*M* SDS present in the buffer.

The migration times of azimilide, F-1054 and F-410 increased with SDS concentration. The resolution of neutral analytes, F-1054 and F-410 increased with SDS concentration as well. When SDS was below 25 m*M*, SDS concentration had a strong impact on the selectivity of the separation. At higher concentrations, the separation time, but not the resolution, increased with SDS concentration (data not shown).

The use of various organic molecules, such as methanol and acetonitrile, as modifiers in CE has been reported in the literature [21]. Adding methanol to the buffer resulted in an increase in viscosity and a significant decrease of EOF. Acetonitrile seemed more compatible with an aqueous buffer solution and had little effect on viscosity. Since our samples contained 10% acetonitrile, for the purpose of dissolving F-1054 and F-410, 10% acetonitrile was added to the separation buffer to mimic the sample matrix electrolyte. This addition of an organic component into the separation buffer also avoided disruption to the local environment of micelles. Hence, better peak shapes and more reproducible migration times were obtained (data not shown).

3.3. Separation of azimilide at acidic pH

3.3.1. CZE of azimilide at acidic pH

After the successful separation of azimilide, NE-11178, F-1292, F-1050 and F-410 at pH 9, attempts were made to separate these compounds at acidic pH. Similar to the CZE results at pH 9 (Fig. 1), azimilide, NE-11178 and F-1292 were easily separated at acidic pH (pH 4–6). The migration of these analytes followed the same order. However, all of the analytes had longer migration times because of reduced EOF (Fig. 4). As expected, the neutrals, F-1054 and F-410, were not separated by CZE.

3.3.2. MECC of azimilide at acidic pH

Separation of the neutral pair, F-410 and F-1054, by both CZE and MECC was not successful at acidic pH. No adequate resolution and acceptable migration time were obtained for this separation even after varying the concentrations as well as the types of surfactants. Different charged surfactants, from negatively charged SDS to neutral Brij® 35, to positively charged CTAC, and at different concentrations, from below to above the critical micellar concentration (CMC), did not result in adequate separations. Since
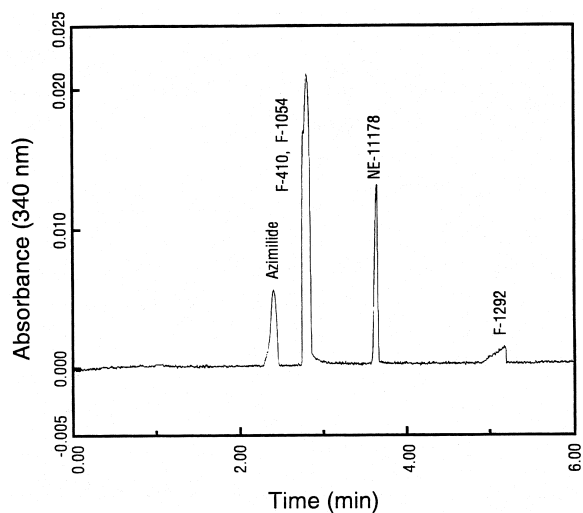

Fig. 4. Separation of azimilide, NE-11178, F-1054, F-410 and F-1292 by CZE in acetate buffer at pH 5. Experimental conditions: 50 cm separation length (75 μm I.D., 363 μm O.D.), 20 m*M* sodium acetate at pH 5 with 1 m*M* CTAC, 30 kV.

the separation of F-410 and F-1054 at low pH was not critical for our needs, because F-410 was a very minor component in real samples, no further exploration was attempted for this separation.

3.3.3. Separation of azimilide and NE-10171 at acidic pH

The separation of azimilide from its desmethyl metabolite, NE-10171, was of primary interest to the project. NE-10171 was the major metabolite present in most of the azimilide-related samples. Separation was very challenging because the structures of azimilide and NE-10171 are very similar, differing by only one methyl group. They have almost the same charge/mass ratio at pH 9. Therefore, NE-10171 tended to co-migrate with azimilide at pH 9, with or without the addition of surfactants, such as Brij® 35 and/or SDS.

Azimilide is a tertiary amine, while NE-10171 is a secondary amine. At pH 9, both of them carried the same charge and migrated almost identically. However, at lower pH values, charge densities increase differentially for these analytes and separation may be feasible. However, even at low pH, the separation of azimilide and NE-10171 proved to be problematic. At pH 5, both CZE and MECC with SDS and/or Brij® 35 were unsuccessful. Separation was finally achieved after CTAC was added, to modify the capillary surface and the separation buffer. Accordingly, under reversed polarity, azimilide was well separated from NE-10171 within 7 min (Fig. 5a). Changing the concentrations of CTAC from below (0.22 mM) to above (2 mM) the CMC did not affect the separation. However, higher CTAC concentrations shortened migration times because of higher reversed EOFs. In addition, adding 0.25% sodium chloride sharpened the peaks at all CTAC concentrations [because of the higher ionic strength, (Fig. 5b)].

The separation of azimilide and NE-10171 was also achieved on an amine-coated capillary (with or without CTAC). One advantage of using the coated capillary was the high reproducibility of migration times. Although the coated capillary alone gave baseline separation of these two compounds (data not shown), the addition of CTAC significantly enhanced the resolution and sharpened the peak shapes, resulting in better quantitation.
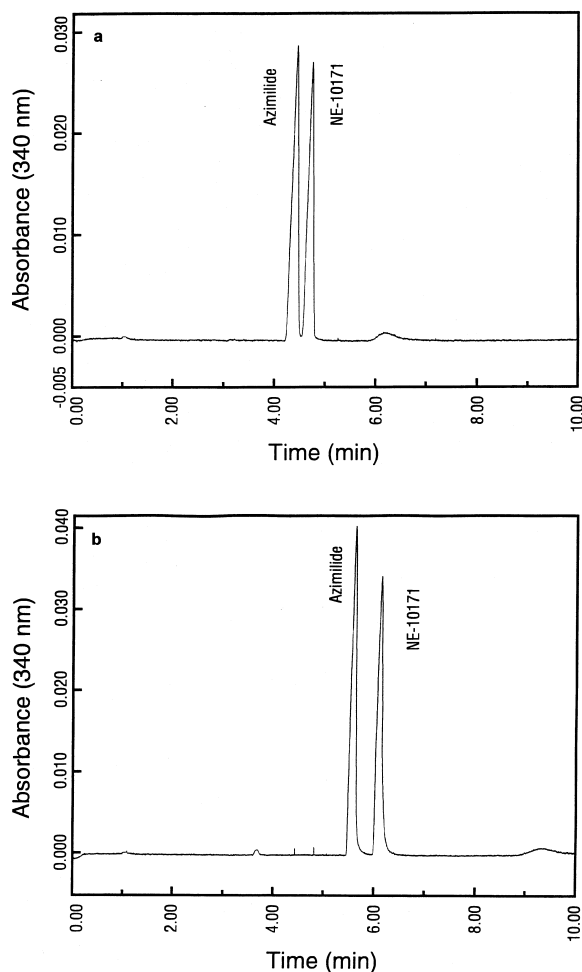

Fig. 5. Electropherograms for the separation of azimilide and NE-10171 by CE with CTAC additive at pH 5. Experimental conditions: the same as those given in Figure 4 and (a) without NaCl; (b) with NaCl added.

3.4. Quantitation and method validation

The goal of this work was to develop a robust method for the quantitation of azimilide and its metabolites and degradants. For comparison, the CE method was evaluated against the same acceptance criteria for validating bioanalytical HPLC methods. These acceptance criteria have been widely adopted in industry for evaluating specificity, sensitivity, linearity, precision, accuracy, recovery and ruggedness. The specificity of this method has been demonstrated by the aforementioned baseline separation of
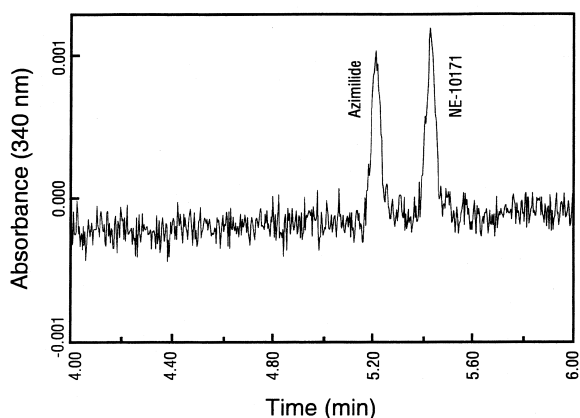
Fig. 6. Electropherogram showing the LOD (1 μg/ml) of azimilide and NE-10171 by CE with CTAC additive at pH 5.

the analytes. The sensitivity, linearity, accuracy and precision, reproducibility and recovery of this method were addressed using the separation of azimilide and NE-10171, as the example.

3.4.1. Sensitivity

The limit of detection (LOD, $S/N=3$) for azimilide was 0.5 μg/ml under optimized conditions at basic pH. Although this sensitivity was enough for some applications, such as stability studies, better sensitivity was desired for clinical studies. Efforts to improve the sensitivity of the CE method using an alternative detection method, such as laser-induced fluorescence (LIF), are currently underway.

The LOD of azimilide and NE-10171 at low pH

was about 1 μg/ml (Fig. 6). For method validation purposes, 10 μg/ml was selected as the lowest non-zero standard in the calibration curve for both azimilide and NE-10171.

3.4.2. Linearity

Linearity was evaluated based on the correlation between the responses (peak areas or heights) and the concentrations of a series of calibration standards. The linearity of the method was judged from both the regression coefficient of correlation and the back-calculated standard concentrations. The regression coefficient of determination (R^2) should be greater than 0.99. The percent relative error (RE%) between the nominal and the back-calculated concentrations of the standards should be within 15%, except the limit of quantitation (LOQ) standard, which should be less than 20%. Standards are rejected from the regression curve if they do not meet these criteria. The final calibration curve should contain standards covering no less than five different levels. At the same time, the pooled % relative error (pooled% RE) of all of the accepted standards should be less that 10%.

The coefficients of determination (R^2) covering a range of 10–1000 μg/ml were 0.9977 and 0.9938 for azimilide and NE-10171, respectively. The back-calculated concentrations of azimilide and NE-10171 standards, along with the pooled % relative errors of these two calibration curves are listed in Table 2. It can be seen that all of the standards met the aforementioned acceptance criteria.

Table 2
Back-calculated concentrations of the calibration standards

Standards	Nominal (μg/ml)	Azimilide		NE-10171	
		Found (μg/ml)	RE (%)	Found (μg/ml)	RE (%)
STD 1	1000	960	−4.0	960	−4.0
STD 2	900	931	3.5	933	3.6
STD 3	750	768	2.4	767	2.3
STD 4	500	499	−0.1	498	−0.3
STD 5	250	246	−1.5	246	−1.6
STD 6	100	100	−0.1	100	0.2
STD 7	50	49	−2.3	48.1	−3.8
STD 8	25	22.5	−10	23.2	−7.2
STD 9	10	9.05	−9.5	9.70	−3.0
		Pooled RE=5.4%		Pooled RE=3.8%	

Table 3
Concentrations of quality control samples

QC		Azimilide		NE-10171	
Samples	Nominal (μg/ml)	Found (μg/ml)	RC (%)	Found (μg/ml)	RC (%)
HQC	800	778	97.3	758	94.7
MQC	400	420	105	411	103
LQC	100	109	109	108	108

3.4.3. Accuracy and precision

The accuracy and precision of the method were determined by the calibration standards and, especially, by the QC samples. QC samples were prepared by independent weighing of azimilide and NE-10171. This ensures that the QC concentrations are independent of the standards. Normally, a relative error of 15 to 20% or less is acceptable for a bioanalytical method. Table 2 indicates that all of the standards had less than 10% RE to their nominal values. QC samples, six each at three different concentration levels across the calibration range, demonstrated that the errors were less than 9% (Table 3).

Table 4 summarizes the results of peak area integration for the standards and QC samples of both azimilide and NE-10171. Both the calibration standard and the QC samples demonstrated reasonable precision. Most of the integration data had a percent coefficient of variation (%C.V.) of less than 4%.

Table 5
Precision of the migration time, at pH 5, with 20 mM acetate buffer and 1 mM CTAC additive

Analyte	Azimilide	NE-10171
Mean	5.23	5.46
S.D.	0.15	0.18
C.V.(%)	2.8	3.2
n	36	36

Table 5 shows that the precision of the migration was within 4% for separations under acidic conditions. It can be concluded that the precision of CE methods can be very high, if the experimental conditions are controlled well.

3.4.4. Recovery

The recovery of the analytes in a method is related to the procedures involved. In general, the closer to 100%, the better. Table 3 shows recoveries of

Table 4
Precision of the integration data for azimilide and NE-10171

Samples		Azimilide		NE-10171	
ID	(μg/ml)	Peak area[a]	C.V.(%)	Peak area[a]	C.V.(%)
1	1000	5.52	3.3	6.03	3.5
2	900	5.36	2.2	5.86	2.0
3	750	4.42	0.9	4.82	0.8
4	500	2.88	0.9	3.14	0.8
5	250	1.43	1.4	1.55	1.4
6	100	0.60	2.1	0.64	2.6
7	50	0.31	2.2	0.31	2.5
8	25	0.15	7.6	0.16	1.9
9	10	0.08	12.0	0.07	9.8
HQC	800	4.48	2.6	4.77	2.3
MQC	400	2.43	1.8	2.59	2.4
LQC	100	0.65	3.7	0.69	2.5

[a] Note: Peak area is the average of three measurements.

selected QC samples. All recoveries were within 9% of their nominal values.

3.4.5. Reproducibility and reliability

In order to achieve high reproducibility and reliability, it is crucial to control accurately and precisely various experimental conditions, such as temperature, electronic potential, buffer pH, ionic strength, electrolyte type, organic solvents, properties of the analytes and the characteristics of the capillary wall. Although it is very hard, if not impossible, to strictly control all of these parameters at the same time, we can minimize the fluctuation of major parameters. In addition, we can perform some corrections to improve reproducibility. The most common correction is a migration time adjustment. For example, in impurity studies, it is necessary to measure the amount of sample in each peak. However, it is also necessary to adjust the migration time in order to make valid comparisons, because different analytes migrate through the detection window with different speeds. Hence, the residence time of each analyte in the detection window is inversely proportional to the velocity of the analyte. Earlier-migrating analytes spend less time, while later-migrating ones take more time to pass through the detection window. Assuming that two analytes have exactly the same absorbance characteristics and the same peak areas, the actual amount of the later-migrating analyte is lower than that of the earlier one. The further away these two analytes are, the bigger the difference is, and the more important the adjustment becomes.

Good linearity and reproducibility were obtained when migration time adjustment was applied to the separation of azimilide. For example, a coefficient of determination (R^2) of 0.9999 was obtained for azimilide at concentrations between 10 to 200 μg/ml at pH 9. Similarly, the coefficients of determination (R^2) for F-410 and F-1054 at concentrations between 0.5 to 10 μg/ml at pH 9 were 0.9986 and 0.9968, respectively.

Migration time adjustment is also important when an internal standard is used, because the internal standard migrates differently from the analytes. However, migration time correction becomes less important if an external calibration curve can be constructed, based on the same analytes. In that case,

analytes are quantitated against themselves in the calibration standards.

4. Conclusions

The above results have demonstrated the usefulness of CE as an alternative or complimentary technique for the development and validation of analytical methods in pharmaceutical research. Accuracy, precision and linearity of those CE methods were found acceptable based on the acceptance criteria for HPLC-based bioanalytical methods. However, sensitivity still remains an issue, and improvements will most likely require a better detection method. This work also shows that a wide range of conditions in CE can be adjusted in order to optimize separations.

5. Acknowledgment

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