High-Performance Capillary Electrophoretic Method for the Determination of Blasticidin S in Formulated Products

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An HPCE method with a UV–vis detector was used to determine blasticidin S content in commercial products. The results indicated that this method was capable of analyzing the blasticidin S content in formulated products with an instrument detection limit of 0.05 μg/mL, a method detection limit of 0.2 μg/mL, and RSD values from 0.61 to 8.11%. The HPCE method was promising in distinguishing between blasticidin S compounds and possible imitation antibiotic fungicides. The small quantity of injection volume and one-day analysis of seven samples indicated the HPCE method is a labor-saving and material-reducing technique. Thus, the present official bioassay method could be replaced by the HPCE method.

Keywords: HPCE; antibiotic fungicide; blasticidin S; detection limit; sensitivity; selectivity

INTRODUCTION

The content of blasticidin S in formulations is traditionally determined by a bioassay which cannot distinguish the actual antibiotic, blasticidin S, from substitute, false products, because the method is based on the growth inhibition of specific bacteria; this inhibition may be caused by many inorganic or organic bactericides. Furthermore, the procedures for bioassay are complex and time-consuming. Thus, a fast, efficient method should be developed for routine analyses.

Capillary electrophoresis is a powerful separation method with great potential for high-resolution separation and purification of biological substances. The method often exceeds 100 000 plates/m (Sepaniak and Cole, 1987). It has been used to analyze a wide range of samples including polypeptides (Cohen and Karger, 1987), proteins (Bruin et al., 1989), nucleosides (Cohen et al., 1987), other amino acid compounds, and vitamin metabolites (Swaile et al., 1988). There are several mechanisms for HPCE. For example, the separation principle of zone electrophoresis is based on electrophoretic mobility and charge. For micellar electrokinetic chromatography, the separation is based on hydrophobicity and complexation. For the isoelectric focusing mode, separation is based on pI value. For gel electrophoresis, separation is based on size and charge (Karger et al., 1989). Among these methods capillary zone electrophoresis (CZE) has been demonstrated as an extremely efficient technique for the separation of charged biological compounds (Jorgenson and Lukacs, 1983).

Blasticidin S consists of a pyrimidine nucleoside, β-amino acid cytosinine, and a blastidic acid group (Figure 1; Prabhakaran et al., 1988). Therefore, capillary zone electrophoresis was used for blasticidin S separation in which charged solutes were differentially transported under the influence of an applied electrical
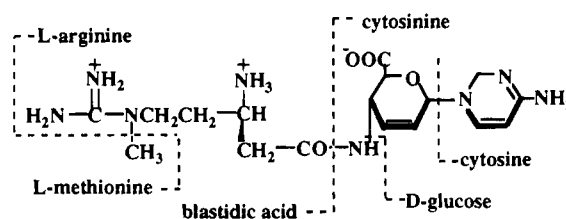

Figure 1. Structure of blasticidin S showing the composition of the cytosinine and blastidic acid groups.

field. We describe here the development of an HPCE method for the determination of blasticidin S in commercial formulated products purchased from markets.

MATERIALS AND METHODS

Solvents and Chemicals. Blasticidin S monohydrochloride salt (MW = 458.9), purchased from Shinung Operation Co., was used as standard. The purity was 96.0% based on HPCE analysis (area percentage). Commercial formulated samples were purchased from markets during the years of 1991 and 1994. Samples A–C were 2% soluble solutions (SL) containing 2% blasticidin salt or 1% blasticidin S. Sample D was 54% wettable powder (WP) containing 50% fthalide and 4% blasticidin salt or 2% blasticidin S. Sample E was 2.4% emulsifiable concentrate (EC) containing 2.4% blasticidin salt or 1.2% blasticidin S. Samples F and G were 21.4% EC containing 20% isoprothiolane and 1.4% blasticidin salt or 0.7% blasticidin S. Solvents and chemicals were of analytical grade.

Capillary Electrophoresis and Analytical Column. High-performance capillary electrophoresis was performed using a Biofocus 3000 automated capillary electrophoretic apparatus. A Biofocus cartridge capillary column coated with hydrophilic polymer (24 cm × 25 μm) for low-viscosity buffer was selected to reduce sample adsorption to the wall and to control electro-osmotic flow (EOF). This capillary column was operated at recommended acidic condition (below pH 3) for best separation. The column used in these studies was placed in circulating water jackets for ambient temperature control.

A regulated dc power supply delivering 8.0 kV was used to provide high voltage between the ends of the column filled with pH 2.4 buffer solution. The elution of a solute was monitored by an on-column UV–vis detector (265 nm) at the negative pole (Figure 2).

The sample was introduced into the capillary vessel using pressure injection mode at 20 psi s, and the volume of sample introduced into the capillary vessel during pressure injection

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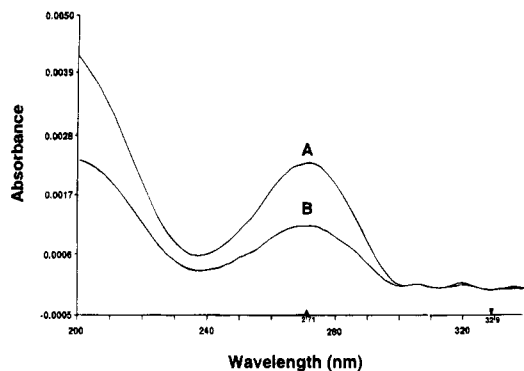
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Figure 2. UV–vis spectra of blasticidin S (curve A) and 2% soluble solution (curve B). Maximum absorption occurred at 271 nm.

was calculated from the expression (Bio-Rad Bulletin 1818, 1993)

$$V = \pi p t r^4 / 8L\eta$$

where V is the volume injected in nanoliter, t is the time in seconds, p is the pressure of the buffer in dyn//cm^2 (1 psi is the 68947.6 dyn/cm^2), L is the capillary length in centimeters, and η is the viscosity in poise or dynes s/cm^2.

Thus, for an injection constant of 20 psi s, the injection volume was calculated as 3.7 nL:

$$V = \frac{(3.1416)(20 \text{ psi s})(68947.6 \text{ dyn/cm}^2/\text{psi})(12.5 \times 10^{-4} \text{ cm})^4}{(8)(24 \text{ cm})(0.015 \text{ dyn s/cm}^2)}$$

Column efficiency is expressed in terms of theoretical plates. The number of theoretical plates N is defined as

$$N = \mu V / 2D$$

where D is the solute's diffusion coefficient, μ is the solute's electrophoretic mobility, and V is the applied voltage (Jorgenson and Lukacs, 1981). Thus, N is directly proportional to the applied voltage and to the ratio of the mobility to the diffusion coefficient and is independent of tube length and analysis time. D and μ were not easily determined.

The theoretical plates (N) were therefore determined by the standard molecular diffusion term in chromatography (Jorgenson and Lukacs, 1981), and the column efficiency was measured by the equation

$$N = 16(t_r/W)^2 = 5.5(t_r/W_{1/2})^2$$

where t_r is the retention time of the peak, W is the peak width at a given peak height (the tangents to the side of the peaks are extrapolated to the base line for W), and $W_{1/2}$ is the width at half peak height. Since the peak was sharp for HPCE, a peak height method (W) was used for theoretical plate calculation.

The reproducibility of retention time (t_r), peak area, linearity, and detection limit was used to evaluate the selectivity, sensitivity, and reliability of the HPCE method.

HPCE Calibration Curve. Blasticidin S monohydrochloride standard (0.0028 g, purity = 96.0%) was weighed into a 25 mL volumetric flask, diluted first with pH 6.0 phosphate buffer to a final concentration of 107.5 μg/mL (stock standard soution), because blasticidin S is stable in acid condition, and then diluted to the proper concentration with pH 7.0 phosphate buffer (working standard solution). Since a preliminary test indicated a break in the slope of the standard curve occurred when the concentration was below 2.3 μg/mL, the final concentrations of 2.3, 4.7, 9.4, 18.7, 28.1, 37.4, and 46.7 μg/mL were used to evaluate repeatability of the HPCE method. Three replications were conducted, and a linear regression was used to determine the suitability of the range selected.

Limit of Detection. The IUPAC limit of detection kS_B/m (Long and Winefordner, 1983) was not applied to determine

the instrument detection limit (IDL), because a previous test indicated that the value obtained by using this formula would be a negative value. Hence, the IDL was determined by injecting a working standard solution to produce a signal that was about 3 times the signal-to-noise ratio (U.S. EPA, 1984).

The concentration of working standard solution that corresponds to 2.5 times the IDL is used to determine the method detection limit (MDL). Repeated HPCE analyses (seven) produced data for the standard deviation (SD); 3 times the SD was used as the MDL. Precision expressed by relative standard deviation (RSD) was used in judging the acceptability of the method.

Matrix Effects and Standard Addition Method. Because the accurate composition of different commercial formulations and their effects on the HPCE method were unknown, the effect of formulations on this approach was analyzed by the standard addition technique. Five equal aliquots of the commercial formulated samples were prepared with standard working solution to final concentrations of blasticidin S of 9.0, 17.8, 26.8, 35.8, and 44.7 μg/mL. These solutions were then analyzed by the HPCE method, and a plot of response vs concentration (blank included) indicated the original concentration of sample. These extrapolated values were used to compare the matrix effects. No interference was noted if (1) the extrapolated concentration was experimentally equivalent to the concentration calculated from the standard calibration curve and (2) the slope of the addition curve was parallel to the slope of the standard calibration curve (Figure 4).

Bioassay. The official bioassay method using *Bacillus cereus* in nutrient agar medium (Difco) was used to determine the potency of sample C. *B. cereus* was obtained from the Food Industry Research and Development Institute (FIRDI 10603), Taiwan. Stock solution of blasticidin S hydrochloride was prepared by weighing 0.0228 g of standard into a 25 mL volumetric flask and diluting with sterile 0.067 M phosphate buffer (pH 7.0) to 200 and 50 μg/mL (standard solution). An aliquot of sample C was accurately weighed and diluted with the same sterile 0.067 M phosphate buffer to the final volume to prepare a sample solution at estimated concentrations of 200 and 50 μg/mL (sample solution), respectively.

A portion of 8 mL of melted agar was added into each sterile petri dish, spread evenly, and allowed to harden. The other melted agar was cooled to 60–62 °C, inoculated with the bacteria, and agitated gently. A portion of 5 mL of the inoculated medium was immediately poured onto each of the above plates, spread evenly, and allowed to harden. Sterile stainless steel wells (10 mm in length, 6 mm i.d., 8 mm o.d., and 4 per plate) were placed evenly on the agar plate prepared as above, and 0.28 mL of the solution to be assayed was placed in each well and incubated at 30 ± 1 °C for 18 h. The diameter of the inhibition zone was measured and used to calculate the antibiotic equivalent from the formulation. S_H and S_L were diameters of the inhibitory zones in millimeters caused by standard solutions at 200 and 50 μg/mL, respectively. U_H and U_L were similarly higher and lower estimated sample concentrations described above. The assay was done in six replications. The ratio (θ) of the potency (P_u) of the test sample to that of the standard (P_s) was calculated as follows

$$\theta = \frac{(\sum U_H + \sum U_L) - (\sum S_H + \sum S_L)}{(\sum U_H + \sum S_H) - (\sum U_L + \sum S_L)} \times \log 4$$

$$\theta = P_u/P_s$$

The official tolerance of the potency is from 85 to 130%; thus, for a formulation with 1% active ingredient, the accuracy should be in the range of 0.85–1.30%.

RESULTS AND DISCUSSION

Chromatogram of Blasticidin S Standard. A typical electropherogram of the blasticidin S standard is shown in Figure 3. The retention times were very
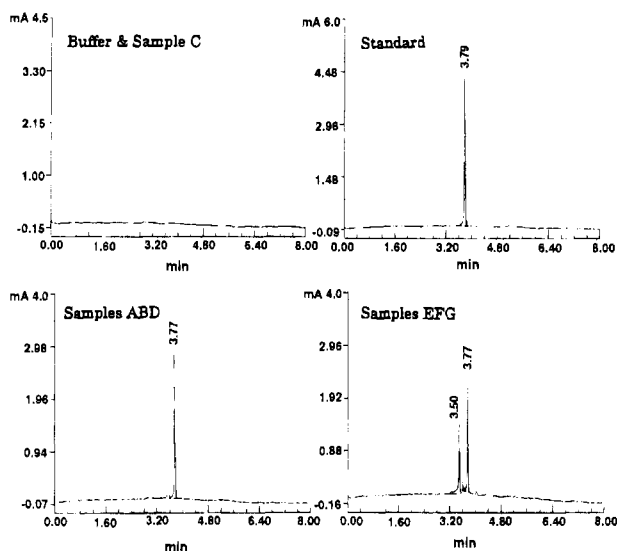
Figure 3. Typical electropherograms of phosphate buffer, blasticidin S standard, and commercial products. All commercial samples showed a blasticidin S peak except sample C.

Table 1. Precision of Retention Time during HPCE Analysis of Blasticidin S Standard[a]

concn (μg/mL)	retention time (min)	precision (% RSD)
2.3	3.69	0.31
4.7	3.68	0.16
9.4	3.71	0.41
18.7	3.68	0.41
28.1	3.64	0.27
37.4	3.62	0
46.7	3.60	0.28

[a] Mean of three measurements.

consistent, ranging from 3.60 to 3.71 min with an RSD from 0 to 0.41%. The retention time was not influenced by the concentration selected (Table 1).

The analysis of the standards from 2.3 to 46.7 μg/mL showed a good correlation between the concentration (X) and peak area (Y), and the coefficient of determination (r^2) averaged 0.9994.

The calculated number of theoretical plates [$N = 16(t_r/W)^2$)] of the column was 8078. The retention time of blasticidin S was 3.82 min (t_r), and the peak width was 0.17 min (W).

Selectivity of HPCE on Blasticidin S Products. The new HPCE method with a UV–vis detector can distinguish actual products from imitation false products. For blasticidin S commercial products of soluble solution (samples A and B) and wettable powder (sample D), only one peak (blasticidin S peak, t_r = 3.8 min) was found (Figure 3). The same electropherograms were observed for blasticidin S commercial products of emulsifiable concentrates (samples E–G), except with several additional peaks compared to samples A, B, and D. These additional peaks were possibly caused by the composition in EC formulation, such as emulsifier, and there was no influence on the analysis of active ingredient.

No blasticidin S peak was observed in sample C (Figure 3). The previous bioassay test indicated that sample C was active and the calculated potency was 1.71% (official tolerance for the bioassay was from 0.85 to 1.35%) (Table 2). This result indicated that sample C was a substitute false product.

Sensitivity of HPCE Method. The instrument limit of detection (IDL), defined as 3 times the baseline noise, was estimated at 0.05 μg/mL, or 0.19 pg/injection

Table 2. HPCE Determination of Blasticidin S in Formulated Products

formulation (% ai claimed)	HPCE (%, RSD)	tolerance (%)
A,[a] 2% SL (1% blasticidin S)	1.11, 1.85	0.80–1.20
B, 2% SL (1% blasticidin S)	1.13, 8.11	0.80–1.20
C, 2% SL (1% blasticidin S)	nd[b]	0.80–1.20
D, 54% WP[c] (2% blasticidin S)	1.60, 5.65	1.60–2.40
E, 2.4% EC (1.2% blasticidin S)	0.97, 0.61	0.96–1.44
F, 21.4% EC[d] (0.7% blasticidin S)	0.59, 4.38	0.42–0.84
G, 21.4% EC[d] (0.7% blasticidin S)	0.67, 3.11	0.42–0.84

[a] Different samples arranged in alphabetical order. [b] Not detected at a concentration of 0.2 μg/mL. [c] Mixed fungicides (Fthalide 50% + 4% blasticidin salt). [d] Mixed fungicides (isoprothiolane 20% + 1.4% blasticidin salt).

for 3.7 nL of injection volume. The method detection limit (MDL) was about 0.2 μg/mL.

Determination of Blasticidin S Content in Commercial Formulated Products. The official tolerance in commercial formulation ranged from +20 to −20% for active ingredient lower than 10% and from +20 to −8% for active ingredient content higher than 10%. The HPCE analysis of blasticidin S showed that the active ingredient contents for samples A, B, and D–G were all within the official tolerance range.

The precision of the HPCE method as measured by RSD values in determination of blasticidin S in commercial formulated products ranged from 0.61 to 8.11%. The RSD values were less than 10%, indicating that the precision of the method was excellent (McFarren et al., 1970). The method therefore can be used for blasticidin S analysis.

The speed of the HPCE method for determining blasticidin S in commercial formulated samples was examined. It took about 1 day to analyze all seven samples plus the standard calibration curve by the HPCE method, compared to the 7 days needed to analyze the same samples by the bioassay method. Furthermore, the solvent waste was significantly reduced by the HPCE method.

Influence of Formulations on HPCE Performance. The analysis of blasticidin S in commercial formulation products was validated by the standard addition method. Commercial samples were fortified with blasticidin S standard, and the total concentrations of blasticidin S were analyzed. A plot of response vs concentration extrapolated back gave the original concentration in the sample solution. A typical standard curve is shown in Figure 4. The active ingredient content of blasticidin S in the formulation (ai%) can be obtained by using the equation

$$\text{ai\%} = \frac{\text{extrapolated concn} \times 25 \text{ mL} \times \text{dilution factor[10]}}{\text{sample wt (g)}} \times 100\%$$

The calculated concentrations of blasticidin S in samples are shown in Table 3. There were only small differences, ranging from 2.4 to −4.3%, between the extrapolated and unspiked concentrations (Table 3). For sample G, no difference existed (Table 3).
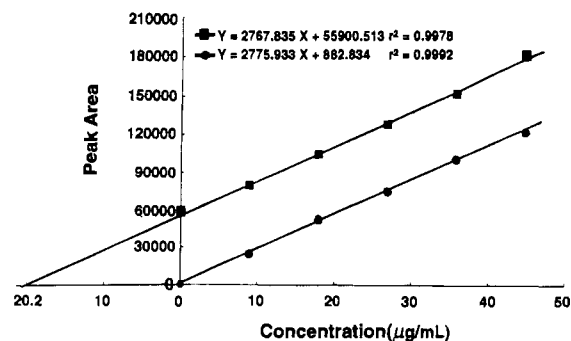
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Figure 4. Comparison of standard curve (●) and the standard addition method (■) for the blasticidin S product of D (54% WP).

Table 3. Effect of Commercial Formulation on the Analysis of Blasticidin S by the HPCE Method Evaluated by Standard Addition

formulation (% claimed)[a]	extrapolated concn (%)	unspiked concn (%)	diff[b] (%)
A, 2% SL (1% blasticidin S)	1.01	1.02	−1.0
B, 2% SL (1% blasticidin S)	1.11	1.16	−4.3
D, 54% WP[c] (2% blasticidin S)	1.70	1.66	2.4
E, 2.4% EC (1.2% blasticidin S)	0.99	0.97	−1.0
F, 21.4% EC[d] (0.7% blasticidin S)	0.71	0.72	−1.4
G, 21.4% EC[d] (0.7% blasticidin S)	0.70	0.70	0

[a] Blasticidin S concentration in commercial formulation claimed by manufacturer. [b] Difference between extrapolated concentration and unspiked concentration. [c] Mixed fungicides (Fthalide 50% + 4% blasticidin salt). [d] Mixed fungicides (isoprothiolane 20% + 1.4% blasticidin salt).

Table 4. Effect of Formulation on the HPCE Analysis of Blasticidin S Evaluated by Linear Regression

formulation	slope	intercept	r^2
standard solutions	2775.933	882.834	0.9992
A, 2% SL	3094.059	75713.943	0.9984
B, 2% SL	2974.989	83659.738	0.9899
D, 54% WP[a]	2767.835	55900.513	0.9978
E, 2.4% EC	2818.967	66418.269	0.9995
F, 21.4% EC[b]	2873.928	66411.784	0.9914
G, 21.4% EC[b]	2992.174	68414.507	0.9894

[a] Mixed fungicides (Fthalide 50% + 4% blasticidin salt). [b] Mixed fungicides (isoprothiolane 20% + 1.4% blasticidin salt).

The slopes of standard addition curves were also about the same value as the slope of the standard calibration curve (Table 4).

The similarity of the calculated ai% of blasticidin S with the unspiked commercial products (Table 3) and the similarity of slopes between the normal standard curve and the standard addition curves indicated that the adjuvants in the commercial formulation would not interfere with the HPCE method in the concentration range tested. Thus, a simple recovery of fortified sample method instead of standard addition method could be used for the quantitation of blasticidin S in commercial formulation products.

In summary, this study has illustrated important advantages for using the HPCE method for determining blasticidin S. The HPCE method can distinguish actual from commercial false products. The retention times of blasticidin S during HPCE were consistent. The instrument detection limit was 0.05 μg/mL, and the method detection limit was 0.2 μg/mL. The RSD values of the HPCE method in determining blasticidin S content in the commercial products were less than 10% and ranged from 0.61 to 8.11%. The 3.7 nL injection volume and the capability of 1-day analysis of seven samples clearly demonstrated HPCE efficiency in saving labor and materials. Therefore, the present official bioassay method could be replaced by the new HPCE method.

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