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Development and validation of a capillary electrophoresis method for the characterization of protegrin IB-367

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Abstract

A capillary electrophoresis (CE) method was developed to characterize protegrin IB-367, an antimicrobial peptide being developed for the treatment of oral mucositis and for other topical applications. The electrophoretic purity and levels of potential impurities/degradation products of IB-367 drug substance are determined by CE using area normalization. Electrophoresis parameters were optimized to allow optimal resolution, reproducibility and minimal analysis time. The separation and resolution between this polycationic peptide and truncated analogs determined by the CE method was much greater than those by the HPLC methods. In addition, the CE methods separates the potential impurities/degradation products from each other while the HPLC methods failed to resolve them. The CE method was validated in the aspects of accuracy, precision, linearity, range, limit of detection, limit of quantitation, specificity, system suitability and robustness. An internal standard was used for the quantitation purpose. The selection criteria of the internal standard as well as the method validation results are presented. The truncated peptide analogs were used to demonstrate the specificity of the method. These analogs were also used to evaluate the limit of quantitation of potential impurities. The relative response factors of these analogs were assessed to determine area normalization feasibility. System suitability tests were established.

Keywords: Validation; Peptides; Protegrins

1. Introduction

Protegin IB-367 is a polycationic peptide displayed a wide range of antimicrobial activities [1,2]. It is currently being developed to treat oral mucositis, a painful oral complication associated with aggressive cancer chemotherapy such as used in bone marrow transplantation [3]. Protegrin IB-367 is a 17-amino-acid peptide rich in arginine and cystine residues (sequence shown in Table 1). It has been chemically synthesized by conventional solid-phase and solution phase peptide methods and purified by high-performance liquid chromatography (HPLC). Reversed-phase HPLC has also been used to characterize IB-367 to assess its purity and potential impurities and degradation products [4]. It is preferred by the regulatory agency to characterize a new chemical entity using two different separation techniques [5], a second method thus needs to be developed to analyze this drug substance. Since capillary zone electrophoresis was known to separate peptides based on charge-size difference [6–13], it was therefore developed to characterize protegrin IB-367 because the peptide is fairly basic and has great intrinsic electrophoretic mobility due to multiple arginine residues. However, capillary electrophoresis has only limited application for quantitative pharmaceutical analysis to date [14–16], as opposed

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Table 1
Peptide sequences and charge/size parameters[a]

Peptide	Sequence	*M*	*Z*	$ZM^{(-2/3)}$	Estimated RMT
IB-300	LCYCRGRFCVCVGR (C2–C11, C4–C9)	1629	4	0.02889	
IB-468	GGLCYCRGRFCVCVGR (C4–C13, C6–C11)	1743	4	0.02762	
IB-469	GLCYCRGRFCVCVGR (C3–C12, C5–C10)	1686	4	0.02824	
IB-367	RGGLCYCRGRFCVCVGR (C5–C14, C7–C12)	1900	5	0.03259	1
Benzylamine		119	1	0.04133	0.85
Gly–Phe	GF	221	1	0.02736	1.10
Gly–Tyr	GY	237	1	0.02611	1.12
Lys–Tyr	KY	309	2	0.04376	0.81

[a] RMT = Relative migration time; *M* = molecular mass; *Z* = valence charge.

to the traditional HPLC methods in biotechnology industry. This paper will demonstrate the quantitative nature of the capillary electrophoresis (CE) method and show validation results in the aspect of accuracy, precision, linearity of response, range, limit of detection, limit of quantitation and robustness. Protegrin IB-367 analogs (truncated peptides) were used to evaluate the resolution of the method. Table 1 lists the peptide sequence of IB-367 and its analogs.

2. Experimental

2.1. Capillary electrophoresis

Sodium monobasic monohydrate phosphate (USP grade) was obtained from EM, CAS 10049-21-5. Phosphate acid in 85% syrup was from EM, hydrochloric acid was from Aldrich. Dipeptide α-amino glycylphenanaline amide (G-2185) was purchased from Bachem (CA, USA). Capillary regenerator solution A (0.1 *M* NaOH) and fused-silica capillaries were obtained from Beckman Instruments (Palo Alto, CA, USA). CE was performed on a P/ACE MDQ CE instrument (Beckman Coulter, Palo Alto, CA, USA). The electrophoresis conditions are: capillary temperature: 25°C; detection: UV detection at 200 nm; run buffer: 100 m*M* phosphate, pH 2.6; injection: 0.5 p.s.i./5 s (1 p.s.i.=6894.76 Pa); capillary: bare silica, 45 cm effective length×50 μm I.D.; separation condition: 20 kV in 15 min; separation timetable:

No.	Time (min)	Event	Value	Duration
1		Rinse-pressure (running buffer)	20.0 p.s.i.	4.00 min
2		Inject-pressure (sample)	0.5 p.s.i.	5.0 s
3	0.00	Separate-voltage	20.0 kV	15.0 min
4	1.00	Autozero		
5	15.00	Stop data		
6		Rinse-pressure (0.1 *M* NaOH)	20.0 p.s.i.	1.00 min
7		Rinse-pressure (water)	20.0 p.s.i.	2.00 min
8		Rinse-pressure (0.1 *M* HCl)	20.0 p.s.i.	1.00 min
9		Rinse-pressure (water)	20.0 p.s.i.	2.00 min
10		End.		

2.2. Peptide synthesis

The syntheses of Protegrin IB-367 and its analogs were accomplished following general solid-phase methodology. All the chemicals and reagents for peptide synthesis was purchased from Bachem. The α-amino group for each amino acid used in the synthesis was protected with the *tert*.-butyloxycarbonyl (t-Boc) group. The sidechain functional groups of these amino acids were protected as follows: a tosyl group for arginine; a 2-bromobenzyloxycarbonyl group for tyrosine; and a 4-methoxybenzyl group for cysteine. The peptide chain was assembled by coupling the first C-terminal amino acid to 4-

methylbenzhydrylamine resin, then removing the Boc group using trifluoroacetic acid (TFA) and coupling the next protected amino acid derivative using dicyclohexylcarbodiimide (DCCI). Repetition of the cycle with the protected amino acids in the desired sequence results in the fully-protected peptide-chain. Couplings were monitored by ninhydrin test. The crude peptide was obtained by treatment of the peptide-resin using liquid hydrogen fluoride with ethyl methyl sulfide and anisole as scavengers. The crude product was extracted by aqueous acetic acid and cyclized by air oxidation in ammonium acetate buffer at pH 7. Purification was accomplished by preparative, reversed-phase HPLC techniques on a Vydac C_{18} silica column using gradient elution (22–42% mobile phase B in 70 min) with 0.1% TFA as mobile phase A and 0.08% aqueous TFA–acetonitrile (30:70) as mobile phase B. The resulting purification fractions with purity greater than 95% monitored by analytical HPLC were then pooled and lyophilized to yield the TFA salt of protegrins.

3. Results and discussion

3.1. Method development

The criteria for an acceptable CE method for pharmaceutical analysis of drug compounds should include linearity, specificity, reproducibility, sensitivity and reasonable run time. The method development for the analysis of IB-367 is described below. Specificity of the CE method was measured by the resolution between IB-367 and its truncated analogs.

3.1.1. Selection of capillary type

To minimize electrostatic interactions, polycationic peptides can be analyzed by coated capillary or fused-silica capillary at low pH values [10]. Protegrin IB-367 is polycationic and has better stability at acidic pH [3], a fused-silica capillary was therefore selected for the method development.

3.1.2. Selection of buffer, separation voltage and capillary I.D.

Initially, a IB-367 solution was spiked with IB-300 and IB-469. The electrophoregrams of the mixture generated on a 75 μm I.D. capillary at different pH values are shown in Fig. 1. The 100 m*M* phosphate buffer (pH 2.6) gave the best separation among the three buffers tested. At pH 3.6, no apparent separation was achieved between IB-300 and IB-469. at pH 4.6, the two analogs were detected as two negative peaks. The separation of three

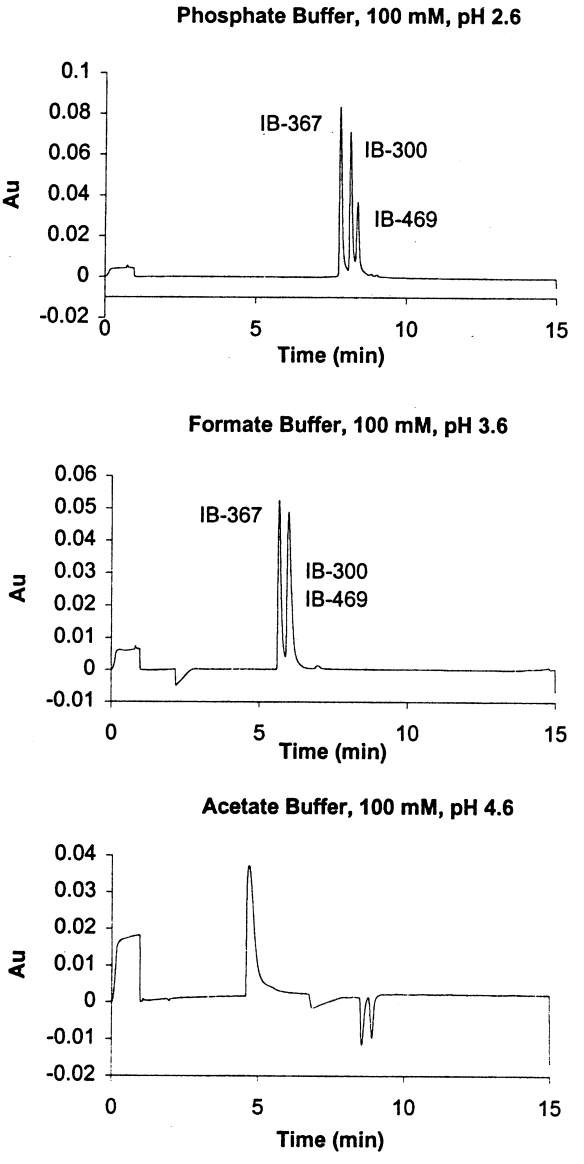

Fig. 1. Separation at different pH values. [IB-367]=0.5 mg/ml, [IB-300]=0.4 mg/ml, [IB-469]=0.2 mg/ml, 25 kV, 50 cm effective length×75 μm I.D. fused-silica, 0.5 p.s.i./5 s injection.

peptides at different voltages using different internal diameter capillaries are compared in Fig. 2. The 20 kV separation voltage on a 50 μm I.D. capillary allowed the greatest resolution among three peptides and it gave reasonable migration time. The current generated by different separation voltages using 50 μm I.D. capillary were plotted against the separation voltages in Fig. 3. It showed that the current started to deviate from the Ohm's law when the separation voltage was greater than 25 kV. The electrophoresis parameters were then determined to be 20 kV separation voltage, 50 μm I.D. fused capillary and 100 m*M* phosphate buffer, pH 2.6 based on good resolution and reasonable analysis time.

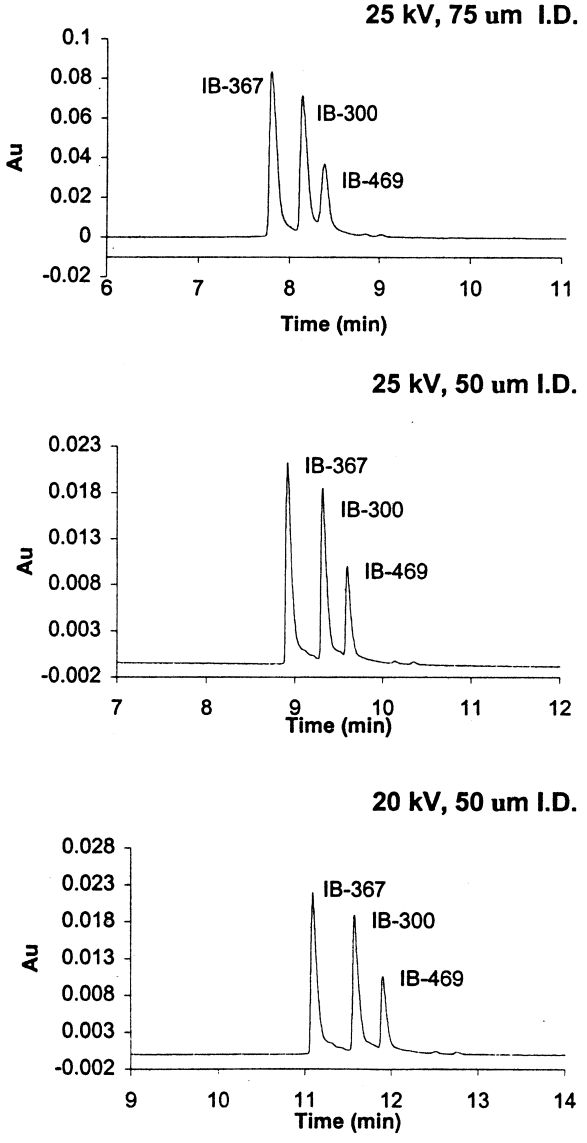

Fig. 2. Separation at different voltage and capillary I.D. Electrophoresis buffer: 100 m*M* sodium phosphate, pH 2.6, fused-silica capillary 50 cm effective length, 0.5 p.s.i./5 s injection. Same samples as in Fig. 1.

3.1.3. Reproducibility

Adequate reproducibility of the CE method was achieved when an internal standard was used to compensate for the injection variability. In order to select an internal standard that migrates close to IB-367, relative migration time was estimated by the Offord equation [7]. Fig. 4 showed a plot of mobility (migration time) of IB-367 and its three analogs versus calculated $ZM^{-2/3}$. The good fit of the data demonstrated in Fig. 4 was used to predict the relative migration time of potential internal standard, as listed in Table 1. The dipeptide α-amino glycylphenylanaline amide was selected as the internal standard for its commercial availability, high purity and its estimated migration time close to that of IB-367. Fig. 5 shows a typical electrophoregram of IB-367 in the presence of the dipeptide internal standard.

The capillary was treated with 0.1 *M* sodium hydroxide, water, 0.1 *M* hydrochloric acid and running buffer consecutively between each run. With the introduction of the internal standard and a consistent capillary treatment procedure, the method achieved reproducibility in relative migration time (RSD=0.13%), relative peak area (RSD=2.75%)

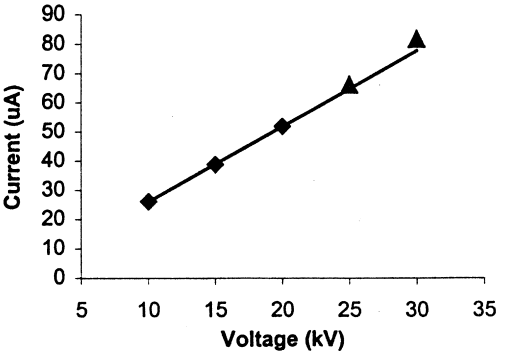

Fig. 3. Ohms law plot. CE buffer: 100 m*M* sodium phosphate, pH 2.6, 50 μm I.D. fused-silica capillary.

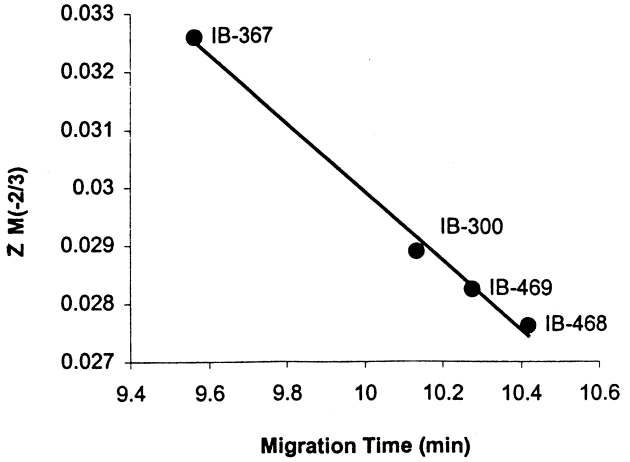

Fig. 4. Migration time vs. theoretical electrophoretic mobility. Separation with 100 m*M* sodium phosphate, pH 2.6, 20 kV, 45 cm effective length×50 μm capillary, detection at UV 200 nm, 0.5 p.s.i./5 s injection.

and relative peak height (RSD=1.90%). Table 2 summarizes the results.

3.1.4. System suitability

System suitability tests serve to define the level of electrophoretic performance necessary to ensure valid CE assay results. System suitability of the method was evaluated by analyzing the symmetry of the IB-367 peak, theoretical plates of the capillary and the resolution between the IB-367 peak and the IB-300 peak. IB-300 was selected to evaluate the resolution criteria since it was the closest peak to IB-367. The concentration of the method was selected at about 0.5 mg/ml to assure symmetry below 3.5 and to assume sufficient sensitivity for detecting low concentration impurities. At 0.5 mg/ml, the limit of

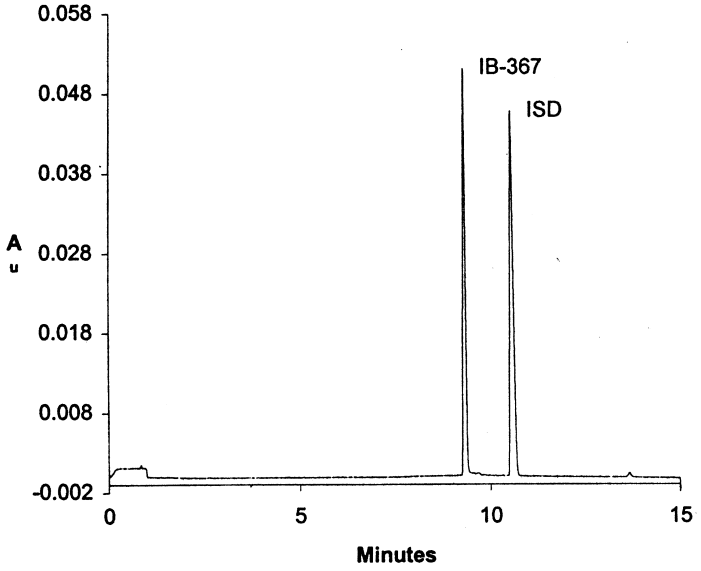

Fig. 5. Electropherogram of IB-367 with the internal standard. [IB-367]=0.5 mg/ml, [I.S.]=0.55 mg/ml, 20 kV, 45 cm effective length×50 μm I.D., 0.5 p.s.i./5 s injection.

Table 2
Reproducibility of relative migration time and relative peak area/height

Day		RMT	Relative peak area	Relative peak height
1		1.1327	0.7913	0.7086
		1.1305	0.8674	0.7396
		1.1306	0.8093	0.7258
		1.1311	0.7793	0.6884
		1.1283	0.8322	0.7234
	RSD (%)	0.14	4.29	2.72
2		1.1340	0.8104	0.7072
		1.1341	0.7998	0.7022
		1.1325	0.7710	0.6978
		1.1313	0.8158	0.7165
		1.1310	0.7939	0.7117
		1.1303	0.8187	0.7223
	RSD (%)	0.14	2.21	1.28
3		1.1335	0.8163	0.7022
		1.1317	0.8017	0.6954
		1.1313	0.8001	0.6975
		1.1308	0.8125	0.7059
		1.1314	0.7947	0.7003
	RSD (%)	0.09	1.12	0.58
	Overall RSD (%)	0.13	2.75	1.90

detection for impurities was 0.1%. (see Section 3.2) The following parameters were summarized from runs with different lots of capillary on a 0.5 mg/ml IB-367 solution: asymmetry <3.5; theoretical plates $>50\,000$; and resolution >2. The calculation of the system suitability parameters are performed by the CE instrument software and the equations are shown in the following:

Tailing Factor. The tailing factor (T) may be defined by the equation:

$$T = W_{5\%}/2f$$

where $W_{5\%}$ = the peak width at 5% of peak height as measured from the front side of the peak to the tailing edge, and f = the width at 5% peak height measured from the leading edge of the peak to a vertical line extrapolated from the peak apex.

Theoretical plates. The number of theoretical plates (capillary efficiency) is a useful measure of capillary performance. The equation used to calculate capillary efficiency (N) is:

$$N = 16\,(t_M/W)^2$$

where t_M = the migration time of the IB-367 peak, in minutes and W = the extrapolated width of the IB-367 peak, in minutes.

Resolution between IB-367 and IB-300. IB-300 migrated closest to the peak of IB-367-03 among three potential impurities (IB-300, IB-468, IB-469), therefore, it was chosen to assess the resolution in the system suitability test. The resolution (R_s) between two peaks is defined as:

$$R_s = 2(t_{M2} - t_{M1})/(W_2 + W_1)$$

where t_{M2} = the migration time of the later eluting peak measured as the distance from the point of injection to the peak maximum, in minutes, t_{M1} = the migration time of the earlier eluting peak measured as the distance from the point of injection to the peak maximum, in minutes, and W_i = the width of peak i measured by extrapolating the relatively straight sides of the peak to baseline ($i = 1$ or 2), in minutes.

Table 3
Validation results: accuracy

Sample solution	Assay (%)		Average assay (%)
(day 1)	Injection 1	Injection 2	
1	100.4	100.7	100.6
2	101.1	101.3	101.2
3	104.1	104.8	104.5
4	105.5	105.7	105.6
5	100.3	99.9	100.1
6	101.8	102.2	102.0
Mean	102.3		
Standard deviation (%)	2.1		
RSD_1 (%)	2.1		

3.2. Method validation

The CE method was validated according to the ICH guideline for validation of analytical procedures [17]. It was evaluated for accuracy, precision, linearity, range, limit of detection, limit of quantitation, specificity and robustness.

3.2.1. Accuracy

The accuracy of the method was evaluated by assaying six independently prepared solutions of IB-367, against two standard solutions of the same lot as external standard. The values, expressed as an average of duplicate injections in percent, are listed in Table 3. The mean of 102.3% met the criteria set in validation protocol (97–103%). The method is considered to be accurate. A typical electrophoregram is shown in Fig. 5.

3.2.2. Intermediate precision (day 2)

The precision of the method was evaluated by assaying six independently prepared solutions of IB-367, against two reference standard solutions used as external standards on two days (RSD_3). Results are shown in Table 4. The RSD of 2.1% for the accuracy test (RSD_1) met the criteria set for repeatability ($<4\%$). The relative standard deviations of 1.9% for the precision test on day 2 (RSD_2) and 2.5% for total 12 samples (RSD_3) met the criteria set for inter-

Table 4
Validation results: precision test

Sample solution	Assay (%)		Average assay (%)
(day 2)	Injection 1	Injection 2	
1	99.9	99.7	99.8
2	96.4	97.2	96.8
3	97.5	98.1	97.8
4	101.1	101.5	101.3
5	97.1	100.6	98.9
6	99.9	99.5	99.7
Mean	99.1		
Standard deviation (%)	1.7		
RSD_2 (%)	1.9		
RSD_3 (%)	2.5		

mediate precision by the validation protocol (<4%). The method is considered to be precise.

3.2.3. Linearity and range

Solutions of IB-367 drug substance at 0.1%, 1%, 10%, 50%, 80%, 100% and 150% of the specified IB-367 concentration (0.5 mg/ml in water) were assayed in duplicate using two reference standard solutions as external standards. The CE method exhibits acceptable linearity from 1% to 150%. The least-squares linear regression equation obtained was $y=1.008x-0.1464$, where x is the expected response and y is the observed response. The coefficient of correlation is 1.00 and standard deviation of the line is 1.6. The method is considered to be linear. The range was determined to be 10% to 150% according to the criteria set by the validation protocol (97–103%). The IB-367 signal at 0.1% concentration in the presence of the internal standard was not observed, possibly due to interactions between IB-367 and glycylphenylanaline at low concentration of IB-367 under the electrophoretic conditions. This possible interaction shall not have any effect on the method since the internal standard will not be used in the method to determine the purity and potential impurities/degradation products of IB-367.

3.2.4. Linearity of potential impurities at low concentrations

Potential impurities IB-300, IB-469 and IB-468 were spiked to a solution of IB-367 (0.5 mg/ml based on powder mass) at 0.05%, 0.1%, 0.2%, 0.5%, 1% (w/w). The mixtures were assayed in duplicate, and the relative corrected area of impurities to that of IB-367 were plotted against the expected percent concentration. The least-squares linear regression equation for IB-300 is $y=1.03x-0.075$, where x is the expected percent concentration based on sample preparation and y is the observed percent concentration based on CE analysis. The coefficient of correlation (r^2) for IB-300 is 0.983. The least-squares linear regression equation for IB-469 is $y=1.10x-0.075$, with $r^2=0.990$. For IB-468, the truncated analog that migrates the furthest from IB-367, the least-squares linear regression equation is $y=1.15x-0.049$, with $r^2=0.997$. The slopes of response relative to IB-367 are all close to 1 for these three potential impurities, which allows the assessment of these impurities by area normalization method. The CE method exhibits acceptable linearity of impurities from 0.05% to 1%.

3.2.5. Limit of detection and limit of quantitation for IB-367

The limit of detection for IB-367 was determined to be a concentration giving a peak height at least twice of the noise level. The limit of detection of the CE method is 0.1% (100% being 0.5 mg/ml IB-367). The limit of quantitation was determined to be 0.5%, by the criteria of signal-to-noise ratio of at least ten.

3.2.6. Limit of detection (LOD) and limit of quantitation (LOQ) for potential impurities/degradation products

The LOD for potential impurities/degradation products was determined to be a concentration giving a peak height at least twice of the noise level. The signal-to-noise ratio for IB-468 peak was used to determine LOD and LOQ, since it is the impurity peak that migrates the farthest from the IB-367 peak and has the least interference due to the main IB-367 peak. The LOD for IB-468 in the presence of IB-367-03 is 0.05%. The LOQ was determined to be 0.5%, by the criteria of signal-to-noise ratio of at least ten. The method is considered sensitive to detect low-concentration potential impurities.

3.2.7. Specificity

The specificity of the method was evaluated to ensure separation of IB-367-03 drug substance and several potential impurities/degradation products, IB-468 ([des-Arg^1]), IB-469 ([des-Arg^1Gly^2]) and IB-300 ([des-$Arg^1Gly^2Gly^2$]). These potential impurities/degradation products were individually synthesized and established as authentic samples. The specificity of the method was demonstrated by assaying a solution of IB-367 reference standard at concentration of 0.5 mg/ml spiked with 5% IB-300, IB-469 and IB-468. The electrophoregram of this mixture is shown in Fig. 6. The CE method demonstrated not only the resolution between IB-367 and the potential impurities/degradation products, but also the resolution among the potential impurities/

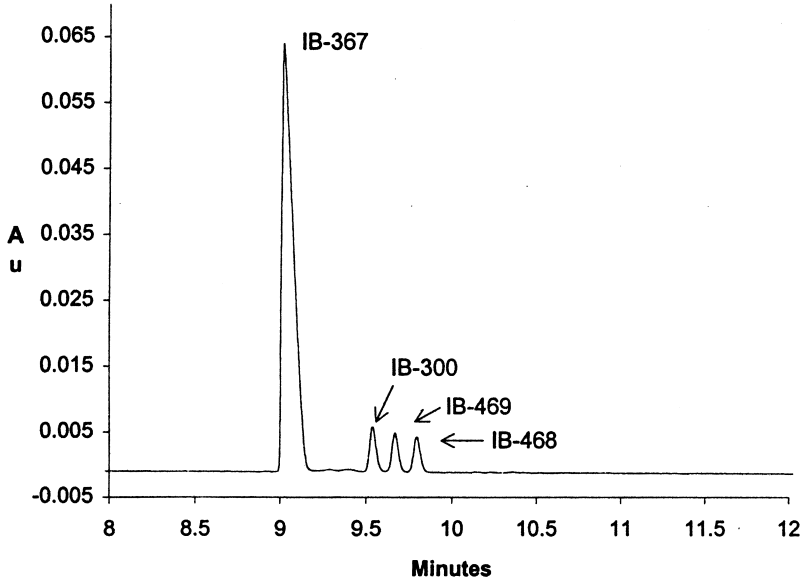

Fig. 6. Electropherogram of IB-367 and its analogs. 100 m*M* sodium phosphate, pH 2.6, 20 kV, 0.5 p.s.i/5 s injection, 45 cm effective length×50 μm, 200 nm detection.

degradation products themselves. The method is considered to be specific for all of the potential impurities/degradation products evaluated.

3.2.8. Robustness

The robustness of the CE method was evaluated by four electrophoresis parameters. The relative migration time of IB-300, IB-469 and IB-468 relative to that of IB-367 and the resolution among these four peptides were monitored by changing the buffer strength, pH of the running buffer, separation voltage and capillary temperature. The results for the robustness experiment are tabulated in Table 5. The method is considered to be robust.

Table 5
Validation results: robustness

Parameter change		Relative migration time			Resolution		
		IB-300	IB-469	IB-468	IB-367/IB-300	IB-300/IB-469	IB-469/IB-468
Buffer strength	90 m*M*	1.060	1.076	1.091	4.7	1.7	1.6
	100 m*M*	1.059	1.074	1.089	4.9	1.9	1.7
	110 m*M*	1.057	1.072	1.086	4.7	1.8	1.7
pH	2.4	1.059	1.073	1.088	4.8	1.9	1.7
	2.6	1.059	1.074	1.089	4.9	1.9	1.7
	2.8	1.058	1.073	1.087	4.7	1.7	1.7
Capillary temperature	23°C	1.059	1.073	1.088	4.9	1.8	1.7
	25°C	1.059	1.074	1.089	4.9	1.9	1.7
	27°C	1.058	1.073	1.087	4.8	1.8	1.7
Separation voltage	19 kV	1.059	1.074	1.089	5.1	2.1	2.0
	20 kV	1.058	1.073	1.088	5.0	2.1	2.0
	21 kV	1.060	1.075	1.089	5.0	2.1	2.0

4. Conclusion

A CE method has been developed for the characterization of protegrin IB-367. The method is specific to IB-367 and showed excellent resolution among analogs. The method was validated to demonstrate its accuracy, precision, linearity and robustness. The method exhibits linearity over a sample concentration range of 1–150%, a limit of detection of 0.1% and a limit of quantitation of 0.5% for IB-367. The method also exhibits linear response for tested potential impurities/degradation products over the range of 0.05–1%. The limit of detection for impurity/degradation products in the presence of IB-367 is 0.05%, and the limit of quantitation for potential impurity/degradation products in the presence of IB-367 is 0.5%. The slopes of the linearity plots of the impurities/degradation products are close to 1, therefore, the level of potential impurities/degradation products can be established by area normalization to IB-367. The performance of the electrophoretic system used in this work has been characterized through measurement of capillary efficiency, peak tailing, and the resolution between IB-367 and IB-300.

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