BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **19**: 68–79 (2005)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.419

ORIGINAL RESEARCH

Use of capillary electrophoresis in drug quality assessment of synthetic porcine secretin

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Received 9 April 2004; revised 27 May 2004; accepted 4 June 2004

ABSTRACT: The purity profile for porcine secretin attributable to contamination by equilibrium products such as aspartoyl[3] secretin has been shown to be dependent on the pH of the analytical system. Capillary zone electrophoresis (CZE) methods have been developed for the efficient separation of synthetic porcine secretin, its equilibrium products and other impurities in aqueous solutions at both acidic and alkaline pH. These conditions are more representative of those used for the reconstitution and administration of porcine secretin, and good results cannot be achieved using HPLC due to poor peak shape above pH 5.8. The influence of various CZE operational parameters was systematically examined. The methods were validated for accuracy, precision, linearity, LOD and LOQ. A comparative evaluation of the stability of test solutions was determined using CZE and HPLC over a range of pH values. HPLC and CZE methods produced similar results at low pH. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: capillary electrophoresis; CE; porcine secretin

INTRODUCTION

Capillary electrophoresis (CE) is a relatively new analytical technique for the separation and analysis of small and large molecules such as peptides, and is complementary to existing approaches such as high performance liquid chromatography (HPLC) (Dunn and Pennington, 1994). The advantages of CE over HPLC include relatively small sample volume requirements, a simpler experimental set-up, easy and rapid method development, reduced sample pre-treatment and running costs, lower solvent demand and rapid separation (Baker, 1995; Landers, 1997). The separation mechanisms are widely different in CE and HPLC, and are based on electrophoretic mobility and partitioning differences, respectively. These two techniques are often used in combination when assessing drug quality. Good agreement between CE and HPLC results would support a comprehensive evaluation of the quality of therapeutic peptide dosage forms.

In this study CE is employed to develop a quantitative analytical method that would be beneficial in the assessment of drug quality of synthetic porcine secretin, which in turn might be useful in the analysis of other peptides. Secretin is a gastrointestinal hormone produced by endocrine cells in the duodenum and jejunum of man and many animals in response to the acidification by gastric HCL. It is a basic linear polypeptide consisting of 27 amino acids with of 12 different types of amino acids and a molecular weight of 3055 (Tsuda *et al.*, 1989; Jorpes, 1968). The amino acid sequence of porcine secretin is His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2. It has a single histidine residue in the N-terminal position and no C-terminal residue with a free carboxyl group (Bodanszky, 1993).

Crude secretin has been analyzed by reverse-phase chromatography (HPLC) in order to determine its purity. Two diastereoisomers, D-Leu[10] secretin and D-Ala[17] secretin have been identified and a study of the degradation of secretin in acid and neutral aqueous solutions was reported by Tsuda *et al.* (1990a,b). The main product produced in neutral solution was identified as β-aspartyl[3] secretin while aspartoyl[3] secretin was found to be produced in acidic solution (Tseuda *et al.*, 1990a,b). The proposed mechanism of degradation involves intramolecular rearrangement of secretin to the corresponding cyclic succinimide, aspartoyl[3] secretin, which is then hydrolysed to β-aspartyl[3] secretin. Secretin was found to be more stable at pH 7.0 but less stable in alkaline and acidic solutions (Beyerman *et al.*, 1981; Tsuda *et al.*, 1990a,b).

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Abbreviations used: As, peak asymmetry; Au, absorbance units; PTF, peak tailing factor.

Published online 15 September 2004

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rinse. Two injections of the run buffer were made before starting the analysis to stabilize the capillary system at both pH values.

To ensure optimal precision, capillaries were flushed daily using two water/buffer rinsing cycles prior to shutdown. The buffers were replaced daily and the pH of the solutions was checked to confirm that the electrolytic formation of hydrogen ions and/or hydroxide ions solutions did not alter the solution pH and cause irreproducible migration times. For long-term storage the CE capillaries were purged using 10 water rinse cycles and stored containing distilled de-ionized water.

RESULTS AND DISCUSSION

The influence of various parameters on the separation of secretin, such as buffer pH and concentration, applied voltage, injection time and capillary length were investigated to optimize the separation of porcine secretin and its impurities.

Influence of buffer pH

Secretin was dissolved in phosphate buffer solutions between pH 2.0 and 8.0 and evaluated on the Dionex system to evaluate the effect of pH on peak resolution, peak shape and peak response. This pH range covered values near the pK_as of the amino acid side chains (Dunn and Pennington, 1994). The separation of porcine secretin, its degradation products and impurities under the influence of different pH conditions are illustrated by the electropherograms shown in Fig. 1.

No distinct peaks were observed at pH 2.0 and noisy backgrounds were obtained due to the low electroosmotic flow evident at this pH for fused-silica capillaries. Secretin was resolved into two peaks at pH 3.0 and 3.5. Published studies have identified aspartoyl[3] secretin and β-aspartyl[3] secretin as the major impurities found in secretin (Tsuda *et al.*, 1990a,b). These are equilibrium products resulting from a reversible reaction between secretin and aspartoyl[3] secretin and between aspartoyl[3] secretin and β-aspartyl[3] secretin in aqueous solution. The presence of these equilibrium products appears to be greatly influenced by the pH of the buffer. The two peaks obtained under acidic conditions were identified as aspartoyl[3] secretin and secretin by matching the relative migration times of the components of the analyte to that of reference standards.

Confirmation of peak identity was achieved by spiking a solution of secretin with standard aspartoyl[3] secretin. An increase in the pH of the buffer to 4.0 resulted in the disappearance of aspartoyl[3] secretin, which then increased significantly when the pH was adjusted to 5.0. A decline in the proportion of aspartoyl secretin occurred with additional increases in pH up to 7.0. Increasing the pH of the run buffer to pH 7.4 and 8.0 resulted in a decrease in the size of the aspartoyl secretin and the emergence of a small peak presumed to be β-aspartyl[3] secretin. This was not confirmed due to the lack of reference material for β-aspartyl[3] secretin. The change in the proportion of aspartoyl[3] secretin with pH is shown in Fig. 2.

The equilibrium products have been reported to have the same amino acids as secretin and only differ from secretin in terms of sequence (Beyerman *et al.*, 1981). The mass to charge ratio (m/z) for the parent ion, which consisted of the S_{1-6} analysis of the fragments obtained from digestion of secretin, β-aspartyl[3] secretin and aspartoyl[3] secretin with α-chymotrypsin, suggested that secretin is converted to β-aspartyl[3] secretin via the hydrolysis of aspartoyl secretin (Tsuda *et al.*, 1990a,b). β-Aspartyl[3] secretin and aspartoyl[3] secretin were reported to be of low biological activity (Tsuda *et al.*, 1990a,b; Beyerman *et al.*, 1981). It reasonable to expect aspartoyl[3] secretin to elute first since it has a small charge-to-mass ratio as compared with secretin and aspartyl[3] secretin (Tsuda *et al.*, 1990a,b). Secretin and β-aspartyl[3] secretin have the same charge-to-mass ratio and therefore should be expected to have the same migration time. The difference in their mobility is probably due to the conversion of aspartyl[3] secretin between the α- and β-conformations, which can result in increases in its hydrodynamic radius, thus resulting in slower migration times (Cifuentes and Poppe, 1997). In addition, changes from the β- to the α-form may modify the overall shape of the aspartyl[3] secretin thus affecting its electrophoretic mobility. Despite this, the reproducibility in migration times for replicate evaluations of aspartyl[3] secretin was 2.8% relative standard deviation (RSD; 13.06 ± 0.37 min) at pH 3.5 and 4.2% RSD (5.68 ± 0.24 min) at pH 3.5. The migration times for aspartoyl secretin were 12.04 ± 0.32 min with a %RSD of 2.6 at pH 3.5 and 5.43 ± 0.18 min with a %RSD of 3.3 at pH 7.4.

Of the various pH conditions studied, pH 3.5 was determined to be the optimal pH for the separation of secretin. This is in agreement with published work which indicates that peptide separations at acidic pH by HPLC are preferred to those conducted under basic conditions (Messana *et al.*, 1997). A plot of the resolution of porcine secretin and aspartoyl[3] secretin as a function of pH is shown in Fig. 3. The better separation at low pH is attributable to the lower EOF occurring at lower pH values, which should give the analytes more time to separate. These results also illustrate the importance of pH control in the separation of structurally related peptides. Peak asymmetry (As) and peak tailing factor (PTF) observed at pH 7.4 were 0.82 and 0.86, respectively, which are close to the acceptable value of 1 as shown in Fig. 3. The As and PTF for the secretin peak at pH 3.5 were slightly higher than 1 and resulted in its full resolution from the aspartoyl[3] secretin peak.

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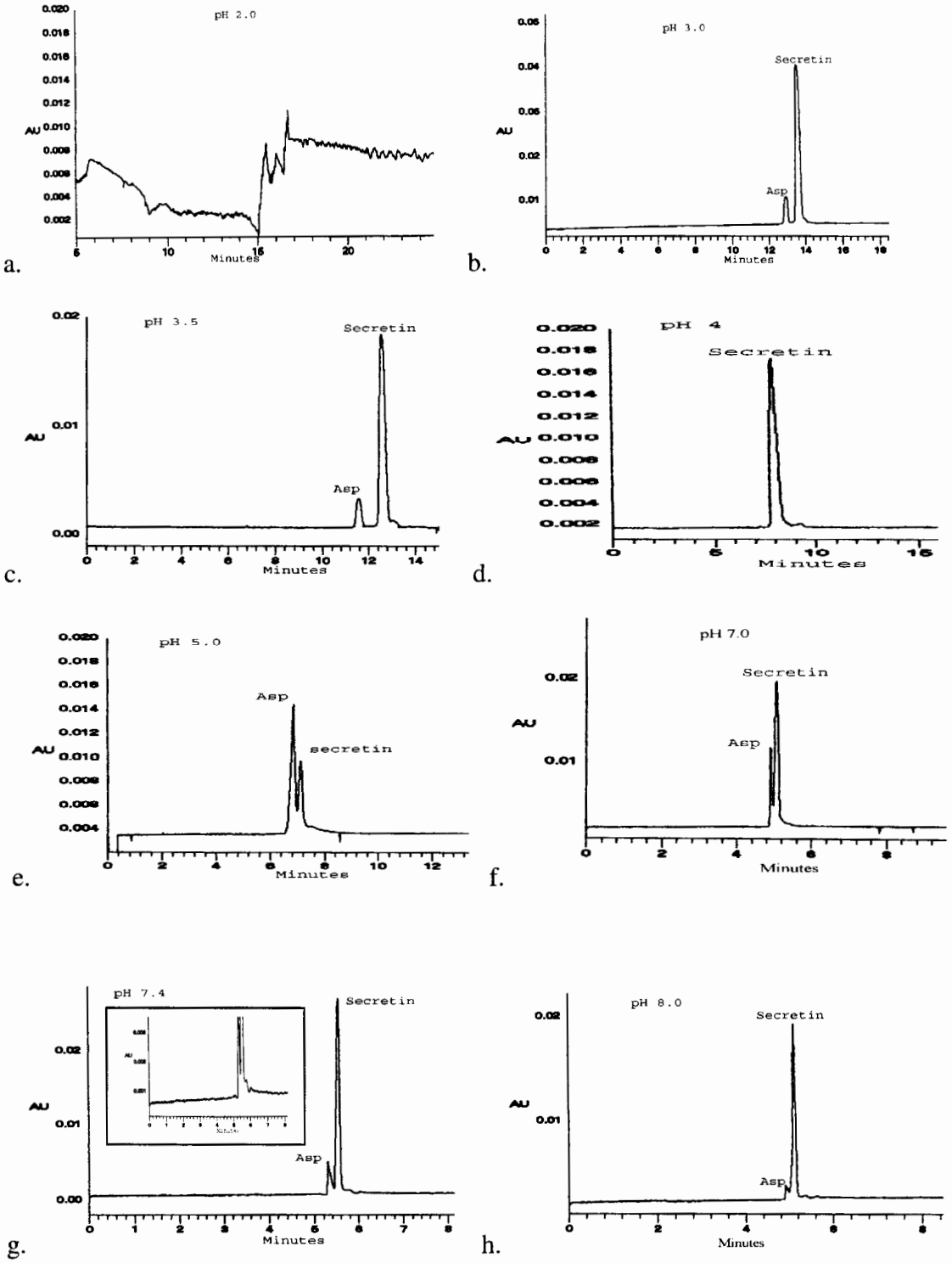

Figure 1. Electropherograms of 294 µg/mL porcine secretin at pH 2.0 (a), 3.0 (b), 3.5 (c), 4.0 (d), 5.0 (e), 7.0 (f), 7.4 (g) and 8.0 (h), using a 47 cm long capillary and the Dionex CES-1.2.2.

The PTF was used to evaluate the peak shape because of its significant impact on the integration parameters. PTF values greater than 1.5, as observed at pH 3.0, were considered to be poor and resulted in imprecise quantitation.

Influence of buffer concentration

An increase in the buffer concentration resulted in an increase in migration time as would be expected since higher ion concentrations result in a decrease

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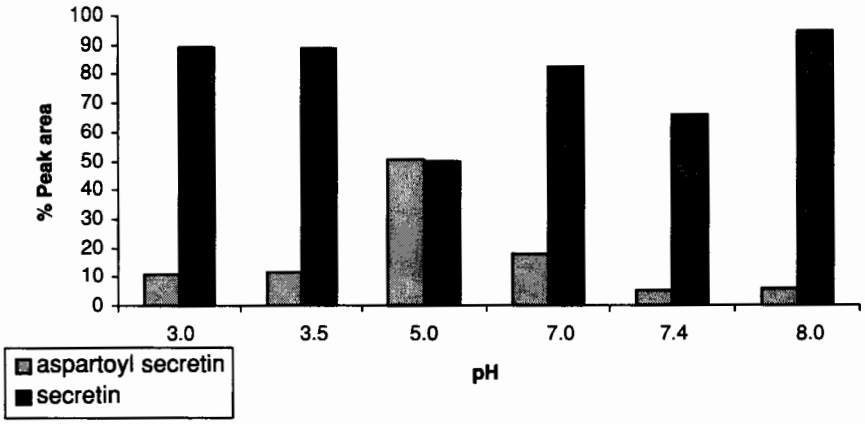

Figure 2. Effect of the pH of the background electrolyte on the proportion of secretin and its equilibrium peptide aspartoyl[3] secretin.

Table 1. Comparison of migration times, resolution, and efficiency (*N*) of separation of porcine secretin in 25 and 50 mM phosphate buffer solutions at various pH values (47 cm, 75 μm i.d., 375 μm o.d. capillary)

	Migration time (min ± SD)		*N* (theoretical plates)		Resolution	
pH	25 mM	50 mM	25 mM	50 mM	25 mM	50 mM
3.0	8.06 ± 0.613	13.33 ± 0.323	8613	13,809	1.02	1.23
5.0	6.07 ± 0.325	7.08 ± 0.110	7832	12,265	0.96	0.98
7.4	5.49 ± 0.049	5.68 ± 0.243	10,891	29,097	1.29	1.94
8.0	5.08 ± 0.011	5.11 ± 0.030	8930	30,875	1.14	1.63

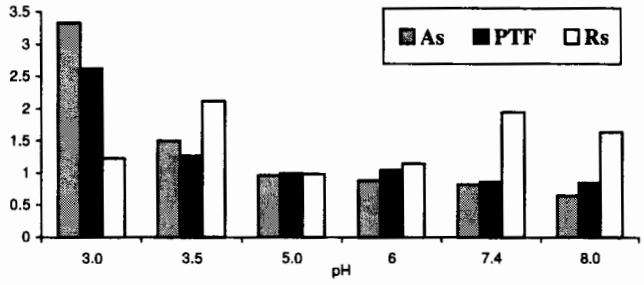

Figure 3. Illustration of resolution (Rs), As and PTF parameters used for the evaluation and selection of the optimal pH for the separation of 294 μg/mL of porcine secretin (47 cm capillary).

Table 2. Comparison of peak shape parameters, peak asymmetry and PTF for porcine secretin in 25 and 50 mM phosphate buffer solutions at various pH values (47 cm, 75 μm i.d., 375 μm o.d. capillary)

	Peak asymmetry		Peak tailing factor	
pH	25 mM	50 mM	25 mM	50 mM
3.0	2.30	2.33	1.58	2.63
5.0	1.13	0.99	1.11	0.97
7.4	1.02	0.82	1.31	0.86
8.0	2.00	0.83	1.81	0.85

in the potential across the diffuse double layer and a subsequent drop in the EOF (Cifuentes and Poppe, 1997; Weinburger, 2000). An increase in the concentration also will increase conductivity, which in turn produces heat and zone band broadening (Kemp, 1998; Kelley *et al.*, 1997). The peak width has also been shown to be proportional to the concentration ratio of sample ions to buffer ions (Harrold *et al.*, 1993) and it has been reported that the concentration of the buffer should be at least 100 times that of the analyte (Baker, 1995). Taking this into consideration, the 50 mM phosphate buffer was selected as the optimal concentration for the separation of porcine secretin since it produced better results in terms of the efficiency of separation, resolution, peak symmetry and peak tailing factor as shown in Tables 1 and 2.

Effect of capillary length on separation of porcine secretin and its equilibrium products

In this study the length of the capillary was investigated by employing three lengths, 47, 57 and 67 cm. The results are shown in Table 3. The shorter length resulted in less interaction length and also shorter analysis time due to the increase in field strength (V/cm), which in turn led to increased electro-osmotic flow and faster migration of the analytes. The 57 cm capillary was selected as the optimal length since it produced better analysis time, resolution and a peak asymmetry factor of 0.82 that was closer to the ideal factor of 1.

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Table 3. Electrophoretic parameters for the 294 μg/mL porcine secretin peak in 50 mM phosphate pH 7.4 using various capillary lengths

Length (cm)	Migration time (min)	Efficiency (theoretical plates)	Resolution	Peak asymmetry	Peak tailing factor
47	5.05	24,448	1.23	0.74	0.92
57	8.79	28,267	1.48	0.82	0.86
67	12.76	30,037	1.61	0.52	0.78

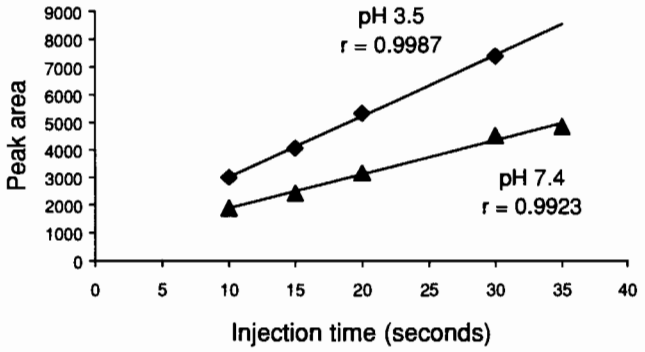

Figure 4. Effect of sample injection time on the peak response of porcine secretin in 50 mM phosphate buffer pH 3.5 and 7.4, using gravity injection at 100 sampler head height.

Influence of the sample injection volume on the separation efficiency

One of the drawbacks of CE is low concentration detection due to short optical path lengths for light absorption through the capillary (Messana *et al.*, 1997; Issaq *et al.*, 1991). The injected volume has a major influence on the resolution and efficiency of separation. Large sample volumes may overload the sample on the capillary and result in poor peak shape and poor precision. Major factors affecting peak width are the injection length of the sample and longitudinal diffusion (Huang *et al.*, 1989). To investigate this, the gravity injection period was varied from 10, 15, 20, 30 and 35 s.

There was a positive linear relationship between the peak area and the injection times at both pH values investigated (Fig. 4). At pH 7.4 there was an increase in peak height with an increase in injection time. However, results obtained at pH 3.5 had broad peaks with a flat apex, probably due to electromigration depression or overloading (Jorgenson and Lukacs, 1981). There was very low electroosmotic flow at pH 3.5 and as a result the analytes migrated slowly, allowing the capillary to equilibrate and leaving more time for the zones to separate (Dionex, 1994). The resulting peaks were broad and demonstrated low efficiency. The sample plug length at 30 s injection time was greater than 1% of the total length and therefore an injection time of 30 s was not appropriate. It has been reported that sample volumes greater than 10 nL often lead to a breakdown in resolution and peak shape deformation (Aebersold and Morrison, 1990). The injected sample amount should not be so high as to result in capillary overloading. Taking these factors into consideration, the maximum injection time that can be used without deformation of the peak was determined to be 10 s.

Comparison of separation of porcine secretin using CE and HPLC

Higher efficiency and better resolution were observed with CZE as compared with HPLC, as indicated in Table 4. Separation of the same analyte by HPLC required an organic solvent which may compromise the resolution of secretin and its equilibrium products. In addition, no organic solvents were used in CZE, making it more representative of the dosage form, more environmentally friendly and more economical than HPLC. Although different, the results from the two approaches are complementary (Khaledi, 1998). The complementary nature is illustrated in Fig. 5, which shows a comparison of an electropherogram of porcine secretin alone with an HPLC chromatogram. The fact that these two methods demonstrated different elution patterns at similar pH values for the same sample suggests that CE may provide additional confirmatory information to that provided by HPLC.

Table 4. Comparison of analysis time and efficiency of the separation of porcine secretin by CZE and FDA-approved HPLC method

Technique	CZE	HPLC
Sample	200 μg/mL porcine secretin	300 μg/mL porcine secretin
Migration/retention time	19.29 min	19.91 min
Efficiency (theoretical plates)	28,989	9680
Resolution	4.16	2.89

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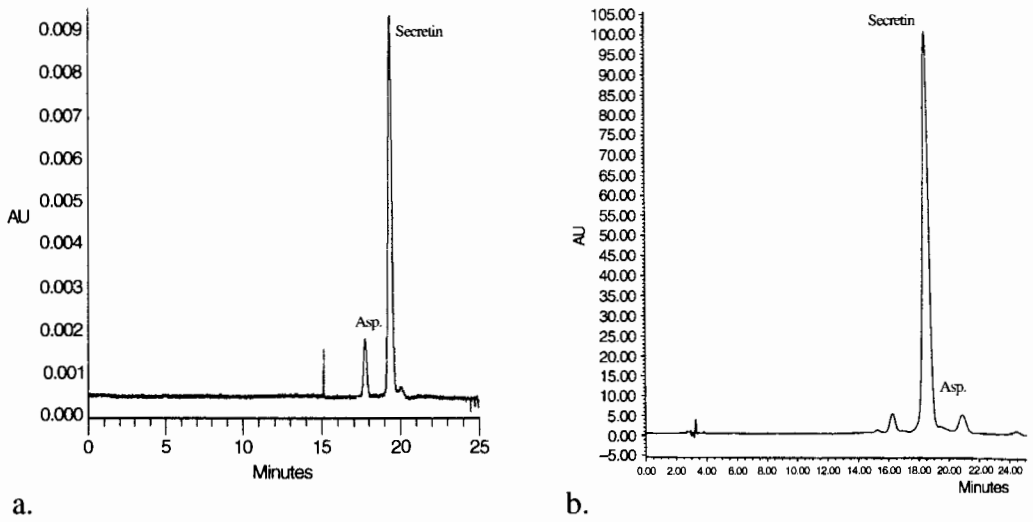

Figure 5. Electropherogram and chromatogram of porcine secretin by CE (a) 200 µg/mL porcine secretin, 50 mM phosphate buffer pH 3.5 (75 µm i.d., 375 µm o.d., 57 cm capillary), and HPLC (b) 300 µg/mL porcine secretin, FDA-approved method.

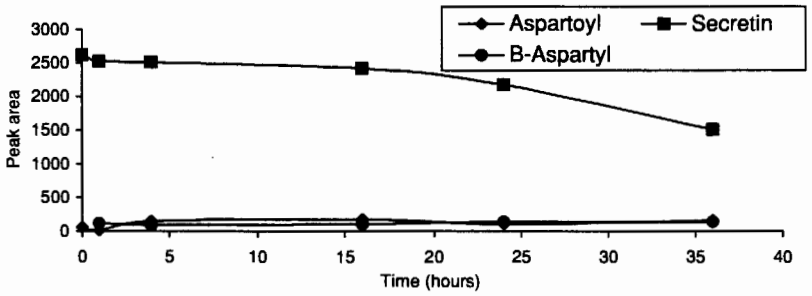

Figure 6. Stability of the test solution of porcine secretin in phosphate buffer, pH 7.4, at 60°C heat for 36 h.

Purity of synthetic porcine secretin

The purity of lyophilized porcine secretin powder was determined by integrating the peaks and calculating the main peak's percentage of the total area. The total level of porcine secretin was 86.8 and 89.0% by CE in phosphate buffer at pH 3.5 and 7.4 respectively, and 87.3% by HPLC. The total levels of β-aspartyl[3] secretin and aspartoyl[3] secretin were 1.6 and 10.8% at pH 3.5 and 3.3 and 6.1% at pH 7.4, respectively as measured by CZE. The corresponding levels of were found to be 0.8 and 10.3% when evaluated by HPLC. The level of β-aspartyl[3] secretin at pH 7.4 was slightly higher than at pH 3.5, while the level of aspartoyl secretin was higher at pH 3.5. The levels of porcine secretin, β-aspartyl[3] secretin, aspartoyl[3] secretin and unknown impurities obtained for CE (under acidic conditions) and HPLC were similar. This level of agreement in results obtained between the validated and accepted HPLC method and the new CZE method presented engenders a greater level of confidence in the latter.

Stability of the test solution

To evaluate the stability of porcine secretin in aqueous solution as a function of pH, the test solution was stored in a heating block at 60°C for 36 h at pH 7.4 and 48 h at pH 3.5. Samples were withdrawn at set time intervals and evaluated by CZE. It was observed that there was very little change in the percentage of secretin, β-aspartyl[3] secretin and aspartoyl[3] secretin produced in phosphate buffer at pH 7.4, as shown in Fig. 6 However, acidic conditions led to the rapid re-equilibration of secretin to aspartoyl[3] secretin, as shown in Fig. 7. Additional peaks were observed which were not seen at zero time.

The stability of the test solution of secretin was also studied using HPLC. Two samples of secretin prepared in 2% acetic acid (pH 3.0) and distilled water (pH 7.0) were stored at 60°C and analyzed at time 0 and 17 h An increase in the proportion of aspartoyl secretin and a decrease in the proportion of secretin were observed under acidic conditions after 17 h. There was

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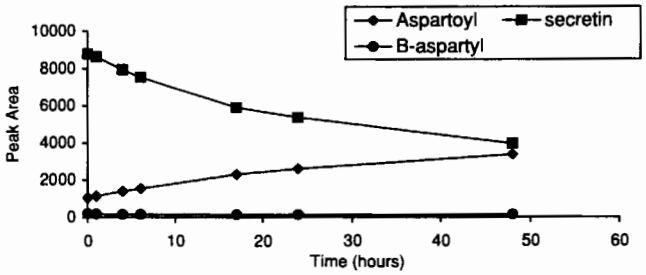

Figure 7. Stability of the test solution of porcine secretin in phosphate buffer, pH 3.5, at 60°C heat for 48 h.

very little or no change in the response of β-aspartyl[3] secretin. These results indicated that aspartoyl[3] secretin was the major impurity at low pH but is hydrolyzed to β-aspartyl[3] secretin upon storage. These results are in agreement with published work by Tsuda *et al.* (1990a,b).

A comparison of stability data obtained using CE and HPLC methods is shown in Table 5. It was observed that the percentage decrease in secretin was higher in acidic conditions for both CZE (33.1%) and HPLC (34.1%) as compared with neutral and slightly alkaline conditions where the decreases were 6.1 and 5.6% for CZE and HPLC, respectively. These results indicate that secretin is more stable in neutral aqueous solution at physiological conditions than in acidic aqueous solution. Further assessment of the data showed that the percentage difference between results obtained between CE and HPLC in acidic conditions is 2.3 and 3.3% at 0 and 17 h, respectively. The same comparison between the two methods at neutral pH was 5.0 and 4.5% at 0 and 17 h, respectively. The good agreement observed between the CE method and the HPLC method at low pH, where sample stability is minimal, further validates the use of CE as an alternative analytical tool for the quantification and characterization of porcine secretin.

Method validation experiments

The optimized method was validated according to the requirements of ICH and the *United States Pharmacopia* (USP) using the P/ACE™ MDQ system from Beckman-Coulter. The P/ACE™ MDQ system was used for validation because it had better temperature control and provided higher signal-to-noise. The operating conditions were adjusted slightly to achieve similar separation due to the differences in instrument cooling capacities (Faller and Engelhardt, 1999) and method validation was therefore done under the conditions described above.

Conformance of calibration data to the linear model. Six standard solutions of porcine secretin at 3.5, 7.5, 10.0, 15.0, 20.0 and 25.0 µg/mL in 50 mM phosphate buffer pH 3.5 and six standard solutions at 3.0, 5.0, 10.0, 15.0, 20.0 and 25.0 µg/mL in phosphate buffer pH 7.4 were used to assess the linearity of the method response. The effect of fluctuations in migration times on the peak area was reduced by using normalized or corrected area which was determined by dividing the observed peak area responses by the migration times (Baker, 1995; Watzig and Dette, 1993). The normalized peak areas were plotted against the concentrations of the standard. A weighted ($1/x$) linear regression was used to prepare the calibration curve using JMP IN statistics software, version 4.40. The observed calibration equations were $y = 392.96x - 444.77$ at pH 3.5 and $y = 313.22x - 202.21$ at pH 7.4. The correlation coefficients were 0.9980 and 0.9910 for pH 3.5 and 7.4, respectively, indicating that there is a positive linear relationship between the concentrations and the peak responses. A linear relationship was also observed between the normalized area of aspartoyl[3] secretin and the concentration of the solution. The calibration equation was $y = 45.47x - 27.54$ with a correlation coefficient of 0.9938. Residual plots of the peak response vs the concentration of porcine secretin showed residuals that were randomly spread above and below the zero line, and the percentage residuals of the concentrations ranged from −11.82 to 3.02 at pH 3.5 and from −8.33 to 15.18 at pH 7.4. β-Aspartyl[3] secretin was not observed under these conditions, probably due to the cooling capacity of the system to maintain the temperature of the sample compartment at 4°C, thus minimizing sample decomposition.

Accuracy. Accuracy may be expressed as the percentage difference between the measured and the accepted ('true'), and is identified in this study as the 'percentage error' (Unites States Pharmacopia, 2000). Six standard

Table 5. Comparison of the stability data obtained from analysis of porcine secretin at 60°C in 50 mM phosphate buffer (pH 3.5) and pH 7.4 by CZE and in 2% acetic acid (pH 3.0) and pH adjusted distilled water (pH 7.0) by HPLC

Time (h)	CZE, pH 3.5	HPLC, pH 3.0	Difference (%)	CZE, pH 7.4	HPLC, distilled water	Difference (%)
0	90.0	87.7	2.3	92.7	87.7	5.0
17	56.9	53.6	3.3	86.6[a]	82.1	4.5
Decrease (%)	33.1	34.1		6.1	5.6	

[a] Sample was withdrawn after 16 h at pH 7.4.

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solutions of porcine secretin (3.5, 5.0, 10.0, 15.0, 20.0 and 25.0 μg/mL) prepared in 50 mM phosphate buffer at pH 3.5 and six standard solutions (3.0, 7.5, 10.0, 15.0, 20.0 and 25.0 μg/mL) prepared in pH 7.4 phosphate buffer were used to construct the calibration curves. Three porcine secretin quality control samples prepared in pH 3.5 (7.5, 12.0 and 17.5 μg/mL) and pH 7.4 (5.5, 12.0, and 17.5 μg/mL) phosphate buffer were analyzed in replicate (n = 6 at each concentration). The percentage error results for the three standards at both pH 3.5 and 7.4 ranged from 2 to 7%, which was comparable to results reported using HPLC (1–10%), indicating that the accuracy of the method was acceptable.

Precision. Precision in CE and its employment for routine analysis depend on the repeatability of the migration time and the accuracy of quantification (Khaledi, 1998). The ICH guideline defines three types of precision: *repeatability*, *intermediate precision* and *reproducibility*. *Repeatability* refers to the precision of the method under the same conditions over a short period of time. The *intermediate precision* is determined by comparing the results obtained in repeatability studies with those obtained using a procedure with some analytical variable change, such as capillary dimension, different days, analysts or equipment within the same laboratory. The *reproducibility* compares results taken from different laboratories. Repeatability and intermediate precision are essential in a CE procedure because of relatively irreproducible migration times, which affect the peak area of the analyte thus contributing to inaccurate results. Repeatability was assessed in this study using six determinations included in the specified range for the procedure. The precision of the method was demonstrated by analysis of six porcine secretin replicates in pH 3.5 phosphate buffer at concentrations of 7.5, 12 and 17.5 μg/mL and pH 7.4 phosphate buffer at 5.5, 12.0 and 17.5 μg/mL. The percentage relative standard deviations for six replicates (%RSD) ranged from 4 to 6% at pH 7.4 and from 2 to 7% at pH 3.5, which were considered to be within acceptable limits.

Repeatability. For an analytical method to be useful for routine analysis of drugs the migration times should be reproducible (Scaepere and Sepaniak, 2000). Two porcine secretin standard solutions (7.5 and 16.0 μg/mL) were prepared at pH 3.5 and two samples (5.5 and 16.0 μg/mL) were prepared in pH 7.4 buffer. Each of the solutions was injected five times and evaluated by CZE on the same day. The %RSD in migration time at pH 3.5 were 0.87 (7.5 μg/mL) and 4.60 (16 μg/mL). At pH 7.4 the %RSD in migration times were 0.19 (5.5 μg/mL) and 0.15 (16 μg/mL). The migration times at pH 7.4 were more reproducible than at pH 3.5, which can be attributed to the larger electroosmotic flow and higher sample stability expected at pH 7.4. Good repeatability was also observed in the peak area responses, which yielded %RSD of 4.40 (7.5 μg/mL) and 1.94 (16 μg/mL) at pH 3.5 and 4.36 (5.5 μg/mL) and 2.83 (16 μg/mL) at pH 7.4. The peak height results showed similar variability to peak areas.

Limit of detection. The limit of detection (LOD) of an individual analytical procedure is the lowest concentration of an analyte that can be distinguished from the blank, but not necessarily quantified. A factor of 2–3 times the noise is generally considered to be acceptable for estimating the detection limit. Noise refers to the random fluctuations of the detector output with time arising from blank injection variability due to lamp fluctuations and spurious electronic signals (Khaledi, 1998). This was obtained by analysis of the buffer solution six times, and injecting 3.5, 5.0 and 10 μg/mL porcine secretin in pH 3.5 phosphate and 3.0, 7.5 and 10 μg/mL porcine secretin in pH 7.5 phosphate buffer. The peak responses in absorbance units (AU) were used to construct a calibration curve. The LOD was determined as 3-times the standard deviation of the blank noise divided by the slope of the curve. The LOD for the CZE method was determined to be 1.5 and 1.0 μg/mL at pH 3.5 and 7.4 respectively, while that obtained using HPLC was 0.044 μg/mL. Solutions containing 1.5 and 1.0 μg/mL of porcine secretin in phosphate buffer pH 7.4 were analyzed using CE, and the signal obtained could not be reproducibly integrated.

Limit of quantification. The limit of quantification (LOQ) of an individual analytical procedure is the lowest concentration of the analyte that can be quantitatively determined with acceptable accuracy and precision. It was estimated in this work as 10-times the signal-to-noise ratio (S/N). LOQ and LOD determinations for secretin were evaluated in solutions whose concentrations ranged from 3.5 to 10.0 μg/mL at pH 3.5 and 3.0 to 10.0 μg/mL at pH 7.4. The estimated LOQs for secretin were found to be 5.0 and 3.3 μg/mL at pH 3.5 and pH 7.4, respectively, by CZE. This compared with an estimated LOQ for secretin of 0.15 μg/mL by HPLC. The observed percentage relative standard deviations for test solutions ranged from 0.30 to 4.94 at pH 3.5 and 0.04 to 2.38 at pH 7.4. These were all considered acceptable, indicating that these values were above the LOQ.

Selectivity of the method. The method should be capable of resolving porcine secretin from aspartoyl[3] secretin, β-aspartyl[3] secretin and other components likely to be present. Lyophilized porcine secretin powder for injection contains porcine secretin, mannitol and L(+)-cysteine hydrochloride. Selectivity of the method was demonstrated by analysis of the porcine secretin solution spiked with L(+)-cysteine hydrochloride.

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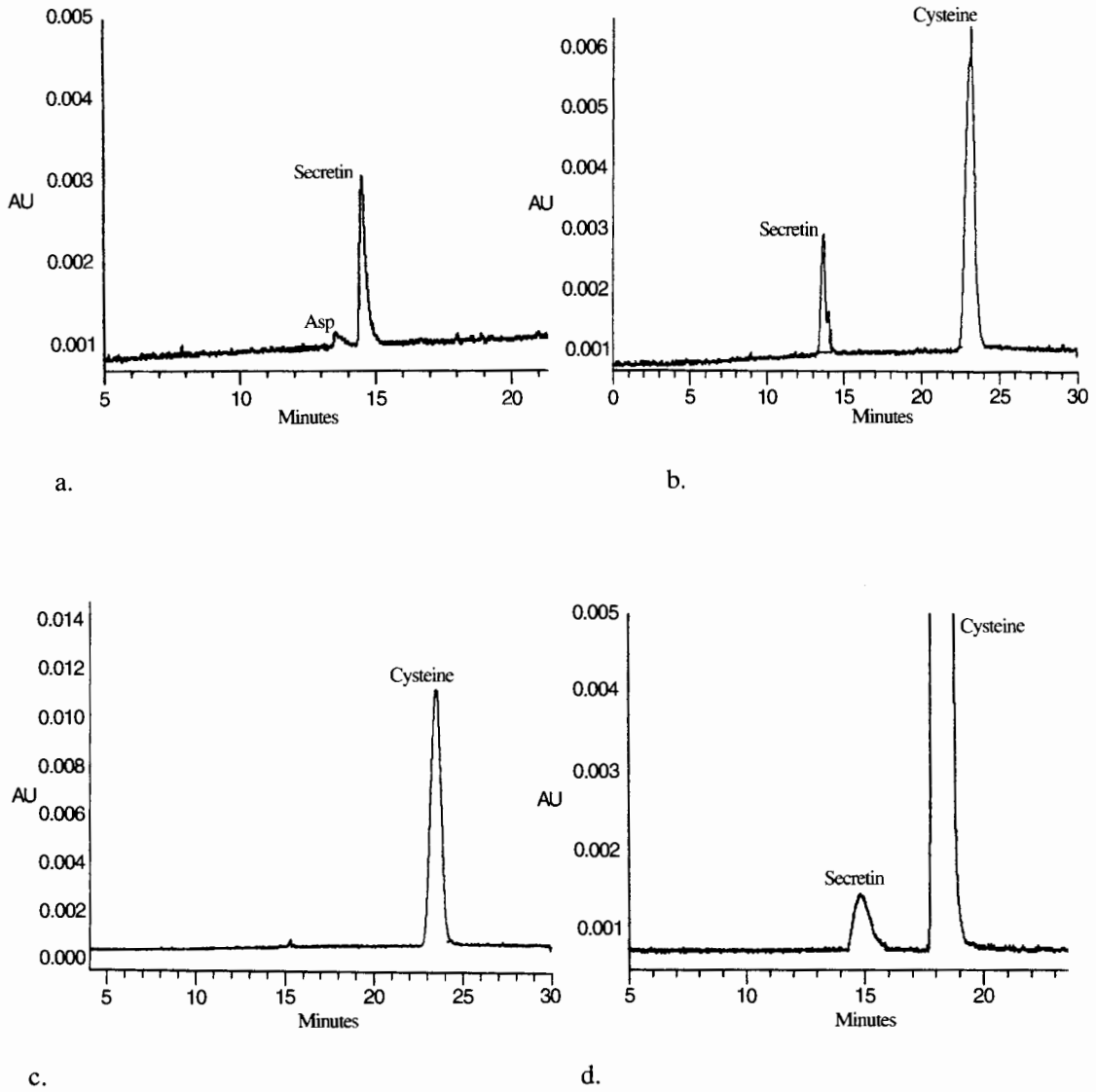

Figure 8. Electropherogram of (a) porcine secretin, (b) porcine secretin spiked with L(+)-cysteine HCL, (c) L(+)-cysteine HCL and (d) lyophilized porcine secretin powder in pH 3.5 phosphate buffer.

Representative electropherograms are shown in Figures 8 and 9. No peak was observed for mannitol. Secretin and its equilibrium products migrated before well before L(+)-cysteine hydrochloride, indicating that the method exhibits an appropriate degree of selectivity for lyophilized porcine secretin powder.

CONCLUSIONS

Two capillary zone electrophoresis methods for the efficient separation of synthetic porcine secretin, its equilibrium products and impurities have been developed using acidic conditions more representative of reconstituted synthetic porcine secretin and at physiological pH. The aqueous conditions used in this study mimic the matrix at which porcine secretin would exist after reconstitution and after administration, and cannot be duplicated with HPLC due to the presence of the needed organic solvent and the poor peak shape obtained for this analyte at high pH. These CE conditions should more accurately reflect purity. Optimal CE separation was obtained at pH 3.5. In addition, the influence of the buffer pH and concentration, length of capillary and sample injection time on the separation of porcine secretin were systematically examined. The buffer pH was shown to be an important parameter that can be manipulated to optimize selectivity for porcine secretin by CE. A method for evaluation of the impurity profile of porcine secretin by means of CE at pH 3.5 and 7.4 has been presented. Aspartoyl[3] secretin and aspartyl[3] secretin were found to be the major

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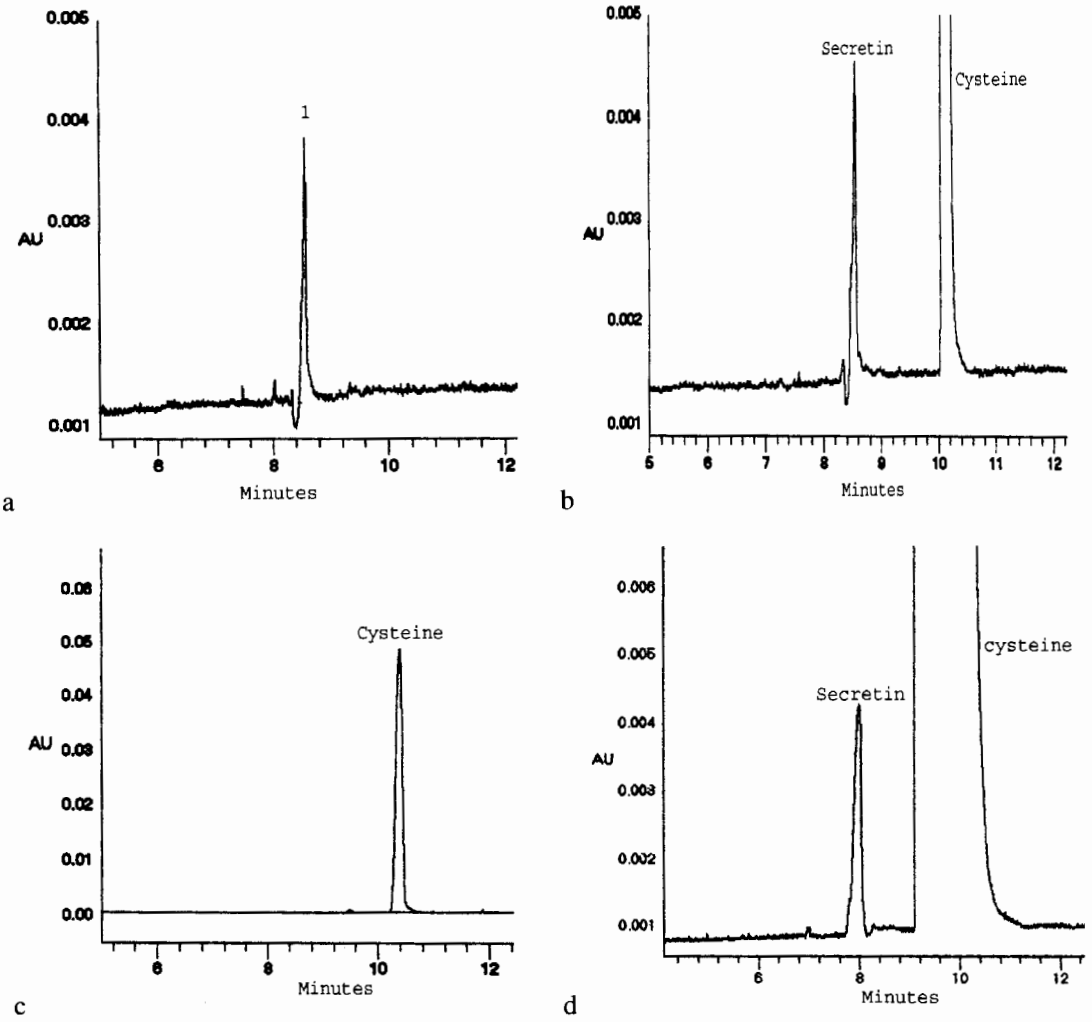

Figure 9. Electropherograms of (a) porcine secretin, (b) porcine secretin spiked with L(+)-cysteine HCL, (c) L(+)-cysteine HCL and (d) reconstituted lyophilized synthetic porcine secretin powder (d) in phosphate buffer pH 7.4.

impurities found in porcine secretin and their proportion was greatly influenced by the pH of the background electrolyte.

The method was validated according to ICH guidelines and USP 24 NF 19, 2000. Linearity, accuracy, precision, limit of detection, limit of quantification and selectivity were determined to assess the suitability of the method. The method was found to be highly selective, accurate and linear for both CE methods. The sample injection volume for CE was 15.8 nL, whereas considerably larger sample volumes (as high as 100 μL) were needed with the HPLC method. An additional advantage of CE over HPLC was the use of relatively small volumes of the background electrolyte, (less than 50 mL/day as compared with 1000 mL/day of a mixed organic mobile phase for HPLC). CE is therefore more economical and environmentally safe than HPLC. The simplicity associated with CZE and the use of inexpensive capillary columns, coupled with the above advantages, makes capillary electrophoresis a valuable compliment to existing techniques for analysis of porcine secretin.

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