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Simultaneous determination of regular insulin and insulin aspart by capillary zone electrophoresis and application in drug formulations

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ABSTRACT

A rapid and simple capillary zone electrophoresis (CZE) with ultraviolet detection has been developed for simultaneous determination of regular insulin and insulin aspart in bulk and commercial injection dosage forms. The simultaneous analysis of the tested drugs was performed in phosphate buffer (80 mM; pH 6.5). The separation of regular insulin and insulin aspart was achieved at 13 kV and detection at 200 nm within 7 min with RSD for the absolute migration time reproducibility of less than 3.8% ($n = 10$). Selectivity, linearity, precision, accuracy, limits of quantification (LOQ) and limits of detection (LOD) were evaluated as the method validation. Calibration plots were linear ($r > 0.999$) over a range of 2.0–60.0 μg/mL for regular insulin and insulin aspart. The LOD were all 1.0 μg/mL (signal-to-noise ratio = 3; injection 6.89 kPa, 7 s). The small amount of sample required and the expeditiousness of the procedure can be an advantageous alternative to traditional methodology for the quantification of tested drugs in individual pharmaceutical products.

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1. Introduction

Human insulin, a pancreatic peptide hormone, is synthesized from beta-cells of the islets of Langerhans, and is secreted into the bloodstream [1]. It plays an important role in regulating the metabolic activities of the body, particularly the homeostasis of the blood glucose. Insulin secretion is a tightly regulated process providing stable concentration of glucose in blood during fasting and feeding. The human insulin consists of 51 amino acids contained within two peptide chains: an A chain, with 21 amino acids; and a B chain, with 30 amino acids. The A and B chains are connected by two disulfide bridges as shown in Fig. 1.

Diabetic mellitus (DM) is one of the most common metabolic diseases in the world. It is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The disease can be treated by administration of human regular insulin produced by recombinant DNA techniques which is structurally identical to insulin produced by the pancreas in the human body. The aim of treatment of DM is to achieve normoglycemic control 24 h per day to prevent or delay the development of late chronic retinopathy, nephropathy, neuropathy and cardiovascular complications [2]. This is difficult to achieve via treatment with human regular insulin preparation due to the natural tendency of human insulin to self-associate with hexamers which delays the absorption of subcutaneous injection. In an attempt to make the absorption rate profile of injected insulin similar to the normal postprandial insulin response, fast-acting monomeric insulin analogues have been developed. Insulin aspart, ultra-short-acting insulin, is a single substitution of proline by aspartic acid at B 28. This change results in insulin aspart having fewer tendencies to self-associate to form hexamer, resulting in being absorbed three times more rapidly from subcutaneous sites into the systemic circulation, leading to improved postprandial glycemic control and more flexibility in meal composition. Human regular insulin is now produced by recombinant DNA techniques. Several methods of large-scale recombinant human regular insulin production using *Esherichia coli* or *yeast* that have been genetically altered by the addition of the gene for human insulin production [3–5] are successfully employed. The production methods include several steps of enzymatic cleavage. Improvements in purification procedures for insulin have reduced or eliminated contaminating insulin precursors that are capable of inducing anti-insulin antibodies. On the other hand, many potential degradation parameters for protein drugs are a complex undertaking, therefore, checking the real amount of insulin products to evaluate the steps throughout the process for correct technology and storage to assure optimal maintenance of safety and efficacy of the protein drugs is very important. Separation of human regular insulin and insulin analogues is difficult due to very slight differences in their mass and charge, however, immunochemical [6] and instrumental analytical methods have been developed for the determination of insulin presence in pharmaceutics or biological samples. Several studies

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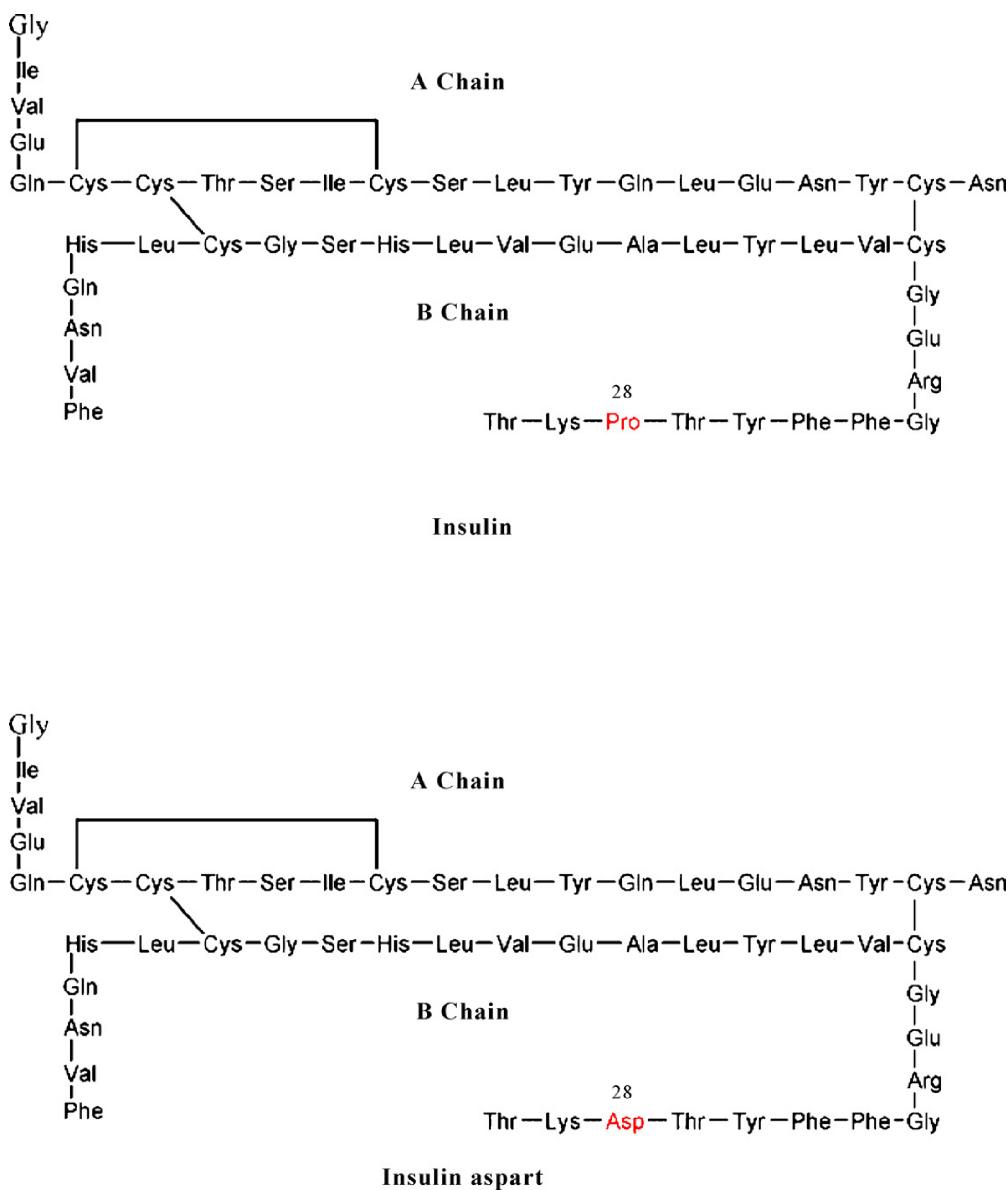

Fig. 1. Chemical structures of regular insulin and insulin aspart.

have been reported in the literature on the use of high performance liquid chromatography in the study of concentration of insulin or insulin analogues with UV detection [7–11], or mass spectrometry [12,13]. With the advantage of a small sample size, high separation efficiency, fast analysis and the low volume of running buffers needed, capillary electrophoresis (CE) has become an attractive alternative for the analysis of therapeutic peptides and proteins. Recently, some CE methods have been used to investigate the separation of insulin and/or insulin analogues with a purity test of insulin [14–17]. CZE mode was used for the separation of insulin and its impurity in solutions [14], and compared with HPLC for the analysis of human insulin and its degradation products [15], or the separation of human and bovine insulin in biological matrix with on-line SPE sample treatment [16]. Ortner et al. [17] described a MEKC with sodium dodecyl-sulphate (SDS) as a micelle-forming agent for the separation of human insulin with five synthetic analogues and no separations could be obtained for all analytes in CZE mode, even human regular insulin with insulin aspart. Regular insulin and insulin aspart are very common in clinical use as short-acting and ultra-short-acting agents, respectively, to achieve acceptable control of blood sugar. So far, no simple and fast separation of CZE method for simultaneous determination of regular insulin and insulin aspart for monitoring the label amount purity of the drugs in pharmaceutical products has been reported.

In this paper, we demonstrate a simple, rapid, accurate CZE method for simultaneous determination of regular insulin and insulin aspart. The method was applied to the determination of regular insulin or insulin aspart in five kinds of commercial insulin injections.

2. Experiments

2.1. Instrumentation

A Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with an UV detector and a liquid-cooling device was used. CZE was performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 40.2 cm (effective length 30 cm) × 50 μm I.D. The temperature of the separation was controlled at 25 °C by immersion of the capillary in cooling liquid circulating in the cartridge, and the temperature of the sample tray was maintained at room temperature. The results were monitored by the on-column measurement of UV absorption at 200 nm (cathode at the detection side). The samples were hydrodynamically injected by 1.0 psi for 7 s, and keeping the separation voltage at 13 kV (anode at the injection end). A Beckman P/ACE MDQ Microsoft Software system was used for data processing.

2.2. Chemicals

Regular insulin and insulin aspart (100 IU/mL, 3.5 mg/mL) were kindly supplied by Novo Nordisk (Denmark) and cefazolin as the internal standard (IS) was purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH), di-sodium hydrogen phosphate dihydrate and phosphoric acid (H_3PO_4, 85%) were supplied by Merck (Darmstadt, Germany). Other agents were analytical-reagent grade. Milli-Q (Millipore, Bedford, MA, USA) treated water was used for the preparation of buffer and related drugs.

2.3. Preparation of electrolyte solutions

The stock solution of 100 mM phosphate buffer was prepared by dissolving 1.78 g of disodium hydrogen phosphate in 100-mL volumetric flask with 50 mL de-ionized water and diluting to volume. Solutions of various phosphate buffers at different pH levels were prepared by neutralizing phosphate solution with 85% H_3PO_4. The final electrolyte solutions containing phosphate buffer (80 mM, pH 6.5) were used for sample analysis. New capillary (50 μm I.D.) conditioning before startup was undertaken with methanol for 10 min, 1 M HCl solution for 10 min, de-ionized water for 2 min, 1 M NaOH for 10 min, de-ionized water for 2 min and running electrolyte for 10 min. The conditioning procedure between runs was rinsing with 0.1 M NaOH (3 min), de-ionized water (2 min) and electrolyte solution (5 min) under positive pressure applied at the injection end.

2.4. Reference and method validation

Stock solutions of regular insulin, insulin aspart and cefazolin were prepared in de-ionized water and suitably diluted with de-ionized aqueous water as reference solutions and as internal standard (IS), respectively. The internal standard was added to decrease the variation of injection. The calibration graphs over the range of 2.0–60.0 μg/mL of regular insulin and insulin aspart with 25.0 μg/mL of cefazolin as IS were established with the peak area ratio of regular insulin, and insulin aspart to cefazolin (IS) as ordinate (y) versus the concentration of regular insulin and insulin aspart in μg/mL as abscissa (x). The precision and accuracy of the method were estimated at low, medium and high concentrations. The intra-day mean precision and accuracy were defined by relative standard deviation (RSD) and relative error (RE) from analyses on the same days. The inter-day precision and accuracy were calculated from repeated analyses of identical samples on five consecutive days for these concentrations of regular insulin, and insulin aspart, and expressed as RSD and RE. The limit of quantification (LOQ) is the minimum injected amount that gives precise measurements and is defined as the sample concentrations generate a peak height ten times the level of the baseline noise. The limits of detection (LOD) were calculated on the basis of the baseline noise, which was defined as the sample concentration generating a peak of height three times the level of the baseline noise (signal-to-noise ratio of 3).

2.5. Applications

Various types of insulin and insulin combinations are currently in clinical use for tight glycemic control. Various premixed combinations of human insulin, such as 70% NPH (insulin protamine) plus 30% regular insulin (Mixtard 30®) or 70% insulin aspart protamine plus 30% insulin aspart (NovoMix 30®), are also available. Each insulin preparation contained 100 international units (IU) of insulin or insulin analogue, which correspond to approximately 3.5 mg/mL insulin or insulin analogue. For the assay of regular insulin in commercial injections (100 IU/mL, 1 IU = 0.035 mg human insulin), including rapid-acting insulin, Actrapid®, intermediate-acting insulin, Insulatard®, and premixed insulin, Mixtard 30®, and the assay of insulin aspart in commercial injections (100 IU/mL, 1 IU = 0.035 mg human insulin aspart), including rapid-acting insulin, NovoRapid®, and premixed insulin, NovoMix 30®, the sample solutions were prepared as follows: accurate measurement of 1 mL of the commercial injections in 10 mL volume flask containing 2 μL of perchloric acid ($HClO_4$) and diluted with milli-Q water to volume. In addition, 85.7 μL mixed solution equivalent to 300.0 μg insulin or insulin analogue was transferred to a 10-mL volumetric flask containing 5 mL 50.0 μg/mL of cefazolin (IS) and then diluted to volume. Perchloric acid was added to precipitate protamine for avoiding the interference of insulin determination.

3. Results and discussion

The strategy of selection of conditions in CE separation of proteins takes into account the specific protein properties which results from their primary structure, number and sequence of amino acid residues. In addition, the protein structure determines its electric charge, size, conformation, hydrophobicity and binding property. Therefore, preliminary testing of regular insulin and insulin aspart standards by capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) was studied at phosphate buffer (pH 8) and phosphate buffer (pH 8) with SDS, respectively. Presumably, the hydrophobic tail of anionic surfactant strongly interacted with regular insulin and insulin aspart; as a result, the analytes were electrophoretically attracted toward the anode and not detected within 8 min. The CZE mode is preferably used for analysis of ionogenic protein and it is based on differences in electrophoretic mobilities which are determined by charge, size and conformation. CZE can provide suitable separation, shorter analysis time and better efficiency than MEKC in simultaneous determination of regular insulin and insulin aspart. Regular insulin and insulin aspart both have two peptide chains (A and B). The only difference is at the 28th amino acid in B chain between regular insulin (MW: 5808) and insulin aspart (MW: 5826). Under alkaline condition (pH 8), insulin aspart possesses higher negative charge and higher ionic volume compared to regular insulin, and the difference of charge/mass ratio between the test drugs makes them effectively separate by CZE. Experiments were performed to determine the optimum conditions that have differences in the effective mobilities of different substances under the electric field. Electrophoretic separation may be carried out in continuous electrolyte systems. The background electrolyte is usually a buffer which can selectively influence the effective mobilities. The dielectric constant, ionic strength and concentration of buffer has

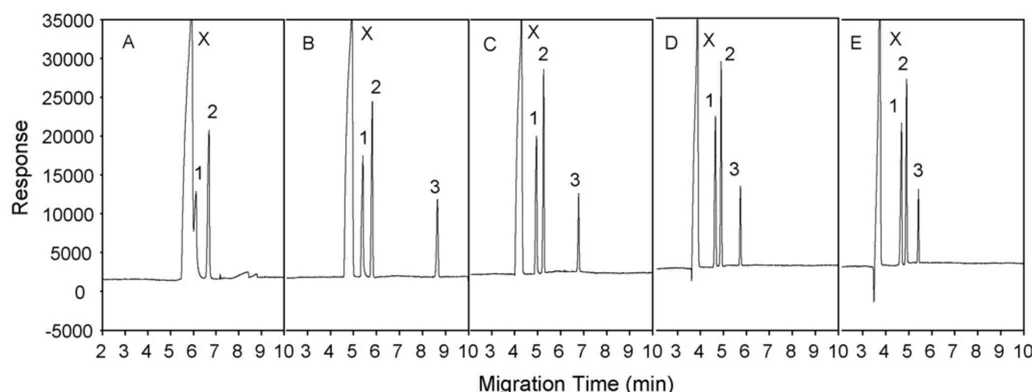

Fig. 2. Effect of pH of phosphate buffer (80 mM) on the migration of regular insulin, insulin aspart and cefazolin (IS). (A) pH 5.5, (B) pH 6.0, (C) pH 6.5, (D) pH 7.0 and (E) pH 7.5. Peaks: 1, regular insulin; 2, insulin aspart. CE conditions: applied voltage, 13 kV (detector at cathode side); uncoated fused-silica capillary, 40.2 cm (effective length 30 cm) × 50 μm I.D.; injection, 6.89 kPa, 7 s; wavelength, 200 nm.

significant effects on solute mobilities and separation efficiency. First, three electrolyte systems, including 50 mM of borate, Tris at pH 9.0 and phosphate at pH 7.0, were tested on the separation of insulin variants in our preliminary study. As compared to the borate and Tris, the phosphate buffer has many significant features such as providing better separation selectivity, peak shape, resolution and sensitivity of the test analytes. Therefore, the phosphate buffer was chosen for the further study.

3.1. Optimization of the separation buffer

The separation of CZE mode is based on the differences in the electrophoretic mobilities resulting in different velocities of migration of ionic species in the background electrolyte contained in the capillary. The charge of species present in the electrolyte system depends on the pH of the solution. Insulin is an acidic protein with a pI value about 5.5. Insulin aspart substitutes aspartic acid for proline at position B 28. The pI values of aspartic acid and proline are 2.97 and 6.10, respectively. With this single acidic amino acid substitution, the isoelectric point of insulin aspart is shifted significantly toward a lower, more acidic pH. The pI value of insulin aspart is about 5.0 as calculated by Software system MassLynx 4.0 from Waters-Micromass (Milford, MA, USA). Insulin aspart possesses higher negative charge than regular insulin. According to the difference of negative charge and molecular weights between regular insulin and insulin aspart, the separation of these insulin analogues is based on the different charge/mass ratio. Above the pI of regular insulin, pH 5.5, insulin aspart was more negatively charged than regular insulin, and it was supposed that insulin aspart would migrate slower than regular insulin due to the higher negative charge.

Separation of tested insulins with a bare capillary in running buffer under the pH higher than pI values is evaluated. The 80 mM phosphate buffers at different pH levels (5.5, 6.0, 6.5, 7.0 and 7.5) for simultaneous determination of the tested drugs were studied. For regular insulin and insulin aspart, therefore, the anionic species dominates at the tested pH in electrolyte solution except for regular insulin at pH 5.5. The peak shape and good resolution of the tested drugs; regular insulin and insulin aspart, showed no significant changes at various pH values, but a significant effect of the peak efficiency and resolution of regular insulin and m-cresol (as a preservative) was obtained at pH 5.5. Owing to the phosphate buffer system of less than pH 5.5, regular insulin is nearly neutral and migrated closely with electro-osmotic flow (EOF), therefore, it was seen overlapped with m-cresol (X) as shown in Fig. 2A. In the pH range of 3.0–7.0, small changes of the buffer pH have an immense effect on the EOF mobility. Above pH 5.5, the insulin and insulin aspart bearing negative charge and their electrophoretic mobilities are lower than EOF. Good resolution of human insulin with m-cresol was obtained under pH > 5.5. However, migration time decreased with increasing pH. Closer migration between regular insulin and insulin aspart was observed above 6.5. When the pH values were 5.5, 6.0, 6.5, 7.0 and 7.5, the resolutions (R_S) between regular insulin and m-cresol, X, were 0.35, 1.27, 2.11, 2.88 and 3.32, respectively; the R_S of regular insulin and insulin aspart were 1.41, 1.60, 1.50, 1.42 and 1.02. The R_S between regular insulin and insulin aspart was 1.50 at pH 6.5 slightly better than 1.42 at pH 7.0. Otherwise, theoretical plate numbers of the tested drugs at pH 6.0, 6.5, 7.0 and 7.5 are 20,100, 30,100, 30,900 and 30,000 for regular insulin and 42,300, 54,900, 50,000 and 47,200 for insulin aspart, respectively. Therefore, pH 6.5 of phosphate buffer was chosen as the optimal pH for simultaneous determination of regular insulin and insulin aspart.

Ionic strength or concentration of the buffer has significant effects on solute mobilities and separation efficiency. The effect of the concentrations of phosphate buffer (60–100 mM) at pH 6.5 on the separation of regular insulin and insulin aspart each at 50.0 and 25.0 μg/mL cefazolin (IS) is demonstrated. The value of R_S obtained for simultaneous determination of regular insulin and insulin aspart in sodium phosphate buffer was 0.9, 1.32, 1.34, 1.43 and 1.48, respectively, at 60, 70, 80, 90 and 100 mM of phosphate buffer. Peak efficiency of the tested drugs showed no significant

Table 1
Regression analyses for determination of regular insulin and insulin aspart.

Concentration range 2.0–60.0 μg/mL	Regression equation	Correlation coefficient (r)
Regular insulin		
Intra-day[a]	$y = (0.0663 \pm 0.0035)x - (0.1134 \pm 0.0171)$	0.999
Inter-day[a]	$y = (0.0677 \pm 0.0024)x - (0.1431 \pm 0.0196)$	0.998
Insulin aspart		
Intra-day[a]	$y = (0.0693 \pm 0.0011)x - (0.0857 \pm 0.0125)$	0.999
Inter-day[a]	$y = (0.0707 \pm 0.0014)x - (0.1011 \pm 0.0153)$	0.999

[a] Intra-day data were based on five replicate analyses and inter-day were from five consecutive days.

changes under 70–100 mM of buffers. The values of generated current were increased with increasing buffer concentration. In order to prevent the generation of extensive Joule heat, a simple CZE mode at phosphate 80 mM, pH 6.5, was used as the optimized condition. Under this CZE condition and 13 kV as a separation voltage, the current produced was about 80 μA in this background electrolyte. The typical electropherogram of the CZE simultaneous separation of regular insulin, insulin aspart and cefazolin (IS) by CZE is shown in Fig. 3A. The repeatability of migration times of regular insulin and insulin aspart was investigated; the observed migration time of regular insulin, insulin aspart and cefazolin were 4.80 ± 0.12, 5.30 ± 0.19 and 6.84 ± 0.20 min, respectively. For regular insulin and insulin aspart, the accuracy of migration time was presented by RSD of 2.5% and 3.5% ($n = 10$), respectively.

3.2. Method validation

The CZE method was validated in terms of linearity, LOD, LOQ, precision and accuracy. To evaluate the linearity of the proposed method, five different concentrations of regular insulin and insulin aspart over the range of 2.0–60.0 μg/mL with fixed concentration of 25.0 μg/mL cefazolin as IS were studied. The linearity between the peak area ratios (y) of the analytes to IS and the concentration of the analytes (x, μg/mL) was investigated. The linear regression lines obtained are listed in Table 1. Good correlation coefficients higher than 0.998 were observed for all straight lines. The precision and accuracy of the proposed method for simultaneous determination of regular insulin and insulin aspart were studied and evaluated as RSD and RE. The repeatability of the proposed method was evaluated by RSD value of the slope of the regression equations. For insulin, the RSD values of the slopes for intra- and inter-day assays were 2.2% and 3.5% ($n = 5$); for insulin aspart, the RSD values of the slopes for intra- and inter-day assays were 1.5% and 1.9% ($n = 5$). The precision and accuracy of the method for simultaneous determination of regular insulin and insulin aspart on intra- and inter-day analyses with three different amounts at low, medium and high concentration levels are shown in Table 2. RSD for precision and RE for accuracy were below 5.8% and 7.2% ($n = 5$). The LOD was determined as concentration giving three times the signal-to-noise ratio and LOQ as concentration causing 10 times the signal-to-noise ratio. Detected at 200 nm, the LOD and LOQ for both insulin analogues were 1.0 and 2.0 μg/mL. The selectivity of the proposed method was briefly tested on the separation of regular insulin and insulin aspart with other insulin analogues including long-acting insulin, insulin glargine and insulin detemir. Under present CZE conditions, a complete separation of regular insulin and insulin aspart from other insulin analogues was obtained as shown in Fig. 4. Peaks 1, 2, 3 and 4 represent regular insulin, insulin aspart, cefazoline (IS) and insulin glargine, respectively; insulin detemir is not seen under this CE condition. Insulin glargine is characterized by an extension of the B chain with two additional arginine molecules (pI 7.0) [18]. In insulin detemir, a fatty acid–myristic acid is connected to the amino acid at position B 29, while B 30 is missing.

3.3. Application

It is important to maintain tight glycemic control close to the normal range, because hyperglycemia can lead to several complications such as retinopathy. A number of tailored regimens can be employed by using short- and intermediate-acting insulin. Therefore, various types of insulin and insulin combinations are currently in clinical use for this propose. The application of the proposed method for the assay of regular insulin and insulin aspart in commercial injections was studied. The uniformity test (a test to evaluate the content variation of the drug in formulations) is usually required by an official pharmacopoeia for quality control

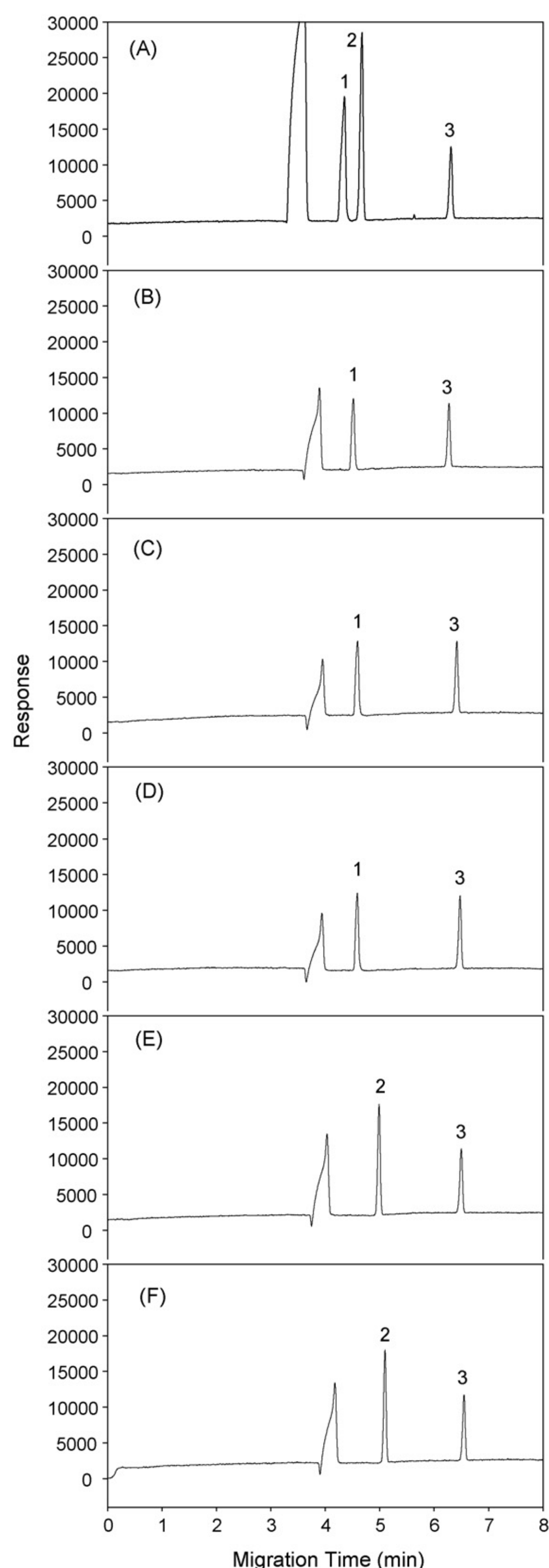

Fig. 3. Electropherograms of (A) regular insulin, insulin aspart and cefazolin (IS) standard at 50, 50 and 25 μg/mL, respectively; (B) regular insulin in Actrapid commercial injection; (C) regular insulin in Insulatard commercial injection; (D) regular insulin in Mixtard commercial injection; (E) insulin aspart in NovoRapid commercial injection and (F) insulin aspart in NovoMix commercial injection. CE conditions: 80 mM phosphate buffer (pH 6.5). Peaks: 1, regular insulin; 2, insulin aspart; 3, cefazolin.

Table 2
Precision and accuracy for the analysis of regular insulin and insulin aspart.

Concentration known (μg/mL)	Concentration found (μg/mL)	RSD (%)	Recovery (%)	RE (%)
Regular insulin				
Intra-day[a]				
5.0	5.19 ± 0.17	3.3	104%	3.8
30.0	31.14 ± 0.10	3.2	104%	3.8
60.0	61.17 ± 1.49	2.4	102%	2.0
Inter-day[a]				
5.0	5.36 ± 0.28	5.2	107%	7.2
30.0	29.39 ± 1.42	4.8	98%	−2.0
60.0	61.88 ± 2.82	4.6	103%	3.1
Insulin aspart				
Intra-day[a]				
5.0	5.16 ± 0.16	3.1	103%	3.2
30.0	29.20 ± 0.30	1.0	97%	−2.7
60.0	60.61 ± 0.90	1.5	101%	1.0
Inter-day[a]				
5.0	5.13 ± 0.30	5.8	103%	2.6
30.0	29.53 ± 0.84	2.8	98%	−1.6
60.0	61.30 ± 1.26	2.1	102%	2.2

[a] Intra-day data were based on five replicate analyses and inter-day were from five consecutive days.

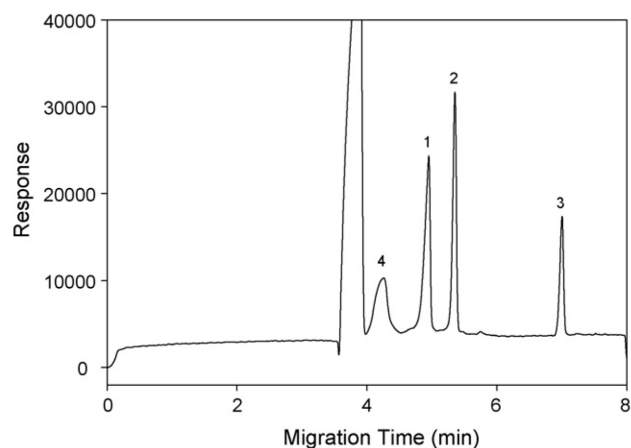

Fig. 4. Electropherogram of the selectivity study. Peaks: 1, regular insulin; 2, insulin aspart; 3, cefazolin; 4, insulin glargine at 50, 50, 25.0 and 50.0 μg/mL, respectively.

of the drug in formulation. The typical electropherogram for the analysis of standard solution of regular insulin and insulin aspart is shown in Fig. 3A. The tested drugs in commercial preparation of Actrapid®, Insulatard®, Mixtard®, NovoRapid® and NovoMix® are shown from Fig. 3B to F, respectively. The results of percentage of claimed content (%) of insulin in commercial insulin injections (Actrapid®, Insulatard® and Mixtard®) and insulin aspart in commercial insulin aspart injections (NovoRapid® and NovoMix®) are shown in Table 3. The analytical values of regular insulin in commercial preparations of Actrapid®, Insulatard® and Mixtard® fell within labeled amounts of 95–102% and insulin aspart in commercial preparations of NovoRapid® and NovoMix® was within labeled amounts of 98% and 96%, respectively.

Table 3
Analytical results for content uniformity of regular insulin and insulin aspart in preparations obtained from commercial sources[a].

Commercial preparation[a]	Concentration found (mg/mL)[b]	Percentage of claimed content (%)[c]
Actrapid®	3.56 ± 0.03	102
Insulatard®	3.45 ± 0.17	99
Mixtard®	3.31 ± 0.22	95
NovoRapid®	3.42 ± 0.13	98
NovoMix®	3.35 ± 0.12	96

[a] Labeled amount of regular insulin and insulin aspart in each commercial injection are 3.5 mg/mL.
[b] Mean ± SD of ten individual analyses.
[c] Content uniformity test is used to check the variation of drug in each dosage form.

4. Conclusion

In the study, we demonstrated a simple, speedy and specific CZE method for simultaneous determination of insulin and insulin aspart. The CZE method has been successfully demonstrated for the assay of insulin and insulin aspart in commercial preparations. We offer a selective and simple method for the separation of structurally highly similar insulin analogues.

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