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Application of the capillary zone electrophoresis (CZE) and capillary gel electrophoresis (CGE) for the separation of human insulin, insulin lispro and their degradation products

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Two capillary electrophoresis (CE) methods have been developed for the separation of charge and mass variants of human insulin and its recombinant analogue lispro. Since the capillary zone (CZE) and Capillary gel electrophoresis (CGE) are based on different principles of separation, they can be used to detect different impurities of insulin and its analogues. Application of CZE enabled a separation of compounds with different m/z ratio, therefore CZE is a suitable method for the separation of deamidation products of insulin. After the optimization, this method is validated according ICH requirements. CGE method was used for the separation of higher molecular weight transformation products. Experimental data have shown that CZE and CGE are simple, fast and robust methods which could be used as a routine analysis for quality control of insulin formulations.

1. Introduction

Human insulin is a globular protein with a molecular mass (*Mr*) of ~5,800 Da, consisting of 51 amino acid residues organized in two polypeptide chains (A and B), linked by two inter-chain disulfide bonds and one intra-chain disulfide bond in chain A (Fig. 1). Insulin exists as a monomer only at low concentrations, while it shows propensity to aggregate into stable dimers at higher concentrations and aqueous solutions at pH 2-8, and into hexamers in the presence of zinc ions. The molecule of insulin consists of ten acidic groups (six carboxylic and four phenolic groups) and six basic groups resulting into a polyvalent acid with *pI* 5.3. (Katsoyannis 1964).

Insulin lispro [INN (28(B)-Lys-29(B)-Pro-insulin)] is an insulin analogue in which the amino acids residues lysine (K) and proline (P), in positions B28 and B29, respectively, have been inverted (Fig. 1). The consequence of this sequence change in the molecule prevents its natural tendency toward aggregation to hexamers, since position B28 is essential for the spatial configuration of the insulin molecule. This leads to insulin lispro characterized by more rapid absorption and shorter duration of action compared with human insulin (Gualandi-Signorini and Giorgi 2001).

In pharmaceutical preparations, during storage and use, insulin can be degraded by two main chemical reactions: deamidation due to hydrolytic reaction and polymerization due to formation of intra-covalent bonding. As a result, the formation of higher molecular weight transformation (HMWT) products takes place (Oliva et al. 2000).

Deamidation is the most prominent non-enzymatic degradation reaction of insulin. It occurs as a result of the removal of the side-chain amide groups in glutamyl (Q) or asparaginyl (N) residues by hydrolysis resulting in free carboxylic acid groups. Consequently, the pI shifts toward the acidic range. Insulin contains six possible residues where this reaction could take place: Q(A5), Q(A15), N(A18), N(B3), and Q(B4). From these residues, three asparagine residues are likely to be the most labile sites, in particular the C-terminal asparagine residue at A21, especially in a low pH environment (Bischoff 1994).

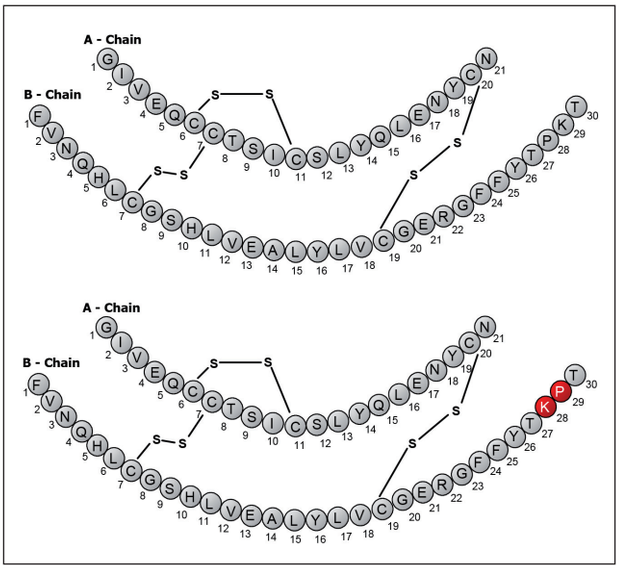

Fig. 1: Structure of human insulin (top) and the insulin analogue, lispro (bottom). The changes in the amino acid sequence are marked in red. (Slightly modified from (www.humalog.com)). The inter-chain disulfide bonds are formed between cysteine residues of chains A and B (residues A7-B7 and A20-B19) whereas the intra-chain disulfide bond is formed between residues, A6-A11, in chain A.

The degradation products formed during this reaction cause a change of the charge and hydrophilic/hydrophobic properties of insulin, which are the key forces controlling its tertiary structure and are responsible for its biological activity (Nilsson and Dobson 2003).

Several analytical techniques such as spectroscopy, chromatography, thermal analysis, electrophoresis, immunoassays, and bioassays are required to completely characterize a protein and examine its degradation profile (Banga 2006). CE has several

Table: Validation data for the CZE method

Pharmaceutical formulation	LOD (mg/mL)	LOQ (mg/mL)	Linear range (mg/mL)	RSD%, Time		RSD%, Area		R^2
				Without IS	With IS	Without IS	With IS	
Human insulin	0.00349	0.01165	0.35-3.5	0.38	0.36	2.63	2.71	0.9958
Insulin analogue Lispro	0.00378	0.01260	0.35-3.5	0.27	0.23	3.1	4.01	0.9982

C = 0.7 mg/ml, n = 10

advantages including simplicity, high speed, excellent resolving power, sensitivity, low sample size requirements, low solvent consumption and ease of automation (Voeten et al. 2018). Moreover, it is an adequate alternative to be employed for peptide and protein separation (Huang et al. 2006; Jacob 2014). Literature data reveal that capillary and related techniques have been recently used for the separation of insulin and its analogues (Haunschmidt et al. 2010; Lamalle et al. 2014; Ortner et al. 2008). However, the data to address the application of these techniques for the degradation study of these products are insufficient. Other advantages of CE, CE-MS methods compared to HPLC include: tiny sample consumption, direct injections of protein samples having high molecular weight, and the possibility of "top-down" proteomics.
The principal aim of this study was to develop an easy and fast CZE/CGE technique for the separation of human insulin, insulin lispro and their degradation products. Aiming to assess the suitability of the aforementioned techniques for the study of the quality of insulins, selectivity, precision, linearity and robustness of the CZE method were assessed.

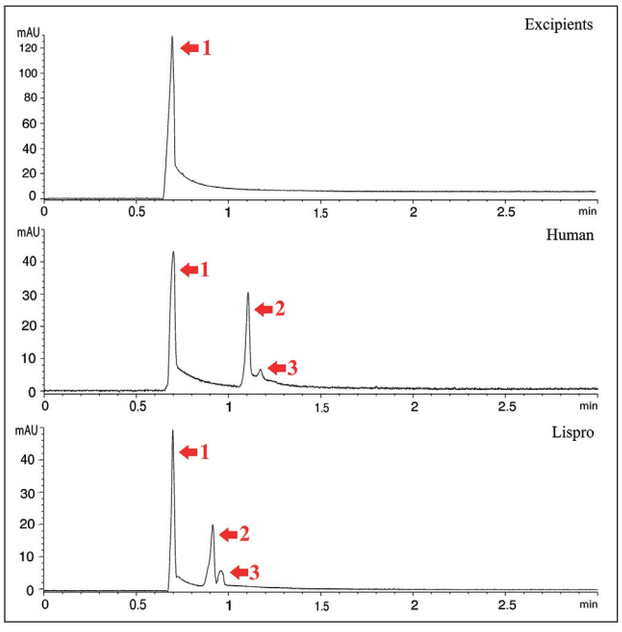

Fig. 2: Selectivity of the method. Measurement conditions: 50 mM ammonium acetate buffer, pH9, l_{eff}=8 cm, i_d.=50 µm, injection: -50 mbar 4 s, -25 kV, λ=210 nm. Peaks: 1=phenol, m-crezol, 2= insulin, 3=desamido insulin.

2. Investigations, results and discussion

2.1. Optimization of the separation buffer

Different capillary zone buffers were used during the optimization step, including phosphate, borate and ammonium formate. The best results were achieved when ammonium acetate was used. An additional reason for selecting this buffer was its compatibility with CE-MS, because of its ability to evaporate. Higher pH was applied (pH 9), since under these conditions the separation of the main impurity was improved. Furthermore, under these conditions the protein adsorption to the capillary wall is prevented. Application of BGE with extremely low or high pH conditions results in reduced interactions of proteins with capillary wall (Dawod et al. 2017). Moreover, the buffer strength that delivered the best EOF was 50 mM.

2.2. Validation of CZE method

In order to assess the suitability of the method for its intended use, the following validation characteristics were assessed: selectivity, precision (migration time and peak area), linearity range, limits of detection (LOD) and limits of quantitation (LOQ)(ICH 1994).

2.2.1. Selectivity

The selectivity of this method was proven by its ability to separate the main compound from its excipients and the main degradation products. To demonstrate the selectivity of this method, diluting solutions which contained the same excipients as those present in the pharmaceutical preparations were used. Representative electropherograms were generated to show that excipients present in the diluting fluid were not interfering with the signal from insulin and desamido insulin degradation products (Fig. 2).

2.2.2. Precision

To characterize the repeatability of the analysis, the precision measured under identical conditions was studied. The use of internal standards (IS) can be essential to improve precision in CE. The internal standards are usually used to compensate injection errors, temperature and viscosity variations (Wätzig et al. 1998).

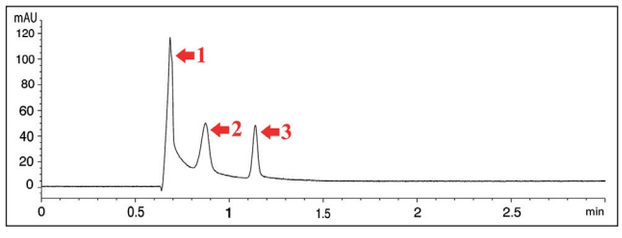

Fig. 3: Electropherogram of human insulin analysis using cinnamic acid as an internal standard. Measurement conditions: 50 mM ammonium acetate buffer, pH9, l_{eff}=8 cm, i_d.=50 µm, injection: -50 mbar·4 c, -25 kV, λ=210 nm. Peaks: 1=phenol, m-crezol, 2= insulin, 3=cinnamic acid.

For enhanced compensation of changes rate of EOF, it is preferred to use two IS, which migrate one before, and the other after the main component. But, in this case it was not possible because the excipient migrates before the main component of the formulation (phenol, m-crezol), hence the IS could interfere with them. Therefore, cinnamic acid was selected as IS. The cinnamic acid migration time was subsequent to the migration times of insulin and its degradation product (Fig. 3). In the Table, the data from comparison of RSD values of migration times and peak areas using IS, and without using IS for compensation are presented. These data were obtained from elecropherograms of 10 replicates. In both cases,

for human insulin and lispro, slightly improved compensation in migration times could be observed. Thus, this indicates that the application of IS provides improved reproducibility of the analysis. On the other hand, no considerable differences were found in RSD values of peak areas obtained with and without internal standards. The reason for the absence of improvement in the compensation of peak areas might be that the application of the internal standard that migrates before the peak of the active ingredient was not possible. Given that the purpose of the analysis primarily consisted on the identification purpose and not the quantitation, the internal standard used was appropriate.

2.2.3. Linearity range

To determine the linear range of the method, seven standard solutions were prepared at increasing concentrations of 0.035, 0.175, 0.35, 0.875, 1.75, 2.625 and 3.5 mg/ml, for both human insulin and insulin lispro. In order to quantify each insulin, the total peak area was used for calculation. The peak areas were found to be linear (R^2>0.998) in the concentration range specified in Table 1.

2.2.4. Limit of detection and quantitation

Limit of detection was calculated based on the standard deviation of the analytical response and slope of the calibration curve, according to the ICH criteria (www.ich.org).

2.2.5. Robustness

A robustness test was performed to prove the reliability of the method. Changes in buffer strength (50±5 mM), temperature (25±2 °C), pH of the buffer (9±0.3) and voltage (25±2 kV) were tested. Retention times and peak areas were measured for both analyzed products subjected to these operational changes. Experimental data showed that any deliberate variation of these experimental factors could not influence the analytical results.

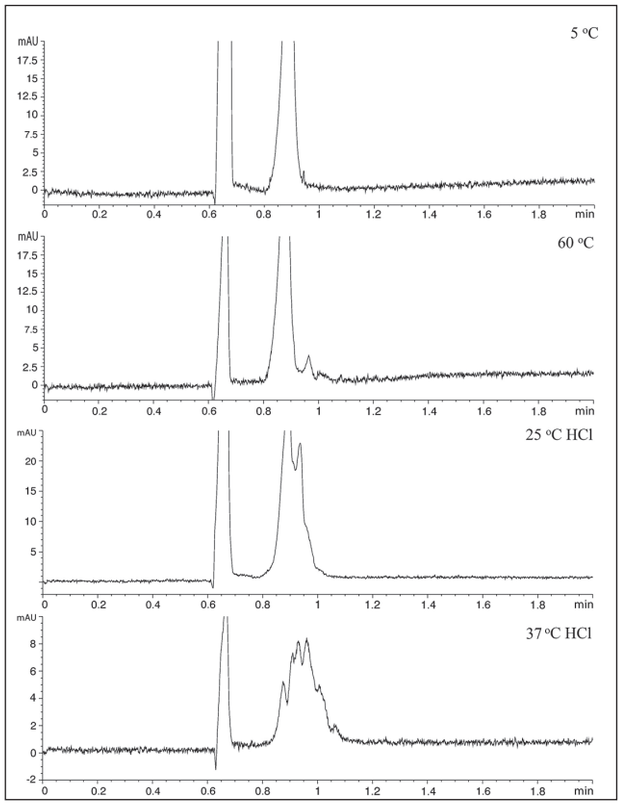

Fig. 4: Degradation of human insulin under different storage conditions. Measurement conditions: 50 mM ammonium acetate buffer, pH9, l_{eff}=8 cm, i_d=50 µm, injection: -50 mbar·4 s, -25 kV, ⊠=210 nm

2.3. Separation and identification of desamido insulin

Experimental data showed that CZE was able to separate charge variants of insulin and its analogues. Desamido insulin is a negatively charged variant of insulin; therefore, it is expected to migrate after the active compound, insulin.

In order to analyze the main impurities, pharmaceutical formulations of insulin were stored under acidic conditions, pH 2, at room temperature, and at 37 °C. In addition, one sample of each formulation was stored in pH 7, at 60 °C. After 3 days of incubation, several degradation products could be detected.

Deamidation is one of the most common protein degradation pathways. This reaction has been used as a marker for the stability of protein drugs during the stability indicating assays. Deamidated degradants have no influence on the potency and immunogenicity of the final product, and according to the literature data they demonstrate almost the same potency as native human insulin and/or its analogues (Moslemi et al. 2003). As seen in Figs. 4, 5, application of the developed and optimized capillary electrophoresis method resulted in the efficient separation of insulin/insulin analogue and their main impurities in less than 2 min, and analysis time is the obvious advantage in comparison to RP-HPLC which currently is an official pharmacopoeia method (European Pharmacopoeia 9th Edition 2016).

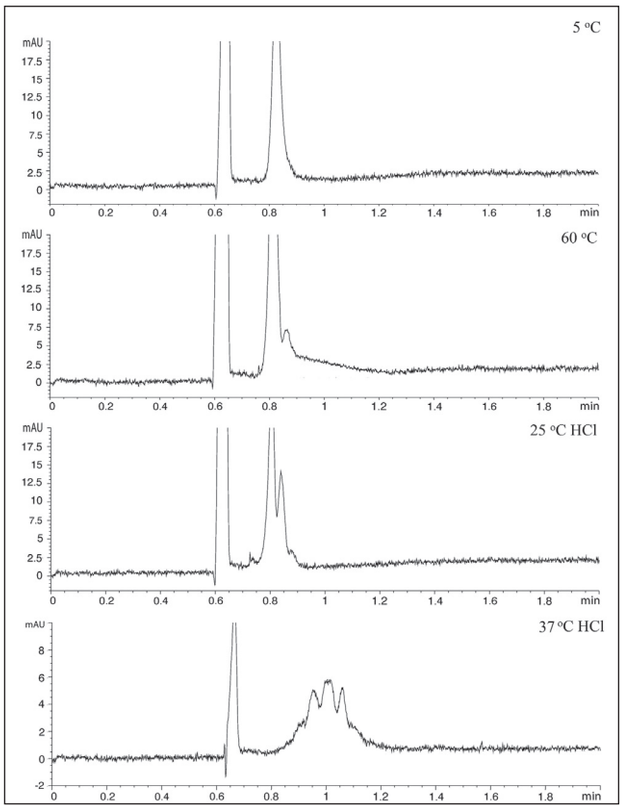

Fig. 5: Degradation of insulin lispro in different storage conditions. Measurement conditions: 50 mM ammonium acetate buffer, pH 9, l_{eff}=8 cm, i_d=50 µm, injection: -50 mbar·4 s, -25 kV, λ=210 nm

CZE analyses were performed using short end capillaries in order to decrease the analysis time.

CZE/MS analysis was used for the identification of the separated products. However, for CZE/MS analysis, effective length of capillary was longer due to its linked part with the MS instrument. Therefore, in these analyses the migration times of components are longer than using CE-UV measurements.

Insulin and its degradation products were identified by a simulation of the isotopic pattern for each molecule using empirical formulae.

Since they contain the same amino acid residues, both insulin and its analogue lispro have the same empirical formula, provided that in the lispro analogue lysine and proline have switched their positions. The empirical formula of human insulin and lispro-insulin is $C_{257}H_{383}N_{65}O_{77}S_6$. Empirical formula of desamido products is $C_{257}H_{382}N_{64}O_{78}S_6$. The change in the *Mr* during this reaction is less than 1 Da. Insulin and its degradation products were identified by using the software to simulate the isotopic pattern based on its empirical formula. Therefore, for the identification purpose, the simulated isotopic pattern was compared with the measured isotopic pattern.

Since the applied MS methods could only identify 1200-5000 m/z and 300-2200 m/z, in these measurements only insulin and its degradation products were identified. However, excipients such as phenol and m-cresol were too small to be noticed in the electropherograms.

As presented in the Fig. 6, in the human insulin sample, insulin and its degradation product have been separated. Simulation of the isotopic pattern suggests that the second peak pertains to the deamidation product of human insulin.

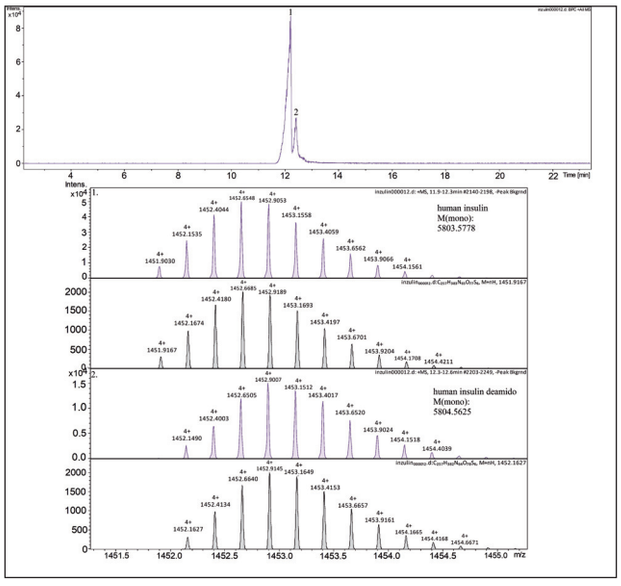

Fig. 6: Electropherogram of analysis of human insulin using CE-MS (top) and simulation of the isotopic pattern to identify the peaks (bottom).

Similarly, for insulin lispro, in both cases (high temperature and low pH) the identification of the formation of desamido insulin-lispro was possible.

2.4. Application of capillary gel electrophoresis (CGE)

CGE was used to separate the higher molecular weight transformation products. These products are unable to be separated by CZE, because in the case of dimer and other polymeric complexes of insulin, the *m/z* ratio is still the same as the monomer of insulin. CGE is able to separate components depending on their mass.

In Fig. 7, the eletropherograms of insulin formulations subjected to different storage conditions are presented. The separation of human insulin from its impurities of different *Mr* is evident. A higher amount of insulin covalent dimer is detected in samples irradiated with UV light and samples stored at 60 °C. The covalent dimer could be detected since CGE separation was carried out by non-reducing conditions, therefore the aggregates remain linked by disulfide bonds. In addition, compounds of lower *Mr* than insulin which are likely to be the result of fragmentation of molecule are observed in electropherogram obtained from the formulation samples subjected to shear stress (shaken for 3 days using the magnetic stirrer at 880 rot/min).

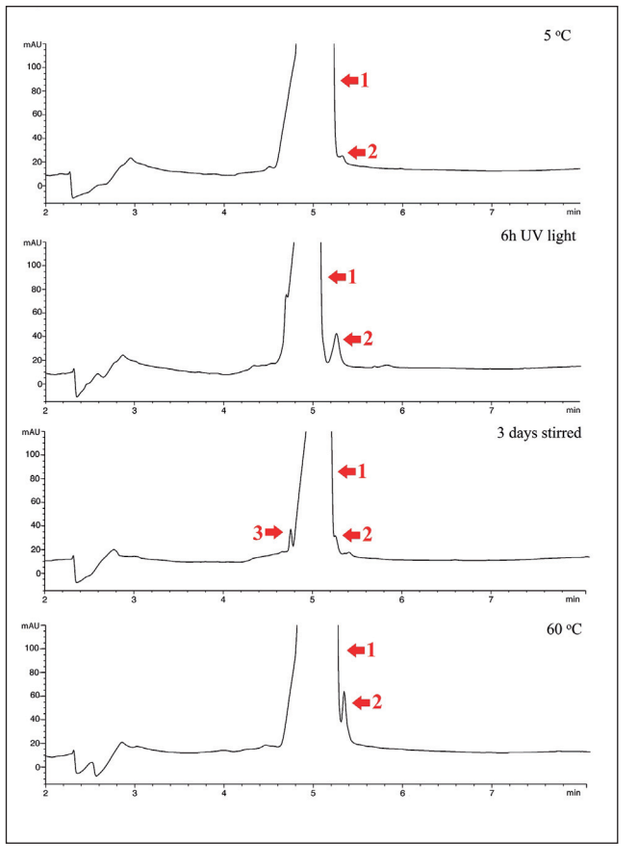

Fig. 7: Degradation of human insulin in different storage conditions. Measurement conditions: Beckman-SDS-MW buffer, l_{eff}=8 cm, i.d.=50 µM, injection: +7.5kV·50 s, 15 kV, λ=200 nm. Peaks: 1=human insulin, 2=covalent insulin dimer, 3=fragmentation product.

In summary, in the present study the suitability of CZE and CGE method could be successfully demonstrated for the assessment of quality of pharmaceutical formulations containing human insulin short-acting analogue, lispro. In order to assess the stability, in regard to degradation caused by deamidation of active ingredients, pharmaceutical formulations in this study were subjected to exaggerated storage conditions. The study results showed that, apart from the active ingredients, these methodologies were suitable for the identification of their deamidated degradation products. The deamidation products separated using CZE were successfully identified with CE-MS. Experimental data documented that CZE can be applied as a simple, fast and robust method for the quality assessment of insulin preparations, in regard to the identification of charge variants. On the other hand, CGE can be suitable for the study of molecular mass variants of insulin and its analogue, lispro.

3. Experimental

3.1. Chemicals and reagents

All reagents used were of analytical grade. Ammonium acetate, ammonia, isopropanol, acetic acid, hydrochloric acid and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). CGE separations were performed in sieving matrix gel buffer (SDS-MW, Beckman Coulter, Inc., Fullerton, CA, USA) and samples were diluted with Beckman sample buffer. All samples were filtered using the syringe membrane filter. Pharmaceutical formulations containing insulin and analogues-Actrapid® Penfill®(Novo Nordisk) and Humalog®(Lilly), NovoRapid® Penfill®(Novo Nordisk) were used.

3.2. Instrumentation

The CE instrument was a 7100 model (Agilent, Waldbronn, Germany). Electrophoretic separations were carried out using fussed silica capillaries (Polymicro Technology, Phoenix, AZ, USA) with an internal diameter of 50 µm and a total length of 50 cm (8 cm effective length) in negative mode using constant voltage (25kV for CZE and 15kV for CGE).

For CE-MS analysis capillary electrophoresis instrument (7100 CE System, Agilent, Waldbronn, Germany) coupled with an electrospray mass spectrometer (maXis II UHR ESI-QTOF MS instrument, Bruker, Karlsruhe, Germany) was used. Hyphenation was performed with a CE-ESI Sprayer interface (G1607B, Agilent). Sheath liquid was transferred with a 1260 Infinity II isocratic pump (Agilent). The CE instrument was operated by OpenLAB CDS Chemstation software.

3.2.1. CZE method

Before the first use the capillary was washed with 0.1 M NaOH (15 min), with water (15min) and with the buffer electrolyte (10 min). At the beginning of each working day, the capillary was flushed with 0.1M NaOH for 15 min. For CZE separations between each injection, the capillary was preconditioned for 5 min with the running buffer and post conditioned with 70 mM SDS for 3 min, 1 M NaOH for 5 min and the buffer electrolyte for 4 min to remove all possible adsorbed materials. The samples were introduced at the cathodic end of the capillary; injections were performed using -50mbar pressure for 4s. Since the proteins tend to adsorb on the inner surface of the capillary, high pH (pH 9.0) was applied.

3.2.2. CGE method

For CGE separations the precondition procedure was 1 M NaOH for 10 min, 1 M HCl for 7 min and the sieving matrix for 25 min. The samples introduced using +7.5kV for 50 s. The detection of insulin and insulin analogs was carried out by the on-column DAD measurement at 214 nm. The electropherograms were recorded and processed by the Agilent ChemStation B.04.02 computer program.

3.2.3. CE-MS analysis

The parameters for the capillary zone electrophoretic separation were: capillary: 90 cm × 50 µm fused silica; background electrolyte: 50 mM ammonium acetate; applied voltage: 25 kV; hydrodynamic injection: 200 mbar; sheath liquid: isopropanol:water = 1:1 containing 0.1% acetic acid; and sheath liquid flow rate: 6 µL/min.
Two different MS methods in the positive mode were used to follow the separations in two different mass ranges: 300-2200 m/z and 1200-5000 m/z, that were tuned according to these mass ranges. Applied spectra rate was 3 Hz in both cases. Some notable parameters for the ion source for 1200-5000 m/z method were: capillary voltage: 4500 V; nebulizer pressure: 0.3 bar; dry gas temp: 200 °C; and dry flow rate: 5 L/min. For 300-2200 m/z method, notable parameters were: capillary voltage: 4500 V; nebulizer pressure: 0.3 bar; dry gas temp: 220 °C; and dry flow rate: 8 L/min.
The calibrant was injected after each separation, enabling the internal calibration of each individual analysis. Mass spectra were recorded by otofControl version 4.1 (build: 3.5, Bruker) and processed by Compass Data Analysis version 4.4 (build: 200.55.2969).

3.3. Buffers and BGE

The buffer used for CZE was 50 mM ammonium acetate pH 9. It was prepared by dissolving ammonium acetate at a concentration of 50mM, and pH was adjusted with ammonia. For CGE, the buffer used was a commercially available sieving matrix gel buffer (SDS-MW) by Beckman Coulter.

3.4. Sample preparation

The concentration of pharmaceutical formulation used was 100IU/ml. This corresponds to 3.50 mg/ml of both human and lispro insulins. For CZE, applied samples were diluted 5 folds with distilled water, to obtain a final concentration of approximately 0.7 mg/ml. For CGE, samples were diluted 2 folds; therefore, the final concentration was 1.75 mg/ml. In this case, for dilution a Beckman sample buffer was used.

3.5. Degradation studies

For the degradation studies, insulin pharmaceutical formulations were subjected to different storage conditions, including acidic conditions (pH 2) at room temperature and at 37 °C, high temperature (60 °C) and UV irradiation (365 nm). Samples were subjected to a continuous motion system at 880 rot/min at room temperature and they were analyzed at different time points, depending on storage conditions.

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Conflicts of interest: All authors declare that they have no conflict of interest.

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