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Comparative study on the deamidation of three recombinant human insulins using capillary electrophoresis

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ABSTRACT

The applicability of capillary zone electrophoresis (CZE) for the separation of different recombinant human insulins and their deamidated isoforms was studied. The high resolving power of CZE is demonstrated by its ability to separate insulin isoforms differing only by 0.984 Da (different-fold deamidated forms) and even components having the exacts same mass but slightly different shapes (same-fold deamidated forms). From among the several insulins available, humulin, glargine and glulisine were selected for our study because their sequences and chemical parameters are quite similar, however, the small differences present in their amino acid sequences influence the deamidation processes. Using a background electrolyte with basic pH was favourable not only for the separation of the different types of insulin but also for the separation of deamidated protein forms even in a bare fused silica capillary. The LOD values ranged between 0.6 - 0.93 mg/L and 2.17 - 4.37 mg/L for UV and ESI-MS detection, respectively. At -20 - -80 °C, the deamidation is minimal, but at temperatures above +5 °C deamidation is accelerated. At +5 °C only 1-fold deamidation forms could be observed for each insulin. Acidified samples incubated for 1-month at room temperature showed varying levels of deamidation: 1-fold, 1–2-fold and 1–2–3-fold forms for glargine, glulisine and humulin, respectively.

1. Introduction

Insulin is a protein hormone consisting of 51 amino acid residues and is a lifesaving drug for people diagnosed with diabetes. It is a key player in maintaining proper metabolism, cell growth and glucose homeostasis [1]. The most extensively used technique to produce insulin as a pharmaceutical is recombinant DNA technology, which results in the formation of several post-translational modifications (PTMs) during the different stages of insulin manufacturing and storage. These modifications can lead to altered biological activity, stability and immune response of the final drug. It is highly important to control these PTM formations to ensure final product quality and patient health. From among the major PTMs (glycosylation, deamidation, methionine oxidation, etc.), the most common type of (non-enzymatic) degradation is deamidation [2,3]. The mechanism of deamidation involves the formation of a free carboxylic acid by the removal of an amide group from glutamine (Q) and asparagine (N) residues via hydrolysis. As a consequence of deamidation a + 0.984 Da shift in the mass spectrum associated with the conversion of $–NH_2$ to –OH can be observed. The rate of formation of aspartic acid and isoaspartic acid from asparagine via succinimide as an intermediate is faster than that of glutamic acid from a glutamine residue through the glutarimide intermediate. Both residues undergo the same mechanism but at different rates, therefore deamidation of asparagine is more common than that of glutamine [3]. The structure of human insulin consists of chain A (21 amino acid residues) and chain B (30 amino acid residues). Insulin contains two interchain disulfide bonds between the A and B chains (A7-B7 and A20-B19), and one intrachain linkage in the A chain (A6-A11). The possible deamidation sites in insulin are A5(Q), A15(Q), A18(N), A21(N), B3(N) and B4 (Q). However, predominantly the A21(N) product variant is formed in the production process and storage of human insulin. This is due to the positioning of A21(N) at C-terminus, which is the most exposed region in the hexameric structure of human insulin [4]. Deamidation of glutamine at position B4 has also been reported in the case of human insulin [5] and insulin glargine [6]. Multiple factors affect the amount and rate of the deamidation of insulin [3]. These factors can be the primary and higher order structure of the protein at the time of manufacturing and storage, pH of formulation, shaking while handling and transportation, storage temperature, etc. [3,7,8].

The separation of the different-fold deamidation forms from each

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other and from the non-deamidated form is not easy (even if the deamidation generates an extra charge in the molecule) because the molecular mass or the hydrophilic/hydrophobic features of these components are only marginally different. However, if the components are the same-fold deamidated forms, then the net charges and the molecular masses are identical and only the shapes of the molecules may differ slightly. There have been several chromatographic techniques applied for deamidation studies. Ion exchange (IEX) liquid chromatography in the form of both cation [9–11] and anion exchange modes [4, 12] is widely used for detecting deamidation. Although reverse phase liquid chromatography is compatible with MS and is extensively used, its separation capability of charge variants remains an issue [13]. Electrophoresis is yet another powerful approach for the separation of charge variant species. The principle of the electrophoretic separation is based on isoelectric point or net charge of the components for capillary isoelectric focusing (CIEF) [14,15] and capillary zone electrophoresis (CZE) [16–24], respectively. Both techniques allow high resolution separation, with CZE having the added advantage of being more MS-compatible [13]. There are studies where CZE was employed for human insulin fragment analysis [25] and for assessing the binding constant of hexameric human insulin with other components [26]. CE-MS was only recently applied for the determination of human insulin deamidation [17].

In our earlier work we demonstrated a CZE method that can be efficiently applied for monitoring the degradation of human insulin and even 10 deamidated forms were separated and quantitatively determined. For this present study, along with human insulin, we took two other insulin analogues, glargine and glulisine (a schematic comparison of the structures of three recombinant human insulins is shown in Fig. 1 and Table S-1). These insulin variants are protected against deamidation at the A21 or B3 residues, respectively. Deamidation is supposed to be the highest in the case of human insulin followed by glulisine and glargine when incubated in acidic medium, since the degradation of the A21 residue is faster than that of B3 [6-8,27-29]. To confirm this assumption and to study hydrolysis patterns at the different sites in comparison to human insulin, these two analogues seemed to be good choices for our study. The goal of our work was also to study the effect of temperature on the deamidation of the three insulins studied and to compare the rates of deamidation occurring in these insulin analogues.

2. Materials and methods

2.1. Reagents and materials

All reagents used were of analytical grade. Methanol, ammonium acetate, acetonitrile (ACN), propan-2-ol (isopropyl alcohol, IPA), sodium hydroxide (NaOH), hydrochloric acid (HCl) and ammonium hydroxide solution were procured from Sigma Aldrich (St. Louis, MO, USA), and dilutions were prepared prior to use in MilliQ water (Millipore Synergy UV). Human insulin (Humulin R, 3.5 mg/mL) was purchased from Lilly (France), glulisine (Apidra, 3.5 mg/mL) and glargine (Lantus, 3.5 mg/mL) were purchased from Sanofi (Germany). Most often the background electrolyte (BGE) was 50 mM ammonium acetate (Sigma) in 20% v/v 2-propanol at pH=9.0. For the preparation of other BGEs, formic acid (Sigma) was diluted to 1 M concentration; solid ammonium acetate was dissolved and its pH was adjusted to the desired value by titration with 25% m/m ammonium hydroxide solution. A membrane filter (0.45 μm pore size) was used to filter all solutions and filtered solutions were stored at +4 °C. Running buffers were kept in ultrasonic bath for minimum 5 min to remove gasses. For first time use, the fused silica capillary was conditioned with 1 M NaOH (20 min) followed by de-ionized water (10 min) and then running buffer (20 min).

2.2. Degradation of insulin samples

Forced degradation of the human insulins was performed by acid catalysis. Final working concentration (0.1 M HCl) was obtained by mixing insulin with 6 M HCl solution. The acidified insulin solutions kept at −80 °C, −20 °C, +5 °C and +25 °C were allowed to be forcedly degraded for 31 days and then analyzed at different time points.

2.3. Measurements with CE

For analyses, CE instrument (7100 model, Agilent, Waldbronn, Germany) with UV and MS (maXis II UHR ESI-QTOF MS, Bruker, Bremen, Germany) detection was used. For CE-UV measurements, fused silica capillaries (Polymicro, Phoenix, AZ, USA) of 85 cm x 50 μm I.D., 370 μm O.D. and effective length of 76.5 cm were used. On-capillary photometric measurement (detection wavelength: 200 nm) was utilized. Samples were injected hydrodynamically (50 mbar, 2 s) at the anodic side of the capillary. BGE contained 50 mM NH_4Ac with 20% (v/v) IPA. Capillaries were preconditioned with 1 M NaOH for 10 min, followed by ACN for 5 min and finally with BGE for 8 min. +25 kV voltage was applied for the separations. OpenLAB CDS Chemstation (Agilent) software was used for both controlling the CE instrument and data processing.

For MS detection, a CE-ESI interface (G1607B, Agilent) provided online hyphenation to the CE instrument. An isocratic pump (1260 Infinity II, Agilent) was used for transferring the sheath liquid. MS instrument was operated by otofControl version 4.1 (build: 3.5, Bruker). For CE-MS measurements, the following parameters were used: fused silica capillary: 120 cm x 50 μm I.D. and 370 μm O.D; voltage applied: +25 kV;

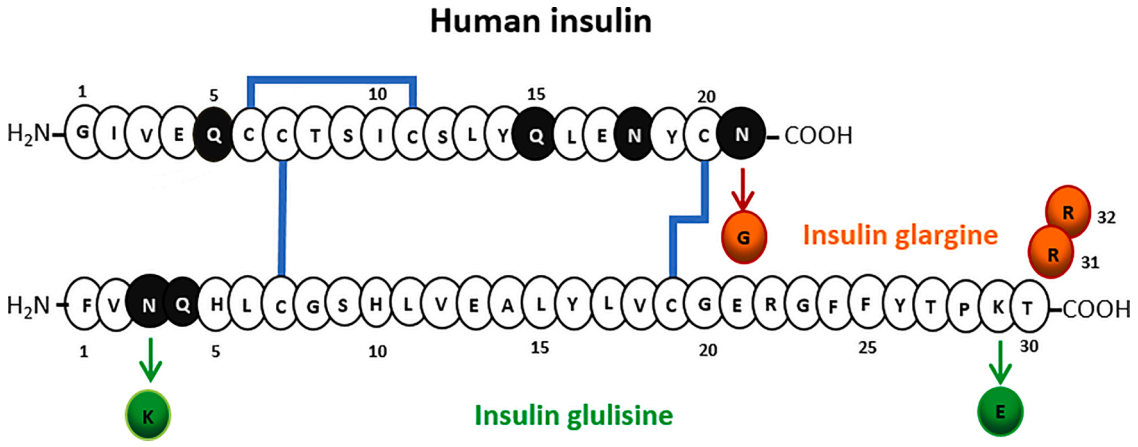

Fig. 1. Structure of human insulin and its analogues. Alterations for insulin glargine and glulisine are shown in red and green, respectively. Blue lines represent the disulfide bridges within the molecule. The six possible sites of deamidation of human insulin are marked in black.

sample injection (hydrodynamic): 50 mbar for 6 s; sheath liquid (SL): IPA:water= 1:1 with 0.1% formic acid (v/v); SL flow rate: 7 μL/min. The capillaries were preconditioned with the BGE and post-conditioned with ACN and BGE for 2 min each. MS parameters: positive ionization mode; dry gas flow rate: 8 L /min; dry gas temperature: 220 °C; nebulizer pressure: 0.4 bar; capillary voltage: 4500 V; spectra rate: 3 Hz; end plate offset: 500 V; mass range: 800–2200 *m/z*. The syphoning effect generated by the nebulizer gas flow was decreased by turning off the nebulizer gas pressure for 5 min at the beginning of each run. For internal *m/z* calibration, Na-formate calibrant was introduced after each separation. Compass Data Analysis version 4.4 (build: 200.55.2969) was used for processing the mass spectra obtained.

3. Results and discussions

3.1. CZE separation of three recombinant human insulins and their deamidation isoforms

It is widely accepted that the separation of intact proteins is to be carried out in coated capillaries to avoid the adsorption effects, although proteins can often show wide, distorted peaks even in these capillaries. In our earlier work we demonstrated that the separation in bare fused silica capillaries using low pH (pH=1.8–2) can provide similar separation efficiencies to those of different coated capillaries (linear polyacrylamide (LPA) or polybrene (PB)) [17]. In bare fused silica (BFS) capillaries the adsorption effects can also be kept low by applying basic BGEs, since the electrostatic interactions are limited between the negatively charged capillary surface and the proteins having a net negative charge (the pI values of insulins are below 7).

In CZE, the separation is based on the difference in charge relative to molecular size. While at neutral pH (close to the pI of the insulins) only one peak appears at the EOF, which includes all insulins (and the m-cresol preservative), the use of more extreme pH values (pH=2 and pH=10) allows the partial separation of the three insulin analogues (Fig. 2). The complete separation could be achieved at pH=9 when the BGE included 20% 2-propanol, as well (adding organic solvents like acetonitrile, methanol or 2-propanol to the BGE is advisable [29,30]). Proteins are denatured/coagulated at highly alkaline conditions, the larger peak broadening (compared to the peak shapes obtained at pH=9) is probably a consequence of the changes of structural stability of the proteins (Fig. 2A and 2B). The considerably smaller migration times obtained at pH=10 are caused by the increase of the EOF in more alkaline media (Fig. 2B), but the EOF was largely reduced by the presence of organic solvent in the BGE (Fig. 2A). Since our intent was to separate not only the three insulins but possibly also their numerous deamidation isoforms, a large resolution was demanded.

The basic pH was favourable for the separation of the insulins as well as their deamidated isoforms, because the carboxyl group(s) formed via deamidation added negative charge(s) to the peptide at this pH. In Fig. 3, the separations of insulins and their deamidated isoforms are shown. The separation of these components demonstrate the high resolving power of CZE, since the separated components differ only by 0.984 Da (different-fold deamidated forms) and even components having slight alterations only in their shapes (same-fold deamidated forms) could be resolved. The three insulins studied showed different levels of deamidation (1–3). The variability in the number of deamidated forms follows from the different numbers of asparagine and glutamine residues present in the studied insulins. If there are 6 potential deamidation sites then theoretically 6 different one-fold deamidated forms might be separated/detected, however, the probability of deamidation (the obtained detection signal intensity for a given deamidation form) can be very small in some cases due to the chemical structure around the deamidable group. In the electropherogram corresponding to humulin, 5 one-fold deamidated components could be clearly separated and detected (Fig. 3A). If the number of the deamidable groups is 5, there are 10 theoretical variations in the 2-fold deamidation forms. Since the majority of these large number of components are present in low concentration and those might be overlapped, a lower number of components could be detected (e.g., 8 components form the theoretical 10 (Fig. 3C).

We tried to separate the three insulins and their deamidated forms in the same CZE run, however, it was possible only for two insulins that

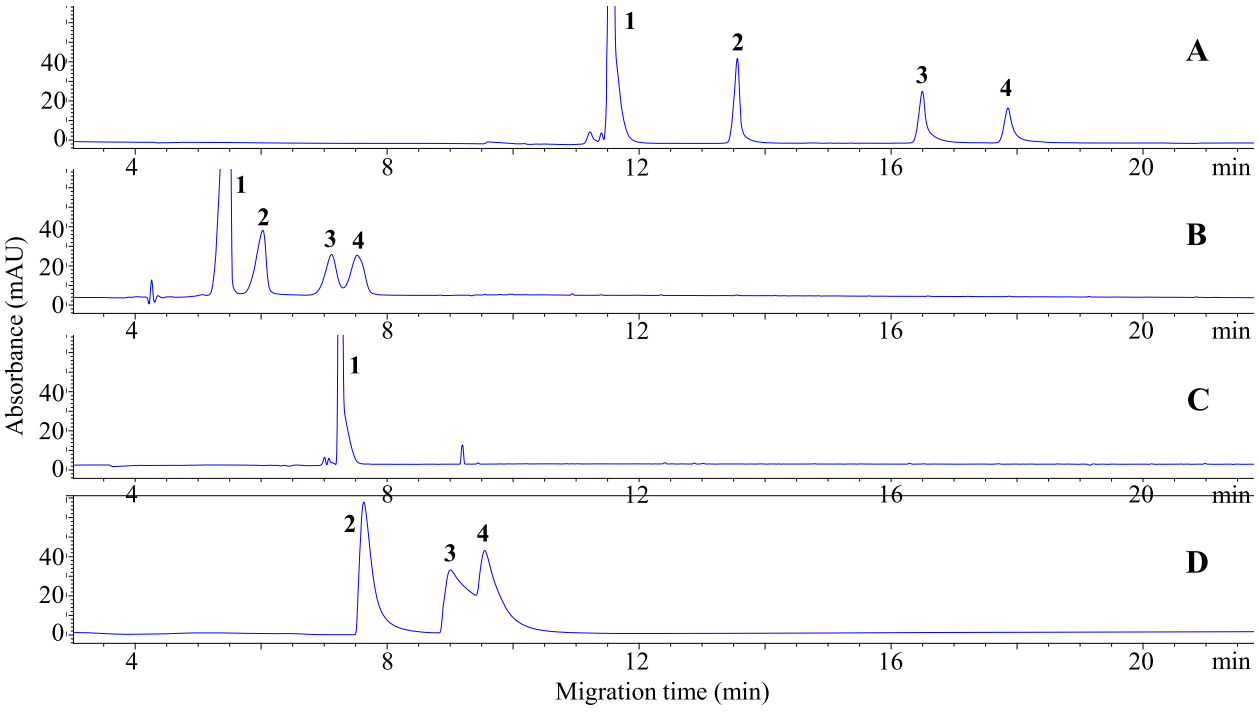

Fig. 2. The separations of three recombinant insulins by CZE. Conditions: capillary l_{tot}: 65 cm x 50 μm id, l_{eff}: 56.5 cm, BGE: 50 mM NH_4CH_3COO + 20 v/v% IPA, pH = 9.0 (A), pH = 10.0 (B), pH = 7.0 (C); 1 M HCOOH, pH = 2.0 (D), separation voltage: 30 kV, injection: 50 mbar x 2 s, preconditioning: 5 min 1 M NaOH, 5 min 1 M HCl, 8 min BGE washing, detection was performed with UV detection at 200 nm. Sample: 1: m-cresol, 2: glargine, 3: humulin, 4: glulisine (c = 1.1 mg/mL).

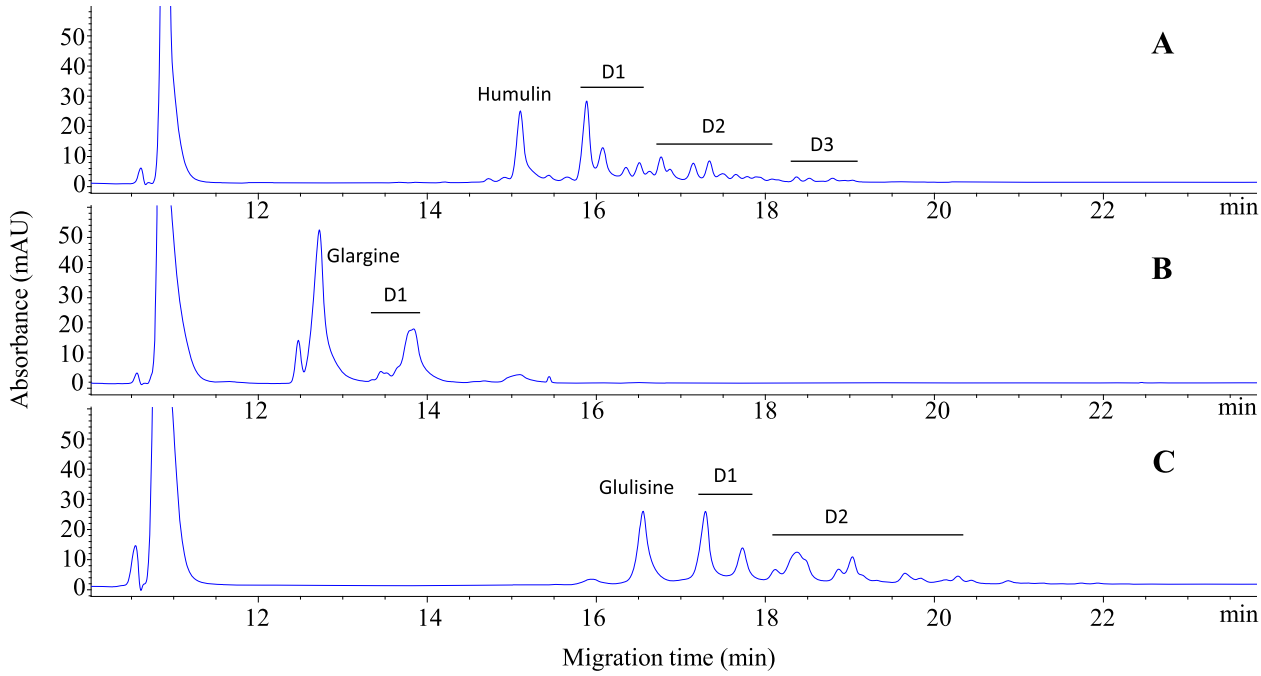

Fig. 3. Study of the formation of deamidated products of recombinant insulins (humulin (A), glargine (B), glulisine (C)). The analyzed insulin samples were acidified (pH = 1) and stored for 31 days at room temperature. Deamidated isoforms are marked as D1-D3. Analysis conditions were the same as stated in Fig. 2.A.

have larger differences in their mobilities (e.g., glargine and humulin or glargine and glulisine) (Fig. S-1). It is interesting to note that applying a lower separation voltage (20 kV) resulted in a considerable overlapping of the widened peaks, since the slower migrating proteins tend to have stronger adsorption (Fig. S-2).

The CZE method developed for the three insulins was evaluated for its analytical performance (Table 1). The linear ranges of the calibration diagrams based on the CZE-UV measurements covered the concentration range between 2 - 1000 mg/L. These calibration graphs gave satisfactory linearity values, with R^2 being better than 0.99. The LOD values ranged between 0.6 - 0.93 mg/L. In the case of MS detection, the LOD values based on base peak electropherograms (BPE) ranged between 2.17 - 4.37 mg/L. The surprisingly good sensitivity of UV compared to MS detection can be attributed to the wide charge distributions of the proteins, which lead to a lower detection signal intensity of a given charged form. The precision values of the three insulins were studied based on 12 successive measurements (Fig. S-5 and S-6), showing good repeatability in time with a maximum of 1.2 RSD%, while the RSD% of peak areas were poorer (5 - 9 RSD%). When internal standard (m-cresol) correction was applied, the migration time precision could be improved to below 0.53 RSD% but not the peak area precision. The larger RSD% values were mainly caused by the small fluctuations in adsorbed proteins and the larger integration errors due to the tailed peaks.

Table 1
The main characteristics and analytical performance data of human insulin and its two analogues obtained by CE-UV/MS.

	Humulin	Glargine	Glulisine
RSD% (migr. time)	1.05	0.84	1.16
RSD% (peak area)	5.80	8.82	7.90
corr. RSD% (migr. time)	0.35	0.074	0.53
corr. RSD% (peak area)	5.61	12.67	5.16
Equation for calibration graphs*	$y = 377.8x - 16.95$	$y = 248.7x - 15.16$	$y = 352.4x - 8.59$
R^2	0.994	0.9916	0.9959
Linear range (mg/L)	2 - 1000	3 - 1000	2 - 1000
LOD (UV, mg/L)	0.63	0.93	0.60
LOQ (UV, mg/L)	2.09	3.1	2.00
LOD (MS, mg/L)	4.37	2.17	3.73
LOQ (MS, mg/L)	14.6	7.23	12.4

* y: absorbance (mAU), x: concentration (mg/L).

3.2. *Effect of temperature on the deamidation of insulins*

In addition to pH, the incubation temperature also has a strong effect on the rate of insulin deamidation. Elevated temperatures increase the rate of deamidation and above 30 °C even conformational changes can happen [3,6-8,27,28]. The recommended storage temperature of insulin is 2 - 8 °C to keep it fit for use until expiration date. In the case of insulin glargine, the manufacturer claimed its stability for about a month if stored between −15 °C and +25 °C temperature cycle (including different preservatives and matrix materials) [31].

In our experiments the effect of temperature on deamidation was studied in insulin samples incubated for one month after their acidification (Fig. 4). As it was expected, lower temperatures considerably hindered the deamidation processes. At −20 - −80 °C the deamidation is minimal, but above +5 °C the deamidation hastens. At +5 °C only 1-fold deamidation forms could be observed for each insulin.

At room temperature incubation, in the case of humulin and glulisine the signal intensities (peak areas) are the largest for the 1-fold and a few 2-fold deamidated forms (D1, D2) as the formation of those has the highest probability and the 2–3-fold deamidation products showed much smaller peak intensities (in our earlier work even 4-fold deamidation was detected for humulin after a 51-day long incubation [17]). Glargine presented only 1-fold deamidated forms and since fewer numbers of degradation components are formed, their intensities are larger than those of humulin or glulisine. One month after acidifying the solution, the amount of humulin and glulisine largely declined to less than 20% of their initial amount, but the decomposition of glargine was less than 50%. The one-fold deamidation reached the highest concentration in 22 days for humulin and glargine, but in the case of glulisine, the 1-fold deamidation form reached maximum concentration after 1 week of incubation (Fig. 5). The higher stability of glargine in acidic conditions is explained by the replacement of the highly susceptible residue A21 asparagine with glycine [32]. These modifications of human insulin result in a stable molecule, which is soluble in slightly acidic conditions and precipitates in the neutral pH of subcutaneous

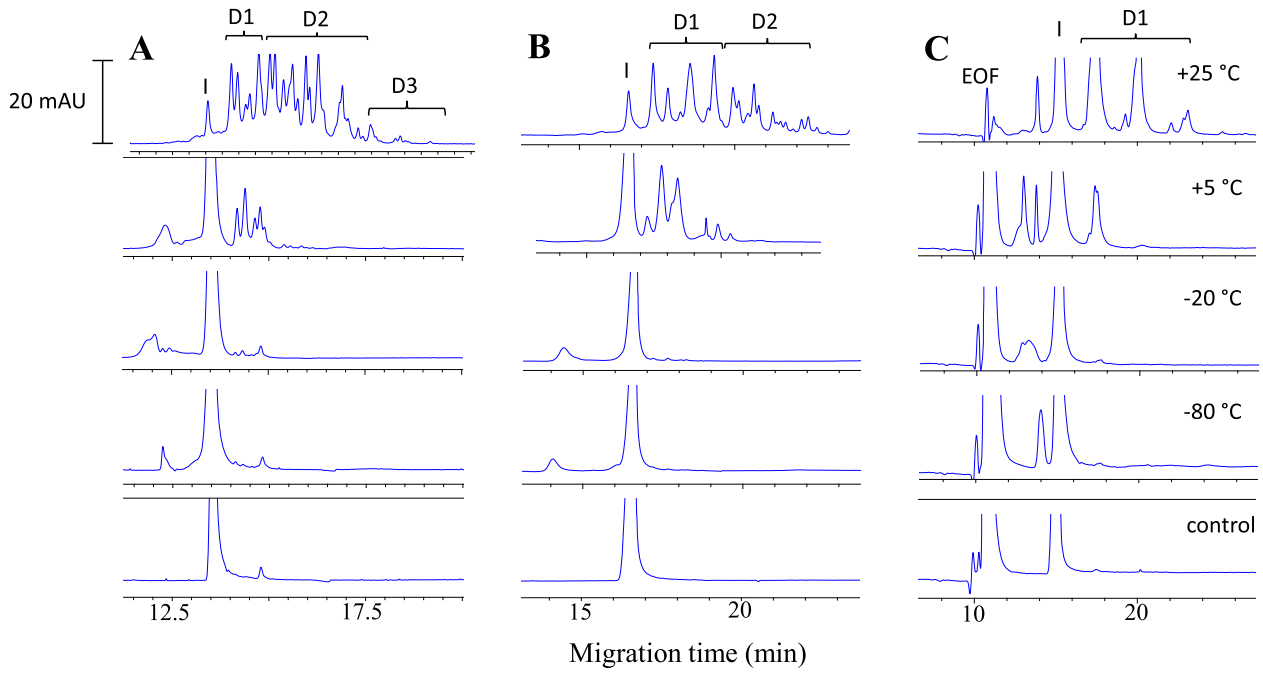

Fig. 4. Effect of temperature on the formation of deamidated isoforms of humulin (A), glulisine (B) and glargine (C). The samples were acidified (pH = 1) and stored for 22 days at room temperature, +5 °C, −20 °C, −80 °C. Deamidated isoforms are marked as D1-D3. Analysis conditions were the same as stated in Fig. 2.A.

tissues. Because of these properties, absorption of glargine is delayed, therefore glargine is a once-daily, long-acting analogue [33]. Two months after the acidification of humulin and glulisine samples, more than thirty peaks could be observed, because presumably, degradation products other than deamidated components were formed, as well. The total intensities of the different forms at given times were nearly the same (Fig. S-12), although a full match is difficult to obtain due to the following reasons: inaccurate integration of the large number of merged peaks, the different samples were stored in very different times up to the analyses, the variance of the migration times of the components (peak areas increase with the migration times), slight difference between the absorbance coefficient values of the deamidated components compared to that of the non-deamidated insulins.

3.3. Mass spectrometric determination of deamidation isoforms

The determination of deamidation isoforms is a challenging analytical task, which requires not only a high performance separation technique, but also the utilization of a high resolution, selective detector. For such endeavours the CE-MS coupling can provide a promising solution. In the 1-month old samples of the three studied insulins, different numbers of deamidated forms were generated and determined: 1-fold, 1–2-fold and 1–2–3-fold forms for glargine, glulisine and humulin, respectively. The isotopic distributions and the exact masses of the components in the spectra enable the identification of a given insulin and its different-fold deamidated forms.

In humulin and glulisine, the desamido-(A21)-insulin (more exactly, two L-aspartate isoforms as L- aspartic acid (Asp) and isoaspartic acid (isoAsp), but no separation and identification of these two forms has been reported until now) can be formed at the highest rate, but other isoforms such as desamido-(B3)-insulin or isoAsp-(B3)-insulin might be generated, as well [18,27]. Abdelwaly et al. found deamidation at position A18-Asn-and A15-Gln-in their study [34]. Therefore, deamidation of all glutamines and asparagines at positions other than A21 cannot be neglected. The number of deamidated forms are largely increased by multiple deamidations, when two-, three- or four-fold deamidated forms can form as two, three or four asparagine or glutamine residues transform into Asp (or isoAsp) or Glu-in a single molecule (in the case of humulin, 5 deamidable amino acids are present, and in the case of glargine and glulisine there is one asparagine less). While humulin and glargine contain two successive deamidable groups (asparagine and glutamine) in B3-B4 positions, in the case of glulisine there is only one deamidable amino acid (glutamine at B4) which might have a better chance of undergoing deamidation.

Fig. 6 demonstrates the MS spectra of glulisine and D1-D2 deamidated isoforms, which are present in a sample incubated for one month. The first peak in the electropherogram is assigned to glulisine at a monoisotopic mass of 1164.733 *m/z* (5818.629 Da after deconvolution as the molecular ions are present with a charge number of + 5) and the following four peaks having an *m/z* increase of 0.984 Da indicate that these peaks are all 1-fold deamidation forms (D1). From among the 1-fold deamidation forms, we assume that the first peak with the highest intensity (D1a) is the desamido-(A21) form, the adjacent, smaller intensity peak (D1b) corresponds to the desamido-(A18) form and the other two even smaller peaks (D1c and D1d) are the forms obtained by the deamidation of the glutamines in the A15 and B4 positions. The glutamine residue at position A5 is probably more protected against oxidation due to the vicinity of a disulfide bridge.

After the 1-fold deamidation components (D1) of glulisine, four 2-fold deamidated peaks (D2) appear in the electropherogram as an additional carboxyl group was generated (thereby increasing the number of negatively charged side chains, increasing their charge-to-mass ratio). The mass spectra of these D2 peaks are shifted by $a + 0.984$ Da mass change due to an additional deamidation process. Although the possible number of the 1-fold deamidated forms is only 5, there are 10 theoretical variations in the 2-fold deamidation forms. However, from these 2-fold deamidation products, it is very probable that the peak appearing first, having the highest intensity (D2a) is the component having deamidations both at A21 and A18 and each of the other 3 peaks likely includes one deamidation of asparagine and one deamidation of glutamine. Unfortunately, these assumptions could not be supported by our tandem MS investigations with the use of collision-induced dissociation (CID). We observed low fragment ion intensities and could mainly identify fragments corresponding to parts of the sequences that precede or follow the cysteines forming disulfide bonds, mainly from the B20-B30 region which does not contain potential deamidation sites. This

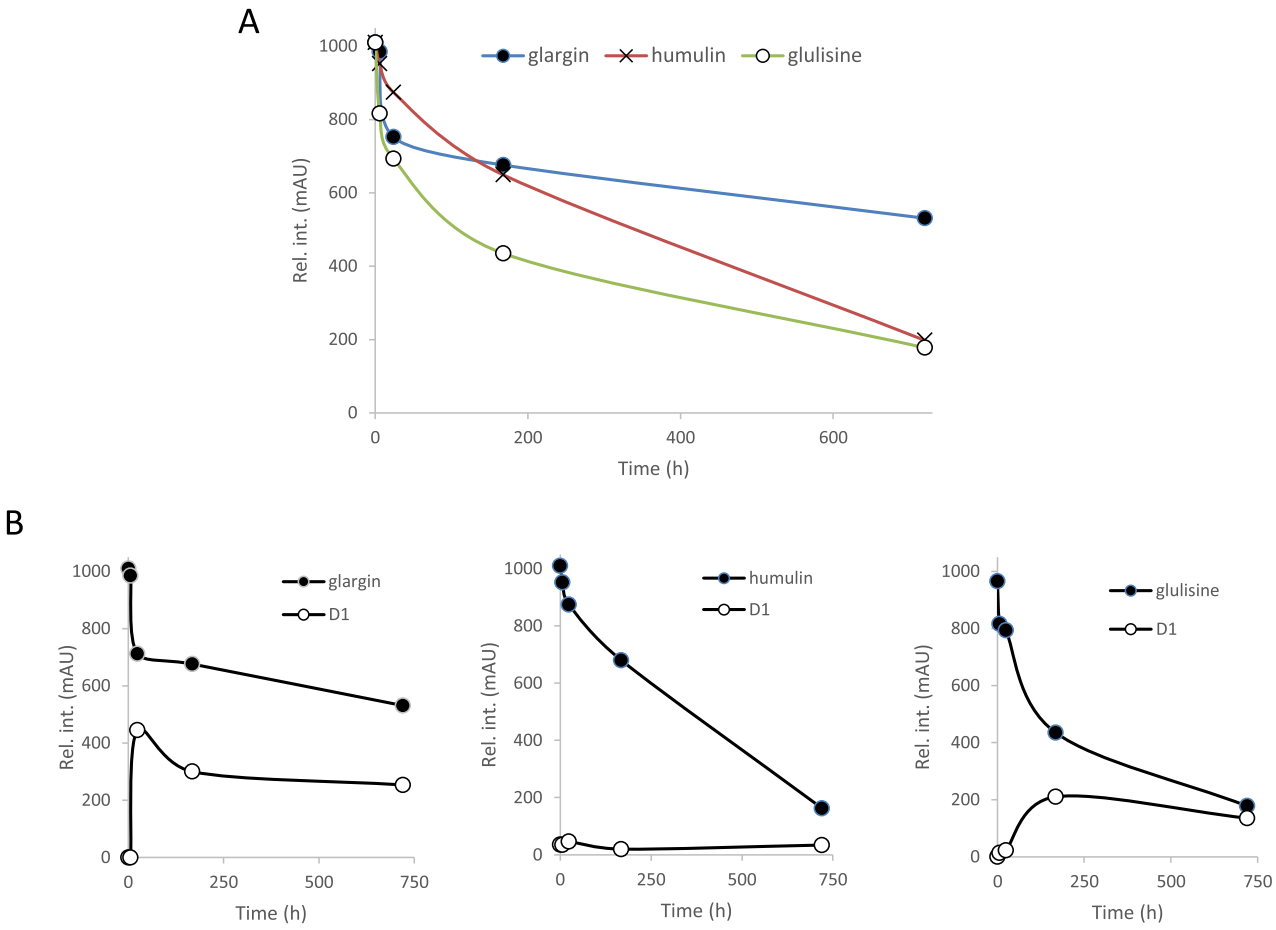

Fig. 5. Concentrations of three recombinant insulins (A) and their one-fold deamidated (D1) isoforms (B) in acidified samples, when the samples were acidified (pH = 1) and stored at room temperature for 31 days. Analysis conditions were the same as in Fig. 2.A, D1 isoforms are the firstly appearing (often the highest intensity) one-fold deamidated components. (The continuous curves in the diagrams only serve to help the representation of the tendencies but those have no mathematical (fitting) meaning.).

is presumably due to the improbable fragmentation of disulfide linkages.

Similarly, the deamidation processes for the other two insulins after 2 weeks and 1 month incubation were monitored by CZE-MS measurements (Fig. S-6 – S-10). In the case of glargine, the absence of asparagine at position A21 largely reduced the possible deamidation events, while humulin represents the largest number of deamidation forms as it includes the largest number of deamidable groups. The experimental identification of the exact deamidation sites in insulin remains a challenge. The CZE-MS analysis can not provide better resolution than CZE-UV, since the UV spectrometry is an on-capillary detection eliminating peak dispersion in connecting/interfacing units. Nevertheless, even with MS detection a large number of different deamidation components could be separated and a great advantage of MS detection is that even if components are not fully separated, the electropherograms obtained at a properly selected m/z range (extracted ion electropherogram, EIC) may exhibit well distinguishable MS signals for the co-migrating components.

The surprisingly good sensitivity of UV compared to MS detection can be attributed to the charge distributions of the proteins, which lead to a lower detection signal intensity of a given charged form. Since the electrospray ionization of the MS system generates a large number of forms of the protein with different charge states, these forms share the overall analytical signal. Thus, the larger number of the different forms may result in poorer detection sensitivity of the given protein form.

4. Conclusions

In our present contribution the deamidation of three recombinant human insulins was studied. Although the main goal of our work was to acquire a better understanding of the deamidation processes, we were also aiming at gaging the analytical capacities of CE equipped with UV and MS detection. From among the several available recombinant human insulins, humulin, glargine and glulisine were selected for our study because even though their sequences and chemical parameters are quite similar, there are small but obvious differences present in these molecules that influence the deamidation processes. Compared to humulin, the asparagine residue at position A21 or B3 is replaced by a non-deamidable amino acid in glargine and glulisine, respectively. In the case of all studied insulins, several isoforms showing different levels of deamidation (1–3) were separated, demonstrating the high resolving power of CZE, since the separated components differ only by 0.984 Da (different-fold deamidated forms) or the masses are exactly the same, but only the shapes of the molecules are slightly different (same-fold deamidated forms). Furthermore, the proteins often contain not only a few but several deamidable amino acid residues (asparagine or glutamine), therefore the number of the possibly different deamidation isoforms can be quite huge. In order to achieve the required high separation power of CZE, a long (120 cm) separation capillary was applied. The surprisingly good sensitivity of UV detection compared to MS detection can be attributed to the wide charge distributions and adduct formation in ESI of the proteins, which lead to a lower analytical signal intensity of

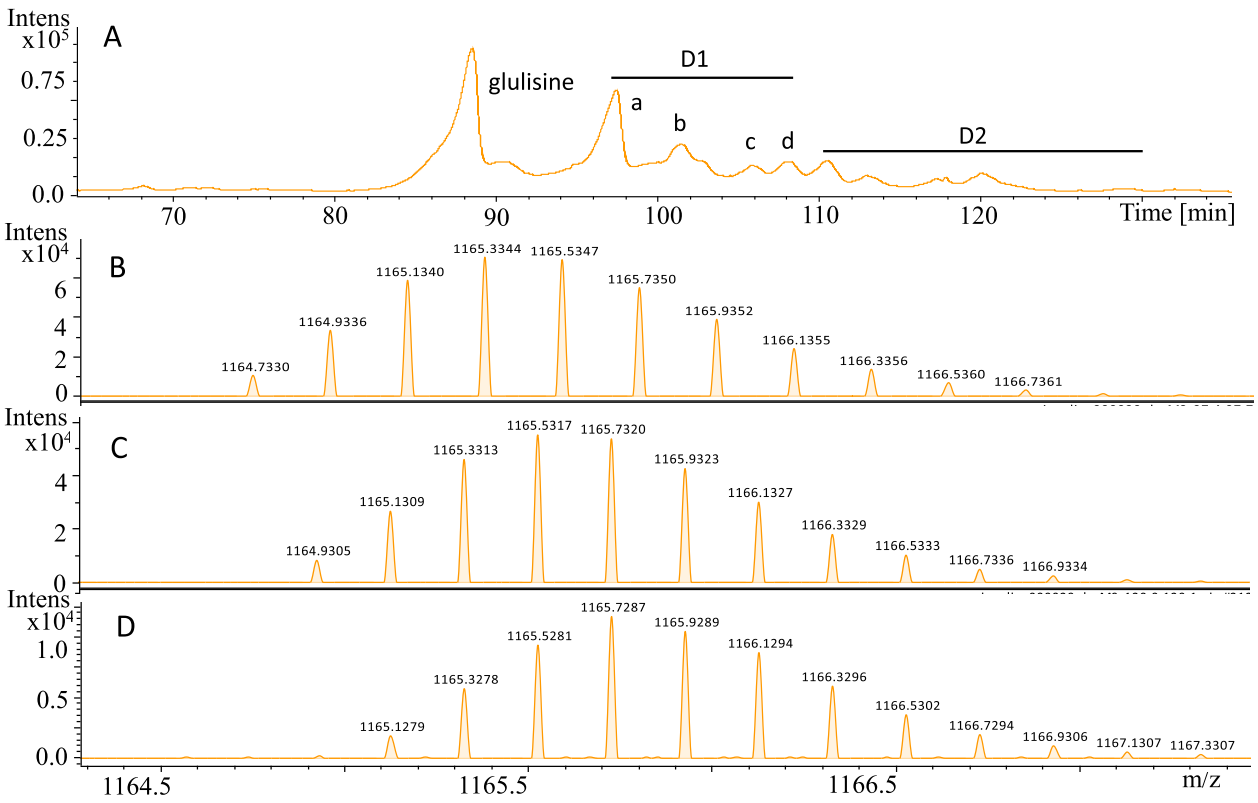

Fig. 6. MS spectra (isotope patterns) of insulin glulisine (B), one-fold (D1) (C) and two-fold (D2) deamidated isoforms (molecular ions with charge number of 5) obtained after CZE-MS separation of the components (A). The acidified insulin (pH = 1) was stored for 31 days before analysis. The four different one-fold deamidation isoforms were marked as a, b, c and d. MS parameters were detailed in the Experimental section.

a given charged form. However, the utilization of high resolution MS detection has undoubtable benefits regarding the identification of insulins and their deamidated forms. We should also mention that the proposed method enables the determination of insulin isoforms without any digestive sample preparation. These merits might allow CZE to become an alternative method in both pharmaceutical research and routine analysis.

After a 22-day long incubation in acidic condition at room temperature, the 1-fold deamidated forms showed the largest signal intensities, as the formation of those has the highest probability. A 1-month long incubation generated 2-fold deamidation forms in appreciable amounts, as well, for humulin and glulisine (for humulin, 3-fold deamidation forms were also detectable). In the case of glargine, only the 1-fold deamidated forms could be identified and since fewer numbers of degradation components are formed, their intensities are larger than those of humulin or glulisine. As it was expected, the lower temperature considerably hindered the deamidation processes for all insulins, at +5 °C only 1-fold deamidation forms could be observed. At −20 - −80 °C the deamidation was found minimal.

Credit author statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464286.

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