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Determination of deamidated isoforms of human insulin using capillary electrophoresis

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ABSTRACT

The applicability of capillary zone electrophoresis (CZE) for the separation of the deamidated forms of insulin has been studied. 50 mM NH_4Ac (pH=9) with 20 % v/v isopropylalcohol was found optimal for efficient separation of insulin from its even 10 deamidated forms. The developed method was efficiently applied for monitoring the degradation rate of insulin and the formation of different deamidation isoforms. Two months after the acidification more than thirty peaks can be observed in the electropherogram, because degradation products other than deamidated components were formed as well. The recorded mass spectra enabled us to assign the exact mass of the components, and thus the identification of insulin isoforms could be accomplished. We think that this study provides useful information on how the determination of several deamidation forms can be carried out with CE-MS, but the identification of the exact position of deamidation sites in the insulin molecule remains a challenge.

1. Introduction

Insulin is an important peptide hormone regulating glucose metabolism. Human insulin consists of two chains (chain-A and chain-B containing 21 and 30 amino acid residues, respectively), two interchain disulphide bonds and one intra disulphide bond within chain-A. Currently, the majority of insulin used for medicinal purposes is produced by recombinant DNA technology, which can undergo several post-translational modifications (PTM) including deamidation, glycosylation, aggregation or oxidation of methionine [1,2]. The most common non-enzymatic degradation of insulin is deamidation, which occurs as a result of the removal of amide groups in asparagine (N or Asn) and glutamine (Q or Gln) residues by hydrolysis resulting in free carboxylate groups (there are six possible residues where deamidation can occur (A5(Q), A15(Q), A18(N), A21(N), B3(N), B4(Q)). Asparagin is converted to aspartic acid and iso-aspartic acid through the formation of a succinimide intermediate. The deamidation of glutamine residue can undergo via the same mechanism through the formation of glutarimide intermediate but at a slower rate, therefore the deamidation is often more common in Asn residues than in Gln residues [1]. PTMs cause alterations in biological activity, immune response and stability, therefore their characterization during manufacture and storage is essential [2].

The deamidation of insulin depends on multiple factors such as pH, temperature, shaking, amino acid sequence, higher structure of proteins and it can occur during pharmaceutical preparation or storage [3,4]. Based on several works, it can be concluded that deamidation of insulin can be forced by low pH [3–8].

Brange found that in strong acidic conditions deamidaton can take place in position A21 [5], while in weak acidic or neutral solutions residue B3 is the most susceptible [6,7].

Several chromatographic and electrophoretic techniques were used to reveal insulin heterogeneity. The importance of these studies is given by the requirement that the ratio of deamidated isoforms in the pharmaceuticals must not exceed 3% [9]. Besides reversed phase HPLC techniques [8,10,11], ion chromatography (IC) [12] was used to study the charge variants including deamidation. The different techniques of capillary electrophoresis (CE) such as capillary isoelectric focusing (CIEF) [13,14] and capillary zone electrophoresis [13,15–19] were found to be useful in the analysis of charge variants. CZE separates deamidated isoforms by their mass to charge ratio. The appropriate choice of pH and different additives of the background electrolyte (BGE) can reduce the interaction between the analytes and the capillary surface enhancing the efficiency and reliability of separations [20]. Determination of deamidated peptides were performed with PVA-coated capillary

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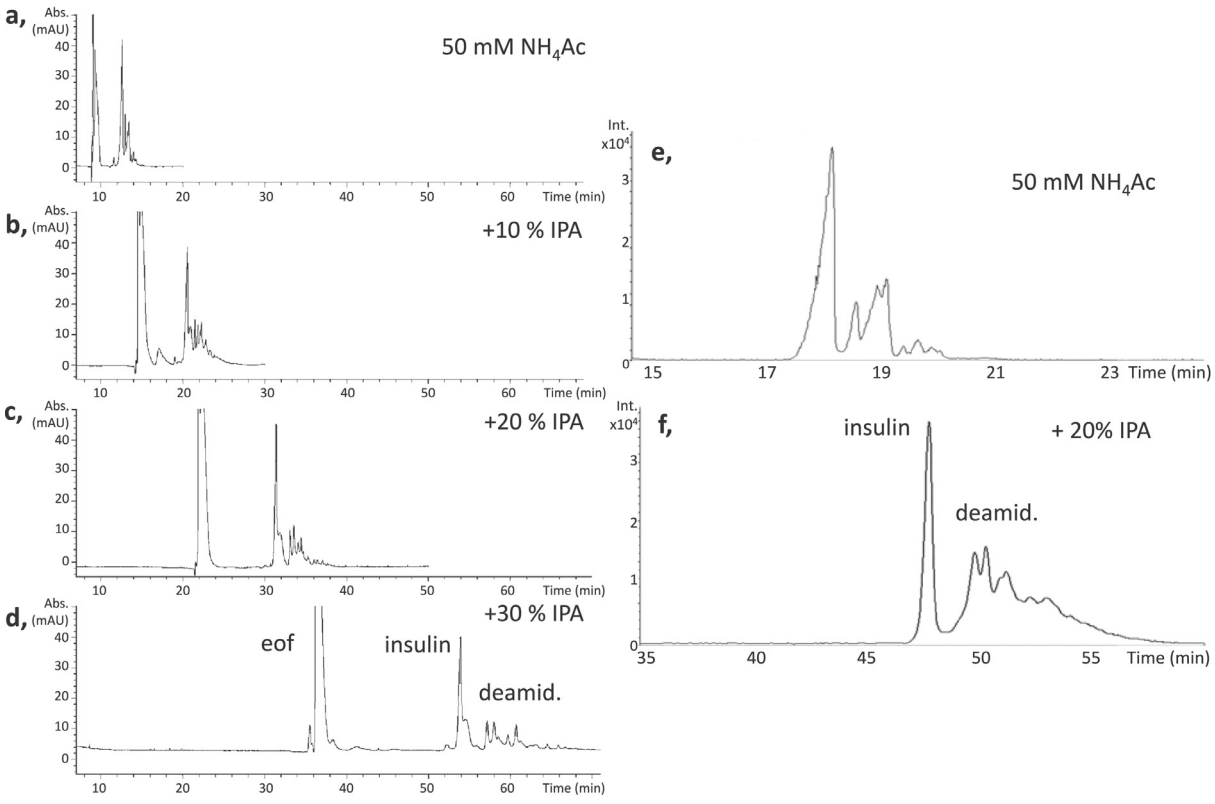

Fig. 1. CZE electropherograms of insulin and its deamidated isoforms with UV detection using running buffer of 50 mM NH_4Ac pH= 9 (a,) and with different concentration of isopropylalcohol 10 % v/v (b,), 20 % v/v (c,) and 30 % v/v (d,). The CZE electropherograms obtained with 50 mM NH_4Ac pH= 9.0 (e,) and 50 mM NH_4Ac, 20 % v/v isopropylalcohol pH= 9.0 (f,) were also detected with MS. Conditions: capillary 85 cm x 50 µm i.d., l_{eff}: 77 cm, hydrodynamic sample injection: 100 mbar•s, U= +25 kV, λ = 200 nm. For MS detection: capillary length: 100 cm, sheath liquid: isopropylalcohol:water=1:1 with 0.1% formic acid; flow rate: 7 µL/min. ESI voltage: 4500 V; end plate offset: 500 V. The 3,43 mg/ml insulin was stored in acidic condition at pH= 1 for 8 days at room temperature.

in acetic acid buffer [21] and with a polybrene-dextrane sulfate coated capillary [22]. There are several CE works about deamidation of antibodies [14,15,20] or small proteins other than insulin [21,22]. In a recent paper [22] a 4.5 kDa peptide drug containing five closely-positioned potential deamidation sites was exposed to acidic conditions for 1-14 h and 6 deamidated components could be separated. However, only a very few papers [19,23,24] are dealing with CE analysis of insulin deamidation, and in these works only one or two deamidated forms (desamido A21-insulin and/or desamido-B3-insulin) have been detected and the components were identified by adding standards. Mandrup monitored the degradation of insulin by IC and CZE, and excellent correlation was established between these techniques [19]. Insulin and desamido insulin were separated using tricine-morpholino buffer at pH=8 [23] and adding acetonitrile and several zwitterions or different organic solvents to the BGE [16].

The deamidation of one amino acid results in a mass increase of 1 Da to the molecular mass of a protein, which can be detected by mass spectrometry (MS) [23,25]. Different types of charge variants of proteins/peptides including deamidated forms were identified by LC-MS [12,26] or CZE-MS [22,27]. Recently, for the first time Dominguez-Vega demonstrated the usefulness of the CE - MS/MS method for compositional and site-specific assessment of multiple peptide-deamidation [22], but according to our best knowledge, CE-MS was not applied so far for the determination of insulin deamidation.

Although CZE is a very efficient tool for the separation of charge variants, only the 1-2 deamidated forms have been separated from insulin and no multiply deamidated forms have been detected using this technique. In this work we developed a CE method which can be efficiently applied for monitoring the degradation of insulin and the formation of a large number of different deamidation isoforms. This is the first work in which even 10 deamidated forms have been separated and quantitatively determined, thus the determinations could be applied to study the formation of deamidated insulin isoforms in time. The aim of the present study was to optimize CZE for UV and MS detection, which would enable the separation and determination of a large number of deamidation isoforms of human insulin.

2. Materials and methods

2.1. Reagents and materials

All chemicals were of analytical grade. Ammonium-acetate, methanol, acetonitrile, isopropylalcohol, ammonium hydroxide solution, NaOH, HCl were purchased from Sigma Aldrich (St. Louis, MO, USA), and diluted with de-ionized water (Millipore Synergy UV) prior to use. The 3.5 mg/mL human insulin (Humulin R) solution was obtained from Lilly (France). The pH of the background electrolyte (50 mM ammonium acetate in 20 % v/v isopropylalcohol for CE-UV and 50 mM ammonium acetate for CE-MS) was 9.0. The buffer was prepared by dissolving solid ammonium acetate, which was then titrated by 25 % m/m ammonium hydroxide solution. All solutions were filtered using a membrane filter of 0.45 µm pore size and stored at +4°C. Running buffers were degassed in an ultrasonic bath for at least 5 min. Prior to first use, the fused silica capillary was rinsed with 1 M NaOH for 20 min, de-ionized water for 10 min and running buffer for 20 min.

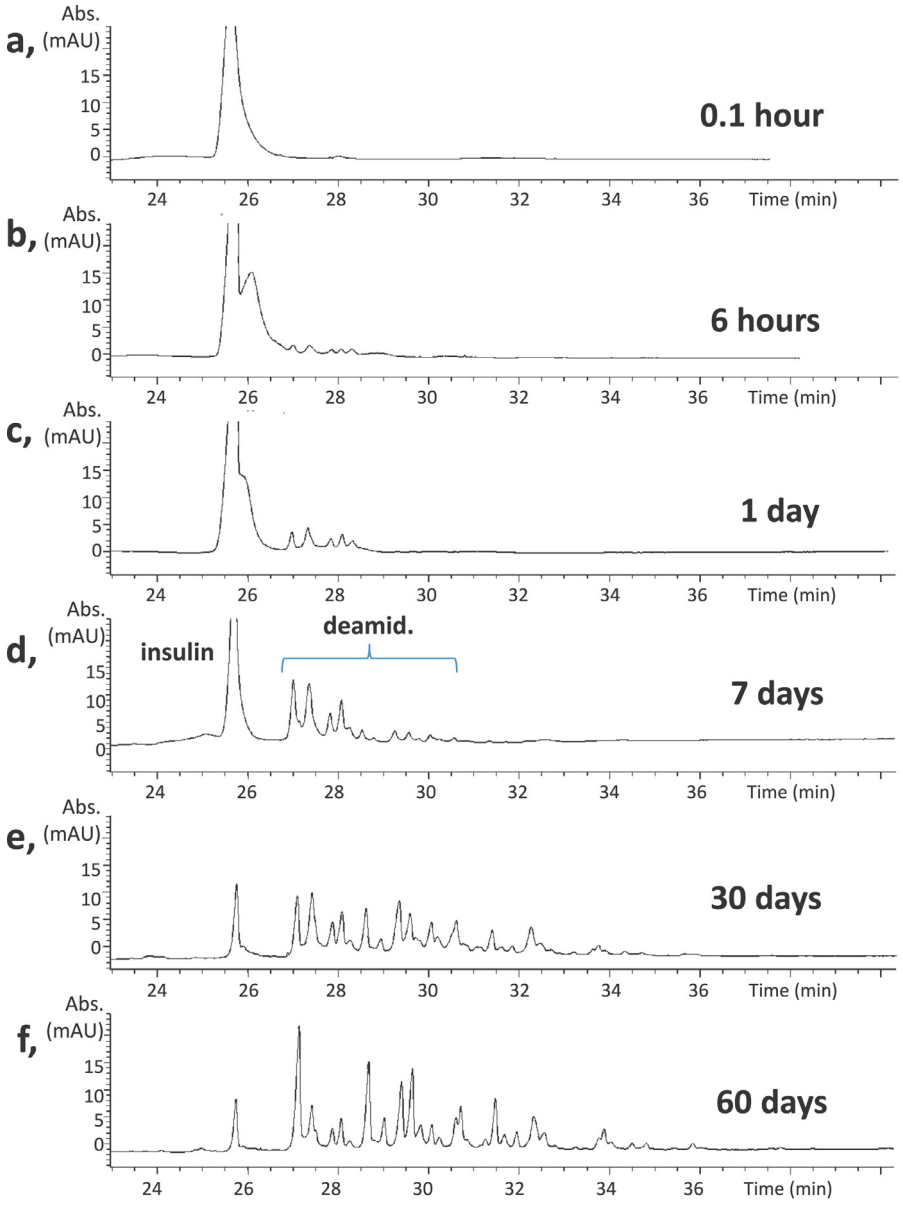

Fig. 2. Study of the formation of deamidated products of insulin. The analysed insulin sample was acidified (pH= 1) and stored for 0.1 hour (a,), 6 hours (b,), 1 day (c,), 7 days (d,), 30 days (e,) and 60 days (f,) at room temperature. Conditions were same as in Fig. 1.b.

2.2. Degradation of insulin samples

Acid catalyzed forced degradation of human insulin was carried out. Stock solution of insulin was mixed with 6 M HCl solution to get a final concentration of 0.1 M HCl. The acidified insulin solution was kept at room temperature for 60 days and analyzed at different times.

2.3. Measurements with CE

Analyses were conducted using a 7100 model CE instrument (Agilent, Waldbronn, Germany) with UV and MS (maXis II UHR ESI-QTOF MS instrument, Bruker, Bremen, Germany) detection. For CE measurements with UV detection, fused silica capillaries of 85 cm x 50 µm I.D. and 370 µm O.D. (Polymicro, Phoenix, AZ, USA) were used (L_{eff} = 77 cm). UV detection was carried out by on-capillary photometric measurement (detection wavelength: 200 nm). Samples were introduced hydrodynamically (50 mbar, 2 s) at the anodic end of the capillary. The BGE consisted of 50 mM NH_4Ac with 20 % v/v isopropylalcohol. The applied voltage was +25 kV. The capillaries were preconditioned with 1 M NaOH for 10 min, acetonitrile for 5 min and finally with BGE for 8 min. OpenLAB CDS Chemstation (Agilent) software was used for both controlling the CE instrument and processing the obtained electropherograms.

As concerns MS detection, a CE-ESI sprayer interface (G1607B, Agilent) provided on-line hyphenation to the CE instrument. Sheath liquid was transferred with a 1260 Infinity II isocratic pump (Agilent). MS instrument was controlled by otofControl version 4.1 (build: 3.5, Bruker). The following analysis conditions were used for CE-MS determinations: 100 cm x 50 µm I.D. and 370 µm O.D fused silica capillary; hydrodynamic sample injection (50 mbar, 6 s), BGE: 50 mM NH_4Ac, pH=9.0; sheath liquid (SL): isopropylalcohol:water= 1:1 with 0.1 % v/v formic acid; SL flow rate: 7 µL/min; applied voltage: +25 kV. The capillaries were preconditioned with the BGE and postconditioned with acetonitrile and BGE for 2-2 min. MS parameters: positive ionization mode;

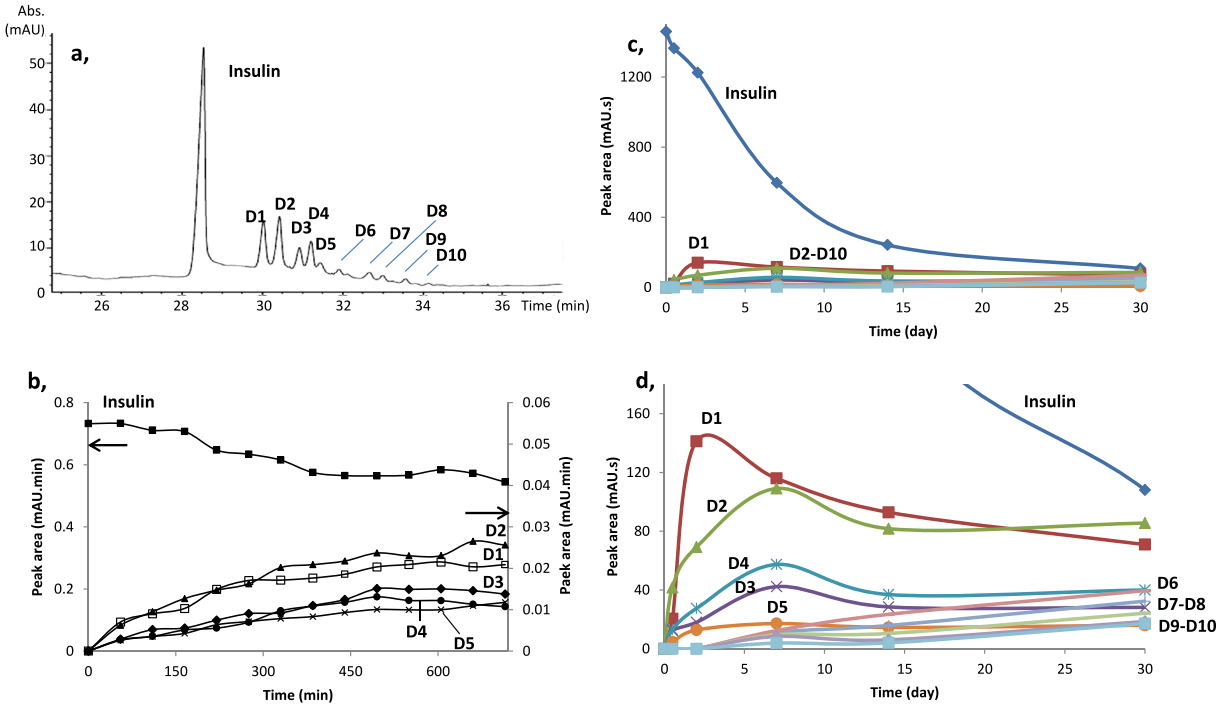

Fig. 3. CZE separation of insulin and its deamidated isoforms. The sample was acidified (pH= 1) and stored at room temperature for 10 days (a,). Separation conditions were same as in Fig. 1.b. Effect of time on the formation of deamidated insulin isoforms studied up to 700 min (b,) and 30 days (c, d,). Deamidated isoforms are marked as D1-D10. Conditions were same as in Fig. 1.b.

nebulizer pressure: 0.4 bar; dry gas temperature: 220°C; dry gas flow rate: 8 L min^{-1}; capillary voltage: 4500 V; end plate offset: 500 V; spectra rate: 6 Hz; mass range: 800-2200 m/z. Nebulizer gas pressure was turned off for 5 min at the beginning of each run in order to reduce the syphoning effect generated by the nebulizer gas flow, thereby improving the resolution of peaks and providing constant current during the electrophoresis. Na-formate calibrant was injected after each separation, which enabled internal m/z calibration. Mass spectra were processed by Compass DataAnalysis version 4.4 (build: 200.55.2969).

3. Results and discussions

3.1. CZE separation of deamidation isoforms of insulin

Insulin is a peptide hormon of 5.8 kDa, that is, it is a quite small protein. Large proteins (above 30 kDa) often strongly adsorb on the bare (non-modified) fused silica capillary, but the separation of peptides and small proteins are quite common in such capillaries without their considerable adsorption. However, to keep the possible adsorption effects to a minimum, acidic or basic conditions for the separations are suggested. Insulin has a pI= 5.3, thus its adsorption in basic electrolyte (having negative net charge) onto the negatively charged capillary wall should not be significant. In CZE, the separation is based on the difference in electric charge relative to molecular size. The electric charge depends on the number of carboxyl and amino groups of the component, but also on the pH of the electrolyte which controls the dissociation of these groups. Basic buffer electrolyte is preferred, because at this pH the carboxyl group(s) formed via deamidation add negative charge(s) to the peptide. Using pH below 4, no separation of the deamidated forms could be achieved (Fig. SM-2). In our work we intended to use both UV photometric and MS detection, the choice of electrolytes were limited by the fact that those should be compatible with MS. Based on the above considerations ammonium acetate buffer of pH=9 seemed suitable. Although this BGE is much simpler than those applied in the literature of deamidation isoforms [19–23], insulin and several deamidated variants could be well separated (in order to develop an electrophoretic method able to separate the deamidation isoforms, an acidified insulin solution incubated for 1 week was employed as test sample (Fig. 1.a and e)). 50 mM concentration of NH_4Ac was found optimal for the separation as it ensured proper ionic strength but current less than 30 μA. Although various (statically or dynamically) coated capillaries are commonly and efficiently used for the separation of proteins [16–18], in our measurements the application of non-modified bare fused silica capillaries provided proper resolving power for the separation of the deamidation isoforms of insulin. Similarly, other works [12,19,23,24] dealing with CZE analysis of insulins used bare fused silica capillaries.

For enhancing the separation efficiency and resolution of the deamidated isoforms, it was suggested to add organic solvents like acetonitrile, methanol and isopropylalcohol (IPA) to the BGE [22]. Separation could be improved with these solvents; the best resolution but longest separation was obtained with IPA (Fig. SM-3). Since IPA content above 30 % v/v started to broaden the peaks and led to long analysis time, 20 % v/v IPA was found optimal (Fig. 1.a-d). Careful postconditioning (washing with 1 mM NaOH for 10 min and with BGE for 8 min) and application of cresol as a time reference component for the normalization led to repeatable separations, the precision data of insulin were 0.36 RSD% and 2.63 RSD% for migration times and peak areas, respectively (the application of internal standard (cinnamic acid) did not improve the data). The precision study for the acidified insulin showed similar data for the migration times: 0.49 RSD% and 0.14 RSD% for insulin and the D1 deamidation form. However, peak areas continuously decreased for insulin and increased for D1, which makes the repeatability measurements meaningless (Fig. SM-4).

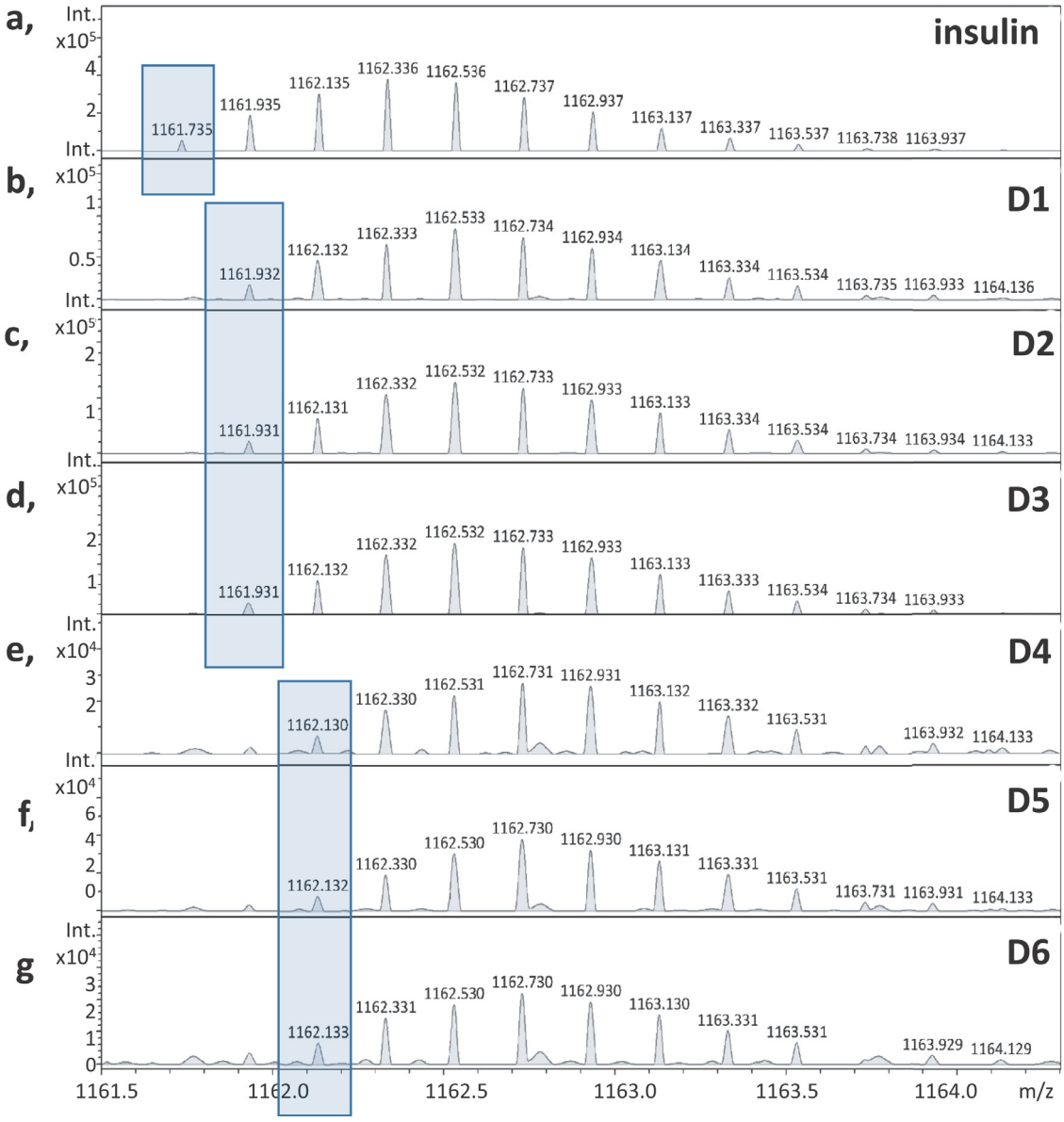

Fig. 4. MS spectra (isotopic distribution) of human insulin and D1-D6 deamidated isoforms (molecular ions with charge number of 5) obtained after CZE separation of the components. The acidified insulin (c= 3.43 mg/ml, pH = 1) was stored for 7 days before analysis. MS parameters: positive ionization mode; nebulizer pressure: 0.4 bar; dry gas temperature: 220°C; dry gas flow rate: 8 L min-1; capillary voltage: 4500 V; end plate offset: 500 V; spectra rate: 6 Hz.

In the case of CE-MS analysis, the length of the separation capillary was 100 cm, long enough for the proper and convenient hyphenation between the CE and MS. It was found that this long separation distance (migration times around 50 min) made analyte peaks wider compared to the separation with no IPA content (Fig. 1.e-f). Therefore, no IPA modifier was added to the BGE for the CE-MS measurements.

In an earlier work, where a single desamido peak was separated from the insulin, it was stated that this peak probably contained several monodesamido insulin derivatives which would not be separated from each other by CZE [23]. Our results show that using a simple and MS compatible ammonium acetate buffer of pH=9.0 probably all possible (3 different) monodesamido (and even several two, three or four-fold) insulin degradants were properly separated. The MS measurements revealed that the D1-D10 peaks were indeed of only a given molecular mass, verifying the separation efficiency of the proposed method.

In a recent work the separation efficiency for the characterization of multiple deamidated degradation products of a peptide therapeutic [22] was similar to that of our measurements. Dominguez-Vega et al used ammonium formate (pH 6.0) BGE in combination with a capillary coated with a bilayer of Polybrene-dextran sulfate. MS detection made it possible to easily distinguish the deamidated from deacetylated-deamidated degradation products.

3.2. Study of the deamidaton of insulin

The rate of deamidation reactions of insulin is mainly influenced by temperature and pH [3–8]. Upon deamidation, asparagine is first converted to a five carbon cyclic intermediate, which is then hydrolysed to form either iso-aspartate or aspartate. At low pH the hydrolysis of the side chain amide generates mainly aspartate [12]. It is widely accepted that the desamido-(A21)-insulin is formed at

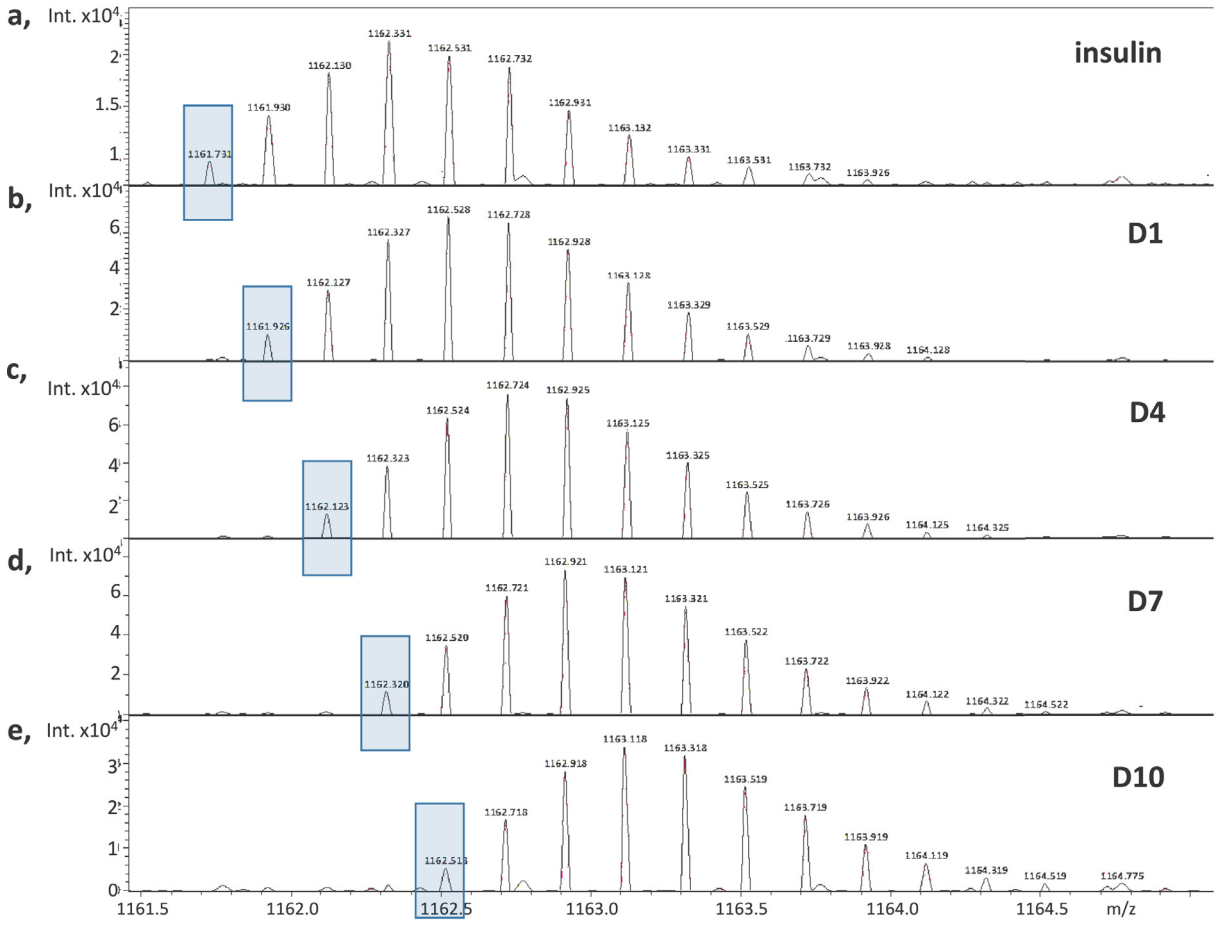

Fig. 5. MS spectra (isotopic distribution) of human insulin and D1, D4, D7, D10 deamidated isoforms (molecular ions with charge number of 5) obtained after CZE separation of the components. The acidified insulin (c= 3.43 mg/ml, pH = 1) was stored for 51 days before analysis. MS parameters were same as in Fig. 4.

the highest rate and two L-aspartate isoforms can be formed: L-aspartic acid (Asp) and isoaspartic acid (isoAsp). Besides, other isoforms such as desamido-(B3)-insulin or isoAsp-(B3)-insulin can be created as well [6,28]. No data about the desamido-(A18)-insulin or the deamidation of Glu were found, but their occurance cannot be excluded during the deamidation processes. It is also known that isoAsp can be generated by spontaneous isomerisation of Asp residues via succinimide ring formation [29]. The number of the deamidation variants of insulin is further increased by multiple deamidation, when two- or three-fold deamidated forms can form as two or three Asn or Gln transform to Asp (or isoAsp) or Glu in a single molecule. These possible processes suggest that not only a few but quite a large number deamidation isoforms can be formed from insulin.

After the insulin sample was acidified with HCl to pH=1, fast formation of deamidated isoforms could be experienced, which was followed in time up to 2 months (Fig. 2). Within 24 h after acidification, the peak of insulin was resolved to two overlapped peaks (Fig. 2.b-c.) with the same mass, but then only a single peak appeared. This interesting phenomenon is perhaps caused by a change in the tertiary structure of insulin (eg. T→R insulin transformation [6]). Here further investigation is required. Within 10 days after acidification, 10 degradation products (D1-D10) could be clearly separated from insulin (Figs. 2 and 3.a). These D1-D10 components should be deamidated forms since the molecular masses of these components are 1, 2, 3 or 4 Da larger than insulin and their migration rates gradually decrease, as the negative charge of the molecule increases with the degree of deamidation. The intensities (peak areas) are the largest for the D1-D3 (monodeamidated forms) as the formation of those has the highest probability. Since asparagine deamidation at A21 resulted in the formation of aspartic acid and iso-aspartic acid, these degradation products probably correspond to two of the D1-D3 peaks (the rate of deamidation is much lower at position B3 because Asn is followed by valine, which has a large side chain (compared to cysteine at A20) [1]). The third peak from among D1-D3 most probably indicates the product of B3 deamidation.

Two months after acidification more than thirty peaks can be observed in the electropherogram, because presumably, degradation products other than deamidated components were formed, as well. The peak of insulin largely declined to 10% of its initial area 1 month after acidification of the solution. The D1-D3 (one-fold deamidated) and D4 (two-fold deamidated) forms reached the highest concentration in 2-8 days, additional two-fold or three-fold deamidated components (D5>) are slowly formed after 1 month (Fig. 3.c-d). However, the quantitation of the degradation forms in these samples is difficult due to the overlapping of a large number of peaks.

3.3. Identification of deamidation isoforms with MS

The identification of deamidation isoforms is a challenging analytical task, which requires high performance separation technique and high resolution, selective detector. The best strategies may be developed on a case-by-case basis and the hyphenation of CE with MS can provide a promising solution [2]. The mass spectra (iso-

topic distributions) are clearly applicable to determine the exact mass of the components, and through this the identification of insulin and its deamidated isoforms can be accomplished (Fig. SM-6, SM-7).

Fig. 4 demonstrates the MS spectra (isotopic distribution) of human insulin and D1-D6 deamidated isoforms obtained within a CZE run shown in Fig. 3.a. The first peak in the electropherogram is assigned to human insulin at a monoisotopic mass of 1161.692 m/z (5808.675 Da after deconvolution as the molecular ions are present with charge number of +5) and the following peaks D1-D3 show an m/z increase of approximately 0.197 Da (0.984 Da after deconvolution). The mass spectra of D4-D6 peaks are further shifted with an additional mass difference which corresponds to the mass change due to an additional deamidation process. D1-D3 and D4-D6 are supposed to be the one and two-fold deamidated insulins, respectively, the determination of which would be problematic without the proper separation of these isoforms, due to their overlapping isotopic distributions. The CZE separation of these isoforms is made possible by the introduction of additional carboxyl groups (thereby increasing the number of negatively charged side chains, affecting their mass to charge ratio) as well as alterations in their molecular shape induced by deamidation. The mass spectra obtained from an electropherogram of the sample incubated at pH=1 for 2 months (Fig. 2.f) is shown in Fig. 5. It can be clearly observed that the isotopic distributions (monoisotopic peaks) of D1, D4, D7 and D10 are successively shifted with approximately 0.197 Da (0.984 Da after deconvolution), that is the method is applicable to separate the one, two, three and four-fold deamidated insulins from insulin.

Although the above CE-MS measurements demonstrate the degree of deamidation by retrieving the mass spectra of each electrophoretic peak, the determination of the exact positions of deamidation would be of crucial importance, as well. A sensible approach would be the dissociation of molecular ions, preferably those having higher charge states, using the coulombic repulsion in our favor. However, our CE-MS/MS experiments using collision-induced dissociation (CID) yielded no fragment ions that would show the expected 0.984 Da mass increase (the most abundant ions of 1162 m/z were selected for CID). Despite applying collision energies ranging between 15-150 eV, only poor fragmentation could be observed (Fig. SM-8). We presume this high resistance to fragmentation can be derived from the presence of 2 interchain and 1 intrachain disulphide bond. It has been shown in the literature, as well that disulphide linkages are less prone to fragmentation under CID conditions [30], especially in the case of insulin [31].

A convenient strategy for the investigation of insulins with MS/MS could be the pretreatment of the oxidized cysteins with a reducing agent in order to separate the A and B chains of the molecule [32,33]. Generally, it is the peptide bonds that cleave upon CID in positive ion mode. However, the immediate reduction of disulphide bridges cannot be achieved in the on-line system of HPLC/CE-MS. On the other hand, electron capture dissociation (ECD) [34], electron transfer dissociation (ETD) [30] and ultraviolet photodissociation [35] might be more suitable candidates for the fragmentation of disulphide bonds. However, no site-specific identification of multiple peptide-deamidation was demonstrated in insulin by HPLC or CE and MS combinated system. Our ongoing research aims at developing a CE-MS/MS method, which enables the determination of exact deamidation sites.

4. Conclusion

In pharmaceutical products deamidation is often observable and it causes problems as the structure of the protein goes through changes. In this work we studied the possibilities of determining the potential deamidation isoforms of human insulin. CZE provided a proper separation of a large number of components having only a minimal difference in their molecular mass (0.017%) or shape of the molecules. It was found that the use of UV detection provided a slightly better separation for these components than MS detection. This can be explained by the peak broadening due to long migration length of the analytes, the laminar flow induced by suction effect of the ESI nebulization and the off-capillary feature of the detection. However, MS detection made possible the determination of the exact mass of the components, and through this the identification of insulin and its deamidated isoforms. Therefore, both UV and MS detections (separately) are advised to use.

The developed CZE separation method can be efficiently applied for monitoring the degradation of insulin and the formation of different deamidation isoforms. This is the first work in which even 10 deamidated forms have been separated and determined. However, developing a CE-MS/MS method, which enables the determination of the exact deamidation sites in insulin is still challenging.

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.

CRediT authorship contribution statement

M. Andrasi: Data curation, Investigation, Methodology, Supervision. **B. Pajaziti:** Conceptualization, Data curation, Supervision. **B. Sipos:** Data curation, Supervision. **C. Nagy:** Supervision, Writing - original draft. **N. Hamidli:** Data curation. **A. Gaspar:** Conceptualization, Methodology, Supervision, Writing - original draft.

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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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461344.

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