Journal of Chromatography A 1678 (2022) 463351

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of human insulin and its six therapeutic analogues by capillary electrophoresis – mass spectrometry

Narmin Hamidli, Blerta Pajaziti, Melinda Andrási, Cynthia Nagy, Attila Gáspár*

Department of Inorganic and Analytical Chemistry, University of Debrecen, Egyetem tér 1, Debrecen H-4032, Hungary

ARTICLE INFO

Article history:
Received 10 May 2022
Revised 12 July 2022
Accepted 17 July 2022
Available online 18 July 2022

Keywords:
Insulin
Insulin analogues
Therapeutics
Capillary electrophoresis
Mass spectrometry

ABSTRACT

In this work, human insulin and its 6 analogues were separated and determined using CZE-MS. Three different capillaries (bare fused silica, successive multiple ionic-polymer layer (SMIL) and static linear polyacrylamide (LPA) coated) were compared based on their separation performances in their optimal operating conditions. Coated capillaries demonstrated slightly better separation of the components, although some components showed wide, distorted peaks. The highest plate number could be obtained in the SMIL capillary (192 000/m). For UV and ESI-MS detection relatively similar LOD values were obtained (0.3–1.2 mg/L and 1.0–3.4 mg/L, respectively). The application of MS detection provided useful structural information and unambiguous identification for insulins having similar or the same molecular mass. This work is considered to be important not only for the investigation of insulins but also for its potential contribution to the top-down analysis of proteins using CE-MS.

1. Introduction

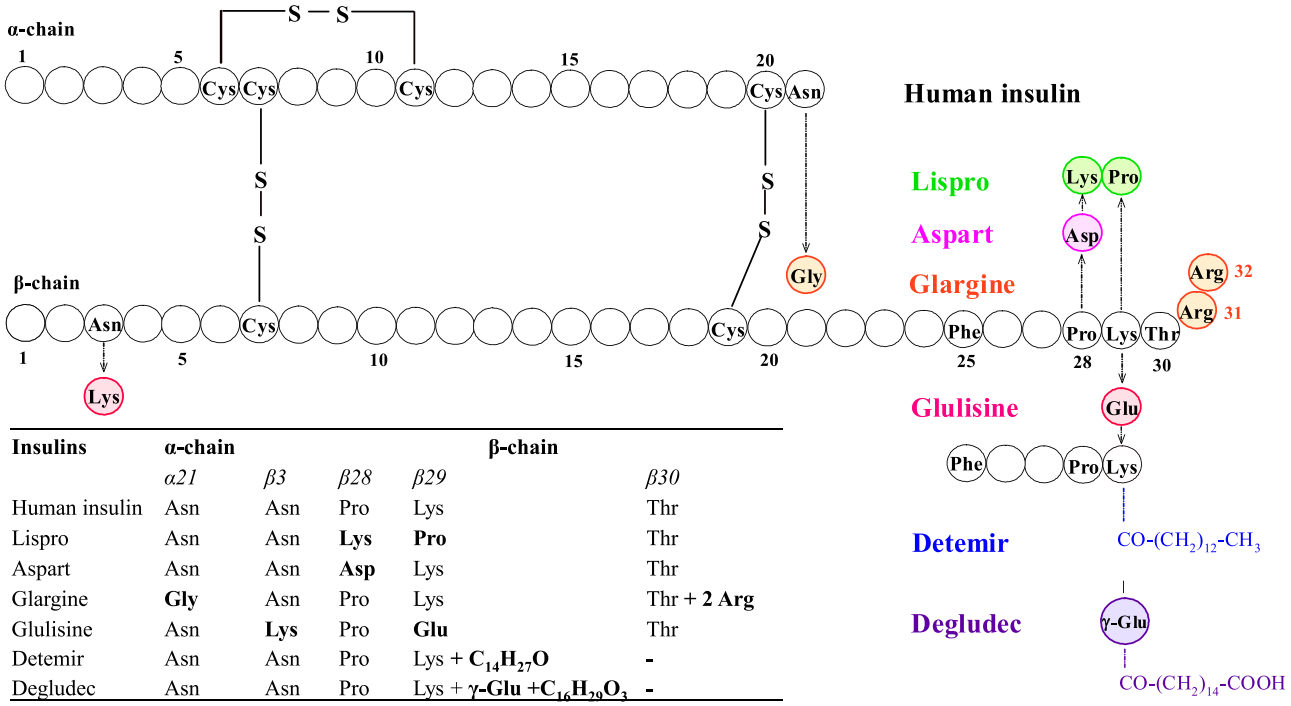
Human insulin plays a major role in the body by regulating blood glucose homeostasis [1]. The disruption of insulin metabolic activities due to decreased amounts of insulin, autoimmune responses or insulin resistance leads to diabetes mellitus [2]. Nowadays this illness is treated by administering insulin in the form of injection. Recombinant DNA technology enables the synthesis and development of human insulin analogues with different effects but they can also be a frequent target for adulteration [3,4]. For pharmaceutical, clinical or forensic applications, robust and straightforward analytical and quality control techniques are needed.

A variety of methods for the separation and detection of recombinant insulin formulations [1,3-5] and their degradation products [6–8], quantitation [2], impurity examinations [9,10] were described in the literature. These methods can be classified as immunochemical and instrumental analytical methods [3]. Since immunoassays like ELISA [11] or radioimmunoassays lack the selective identification of different insulin analogues [12], instrumental techniques such as HPLC [2,8-10,13] with UV or MS detectors gained popularity in this area. The current European Pharmacopoeia method [14] for the analysis of individual insulins is also based on HPLC-UV approach.

Several capillary electrophoresis (CE) methods, predominantly capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary gel electrophoresis (CGE) have also been reported for the determination of insulins. Lamalle et al. [4] and Haunschmidt et al. [15] utilized MEKC for the analysis of human insulin and 5 of its analogues. However, since MEKC separations necessitate the use of micelle forming detergents (e.g., sodium dodecyl sulfate) in the background electrolyte (BGE) [16], its coupling with MS detection is problematic. Ortner et al. [3] successfully separated an insulin mixture with MEKC coupled to MS by using a volatile detergent (perfluorooctanoic acid) in the buffer solution, however, the suppression of the MS signal could not be completely avoided. Similarly, CGE analysis [7] uses a polymer sieving matrix, which facilitates the separation of components by their size. The use of such a matrix in the BGE hinders the chance for hyphenation with MS and can lead to peak overlapping of insulin analogues having the same (e.g., human insulin and insulin lispro) or very similar molecular mass. Therefore, CGE and MEKC frequently employ UV detection, however, that does not allow the clear identification (eg.: molecular mass, sequence, structure) of proteins [5]. Although CZE is suitable for coupling with MS, only a relatively few works utilize CZE for the separation of insulins. Early studies demonstrated the separation of human insulin and human growth hormone [8] as well as the quantification of human insulin [17]. Later, separation performance of CZE for 6 insulin formulations was compared to MEKC where the apparent advantages of MEKC on selectivity and resolution over CZE were presented

* Corresponding author.
E-mail address: gaspar@science.unideb.hu (A. Gáspár).

https://doi.org/10.1016/j.chroma.2022.463351



Insulins	α-chain			β-chain	
	α21	*β3*	*β28*	*β29*	*β30*
Human insulin	Asn	Asn	Pro	Lys	Thr
Lispro	Asn	Asn	**Lys**	**Pro**	Thr
Aspart	Asn	Asn	**Asp**	Lys	Thr
Glargine	**Gly**	Asn	Pro	Lys	Thr + **2 Arg**
Glulisine	Asn	**Lys**	Pro	**Glu**	Thr
Detemir	Asn	Asn	Pro	Lys + $\mathbf{C_{14}H_{27}O}$	-
Degludec	Asn	Asn	Pro	Lys + **γ-Glu** +$\mathbf{C_{16}H_{29}O_3}$	-

Fig. 1. The structure of human insulin and its analogues.

[3,15]. CZE was applied for analyzing the fragments of human insulin [18] and for determining the binding constants of human insulin hexamer complexes with different components [19]. A successful application of CZE was implemented by Yeh et al. [1] where authors achieved the baseline separation of human insulin and two analogues in a single run. Including the earlier mentioned studies with CZE, the majority of separations employ BGE solutions with pH values higher than the isoelectric points (pI) of the components (pH 6.5–9.2) to minimize the protein-capillary surface interactions. The application of BGEs having low pH values or the modification of the capillary surface with dynamic or static coatings for the separation of insulin mixtures are also considered as good alternatives but tend to be overlooked in the literature. Recently, we have demonstrated that the adsorption of proteins (of varying sizes, including insulin) can be efficiently suppressed during CZE even in bare fused silica (BFS) capillaries if very low pH BGEs (pH=1.8) are used [20].

In this work, we studied the separation of human insulin and its 6 analogues using semipermanent physically (electrostatically) coated (successive multiple ionic-polymer layer (SMIL) [21]) and static coated (linear polyacrylamide, LPA) capillaries in acidic pH ranges and compared their performance with that of BFS capillaries. Although the main goal of this work was to determine insulin and its analogues, in a broader sense, the separation of seven very similar proteins (Fig. 1) is considered as an analytical challenge. There are several reasons why such an analysis of insulin analogues is valuable: (1) recently, top-down proteomics is a hot research field demanding new experiences about the CZE separation of intact proteins mixtures and their MS studies, (2) since these 7 analogues are small proteins with very little differences, it is important to consider, whether the CZE-MS method is useful in differentiating and quantifying such components, (3) the developed analytical method, which was proved to be suitable for the qualitative and quantitative analysis of 7 insulin analogues, is obviously suitable for the determination of each analogue in pharmaceutical formulations, (4) the method is likely applicable for the analysis of counterfeit insulin mixtures, where only minimal differences compared to the 7 studied analogues can be expected [5] and (5) surprisingly, an evaluative comparison of the detection sensitivities of proteins obtained with CZE-MS and CZE-UV is missing in the literature. The identification of the separated components by MS detection was also studied.

2. Material and methods

2.1. Reagents, samples

All chemicals were of analytical grade. Acetic acid (AA), formic acid (FA), ammonium hydroxide, ammonium acetate (NH_4Ac), acetonitrile, isopropyl alcohol (IPA), hydrochloric acid, sodium hydroxide, methanol, 3-(trimethoxysilyl)propyl methacrylate were obtained from Sigma Aldrich (St. Louis, MO, USA). SMIL coating agents hexadimethrine bromide (polybrene, PB) and dextran sulfate (DS) were purchased from Sigma Aldrich and Merck Millipore (Darmstadt, Germany), respectively. Tris–HCl, N,N,N',N'-tetramethyl ethylenediamine (TEMED), ammonium persulfate and acrylamide used for the LPA coating generation were purchased from Sigma Aldrich. Polymerization solution used for LPA capillary coating procedure contained 1 mL degassed 4% m/m acrylamide dissolved in Tris–HCl (pH=7.0), 1 μL TEMED and 10 μL 10% m/v APS solution (dissolved water).

Solutions of human insulin (Humulin R) and lispro (Humalog) by Lilly (France); glargine (Lantus) and glulisine (Apidra) by Sanofi (France) and aspart (Novorapid), degludec (Tresiba), and detemir (Levemir) by Novo Nordisk (Denmark) with 100 units/mL (3.47 mg/mL) concentration each were used for the analysis. The pH of the solutions, the isoelectric points and other characteristics of the studied insulins can be found in the Table 1. All sample solutions were diluted in deionized water (Millipore Synergy UV) to obtain the final concentration of 0.76 mg/mL. Sample, BGE and SMIL coating solutions were filtered by using a membrane filter of

Table 1
The main characteristics and analytical performance data of human insulin and its analogues obtained in SMIL capillary.

	Degludec	*Detemir*	*Aspart*	*Humulin*	*Lispro*	*Glulisin*	*Glargine*
pH *of formulation* [a]	7.6	7.4	7.2–7.6	7.0–7.8	7.0–7.8	7.3	4
pI	5.5	5.5	5.1	5.4	5.6	5.5	6.7
Average mass value (Da)	6102.3	5916.7	5825.6	5807.6	5807.6	5821.2	6063.8
Monoisotopic mass value (Da)	6099.8	5912.7	5821.6	5803.6	5803.6	5818.6	6058.8
Equation for calibration graphs[b]	$y = 19.766x - 0.0859$	$y = 89.764x - 0.2457$	$y = 45.381x - 0.016$	$y = 73.111x + 0.0117$	$y = 14.667x + 1.9733$	$y = 19.533x + 0.3645$	$y = 58.496x - 0.1546$
x-intercept[b]	−0.00435	−0.00274	−0.00035	−0.00016	−0.13454	0.01866	−0.00264
R^2	0.9988	0.9901	0.9984	0.9998	0.9908	0.9917	0.9997
Linear range (mg/L)[b]	4–500	1–300	2–500	2–500	3–500	3–500	2–500
LOD (mg/L)[b]	1.2	0.3	0.6	0.5	0.9	1.1	0.5
LOD (MS) (mg/L)	3.4	1.4	1.1	1.1	1.9	2.9	1.0
*RSD% (*min*)*	0.21	0.5	0.08	0.1	0.2	0.33	0.37
RSD% (area)[d]	7.5	7.3	8.5	5.8	7.7	8.8	7.9
Resolution[b]	26.1[c]	2.07	2.32	0.51	0.43	0.48	15.3
Number of theoretical plates/m (SMIL)	62,115	17,218	161,018	139,076	89,815	62,783	192,216
Number of theoretical plates/m (BFS)	11,158	8051	54,464	56,464	76,162	23,237	142,912
Number of theoretical plates/m (LPA)	938	3635	40,598	50,762	23,814	4465	42,600

[a] Provided by the producers.
[b] Obtained with UV detection.
[c] Calculated between m-cresol and degludec peaks.
[d] m-cresol is used as internal standard.

0.45 μm pore size before analysis. The stock solutions were stored at +4 °C.

BGEs applied for the analyses in uncoated (BFS) capillaries were 1 M FA (pH=1.8) and 50 mM NH_4Ac (pH= 7.0 and pH= 10.0); for SMIL and LPA coated capillary measurements 0.3 M FA (pH=2.3) and 50 mM FA (pH=2.6) solutions were employed, respectively. The current values never happened to exceed 30 µA (25, 29 and 10 µA for BFS, SMIL and LPA coated capillaries, respectively).

2.2. CE capillaries

BFS capillaries of 65 cm x 50 μm I.D. and 370 μm O.D. (Polymicro, Phoenix, AZ, USA) were used without coating and with SMIL or LPA coatings. Prior to first use, the BFS capillary was rinsed with 1 M NaOH for 20 min, water for 5 min and with the BGE of choice for 20 min.

SMIL preparation was carried out based on the procedure rendered by Haselberg et al. [22]. As coating solutions, 10% (m/v) PB and 3% (m/v) DS (prepared with deionized water) were used after being filtered. Prior to the coating procedure, the capillary was rinsed with 1 M NaOH for 30 min and water for 15 min. This was followed by 20 min PB, 10 min water, 20 min DS, 10 min water, 20 min PB and 10 min water rinsing at 1 bar to generate the 3 ionic layers of the coating. The capillary was then directly used in a CZE analysis. Between runs a 3 min-long BGE preconditioning was applied. To activate the coating after overnight disuse, 5 min water and 5 min BGE rinse was performed, followed by the application of +10 kV for 10 min. Capillary was stored in water following a 10 min-long water rinse [23].

The preparation of LPA was the same as in our earlier work [20], which was based on the technique suggested by Hjerten [24].

2.3. Instrumentation

CE separations were carried out using CE 7100 System (Agilent, Waldbronn, Germany) coupled to UV and high resolution MS detectors. For UV on-capillary (L_{eff} = 57 cm) detection, 200 nm detection wavelength was chosen. Hydrodynamic sample injection (50 mbar, 2 s) was carried out at the anodic end of the BFS and LPA coated capillaries and at the cathodic end of SMIL coated capillaries. For the electrophoretic separation +25 kV for BFS, +30 kV for LPA capillaries and −30 kV for SMIL capillary were used. CE instrument was operated and results were processed by OpenLAB CDS Chemstation version B.04.02 software (Agilent).

Mass detection was performed by MaXis II UHR ESI-QTOF MS (Bruker, Karlsruhe, Germany) MS instrument. CE-ESI sprayer interface (G1607B, Agilent) allowed the hyphenation of CE with MS. 1260 Infinity II isocratic pump (Agilent) was utilized for the transfer (4 µL/min) of the sheath liquid, which contained isopropyl alcohol:water (1:1) with 0.1% v/v FA. The following parameters were employed for the electrospray ion source (positive ionization mode): capillary voltage: 3.5 kV; end plate offset: 500 V; nebulizer pressure: 0.3 bar (during and 500 s after the injection it was switched off); dry gas temperature: 200 °C and dry gas flow rate: 4.0 L/min. The MS method was tuned according to the 600–2500 *m/z* mass range and 3 Hz spectra rate was applied. For the seven insulin species mass resolutions were in the range of 65,000–94,000 (FWHM). For MS/MS analyses the spectra rate was changed to 1 Hz and 20–1800 *m/z* mass range was used. The most abundant ions (5+ charged state) were selected as precursor ions and the collision energy was set to 45 eV. External mass calibration was ensured by ESI-MS Tuning mix calibrant solution (part No: G2431A, Agilent) for MS and by Na-formate for MS/MS analyses. Electropherograms were background corrected. The measurements were controlled by otofControl software version 4.1 (build: 3.5, Bruker) and the data was handled by Compass DataAnalysis version 4.4 (build: 200.55.2969).

3. Results and discussion

3.1. CZE separation of insulin and its analogues

To obtain the selective separation of the 7 insulins in a BFS capillary, the analysis of the insulin mixture was performed in both high (pH=10.0) and quite low (pH=1.8) pH separation media. In the case of high pH BGEs, both the proteins and the capillary surface possess a large net negative charge, by which – theoretically - the adsorption challenge can be overcome. (In spite of the large net negative charge of the components, the counter directed EOF drives them toward the cathode.) Similarly, low pH values ensure a protonated capillary surface (minimal or zero EOF) and thus proteins having a large net positive charge can readily migrate to the detector without being exposed to adsorption. The analysis of the insulin mixture in high pH BGE indicated an incomplete separation profile (Fig. 2c), where some components displayed narrow and decent peak shapes, while others could not be resolved. It should be noted that m-cresol (common additive in insulin pharmaceuticals) possesses negative charge at pH=10, thus its peak appears close to the insulin analogue migrating first. However, the BGE solution with very low pH value offered better selectivity, since strong acidic medium could seemingly separate more insulins in a BFS capillary (Fig. 2a). On the contrary, upon the use of neutral pH (pH=7.0), poor resolution was acquired due to the co-migration of insulins (Fig. 2b), which is the consequence of the very similar charge-to-size ratios (the pH of BGE is close to the pI values of proteins) and the strong adsorption of insulins onto the capillary surface. (At pH=7 all insulin analogues have small net negative charge, but those also include some positively charged functional groups which can interact with the negatively charged capillary surface.)

Although the use of strongly acidic pH (pH=1.8) seems promising, poor separation efficiency can be reported (theoretical plate numbers are given it Table 1) due to wide insulin peaks (especially for degludec and detemir). The triangular shape of the peaks reminds us of electrodispersion, which normally occurs when there is a considerable difference between the mobilities of the analyte and co-ion of the background electrolyte. Distorted (slightly right-angled triangle) peak shape for insulin was found in the literature, as well [5]. By decreasing the analyte concentrations, the resolution could be increased but the distorted character of these peaks remained.

The separation of the insulin mixture was also studied in neutral LPA and positively charged SMIL capillaries. LPA is a covalent coating ensuring the neutral surface of the capillary wall and the suppression of electroosmotic flow (EOF). Thereby the charged proteins are expected to be separated without interacting with the capillary surface. Although LPA coating is predominantly applied for the analysis of large proteins, its use for small peptides has also been documented in the literature [25,26], which makes it a proper choice for the separation of insulin analogues, as well. The main limitations of LPA coated capillaries are the operating pH range and incompatibility with organic solvents. The performance of LPA is efficient from slightly higher acidic pH values (above pH=2.3 [27]) up to pH 8. By using BGEs of moderate pH, the detection of proteins with pI values in a neutral range (e.g., insulins, especially glargine pI~6.7), would not be attainable owing to their minimal mobility and the lack of EOF. The separation of the 7 components could be achieved at pH=2.7 (50 mM FA) (Fig. ESM-1). However, similar to the performance of BFS, wide and triangular peak shapes were observable with the LPA coated capillary, as well.

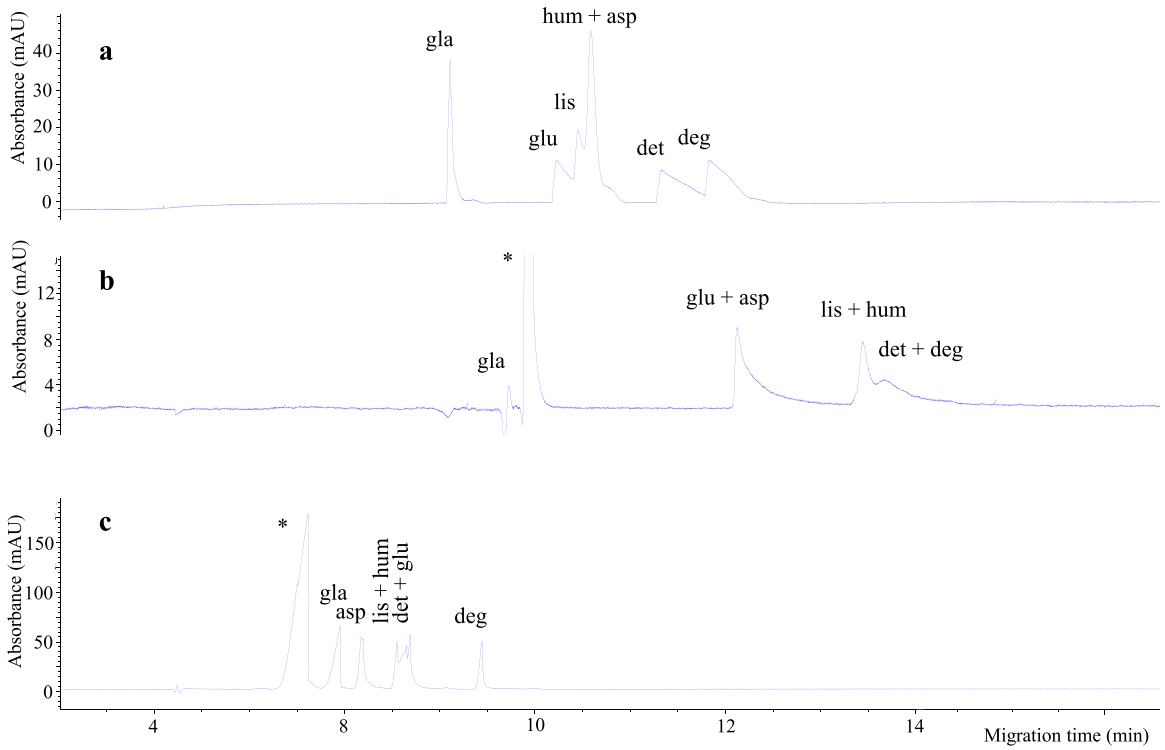


Fig. 2. The analysis of the 7 insulin mixture in BFS capillary using acidic, neutral and basic BGE: (**a**) 1 M FA (pH=1.8), (**b**) 50 mM NH_4HAc (pH=7.0), (**c**) 50 mM NH_4HAc (pH=10.0).
Conditions: separation voltage: +25 kV, injection: 50 mbar x 2 s, preconditioning: 3-step washing (18 min 1 M NaOH, 6 min acetone and 24 min BGE), UV detection at 200 nm. *m-cresol Sample: hum=human insulin; lis=lispro; gla=glargine; glu=glulisine; asp=aspart; deg=degludec; det=detemir.

To improve peak tailing, the effect of sample dilution was studied in the LPA coated capillary (Fig. ESM-2). Unfortunately, peak narrowing upon sample dilution was not considerable (e.g., the wide peaks of degludec and detemir (N = 938 and N = 3635, respectively) could not be narrowed) (Fig. ESM-2b). The wide peaks lead not only to overlapping but also to poor detection sensitivity. The general belief is that large proteins have a larger tendency to adsorb onto capillary walls. However, when human serum albumin (HSA) - a larger protein of ~66 kDa molecular mass and pI = 4.7 - was analyzed using the same LPA capillary, there was no sign of excessive peak broadening. In fact, most insulins (glulisine, lispro, degludec and detemir) showed wider peaks than HSA (the size of which is more than ten times larger) (Fig. ESM-3). This suggests that wall adsorption is influenced not by the size but rather by the pI of the component and the charge of the capillary wall. The slow migration of the components (smaller charge-to-size ratio) obviously contributes to zone broadening, but other effects (e.g., interactions or pH differences between insulin solutions and the electrolyte ions [28]) are also important. The aforementioned results on wider and narrower insulin peaks were reproducible for different LPA capillaries and for varying lengths (Fig. ESM-4). Although the longer (100 cm) LPA capillary was expected to give a better separation of insulins, the increased analysis time did not lead to enhanced resolution. Therefore, a capillary length of 65 cm (shortest length possible in the case of our CE-MS system) was applied in our studies.

Compared to LPA coated capillaries, the coating preparation and capillary conditioning procedures for SMIL capillaries are considerably simpler. The semipermanent physically adsorbed coating generated by the 3-step successive rinse with polycationic PB, polyanionic DS and PB, provides a stable, positively charged capillary surface [21]. The cationic surface provokes an anode-directed, strong EOF. As SMIL operates mainly in acidic medium, EOF opposes the electrophoretic mobilities of counter (cathode) directed proteins. Even a slight alteration in pH caused a considerable change in the separation of the 7 insulins using the SMIL capillary (Fig. ESM-5). The best resolution between human insulin and aspart was achieved using pH 2.3, while at pH 2.1 their co-migration is apparent.

The preparation time of the capillary can be reduced by using a single layer PB coating, the performance of which does not lag far behind that of the multilayer coated capillary (Fig. ESM-6). However, single layer PB capillary demonstrated lower precision data in migration times and peak areas due to the incomplete and thin (~1 nm) coverage of the fused silica surface [29], which necessitates the regenaration of the coating prior to each run (this is time-consuming between runs and troublesome in the case of MS detection). Nevertheless, by having a thicker polymer layer (5 nm in the case of three-layer SMIL [29]) on the BFS wall, these difficulties can be eliminated. The electropherograms obtained with the optimal separation performances in the three different capillaries are compared in Fig. 3. The best resolutions for the 7 insulins were achieved in the SMIL capillary.

Considering the studies by Katayama [21] and Haselberg [22] as well as our own experience, SMIL coating presents good stability up to 40 runs without regeneration and can be used up to a month when appropriate storage and reactivating conditions are applied. In addition, SMIL coating demonstrates high capillary-to-capillary reproducibility with 0.63% RSD in acidic media. In optimized conditions, LPA coating proved its stability over 100 runs. High precision values of ~0.5% and ~4.9% were observed for migration time and peak area, respectively (n = 25).

3.2. Mass spectrometric detection of insulin and its analogues

Although MS detection for large proteins is often less sensitive than simple UV spectrophotometry (due to the wide charge distributions and different adducts of proteins), in the case of small

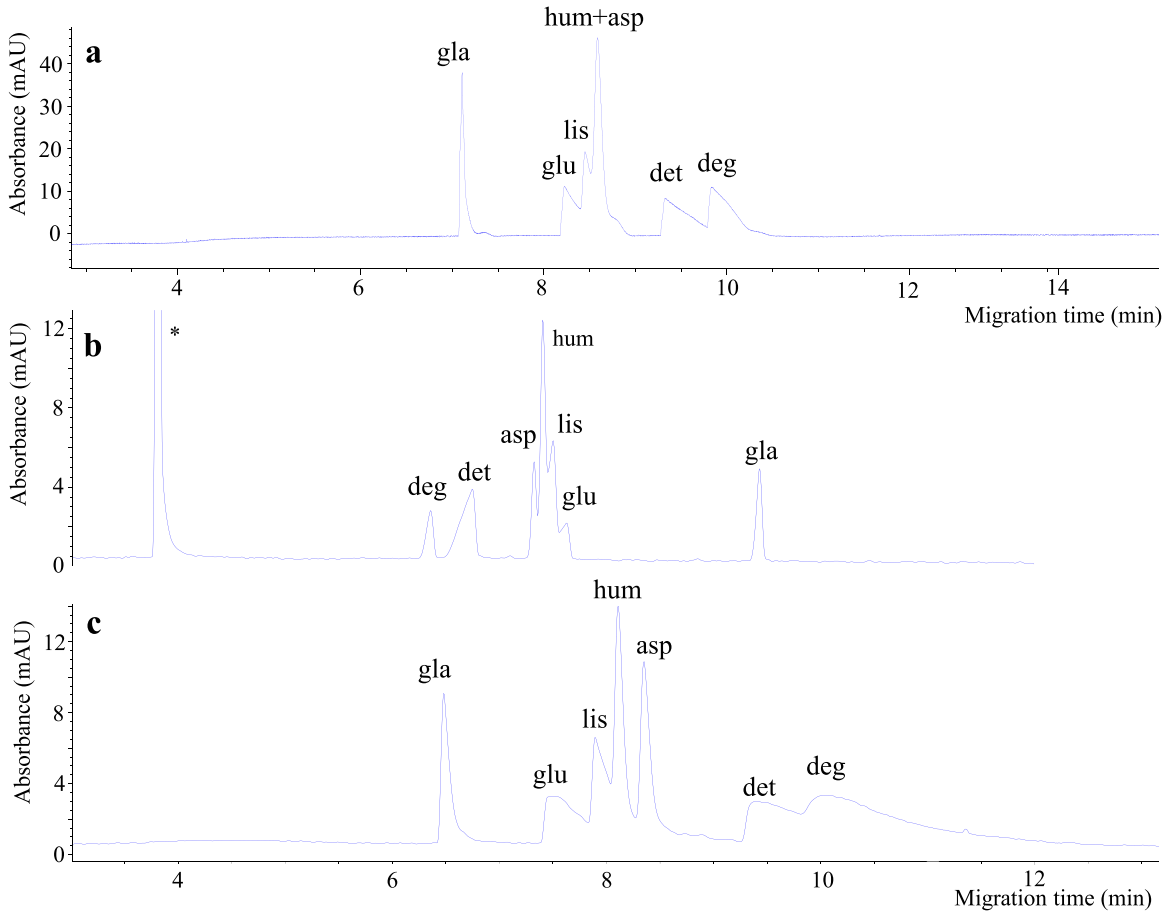


Fig. 3. The CZE electrophorerograms obtained for insulins in three different capillaries using optimized conditions. (**a**) BFS capillary, BGE: 1 M HCOOH (pH 1.8), the other parameters are the same as stated at Fig. 2. (**b**) SMIL coated capillary, BGE: 0.3 M FA (pH 2.3), separation voltage: −30 kV, injection: 50 mbar x 2 s, preconditioning: 3 min BGE washing. (**c**) LPA coated capillary, BGE: 50 mM FA (pH 2.6), separation voltage: +30 kV, injection: 50 mbar x 2 s, preconditioning: 5 min BGE washing. UV detection was performed at 200 nm. *m-cresol.

proteins such as insulins, MS offers similar sensitivity in addition to the extensive qualitative information. Insulin, being a small protein, possesses a mass spectrum with relatively simple isotopic distribution and only a few charged forms. In acidic medium (pH=2.1) glargine is present up to the $[M + 8H]^{8+}$ charged form, whereas the highest charged form for other insulins is limited to $[M + 6H]^{6+}$ (Fig. ESM-7), unlike the basic medium (pH 9.0), where the highest changed form is $[M + 5H]^{5+}$ (Fig. ESM-8).

The electropherogram obtained for the mixture of the 7 insulins with MS detection is shown in Fig. 4. The experimental mass values of the separated insulin analogues agreed within 1 ppm accuracy with the theoretical masses (Fig. ESM-9). A better resolution of insulin peaks could be acquired by disabling the ESI nebulization pressure for the first 500 s of the electrophoretic run, which hindered the siphoning effect (little vacuum at the outlet end of the CE capillary).

Besides the separation, information about the structure would also be necessary when analyzing insulin mixtures. Structural information can be acquired from the dissociation of molecular ions. Several fragmentation techniques exist, the most important ones being the collision induced dissociation (CID), electron transfer dissociation (ETD), electron capture dissociation (ECD) and UV photodissociation (UVPD) – each yielding well-defined, characteristic ion series. Utilizing a combination of these strategies can provide complementary data sets, which facilitates structural elucidation. The top-down investigation of intact proteins is quite a challenging task, especially in cases where several disulfide bridges are present in the molecule. Certain fragmentation techniques (e.g., ECD) allow the rupture of the S-S bond [30], however, CID is generally not amenable for such purposes. Under CID conditions, the preferential cleavage sites are at the peptide backbone outside the disulfide loop, potentially leaving a considerable part of the molecule intact and inaccessible. However, there are works describing the rupture of disulfide bridges using positive CID conditions [31,32]. The preliminary reduction of proteins (e.g., with tris(2-carboxyethyl)phosphine-HCl [33,34]) alleviates the difficulties associated with poor fragmentation coverage at the cost of increased analysis time.

Regardless of the three disulfide bonds present in insulins, the MS/MS analysis of the intact molecule with ESI-CID can, in fact, be useful for differentiating insulins having very similar (or the same) masses and structures. This is because the alterations in amino acid residues are located outside the disulfide loop. The utility of MS/MS is demonstrated by its ability to discern insulin analogues differing only in the sequential order of 2 amino acids. The appearance of diagnostic fragments enabled the unambiguous differentiation of these analogues (human insulin and lispro) [33,34]. Apart from these diagnostic ions, there was a scarcity in product ion peaks when samples were not reduced prior to analysis.

The restricted fragmentation behavior of insulin due to the presence of disulfide bonds is demonstrated in our experiments, as well (Fig. 5). Aspart and human insulin were chosen for the comparative MS/MS analysis, which differ only in the amino acid at the B28 position (Asp → Pro). The Asp→Pro-change causes a mass

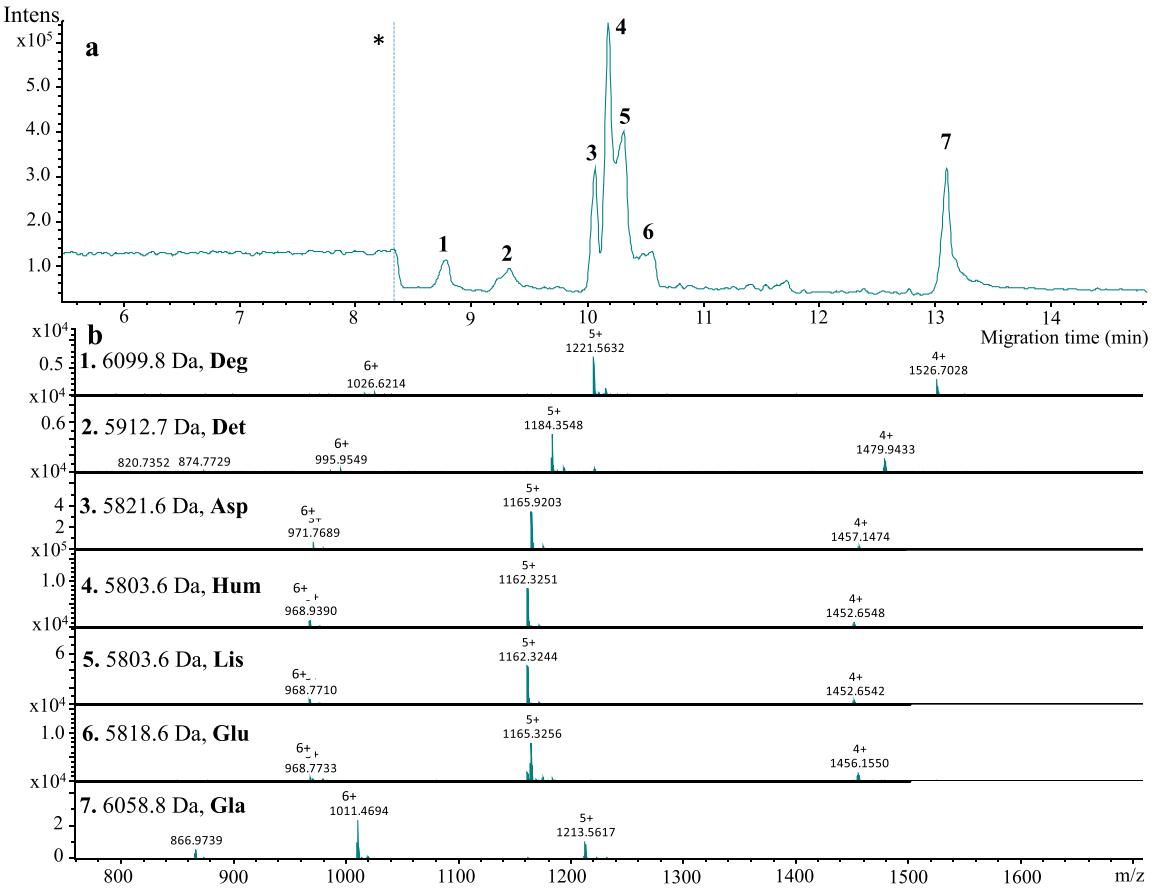


Fig. 4. The CZE–MS separation of the insulin mixture in SMIL capillary: Base peak electropherogram (**a**) and mass spectra of separated insulins (**b**). *Switching on the ESI nebulization pressure. Conditions: 65 cm SMIL coated capillary, BGE: 0.3 M FA (pH 2.3), separation voltage: −30 kV, injection: 50 mbar x 2 s, preconditioning: 3 min BGE washing, nebulization ESI pressure: 0.3 bar, sheath liquid flow rate: 0.4 mL/min, dry gas temp.: 200 °C, spectra rate: 3 Hz, *m/z* range: 600–2500.

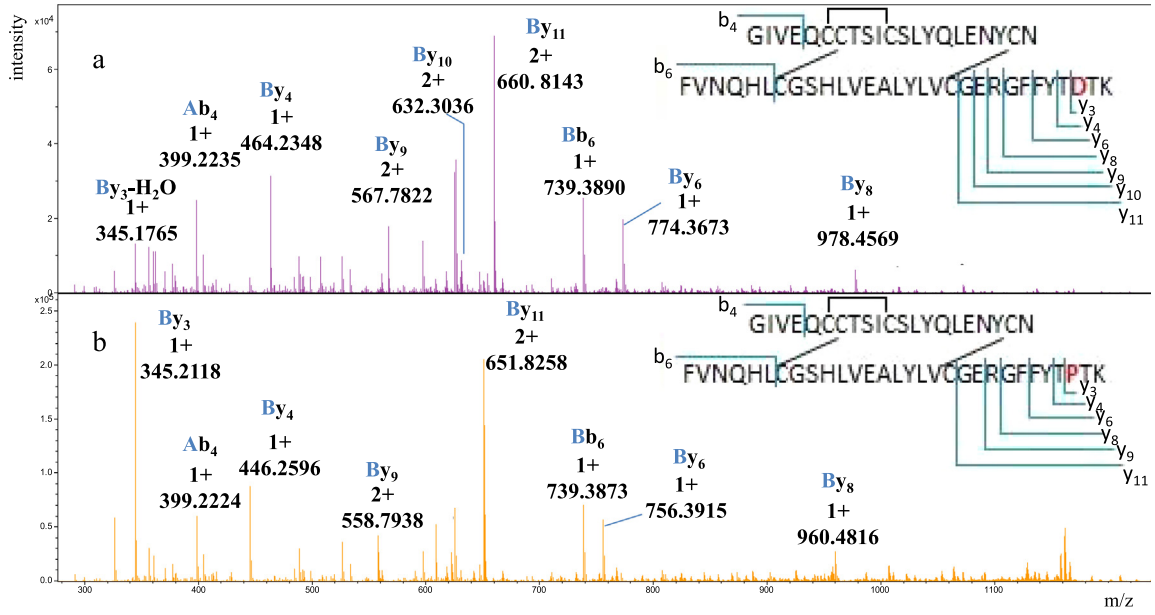


Fig. 5. The spectra of aspart (**a**) and human insulin (**b**) from MS/MS analysis. The legends for the annotated peaks contain the chain (in blue) and the fragment type. Structure of the analogues are indicated, highlighting the difference in amino acid sequences in red as well as identified fragment types.

Table 2
List of peaks assigned on the MS/MS spectrum of aspart.

Experimental m/z	*Theoretical m/z*	*Fragment type*	*Chain*
345.1765	345.1769	y_3 -H_2O	B
363.1875	363.1874	y_3	B
399.2235	399.2238	b_4	A
446.2245	446.2245	y_4 – H_2O	B
464.2348	464.2351	y_4	B
567.7822	567.7826	y_9^{2+}	B
632.3036	632.3039	y_{10}^{2+}	B
660.8143	660.8146	y_{11}^{2+}	B
739.3890	739.3886	b_6	B
774.3673	774.3668	y_6	B
851.0464	851.0471	$[A_{(16-21)}B_{(16-30)}]y^{3+}$	A-B
893.7325	893.7333	$[A_{(15-21)}B_{(16-30)}]y^{3+}$	A-B
934.9183	934.9183	$[A_{(20-21)}B_{(17-30)}]y^{2+}$	A-B
944.4275	944.4306	$[A_{(1-14)}B_{(1-12)}]b^{3+}$	A-B
954.0928	954.0940	$[A_{(1-15)}B_{(1-11)}]b^{3+}$	A-B
978.4569	978.4567	y_8	B
1016.4501	1016.4506	$[A_{(20-21)}B_{(16-30)}]y^{2+}$	A-B
1137.9930	1137.9934	$[A_{(17-21)}B_{(17-30)}]y^{2+}$	A-B
1155.0043	1155.0037	$[A_{(18-21)}B_{(16-30)}]y^{2+}$	A-B

shift of −17.9742 Da. The mass shifts observable in the product ion spectra indicated the presence of fragments that contain the B28 residue. As can be seen in Fig. 5, a fairly large number of such ions occur in the spectra and there is a clear abundance of fragments that contain smaller peptides excluded from the disulfide-bonded region. Cleavage took place typically at the amide bond, leading to b- and y-type ions (where "b" and "y" denote ions extending from the N- and C-terminus, respectively and subscripts express the amino acid position at which fragmentation occurred (Fig. ESM-10) [35]. Upon a closer inspection of the MS/MS spectra, larger peptides spanning the A-B chains also appear. These peptides show the traditional b or y-type fragmentation [35], only they are held together by inter-/intrachain disulfide linkages. MS/MS fragments assigned for Aspart are listed in Table 2.

3.3. Analytical performance

The CZE-MS method developed for human insulin and its 6 analogues was evaluated for its analytical performance on the separation and detection. The main parameters for method validation are provided in Table 1. The linear ranges of the calibration diagrams based on the CZE-UV measurements conducted with the SMIL capillary covered the concentration range between 1 - 500 mg/L. These calibration graphs gave satisfactory linearity values, with R^2 being the lowest for detemir (0.9901) and the highest for glargine (0.9997). The LOD values ranged between 0.3–1.2 mg/L. In the case of MS detection, the LOD values based on base peak electropherograms (BPE) ranged between 1.0–3.4 mg/L. The LOD data obtained with MS would likely be decreased by using high sensitivity mass spectrometers. 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. Detection sensitivities were further weakened by peak broadening.

The precision values were studied based on 10 successive measurements on the SMIL coated capillary (Fig. ESM-11), showing good repeatability in time with a maximum of 0.5 RSD% value for detemir (m-cresol was used as a time reference marker). The RSD% of peak areas were poorer (5–9 RSD%) even when internal standard (m-cresol) correction was applied. The larger RSD% values were mainly caused by the slight fluctuation in adsorbed proteins and hence larger integration errors due to the tailed and overlapped peaks.

Due to the very similar charge/size ratios of the investigated insulins and peak tailing effects, not all peaks were baseline separated. Therefore, the plate number and the resolution data show large variance (Table 1). The highest plate numbers could be obtained in the SMIL capillary. While peak broadening caused decreased plate numbers for several insulins, the baseline separated and narrower glargine peak shows the highest plate numbers with 192 000/m.

4. Conclusions

In the present work, we studied the relevance and analytical performance of BFS, static LPA and semipermanent coated SMIL capillaries in the analysis of human insulin and its 6 analogues. These studies are considered to be important not only for the investigated insulins but also for their potential contribution to the top-down analysis of proteins using CE-MS. When compared, the coated capillaries showed a better separation of insulin peaks than the BFS capillary, however, BFS utilizing very low pH (pH=1.8) BGEs can also be a simple, proper alternative for the determination of a single insulin in real samples. The separation of several insulins in a single sample would facilitate the analytical and quality control of insulin formulations, particularly the mixed insulin solutions [36]. This is necessary especially for the analysis of counterfeit insulin mixtures [4].

MS can provide useful structural information and unambiguous identification, however, the application of MS detection after CZE separation requires the careful selection of BGE parameters. As a general belief, the sensitivity of CE-MS is typically at least one order of magnitude lower compared to CE-UV. However, this statement is valid only for small molecules. Upon surveying the literature relating to intact protein analysis (Table ESM-1, including [37–39]) we found no report demonstrating that CE-MS yields better LOD values than CE-UV. It is also obvious that the larger the protein the higher the superiority of the CE-UV over the CE-MS in terms of detection sensitivity. Since insulin is a small protein with a mass spectrum showing relatively simple isotopic distribution and only a few charged forms, similar detection sensitivity can be obtained with UV and MS detection. Although the ESI-CID analysis of proteins in positive ionization mode typically generate fragmentation patterns bearing limited information, in our case it enabled the identification of the commercial insulins studied without the incorporation of additional sample preteatment steps, since the variations in amino acid sequences reside outside the disulfide bonded region.

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

Narmin Hamidli: Data curation, Investigation, Writing – original draft. **Blerta Pajaziti:** Conceptualization, Investigation, Data curation. **Melinda Andrási:** Data curation, Investigation, Methodology. **Cynthia Nagy:** Investigation, Data curation, Writing – original draft. **Attila Gáspár:** Conceptualization, Methodology, Supervision, Writing – original draft.

Acknowledgments

The authors acknowledge the financial support provided to this project by the National Research, Development and Innovation Office, Hungary (K127931), Stipendium Hungaricum (#242771) and

the New National Excellence Program of the Ministry for Innovation and Technology (ÚNKP-21–3-II). BP is grateful for the Central European Exchange Program for University Studies (CEEPUS) for her fellowship (CIII-RO-0010).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463351.

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