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Research Article

Quantification of buserelin in a pharmaceutical product by multiple-injection CZE

An efficient and rapid separation method based on reversed-polarity multiple-injection CZE (MICZE), has been developed for the quantification of buserelin in a pharmaceutical product. The determinations were performed by serially injecting five standard solutions of buserelin (50–300 μg/mL) and one reference analyte into a Polybrene-coated capillary. All the samples contained goserelin, an analog peptide to buserelin, as internal standard (IS). Immediately after pressure injection, the applied sample plugs were subjected to electrophoresis for 2 min at −25 kV. Consequently, each sample plug became isolated from its neighboring plugs by the BGE, composed of 100 mM phosphate-triethanolamine buffer at pH 3.0 containing 10% v/v ACN. During separation the individual sample components migrated at similar velocities and as distinct zones through the capillary giving 24 peaks, 12 from the analyte and the IS and 12 from the sample matrix. The buserelin content of the pharmaceutical product was determined to be 0.94 ± 0.05 mg/mL, which is only a slight deviation from the declared concentration (1 mg/mL).

Keywords:
Buserelin / Multiple injection / Peptides / Polybrene / Quantitative analysis
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1 Introduction

The increasing use of synthetic peptides as therapeutical agents is a result of their high specificity [1, 2]. Buserelin and goserelin are examples of such important peptides which reduce the plasma level of testosterone and estradiol for treatment of prostate and breast cancer [3–5]. Buserelin and goserelin (Fig. 1) are analogs with different modifications at the *C*-terminal. Incorporation of a D-serine residue, which does not occur naturally, into the peptide chains confers a higher metabolic stability on the peptides [2].

CE has been demonstrated to be an excellent technique for the analysis of peptides and proteins [6–11]. CZE has been applied to analyses of buserelin [12] and similar drugs, *e.g.*, goserelin [13] and lecirelin [14]. However, quantitative analysis in the conventional single-injection mode is time-consuming, since each analyte or standard sample has to be analyzed separately and at least in triplicate. Furthermore, the construction of a calibration graph for the quantification requires analysis of two or more standard samples.

In order to speed up the quantification of buserelin, the potential of multiple-injection CZE (MICZE) was explored. In MICZE, several analyte samples are sequentially introduced into the capillary. By application of an electrical field over the capillary for a short period of time between each injection, the zones become isolated by buffer plugs. MICZE is an efficient separation technique which has been used for evaluation of association constants [15, 16], DNA analysis [17–20], microfluidic sample handling [21], as well as enantiomeric separation [22].

The aim of this paper has been to develop a CZE method based on multiple injection technique for the quantification of buserelin and goserelin in pharmaceutical products.

2 Materials and methods

2.1 Chemicals

Reference standards of goserelin acetate and buserelin acetate were supplied by the European Directorate for the Quality of Medicines (EDQM). Triethanol amine (98%), hexadimethrine bromide (Polybrene), and ACN of HPLC reagent grade were purchased from Sigma-Aldrich Chemie

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Abbreviations: IS, internal standard; **MICZE,** multiple-injection CZE; **RMD,** relative mobility difference; **TEA,** triethanolamine

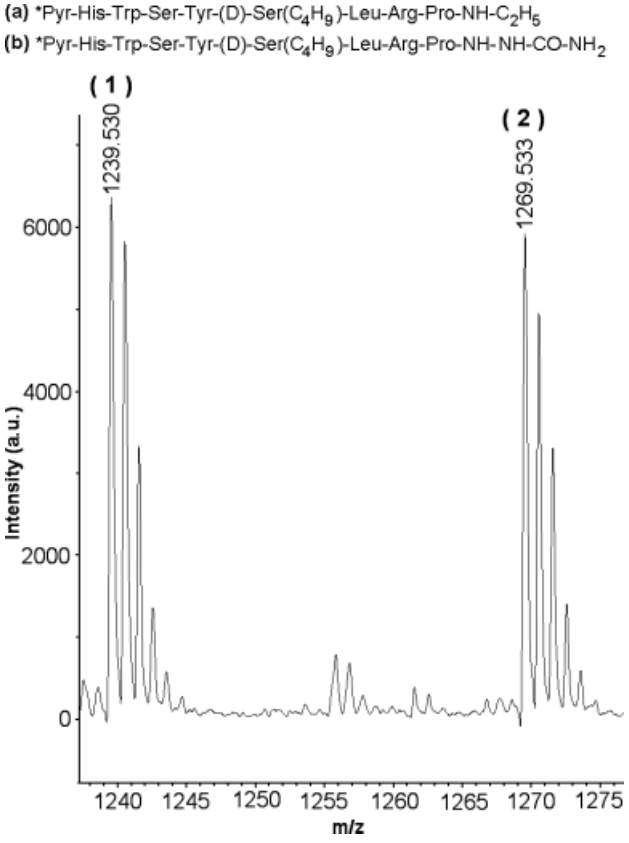

Figure 1. Molecular structures and MALDI-TOF-MS spectrum of (A) buserelin and (B) goserelin. (1) [Buserelin + H]$^+$ and (2) [goserelin + H]$^+$. Experimental conditions as given in Section 2. * Pyr stands for pyroglutamic acid.

(Steinheim, Germany). α-Cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma (St. Louis, MO, USA). Phosphoric acid, hydrochloric acid (37%), and sodium hydroxide were obtained from Merck (Darmstadt, Germany). TFA (99%) was supplied by Pierce (Rockford, IL, USA). The ProteomeLab™ kit for CIEF was supplied by Beckman Coulter (Beckman Coulter, CA, USA). Mesityloxide of technical grade (>90%) was purchased from Fluka Chemie, (Buchs, Switzerland). All the solutions were prepared from double-distilled water, produced by a Maxima water purification system from USF Elga (Bucks, UK) and were filtered through 0.22 μm polyvinylidene fluoride syringe filters (Millipore, Cork, Ireland).

2.2 CE

CE experiments were performed using a P/ACE™ MDQ system (Beckman Coulter), equipped with either a DAD or a conventional UV detector, monitoring at 220 or 214 nm. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) or a neutrally coated capillary, eCAP™ (Beckman Coulter), 60 cm (50 cm effective length) × 50 μm id, were kept at a constant temperature of 20.0°C and the applied voltage was −25 kV unless otherwise stated. The sample storage temperature was 10°C. Integration of the peaks was performed by using 32 Karat software version 7.0 Build 1048 (Beckman Coulter). The BGE was composed of either 65 mM sodium phosphate at pH 3.0 or 10% ACN v/v in 100 mM phosphoric acid titrated to pH 3.0 with triethanolamine (TEA).

The samples were injected for 3 s by applying a pressure of 3.4 kPa at the cathodic end of the capillary. In MICZE mode, the separations were carried out by injecting six different analyte samples. Between each injection the injected sample plug was electrophoresed for 2 min at −25 kV in order to get the sample plugs well-separated, Fig. 2. Thus, the electrophoretic migration distance ($\sim L_d$) for all the sample plugs was approximately the same.

In addition, a Hewlett Packard^{3D} CE system (Waldbronn, Germany) equipped with a fused-silica capillary of dimensions 58.5 cm (50 cm effective length) × 50 μm id, was used to verify the robustness of the method. This system was used under the same conditions as for the P/ACE MDQ system and data analysis was performed using Agilent ChemStation software Revision A.10.01 (Waldbronn, Germany).

2.3 CIEF

CIEF of buserelin and goserelin was carried out by using the ProteomeLab kit for CIEF from Beckman Coulter (Beckman Coulter). The solutes, ampholytes (3–10), and p*I* markers; ribonuclease A (p*I* = 9.45) and carbonic anhydrase (p*I* = 5.9), were dissolved in the CIEF-polymer solution. The capillary

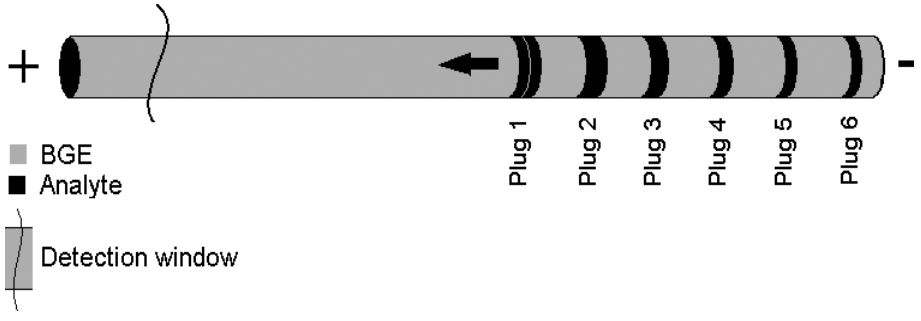

Figure 2. Schematic illustration of MICZE. Immediately after pressure injection each sample is subjected to a short period of electrophoresis. The applied sample plugs are therefore separated by the BGE. As seen, the sample plugs become broader as the sample components separate during the electrophoresis process.

was filled with this mixture by applying a pressure of 200 kPa at the injection end of the capillary for 1 min. The anolyte and the catholyte solutions were composed of 10 mM H_3PO_4 in the separation polymer medium and 20 mM NaOH in water, respectively.

The separations were performed at 20°C in a neutrally coated capillary from Beckman Coulter. The dimensions of the capillary were 30 cm (20 cm effective length) × 50 μm id The focusing step was carried out at 15 kV for 6.0 min, followed by a mobilization step at a pressure of 3.4 kPa at the injection (anodic) end of the capillary. During the mobilization a voltage of 21 kV was applied over the capillary to counteract parabolic zone broadening caused by the pressure-driven flow. Detection was performed at 214 nm. Between each injection the capillary was rinsed at 200 kPa with 10 mM H_3PO_4 for 1 min, followed by a 1-min water wash.

2.4 MALDI-TOF-MS

MALDI analysis of buserelin and goserelin was performed on an Autoflex (Bruker Daltonics, Bremen, Germany) reflector type TOF mass spectrometer, equipped with a pulsed nitrogen laser working at 337 nm. The instrument was operated in the positive ion mode $[M + H]^+$ with delayed extraction at an accelerating voltage of 20 kV and with a variable voltage reflectron. The peptides were dissolved in 50% v/v ACN in water containing 0.1% v/v TFA. One microliter of the solute–matrix mixture was applied on the MALDI sample plate and allowed to air-dry before being placed in the mass spectrometer. The spectrometer was calibrated using Bruker standard peptide mixture, consisting of seven peptides ranging from m/z 1046.51 to 3147.47.

2.5 Capillary coating

Regnier and others [23, 24] originally reported noncovalent coating of fused-silica capillaries with polycationic polymers. The coating creates a temporarily chemically stable layer on the inner surface of the capillary. Cationic polymers are adsorbed onto the capillary wall through hydrogen bonding, electrostatic, and hydrophobic interactions, depending on the chemical structure of the polymers. The modification of the capillary surface eliminates the adsorption of positively charged proteins and peptides onto the surface [25], provided that the coating does not contain hydrophobic groups. Dynamic modification of the capillary wall has the advantage over covalent coating in that it can be replaced or removed faster. The drawback, however, is that a dynamic coating is generally not stable enough for several runs [26].

In this study, the capillary surface was dynamically coated with either TEA or Polybrene. TEA and Polybrene reverse the net charge of the capillary wall and thus, change the direction of the EOF [26, 27]. The TEA coating was performed by filling the capillary with TEA containing BGE, prior to the injection of the sample. The Polybrene coating was performed in several steps as follows:

(i) The capillary was washed with 0.1 M HCl for 5 min, 2% m/v SDS for 4 min and 0.1 M NaOH for 5 min to remove possible residual peptide molecules and to regenerate the capillary surface in preparation for the coating. These washing steps were performed by applying a high pressure (140 kPa) at the injection end of the capillary; (ii) 0.25% m/v aqueous Polybrene solution was then introduced into the capillary for 4 min; and (iii) The coated capillary was, finally, rinsed and then filled with the BGE for 4 min before the injection of the analyte (at 3.4 kPa for 3 s).

2.6 Quantitative analysis

The internal standard (IS) method was used for the quantitative analysis. The structural similarity between buserelin and goserelin, Fig. 1, prompted us to employ goserelin as an IS for the quantitative determination of buserelin and *vice versa*. The presence of the IS compensates for variations in injection volume and thereby improves the injection precision [28]. The standard curve correlates the relative peak area between the standard and the IS, with the corresponding amount (concentration) of the standard. Because of the small mobility difference between buserelin and goserelin, the peak areas were not corrected by their corresponding migration times [29]. Standard solutions of buserelin and goserelin were prepared at different concentrations, ranging from 50 to 300 μg/mL in water containing 10% v/v BGE. As IS, goserelin (or buserelin) was added to the standards and samples at a constant concentration, *i.e.*, 50 μg/mL.

Each MICZE run yielded an IS curve (with five concentrations), that was used for the determination of the analyte concentration. The analyte was analyzed together with the standards.

The solute concentrations which gave S/N of 3 and 10 were selected to be the LOD and LOQ, respectively.

3 Results and discussion

3.1 Separation of buserelin and goserelin

A CZE method for quantitative analysis requires calibration standards and an IS which should be chemically and physically similar to the analyte. Buserelin and goserelin are two peptide analogs which can be used as IS for the quantification of each other in pharmaceutical formulations. Therefore, initial experiments aimed at separating buserelin and goserelin by CZE. These peptides contain nine amino acids with the same sequence. As Fig. 1 shows, the carboxyl groups at the C-terminals of buserelin and goserelin are chemically blocked with ethylamine (C_2H_5–NH_2) and semicarbazide (NH_2–NH–CO–NH_2), respectively. This makes goserelin 30 amu heavier than buserelin. The calculated monoisotopic molecular masses of buserelin and goserelin are 1238.63 and

1268.62 Da, respectively. These masses were in good agreement with those determined by MALDI-MS, *i.e.*, 1238.53 and 1268.53 Da, respectively, Fig. 1.

Ethylamine and semicarbazide, attached to the *C*-terminals, cannot be ionized and thus do not contribute to the p*I*s of the peptides. The identical amino acid sequence of the peptides (see Fig. 1) brings about similar conformation and similar p*I*s, which was confirmed by a CIEF analysis, Fig. 3. The high p*I* value (approximately 9.6) is explained by the amino acid composition and sequence of the peptides, particularly by strongly basic Arg-residue and weakly acidic Tyr-residue present in the peptide chains.

Thus, the absence of a charge difference between the peptides implies that they can be electrophoretically separated only on the basis of their mass difference. The larger molecular mass of goserelin results in a lower electrophoretic mobility (μ_{ep}) according to the following equation [30]:

$$\mu_{ep} = CZ/M^{2/3} \quad (1)$$

where C is a constant, Z is the peptide valency (or charge number), and M is the molecular mass.

The CZE separation of the peptides was investigated at pH 3.0, where the peptides are positively charged and the EOF is very low. At these conditions, electrostatic interactions between the positively charged peptides and the capillary wall are also minimized. The analysis at pH 3.0 resulted in only a partial resolution of the peptides, Fig. 4A. The resolution of the peaks was improved by the addition of 10% v/v ACN to the BGE, Fig. 4B. ACN has been shown to be a useful organic additive for peptide analysis [31]. Baseline separation of the solutes was achieved when TEA was used to adjust the pH of the BGE before the addition of 10% v/v ACN, Fig. 4C. TEA also worked as a co-ion source in the BGE and as a capillary surface modifier [27]. The TEA-modified capillary wall generated an anodic EOF which reduced the apparent mobility of the solutes toward the cathode and thereby improved their resolution by enhancing the relative mobility difference (RMD, see Eq. 3) from 0.022 to 0.026, Figs. 4B and C. This small increase in the RMD value enhanced the resolution factor (R_s) by as much as 18%, assuming that the separation efficiency (N) in the TEA-coated capillary and fused-silica capillary were similar. In order to further enhance the resolution by increasing the anodic EOF, the capillary surface was dynamically coated with Polybrene. Polybrene is a quaternary hexylamine polymer which produces a strong anodic EOF [24, 26]. The Polybrene coating improved the resolution and the peak efficiency, Table 1. The strong anodic EOF generated by the Polybrene coating reversed the migration direction and the migration order of the peptides. At these conditions, goserelin migrated faster than buserelin toward the anodic end of the capillary, Fig. 4D.

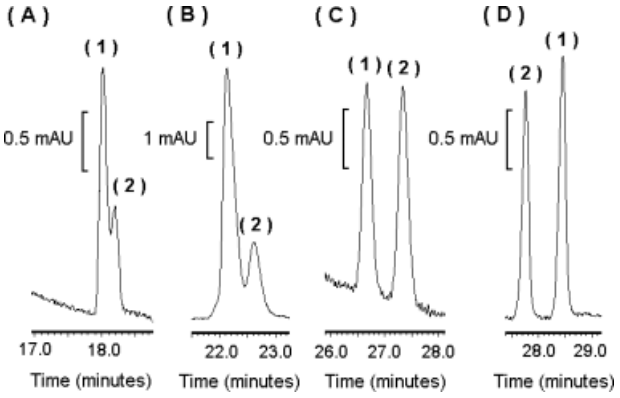

Figure 4. CZE separation of buserelin and goserelin at different conditions. The separations were performed by using: (A) 65 mM phosphate buffer at pH 3.0; (B) 65 mM phosphate buffer at pH 3.0, containing 10% v/v ACN; (C and D) 100 mM phosphate-TEA buffer at pH 3.0 and containing 10% v/v ACN. Separations were performed in (A, B) noncoated fused-silica capillaries, (C) a TEA-coated capillary, and (D) a Polybrene-coated capillary. The applied voltage was 25 kV in (A), (B), and (C), and −25 kV in (D). The capillary temperature was kept constant at 20°C with UV detection at 220 nm. For other conditions, see Section 2. (1) and (2) denote buserelin and goserelin peaks, respectively.

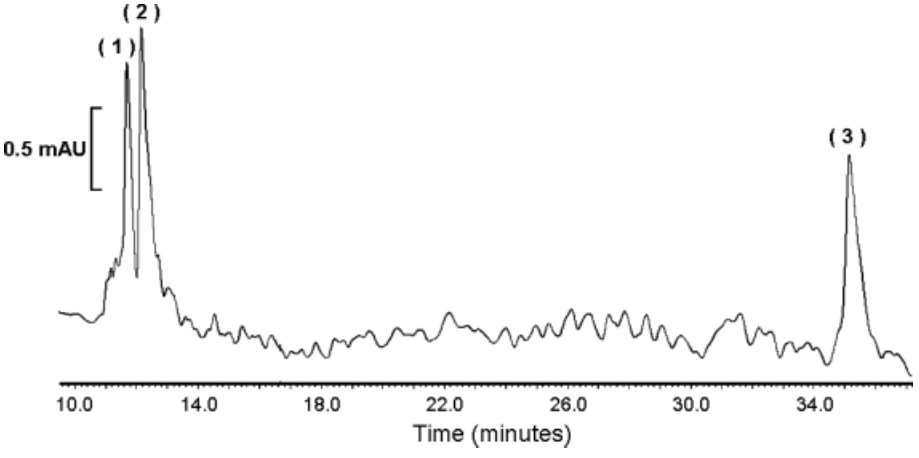

Figure 3. CIEF of buserelin and goserelin. Experimental conditions as given in Section 2. (1) Buserelin and goserelin, (2) ribonuclease A (p*I* = 9.45), and (3) carbonic anhydrase II (p*I* = 5.9).

The peptides are transported in the capillary through a combination of their own electrophoretic mobilities (μ_{ep}) and the EOF. The apparent or observed mobility (μ_{app}) is the vectorial sum of the electrophoretic mobility (μ_{ep}) and the electroosmotic mobility (μ_{eo}), *i.e.*, $\mu_{app} = \mu_{ep} \pm |\mu_{eo}|$, where the negative sign indicates anodic direction of the EOF. The influence of the EOF on the apparent mobility of the peptides provides the possibility of improving the peak efficiency and resolution by changing the magnitude and/or direction of the EOF. The EOF *per se* only displaces the zones and therefore cannot affect the width of the zones (w_d) in the capillary, provided that the EOF does not cause extra zone broadening [32]. However, EOF does affect the migration rate of the zones and thereby the resolution and the peak width (w_t) in the electropherogram. The relationship between the peak width, the anodic electroosmotic velocity (v_{eo}), the electrophoretic velocity (v_{ep}), apparent velocity (v_{app}), and the zone width in the capillary can be described by the following equation:

$$w_t = w_d/|v_{app}| = w_d/|(v_{ep} \pm |v_{eo}|)| \quad (2)$$

As mentioned, the width of the zone in the capillary is not affected by the EOF, provided that the EOF does not cause extra zone broadening. When the magnitude of the anodic EOF is larger than the electrophoretic velocity of an analyte ($v_{ep} - |v_{eo}| < 0$), the peak width (w_t) decreases with increasing EOF.

The peak efficiency and the peak resolution are affected by a change in the peak width, $N = 16\,(t_{mig}/w_t)^2$ (where t_{mig} is the migration time of the solute). The relation between the resolution factor (R_s), peak efficiency, and apparent mobilities is [33]:

$$R_s = 1/4(N_{average})^{1/2}(\Delta\mu/\mu_{app(average)}) \quad (3)$$

The equation shows that the resolution factor depends on two terms: (i) an efficiency term and (ii) a selectivity term ($\Delta\mu/\mu_{app(average)}$), *i.e.*, RMD. The selectivity term has a higher influence on the resolution factor, since the resolution factor only increases by the square root of the efficiency.

The results summarized in Table 1 demonstrate the impact of the capillary surface modifications on the peak efficiency and thereby on the resolution factor. Compared with the TEA coating, the Polybrene coating enhances the peak resolution and the separation efficiency by approximately 30 and 50%, respectively. The higher resolution factor achieved by using the Polybrene-coated capillaries could not be attributed to a higher RMD value, since the difference between the RMD values obtained in the TEA and the Polybrene-coated capillaries was only 1.21×10^{-3}. Thus, the major contribution to the resolution factor came from the higher separation efficiency.

The absolute values of the apparent mobility of the peptides were comparable in the capillaries coated with TEA and Polybrene, *i.e.* 0.714×10^{-8}–0.769×10^{-8} $m^2 \cdot V^{-1} \cdot s^{-1}$, respectively, see Table 1. As predicted by Eq. (2), the efficiencies obtained in these capillaries should also be comparable. However, the efficiency obtained in the capillary coated with TEA was approximately 1.5 times lower than for the capillary coated with Polybrene. The main reason for this inconsistency may be zone dispersion, which can be ascribed to the adsorption of the peptides onto the inner surface of the TEA-coated capillary.

Table 1. CZE analysis of buserelin and goserelin in TEA-coated, Polybrene-coated, and neutrally coated capillaries ($n = 7$)

Capillary	TEA-coated	Polybrene-coated	Neutrally coated
$\mu_{eo} \times 10^8$ ($m^2 \cdot V^{-1} \cdot s^{-1}$)	−0.212 ±5%[a)]	−1.740 ±2%	Not measured
$\mu_{app} \times 10^8$ ($m^2 \cdot V^{-1} \cdot s^{-1}$) (Buserelin)	0.769 ±4%	−0.714 ±4%	1.010[b)] ±0.1%
$\mu_{eff} \times 10^8$ ($m^2 \cdot V^{-1} \cdot s^{-1}$) (Buserelin)	0.981 ±6%	1.026 ±4%	–
N (Buserelin)	148 000 ±26%	209 000 ±14%	244 000 ±3%
$\mu_{app} \times 10^8$ ($m^2 \cdot V^{-1} \cdot s^{-1}$) (Goserelin)	0.751 ±4%	−0.732 ±4%	–
$\mu_{eff} \times 10^8$ ($m^2 \cdot V^{-1} \cdot s^{-1}$) (Goserelin)	0.963 ±6%	1.008 ±4%	–
N (Goserelin)	124 000 ±21%	205 000 ±13%	–
Resolution[c)]	2.18 ±8%	2.83 ±9%	

Separation conditions: buserelin and goserelin were dissolved in a ten-fold diluted BGE at 50 μg/mL and analyzed at either 25 or −25 kV. For other separation conditions, see Section 2.

a) ±RSD %.

b) The apparent mobility of buserelin is smaller than its effective mobility (≈1.026×10^{-8} $m^2V^{-1}s^{-1}$), which may depend on the presence of a very weak anodic EOF, being caused by the adsorption of TEA onto the capillary wall.

c) In addition to Eq. (3), the resolution factor was calculated by: $R_s = 2\Delta t_{mig}/\Sigma w_t$.

In order to confirm the adsorption of the solutes in the TEA-coated capillary, buserelin was analyzed in a neutrally coated capillary. The efficiency in the neutral capillary was found to be 1.6 times higher than in the capillary coated with TEA, Table 1. From these results it can be concluded that adsorption was the main source of the peak broadening in the TEA-coated capillaries as well as in the fused-silica capillaries.

In comparison to TEA, the molecular structures of the peptides are more complex which may account for a stronger interaction with the capillary wall. The peptides consist of nine amino acid residues with diverse physiochemical properties and are able to interact with the silanol groups of the capillary wall through hydrogen bonds as well as electrostatic and hydrophobic interactions. Additionally, the butyl group attached to the D-serine residue makes these peptides more hydrophobic, resulting in a tighter adsorption onto the capillary wall.

Polybrene, on the other hand, interacts strongly with the silanol groups and thus, suppress the adsorption of the solutes. This resulted in high separation efficiency, comparable to that achieved in the neutral capillary, Table 1. In addition, the presence of 10% v/v ACN in the BGE reduces hydrophobic interactions between the solutes and the hydrophobic moiety of the Polybrene coating.

The highest resolution of the peptides was achieved in reverse polarity electrophoresis mode using Polybrene-coated capillaries with a BGE consisting of 100 mM phosphate-TEA buffer, pH 3.0, containing 10% v/v ACN at 20°C. Therefore, the Polybrene coating was employed for the quantitative analyses.

3.2 MICZE analysis of the peptides

Figure 5A shows a separation of buserelin and goserelin using CZE in the conventional single-injection mode, where four peaks are detected within an analysis window smaller than 30 min, *i.e.*, two small peaks arising from the sample matrix and two much larger peaks corresponding to goserelin and buserelin. The matrix peaks are virtually stationary and therefore the larger matrix peak was used as the EOF marker [34].

In order to shorten the average analysis time for each analyte and to utilize the whole separation space between the goserelin peak and the matrix peaks, the potential of MICZE was explored. A successful transfer from a single injection analysis to a multiple-injection mode requires a CZE method with a high peak capacity [35]. In addition, it is important that the analytes separate within a narrow elution window, *i.e.*, the solute and IS should have mobilities which differ only slightly. The developed method coupled together with the structural similarity between buserelin and goserelin ($\Delta\mu = 1.8 \times 10^{-10}\ m^2V^{-1}s^{-1}$) fulfilled these requirements, Fig. 5A and Table 1. Accordingly, the CZE method could be easily adapted to six consecutive sample injections.

The presence of the matrix peaks reduced the sample capacity of the MICZE method. During the MICZE analysis, the counter-EOF-migrating peptide zones ($\nu_{ep} < |\nu_{eo}|$) passed through $n - 1$ stationary matrix zones on the way toward the detection point. This meant that the peptides from the last injected sample did not encounter any stationary matrix zone. The number of applied plugs ($n = 6$) and the time period (2 min) for the electrophoresis of the applied plugs were both adjusted so that the matrix peaks from the last injection were detected ahead of the goserelin peak from the first injected sample, see Fig. 5B. Thus, in order to keep the analyte and matrix peaks separated, only half the length of the capillary ($L_d/2$) was utilized for the multiple injections. Separation parameters which govern multiple-injection analysis will be discussed further in a forthcoming paper.

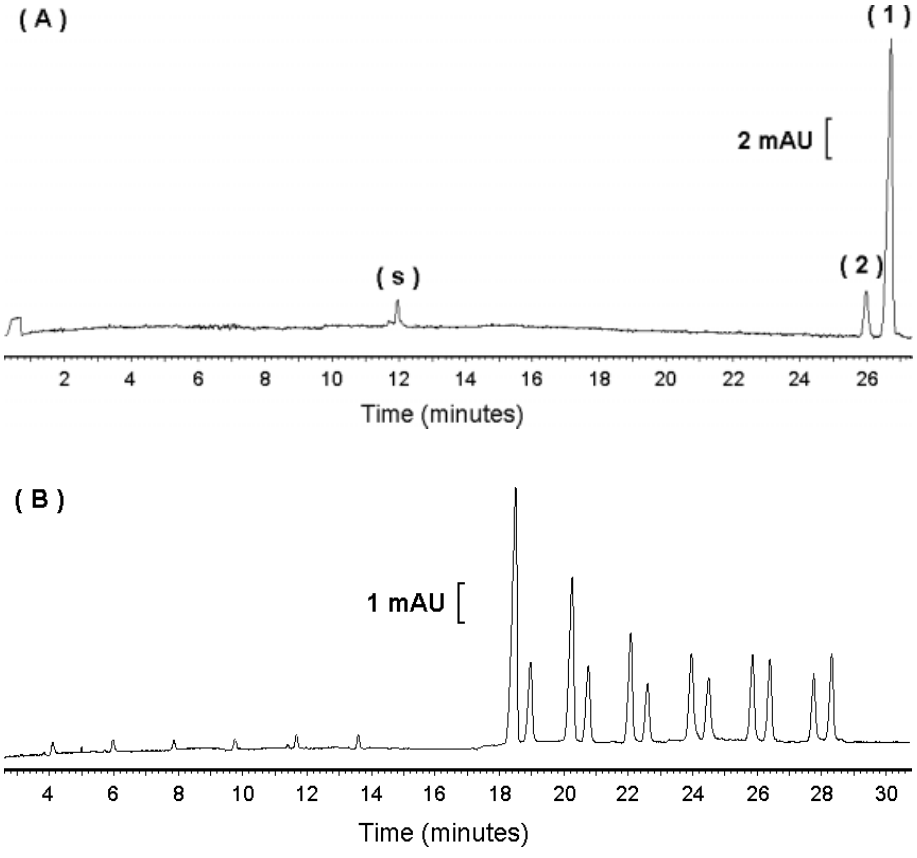

Figure 5. CZE and MICZE of goserelin using buserelin as an IS. (A) Separation of buserelin and goserelin using the single-injection mode in a Polybrene-coated capillary. (B) Determination of goserelin by MICZE in a Polybrene-coated capillary. Goserelin standards were dissolved in a ten-fold diluted BGE at different concentrations (300, 190, 150, 100, 75, and 50 μg/mL). All these standards contained 50 μg/mL buserelin as the IS. The separations were performed at 25 kV in reversed electrophoresis polarity mode, using phosphate-TEA buffer (100 mM, pH 3.0) containing 10% v/v ACN as the BGE. Other conditions are described in Section 2. (1) Buserelin, (2) goserelin, and (s) peaks from the sample matrix.

3.3 Validation

The suitability of the separation system for quantitative analysis was studied by examining linearity, LOD and LOQ, repeatability, and reproducibility.

3.3.1 Linearity

The UV response of the peptides was found to be linear in the concentration range of 10 to at least 400 μg/mL. Good linearity was observed between the peak area ratios (analyte/IS) and the corresponding solute concentrations. The calibration curves given by the linear regression method were

$y = 0.024$ (±1.2%) $x - 0.054$ (±395%), $r^2 \geq 0.996$ ($n = 6$) for buserelin.

$y = 0.014$ (±2.2%) $x - 0.013$ (±185%), $r^2 \geq 0.993$ ($n = 6$) for goserelin.

LOD (S/N = 3) and LOQ (S/N = 10) for both buserelin and goserelin were estimated to be 4 and 11 μg/mL, respectively.

3.3.2 Interday and intraday reproducibility

Initially, a RSD of 1.4% between peak area ratios was obtained when a buserelin standard solution, containing goserelin as IS, was analyzed five times by MICZE with six injections *per* analytical run. These results indicated that the precision of the measurements was high enough to employ the method for the quantitative analysis of the peptides.

It is known that the stability of the polybrene coating is low at acidic pH's [26, 36]. Therefore, the coating was regenerated prior to each run. Despite this, the interday variations in the migration times were relatively high, Table 2A. However, a good migration time reproducibility was achieved when the solute and the IS were analyzed within the same day (RSD <5%), Table 2B. Variations in the interday and intraday performances of the peak area ratios were comparable to those for the migration times. However, since the analyte and the standards were analyzed simultaneously in MICZE mode, the linearity of the standard curves and thus the determinations were not impaired by these variations. The results tabulated in Table 3 show the quantification of two different standard solutions containing 75 and 100 μg/mL buserelin within 2 days. The relatively small RSD value (<5%) for the concentration measurements, as well as good agreement between the true and the experimentally determined concentrations, indicated good precision and accuracy of the MICZE method.

Table 3. MICZE determination of the buserelin content of the two standard samples

Real concentration (μg/mL)	Intraday determinations ($n = 4$)	Interday determinations ($n = 8$ in 2 days)
(A) 75.0	73.1 μg/mL ±4.3%	76.3 μg/mL ±4.9%
(B) 100.0	99.8 μg/mL ±2.4%	100.0 μg/mL ±2.3%

The buserelin concentrations were determined by using calibration curves constructed by the simultaneous analysis of five buserelin standard solutions at 300, 200, 150, 100, and 50 μg/mL (A) or 300, 200, 150, 75, and 50 μg/mL (B), containing 50 μg/mL goserelin as the IS.

Table 2. Repeatability and reproducibility of the migration times in MICZE using Polybrene-coated capillaries

Analyte	t_{mig} 1[a)] (min) (±RSD %)	t_{mig} 2 (min) (±RSD %)	t_{mig} 3 (min) (±RSD %)	t_{mig} 4 (min) (±RSD %)	t_{mig} 5 (min) (±RSD %)	t_{mig} 6 (min) (±RSD %)
A: Interday precision (reproducibility) of the migration times ($n = 41$ in 6 day)						
Goserelin	17.1 ±13%	18.9 ±12%	20.9 ±11%	22.8 ±10%	24.6 ±9%	26.0 ±7%
Buserelin	17.8 ±13%	19.6 ±12%	21.5 ±11%	23.4 ±10%	25.3 ±9%	26.6 ±7%
B: Intraday precision (repeatability) of the migration times ($n = 11$)						
Goserelin	15.8 ±4%	17.6 ±4%	19.5 ±3%	21.4 ±3%	23.2 ±3%	25.3 ±2%
Buserelin	16.3 ±4%	18.1 ±4%	20.0 ±3%	21.8 ±3%	23.8 ±3%	25.8 ±2%

Separation conditions are given in Section 2.

a) The numbers 1–6 indicate the injection (plug) numbers.

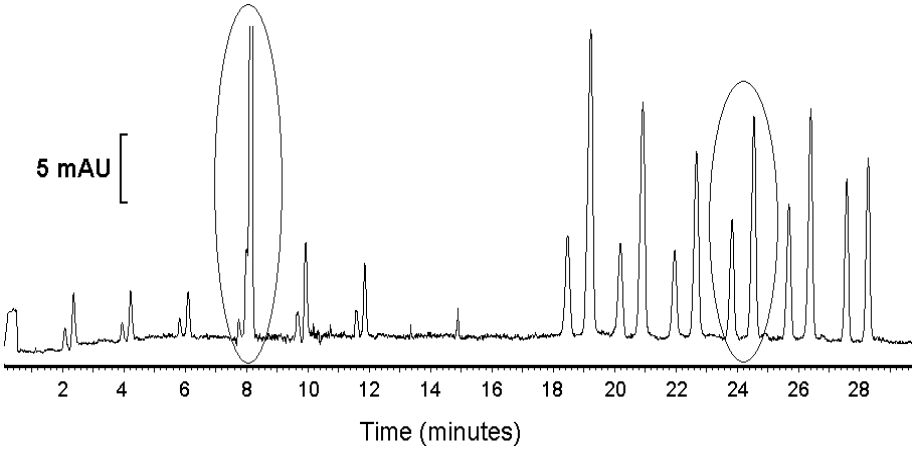

Figure 6. Determination of buserelin in a commercial pharmaceutical product by MICZE. The buserelin standards and the analyte samples were dissolved in water containing 10% v/v BGE at 150, 125, 100, 90 (analyte sample), 75, and 50 μg/mL buserelin, containing 50 μg/mL goserelin as the IS. Injection 4 in the electropherogram contains the pharmaceutical sample whose matrix peak, originating from benzyl alcohol (for conserving buserelin), is larger than those of the standards. The separation conditions were as in Fig. 5.

3.4 Quantitative analysis

The MICZE method was used to determine the concentration of buserelin in a pharmaceutical product, Fig. 6. For the analyses, five different standard solutions of buserelin were analyzed at concentrations 300, 200, 100, 75, and 50 μg/mL along with the buserelin sample diluted to 90 μg/mL. All the standards as well as the sample contained 50 μg/mL goserelin as an IS. In all the other aspects, the determination was carried out as described in Section 3.2. The peak area of buserelin relative to that of the IS was used to determine the buserelin concentration in the sample and hence in the pharmaceutical product. The buserelin content was determined to be 0.94 ± 0.05 mg/mL (the errors are given as SEM, $n = 12$). The declared concentration of the commercial product was 1 mg/mL.

3.4.1 Ruggedness

In order to examine the ruggedness of the method, an interlaboratory assay of the buserelin content of the same sample was performed. The buserelin content was determined to be 0.98 ± 0.03 mg/mL ($n = 3$), which is comparable with the concentration determined above. All the determined concentrations were within the specified limits.

4 Concluding remarks

A fast and accurate quantification of the synthetic peptide buserelin using MICZE has been demonstrated in Polybrene-coated capillaries. By utilizing MICZE, the average analysis time for each analyte sample was reduced to approximately 9 min from 40 min, when using conventional single injection mode. The determinations were easily carried out by using goserelin, an analog to buserelin, as an IS. The small mobility difference ($\Delta\mu = 1.8 \times 10^{-10}\ m^2 \cdot V^{-1} \cdot s^{-1}$) between buserelin and goserelin permitted six consecutive sample injections.

Essential to the successful separation of the solutes at pH 3.0 was the presence of ACN in the BGE and the anodic EOF. The strong anodic EOF generated by the Polybrene coating provided high separation efficiency and high peak resolution.

When employing MICZE for quantitative analysis, it is important to avoid excessively high solute concentrations with the attendant high impurity concentrations, which may reduce the sample capacity of MICZE. In this study, no interfering impurities were observed.

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