Development and validation of a micellar electrokinetic capillary chromatography method for the determination of goserelin and related substances

An MEKC method for the analysis of goserelin and related substances has been developed using a combination of additives including CTAB, β-CD, and sodium hexanesulfonate. For this assay, the running buffer (pH and additives) and separation conditions (voltage and temperature) were optimized. The optimized system was the following: 200 mM 6-aminocaproic acid buffer (pH 4.2) supplemented with 175 mM CTAB, 3.0% w/v β-CD, and 20 mM sodium hexanesulfonate; the voltage was 10 kV in reverse polarity mode, the temperature was 20°C, and UV detection was measured at 220 nm. The method was qualified by evaluating the specificity, precision, linearity, accuracy, LOD, and LOQ. According to validation experiments, the optimized method was specific, accurate, and repeatable and satisfied the requirements for the analysis of goserelin and related substances. Compared with the RP-HPLC method, the MEKC method better solved the problem of overlapping impurity signals, and the migration time required was shorter. This method can be used for quality control and for the analysis of goserelin and its related substances.

1 Introduction

In recent years, the development of peptide drugs has attracted increasing attention in the pharmaceutical industry. An increasing variety of peptides has been produced, and these compounds have been approved as therapeutic agents to treat diseases, such as cancer, autoimmune diseases, and diabetes [1, 2]. Goserelin is a synthetic decapeptide analog of luteinizing hormone-releasing hormone, which is used for the palliative treatment of advanced prostatic carcinoma, breast cancers, and several benign gynecological disorders, such as endometriosis and uterine fibroids [3, 4].

Inevitably, closely related impurities of peptides, such as diastereoisomers and acetylated products, are produced during the processes of synthesis, manufacture and storage, and these impurities can affect the biological activity and safety of the final product [5]. Therefore, demand is increasing for the introduction of methods that are suitable and reliable for the analysis of goserelin and its related substances.

Various methods such as HPLC, LC-MS, and CE-MS have been developed to analyze the impurities in goserelin [6–8]. An HPLC method was used to analyze the related substances of goserelin in European Pharmacopoeia [6]. But the peaks representing (D-Tyr5)-goserelin and (D-His2)-goserelin were overlapped. Sanz-Nebot et al. used an LC-ES-MS method for the analysis of synthesis mixtures of goserelin [7]. Based on the determination of their molecular masses, the sequences of various goserelin side products were proposed. In another work, Hoitink et al. characterized the degradation products of goserelin at pH 5 and 9 using CZE-MS [8]. Compared to RP-HPLC, diastereomers in CZE achieved lower resolution because these isomers exhibited identical charge-to-mass ratios. However, the degradation products of the semicarbazide moiety and des-pyroglutamyl...
goserelin were identified using CZE-MS but were not visible when using LC–MS. Thus, CE represents a useful complementary technique to HPLC, particularly for impurity profiling.

CE is a powerful tool for the quality control of peptide drugs [9–15] because it has its own advantages including high separation efficiency, high peak capacity, low sample and reagent consumption, high speed in analysis, instrumentation simplicity, and use of various separation modes. Previously, we developed a CZE method to determine the purity of exenatide and conducted a preliminary study on its stability. The CZE method provided better resolution and shorter migration times than RP-HPLC [16]. Several CE-based methods have been used for the analysis of luteinizing hormone-releasing hormone analogs. Tamizì et al. developed a simple and rapid CZE method for the separation of buserelin and its degradation products in 26.4 mM phosphate buffer, pH 3.0 [17]. This method was then applied to monitor the stability of the drug. Stanovà et al. successfully analyzed the synthetic peptide buserelin in a complex biological matrix (urine) in 50 mM formic acid BGE using CZE-ESI-MS [18]. Corran and Sutcliffe compared the selectivity obtained to separate gonadorelin and five therapeutically analogs using RP-HPLC, with CZE in 0.2 M sodium phosphate and MEKC involving the use of various surfactants [19]. The RP-HPLC system was found superior to the CZE and MEKC systems used in this study, but the MEKC system with CTAB was potential to analyze the analogs based on the differential distribution coefficient of the analytes between micelles and water.

The separation principle of MEKC is based on differences in the combination of charge-to-mass ratio, hydrophobicity, and charge interactions at the surface of micelles [20, 21]. The main objective of this study was to develop an MEKC method to separate goserelin and its main related substances. The MEKC method was optimized for experimental parameters (e.g., surfactant, additives, and running buffer pH) and separation parameters (e.g., temperature and voltage). Under optimal separation conditions, better resolution was achieved by adding the surfactant CTAB and the additives β-CD and sodium hexanesulfonate to 6-aminocaproic acid (EACA) buffer. Importantly, (D-Tyr)-goserelin and (D-His)-goserelin were successfully separated, a result that cannot be achieved using the standard HPLC method described in the European Pharmacopoeia [6].

2 Materials and methods

2.1 Chemicals and reagents

Goserelin and various impurities were purchased from Bachem (Torrance, CA, USA). EACA and SDS were supplied by Sigma-Aldrich (St. Louis, MO, USA). CTAB, CHAPS, and sodium hexanesulfonate were purchased from Aladdin (Shanghai, China). Several CDs were tested for the CE system. Hydroxypyropyl-β-cyclodextrin, α-CD, β-CD, γ-CD, (2,6-di-O-methyl)-β-cyclodextrin, and carboxymethyl-β-cyclodextrin were supplied by Aladdin. Acetic acid was obtained from Fluka (Buchs, Switzerland). Sodium hydroxide and hydrochloric acid were obtained from Beckman. All solutions were prepared using Milli-Q water.

2.2 Instrumentation

Experiments were performed using an Agilent 7100 capillary electrophoresis instrument, which was equipped with diode array detector (DAD) system; the UV detection monitored at 220 nm. Data were collected and analyzed using the included Agilent Chemstation software. A bare fused silica capillary (total length: 48.5 cm; effective length: 40 cm; 50 μm i.d.) was obtained from Agilent Technology.

2.3 Solutions and sample preparation

All samples were dissolved in Milli-Q water. A mixture solution of goserelin and eight impurities were prepared to achieve a final concentration of 0.2 mg/mL for each compound, this solution was used during the optimization of the separation system. Another mixture solution of goserelin (2 mg/mL) and impurities (20 μg/mL) was prepared to assess the optimal condition. The optimal running buffer system was 200 mM EACA buffer containing 175 mM CTAB, 3.0% β-CD and 20 mM sodium hexanesulfonate; the pH of the running buffer was adjusted to 4.2 using acetic acid.

2.4 Analysis method

New capillaries were conditioned before use by pretreatment with 0.1 M NaOH for 10 min, 0.1 M HCl for 10 min, and Milli-Q water for 10 min at a pressure of 100 kPa. After each injection, the capillary was sequentially rinsed with 1 M NaOH, 0.1 M NaOH, and Milli-Q water for 2 min each time; subsequently, the capillary was rinsed with running buffer for 5 min at a pressure of 100 kPa. The sample was injected for 10 s at a pressure of 5 kPa; then, separation was performed at 10 kV using negative polarity. All steps were conducted at 20°C, and the detection wavelength used was 220 nm.

The system was shut down at the end of each day by sequentially rinsing the capillary (at a pressure of 400 kPa) with 0.1 M NaOH and 0.1 M HCl for 5 min. The capillary was then rinsed with Milli-Q water for 10 min. Finally, the capillary was immersed in Milli-Q water.

3 Results and discussion

3.1 Method development and optimization

Goserelin and its main impurities were used as models in this study to optimize separation conditions. The amino
acid sequences of the analyzed peptides and their relative molecular masses (Mr) are presented in Table 1; all studied peptides have identical numbers and types of ionizable residues (the phenol group of tyrosine, imidazole group of histidine, guanidine group of arginine, and N-terminal amino group). Because of these similarities of structure and Mr, these peptides exhibit only subtle differences in charge-to-size ratio and are difficult to separate. According to Supporting Information Fig. 1, the differences in migration behavior between these peptides were insufficient to separate these peptides using this system.

Unlike CZE, MEKC can be used to separate both charged and neutral analytes because hydrophobicity is used as an additional differentiating parameter [20, 21]. Thus, MEKC provided an effective means to analyze peptide drugs and related impurities. Several separation parameters (surfactant, β-CD, sodium hexanesulfonate, and running buffer pH) and instrumental parameters (temperature and voltage) were optimized.

### 3.1.1 Effect of surfactant

Various surfactants (SDS, CTAB, and CHAPS) were screened in this study. As the anionic surfactant, SDS is the most popularly used surfactant in MEKC. However, the SDS-MEKC system provided poor separation, probably because a strong electrostatic attraction existed between positively charged peptides and negatively charged SDS micelles, causing the peptides to migrate similarly [19]. The CTAB system separated the tested peptides better than the other tested systems. Considering that resolution was crucial for impurity analysis, CTAB was selected for use as the surfactant. The effect of CTAB concentration (100–200 mM) on resolution was investigated. As shown in Fig. 1, the efficiency at separating peptides 1, 4, 5, 6, and 7 increased with CTAB concentration because the probability of peptide contact with micelles increased, then the distribution coefficient of the analytes between the micelle and bulk phases increased [22]. The best separation profile was achieved when using 175 mM CTAB. Concentrations above 200 mM were not selected because peaks 8 and 9 were poorly shaped under these conditions, and CTAB exhibited limited solubility in BGE.

### 3.1.2 Effect of β-CD

In MEKC, the addition of CDs introduces an additional equilibrium, reducing migration time, and improving selectivity. CDs contain an internal hydrophobic cavity that can bind other compounds through hydrophobic interaction, hydrogen bonding, steric factors, and electrostatic interaction [23]. Because of the chirality of glucose hydroxyl groups, which form the rim of the CD cavity, the inclusion complex formation is chiralselective. Because hydrophobic analytes can be incorporated into either CD or micelles, the addition of CDs reduces the distribution coefficient of such analytes between the two phases [20]. The migration behavior of analytes depends on their competitive distribution among water, CDs, and micelles, thereby increasing resolution.

Various CDs, having cavities that differ in size, shape, and other properties, can include different analytes [24]. Therefore, various CDs were tested: α-CD, β-CD, γ-CD, hydroxypropyl-β-cyclodextrin, carboxymethyl-β-cyclodextrin, and (2,6-di-O-methyl)-β-cyclodextrin. Among all tested CDs, β-CD afforded the best resolution. The effect of β-CD concentration on resolution was evaluated from 2.0 to 3.5% (Fig. 2). As the concentration increased, the separation efficiency of the analytes improved. A concentration of 3.0% provided adequate resolution. Concentrations above 3.5% were difficult to achieve due to the limited solubility of β-CD. Therefore,
3.0% β-CD was selected. Adding CDs can reduce analysis time because the distribution coefficient of representing analyte between the micelle and bulk phases decreases. However, at 3.5% β-CD, the migration time unexpectedly increased, possibly due to increased viscosity.

3.1.3 Effect of sodium hexanesulfonate

Ion-pair reagents can be used to improve the resolution obtained using MEKC. Because the tested peptides are positively charged at acidic pH, sulfonate was used as an ion-pair reagent. Cationic analytes can form paired ions with sulfonate, thus decreasing electrostatic repulsion between the cationic micelle and the cationic analytes. Consequently, the analytes were more efficiently incorporated into the micelles [21, 22, 25]. In this study, sulfonates with different alkyl chain lengths were investigated including sodium pentanesulfonate, sodium hexanesulfonate, sodium heptanesulfonate, and sodium octanesulfonate. The resolutions obtained using these sulfonates did not significantly differ. Sodium hexanesulfonate was selected for further study at different concentrations. As shown in Fig. 3, when the sodium hexanesulfonate concentration was increased from 0 to 30 mM, the separation of the peptides (particularly 1 and 6) increased, and the peak shapes obtained for peptides 8 and 9 were significantly improved. However, when the sodium hexanesulfonate concentration was increased from 20 to 30 mM, the signal responses of all peptides obviously decreased. Considering resolution, signal response and peak shape, 20 mM was considered the optimal sodium hexanesulfonate concentration.

3.1.4 Effect of buffer pH

pH affects the sample charge density and ionization state of the inner capillary wall, which is related to EOF [22, 24]. Thus, pH should be considered when attempting to improve selectivity. At the fixed EACA concentration of 200 mM (including 175 mM CTAB, 3.0% β-CD, and 20 mM sodium hexanesulfonate), the effect of pH on resolution was evaluated in the range from 3.6 to 4.6. As shown in Fig. 4, migration time gradually decreases with pH increased because of the increase in EOF. The efficiency at separating peptides 1, 5, and 6 was decreased at pH of less than 4.0 due to the strong electrostatic repulsion between positively charged micelles and cationic analytes at low pH [20, 22]. Good resolution was obtained at pH 4.2 because of the low magnitude of the EOF, which allowed appreciable differentiation between the electrophoretic mobility of all peptides [26]. At pH 4.4–4.6, the peak shapes obtained for peptides 8 and 9 were obviously poor, and the signal response was low. In this study, pH 4.2 was selected as the experimental condition.

3.1.5 Effect of instrumental parameters

The effects of temperature and voltage on resolution were evaluated. Temperature was studied in the range from 18 to 25°C (Supporting Information Fig. 2). As temperature was increased, migration time and resolution decreased because
the distribution coefficient decreased; the velocity of the EOF and the electrophoretic velocity of the micelle increased because the viscosity of the micellar solution decreased [21]. However, the temperature cannot be reduced too much due to reduction in solubility. The optimum temperature was determined to be 20°C. As shown in Supporting Information Fig. 3, lower applied voltages can provide better resolution; however, the migration time lengthened. Thus, −10 kV was considered the best compromise between resolution and migration time.

3.1.6 Optimum results

The following conditions were considered optimal: the running buffer was 200 mM EACA buffer (pH 4.2), containing 175 mM CTAB, 3.0% w/v β-CD, and 20 mM sodium hexanesulfonate. The separation voltage was 10 kV in the negative polarity, and temperature was 20°C. The detection was monitored at 220 nm. Goserelin and eight impurities were separated under these conditions (Fig. 5). (D-Tyr⁵)-goserelin and (D-His²)-goserelin were efficiently separated using this MEKC method; in contrast, the peaks representing these two impurities were overlapped using RP-HPLC. Moreover, MEKC required shorter analysis time than RP-HPLC (Supporting Information Fig. 4). MEKC provided complementary information to RP-HPLC and presented advantages over RP-HPLC when analyzing goserelin and its related substances.

3.2 Method validation

The optimized method was preliminarily validated in terms of specificity, precision, linearity, accuracy, LOD, and LOQ, in accordance with ICH guidelines.

3.2.1 Specificity

Specificity, which is described as the ability of a method to discriminate between an analyte and potential interfering substances and between a target peptide and its impurities, was evaluated by comparing the electropherograms of Milli-Q water that dissolved sample. The target peptide and impurity peaks did not interfere under the selected operating conditions and were considered sufficiently separated.

3.2.2 Precision

A precision study was performed for intraday (n = 6) and interday (n = 9) assays for eight impurities (20 μg/mL of impurity 6 and 10 μg/mL of other impurities) and goserelin (2 mg/mL). The experimental results, which were expressed as RSDs for the migration time and corrected area (A/t), are shown in Table 2. The intra- and interday RSDs for migration time and corrected areas for the eight impurities and goserelin were less than 3.0%. Thus, the method described here exhibited good precision for the analysis of impurities of goserelin.

3.2.3 Linearity

The linearity of impurity 6 in the concentration range 0.005–0.03 mg/mL and other impurities in the concentration range 0.0025–0.02 mg/mL were assessed. The linearity for goserelin was evaluated in a concentration range from 0.0025 to 3 mg/mL. Samples at each concentration were injected in triplicate. Corrected area was plotted versus concentration. Table 2 showed the linearity obtained for goserelin and each impurity. The linearly dependent coefficients of goserelin and impurities were larger than 0.99.

3.2.4 Accuracy

Recovery studies were performed to assess accuracy. Recovery studies of impurities were evaluated by spiking the known impurities at 50, 100, and 150% concentration levels of their specified limits. Impurity 6 was assessed at 0.01, 0.02, and 0.03 mg/mL and the other impurities were evaluated at 0.005, 0.01, and 0.015 mg/mL. The samples were tested in triplicate. As shown in Table 2, all impurities and goserelin were recovered at 80.0–120.0%.

3.2.5 LOD and LOQ

The LOD and LOQ values for the known impurities and goserelin were determined based on S/N. A ratio of 3:1 was used to estimate the LOD, whereas a ratio of 10:1 was used for the LOQ. The results illustrated that the method was sensitive to detect impurities (see Table 2).
Table 2. Parameters of validation of the method developed

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Intraday RSD%</th>
<th>Interday RSD%</th>
<th>Intraday RSD%</th>
<th>Interday RSD%</th>
<th>Linear regression equations</th>
<th>Accuracy (%)</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
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<tr>
<td></td>
<td>Intrday</td>
<td>Interday</td>
<td>Intrday</td>
<td>Interday</td>
<td>Linear regression equations</td>
<td>accuracy (%)</td>
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<td>1</td>
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<td>2</td>
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<td>1.98</td>
<td>1.58</td>
<td>Y = 95.673X - 0.0265</td>
<td>104.1</td>
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<td>3</td>
<td>1.98</td>
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<td>1.75</td>
<td>1.79</td>
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<td>103.9</td>
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<td>4</td>
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<td>2.21</td>
<td>Y = 68.412X - 0.0095</td>
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<td>Y = 59.543X - 0.0022</td>
<td>97.9</td>
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<tr>
<td>6</td>
<td>2.24</td>
<td>2.07</td>
<td>1.87</td>
<td>2.88</td>
<td>Y = 47.933X - 0.0325</td>
<td>91.6</td>
<td>1.15</td>
<td>3.73</td>
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<td>7</td>
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<td>1.61</td>
<td>2.25</td>
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<td>91.9</td>
<td>0.90</td>
<td>2.02</td>
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<td>8</td>
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<td>2.18</td>
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<td>9</td>
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<td>2.42</td>
<td>1.85</td>
<td>1.89</td>
<td>Y = 54.009X - 0.0287</td>
<td>115.7</td>
<td>1.36</td>
<td>2.63</td>
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</table>

Table 3. Results of the content of related substances in goserelin

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<th>Impurity sample</th>
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<th>3</th>
<th>5</th>
<th>6</th>
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<th>8</th>
<th>Total impurity</th>
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<tr>
<td>2</td>
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<td>0.09</td>
<td>0.07</td>
<td>0.10</td>
<td>0.72</td>
</tr>
</tbody>
</table>

3.2.6 Robustness

Robustness testing studies the capacity of the method to remain unaffected by making small but deliberate variations in electrophoretic conditions. The robustness of this method was determined during its development by means of variations of certain parameters. The electrophoretic parameters evaluated were buffer pH, CTAB concentration, β-CD concentration, sodium hexanesulfonate concentration, capillary temperature, and separation voltage. Considering the resolution among impurities was the major criteria, evaluation of the robustness was based on the resolution. The data obtained in these experiments showed that the method allowed slight adjustment for experimental parameters. The method was robust when the parameters were controlled carefully during analysis.

3.3 Application: Analysis of related substances of goserelin

The electrophoretic system was used to evaluate related substances of goserelin in raw synthesis products. The samples were prepared by triplicate and analyzed. Two lots of bulk drug of goserelin were studied and the data are listed in Table 3. The impurity 4 and impurity 9 were not found in the samples under these conditions. Moreover, there was some difference in the impurity 5 between the two lots. The method was capable of analyzing the related substances of goserelin and lots consistency.

4 Concluding remarks

The MEKC method described here was developed to analyze goserelin and its related substances. Goserelin and eight main impurities were separated using the MEKC method. In this study, the separation efficiency was improved by using a combination of additives (CTAB, β-CD, and sodium hexanesulfonate) in the MEKC. The results of a validation study imply that the proposed MEKC method is simple, rapid, efficient, and accurate. In addition, the proposed method can separate the impurities (D-Tyr5)-goserelin and (D-His2)-goserelin, which cannot be achieved using the standard European Pharmacopoeia method. Thus, the outlined MEKC method is useful for the impurity profiling of peptide drugs.

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The authors have declared no conflict of interest.

5 References
