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Determination of the isoelectric point of shortened glucagon-like peptide-1 by capillary isoelectric focusing with whole column imaging

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ABSTRACT

The isoelectric point of shortened glucagon-like peptide-1, a promising new drug to therapy of type 2 diabetes, was determined using a new generation of capillary isoelectric focusing-whole column imaging. Urea was added as a solubilizer to enhance the stability and repeatability of shortened glucagon-like peptide-1. The isoelectric point of shortened glucagon-like peptide-1 measured by capillary isoelectric focusing-whole column imaging was 4.49, which was similar to the theoretical value of 4.40. Furthermore, the accuracy and repeatability of capillary isoelectric focusing-whole column imaging were investigated. The results showed that this approach provided good accuracy (bias less than 2%) and excellent repeatability (coefficients of variation less than 0.25%). The isoelectric focusing coupled with whole column imaging required only a few minutes for an assay. The study demonstrated that the capillary isoelectric focusing-whole column imaging is useful for the quality control and characterization of peptide and proteins because of its accuracy, repeatability, high performance, and high throughput.

KEYWORDS

Capillary isoelectric focusing; isoelectric point; shortened glucagon-like peptide-1; whole column imaging detection

Introduction

The determination of isoelectric point of peptide and protein drugs plays an important role in the quality control and research. Currently, gel isoelectric focusing, and capillary isoelectric focusing are major methods for proteins and peptide isoelectric point analysis.[1–3] The gel isoelectric focusing method is inherently time-consuming and poorly suited for automation. As for the traditional capillary isoelectric focusing, the focused zones will have to be pushed though the detection point, which may cause the distortion of pH gradient, loss in resolution and high risk of protein precipitation that affect the results.[4]

The new generation of capillary isoelectric focusing-whole column imaging detection[5–8] can solve those problems, which combines the capillary

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isoelectric focusing electrophoresis with the whole column imaging detection. A complementary metal-oxide-semiconductor camera is used to monitor the whole separation channel and record the real-time focusing process. The application of whole column imaging eliminates the mobilization of focused zone, which maintains the high efficiency of separation and increases the accuracy and repeatability of the method. Moreover, the running time is reduced to less than 10 min, so the optimization of separation condition is simplified.[9,10]

Figure 1 shows a schematic diagram of capillary isoelectric focusing-whole column imaging detection. The inlet of the capillary column is connected to autosampler and the outlet of the capillary column is connected to a waste vial. When high voltage direct current is passed into the cathode and anode electrolyte, a stable pH gradient is established by carrier ampholytes.[11] The sample moves toward the anode or cathode in the 50 mm capillary column based on its charge. When the protein reaches its isoelectric point, which is equal to the pH value, the protein movement stops and the isoelectric focusing is completed. During the focusing process, a complementary metal-oxide-semiconductor camera is used to record the entire dynamic imaging in this capillary column and ultraviolet absorption is used as the detection signal.

Glucagon-like peptide-1 is an endogenous peptide which is secreted from intestinal endocrine l-cells. Glucagon-like peptide-1 exerts glucose regulation actions through secretion of glucose-dependent insulin, induction of β-cell proliferation, enhancement resistance to apoptosis, slowing of gastric emptying, and glucose-dependent inhibition of glucagon secretion.[12] However, due to its short half-life, the clinical application of glucagon-like peptide-1 is restricted.[13] Shortened glucagon-like peptide-1 is a glucagon-like peptide-1 analog with a composition of 26 amino acids. Five amino acids from glucagon-like peptide-1 C-terminus are deleted to destruct binding domain with clearance receptor to prolong the half-life. Hence, the shortened

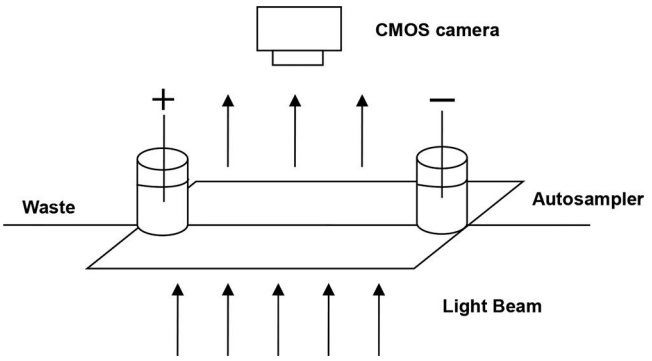

Figure 1. Schematic of capillary isoelectric focusing-whole column imaging.

glucagon-like peptide-1 is more effective in decreasing blood glucose and promising for the treatment of type 2 diabetes.[14]

As important information for quality control of shortened glucagon-like peptide-1, the isoelectric point determination plays a key role. In this study, the capillary isoelectric focusing-whole column imaging detection method was used to determine the isoelectric point of shortened glucagon-like peptide-1. Different experimental factors were investigated. The accuracy and precision of capillary isoelectric focusing-whole column imaging detection method were also examined.

Materials and methods

Instrumentation

The capillary isoelectric focusing-whole column imaging detection was performed on the CEInfinite instrument (Advanced Electrophoresis Solutions, ON, Canada). The cartridge was a 50 mm × 100 μm internal diameter silica capillary with fluorocarbon coating inside. Two pieces of 3 mm dialysis hollow fiber membrane were fixed at the ends of the separation cartridge. The system was equipped with a complementary metal-oxide-semiconductor camera for whole column optical absorption imaging.

Materials

Shortened glucagon-like peptide-1 was obtained from Dili Biological Engineering (Dalian, China). Carrier ampholytes (pH 3-10) and markers (isoelectric point 4.22, 4.65, 5.91, 6.14, 7.05, 7.40, 7.90, 9.22) were provided by Advanced Electrophoresis Solutions (ON, Canada). Reference peptides (isoelectric point 6.40, 7.00, 8.40) were purchased from Bio-Rad (Hercules, CA, USA). Urea and sucrose were obtained from Guangzhou Chemical (Guangzhou, China). Glycerol were purchased from Beijing Chemical (Beijing, China). Water used in the experiments was prepared by a Milli-Q system (Millipore, Bedford, MA, USA).

Sample preparation

The concentration of shortened glucagon-like peptide-1 was 0.2 mg/mL in phosphate buffer. To prepare shortened glucagon-like peptide-1 for analysis with capillary isoelectric focusing-whole column imaging, 50 μL of shortened glucagon-like peptide-1 were mixed with 2 M urea, carrier ampholytes and two markers (isoelectric point 4.22, 4.65) to make a final volume of 200 μL. The sample was centrifuged for 1 min at 13,200 rpm and the supernatant liquid was used for analysis.

Isoelectric focusing and calculation of isoelectric point

The samples were injected by an autosampler and the injection volume was 50 μL. A total of 0.1 M phosphoric acid and 0.1 M sodium hydroxide were used as the anolyte and catholyte, respectively. The initial focusing voltage was set as 1000 V for 1 min followed by 2000 V for 2 min, and then the voltage was altered to 3000 V and maintained for 4 min. During this period, complementary metal-oxide-semiconductor camera as imaging detector recorded the process of isoelectric focusing every 20 s. The detection wavelength was set to 280 nm. Two markers with known isoelectric point values were used as calibration points and the isoelectric point of sample was calculated according to the distance from anode to focused zones.

Accuracy

The accuracy of the capillary isoelectric focusing-whole column imaging detection method was determined by analyzing reference peptide samples with isoelectric point values of 6.40, 7.00, and 8.40. These peptides were mixed with carrier ampholytes (pH 3–10) and corresponding markers, respectively. The mixtures were centrifuged for 1 min at 13,200 rpm and the supernatants were used for analysis. Each reference peptide sample was analyzed thrice and the biases of the mean isoelectric point values from the reference values served as the measures of accuracy.

Repeatability

The intra- and inter-assay precisions were used to assess analytical method repeatability. The reference peptide samples used in these tests and the preparations of analytical samples were as same as the accuracy experiments. The intra-day precision was estimated by six replicate analyses in one day and the inter-day precision was evaluated by six replicate analyses in 6 days (one per day).

Results and discussion

Isoelectric focusing process

Peptide (isoelectric point 7.00) and two markers (isoelectric point 6.40 and 8.40) were focused from anolyte and catholyte sides to their respective isoelectric point position. The dynamic focusing process was recorded by a complementary metal-oxide-semiconductor camera every 20 s and the typical imaging at times 0, 40, 100, and 140 s are shown in Figure 2. The real-time scanning greatly reduced the analytical time. In this case, the focusing was completed within 3 min. Another advantage of capillary isoelectric focusing-whole column imaging was that the real-time scanning eliminated possible

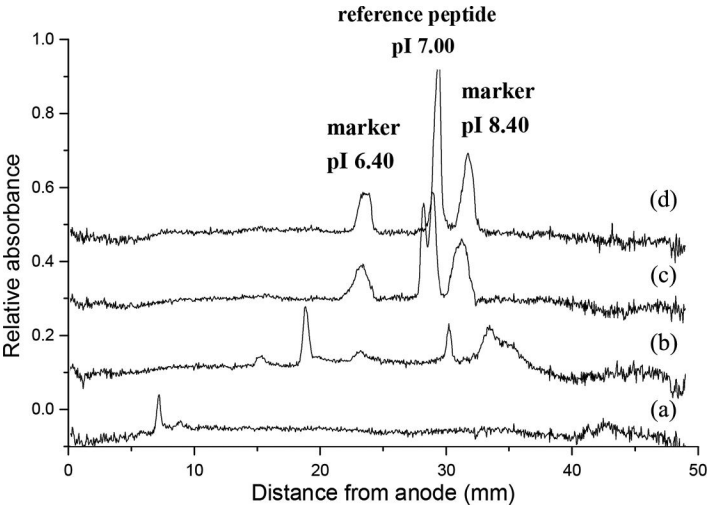

Figure 2. Imaging of the capillary isoelectric focusing process at the time (a) 0 s, (b) 40 s, (c) 100 s, and (d)140 s. The scanned distance from anode to cathode was 50 mm; analyte mixtures including one peptide sample (5 μg/mL) and two makers (2 μg/mL each) with isoelectric point 6.40 and 8.40 in ampholyte (pH 3-10) buffer.

error induced by the diffusion of focused zone when pushed though the detection point.

Accuracy

Three peptides (isoelectric point 6.40, 7.00, 8.40) were prepared separately to assess the accuracy of isoelectric point determination. Markers as internal standards were added to calibrate the isoelectric point scale for each measurement and the distance from anode to focused zone was determined to calculate the isoelectric point of reference peptide. For the reference peptide (isoelectric point 7.00), the mean value for three measurements was 7.05 with a bias of 0.57%. The calculated isoelectric point values for another two peptides (reference isoelectric point 6.40 and 8.40) were 6.52 and 8.36, respectively. The biases between test values and the reference values were both within 2% (Table 1). The coefficients of variation for assays were from 0.08 to 0.14%. These results show the high accuracy of isoelectric point measurements using capillary isoelectric focusing-whole column imaging.

Table 1. Accuracy assessment for isoelectric point determination using capillary isoelectric focusing-whole column imaging detection. Three peptides (2 μg/mL) with isoelectric points of 6.40, 7.00, and 8.40 were used as references. The pH range of ampholytes was 3–10.

Reference isoelectric point	Mean ($n = 3$)	Coefficient of variation (%)	Bias (%)
6.40	6.52 ± 0.01	0.09	1.93
7.00	7.04 ± 0.01	0.14	0.57
8.40	8.36 ± 0.01	0.08	−0.52

Repeatability

To assess the repeatability of the capillary isoelectric focusing-whole column imaging, peptides with isoelectric points of 6.40, 7.00, and 8.40 were determined consecutively. Typical electropherograms for consecutive injections of peptide (isoelectric point 7.00) are given in Figure 3 and the precision values are shown in Table 2. The coefficients of variation values for intra- and inter-day replicate analyses were both less than 0.25%, which demonstrates that the repeatability of capillary isoelectric focusing-whole column imaging was excellent.

Isoelectric point of shortened glucagon-like peptide-1

The capillary isoelectric focusing-whole column imaging was used to detect the isoelectric point of shortened glucagon-like peptide-1. Unfortunately, satisfactory results were not obtained was due to the hydrophobicity of shortened glucagon-like peptide-1. Precipitation of protein and peptide at isoelectric point may affect the stability of sample and, by extension, the measurement of isoelectric point.

To address this problem, solubilizers including urea, glycerol, and sucrose were added to increase the solubility of shortened glucagon-like peptide-1. The results are showed in Figure 4. The addition of 2 M urea, 20% (v/v) glycerol and 10% (w/v) sucrose provided better results than in the absence of solubilizer. The sharpest peaks and highest response for shortened

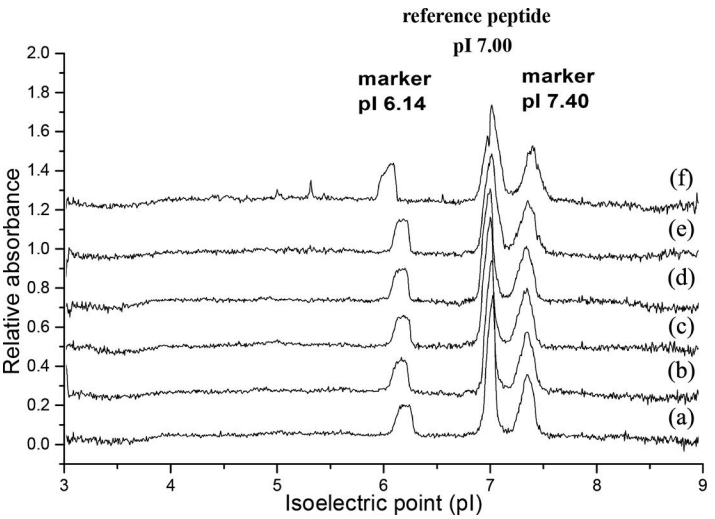

Figure 3. Replicate electropherograms of the reference peptide (isoelectric point 7.00, 2 µg/mL) determined by capillary isoelectric focusing-whole column imaging ($n = 6$). Markers with isoelectric points of 6.14 and 7.40 were used to calibrate the isoelectric point scale. The pH range of ampholytes was 3–10.

Table 2. Intra- and inter-day precision of isoelectric point determination by capillary isoelectric focusing-whole column imaging. Three peptides (2 μg/mL) with isoelectric point 6.40, 7.00 and 8.40 were used as references. The pH range of the ampholytes was 3–10.

	Mean ($n = 6$)		Coefficient of variation (%)	
Reference isoelectric point	Intra-day	Inter-day	Intra-day	Inter-day
6.40	6.51 ± 0.01	6.51 ± 0.02	0.12	0.24
7.00	7.04 ± 0.01	7.05 ± 0.02	0.11	0.22
8.40	8.36 ± 0.01	8.36 ± 0.01	0.08	0.10

glucagon-like peptide-1 were obtained in the presence of urea. The concentration of urea influenced the determination of the analyte as shown in Figure 5. When the concentration of urea was 2 M, improved repeatability and satisfied peak width at half height were achieved compared to the addition of 1 M urea. However, there were no significant improvement in the results by addition of urea concentrations from 3 to 8 M. hence, 2 M urea was selected to be the solubilizer due to its slightly narrower peak width at half height.

The determination of the isoelectric point of shortened glucagon-like peptide-1 was performed at a low concentration of 50 μg/mL. Two markers with isoelectric point values of 4.22 and 4.65 were added for calibration. The isoelectric point of shortened glucagon-like peptide-1 was determined thrice and the values were 4.49, 4.50, and 4.49, respectively (Figure 6). The average isoelectric point value was 4.49, which was similar to the theoretical value 4.40 calculated by ExPASy software with a coefficient of variation of only 0.13%. These results show that the addition of urea increased the stability

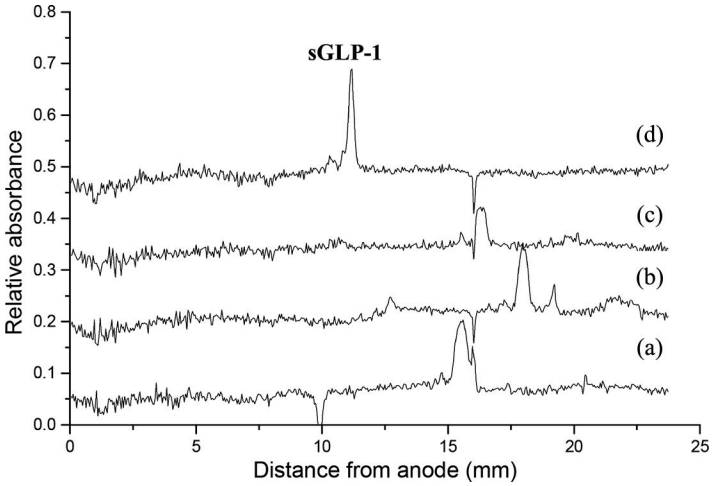

Figure 4. Effect of various solubilizers to the isoelectric focusing of shortened glucagon-like peptide-1 (sGLP-1). Sample preparation, the sGLP-1 was mixed with ampholytes (pH 3-10) to a final concentration of 50 μg/mL: (a) no solubilizer, (b) 10% (w/v) sucrose, (c) 20% (v/v) glycerol, and (d) 2 M urea.

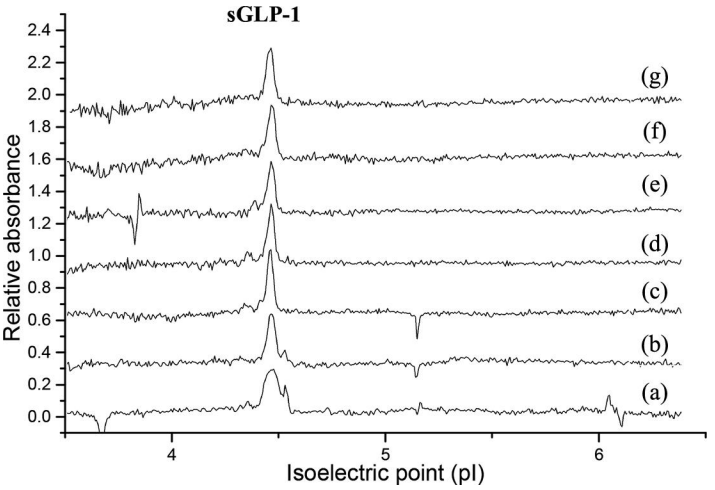

Figure 5. Effect of various urea concentrations on the isoelectric focusing of shortened glucagon-like peptide-1(sGLP-1). The sGLP-1 concentration was 50 µg/mL in ampholytes (pH 3-10). The final concentrations of urea were from 1 to 8 M (a to g).

of shortened glucagon-like peptide-1 and improved the repeatability and response intensity. The determination of the isoelectric point of shortened glucagon-like peptide-1 by capillary isoelectric focusing with whole column imaging was very rapid. The analysis in a few minutes of the isoelectric point for proteins and peptides is highly convenient allows high throughput.

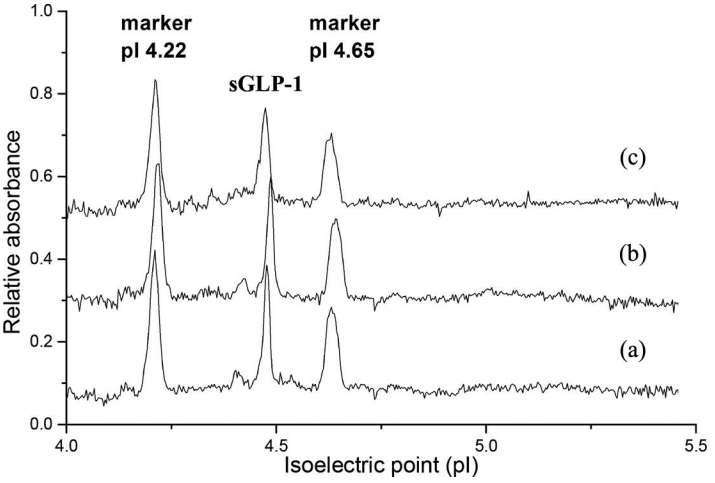

Figure 6. Isoelectric point of shortened glucagon-like peptide-1 determined by capillary isoelectric focusing-whole column imaging ($n = 3$). 50 µg/mL of shortened glucagon-like peptide-1 (sGLP-1) was in ampholyte (pH 3-10). The solubilizer was 2 M urea and the isoelectric points of the calibration marker were 4.22 and 4.65.

Conclusion

Unlike traditional electrophoresis, capillary isoelectric focusing-whole column imaging avoids the diffusion of sample zone caused by mobilization and provides more reliable result. The dynamic focusing process is scanned in real-time, which is helpful for visualization of protein separation mechanisms and characterization of the desired separation.[4] Also, compared with other electrophoresis methods, the detection time is significantly reduced.

In this study, capillary isoelectric focusing-whole column imaging was shown to accurately measure the isoelectric points of proteins and peptide. Moreover, the isoelectric point values showed no significant changes for replicate injections. The isoelectric point of shortened glucagon-like peptide-1 was difficult to measure due to its hydrophobicity. However, the addition of 2 M urea enhanced the solubility of shortened glucagon-like peptide-1, so narrower peak width at half height and better repeatability were observed. The determined isoelectric point value of shortened glucagon-like peptide-1 was 4.49, which was similar to its theoretical value.

Furthermore, the development of analytical condition was more convenient and simpler than previous approaches due to the reduced analysis time. This study has demonstrated that capillary isoelectric focusing-whole column imaging may be used to provide high performance, high throughput, high resolution, and reproducible protein or peptide analysis. Capillary isoelectric focusing-whole column imaging has been applied for the separation and determination of complex samples,[15,16] protein stability research,[17] molecular mass estimate,[18] and characterization of drugs and proteins interactions,[19,20] which may be a promising alternative to traditional isoelectric focusing detection.

Funding

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References

[1] Issaq, H. J. A Decade of Capillary Electrophoresis. *Electrophoresis* **2000**, *21*, 1921–1939. DOI: 10.1002/1522-2683(20000601)21:10<1921::aid-elps1921>3.0.co;2-y.

[2] Kilár, F. Recent Applications of Capillary Isoelectric Focusing. *Electrophoresis* **2003**, *24*, 3908–3916. DOI: 10.1002/elps.200305650.

[3] Rabilloud, T. Detecting Proteins Separated by 2-D Gel Electrophoresis. *Anal. Chem.* **2000**, *72*, 48A–55A. DOI: 10.1021/ac002709u.

[4] Mao, Q.; Pawliszyn, J. Capillary Isoelectric Focusing with Whole Column Imaging Detection for Analysis of Proteins and Peptides. *J. Biochem. Biophys. Methods* **1999**, *39*, 93–110. DOI: 10.1016/s0165-022x(99)00006-8.

[5] Wu, J.; Pawliszyn, J. Universal Detection for Capillary Isoelectric Focusing without Mobilization using Concentration Gradient Imaging System. *Anal. Chem.* **1992**, *64*, 224–227. DOI: 10.1021/ac00026a024.
[6] Wu, J.; Pawliszyn, J. Absorption Spectra and Multicapillary Imaging Detection for Capillary Isoelectric Focusing Using a Charge Coupled Device Camera. *Analyst* **1995**, *120*, 1567–1571. DOI: 10.1039/an9952001567.
[7] Wu, J.; Tragas, C.; Watson, A.; Pawliszyn, J. Capillary Isoelectric Focusing with Whole Column Detection and a Membrane Sample Preparation System. *Anal. Chim. Acta* **1999**, *383*, 67–78. DOI: 10.1016/s0003-2670(98)00489-9.
[8] Liu, Z.; Pawliszyn, J. Capillary Isoelectric Focusing of Proteins with Liquid Core Waveguide Laser-Induced Fluorescence Whole Column Imaging Detection. *Anal. Chem.* **2003**, *75*, 4887–4894. DOI: 10.1021/ac034587m.
[9] Bo, T.; Pawliszyn, J. Role of Calcium Binding in Protein Structural Changes and Phospholipid–Protein Interactions Studied by Capillary Isoelectric Focusing with Whole Column Imaging Detection. *Anal. Chim. Acta* **2006**, *559*, 1–8. DOI: 10.1016/j.aca.2005.11.047.
[10] Bo, T.; Pawliszyn, J. Characterization of Phospholipid-Protein Interactions by Capillary Isoelectric Focusing with Whole-Column Imaging Detection. *Anal. Biochem.* **2006**, *350*, 91–98. DOI: 10.1016/j.ab.2005.11.039.
[11] Hjertén, S.; Liao, J. L.; Yao, K. Theoretical and Experimental Study of High-Performance Electrophoretic Mobilization of Isoelectrically Focused Protein Zones. *J. Chromatogr. A* **1987**, *387*, 127–138. DOI: 10.1016/s0021-9673(01)94519-4.
[12] Baggio, L. L.; Drucker, D. J. Biology of Incretins: GLP-1 and GIP. *Gastroenterology* **2007**, *132*, 2131–2157. DOI: 10.1053/j.gastro.2007.03.054.
[13] Mentlein, R.; Gallwitz, B.; Schmidt, W. E. Dipeptidyl-Peptidase IV Hydrolyses Gastric Inhibitory Polypeptide, Glucagon-Like Peptide-1 (7–36) Amide, Peptide Histidine Methionine and is Responsible for their Degradation in Human Serum. *Eur. J. Biochem.* **1993**, *214*, 829–835. DOI: 10.1111/j.1432-1033.1993.tb17986.x.
[14] Li, Y.; Shi, C.; Lv, Q.; Zhang, H.; Li, B.; Bian, G.; Huang, Q.; Zhang, W.; Xue, X.; Ren, X.; et al. GLP-1 C-Terminal Structures Affect Its Blood Glucose Lowering-Function. *J. Pept. Sci.* **2008**, *14*, 777–785. DOI: 10.1002/psc.997.
[15] Thomassen, Y. E.; van Eikenhorst, G.; van der Pol, L. A.; Bakker, W. A. Isoelectric Point Determination of Live Polioviruses by Capillary Isoelectric Focusing with Whole Column Imaging Detection. *Anal. Chem.* **2013**, *85*, 6089–6094. DOI: 10.1021/ac400968q.
[16] Dou, P.; Liu, Z.; He, J.; Xu, J. J.; Chen, H. Y. Rapid and High-Resolution Glycoform Profiling of Recombinant Human Erythropoietin by Capillary Isoelectric Focusing with Whole Column Imaging Detection. *J. Chromatogr. A* **2008**, *1190*, 372–376. DOI: 10.1016/j.chroma.2008.03.001.
[17] Bo, T.; Pawliszyn, J. Protein Thermal Stability and Phospholipid-Protein Interaction Investigated by Capillary Isoelectric Focusing with Whole Column Imaging Detection. *J. Sep. Sci.* **2006**, *29*, 1018–1025. DOI: 10.1002/jssc.200500456.
[18] Liu, Z.; Lemma, T.; Pawliszyn, J. Capillary Isoelectric Focusing Coupled with Dynamic Imaging Detection: A One-Dimensional Separation for Two-Dimensional Protein Characterization. *J. Proteome. Res.* **2006**, *5*, 1246–1251. DOI: 10.1021/pr060023y.
[19] Lemma, T.; Pawliszyn, J. Human Serum Albumin Interaction with Oxaliplatin Studied by Capillary Isoelectric Focusing with the Whole Column Imaging Detection and Spectroscopic Method. *J. Pharm. Biomed. Anal.* **2009**, *50*, 570–575. DOI: 10.1016/j.jpba.2008.10.028.
[20] Lemma, T.; Mandal, R.; Li, X. F.; Pawliszyn, J. Investigation of Interaction between Human Hemoglobin A_0 and Platinum Anticancer Drugs by Capillary Isoelectric Focusing with Whole Column Imaging Detection. *J. Sep. Sci.* **2008**, *31*, 1803–1809. DOI: 10.1002/jssc.200700418.