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Reference


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Chapter 15

Capillary Electrophoresis-Ultraviolet-Mass Spectrometry (CE-UV-MS) for the Simultaneous Determination and Quantification of Insulin Formulations

Julie Schappler and Serge Rudaz

Abstract

This chapter describes a CE-UV-MS method for the identification and quantification of insulin in pharmaceutical formulations in a single run. The CE conditions are optimized to avoid the adsorption of the protein onto the capillary wall. Particular attention is paid regarding the choice of the internal standard. A strategy based on multiple injections is applied to correct both ionization and injection variabilities. The methodology is validated according to international guidelines and the obtained accuracy profile demonstrates the ability of the CE-UV-MS method to quantify insulin in pharmaceutical formulations within a ±5% acceptance range. This strategy can be implemented in the field of quality control, as well as in the detection of counterfeits.

Keywords: Capillary electrophoresis, Identification, Insulin, Intact protein, Multiple injections, Quality control, quantification, Mass spectrometry

Abbreviations

ACN Acetonitrile
BGE Background electrolyte
CE Capillary electrophoresis
dH₂O Double-distilled water
EOF Electrophoretic flow
ESI Electrospay ionization
FS Fused silica
I.D. Internal diameter
ICH International Conference of Harmonization
iPrOH Isopropanol
LOD Limit of detection
m/z Mass-to-charge ratio
MeOH Methanol
MS Mass spectrometry

185
1 Introduction

The amount of recombinant proteins produced by biotechnology has grown considerably in the last few years [1]. During the biopharmaceutical development and production processes, numerous parameters are needed for regulatory purposes, regarding the identity, quantity, quality, and purity of the products. Since unoffical channels exist to obtain these products without prescription or without extensive evidence for quality control, the analysis of therapeutic proteins is also relevant for the parallel market and counterfeit drugs. Therefore, it is essential to have analytical methods that quickly monitor the identity, quality, and quantity of these biopharmaceuticals.

For the identification and quantification of protein formulations, the analysis of proteins in their intact form is a promising approach because no tedious sample preparation, such as a digestion step, is required [2]. In order to perform the simultaneous identification and quantification of an intact protein, whether in a pharmaceutical formulation or in another matrix, the hyphenation of capillary electrophoresis (CE) and mass spectrometry (MS) via an electrospray ionization (ESI) source appears as an attractive option [3]. CE offers high speed, great efficiency, and low solvent and sample consumptions, while MS provides selectivity, sensitivity, and identification features. Due to its high mass range and mass accuracy, the time-of-flight (TOF) analyzer is particularly well suited for the detection of multi-charged macromolecules when ESI is employed [4].

In this study, a CE-UV-TOF/MS method is developed for the analysis of formulations of recombinant human insulin. Patients suffering from diabetes mellitus often buy insulin online without prescription due to the potentially lower cost, which increases the risk of finding counterfeit drugs in the parallel market. In the context of public health, CE coupled to UV-TOF/MS for quality control of these biopharmaceutical formulations offers nice features with simultaneous identification and quantification of insulin, thanks to an external calibration approach. A multiple-injection technique, used to get both information in one single run, is selected and consists of the successive injection of a reference standard of insulin and the unknown sample. The complete methodology is fully validated according to the guidelines of the International Conference of Harmonization (ICH) and can be further applied to pharmaceutical formulations obtained in drugstores and on the Web without a formal prescription [5].

2 Materials

2.1 Apparatus and Material

1. Experiments are carried out with an HP 1100 CE system from Agilent Technologies (Waldborn, Germany), equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. The instrument is coupled to a 6210 LC/MS TOF mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) via a coaxial sheath flow ESI interface from Agilent.

2. Uncoated fused silica (FS) capillaries are obtained from BGB Analytik AG (Böckten, Switzerland), with an inner diameter (I.D.) of 50 μm, a total length of 80 cm, and an effective length of 22 cm for UV detection.

2.2 Chemical, Reagent, and Sample Solutions

2.2.1 Solutions for Capillary Electrophoresis-Ultraviolet-Mass Spectrometry (CE-UV-MS)

1. Background electrolyte (BGE): Prepare 50 mL of a mixture of ammonium formate 75 mM (pH 9.0)-ACN (90:10, v/v) (see Note 1).

In a beaker, insert 500 μL of ammonia 28% and add ddH₂O up to 40 mL. Adjust the pH to 9.0 with formic acid 99% and add ddH₂O up to 50 mL. Take 40 mL of this solution and add 10 mL of acetonitrile (ACN). This solution can be kept for 1 month at 4 °C.

2. Sheath liquid: Prepare 100 mL of a mixture of 0.07% ddH₂O-formic acid (50:50:1, v/v/v).

In a beaker, insert 50 mL of isopropanol (iprOH) and 50 mL of ddH₂O and add 1 mL of formic acid 99%.

2.2.2 Solutions of Insulin Formulations for the Validation of the CE-UV-MS Procedure

1. Solutions used for calibration (calibration standards): Prepare three independent samples (0.5 mL) of insulin at 5 U.I./mL. Mix 25 μL of insulin at 100 U.I./mL with 250 μL procaine at 100 μg/mL. Add 225 μL ddH₂O (see Note 2).

2. Solutions used for validation (validation standards): Prepare nine independent samples (0.5 mL) of insulin at 3.6 U.I./mL.

Mix 18 μL of insulin at 100 U.I./mL with 250 μL procaine at 100 μg/mL. Add 232 μL ddH₂O (see Notes 2 and 3).

3. Solutions used for validation (validation standards): Prepare nine independent samples (0.5 mL) of insulin at 5 U.I./mL.

Mix 25 μL of insulin at 100 U.I./mL with 250 μL procaine at 100 μg/mL. Add 225 μL ddH₂O (see Notes 2 and 3).
4. Solutions used for validation (validation standards): Prepare nine independent samples (0.5 mL) of insulin at 6.4 U/I/mL. Mix 32 µL of insulin at 100 U/I/mL with 250 µL procaine at 100 µg/mL. Add 218 µL ddH₂O (see Notes 2 and 3).

2.2.3 Solutions of Insulin Formulations for the Routine Use of the CE-UV-MS Procedure

1. Solutions to be quantified (unknown samples): Prepare two independent samples (0.5 mL) of the insulin formulation at 5 U/I/mL. Mix 25 µL of the insulin formulation at 100 U/I/mL with 250 µL procaine at 100 µg/mL. Add 225 µL ddH₂O (see Notes 4 and 5).

3 Methods

3.1 Capillary Electrophoresis-Ultraviolet-Mass Spectrometry (CE-UV-MS) (See Note 6)

1. New capillaries are pretreated by applying a pressure of 1 bar to the capillary inlet and using the following sequence: MeOH, 1 M HCl, ddH₂O, 0.1 M NaOH, ddH₂O, and BGE for 5 min each.

2. The capillary is thermostated at 25 °C and the samples maintained at ambient temperature in the sample tray.

3. The BGE is prepared daily and renewed every three runs.

4. The in-between rinsing run is carried out by pumping fresh BGE through the capillary at 2 bar for 1 min.

5. When the capillary is not in use, it is rinsed with ddH₂O and dry-stored at room temperature.

6. The sample is introduced into the capillary by hydrodynamic injection using the following sequence: the first sample is injected at 50 mbar for 10 s (equivalent to 0.68% of the total capillary length). Then, a plug of BGE is injected at 50 mbar for 130 s (8.90% of the total capillary length). The second sample is injected at 50 mbar for 10 s (0.68% of the total capillary length). Finally, a plug of BGE is injected at 50 mbar for 4 s (0.27% of the total capillary length) (Table 1, see Notes 7–10).

7. The separations are carried out at 30 kV with positive polarity at the inlet and an initial ramping voltage of 5000 V/s.

8. Insulin and procaine are detected with UV detection at 195 nm.

9. The sheath liquid is delivered at a flow rate of 4 µL/min by a syringe pump system.

10. The ESI source parameters are set: the ESI voltage at +4500 V, the nebulizing gas pressure at 4 psi, the drying gas flow rate and temperature at 4 L/min and 150 °C, respectively, and the fragmentor voltage at 400 V.

11. One spectrum is acquired per second (9742 transients/spectrum) in the 900–2500 m/z range.

12. The CE-ESI-MS total ion electropherogram (TIE) shows two peaks (Fig. 1a, Table 2): the first peak (a) corresponds to the insulin standard from the first injected sample, and the second peak (b) is insulin from the second injected sample to be tested. The [M + 3H]⁺ and [M + 4H]⁺ ions are detected as the major extracted ions (1937 and 1453 m/z, respectively, Fig. 1b) on the mass spectrum. The extracted ion electropherogram (XIE) is obtained using both ions (Fig. 1c) and integration achieved on the XIE.

13. The CE-UV-MS electropherogram shows six peaks (Fig. 1d, Table 2): the first three peaks (c, *, e) come from the first injected sample, and the three last peaks (d, **, f) come from the second injected sample. Peaks c and d are procaine migrating before the electroosmotic flow (EOF), peaks * and ** are neutral excipients detected in the EOF (e.g., metacresol and glycerol), and peaks e and f are insulin migrating after the EOF.

3.2 Analysis of Insulin Formulations by CE-UV-MS: Validation of the Procedure

1. Evaluate the method selectivity by comparing typical electropherograms obtained by injecting ddH₂O (CAL 00), procaine at 50 µg/mL in ddH₂O (CAL 0), and a validation standard (VS). No interference should be observed at the migration time corresponding to the procaine and insulin peaks in UV or MS measurements (Fig. 2).

2. Inject the first sample (calibration standard) and then the second sample (validation standard) according to the injection sequence (Table 1), and perform the CE-UV-MS analysis.
Fig. 1 Analysis of insulin by CE-UV-MS. (a) MS detection, total ion electropherogram (TIE), (b) MS detection, extracted mass spectrum, (c) MS detection, extracted ion electropherogram (XIE), and (d) UV detection. (a) and (e) insulin at 3 U/I/mL from the first injection, (b) and (f) insulin at 5 U/I/mL from the second injection, (c) procaine at 50 µg/mL from the first injection, (d) procaine at 50 µg/mL from the second injection, *neutral excipients from the first injection, and **neutral excipients from the second injection. Adapted from [5], with permission.

Table 2
Correspondence between Injected Samples and Detected Peaks

<table>
<thead>
<tr>
<th>Injected samples</th>
<th>Detected peaks</th>
<th>ESI-MS</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a Insulin, c Procaine, e Excipients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>b Insulin, d Procaine, f Excipients, Insulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 Selectivity of the CE-UV-MS method. (a) UV detection and (b) MS detection. Injection of: ddH₂O (CAL 00), procaine at 50 µg/mL (CAL 0), and validation standard (VS) at 6.4 U/I/mL. (a) and (e) insulin at 3 U/I/mL from the first injection, (b) and (f) insulin at 5 U/I/mL from the second injection, (c) procaine at 50 µg/mL from the first injection, (d) procaine at 50 µg/mL from the second injection, *neutral excipients from the first injection, and **neutral excipients from the second injection. Adapted from [5], with permission.

Repeat the analysis with the three independent replicates \((n = 3)\), the three levels of concentration \((k = 3)\), and the three series \((j = 3)\) (see Notes 3 and 11).

3. Correct peak area as follows: The area of insulin peak 2 detected by MS (corresponding to peak b in Fig. 1c) is corrected by the area of the procaine peak 2 detected by UV (peak d in Fig. 1d). The same correction is done for the area of insulin peak 1 detected by MS (peak a over peak c). The ratio \((b/d)\)/(a/c) is used for trueness and precision calculations (see Note 12).

4. Estimate the method trueness for each concentration level (expressed as recovery, %, obtained by the ratio of calculated concentration over true concentration).

5. Estimate the method precision by the variances of repeatability and intermediate precision (expressed as RSD, %), obtained by variance decomposition (ANOVA).

6. Construct the accuracy profile (Fig. 3). The upper and lower confidence limits (plain outer lines, respectively, in Fig. 3) are calculated from the trueness (plain central line in Fig. 3) and represent the total error of the method, based on the confidence interval \((\alpha = 5\%)\) (see Note 13).
7. Make a decision: If the upper and lower confidence limits are included into the acceptance limits (±5%, dotted lines in Fig. 3), the CE-UV-MS method can be considered valid for insulin determination over the investigated concentration range (3.6–6.4 U.I./mL). Quantification of real samples can be performed with an appropriate method uncertainty.

3.3 Analysis of Insulin Formulations by CE-UV-MS: Routine Use of the Procedure

1. Inject the first sample (calibration standard) and then the second sample (unknown sample) according to the injection sequence (Table 1), and perform the CE-UV-MS analysis. Repeat the analysis with the second preparation of the unknown sample.

2. Identify insulin by comparing the mass spectrum of peak a and peak b (Fig. 4c). [M + 3H]⁺ and [M + 4H]⁺ ions should be detected as the major extracted ions (1937 and 1453 m/z, respectively).

3. Correct peak area as follows: The area of the unknown peak detected by MS (corresponding to peak b in Fig. 4b) is corrected by the area of the procaine peak detected by UV (corresponding to peak d in Fig. 4a). The same correction is done for the area of insulin calibration standard detected by MS (peak a over peak c).

4. Calculate the concentration of insulin: The mean ratio (b/d)/(a/c) is used for quantification, expressed as a relative concentration ± confidence interval [%] (see Note 14).

5. Make a decision: The unknown sample can be considered compliant with the expected specifications if its identity is confirmed and its concentration falls within the expected limits ± 5% around the target value (see Note 15).

4 Notes

1. Volatile BGE has to be used to be directly ESI-MS compatible. ACN is added to improve CE performance and reduce the adsorption of the protein onto the capillary wall [6].

2. The exact nature and concentration of the excipients of insulin formulations remain unknown in most cases; for instance, the zinc and glycine quantities are not mentioned in the manufacturer’s datasheet of Actrapid®, hindering the reconstitution of the formulation. To overcome this issue, a reference batch of Actrapid® at 100 U.I./mL (Novo Nordisk A/S, Bagsvaerd, Denmark) is used as a stock solution for the calibration and validation samples.

3. According to ICH guidelines, as well as recommendations from the “Société Française des Sciences et Techniques Pharmaceutiques” (SFSTP), three series (j = 3) of three independent replicates (n = 3) are prepared at each concentration level (k = 3) [7].

4. If the formulation to be quantified is not 100 U.I./mL, the dilution should be adapted so that a sample at 5 U.I./mL is analyzed.
5. Two independent samples are prepared since it has been demonstrated that most of the variability comes from the repeatability.

6. Under these conditions, the limit of detection of the method (LOD, estimated for a S/N = 3) is 0.2 U.I./mL, while the response function is linear over a concentration range of 0.2–7 U.I./mL.

7. The unknown sample is injected with the second injection plug, so the sample to be quantified is less subject to variability caused by the previous hydrodynamic injections of the sample "calibration standard" and BGE.

8. In CE-ESI-MS, the ionization standard should exhibit similar ionization behavior as that of the protein to be quantified. As neither stable isotopically labeled nor structural analogues are easily available, a standard of insulin at a known concentration is first injected (calibration standard), followed by an injection of the sample to be quantified (validation standard or unknown sample) in the same run. This procedure allows decreasing the run-to-run variability of the ionization. Short-term variability of the ionization process can still occur since both peaks do not co-migrate.

9. In addition to the ionization standard, an injection standard is added to both samples to correct for the variability of the hydrodynamic injection between both injections in the same run. Procaine is selected because it migrates before the EOF and UV detection is used to avoid any additional source of ionization variability.

10. With a BGE plug corresponding to approximately 9% of the capillary length between both injections, the procaine peaks are sufficiently resolved to be easily integrated.

11. The double role of the first injected sample (calibration sample and internal standard) improves the throughput of the validation process, resulting in fewer injections.

12. A linear response function without significant intercept is used as calibration function.

13. The accuracy profile is selected as the decision tool to evaluate the method's capacity to quantify samples over the expected concentration range (75–125%, i.e., 3.6–6.4 U.I./mL). The accuracy takes into account the total error of the method and includes the combination of systematic (true) and random (precision) errors. The accuracy profile is constructed by first reporting the trueness at each concentration level (plain central line in Fig. 3). Then the upper and lower confidence limits (UL and LL, respectively, plain outer lines in Fig. 3) are calculated at each concentration level with the following equations and reported on the graph:

$$ UTL = true_{\text{ness}} + t_{c,n} \sqrt{e} \sqrt{\sigma_e^2} $$

$$ LTL = true_{\text{ness}} - t_{c,n} \sqrt{e} \sqrt{\sigma_e^2} $$

where $t_{c,n}$ is the Student constant and $\sigma_e^2$ is the intermediate precision variance determined thanks to the regular ANOVA-based variance decomposition.

14. The confidence interval associated to the result mean ($\overline{x}$) is expressed as

$$ \overline{x} = \overline{\text{true}_{\text{ness}}} \sqrt{\frac{e}{N} + \frac{t_{c,n}^2}{N}} $$

where $t_{c,n}$ is the Student constant, $N$ is the number of analyses performed during routine analysis for each sample, and $s_e^2$ and $s_{ie}^2$ are the repeatability and the inter-day variances, respectively, determined during validation thanks to the regular ANOVA-based variance decomposition.

15. The example is provided with a sample of insulin purchased on the Web. A concentration of 100.2 ± 2.2% is obtained for this sample, which is considered compliant.

Acknowledgments

The authors wish to thank Dr. Aline Staub Spörri from the Food Authority Control of Geneva for technical support and fruitful discussions.

References


