A New High-resolution Approach to the Detection of Insulin Degradation Products

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Introduction

In drug manufacturing, storage studies are needed for safety and efficacy reasons to identify toxic breakdown products and determine the shelf life of drugs such as insulin. In this study, we have looked to compare and contrast capillary electrospray ionization mass spectrometry (CESI-MS) and high flow liquid chromatography-mass spectrometry (LC-MS) in the analysis of degraded insulin.

Standard reverse phase liquid chromatography separates constituents based on their differential interactions with the column stationary phase and an organic mobile phase. CESI-MS is a different separation technique and is the integration of CE and ESI into a single dynamic process and provides highly efficient peptide and protein separations based on their size and charge.[1] The ionization is at the ultra-low nanoflow regime (~25 nL/min) and it simply uses an open capillary (Figure 1), which eliminates some of the challenges of stationary phase-based separations such as protein recovery or conformational changes using reverse phase gradient elution solvents.

The purpose of this work was to compare the breakdown products detected in the presence of high levels of the parent drug using both CESI-MS and LC-MS techniques. In this comparison, the MS method remained the same with the exception of the source conditions, which had to be optimized for the different flow rates used by CESI and LC. The peak height of the parent insulin peak was adjusted by modifying the injection conditions, so levels of the impurities detected were not biased. In the case of the LC method, this meant reducing the injection volume to 2 µL, which also helped reduce peak tailing of the parent insulin.

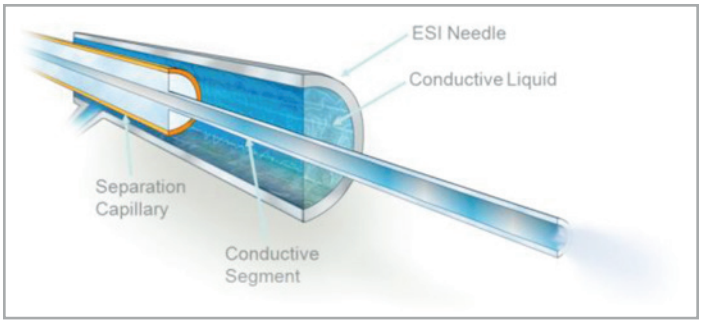

Figure 1. The OptiMS cartridge provides an ultra-low flow ESI interface.

Materials and methods

Chemicals: All chemicals were Reagent Grade and were purchased from Sigma Aldrich.

Sample Preparation: Insulin (Sigma I2643, 5.44 mg dissolved) was dissolved in 2 mM phosphate buffer (1 mL). Hydrochloric acid (2 M, 15 µL) was added and mixed until the insulin had dissolved. Sodium hydroxide (2 M, 15 µL) was added followed by phosphate buffer (4.4 mL). The sample was then degraded for 3 1⁄2 days (50 °C). Before analysis, the sample was diluted with an equal volume of ammonium acetate (200 mM).

Step	Time (min)	Flow rate (µL/min)	% mobile phase A	% mobile phase B
0	0	400	95	5
1	1	400	95	5
2	10	400	50	50
3	12	400	10	90
4	13	400	10	90
5	13.2	400	95	5
6	15	400	95	5

Table 1. HPLC conditions used for reverse phase separation of degradation products of insulin.

LC-MS method: For the LC-MS analysis, 2 µL of the insulin sample was injected onto an Aeris peptide HPLC column (XB- C18, 2.7 µm, 2.1 x 100 mm) that has been developed for the analysis of peptides. For this analysis, the SCIEX TripleTOF® 5600+ LC-MS/MS System was fitted with the DuoSpray™ Turbo V Ion Source. Gas 1 was 50 psi, gas 2 was 60 psi, curtain gas was 30 psi, temperature was 450 °C and the ion-spray voltage was set at 5500 V. The HPLC separation used the gradient elution shown in Table 1, where mobile phase A was water containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid with the column held at 25 °C. Data was acquired using a TOF-MS method over the mass range 600–2000 Da with DP set to 100 V and CE set to 10 V.

CESI-MS method: Sample was injected by pressure (3 psi, 5 s) onto a 30 µm ID x 95 cm bare fused-silica capillary (including porous spray tip) housed in an OptiMS cartridge. The capillary was covalently coated with polyethylenimine (PEI)[2] and thermostatted using recirculating liquid coolant regulated at 25 °C. For this analysis, the SCIEX TripleTOF® 5600+ System was fitted with the NanoSpray® III Ion Source. Gas 1, gas 2 and temperature were not applied (set to 0, 0 and 50, respectively). In order to generate ionization at these very low flow rates, simply apply ion-spray voltage below 2000 V (typically 900–1800 V is sufficient). The curtain gas was set very low at 5 psi (set automatically in the CESI software). The CE separation used the condition shown in Table 2 with a background electrolyte of 10% acetic acid. Data was acquired by TOF MS over the mass range 600–2000 Da, with conditions identical to those used for the LC-MS analysis.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Results

Analytes separate in CE based on their mobility (m/z) and elute in HPLC based on hydrophobic interactions with the stationary phase in the column. Both techniques showed complementary specificity with peaks moving in opposite directions. In the LC-MS experiment, insulin elutes at 6.8 min accompanied by an early eluting sharp peak corresponding to the solvent front and a late eluting broad peak corresponding to the high organic section of the gradient elution (Figure 2). In both of these areas, only singly charged peaks could be found. Neither of these features was present in the CESI-MS electropherogram where the broad peak at 7.5 min contained the majority of the singly charged species and insulin migrated at 9.4 min.

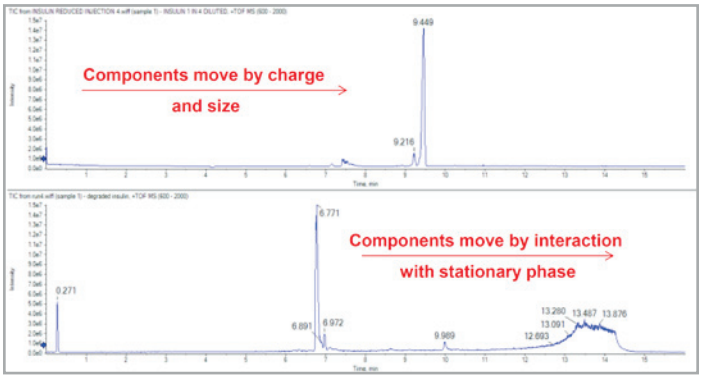

Figure 2. Direct comparison on a CESI-MS electropherogram and a LC- MS chromatogram from the injection of the same insulin sample.

In these experiments, the injection pressure and time in the CESI-MS analysis together with the injection volume of the LC-MS analysis was adjusted so that the response for the insulin peak (the most prominent peak) was the same in both injections.

Both CESI-MS and LC-MS data were studied in depth to compare resolution and the capability of both techniques to identify low-level impurities. In Figure 3, the region around the parent peak for insulin has been expanded to show the resolution for the major impurity peak, which was formed as a result of a deamination modification. In CESI-MS, this peak migrates before the parent peak and for LC-MS this peak elutes after the parent. In both cases, the deamination resulted in a small mass change of 1 Da, but also resulted in a charge alteration. The CESI-MS gave better resolution than HPLC-MS with a baseline separation of this product from the parent insulin. Separation of this major product is important as this small mass change would otherwise be masked by the spectra of the parent ions.

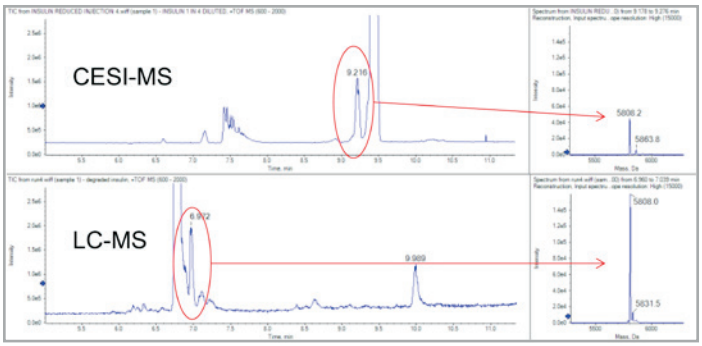

Figure 3. Expanded region around insulin parent peak to compare resolution of major degradation product.

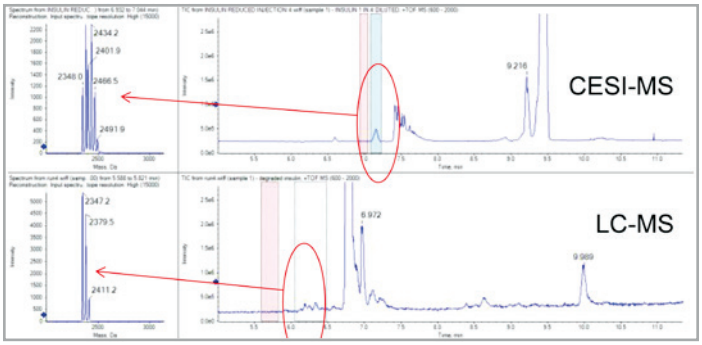

Figure 4. Expanded region containing cleavage products from breakage of the insulin backbone.

Figure 4 shows the region where products from the breakage of the insulin backbone elute and migrate. In the CESI analysis, these cleavage products migrate in a sharp peak easily detected at 7.2 min. In the LC-MS analysis, these constituents elute in a less distinguishable peak at 6.3 min and in addition is missing several of the diagnostic ions (notable peaks at 2434 and 2460 Da) that were present in the CESI-MS analysis.

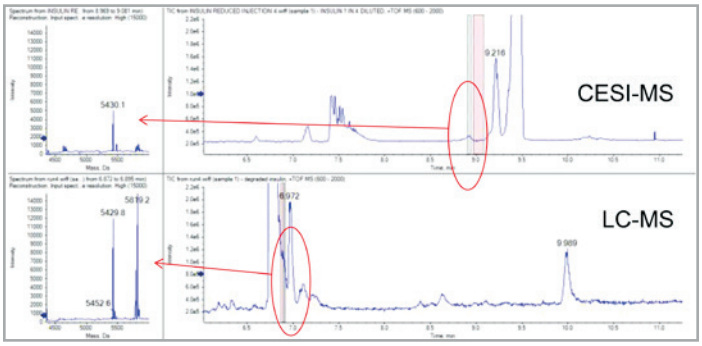

Figure 5. Detection of the loss of terminal amino acids.

In Figure 5, the peak at 8.9 min was the result of loss of terminal amino acids, which generated a component of mass 5430 Da. In the HPLC analysis, this component co-eluted under the parent mass and its retention time was identified using extracted ion chromatograms.

Finally, in Figure 6, 2 components resulting from the degradation process were identified in the CESI-MS that were not detected by LC-MS. The first was a dimer of 2 insulin molecules that migrated as a minor peak at 10.35 min. This peak, as expected, migrated after insulin (9.4 min) as it was twice as big (11615 Da). In addition, there was a shoulder eluting before the parent ion at 9.1 min. This was identified as an additional deamination product that, under the separation conditions used, had a slightly different charge, which caused it to migrate after the major deamination product. This second deamination product could not be distinguished in the LC-MS analysis.

Both LC-MS and CESI-MS analysis were capable of detecting degradation products that were less than 1% of the size of the parent peak.

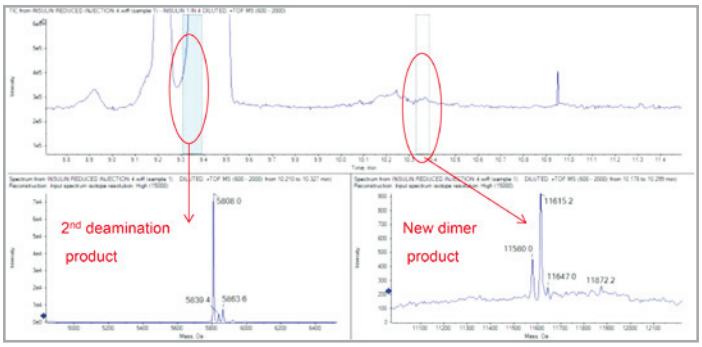

Figure 6. Components identified in the CESI-MS analysis that were not found in the LC-MS analysis.

Conclusions

LC-MS and CESI-MS have been shown to be complementary techniques because of their differing mechanism of separation.

Both techniques were capable of detecting low-level degradation products that were less than 0.5% of the area of the insulin peak.

In this study, CESI-MS exhibited better peak resolution for degradation products of insulin and was capable of detecting additional features not detected by the LC-MS method.

References

1. Busnel, J.-M. et al. High Capacity Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry: Coupling a Porous Sheath less Interface with Transient-Isotachophoresis *Anal. Chem.* 2010, 82, 9476–9483.

2. Santos, M.R. et al. A covalent, cationic polymer coating method for the CESI-MS analysis of intact proteins and polypeptides. SCIEX separations application note, 2015.

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