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Technical Note

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Characterization of a Low-Level Unknown Isomeric Degradation Product Using an Integrated Online−Offline Top-Down Tandem Mass Spectrometry Platform

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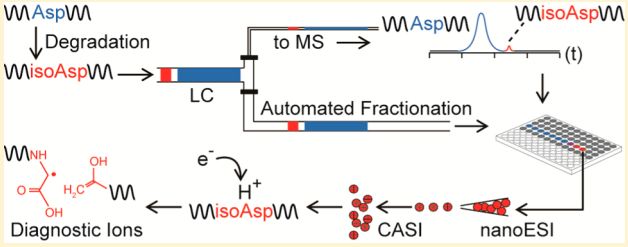
Supporting Information

ABSTRACT: An integrated online−offline platform was developed combining automated online LC-MS fraction collection, continuous accumulation of selected ions (CASI), and offline top-down electron capture dissociation (ECD) tandem mass spectrometry experiments to identify a low-level, unknown isomeric degradant in a formulated drug product during an accelerated stability study. By identifying the diagnostic ions of the isoaspartic acid (isoAsp), the top-down ECD experiment showed that the Asp9 in exenatide was converted to isoAsp9 to form the unknown isomeric degradant. The platform described here provides an accurate, straightforward, and low limit of detection method for the analysis of Asp isomerization as well as other potential low-level degradants in therapeutic polypeptides and proteins. It is especially useful for unstable and time-sensitive degradants and impurities.



Therapeutic peptides and proteins have become a rapidly increasing sector of today's pharmaceuticals market. It is expected that, among the best-selling pharmaceuticals, the ratio of the therapeutic peptides and proteins will increase over the next decade.[1] However, peptide and protein pharmaceuticals are chemically and physically unstable in nature. Degradants and impurities can be generated during manufacturing and storage, leading to inactivation or worse, to toxicological responses. Because of quality and safety concerns, the demand in analytical technologies to rigorously characterize degradants and impurities of these large and complex biomolecules has increased dramatically.[2]

Exenatide is a 39 amino acid synthetic analogue of a glucagon-like peptide-1 (GLP-1) agonist hormone approved for the treatment of type II diabetes mellitus.[3] Exenatide was formulated at pH 4.5 for the best stability profile, because the asparagine (Asn) deamidation rate is lowest at this pH.[4] During the formulation-accelerated stability study, an unknown degradant was observed by liquid chromatography−mass spectrometry (LC-MS) analysis. The LC-MS and collision-activated dissociation (CAD) tandem mass spectrometry (MS/MS) spectra showed that the accurate mass and MS/MS fragmentation patterns of the degradant were very similar to that of the exenatide active pharmaceutical ingredient (API), indicating that this degradant was most likely an isomer of the API with a similar peptide sequence.

In order to identify this low-level, unknown isomeric degradant, an integrated platform was developed by combining automated online LC-MS fraction collection, offline (direct infusion) continuous accumulation of selected ions (CASI), and top-down electron capture dissociation (ECD) MS/MS. This platform is also applicable to the detailed analysis of a large variety of low-level protein post-translational modifications (PTMs) at intact protein level.

MATERIALS AND METHODS

Peptides and Reagents. Exenatide injectable was purchased from Amylin Pharmaceuticals (San Diego, CA). All other chemicals were purchased from Thermo Fisher Scientific (San Jose, CA).

Formulation Conditions. Byetta (exenatide) injection was stored for 1−3 months at 30 °C and 65% relative humidity (RH) in order to study the degradation rate and degradation products formed under these storage conditions. The formulation of Byetta is as follows: 0.25 mg/mL exenatide,

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meta-cresol, mannitol, glacial acetic acid, and sodium acetate trihydrate buffer solution in pH 4.5.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). The mixture of formulated exenatide and its degradants were separated on a Phenomenex Kinetex reverse-phase C18 column (2.1 mm × 50 mm, 100 Å). Mobile-phase A was 100% HPLC grade water with 0.1% formic acid. Mobile phase B was 80:20 (v/v) acetonitrile: water with 0.1% formic acid. For the LC-MS experiments, an Agilent 1200 series HPLC system (Wilmington, DE) was used. The gradient program was as follows: 20% B to 75% B in 45 min; 75% B to 95% B in 0.1 min; and a hold at 95% B for 5 min. The entire gradient program was 55 min at a flow rate of 200 μL/min at 55 °C. For the online fraction collection, the gradient program was as follows: 5% B for 2 min; 35% B to 55% B in 17 min; 55% B to 100% B in 1 min; and hold at 100% B for 1 min. The entire gradient program was 30 min at a flow rate of 250 μL/min at 55 °C. All chromatography was monitored using a 214-nm UV detector.

Online LC-MS Fraction Collection and Offline Direct Infusion. The online LC fraction collection was carried out by a TriVersa NanoMate robot (Advion, Inc., Ithaca, NY). After HPLC separation, the eluent was split, with 0.6 μL/min of the LC flow going to the 1.7 kV nano electrospray ionization source (ESI) and the remainder of the LC flow going to the fraction collector controlled by the NanoMate robot. Each fraction was collected for 10 s, and 41.5 μL of the fraction was collected into each well. After fraction collection, the NanoMate was converted to the offline direct infusion mode. Five to ten microliters (5−10 μL) of the collected fraction was loaded and 1.6−1.7 kV ESI spray voltage was applied for each ECD MS/MS experiment.

Tandem Mass Spectrometry. CAD and ECD MS/MS experiments were performed on a Bruker 9.4-T solariX hybrid Qq−Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an indirectly heated hollow dispenser cathode (Bruker Daltonics, Billerica, MA). In CAD experiments, precursor ions were isolated via the front-end quadrupole and collided with argon in the collision cell. The product ions were transferred to an ion cyclotron resonance (ICR) cell for mass analysis and detection via the ion transfer guide. In ECD experiments, precursor ions were isolated by a front-end quadrupole and accumulated in the collision cell before being transferred to the ICR cell. The precursor ions were then irradiated with ∼1.5 eV electrons for ∼20 ms at a cathode heating current of 1.5 A. A 0.67 s transient was typically acquired for each scan, and each ECD spectrum shown represents the results of 40−100 transients summed to improve the signal-to-noise ratio (S/N).

Data Analysis. The MS/MS spectra were calibrated internally using product ions assigned with high confidence, giving typical mass measurement accuracy better than 2 ppm. Deconvoluted mass lists were generated semiautomatically by the SNAP (Sophisticated Numerical Annotation Procedure) algorithm using the DataAnalysis (Bruker Daltonics, Billerica, MA) software for solariX spectra and were verified manually.

RESULTS AND DISCUSSION

LC-MS and LC-CAD MS/MS experiments were carried out on an exenatide formulation sample after a three months' accelerated stability study at 30 °C and 65% RH. The major degradation products are oxidation, hydrolysis, and dehydration and can be routinely identified based on accurate mass measurement and CAD fragmentation. As shown in the LC-UV chromatogram (Figure 1), one of the degradation products

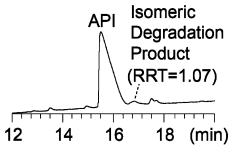
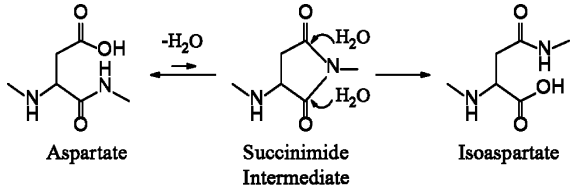


Figure 1. Liquid chromatography−ultraviolet (LC-UV) chromatogram of an exenatide formulation after a three months' accelerated stability study at 30 °C and 65% relative humidity (RH).

at a relative retention time (RRT) of 1.07 is ∼2% of the API in peak area. According to the FDA guidelines (ICH, Q3A, and Q3B), a degradation product must be identified if greater than 0.1% for a dosing of <2 g/day. The LC-MS accurate mass measurement suggests that the degradant ($[M+5H]^{5+}$, m/z 837.8135 (measured)) has the same molecular formula as that of the API ($[M+5H]^{5+}$, m/z 837.8127 (calculated)). Furthermore, the CAD MS/MS spectrum for the degradation product at RRT = 1.07 was very similar to that of the API (see Figure S1 in the Supporting Information). The accurate mass and MS/MS data suggest that the degradation product at RRT = 1.07 has a peptide sequence similar to that of the API and might be an isomer of the API.

A common isomerization in polypeptide/protein is the conversion of Asp to isoAsp through a succinimide intermediate (see Scheme 1).[4] Although the Asp isomerization

Scheme 1. Asp Converts to isoAsp through a Succinimide Intermediate



is the smallest PTM, such conversion alters the protein structure and, thus, changes its bioactivity. For example, the Asp isomerization significantly reduces the binding affinities and efficacy of monoclonal antibodies.[5] Kinetics studies show that the rate of Asp isomerization is dependent on pH with the highest rate at pH ∼4−5, a condition at which most protein pharmaceuticals are formulated to inhibit Asn deamidation.[6] In order to better design and formulate therapeutic polypeptides and proteins, it is important to identify and locate Asp isomerization sites in their degradants and impurities.[7]

However, differentiating Asp from isoAsp is quite challenging, especially when the size of the protein is larger. The identification of isoAsp can be achieved by modifying isoAsp residues using Asp-N or protein isoaspartate methyltransferase (PIMT).[8−10] However, these methods involve enzymatic incubation in a pH 7.8 buffer, which could introduce false-positive isoAsp, because of base-catalyzed Asn deamidation.[11] In addition, the enzymatic approach described in the literature requires a few hundred picomoles of a pure compound, making it even more challenging for the determination of isoAsp when encountered as a low-level drug degradant.

Recently, Cournoyer and co-workers have reported the use of ECD MS/MS to differentiate Asp from isoAsp.[12] In

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comparison to the conventional CAD technique, which mainly forms b and y ions via cleaving the amide bonds, ECD involves the cleavage of the N−C_α bond and produces c and z ions, as illustrated in Scheme S1 in the Supporting Information. In the case of isoAsp, ECD can break not only N−C_β bonds to form c and z ions but also C_α−C_β bonds to form c+57 and z−57 ions, which are unique to isoAsp and can be used as the diagnostic ions for the identification of isoAsp. This approach was employed to detect the presence of the isoAsp in the aged β-2-microglobulin (~11.7 kDa) at intact protein level using a CAD-ECD MS^3 method.[13]

However, one of the major drawbacks for applying the top-down ECD to low-level degradation identification is its low fragmentation efficiency. Performing online LC-ECD MS/MS on a 2% degradant at MW ≈ 4000 Da was not productive, because of the limited LC time scale. Under the offline direct infusion mode, it is also not practical to isolate and enrich this low-level degradant in gas phase by CASI as the degradant has the same molecular weight as that of the API.[14] Another analytical challenge is the limited sample supply, with only 500 μL of 0.25 mg/mL drug product available from each stability study. Although the degradant can be isolated through offline LC fraction collection, the fractions collected were not stable and can be oxidized within a few hours after offline LC fraction collection. As such, it was not practical to use a traditional isolation method to obtain the pure degradant.

In order to practically apply the top-down ECD technique to characterize this isomeric degradant, an integrated analytical platform was developed by combining automated online LC-MS fraction collection, CASI, and offline direct infusion top-down ECD MS/MS experiments. The NanoMate robot is commercially available and can be easily switched between the online LC-MS fraction collection mode and the offline direct infusion mode. The solariX FT-ICR-MS can enrich the ion of interest by the CASI technique, which will facilitate the following top-down ECD MS/MS. As shown in Figure 2, ~40 μg of formulated exenatide was loaded into an HPLC with a flow rate of 250 μL/min. The eluent was then split into two parts: 0.6 μL/min of the total flow was sent to LC-MS for detection, while the remainder of the flow was collected into a 96-well plate by the NanoMate robot. The retention time in the total ion chromatogram (TIC) of the online LC-MS reflects the location of each fraction on the 96-well plate. As the retention time of the unknown isomeric degradant is ~20 s, to obtain the pure isomeric degradants, the rate of the fraction collection was set to 10 s/well. Thus, each well will have ~41.6 μL of solution for the top-down ECD MS/MS (see Figure 2).

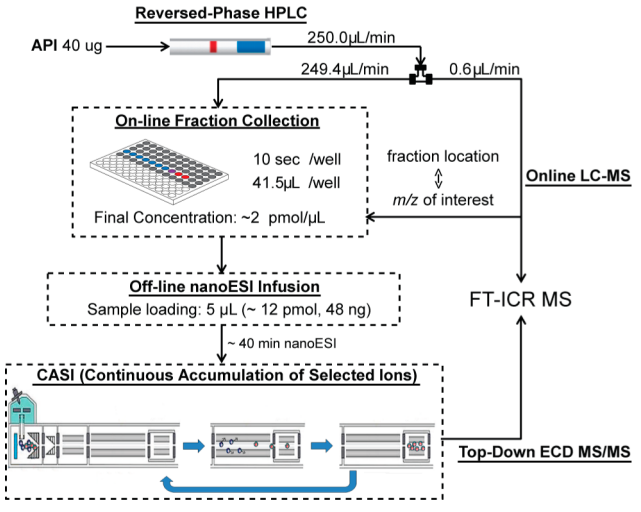

Figure 2. Experimental flow of the integrated online LC-MS fraction collection and offline top-down ECD MS/MS platform.

After online fraction collection, the NanoMate was then switched to the offline direct infusion mode. The final concentration for the fraction containing the unknown isomeric degradant was ~2 pmol/μL for a sample loading of 40 μg, the ion of interest was then accumulated for ~4−16 s in the collision cell using CASI[14] to obtain sufficient precursor ions for the top-down ECD experiment, which eliminated the need for additional lyophilization steps. The signal of the nanoESI lasted for ~40 min for a sample loading of 5 μL, providing sufficient time for CASI and multiple transients averaging, which are necessary for a high-quality top-down ECD experiment. The use of a similar platform has also been reported for the identification of protein PTMs[15,16] and the degradation products of therapeutic antibodies,[17] although the instrument used for those studies do not have the CASI capability.

Figure 3 shows the top-down ECD spectrum of the unknown isomeric degradant. Extensive c and z ions were produced by ECD with only two missed cleavages (excluding cleavages of *N*-terminal to proline residues). In the ECD spectrum of the

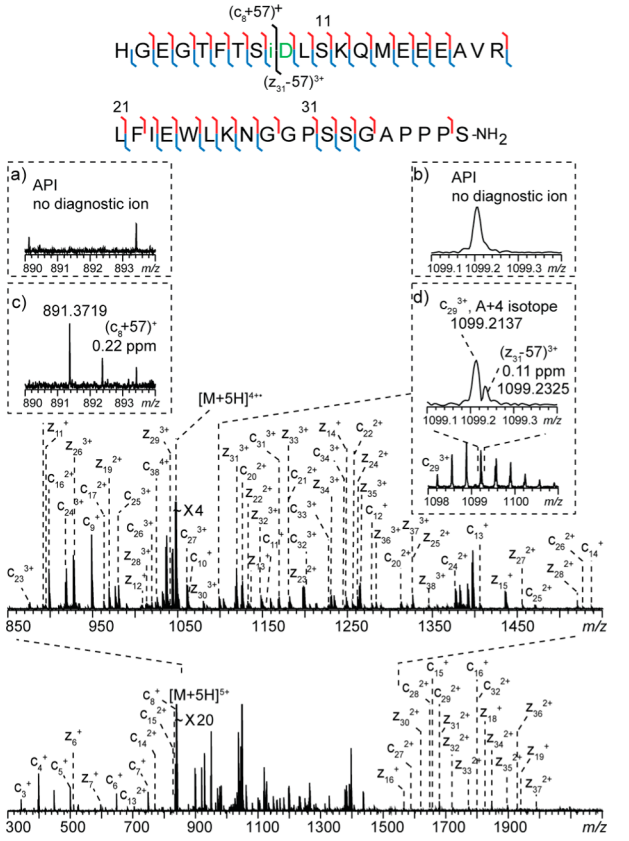

Figure 3. ECD produced isoAsp diagnostic ions suggesting the conversion of Asp9 to isoAsp9 in the unknown isomeric degradant. Insets (a) and (b) show that no isoAsp diagnostic ion was found in the ECD spectrum of the exenatide API. The isoAsp diagnostic ions (c) $(c_8+57)^+$ and (d) $(z_{31}-57)^{3+}$ were detected in the ECD spectrum of the unknown isomeric degradant.

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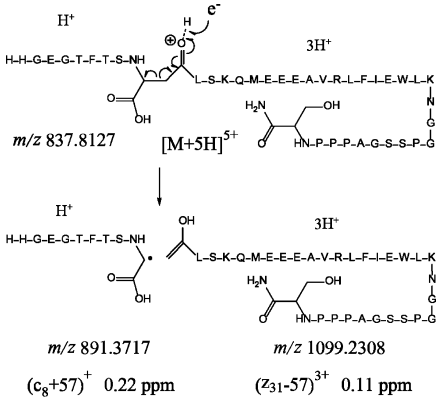
unknown isomeric degradant, two isoAsp diagnostic ions were observed: $(c_8+57)^+$ (m/z 891.3719, 0.22 ppm) and $(z_{31}-57)^{3+}$ (m/z 1099.2325, 0.11 ppm) (see insets c and d in Figure 3). As shown in Scheme 2, during the ECD process, the unknown isomeric degradant at m/z 837.8127 $[M+5H^+]^{5+}$ captured one electron and neutralized the positive charge at the carbonyl adjacent to isoAsp. This process initiated the homolytic cleavage at the $C_\alpha-C_\beta$ bond, which generated the diagnostic ions for isoAsp. For comparison, ECD was also performed on the exenatide API and no diagnostic ions were identified (insets a and b in Figure 3). The above data suggested that the unknown isomeric degradant at RRT = 1.07 resulted from the conversion of Asp9 to isoAsp9.

Scheme 2. Formation of the isoAsp Diagnostic Ions for the Isomeric Degradant during Electron Capture Dissociation (ECD)[a]



[a]The precursor ion $[M+5H]^{5+}$ (m/z 837.8127) captured one electron and produced diagnostic ions $(c_8+57)^+$ (m/z 891.3717) and $(z_{31}-57)^{3+}$ (m/z 1099.2308).

It is worth noting that high resolving power is critical for the unambiguous identification of isoAsp. For example, the mass difference between the diagnostic ion $(z_{31}-57)^{3+}$ (m/z 1099.2325) and the A+4 isotope of the ion c_{29}^{3+} (m/z 1099.2137) is only 18.8 mDa (see inset d in Figure 3). The FT-ICR MS was able to separate these two peaks at a resolving power of 500,000.

CONCLUSIONS

An integrated online liquid chromatography–mass spectroscopy (LC-MS) fraction collection and offline continuous accumulation of selected ions (CASI)/top-down electron capture dissociation (ECD) tandem mass spectrometry (MS/MS) platform was developed to identify a low-level, unknown isomeric degradant in exenatide as the aspartic acid (Asp) isomerization at position 9. This platform is capable of performing solution phase isolation for low sample quantities during online LC-MS fraction collection. CASI enriched the ion of interest in the gas phase, to improve the limit of detection and quality of the top-down ECD MS/MS spectrum. The isoaspartic acid (isoAsp) diagnostic ions were positively identified with high confidence, because of the high resolving power and the high mass accuracy offered by Fourier transform ion cyclotron resonance mass spectroscopy (FT-ICR MS). The integrated platform minimizes the number of analytical steps and makes the characterization of unstable impurity and degradants possible. This platform is robust, fast, and accurate, and should contribute substantially to the characterization of low-level degradants and impurities for a wide variety of polypeptide/protein pharmaceuticals.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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