Altasciences CLINICAL RESEARCH
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The Application of Research Grade MetabolitePilot™ Software for the Determination of Exenatide Catabolites using HRAM with SWATH Acquisition

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Overview

Purpose

To implement research grade MetabolitePilot™ software for the determination of *in vitro* catabolites of exenatide, a 4.2 kDa GLP-1 agonist.

Method

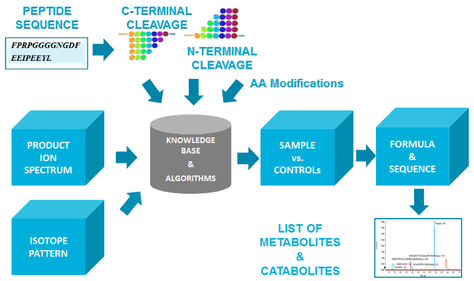
- Incubation of exenatide in rat whole blood at 37°C
- Extraction by protein precipitation in ethanol:acetonitrile (7:3)
- HRAM measurements of SWATH data acquired using a SCIEX TripleTOF5600+

Results

The MS/MS spectra of chromatographically unresolved exenatide(3-39) and (4-39) catabolites whose precursor masses were transmitted through the same SWATH window were successfully identified using the advanced spectral deconvolution algorithm in research grade MetabolitePilot™.

Introduction

The stability of peptide/protein biotherapeutics directly impacts their pharmacokinetic profile, efficacy, and safety, making it essential to characterize potential metabolic soft spots. To facilitate an accurate mass workflow for the confirmation of peptide biotransformations and their profiling across a time course, a research grade version of MetabolitePilot™ software has been engineered with an expanded peak finding strategy that (i) supports higher charge states, (ii) generates putative catabolic products by cleavage of the amide backbone and disulfide bonds, (iii) considers isotopic distribution of catabolic products, and (iv) interprets MS/MS data using conventional peptide fragmentation patterns. In the current investigation, MetabolitePilot™ was implemented to determine the *in vitro* catabolites of exenatide, obtained using HRAM in SWATH acquisition mode (**S**equential **W**indow **A**cquisition of All **The**oretical MS).



Methods

Rat whole blood fortified with exenatide (1 µg/mL) and incubated at 37°C was sampled at t_0, t_{30}, t_{60}, t_{120} and t_{240} and subsequently precipitated with ethanol:acetonitrile (7:3). Extracts were chromatographed on a Halo Peptide ES-C18 column (2.1 x 150 mm, 5 µm) under gradient conditions at 50°C with an acidified water/acetonitrile mobile phase, ramped from 98% aqueous to 70% organic over 30 minutes. Data was acquired with a SCIEX TripleTOF 5600+ operated in SWATH mode using an accumulation time of 100 ms per experiment, where each experiment coincided to fixed 50 Da wide MS/MS windows at 45 eV collision energy for precursor masses 200 - 1250 Da. A research grade version of SCIEX MetabolitePilot™ Software was used for post-acquisition processing.

Results and Discussion

The post-acquisition processing workflow in research grade MetabolitePilot™ initially involved defining potential bio-transformations. In addition to the provided default modifications in the Biologics set, custom bio-transformations can be entered, and for exenatide included deamidation (N/Q), oxidative deamination to alcohol (K), demethylation (A/T) and demethylation + oxidation (A/T). The MetabolitePilot™ processing algorithm always considers hydrolytic cleavages, and for exenatide, generated 702 theoretical catabolites.

The next stage in the MetabolitePilot™ workflow defined the Peak Finding Strategy, which leverages the TOF-MS data derived from the first experiment in the SWATH acquisition. Within the Processing Parameters dialog, the peptide sequence of exenatide was entered along with an MS/MS reference spectrum from which two diagnostic product ions (y_3 ion at *m/z* 299.1717 and y_4 ion at *m/z* 396.2245) were also incorporated into the peak finding algorithm (**Figure 1a, b**).

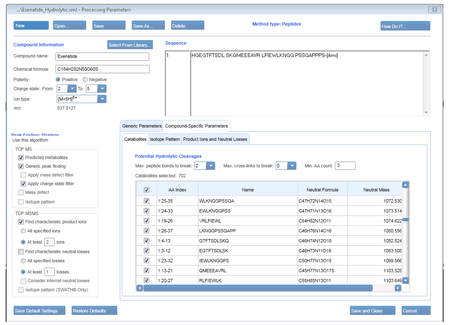


Figure 1a. Processing Parameters workflow in research grade MetabolitePilot™. Both TOF-MS and TOF-MS/MS data acquired in SWATH mode were incorporated in the peak finding strategy for exenatide catabolites. For a minimum of three amino acid residues, 702 putative catabolites were proposed based on hydrolytic cleavages alone.

Results and Discussion (Continued)

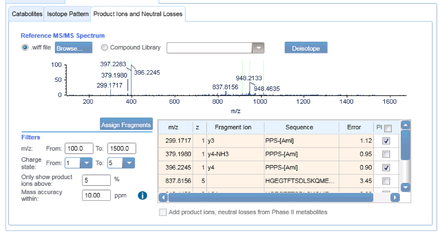


Figure 1b. Compound specific parameters indicating the use of y_3 and y_4 product ions incorporated into the peak finding algorithm.

With the Bio-transformation and Processing Parameters established, incubated samples were interrogated against control samples. Proposed catabolites presented in the Results workspace (**Figure 2**) were considered only if the measured parent mass was within 10 ppm of theoretical and the response was three-fold greater than that observed in control samples. This same mass accuracy was applied to the assignment of product ions measured against theoretical *b*- and *y*-ion masses for proposed catabolite sequences (**Figure 2**).

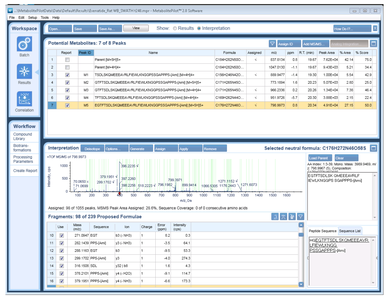


Figure 2. The Results workspace of research grade MetabolitePilot™ highlights proposed catabolite sequences for exenatide including elemental composition, measured *m/z*, parent mass error and chromatographic retention time. The Interpretation pane plots the catabolite MS/MS spectrum correlating fragment ion mass with sequence information, here for exenatide(3-39). The measured mass error for each fragment ion is also reported. In the Results pane, the XIC from a TOF-MS scan can be plotted against control sample and both the isotope pattern and MS/MS spectrum can be displayed and are incorporated into a scoring assignment.

Results and Discussion (Continued)

Results from each time-point were compiled in the Correlation workspace and potential catabolites plotted (**Figure 3**). In the case of exenatide, only one catabolite demonstrated increased response with incubation time, and coincided to the N-terminal HG clipping biotransformation product exenatide(3-39), whose chromatographic profile and MS/MS spectrum are compared to exenatide in Figure 4. Exenatide(4-39), (5-39), and (7-39) catabolites were also detected at incubation times ≥ t_{120}, and therefore could not be fully correlated within the time-frame of the experiment (**Figure 3**).

Figure 3. Incubation time profiles for proposed catabolites are generated in the Correlation workspace. The major catabolite for exenatide resulted from N-terminal HG clipping whilst exenatide(4-39), (5-39), and (7-39) were only expressed after t_{120}. XICs from the TOF-MS scan of the SWATH acquisition can be plotted at each incubation time point as illustrated for exenatide(3-39).

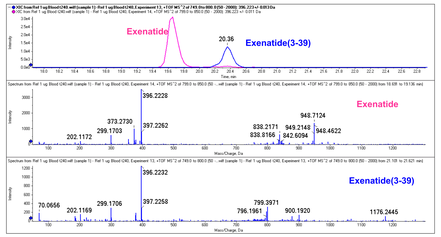


Figure 4. Comparison of chromatographic retention time and MS/MS SWATH spectra for exenatide and its major catabolite, exenatide(3-39) indicating the highly abundant diagnostic y_3/y_4 ions.

Results and Discussion (Continued)

Regardless, each minor putative catabolite generated the diagnostic y_3- and y_4- ions of the exenatide reference spectrum, and each parent mass was measured within 5 ppm of theoretical. Of particular note, exenatide(3-39) and (4-39) were chromatographically unresolved and their $[M+5H]^{+5}$ precursor masses were simultaneously transmitted through the same SWATH window (i.e. *m/z* 749 – 800), thereby generating a mixed MS/MS spectrum. While a formidable challenge to deconvolute this complex scenario in applications such as PeakView, the advanced algorithm used in MetabolitePilot™ successfully re-constructed the MS/MS spectrum derived from each of exenatide(3-39) and (4-39), thereby aligning the TOF-MS XICs with confirmatory *b*- and *y*-fragment ions, as outlined in **Figure 5**.

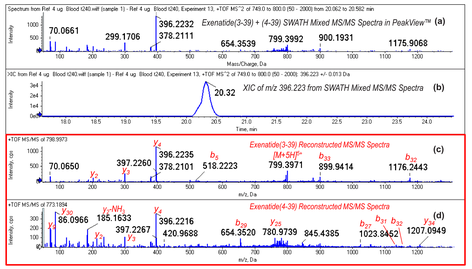


Figure 5. The XIC in PeakView for the y_4 diagnostic ion (*m/z* 396.223) derived from the SWATH MS/MS experiment for *m/z* 749 – 800 (a) demonstrates a chromatographic profile suggestive of co-eluting catabolites (b). Since the SWATH MS/MS spectrum is derived from a 50 Da mass window, it is difficult to correlate specific fragment ions with precursor mass. However, the advanced MetabolitePilot™ algorithm could identify the putative catabolites as exenatide(3-39) and exenatide(4-39) despite their co-elution, and moreover could re-construct the MS/MS spectrum derived from each of the parent masses 798.9973 (c), and 773.1894 (d), respectively. With the MS/MS spectra properly de-convoluted, confirmation of the proposed catabolites was possible.

Conclusions

The research grade version of MetabolitePilot™ software, when combined with accurate mass measurements from a TripleTOF platform operated in SWATH acquisition mode, represents a formidable platform for protein biotherapeutic catabolism studies. The advanced capabilities of the software algorithm were exemplified in properly assigning co-eluting catabolites of exenatide whose parent masses were simultaneously transmitted through the same SWATH window. A commercial version of MetabolitePilot™ (v 2.0) is now available which incorporates the peptide sequencing capabilities presented herein.