Therapeutic Peptide Catabolite Identification using MetabolitePilot™ 2.0 Software and the TripleTOF® 6600 System

Find, Identify and Confirm Peptide Catabolites Using MetabolitePilot™ 2.0 Software and the Advanced Biotransform Solution

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Key Challenges in Peptide Catabolism Studies

- Therapeutic peptides are susceptible to unspecific proteolysis along the peptide backbone and therefore the number of potential catabolites to be considered is much larger than those from a small molecule MetID study
- In addition to unspecific cleavages potential biotransformations of the amino acids of the above species must also be considered
- Multiple charge states of both the parent peptide and potential catabolites must be considered, which increases the complexity of MetID relative to small molecule studies
- Missing, low-level peptide catabolites in complex biological matrices and incomplete catabolite information leading to repeated sample analysis and decreased productivity
- Maintaining data quality for both quantitative and qualitative analysis in a high-throughput environment

Key Features of MetabolitePilot Software for Therapeutic Peptide Catabolite Identification

- A dedicated peptide processing workflow that performs targeted searching for both hydrolytic cleavages and biotransformations for multiply charged species
- Flexible processing parameters that accommodate cross-linked and cyclic peptides plus non-natural amino acids and custom side chain modifications
- Multiple peak finding strategies utilizing MS and MS/MS information for targeted and untargeted searching for drug related metabolites for multiply charged species

Figure 1. The SCIEX Advanced Biotransform Solution featuring the new MetabolitePilot™ 2.0 software with a dedicated peptide catabolism workflow, TripleTOF® 6600 and Exion AD LC system.

- A dedicated peptide sequence assignment workspace with automated catabolite sequence generation plus candidate ranking and catabolite confirmation using a/y/b ion labelling in the MS/MS interpretation workspace
- Integrated correlation function allows comparison of catabolism across multiple samples for time course studies or inter-species comparison, using both MS and analog data.

Introduction

Even though the principles of pharmacokinetics and metabolism of small molecule drugs apply to peptide and protein drugs, the mechanisms of metabolism are very different. Small molecule drug metabolism occurs via the conversion of the molecule or moieties into more hydrophilic species via Phase I and Phase II processes. Peptide and protein drugs are acted upon by serum and tissue proteases that are distributed throughout the body and proteolysis is a major elimination pathway for most therapeutic peptides. Unmodified peptides typically have

very short half-lives. Fortunately there are many approaches available to enhance the stability of therapeutic peptides through structural modifications such as: N and C terminal protection, amino acid substitution, cyclization and amino acid modification.

Using these approaches the ADME properties of therapeutic peptides and proteins have been improved significantly and bio-therapeutic drugs have gained importance in the treatment of critical diseases. As in small molecule drug development there is a need to understand their bio-transformations to ensure that the molecule is safe and efficacious and does not cause toxicity. The ability to find, identify and confirm catabolites as quickly as possible is critical at multiple stages of drug discovery and development. Advancements in accurate mass instrumentation such as the TripleTOF® 6600 have enabled the generation of very information rich raw data but new software to automate the catabolite identification workflow is required to benefit drug metabolism researchers. To address this need SCIEX has introduced the new MetabolitePilot™ 2.0 software that includes a dedicated peptide catabolism workflow. Figure 2 shows an overview of the data processing workflow for peptide catabolism in MetabolitePilot™ 2.0 software. The sequence of the therapeutic peptide (including any amino acid modifications and cross links) is input into the software along with an experimental reference mass spectrum. From this data a list of potential hydrolytic cleavages is generated along with a theoretical isotope pattern, charge state range and a list of product ion and neutral losses. These pieces of data are taken along with a list of potential biotransformations to form the knowledge base used for predicted and targeted metabolite searching. In addition to targeted searching generic peak finding algorithms are also used to find unexpected metabolites. Sample to control comparisons are performed to identify new species and then the MS and MS/MS information is used to generate a list catabolites. MetabolitePilot 2.0 software includes automated sequence generation for catabolites with rankings to speed up the data processing and an interpretation workspace for confirmation of the catabolite sequence using a/y/b fragment ion labelling of the MS/MS.

In this tech note the in-vitro metabolism of a model therapeutic peptide Bivalirudin was investigated to highlight the new peptide catabolism workflow features of MetabolitePilot 2.0 software.

Experimental

Sample Preparation

The synthetic peptide Bivalirudin (FPRPG GGGNG DFEEI PEEYL) was incubated with rat plasma at 37°C at different time points. After incubation, protein precipitation was performed with the addition of 1:1:1 acetonitrile:methanol:water 0.1% formic acid solution. The supernatant was removed and analyzed by LC/MS.

Mass Spectrometry Data Collection

A SCIEX TripleTOF® 6600 LC-MS/MS system with IonDrive™ source was used to collect the information dependent data with dynamic background subtraction, focusing on multiply charged precursor ions. TOF MS spectra (m/z 100 to 2000) were collected with accumulation time of 150ms, the accumulation time for TOF/MS/MS (m/z 100 to 2000, CE=45, CES=15) was 75ms.

Chromatography

Samples were chromatographed on a Phenomenex Aeris 2.1 mm X 50 mm, 3 µm column at 40°C with 6-minute gradient of eluent A, 2% acetonitrile + 0.1% formic acid, and eluent B, 98% acetonitrile + 0.1% formic acid, was

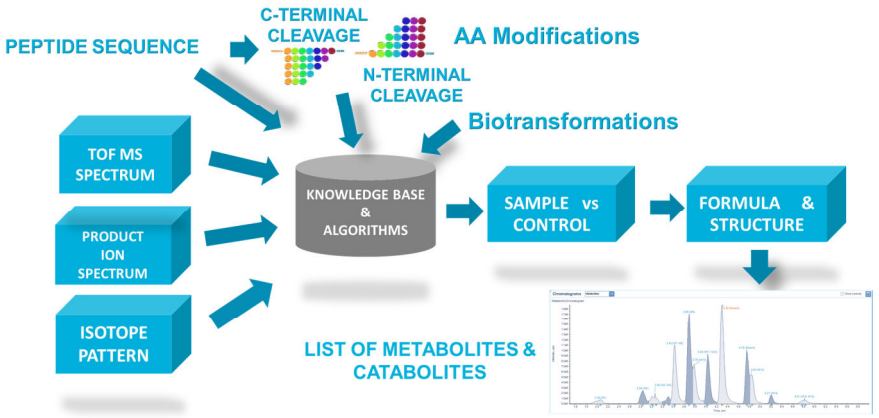

Figure 2. Data mining strategy to find degradation and catabolism products of therapeutic peptides. Therapeutic peptide sequence, its MS/MS experimental or predicted spectrum and a list of potential amino acid modifications are used as an input for series of accurate mass LC/MS peak finding strategies that find peptide – related material in either IDA or SWATH™ data.

used at a flow rate of 400µL/min. The injection volume was 10µL

Data Processing

The peptide specific processing parameters are shown in Figure 3. To identify therapeutic peptide–related material, a peak finding strategy based on predicted catabolites that are proteolytic hydrolysis products (minimum of 5 aa) potentially combined with a set of common modifications, such as reduction, oxidation, methylation and acetylation as well as well as any material with MS/MS fragments matching at least 2 of 6 characteristic parent peptide fragments was used. Matrix blank was used in sample/control comparison.

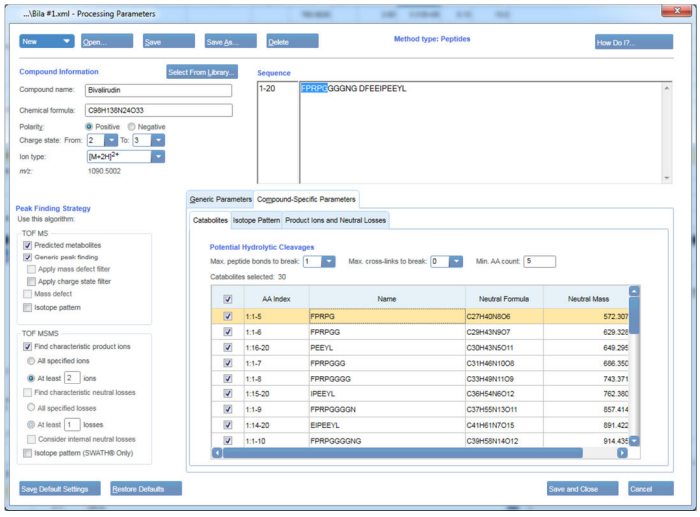

Figure 3A. The processing parameters used for Bivalirudin peak finding showing a portion of the catabolite list generated from sequential hydrolytic cleavage of the N and C terminus.

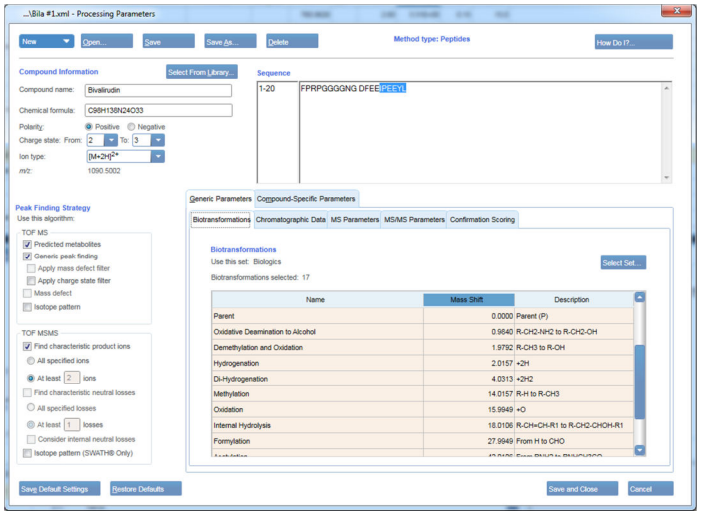

Figure 3B. The processing parameters used for Bivalirudin peak finding showing a portion of the biotransfom list used in peak finding. The biotransformation list is customizable and allows deletions, additions and the creation of new biotransformations.

Results

A total of 61 potential metabolites were found within ±5.0 ppm and in the retention time window 0.5 to 6.0 minutes after 24 hours incubation. The catabolite with the largest peak area was the result of proteolytic cleavage at residues D-F (11,12) and has the sequence FEEI PEEYL which was assigned automatically. The retention time of the catabolite was 2.94 minutes and the retention time of the parent was 3.05 minutes, Figure 4 shows the XIC MS and MS/MS spectra of this catabolite. After identification of the catabolite in the results workspace further confirmation was performed using MS/MS in the interpretation workspace (Figure 5). For this doubly charged catabolite 10 singly charged product ions were assigned to y or b ions within ±4.0 ppm and this resulted in sequence coverage of 5 of 9 consecutive amino acids.

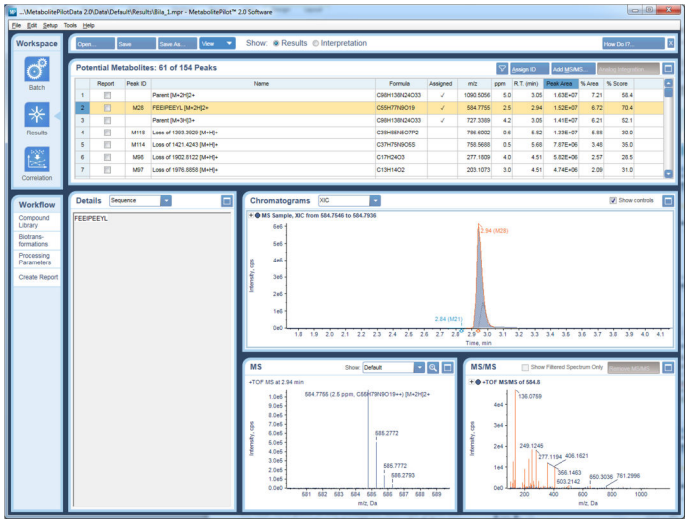

Figure 4. The results workspace of MetabolitePilot 2.0 software displaying results from the incubation of Bivalirudin with rat plasma after 24 hours. The major metabolite was found to be a result of cleavage at residues D-F (11,12) and has the sequence DFEEI PEEYL.

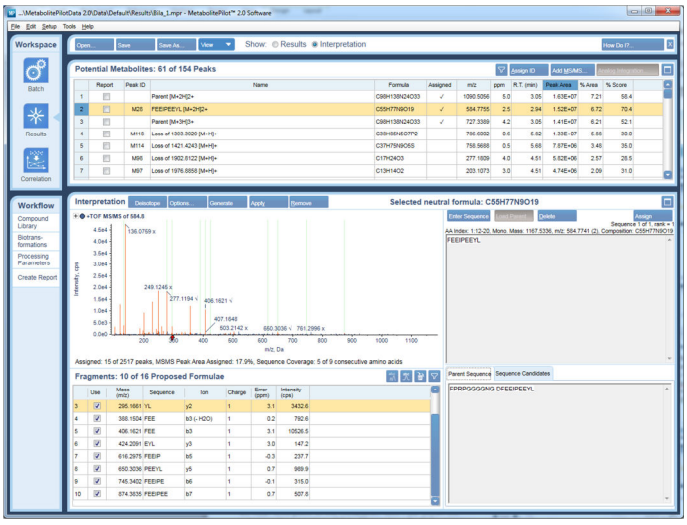

Figure 5. The interpret results workspace of MetabolitePilot 2.0 software displaying results of MS/MS annotation of the major metabolite DFEEI PEEYL. A total of 10 fragments peaks were assigned to b or y ions covering 5 of the 9 amino acids in the sequence.

The next two major metabolites found were methylation of the parent and methylation of the DFEEI PEEYL catabolite. These were found at retention times of 3.11 and 3.17 minutes respectively and both elute after the parent. There are 11 potential methylation sites on Bivalirudin including the N and C terminal. By matching predicted fragments of each of the putative methylation sequences to fragments in TOF MS/MS spectra of the [M+3H]3+ precursor ion, and scoring the matches, the site of modification can be proposed with confidence. MetabolitePilot assigned the most probable site of methylation to glutamic acid 14 which was supported by 22 peptide fragments which covered 10 of 20 consecutive amino acids (Figure 6). Other proteolytic cleavages were observed at residues P-R (2,3) yielding peptide RPG GGGNG DFEEI PEEYL found at retention time 2.90 minutes and P-E (16,17) yielding peptide FPRPG GGGNG DFEEI P found at 3.57 minutes.

The correlation workspace of MetabolitePilot™ 2.0 software was next used to confirm the presence of the major identified metabolites across the assay time points. The correlation workspace has an interactive graph window where results can be displayed as an x, y plot, bar graph or table. In addition to the correlation workspace overlaid XIC's, TOF MS and TOF MS/MS spectra are also displayed for the chosen metabolites/catabolites. Figure 7 displays the correlation plot of the parent, and two major metabolites found in the 5 and 24 hour samples but not in the 1 hour time point.

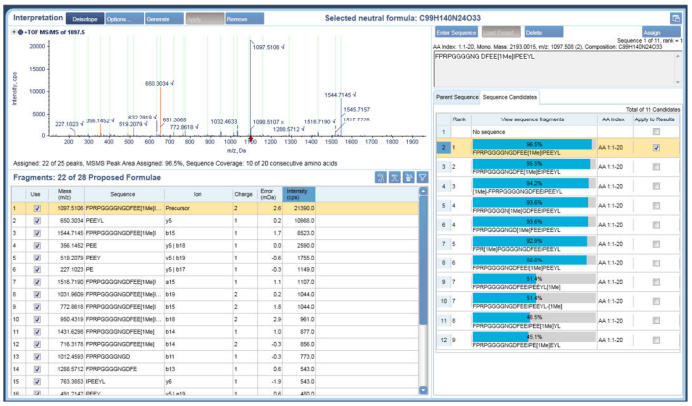

Figure 6. The interpret results workspace of MetabolitePilot 2.0 software displaying results of MS/MS annotation of the methylated metabolite FPRPG GGGNG DFEE[1Me]I PEEYL. A ranking of other proposed catabolites is shown in histogram format below. A total of 22 fragments peaks were assigned to b or y ions covering 10 of the 20 amino acids in the sequence.

Figure 7. The correlation workspace of MetabolitePilot 2.0 software displaying results from the incubation of Bivalirudin with rat plasma after 1, 5 and 24 hours showing the disappearance of the parent and formation of the two major maetabolies.

Conclusions

The TripleTOF™ 6600 system with new MetabolitePilot™ 2.0 software effectively addresses the complex task of performing metabolite and catabolite ID on therapeutic peptides. MetabolitePilot™ 2.0 software efficiently finds metabolites using powerful multiple peak finding strategies applied in parallel, such as non-targeted generic peak finding and targeted searching of hydrolytic cleavage catabolites and biotransformations. MetabolitePilot™ 2.0 software automatically performs catabolite sequence generation using integrated MS/MS fragment interpretation and assignment, all in a single user friendly workspace.

References

Software-Aided Workflow for Investigation of Catabolic Degradation Products During Peptide Therapeutics Compound Optimization. **Eva Duchoslav; Mark Woodward, Jinal Patel; Suma Ramagiri**. ASMS *2015 Poster Presentation.* https://sciex.com/Documents/posters/asms2015_226phrama_Duchoslav.pdf

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