Metabolite Profiling and ID of Cyclic Peptides Using Automated Software Processing and High Resolution Mass Spectrometry

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ABSTRACT

In recent years, peptide and protein drugs have evolved into mainstream therapeutics and are representing a significant portion of the pharmaceutical market. Understanding the metabolism of synthetically or biotechnologically- derived peptides is critical for drug development as metabolism plays an important role in determining the efficacy and safety of the drug. In this paper, we present a Routine Biotransform Solution to conduct metabolite profiling of peptides with varying complexity of cyclization. The solution featured data acquisition by X500R QTOF systems and qualitative analysis with MetabolitePilot™ software to yield major metabolic products for somatostatin and insulin while showing drug stability for daptomycin.

INTRODUCTION

The use of peptide molecules as therapeutic drugs is attractive due to their high selectivity and efficacy. Proteolysis is a major elimination pathway for most therapeutic peptides by proteases that are distributed throughout the body; unmodified peptides typically have very short half-lives. 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 or modification and cyclization. Like small molecule drug development, there is a need to understand the bio-transformations of the enhanced therapeutic peptides to ensure that the molecule is safe and efficacious. The ability to find, identify and confirm catabolites as quickly as possible is critical at multiple stages of drug discovery and development. However, unlike small molecule MetID study, the number of potential catabolites to be considered for therapeutic peptides can be considerably larger. Additionally, the parent molecule and its potential catabolites can form ions with multiple charge state, therefore, increasing the complexity of a MetID study for therapeutic peptides.

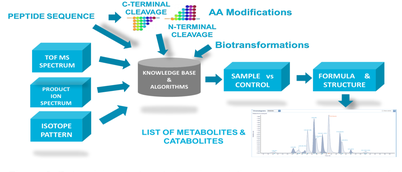

Figure 1: Data mining strategy to find degradation and catabolism products of therapeutic peptides

MATERIALS AND METHODS

Sample Incubations:
Somatostatin, daptomycin and insulin analog were purchased from Sigma-Aldrich. Peptides were prepared in water at 1 mg/mL. The starting incubation volume was 500 µL and contained 50 µg/mL peptide in either rat whole plasma or liver S9 fraction. Somatostatin and daptomycin were incubated in plasma and 50 µL aliquots was taken at 0, 5, 15, 30 and 60 mins and 0, 0.5, 1, and 5 hrs time points, for respective peptides. Insulin was incubated in liver S9 fraction and 50 µL aliquots were taken at 0, 15, 30, 60 and 120 mins . Each aliquot was precipitated with 100 µL of acetonitrile:methanol:0.1% formic acid. The mixture was vortexed for 1 min, centrifuged at maximum *g* for 10 minutes then 40 µL of supernatant was removed and diluted to 160 µL with water.

Chromatography:
Samples were chromatographed on a SCIEX ExionLC™ AD and Agilent Integrated system using a Phenomenex Kinetex C18 column (2.0 x 50 mm), 2.6 µm. Elution was performed using a 19 minutes cycle for Somatostatin and Daptomycin on Shimadzu and a 24 minutes cycle for insulin. The short cycle was a linear gradient from 10% to 60% B over 9 mins, then to 95% B at 11 min, held at 95% B until 13 min and returned to 5% B at 14 min. The long cycle was a linear gradient from 5% to 50% B over 13.5 mins, then to 95% B at 17 min, held at 95% B until 19 min and returned to 5% B at 21 min. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The flow rate was 400 µL/min, the column was kept at 40ºC and 10 µL was injected for daptomycin and 5 µL was injected for somatostatin and insulin .

MS/MS Conditions:
Data was collected on a SCIEX X500R QTOF System with SCIEX OS 1.0 and 1.3. TOF MS acquisition covered *m/z* 100 to 1700 with 100 ms accumulation time for somatostatin and daptomycin and *m/z* 200 to 2000 with 200 ms accumulation time for insulin. IDA data were acquired by the peptide workflow using the charge state filter with dynamic background subtraction and intensity threshold was 500 cps for somatostatin and daptomycin and 100 cps for insulin. Data independent acquisition was performed using SWATH® Acquisition with 23 variable width SWATH ® Acquisition windows for somatostatin and 30 fixed 50 Da SWATH ® Acquisition windows for insulin. The MSMS scan from each SWATH ® Acquisition covered *m/z* 50 to 1700 with a 30 ms accumulation time for somatostatin and *m/z* 200 to 2000 with a 25 ms accumulation time for insulin.

Data Processing:
Data was processed in MetabolitePilot™ 2.0.4 software using the installed biologics biotransformation list. Peptide-targeted catabolite finding approach was opted where the peptide sequence, its hypothetical c-terminal and n-terminal hydrolytic cleavages and biotransformations were used to create a knowledge base to mine the TOF MS by using the predicted metabolite finding algorithm and characteristic product ions of the peptide sequence were also used to mine the TOF MSMS to find metabolites (Figure 1). Peptide information is entered into MetabolitePilot software using single letter codes to represent each standard amino acid, defined symbol for custom amino acid and links, [*#] to represent intra- and inter-chain cyclization bridges. An example of the processing workspace for daptomycin is shown in Figure 2.

For somatostatin, a charge range from +1 to +4 was set and the XIC threshold for MS and MS/MS XIC was set to 5000 and 1000 cps, respectively. The XIC threshold for MS was lowered to 1000 cps for 60 minute sample. For daptomycin, a charge range from +1 to +3 was set, the XIC threshold for MS and MS/MS XIC was set to 5000 and 2000 cps, respectively. For insulin, a charge range from +3 to +6 was set and the XIC threshold for MS and MS/MS XIC was set to 1000 and 500 cps respectively. A sample to control ratio of >3 for plasma matrix and >2 for S9 matrix was used. The MS accuracy was set to 10 ppm for somatostatin and daptomycin and 15 mDa for insulin.

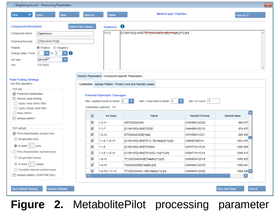

Figure 2. MetabolitePilot processing parameter workspace for the peptide, daptomycin. The sequence is entered using the single letter code for standard amino acids and defined symbols, O and Ky, for O-benzyltyrosine and kynurenine amino acids. The cyclization of C-terminal of the peptide with the R-group of threonine is indicated with the red line. A medium-chain fatty acid group, decanoic acid, is added as N-terminal modification indicated by [C10H19O]-.

RESULTS

For each results table, the potential metabolites peaks were sorted based on retention time (R.T.) and neutral mass. The results with the highest mass were filtered for, using the Report checkbox, then sorted based on MS peak area to identify the top metabolites formed at each unique retention time.

MetabolitePilot software considers the isotopic distribution of the predicted catabolites and uses the most intense isotope during MS peak finding, not just the monoisotopic peak. For larger peptides, this results in more sensitive peak detection of minor metabolite such as 2660.20 Da at 7.48 mins, as shown in Figure 3. Major metabolites of insulin are resulting from cleavages along Chain B and are shown in Table 2. The MS ions of these metabolites and the parent drug account for ~92% of total intensity of the metabolite chromatogram. Table 1 shows the major metabolites found for somatostatin in the 15 minute incubation sample and with the inclusion of parent MS peaks accounts for ~65% of total intensity of the metabolite chromatogram. Each prominent MS peak was confirmed by MSMS fragment ion interpretation in order to be considered a feasible metabolite. The MSMS annotation of daptomycin is shown in Figure 6.
Any co-eluting MS peaks not considered as metabolites were excluded from the result to facilitate timepoints data correlation. The correlation workspace was used to compare and confirm metabolites across multiple incubation timepoints using the reviewed results tables. Figure 4 shows the metabolite profile of prominent somatostatin incubation in plasma and Figure 5 shows the stability profile of daptomycin incubation in plasma.

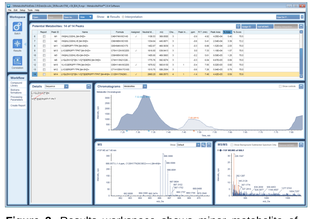

Figure 3. Results workspace shows minor metabolite of insulin identified from S9 incubation of 60 minute.

Figure 4. Correlation workspace shows metabolite profile of somatostatin in plasma incubation from 0 to 60 minutes.

Table 1. A summary of major catabolites for somatostatin observed from plasma incubation of 15 minutes

Sequence	Neutral Mass	Ion m/z, charge	R.T. (min)	Peak Area (%)
FWK	479.25	480.2605 (+1), 240.6340 (+2)	4.06	2
GC[*1]KNFFWK / FTSC[*1]	1482.64	371.6685 (+4), 495.2224 (+3)	4.95	2.2
AGC[*1]KNFFWK / TFTSC[*1]	1654.73	414.6897 (+4), 552.5839 (+3), 828.3725 (+2)	5.09	56.3

Table 2. A summary of top 5 catabolites for insulin observed from s9 incubation for 60 minutes

Sequence	Neutral Mass	Ion m/z, charge	R.T. (min)	Peak Area (%)
FVNQHLCGSHL	1253.60	418.8728 (+3)	5.94	27.4
FVNQHLCGSHLVE	1481.71	494.9100 (+3)	6.28	19.3
FVNQHLCGSHLVEA	1552.74	518.5889 (+3)	6.44	8.4
VNQHLCGSHL	1106.52	445.8871 (+3)	4.92	5.4
VNQHLCGSHLVE	1334.64	468.5639 (+3)	5.41	3.4

Footnotes:
Figure 3: * Peak Index of 1 indicates that the first isotope peak after the monoisotopic peak was most abundant and used to find the catabolite
Figure 4: The results of 5 minute timepoint were excluded due to lack of consistent metabolite identification
Table 1 & 2: The [*1] indicates the intra- or inter-linked cysteine residues on the catabolite

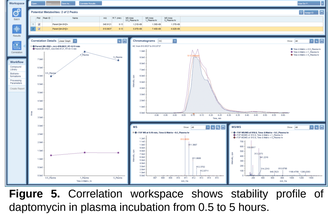

Figure 5. Correlation workspace shows stability profile of daptomycin in plasma incubation from 0.5 to 5 hours.

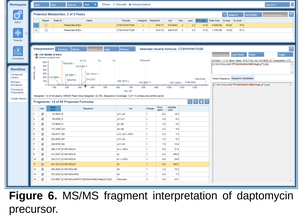

Figure 6. MS/MS fragment interpretation of daptomycin precursor.

The most common problem of identification in high resolution mass spectrometry of peptide data is in identification of isobaric compounds. The interpretation workspace of MetabolitePilot™ in Figure 7 and 8 shows each proposed isobar as a line in the sequence candidates table for a metabolite of somatostatin and insulin. The evidence for each proposal is represented as percentage of total monoisotopic ion count annotated by comparing the MSMS fragment peaks with theoretical CID mass fingerprint within 5 ppm mass tolerance. The top sequence from the assignment algorithm is shown as rank 1. An identical proposal is achieved for somatostatin catabolite, 1654.73 Da, at 5.07min by MSMS annotation of both IDA and SWATH® Acquisition as shown in Figure 8.

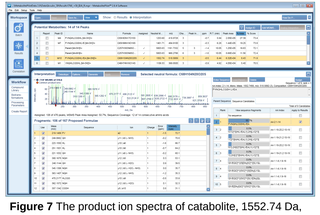

Figure 7 The product ion spectra of catabolite, 1552.74 Da, at 6.44 min from 60 minute incubation of insulin in S9 fraction.

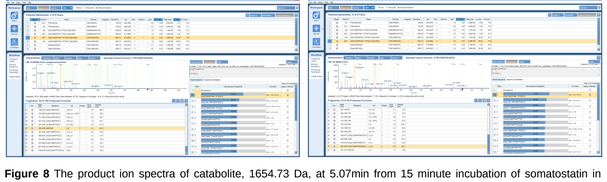

Figure 8 The product ion spectra of catabolite, 1654.73 Da, at 5.07min from 15 minute incubation of somatostatin in plasma, acquired by SWATH® (left) and IDA (right) acquisition. There are 20 possibilities presented as a line in the sequence candidate table. The most-confident MSMS spectral annotation is identical for IDA and SWATH spectra.

CONCLUSIONS

Metabolite profiling for three cyclic peptides was facilitated by detection of metabolite products for somatostatin and insulin. Daptomycin incubation profile exhibits stability of the peptide in plasma over a 5 hour period. Analysis of plasma incubations of somatostatin and daptomycin and S9 incubation of insulin have shown that the X500R QTOF system combined with MetabolitePilot™ software is a powerful solution for catabolism analysis of cyclic peptides. Post-acquisition data processing is enhanced by the automatic sequence proposal feature and integrated MS/MS fragment interpretation tool that performs fragment assignments to easily differentiate isobaric matches.

REFERENCES

- Application Note (2017) - RUO-MKT-02-6114
- T. Uhlig, et al., The emergence of peptides in the pharmaceutical business: from exploration to exploitation, *EuPA Open Proteomics*, 4 (2014), pp. 58-69

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