Rapid Peptide Catabolite ID using the Routine Biotransform Solution

Rapidly Identify Major Catabolites with SCIEX X500R QTOF System and MetabolitePilot™ Software 2.0

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Proteolysis is a major elimination pathway for most therapeutic peptides. Peptide and protein drugs are acted upon by serum and tissue proteases that are distributed throughout the body and 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.

In this work, the Routine Biotransform Solution featuring the X500R QTOF System with MetabolitePilot software 2.0 was employed to study the catabolism of three peptides after incubation with rat blood and plasma. Data was acquired with both data dependent (IDA) and data independent strategies

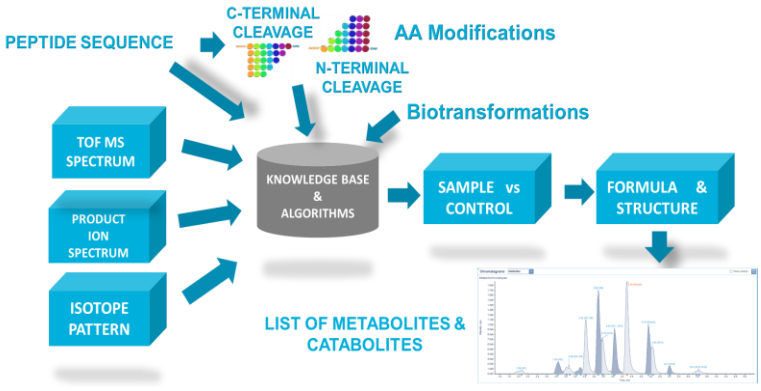

Figure 1. Data Mining Strategy to find Degradation and Catabolism Products of Therapeutic Peptides. Therapeutic peptide sequence, its MS/MS experimental or predicted spectrum and a list of potential amino acid modifications are used as an input for series of accurate mass LC-MS peak finding strategies that find peptide related material in either IDA or SWATH® Acquisition data.

(SWATH® Acquisition) and to increase sample throughput a short 50 mm column and a 5 minute gradient were used.

Figure 1 shows an overview of the data processing workflow for the peptide catabolism workflow in MetabolitePilot 2.0 software.

Key Features of the X500R System with MetabolitePilot™ Software for Peptide Catabolism

- Compact benchtop X500R QTOF system with flexible data acquisition strategies
  - Data dependent acquisition with real-time multiple mass defect filtering (MDF)
  - SWATH® Acquisition for comprehensive sample analysis
  - A dedicated peptide processing workflow with MetabolitePilot Software 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
  - 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

Experimental

Samples: Renin substrate tetradecapeptide (porcine) DRVYIHPFHLLVYS, calcitonin gene related peptide (human) ACDTATCVTHRLAGLLSRSGGVVKNNFVPTN VGSKAF (with C-terminal amidation and disulphide bridge between the cysteine residues) and parathyroid hormone (1-34, Human) SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF were purchased from Sigma-Aldrich.

Sample Preparation Incubations: 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 blood or plasma. At 10, 30 and 60 mins 50 µL aliquots were taken and 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.

Mass Spectrometry: Data was collected on a SCIEX X500R QTOF System with SCIEX OS Software 1.2. TOF MS acquisition covered *m/z* 200 to 2000, with 100 msec accumulation time. IDA data were acquired using the peptide workflow using the charge state filter with dynamic background subtraction and intensity threshold 200 cps. Data independent acquisition was performed using SWATH® Acquisition with 22 fixed 50 Da Q1 windows. The MS/MS scan from each SWATH Acquisition windows covered m/z 200 to 2000 with a 25 msec accumulation time. Total cycle time for the method was 705 msec.

Chromatography: Samples were chromatographed on a SCIEX ExionLC™ AD system using a Phenomenex Kinetex C18 column (2.0 x 50 mm), 2.6 µm. Elution was performed using a linear gradient from 5% to 40% B over 4 mins, then to 95% B at 4.5 min and held at 95% B until 5.25 min. The column was returned to 5% B at 5.4 min. Total cycle time was 7 minutes. 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 40 °C and 10 µL was injected.

Data Processing: Data was processed in MetabolitePilot™ Software 2.0.2 using the installed biologics biotransformation list. A sample to control ratio of >3 was used. TOF MS was used to find metabolites using both the predicted and generic peak finding (charge state filter on) algorithms. Characteristic product ions were also used to mine TOF MS/MS to find metabolites. Peptide information is entered into MetabolitePilot software using single letter codes to represent each amino acid and an example of the processing workspace for cGRP is shown in Figure 2.

For renin substrate tetradecapeptide, a charge state range from +2 to +5 was set and the XIC threshold for MS and MS/MS XIC

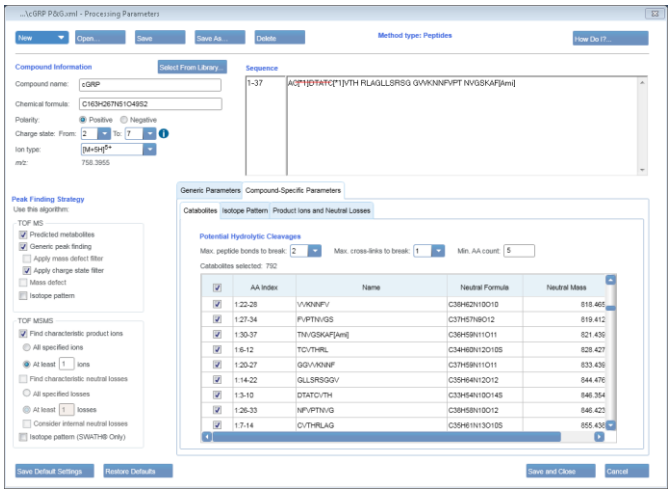

Figure 2. MetabolitePilot Software Processing Parameters Workspace for the Peptide cGRP. The peptide sequence is entered using the single letter amino acid codes and the intra-chain disulfide bond is indicated with the red line. Predicted peak finding of cleavage catabolites with 5 or more amino acids was selected.

was set to 8500 and 500 cps respectively. For calcitonin gene related peptide, a charge state range from +2 to +7 was set and the XIC threshold for MS and MS/MS XIC was set to 6000 and 300 cps respectively. For parathyroid hormone, a charge state range from +2 to +6 was set and the XIC threshold for MS and MS/MS XIC was set to 5000 and 2000 cps respectively.

High Quality LC-MS Data with SWATH® Acquisition

Each peptide eluted between 3.2 and 3.5 minutes. An example chromatogram from the SWATH acquisition data from the 10 min water incubation controls is shown in Figure 3. Each peak has a width of approximately 6 seconds, and the SWATH acquisition approach allowed the collection of at least 7 data points across each peak.

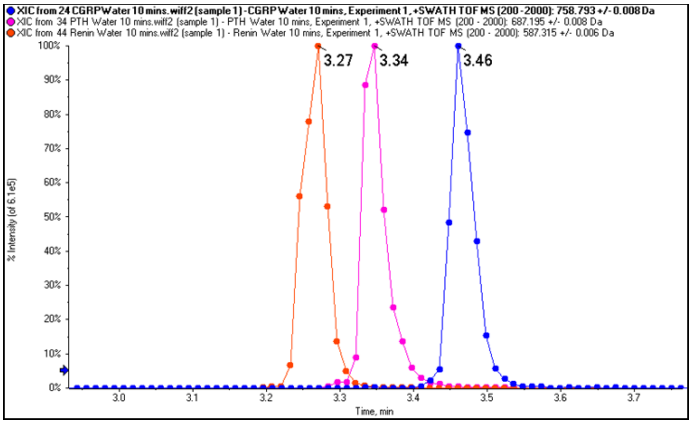

Figure 3. LC-MS Chromatogram from the SWATH Acquisition Data. Control samples from the 10 min water incubation experiment, showing the 3 peptides, renin substrate tetradecapeptide (orange), parathyroid hormone (pink) and calcitonin gene related peptide (blue).

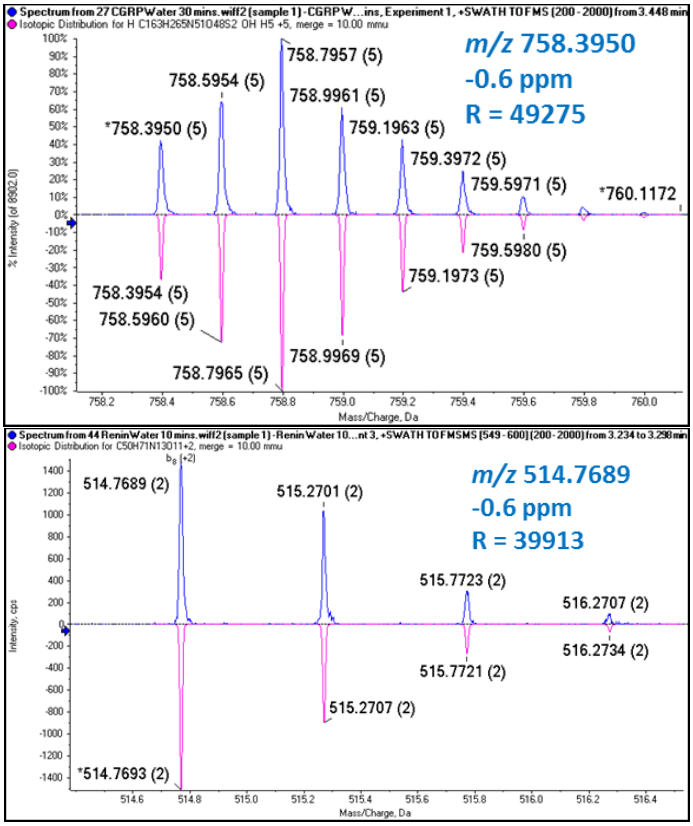

Figure 4. Mass accuracy and resolution in TOF MS and TOF MS/MS. The top panel shows the MS scan of the +5 charge state of cGRP (blue trace) with the ppm error and resolution for the monoisotopic ion and a near perfect match of the theoretical isotope pattern (pink). The product ion spectrum of the b_8 product ion of renin substrate in the bottom panel with the ppm error and resolution for monoisotopic ion mass fragment and a near perfect match of the theoretical isotope pattern (pink). The excellent mass accuracy and > 30K resolution in both MS and MSMS allow easy assignment of elemental composition, even to the product ions.

Excellent mass accuracy and resolution were achieved in both MS and MS/MS scans (Figure 4). The high mass accuracy obtained in MS/MS scans greatly facilitates structure elucidation because it allows unambiguous assignment of elemental composition.

Peptide Catabolism Processing in MetabolitePilot™ 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 ions 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 of catabolites. MetabolitePilot Software 2.0 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 spectrum. Finally, the integrated correlation function allows comparison of catabolism across multiple samples for time course studies or inter-species comparison, using both MS and analog data.

Data were processed with the auto-assign option turned on and using either plasma or blood blanks as controls and each sample took between 2 to 4 minutes to process. Each results table was then sorted based on MS peak area to identify the top metabolites formed at each unique retention time. For co-eluting MS peaks, the results with the highest mass were considered as the metabolite. The auto-assign function of MetabolitePilot software will generate sequence proposals for catabolic products arising from one or more hydrolytic cleavages, one biotransformation and a combination of one biotransformation with one or more hydrolytic cleavages. This feature helps to speed metabolite assignment because the user only needs to review and confirm the proposed metabolite sequence using the MS/MS interpretation tool. An example of the interpretation workspace is shown in Figure 5 for the major cleavage metabolite of renin substrate tetradecapeptide. There are several possible identities of the metabolite and each proposed candidate is represented with a line in the sequence candidates tab. The top sequence from the assignment algorithm is shown as rank 1 and the other possibilities are shown below.

MetabolitePilot Software takes into account the isotopic distribution of the predicted catabolites and uses the most intense isotope for peak finding, not just the monoisotopic peak and for larger catabolites this results in more sensitive peak detection. The top 3 catabolites found for each compound after 30 minutes of incubation are shown in Table 1. For renin, the catabolites involved C-terminal cleavage at tyrosine-13 and serine-14 and N-terminal cleavage at valine-13 and arginine-2. Catabolites of parathyroid hormone involved N-terminal cleavages at histidine-14 and leucine-28 and C-terminal cleavage at lysine-13. Cleavages at N-terminal of glycine-14 and serine-19 and C-terminal of arginine-18 and glycine-33 results in catabolites for calcitonin gene related peptide. The same catabolites were found using both IDA and SWATH Acquisition.

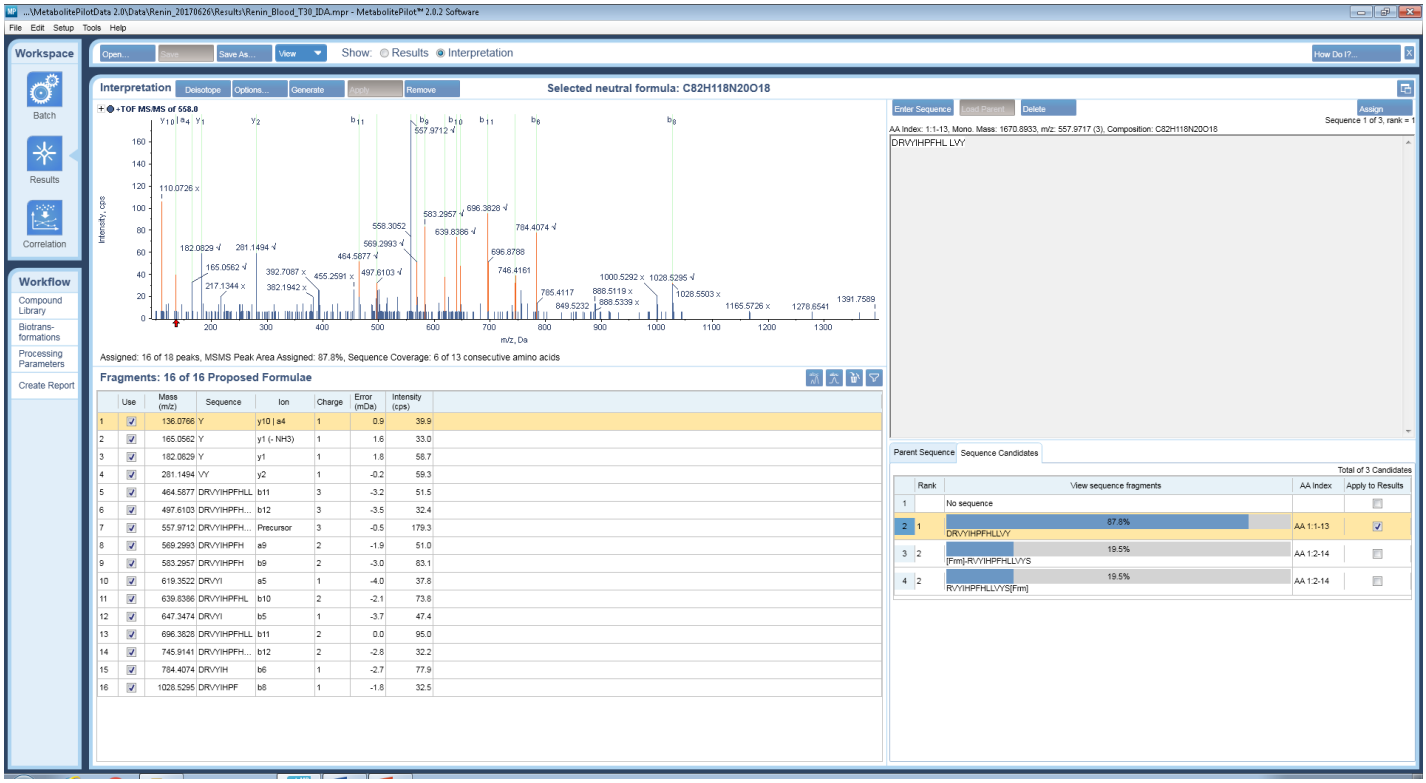

Figure 5. Interpretation Workspace of MetabolitePilot Software. IDA data from the 30 minute incubation of renin substrate tetradecapeptide in blood. There are 6 potential metabolites in this sample; the image displays the product ion spectrum of catabolite at 3.33 min with mass of 1670.89 Da. There are 2 possible MS peak identities for this mass; one is a hydrolytic cleavage (1-13) and other is hydrolytic cleavage (2-14) and formylation. There are 2 possible sites of formylation on the 2-14 cleavage. Each isomer is represented as a line in the sequence candidates table and the evidence for each proposal is represented as percentage of total monoisotopic ion count annotated. The MS/MS spectral annotation shows confidence in cleavage, 1-13, as the metabolite identity.

Table 1: Summary of Top 3 Major Metabolites Observed from Blood or Plasma Incubation of 30 Minutes.

Name	Formula	Neutral Mass	m/z (charge)	Peak Index*	R.T. (min)	Incubation Matrix
Renin Substrate Tetradecapeptide						
VYIHPFHLLVYS	$C_{75}H_{106}N_{16}O_{16}$	1486.79	496.6051 (+3)		3.36	Plasma, blood
RVYIHPFHLLVYS	$C_{81}H_{118}N_{20}O_{17}$	1642.89	548.6381 (+3)		3.12	Plasma, blood
DRVYIHPFHLLVY	$C_{82}H_{118}N_{20}O_{18}$	1670.89	557.9700 (+3)		3.34	Plasma, blood
Calcitonin Gene Related Peptide						
SGGVVKNNFVPTNVGSKAF-[Ami]	$C_{86}H_{137}N_{25}O_{25}$	1920.01	641.0112(+3)	1	2.64	Plasma
*AC[*1]DTATC[*1]VTHRLAGLLSR*	$C_{77}H_{132}N_{26}O_{25}S_2$	1884.92	472.2379 (+4), 629.3137 (+3)		3.25	Plasma
GLLSRSGGVVKNNFVPTNVG	$C_{88}H_{147}N_{27}O_{27}$	2014.11	504.5336 (+4), 672.3749 (+3)	1	3.76	Blood
Parathyroid Hormone						
SVSEIQLMHNLGK	$C_{62}H_{106}N_{18}O_{20}S$	1454.75	485.9246 (+3)		2.7	Plasma
HLNSMERVEWLRKKLQDVHNF	$C_{119}H_{187}N_{37}O_{32}S$	2678.38	536.6837 (+5)	1	2.94	Plasma
LQDVHNF	$C_{39}H_{57}N_{11}O_{12}$	871.42	436.7164 (+2)		2.14	Plasma

* Peak Index of 1 indicates that the first isotope peak after the monoisotopic peak was most abundant and used to identify the catabolite

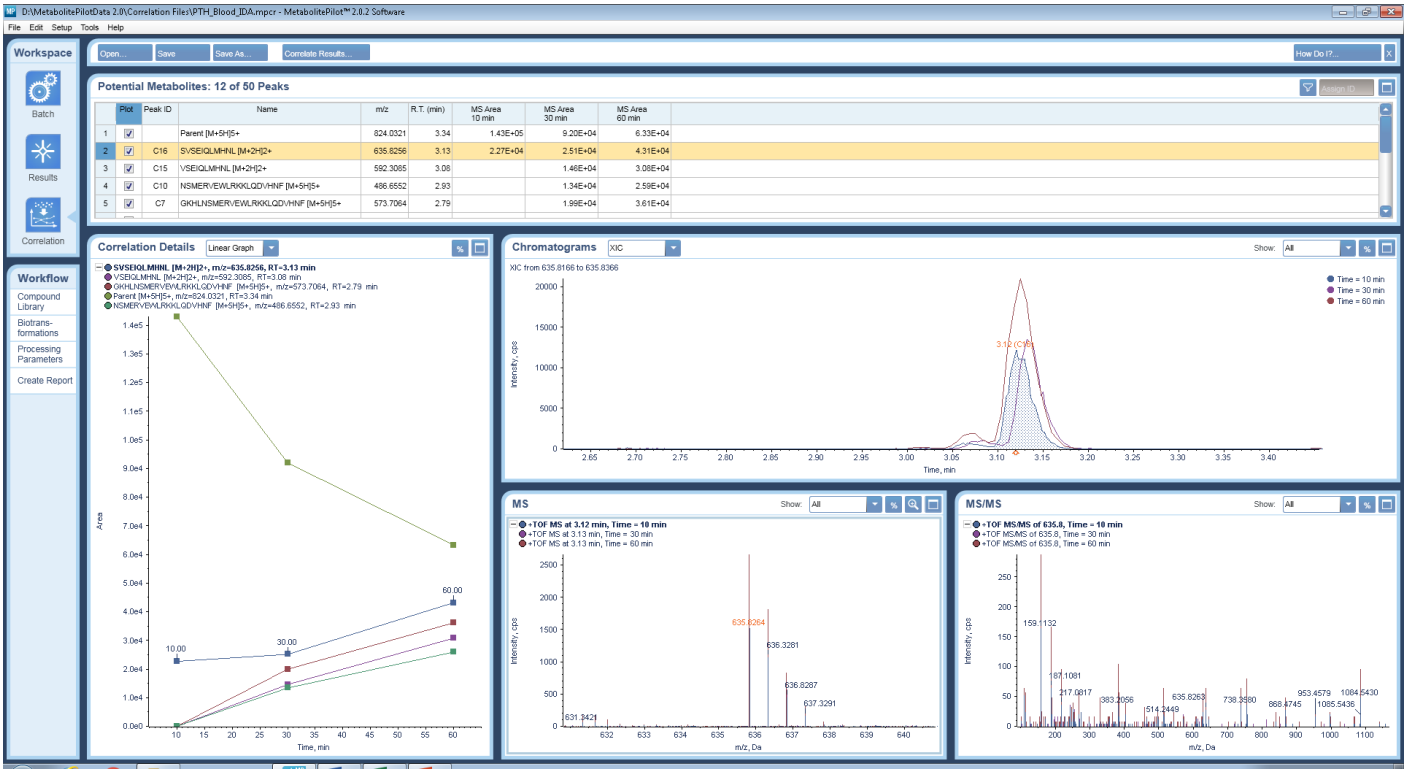

Figure 6. MetabolitePilot Software Correlation Workspace. Correlation plot for the prominent metabolites of parathyroid hormone from 10 to 60 min incubation timepoints. The correlation is shown in a linear plot with overlays of chromatographic. MS and MS/MS data, bar graph and table views are also available in this correlation workspace.

The correlation workspace was used to compare and confirm metabolites across multiple incubation timepoints using the processed results tables. After selecting the samples for correlation, the software automatically tabulates the abundance of each metabolite across all samples and overlays all chromatographic, MS, and MS/MS data. Figure 6 shows an example correlation plot for prominent metabolites of parathyroid hormone from 10 to 60 mins and shows the correlation in a linear plot. A bar graph and table views are also available in the correlation workspace.

Conclusions

Rapid detection and characterization of major metabolites is required for lead optimization in fast paced drug discovery environments. Blood and plasma incubations of renin substrate tetradecapeptide, calcitonin gene related peptide and parathyroid hormone have shown the X500R QTOF system combined with MetabolitePilot Software 2.0 is a powerful solution for rapid catabolism analysis. Data processing is accelerated by the automatic sequence proposal feature and integrated MS/MS fragment interpretation tool that performs fragment assignments all in a single integrated workspace.

Document number: RUO-MKT-02-8529-A

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