Fast and comprehensive detection and characterization of cyclic peptide metabolites using software-aided data processing tools

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Introduction

- Cyclic peptide (CD) has better biological activity compared to their linear due to counterparts the conformational rigidity.
- Resistance to hydrolysis due to the lack of both amino and carboxyl termini.
- Some cyclic peptides can cross the cell membrane
- 9 Cyclic peptide drugs have been approved by FDA and/or EMA from 2006 to 2014

Challenges in identifying cyclic peptide metabolites by LC/MS

- Commonly employed LC/MS data processing tools for finding metabolites of small molecule drugs are not well suited for analysis CP metabolites
- CPs often contain unnatural amino acids and peptide linkages
- CP metabolites are difficult to generate high quality and interpretable fragments due to their unique structures (multiple disulphide linkages)
- The number of theoretical metabolite products of a CP drug is huge, searching for all of predicted metabolites is impractical

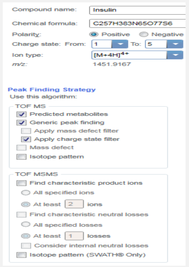
Objective

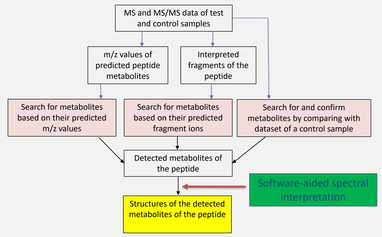
- Apply and test recently expanded tool, MetabolitePilot™ software, for automated detection and structural characterization of cyclic peptide metabolites using insulin as model cyclic peptide

Experimental conditions

- Insulin was incubated with Trypsin and Chymotrypsin in buffer for 0 to 3h
- Chromatography separation was performed on a Waters Acquity BEH C18 column (1.7 um,2.1 x 100 mm) using mobile phases 0.1% formic acid in water and 0.1% formic acid in acetonitrile.
- High-resolution mass spectrometry data were obtained using the TripleTOF® 5600 system (SCIEX) with IDA acquisitions (CE of 50.0).IDA for MS/MS only include the charge state from 2 to 5, and up to 20 MS/MS spectra with dynamic exclusion.
- The data were analyzed with MetabolitePilot™ software 2.0 based on an insulin-tailored processing method using the TOF-MS predicted metabolite peak finder, multiple- charge filter and TOF-MSMS characteristic product ions peak finder.

Workflow for detecting and identifying metabolites of cyclic peptides using MetabolitePilot™ (Sciex)

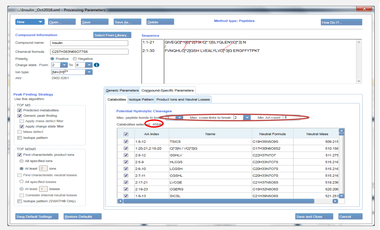




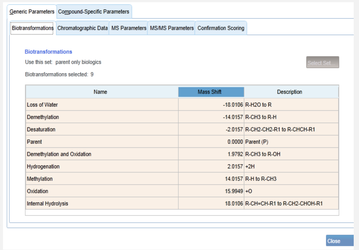
Peak finding strategy from MS datasets

- Predicted metabolites strategy finds peaks in accurate mass XICs for parent compound and predicted hydrolytic cleavage products with or without additional biotransformation.
- Generic peak finder strategy finds 3-dimensional peaks in the LC/MS data, then removes entries that do not have charge state within the user selected range.
- If any control samples are used in processing, peaks that are present in control samples are filtered from the results.
- Accurate m/z and peak charge state are confirmed in the TOF MS spectra.

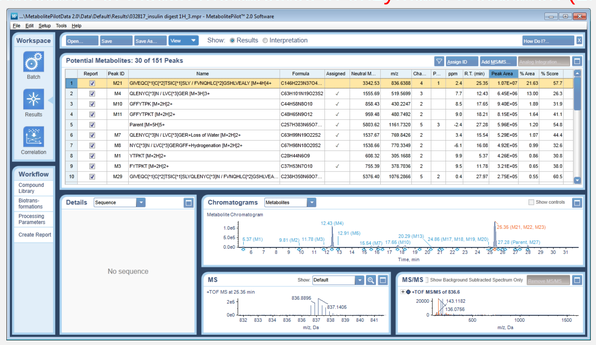
Processing method for searching for peptide metabolites



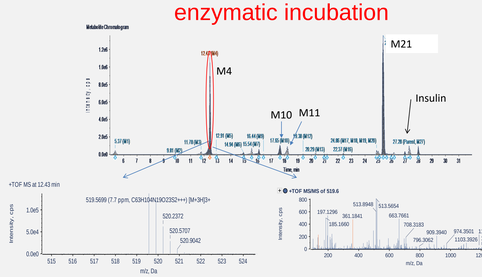
~46000 predicted catabolites

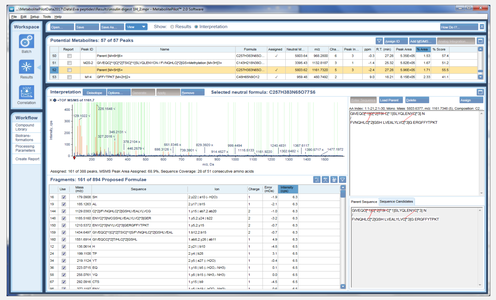


Identification of insulin metabolites in enzymatic incubation (1h)



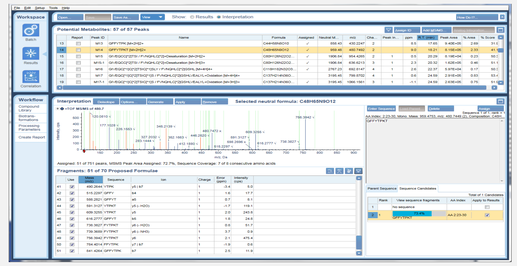
Identification of M4, a major metabolite of Insulin in enzymatic incubation





161 of both singly and multiply charged fragments (~70% TIC) could be associated with insulin sequence. Due to the insulin structure larger fragments had relatively low abundance but they maintained good mass accuracy.

MS/MS Fragment assignment of GFFYTPKT metabolite



51 (~73% TIC) out of 751 peaks with S/N > 5 were assigned as sequence-related fragments.

Summary of insulin metabolites detected and characterized in enzymatic incubation (1 h)

Peak ID	Name	Formula	Neutral Mass	m/z	Charge	R.Time (min)	Peak Area	MS/MS Assigned
M1	YTPKT	C28H49N9O9	608.32	305.1688	2	5.4	4.28E+05	√
M2	QLENYC[*3]N / VC[*3]GER	C57H96N18O25Z	1442.60	722.3065	2	9.8	1.71E+04	
M3	FFTPKT	C39H53N7O10	755.39	378.7036	3	11.8	3.22E+05	√
M4	QLENYC[*3]N / LVC[*3]GER	C63H105N19O35Z	1555.69	519.5699	2	12.4	6.45E+06	√
M5	GIVEQC[*1]C[*2]TSIC[*1]LYQ-Loss of Water	C65H104N16O25Z3	1556.66	779.3393	2	12.9	5.19E+04	
M6	QLENYC[*3]N / YVC[*3]GER	C72H110N20O25Z	1718.75	573.9343	3	14.9	1.62E+05	√
M7 (M4-H2O)	QLENYC[*3]N / LVC[*3]GER-Loss of Water	C63H99N19O22Z	1537.67	769.8426	2	15.5	5.29E+05	√
M8	NYC[*3]N / LVC[*3]GERGFF-Hydrogenation	C67H99N18O25Z	1538.66	770.3349	2	16.1	4.92E+05	√
M9	QLENYC[*3]N / LVC[*3]GERGF	C61H96N18O23Z	1559.77	780.8916	2	16.4	5.28E+05	
M10	GFFYTPK	C46H58N8O10	858.43	430.2247	2	17.7	9.40E+05	√
M11	GFFYTPKT	C50H65N9O12	959.48	480.7482	2	18.2	8.15E+05	√
M12	SLYQLENYC[*3]N / LVLVC[*3]G	C85H127N19O27S2	1909.86	637.6257	3	19.4	2.54E+04	√
M13	QLENYC[*3]N / LVC[*3]GERGFF	C83H122N22O26S2	1906.84	954.4265	2	20.3	8.16E+04	√
M14	GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYC[*3]N-Loss of Water	C90H148N24O31S4	2188.97	730.6457	3	21.0	2.27E+04	√
M15	SLYQLENYC[*3]N / LVLVC[*3]GERGFFY	C125H176N28O38S2	2709.24	678.3180	4	21.2	1.46E+04	√
M16	GIVEQC[*1]C[*2]TSIC[*1]SLY-FVNQHLC[*2]GSHL	C118H162N30O37S4	2767.23	692.8140	4	22.4	5.88E+04	√
M17	GIVEQC[*1]C[*2]TSIC[*1]SL-FVNQHLC[*2]GSHL	C109H173N31O35S4	2604.14	868.0542	3	24.9	2.47E+05	√
M18 (M24+H2O)	GIVEQC[*1]C[*2]TSI-C[*1]SLYQLENYC[*3]N-/ FVNQHLC[*2]GSHLVEALYLVC[*3]GER	C209H322N56O67S6	4880.18	834.3705	6	25.0	2.55E+04	√
M19	GIVEQC[*1]C[*2]TSIC[*1]SLY-FVNQHLC[*2]GSHLVEALYL	C137H216N36O45S4	3179.46	795.8729	4	25.0	1.78E+05	√
M20	GIVEQC[*1]C[*2]TSIC[*1]SLY-FVNQHLC[*2]GSHLVEALY	C123H191N33O39S4	2854.28	952.4339	3	25.1	2.58E+04	
M21	GIVEQC[*1]C[*2]TSIC[*1]SLY-FVNQHLC[*2]GSHLVEALY	C140H223N37O45S4	3342.53	836.6388	4	25.4	1.22E+05	√
M22	GIVEQC[*1]C[*2]TSIC[*1]SLY-[1C*]2GSHLVEALY	C117H184N28O38S4	2717.23	906.7501	3	25.6	2.72E+04	√
M23	GIVEQC[*1]C[*2]TSIC[*1]SLY-FVNQHLC[*2]GSHLVEALY	C140H223N37O45S4	3342.52	836.6369	4	26.0	3.79E+04	√
M24	GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYC[*3]N / FVNHLC[*2]GSHLVEALYLVC[*3]GER	C209H320N56O66S6	4862.17	973.4418	5	26.2	7.62E+04	
M25	CSLYQLENYC[*3]N / GSHLVEALYLVC[*3]GERGFFY	C158H228N38O47S3	3505.58	1169.5354	3	26.2	4.44E+04	
M26	GIVEQC[*1]C[*2]TSIC[*1]SLY-FVNQHLC[*2]GSHLVEALY	C140H223N37O45S4	3342.52	836.6381	4	27.0	1.82E+05	
M27	GIVEQC[*1]C[*2]TSIC[*1]SLY-FVNQHLC[*2]GSHLVEALYL	C152H236N38O46S4	3455.60	864.9078	4	27.4	1.18E+05	
M28	GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYC[*3]N / FVNQHLC[*2]GSHLVEALYLVC[*3]GERGFF	C209H341N59O65S6	5213.33	1043.6723	5	28.0	2.32E+05	
M29	GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYC[*3]N / FVNQHLC[*2]GSHLVEALYLVC[*3]GERGFFY	C238H350N60O77S6	5376.40	1076.2866	5	28.0	2.75E+05	
	Parent [M+5H]5+	C257H383N65O77S6	5803.62	1161.7320	5	27.3	5.96E+05	√

The major enzymatic incubation products have peak areas shaded with dark blue. Details on the disulphide bonds in metabolite sequences are provided with the '[*x]' symbol, chains are separated with '/' symbol.

Major insulin catabolites found in enzymatic incubation (1h)

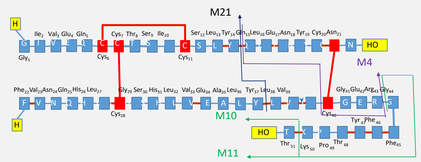
Insulin sequence: 51 AA

Avg. Mol Wt: 5807.7, Accurate mass: 5803.67

Formula: C257H383N65O77S6

Insulin: Sites of Enzymatic Cleavage for Trypsin & Chymotrypsin

Trypsin : C-Term Cleavage at K,R; Chymotrypsin: C-Terminal Cleavage at L,F,Y,W



Major digest products at 60 minutes

- M21 : GIVEQC[*1]C[*2]TSIC[*1]SLY / FVNQHLC[*2]GSHLVEALY (21% total)
- M4: QLENYC[*3]N / LVC[*3]GER (13%)
- M10: GFFYTPK (~2%)
- M11: GFFYTPKT (1.5%)
- Parent (1.2%)

Conclusions

- Detection and structural characterization of CP metabolites in biological matrix represent great analytical challenges
- Recently released MetabolitePilot™ 2.0 software employs multiple search mechanisms and automated peptide spectral interpretation tools
- Results from detection and identification of insulin metabolites formed in incubation with Trypsin and Chymotrypsin demonstrate that the software is capable of rapidly identifying metabolites of cyclic peptides
- the application MetabolitePilot™ software in identifying in-vivo metabolites of CP will be studied further

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