ENHANCED WORKFLOW FOR IDENTIFYING METABOLITES OF LIPID-MODIFIED POLYPEPTIDE THERAPEUTICS USING LC-HRMS

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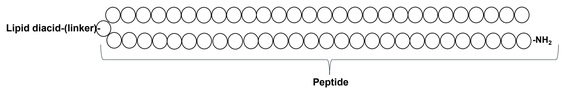
Introduction

The modification of peptide therapeutics through lipidation has gained traction in recent years, showing significant potential to enhance half-life and stability, reduce immunogenicity, and improve membrane permeability for delivery. Several lipid-modified peptide drugs, such as semaglutide and tirzepatide, have received FDA approval [1, 2]. However, challenges arise due to the formation of polar metabolites, matrix interferences, poor ionization, and low abundance, making chromatography for metabolite separation and reliable mass spectral data acquisition difficult during metabolism studies for preclinical and clinical samples [1, 2, 3].

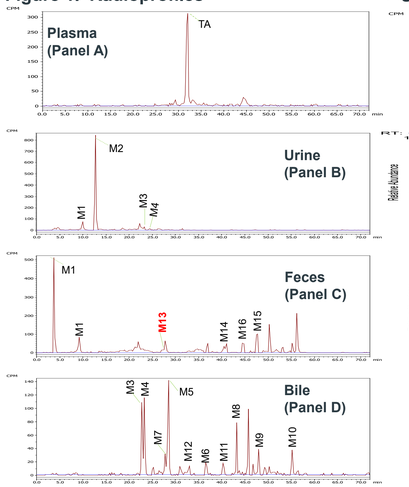


Methods

- A single subcutaneous injection of 0.2 mg/kg (9 µCi/kg) of [^{14}C]TA was administered to bile duct-intact (intact) and bile duct-cannulated (BDC) male Sprague Dawley rats. Plasma, urine, bile, and feces samples were collected and used for radioprofile and metabolite identification.
- Plasma and feces was extracted with mixture of acetonitrile/methanol.
- Bile and urine was centrifuged prior to analysis.
- Radioprofiles were obtained by HPLC separation and offline LSC counting.
- Metabolite identification was achieved using LC-HR-MS and MS/MS.
  LC/MS method A Waters Atlantis Premier BEH C18 AX, 4.6 mm x 150 mm; 2.5 µm) MPA: 0.4% formic acid in water, pH 3.2, MPB: 0.1% FA in acetonitrile. Flow: 0.5 mL/min.
  LC/MS method B Phenomenex Luna Omega, 4.6 mm x 150 mm; 5 µm. MPA: 0.4% formic acid in water, pH3.2, MPB: 0.1% FA in acetonitrile. Flow: 0.5 mL/ min
  LC/MS method C: Same as LC/MS B, except for column size 2.1 mm I.D. x 50 mm, 5 µm. Flow: 0.1 mL/min (micro-flow).
  LC/MS method D: Waters Acquity UPLC Peptide BEH C18, 1 mm x 150 mm; 1.7 µm. MPA: 0.2% formic acid in water, MPB: 0.2% FA in acetonitrile. Flow: 0.1 mL/min (micro-flow).
  LC/MS method E: Thermo PepmapTM Neo C18, 75 µm I.D.x 150 mm; 2 µm, MPA: water, MPB: 80% acetonitrile in water. Flow: 0.3 µL/min (nano-flow).

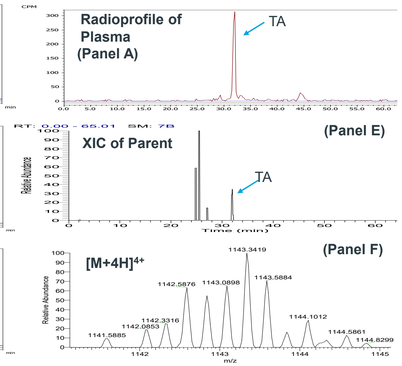
Results

Figure 1. Radioprofiles



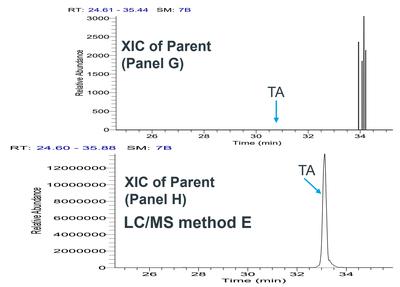
LC/MS A provided good separation of parent from metabolites (Panel A). **LC/MS B** provides good retention of polar metabolites and sufficient separation of both polar and non-polar metabolites in urine, bile and feces. (Panel B-D)

Figure 2. Representative Profiles



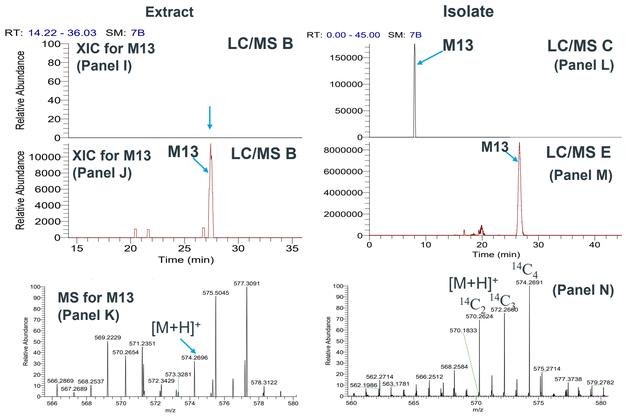
Parent is the most abundant radioactive peak in plasma (Panel A), was separated from metabolites. LC-MS extracted ion chromatogram (XIC) show peak corresponding to radioactive component (Panel E) with MS spectrum expansion of molecular ion shown in Panel F. No MS/MS spectra was acquired during direct analysis using **LC/MS method A**.

Figure 3. Representative Mass Spectral Data



Parent as the most abundant radioactive peak in 24hr plasma, was not detectable by MS during direct analysis (Panel G). Using microflow technique, parent was detected at 1E+7 (Panel H) and expected MS isotopic pattern and MS/MS spectra were acquired.

Figure 4. Representative FT-MS Spectra of Metabolite M13



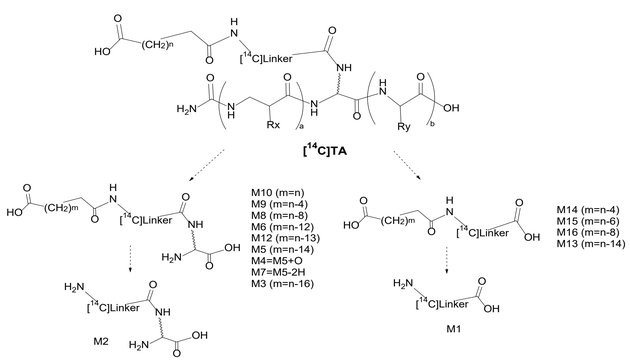
M13 in feces was not detected (Panel I) by MS in direct analysis with split for radioprofile and was detectable without split (Panel J), but at low level without isotopic pattern (Panel K) and MS/MS data.

M13 in isolate from feces was detected by MS in microflow analysis (Panel L) and nanoflow analysis (Panel M), with significant improvement in MS signal (108x), distinguish isotopic pattern (Panel N) and reliable MS/MS spectrum.

Discussion

- Due to the low dose level, ion suppression of interferences in matrices and poor ionization of metabolites, none of the metabolites in plasma, urine, bile and feces can be detected and identified by LC/MS through direct HR-LC/MS analysis.
- Through SPE cleanup and enrichment, only a few metabolites were identified.
- To overcome the challenge, radioactive peaks were isolated through HPLC separation and collection of fractions.
- Microflow and nanoflow LC/MS methods were developed and applied to analyze the fractions collected in plates from radioprofile analysis and preparation HPLC analysis.
- MS sensitivity was significantly improved comparing to traditional LC/MS methods.
- Acquired full scan MS and MS/MS using nanoflow exhibited distinctive $^{14}C_2$:$^{14}C_3$:$^{14}C_4$-isotopic MS ion pattern observed from parent were utilized for metabolite search and identification.

Figure 5. Proposed Metabolites



Conclusion

- Multiple chromatographic methods were utilized to resolve metabolites in radioprofiles for each matrix (plasma, urine, bile and feces).
- Traditional LC-MS columns (4.6 mm id) did not provide MS or MS/MS data for metabolites. Therefore, microflow and nanoflow LC/MS with improved MS sensitivity (10-100x) was used to identify individual metabolites.
- Sixteen metabolites were identified in samples.
  - Parent drug is the predominant circulating component in plasma.
  - Metabolites identified in excreta are predominantly lipid- and linker-related components:
  - Urine, the largest metabolite M2 accounted for 32% and 28% of the dose in intact and BDC rats, respectively.
  - Feces, the largest metabolite M1 accounted for 14% and 2% of the dose in intact and BDC rats, respectively.
  - Bile, nine metabolites were identified, individually accounting for 3-6% of the dose.
- The primary metabolic pathways include proteolytic cleavage of the peptide backbone, following by hydrolysis of the amide bond in the linker region, beta-oxidation and oxidative decarboxylation of the fatty acid, and dehydrogenation

References

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