# Profiling and Identification of Lipid Modified Therapeutic Peptides and Metabolite Using UPLC-HRMS: Challenges and Strategies

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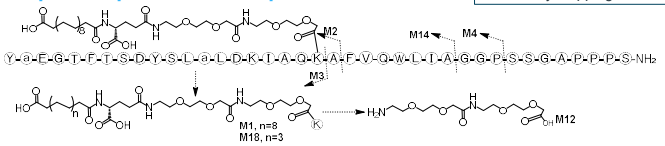
## Introduction

- LC-MS/MS plays a key role in the profiling and identification of therapeutic peptides (TPeps), that eventually contributes to the successful implementation of peptide-based therapies.
- With increased emerging TPeps entering clinical trials, the metabolite identification (MetID) of these TPeps remains challenging [1].
- Here, we present a case study related to an *in vitro/in vivo* metabolism of lipid modified Tpeps.
- Tpeps studied include semaglutide, tirzepatide and other analogues conducted in non-clinical species (rat, dog, monkey, etc.) and humans employ UPLC-HR-MS.
- Here we showcase how advanced sample preparation, chromatographic separation, and bioinformatics tools were integrated to address these challenges.

## Key Challenges in TPeps MetID

- **Structural Complexity:** Amino acid substitutions, sequence variations, post-translational modifications (glycosylation, lipidation), disulfide bonds, and linker modifications alter fragmentation patterns.
- **Low Abundance Metabolites** (often <1% of parent drug) masked by matrix interferences such as endogenous interferences TMAP (N,N,N-Trimethyl-L-alanyl-L-proline betaine) and bile acids, albumin binding etc).
- **Analytical Limitations:** Poor ionization, fragmentation complexity (MS/MS), and lack of reference standards etc.

## Example of Lipid Modified Peptide [2]:




## Metabolite ID Workflow for TPeps

**☐ SAMPLE PREPARATION**
- In vitro/In vivo
- -$^{14}$C/$^{12}$C Co-spiking
- - SPE, PP, LLE/SLE/mixed-mode

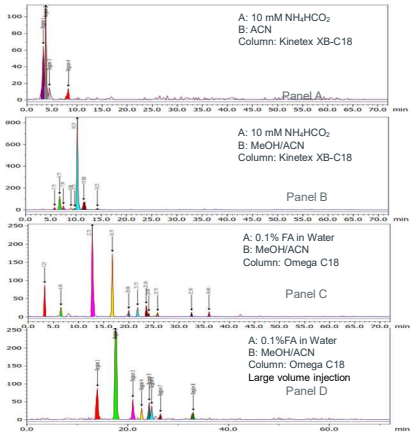
**☐ UPLC SEPARATION**
- RP-C18, HILIC or mixed-mode
- pH-adjusted buffers
- large-volume injection and nano-flow

**☐ HRMS/MS ANALYSIS**
- Q-Exactive Orbitrap
- DDA/DIA
- ESI+/ESI– switching

**☐ METABOLITE CONFIRMATION**
- Co-chromatography of $^{14}$C/$^{12}$C offline Radioprofile
- Bioinformatics tools

**☐ VISUALIZATION & REPORTING**
- Heatmaps
- Annotated M and MS/MS spectra
- Pathway mapping

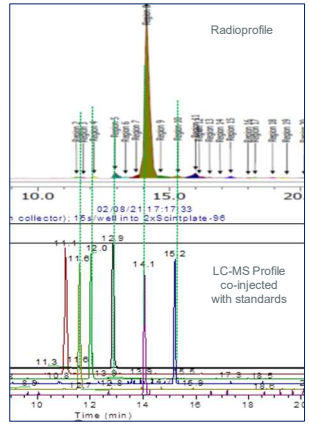
## Chromatographic Optimization




**Radioprofiles of pooled Urine**

Panel A = reverse phase separation with poor retention of radioactive components
Panel B = change strong solvent improved retention of radioactivity
Panel C = change column improved selectivity for separation of radioactive components
Panel D = large volume injection used to load sufficient mass of radioactive components to acquire MS and MS/MS data

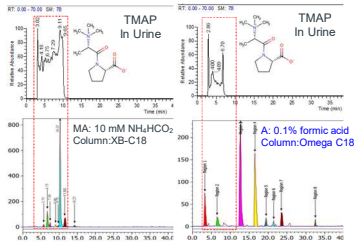
## Co-chromatography




**Radioprofile and LC/MS of pooled plama**

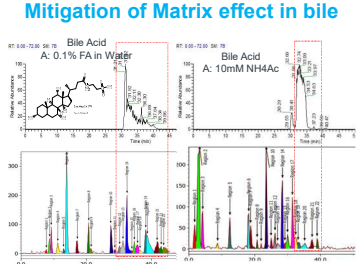
MetID is challenging in studies involving 100% [$^{14}$C]-labeled dosing or low-abundance samples. To aid metabolite identification, $^{12}$C reference standards were co-spiked into plasma/excreta matrices. This strategy enabled differentiation of MS patterns between $^{12}$C/$^{14}$C compounds; retention time alignment between radioprofiles and LC/MS profiles; the unique $^{12}$C/$^{14}$C pattern provided additional confidence in metabolite identification and MS accuracy.

## Mitigation of Matrix effect in urine




**Separation of TMAP from metabolite in urine**

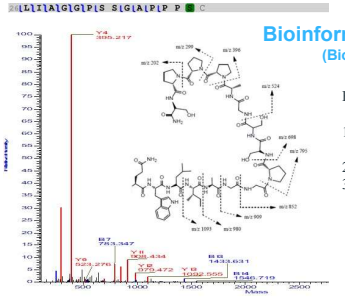
## Mitigation of Matrix effect in bile




**Separation of bile acid from metabolite in bile**

## Bioinformatic tool to assist structure elucidation

**(Biopharma Finder, MMS, Compound Discoverer etc )**



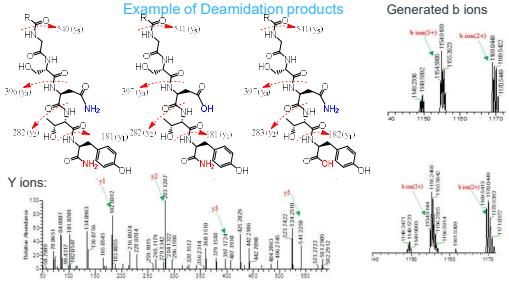
BioPharma Finder used for MetID by providing:

1. Assignment of MS/MS fragment ions associated with peptide backbone moiety of molecule
2. Peptide metabolite sequence coverage
3. Quantitation of peptides by Deconvolution

## Interpretation of MS/MS of metabolites in Plasma

Example of Deamidation products

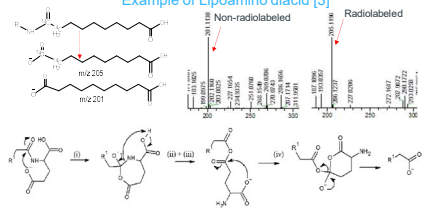
Generated b ions




HRMS combined with sufficient LC separation are critical to identify and quantify peptide metabolites with the higher structural similarity: one or few amino acid difference, or deamidation product with monoisotopic mass output resulted in +1 Dalton mass difference (a 0.20 Da, 0.25Da, 0.33Da mass increase for +5,+4,+3 charged MS or MS/MS fragments); unable to deconvolve separately by BioPharma finder algorithm nor Xtract algorithm without sufficient separation.

## Interpretation of MS/MS of metabolites in Urine

Example of Lipoamino diacid [3]




Proposed stepwise formation mechanism of [lpb+O]$^-$ product ions

Lipid-linker conjugate was detected as one common urinary metabolite of lipid modified peptide. It is interesting to observe that when glutamate included in the linker, a complementary ion pair such as [Lpb + O]$^-$ appeared in negative LC-MS/MS. The particular product ions specific to fatty acyl moiety (carboxylate), annotated as [lpb+O]$^-$ [3].

## Conclusion:

- This integrated UPLC-HRMS workflow enables robust MetID for lipid-modified Tpeps, addressing key challenges through adaptive chromatography, advanced HRMS, and bioinformatic software assisted interpretation of MS, MS/MS data.
- Proteolytic hydrolysis/deamidation are primary metabolic pathway observed in circulation; Additional metabolic pathway in excreta included β-oxidation, oxidation, α-oxidation taurine conjugation etc.

## References

1.He MX, et al, Metabolism and Excretion of Therapeutic Peptides: Current Industry Practices, Perspectives, and Recommendations, 2023 Nov;51(11):1436
2.Martin, JA , et al. Absorption, distribution, metabolism, and excretion of tirzepatide in humans, rats, and monkeys. European J Pharm. Sci. 2024; 202:106895
3.Hueber R. et al. Identification of Bacterial Lipo-Amino Acids: Origin of Regenerated Fatty Acid Carboxylate from Dissociation of Lipo-Glutamate Anion. 2022, Amino Acids, 54:241

## Acknowledgement

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