Peptide Metabolism: Identification of metabolite structures of GLP-1 receptor agonists in different *in vitro* systems using high resolution mass spectrometry.

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Introduction

Peptide drugs are an important class of therapeutics under investigation in various pharmaceutical companies. Assessment of peptide stability in vitro, the identification of cleavage sites and the structural elucidation of degradation products are important tasks of drug metabolism scientists. However, most in vitro systems established to investigate metabolism of small molecules (e.g. microsomes) are not relevant for peptides because most peptides show low cell membrane permeability and are subject to hydrolysis by enzymes expressed on epithelial cell surfaces. In addition to relevant in vitro systems, appropriate mass spectrometry approaches and tailored software tools are required due to the higher molecular weight, presence of multiple-charge stages upon electrospray ionization and increasing molecular complexity (modified amino acids, cyclisation etc.) of peptide drug candidates.

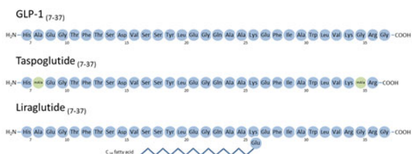
Experimental

Incubations were performed using individually-expressed recombinant enzymes (DPP-IV, neprilysin) or cell cultures of HUVECs (Primary and immortalized), RPTEC-TERT1 and human hepatocytes. The test compounds Glucagon-Like Peptide-1, (GLP-1) and three analogs (taspoglutide, liraglutide, exenatide) were used at a final concentration of 10 µM. Aliquots from each incubation were taken and quenched using cooled acetonitrile. Before injection to the HPLC system the samples were centrifuged and the supernatant was diluted with the same volume of 0.1% formic acid. **Chromatographic separation** of metabolites was performed on a Dionex Ultimate 3000 RS System (ThermoFisher Scientific) using an Acquity CSHT Phenyl-Hexyl Column (1.7 µm, 2.1 x 100 mm). The mobile phases consisted of A: H2O/0.1% formic acid and B: methanol/0.1% formic acid. Start condition was 5 %B at a flow rate of 0.4 mL/min, the LC gradient started at 1 min and was increased at 10 min to 100%. **MS analyses** were run on a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (ThermoFisher Scientific) operated in positive ESI mode. Spray voltage 3.5 kV collision energy 25 V at 10Hz. Mass range 166 – 2500 Da, data dependent mode with inclusion list (generated by Mass MataSite). **Mass-MetaSite** was used in version 3.2.0 and run in peptide mode to detect the peptide related chromatographic peaks and predict the metabolites structures. Multiple charged ions were enabled up to z = 5. The minimum mass of metabolite generation was set to 200 amu with a maximum of two metabolite generations. All results were uploaded and reviewed with **WebMetabase** version 3.1.4 (Lead Molecular Design)

Conclusion

- The peptide mode of Mass-Metasite was successfully applied to process HRMS data to detect and identify metabolites of linear model peptides
- Mass-MetaSite detected and integrated parent related chromatographic peaks and automatically proposed chemical structures of metabolites. Based on these metabolite structures, peptide-bond cleavage sites could be elucidated.
- WebMetabase was able to sort and match metabolites based on retention time and MS² fragmentation across different *in vitro* experiments resulting in an efficient workflow to compare metabolism results from different *in vitro* systems.
- This approach demonstrated to be an effective tool to investigate peptide metabolism in relevant *in vitro* systems.

Substrates

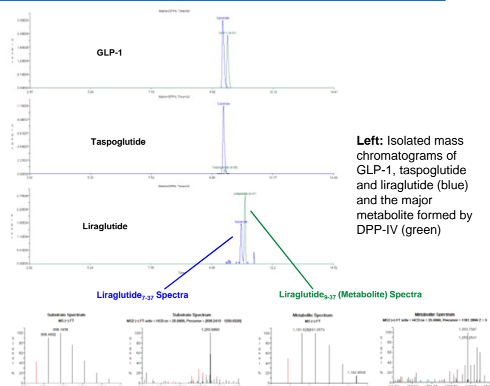


Structures of GLP-1, taspoglutide and liraglutide

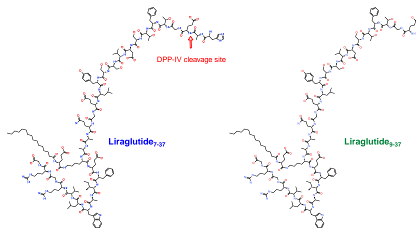
In vitro systems

In vitro systems used for peptide stability/metabolism assessment		
Name	**Abbreviation**	**Comment**
Dipeptidyl peptidase IV	DPP-IV	assay concentration: 1 µM
Neutral endopeptidase	NEP	assay concentration: 1 µM
Cryopreserved human hepatocytes	HH	0.5 * 10⁶ cells per ml
Primary Human umbilical vein endothelial cells	pHUVEC	DPP-IV content: > 0.3 ng/10⁶cells NEP content: > 0.1 ng/10⁶cells
Immortalized Human umbilical vein endothelial cells	iHUVEC	DPP-IV content: > 0.3 ng/10⁶cells NEP content: > 0.1 ng/10⁶cells
TERT1-immortalized renal proximal tubule epithelial cells	RPTEC/TERT1	DPP-IV content: > 1.5 ng/10⁶cells NEP content: > 0.1 ng/10⁶cells

Analysis by Mass-Metasite



Left: Isolated mass chromatograms of GLP-1, taspoglutide and liraglutide (blue) and the major metabolite formed by DPP-IV (green)



Structure display of liraglutide and structure proposal of its metabolite found in DDP-IV incubates by Mass-Metasite

DPP-IV cleavage

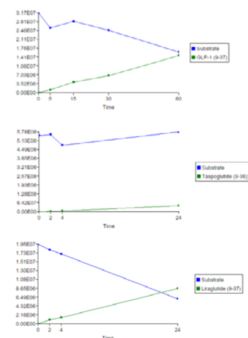
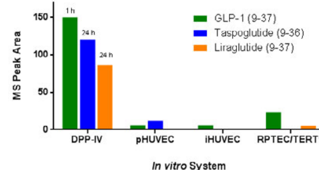


Figure (left): GLP-1 and the analogues were metabolized in the presence of DPP-IV

The main metabolite identified by Mass-MetaSite (< 2 ppm) resulted from N-terminal cleavage after amino acid 8, corresponding to:

$GLP\text{-}1_{9\text{-}37}$ (upper panel) $Taspoglutide_{9\text{-}36}$ (middle panel) and $Liraglutide_{9\text{-}37}$ (lower panel)



Comparing different *in vitro* systems for the formation of DPP-IV metabolites ($GLP\text{-}1_{9\text{-}37}$, $Taspoglutide_{9\text{-}36}$ and $Liraglutide_{9\text{-}37}$)

Peptide Metabolism in different *in vitro* systems Example: Taspoglutide

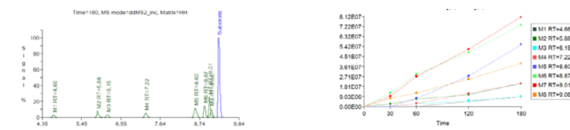


Figure: MS Peak Areas of taspoglutide and its metabolites (right panel) monitored over 3h and isolated mass chromatogram (left panel) in human hepatocytes after 3h of incubation

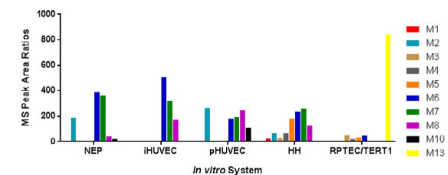


Figure (left): Metabolite appearance of taspoglutide incubated in different *in vitro* systems

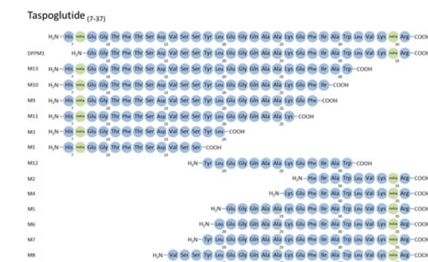


Figure (left): Structure of Taspoglutide and its metabolites identified in different in vitro systems