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Application Note

Biotherapeutic Peptide Mass Confirmation and Impurity Profiling on a SmartMS Enabled BioAccord LC-MS System

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Abstract

This application note demonstrates how BioAccord LC-MS System can be used for peptide mass confirmation and impurity profiling within research, development, and more highly regulated laboratories in manufacturing and QC. The integrated workflows within UNIFI allow accurate mass-based identification, sequence conformation, and relative quantification of biotherapeutic peptides and their impurities on a single platform, with SmartMS functionality making these capabilities accessible to a broad segment of scientists and technicians within the organization.

Benefits

- Compliance-ready qualitative and quantitative workflows for peptide mass confirmation and impurity profiling by optical and mass-based analyses
- SmartMS-based system operation for rapid deployment, training, and routine operation by individuals not necessarily having previous LC-MS experience

Introduction

Today, a wide variety of peptide biotherapeutics are progressing through the development pipeline due to their numerous therapeutic benefits and significant advancements in technologies for synthetic and recombinant production, downstream processing, and bio delivery. While varied in physical properties, most of these molecules have molecular mass below 5000 Da. Ultimately, peptide purity depends upon optimization of various parameters during the production and processing steps, which requires analytical tools capable of assessing these molecules with rapid and reliable results for confirming the main product, any characteristic impurities, and to detect any unexpected product modifications. Generally, conventional liquid chromatography assays with optical detection are utilized to address the impurity profiling, but these assays have certain limitations for unresolved impurities and addressing the cause of newly discovered peaks, which makes the use of more advanced analytical instruments capable of delivering more structural information on these profiles a path for addressing these challenges in quick time.

Mass spectrometry is well suited for establishing the identity and purity of biotherapeutic peptides[1]. The incorporation of high-resolution mass spectrometry into the analytical workflow can provide accurate mass-based confirmation of peptide API, known impurities, along with providing verification of peptide sequences via their fragment ions[2]. The BioAccord LC-MS System is a high performance analytical platform that was designed and developed to be efficiently and easily deployed and operated even by laboratories lacking previous experience with LC-MS technologies. The SmartMS capabilities of the BioAccord are manifested with a simplified user interface, automated startup, and advanced self-diagnostics capabilities. This application note demonstrates how the BioAccord System supports an integrated workflow for biotherapeutic peptide analysis and impurity profiling.

Liraglutide, a glucagon-like peptide -1 receptor agonist, is a therapeutic peptide of 31 amino acids (HAEGTFTSDV SSYLEGQAAK EFIAWLVRGR G) with attachment of palmitic acid chain to a lysine side chain via a glutamic acid linker and has a monoisotopic mass of 3748.9465 Da. It can be produced by both rDNA technology and chemical synthesis. Liraglutide of rDNA origin is used in this study to demonstrate integrated workflow on the BioAccord System. The compliance-ready UNIFI application on the waters_connect Informatics platform enabled a streamlined workflow, combining automated data acquisition, processing, and reporting.

Experimental

Sample Preparation

A solution of liraglutide (rDNA origin) at 6 mg/mL is used.

LC-MS Conditions

LC-MS system:	BioAccord incorporating the ACQUITY RDa Mass Detector ACQUITY UPLC I-Class PLUS and ACQUITY UPLC TUV Detector
Column:	ACQUITY UPLC Peptide CSH C_{18}, 130 Å, 1.7 µm, 2.1 x 150 mm (p/n: 186006938)
Column temp.:	45 °C
TUV wave length:	215 nm
Flow rate:	0.12 mL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Injection volume:	1 µL

Gradient

Steps	Time (min)	Solvent A Composition (%)	Solvent B Composition (%)
1	0.00	95	5
2	2.00	70	30
3	80.00	45	55
4	100.00	5	95
5	101.00	95	5
6	105.00	95	5

MS Conditions

Mode:	Full scan with fragmentation
Mass range:	50–2000 *m/z*
Polarity:	Positive
Capillary voltage :	1.50 kV
Desolation temp.:	550 °C
Cone voltage:	50 V
Fragmentation cone voltage:	95–100 V
Lockmass:	Waters_connect Lockmass Solution (p/n: 186009298)

Data Management

Informatics:	waters_connect platform with UNIFI Application

Results and Discussion

The LC-HRMS strategy for biotherapeutic peptide mass confirmation and impurity profiling utilizes the UNIFI informatics peptide mapping workflow for characterization of the peptide and impurity peaks, and accurate mass screening workflow for targeted impurity profiling and automated relative % measurements. Data from the characterization stage is used to generate a targeted list of peptide species for accurate mass monitoring and quantification.

Peptide Characterization Using Peptide Mapping Workflow

The impurities in liraglutide were first chromatographically separated on an ACQUITY UPLC Peptide CSH C_{18} 130 Å Column using a shallow acetonitrile gradient in 0.1% formic acid. The acquired LC-MS data was then processed by the peptide mapping workflow method to identify the impurities and confirm the peptide sequence of impurities and liraglutide. Figure 2 shows the zoomed total ion chromatogram of the liraglutide sample. Peaks are labeled with respective identifications, and Figure 3 shows the component summary providing details of these identifications. The impurities observed were isomers, N-terminal truncations, additions, and oxidized forms of liraglutide.

Component Summary

	Component name	Protein name	Peptide	Modifiers	Observed mass...	Mass error (ppm)	Observed RT...	Response	Observed m/z	Charge	Matched 1st Gen Primary Ions
1	1:18:GPA K [20], Oxidation...	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20], Oxidation W [25]	3765.9338	-3.9	30.33	4676922	942.2389	4	25
2	1:18:GPA K [22]+H+	LGT_+HA	HAHAEGTFTSDVSSYLEGQAA...	GPA K [22]	3958.0335	-4.1	32.53	16464621	792.4125	5	36
3	1:18:GPA K [20], Oxidation...	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20], Oxidation x2 W [25]	3781.9204	-6.1	35.83	6477567	946.2355	4	32
4	1:18:GPA K [20]+H+	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20]	3749.9370	-4.5	37.51	92108280	938.2397	4	18
5	1:18:GPA K [20]+H+	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20]	3749.9342	-5.2	37.86	119050784	938.2390	4	42
6	1:18:GPA K [20]+H+	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20]	3749.9422	-3.1	38.19	3339716608	1250.6523	3	42
7	1:18:GPA K [20]+H+	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20]	3749.9332	-5.5	40.56	143428640	938.2388	4	42
8	1:18:GPA K [20]+H+	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20]	3749.9360	-4.7	41.40	17017996	938.2394	4	37
9	1:18:GPA K [20]+H+	LGT	HAEGTFTSDVSSYLEGQAAKE...	GPA K [20]	3749.9335	-5.4	42.07	26564040	938.2388	4	36
10	1:18:GPA K [19]+H+	LGT_-H	AEGTFTSDVSSYLEGQAAKEFI...	GPA K [19]	3612.8759	-5.2	46.82	79922800	1204.9635	3	42
11	1:18:GPA K [16]+H+	LGT_-HAEG	TFTSDVSSYLEGQAAKEFIAWL...	GPA K [16]	3355.7830	-3.2	47.49	13810025	839.7012	4	34
12	1:18:GPA K [18]+H+	LGT_-HA	EGTFTSDVSSYLEGQAAKEFIA...	GPA K [18]	3541.8453	-3.5	47.85	49845260	1181.2866	3	40

Figure 3. Component summary of liraglutide LC-MS analysis in peptide mapping workflow.

In one of its modes of operation, the ACQUITY RDa is capable of generating structurally informative fragment ions, adding more confidence to accurate mass-based peptide assignments. This unique feature is achieved by altering MS scans (one with lower collisional energy, and one with higher collisional energy ramping) during data acquisition. As a result, the instrument can be operated in MS only (MS 1) or Full scan with fragmentation (data independent acquisition) modes (MS 2) with collision induced fragmentation. Figure 4 and 5 shows the fragmentation spectra for liraglutide containing palmitic acid chain attached to Lys^{20} sidechain via a glutamic acid linker, and liraglutide truncated impurity (observed at relative level of 0.45% in UV detection) confirming the loss of the N-

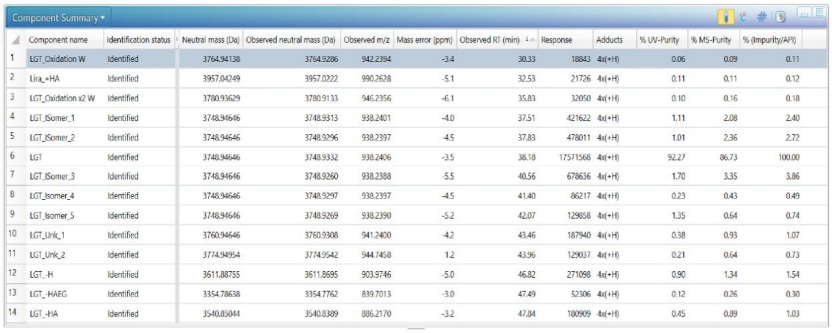
terminal two amino acids 'HA' respectively. The automated data processing generates annotated fragment ions spectra for all detected components.

Creation of Novel Amino Acid Modifications and Unnatural Amino Acids in the UNIFI Scientific Library

Existing amino acid modifications can be selected during UNIFI processing to assign peptide impurities. Novel amino acid modifications can be created to supplement the default set of modifications in the software. Also, unnatural amino acids can be created and saved in UNIFI scientific library. The scientific library application has a simple interface that enables the user to generate these new modifications (Figure 6).

Impurity Profiling Using the UNIFI Accurate Mass Screening Workflow

Impurities identified in characterization stage by the peptide mapping workflow can be added to a scientific library file and imported to the accurate mass screening workflow for targeted impurity profiling. Additional library entries can also be created based on prior knowledge. The accurate mass screening workflow uses XIC of each component targeted based on mass and retention time information on this component target list. The accurate mass screening workflow is used to automate the determination of % purity of peptides based on UV and MS response and %relative abundance to main peak MS response. Figure 7 shows the accurate mass screening workflow's component summary displaying the purity level of liraglutide and impurity components that are monitored using both MS and UV detection. Figure 8 shows the extracted ion chromatogram (XIC) of liraglutide(A) and truncated liraglutide impurity 'LGT-HA' (B).



Component Summary

	Component name	Identification status	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Response	Adducts	% UV-Purity	% MS-Purity	% (Impurity/API)
1	LGT_Oxidation W	Identified	3764.94138	3764.9286	942.2394	-3.4	30.33	18843	4x(+H)	0.06	0.09	0.11
2	Lira_+HA	Identified	3957.04249	3957.0222	990.2628	-5.1	32.53	21726	4x(+H)	0.11	0.11	0.12
3	LGT_Oxidation x2 W	Identified	3780.93629	3780.9133	946.2356	-6.1	35.83	32050	4x(+H)	0.10	0.16	0.18
4	LGT_ISomer_1	Identified	3748.94646	3748.9313	938.2401	-4.0	37.51	421622	4x(+H)	1.11	2.08	2.40
5	LGT_ISomer_2	Identified	3748.94646	3748.9296	938.2397	-4.5	37.83	478011	4x(+H)	1.01	2.36	2.72
6	LGT	Identified	3748.94646	3748.9332	938.2406	-3.5	38.18	17571568	4x(+H)	92.27	86.73	100.00
7	LGT_ISomer_3	Identified	3748.94646	3748.9260	938.2388	-5.5	40.56	678636	4x(+H)	1.70	3.35	3.86
8	LGT_Isomer_4	Identified	3748.94646	3748.9297	938.2397	-4.5	41.40	86217	4x(+H)	0.23	0.43	0.49
9	LGT_Isomer_5	Identified	3748.94646	3748.9269	938.2390	-5.2	42.07	129658	4x(+H)	1.35	0.64	0.74
10	LGT_Unk_1	Identified	3760.94646	3760.9308	941.2400	-4.2	43.46	187940	4x(+H)	0.38	0.93	1.07
11	LGT_Unk_2	Identified	3774.94954	3774.9542	944.7458	1.2	43.96	129037	4x(+H)	0.21	0.64	0.73
12	LGT_-H	Identified	3611.88755	3611.8695	903.9746	-5.0	46.82	271098	4x(+H)	0.90	1.34	1.54
13	LGT_-HAEG	Identified	3354.78638	3354.7762	839.7013	-3.0	47.49	52306	4x(+H)	0.12	0.26	0.30
14	LGT_-HA	Identified	3540.85044	3540.8389	886.2170	-3.2	47.84	180909	4x(+H)	0.45	0.89	1.03

Figure 7. Accurate mass screening workflow's component summary displaying the purity level of liraglutide and each targeted impurity using both MS and UV detection.

A report or multiple reports can be automatically generated post-acquisition using a pre-defined (default or user-defined) template(s) to summarize the final results of this impurity monitoring study (Figure 9). The software has provisions for electronic signatures on these reports, if necessitated by regulatory or organizational requirements.

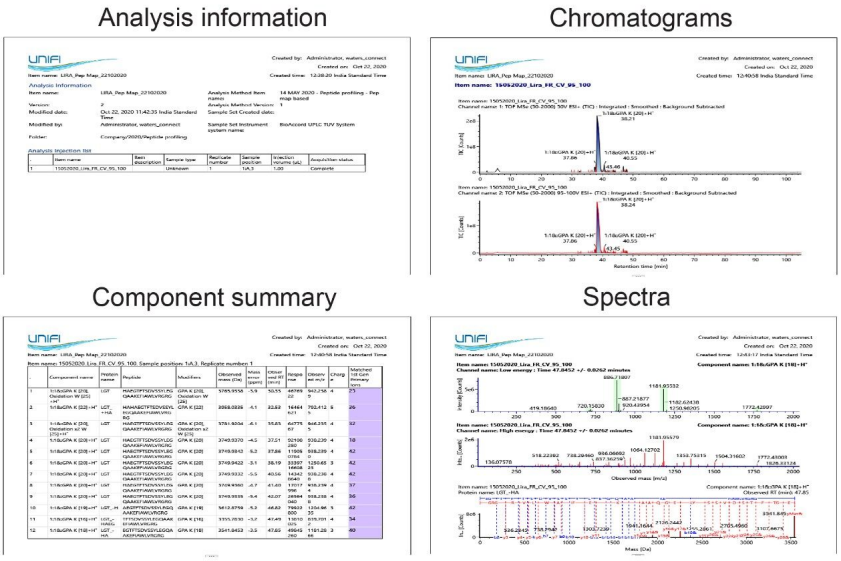


Figure 9. Representative UNIFI report of liraglutide analysis performed in peptide mapping workflow.

Using the peptide mapping and accurate mass screening/monitoring workflows described here with liraglutide, scientists can utilize the BioAccord LC-MS System to perform both mass confirmation and impurity profiling of peptide biotherapeutics in an efficient integrated approach. The significant structural information generated by the addition of the accurate mass data will facilitate more rapid method development, method transfer, and investigations of product deviations, enabling better and faster decision making within peptide biotherapeutic organizations.

Conclusion

This application note demonstrates how BioAccord LC-MS System can be used for peptide mass confirmation and impurity profiling within research, development, and more highly regulated laboratories in manufacturing and QC. The integrated workflows within UNIFI allow accurate mass-based identification, sequence conformation, and relative quantification of biotherapeutic peptides and their impurities on a single platform, with SmartMS functionality making these capabilities accessible to a broad segment of scientists and technicians within the organization.

References

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