Rapid Screening of Peptide Impurities in Calcitonin-Salmon Nasal Spray Using Data-Dependent LC-MS-MS and Data-Independent LC-MS^E

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Overview

This work demonstrates the application of data-dependent (DDA) LC-MS-MS and data independent (DIA) LC-MS^E acquisition to conduct peptide impurity profiling using calcitonin salmon nasal spray drug as an example.

Introduction

Comprehensive characterization of a peptide API is required to assure the quality of this drug class. The presence of impurities may lead to altered efficacy and/or induce unwanted toxicity including immunogenicity. Therefore, the impurity profiling of a peptide drug is critical to its safety and efficacy. Generally, liquid chromatography with UV detection (LC-UV) is used for the identification and quantitation of the impurities. However, the LC-UV method lacks in sensitivity, specificity, and the ability to identify site specific modifications in peptide sequences. While LC-MS is capable of identification and quantitation of low level impurities, the data analysis is usually performed manually. Hence, the method is time consuming and tedious, and it is difficult to detect co-eluting peptides or those that do not exhibit a TIC peak.

^{1}Cys-Ser-Asn-Leu-Ser-Thr-^{7}Cys-Val-Leu-Gly-Lys-Leu-Ser-^{14}Gln-Glu-Leu-His-Lys-Leu-^{20}Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2

Figure 1. The amino acid sequence of calcitonin salmon. Cys 1 and Cys 7 form disulfide bond. The glutamines at positions 14 and 20 can undergo deamidation to form glutamic acid and generate impurities.

To overcome the above challenges, we applied DDA and DIA approach to screen peptide impurities in calcitonin salmon sample.

Experimental

Chromatography

- Column: Waters ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 130 Å, 1.7 µm) maintained at 45 °C
- Mobile phase: A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile: water (95:5)
- Flow rate: 250 µL/min
- Injection volume: 5 µL
- Run time: 66 min with gradient elution

Sample preparation: Calcitonin salmon nasal spray was diluted 10-fold with 0.1% formic acid for LC-MS and 4-fold for DDA LC-MS/MS and DIA LC-MS^E analysis

DDA LC-MS-MS

- Instrumentation: Thermo Sceintific Vanquish UHPLC system coupled with Thermo Scientific Q Exactive HF Orbitrap mass spectrometer
- MS mass range: *m/z* 400-1500 (resolution: 120,000)
- Selection criteria: 1) the charge state: 2 to 5; 2) dynamic exclusion: 10 seconds; 3) intensity threshold: 1000; 4) Exclusion list (API ions) 686.9550 (5^+), 858.4455 (4^+) and 1144.2483 (3^+)
- Isolate selected ions with a 2.0 m/z isolation window, and undergo HCD fragmentation with a NCE value of 30
- MS/MS mass range: *m/z* 200 – 2000 (resolution: 30,000)
- Data analysis:
  - Convert mass spectrometric data to .mgf files using MS Convert
  - Submit to MASCOT for database search and generate peptide summary report
  - Sort and collate the list of monoisotopic masses with corresponding ions at different charge states
  - Quantitation of impurities by comparing the extracted ion chromatogram (XIC) of individual impurity to API's XIC

Experimental (contd.)

DIA LC-MS^E

- Instrumentation: Waters ACQUITY I-class UPLC coupled to a Waters Synapt G2Si mass spectrometer
- Mass range: *m/z* 100-2000
- Lock spray: Glu-fibrinopeptide 785.8426 Da (mass tolerance: 0.25 Da)
- MS^E data acquisition: For low collision energy mode, the trap and transfer collision energy off and for the high collision energy, the trap collision energy was turned on from 20-35 V.
- Data analysis:
  - Software: BiopharmaLynx
  - Data processing: Peptide mapping
  - MS and MS^E ion intensity threshold: 250 and 100 counts, respectively
  - MS and MS^E mass match tolerance: 5.0 and 10.0 ppm, respectively.
  - Digest reagent: None
  - Modifications: based on literature data
  - Quantitation of impurities by comparing the ADC (analog to digital converter) response of individual impurity to that of the API peak

Results

DIA LC-MS^E approach

Table 1. List of selected peptide impurities observed using DIA approach

Impurity	Rt (min)	M.W. (mono)	Δm/z	% ADC
1	5.8	2195.396	-1234.7	0.01
2		2213.408	-1216.7	0.08
3	21.3	3503.072	73.0	0.02
4		3333.996	-96.1	0.13
5	26.1	3412.084	-18.0	0.01
6	28.7	3412.032	-18.0	0.14
7		3315.960	-114.1	6.45
8		3297.972	-132.1	0.41
9		3477.048	47.0	0.01
10		3572.900	142.8	0.15
11		3214.884	-215.2	0.34
12	30.0	3468.080	38.0	2.18
13		3532.128	102.1	0.02
14		3232.920	-197.2	0.03
15		3501.096	71.0	0.02
16		3451.980	21.9	1.65
17	30.4	3430.072	0.0	0.78
18		2912.788	-517.3	0.01
19		3088.856	-341.2	0.09
20		3316.968	-113.1	0.05
21	31.5	2715.680	-714.4	0.04
22		3431.125	1.1	0.01
23		2930.784	-499.3	0.02
24		3316.968	-113.1	0.05
25		1691.986	-1738.1	0.13
26		3430.072	0.0	0.78
27		3453.000	22.9	0.01
28	32.5	3450.495	20.4	0.73

- The LC-MS^E analysis of three lots of calcitonin salmon sample resulted in 505-535 components with charge states 1-5
- In order to simplify data analysis the impurity peaks observed in the TIC and with charge states 2-5 were selected
- Table 1 shows a selected list of 28 impurities observed at various retention times. The %ADC range observed is from 0.01-6.45% and the mass range is *m/z* 1691-3572. Twelve of the 28 were above 0.1%.

DDA LC-MS-MS approach

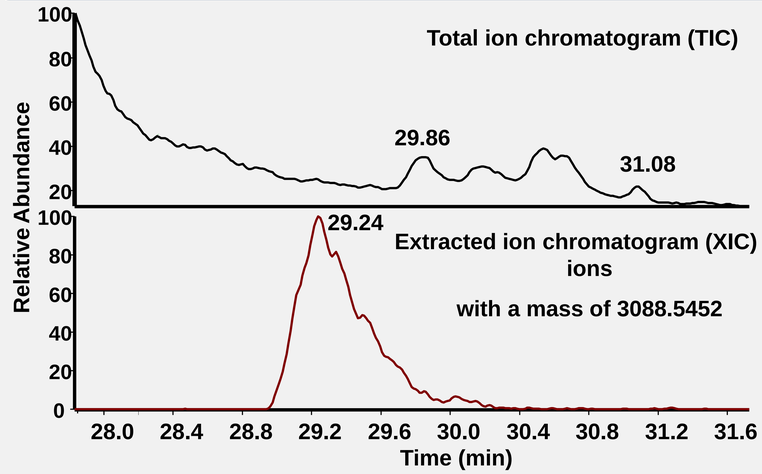

Figure 2. Zoomed TIC (top panel) and XIC of ion with *m/z* 3088.545

- Extracted ion chromatogram (XIC) was used to confirm the identification and determination of retention time of ions of interest

Table 2. List of selected peptide impurities observed using DDA approach

Index	TIC Peak Retention Time (min)	Monoisotopic Mass	Detected by DDA	Area %
1	5.77	2213.1206	Yes	0.06
2	18.49	1321.6378	No	0.009
3	19.44	1578.7394	No	0.005
4	20.6	3447.7259	Yes	1.17
5		1234.6048	Yes	0.05
6		3333.6461	Yes	0.02
7	24.32	1828.8825	No	0.02
8	24.99	3412.6908	Yes	0.23
9	29.86	2930.4755	Yes	0.04
10	30.4	2715.3867	Yes	0.02
11	30.54	2070.0608	Yes	0.12
12	31.08	1691.8229	Yes	0.06
13	32.71	3471.7261	Yes	0.17
14	35.84	3428.6858	Yes	0.08
15	37.99	3471.7261	Yes	0.02
16	57.07	Only Singly-charged ions observed		
17	58.8	Only Singly-charged ions observed		

- The impurities were classified into three groups:
  - Impurities observed in total ion chromatogram (TIC)
  - Impurities co-eluting with the peptide API or eluted at its peak tail (challenging for manual screening)
  - Impurities buried under the TIC baseline (challenging for manual screening)
- The monoisotopic masses of the detected impurities ranged from 1234-3802 amu at 0.01-4.8%. Four out of 17 were above 0.1% by DDA.

Conclusions

- The DDA LC-MS/MS and DIA LC-MS^E can be utilized to facilitate the detection of peptide impurities in a peptide drug, and the DIA LC-MS^E showed the promise for direct impurity quantitation

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