LC-UV/MS Workflows Enabling Orthogonal Impurity Profiling of GLP-1 Analogs

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Introduction

Glucagon-like-peptide-1 receptor agonist (GLP-1 RA)

- Metabolic regulator for type 2 diabetes / weight management
- Analogs: modified to increase resistance to enzyme degradation and reduce clearance rate
- Impurities: amino acid sequence variance, spontaneous (non-enzymatic) post-translational modification
- Market growth drive forces: patent expiry, new clinical indications, multi-agonists

Analytical challenges

- Ubiquitous reversed-phase LC analysis is difficult to detect and resolve critical impurities

Solutions

- Orthogonal separation mode and column screening
- QC-friendly LC/UV-MS workflows with complimentary mass spectrum data

Waters GLP-1 RA Solutions

Experimental

- LC: ACQUITY™ Premier System
- Software: Empower™ 3.8.1
- Samples: exenatide and deamidation impurity standards
- Injection volume: 2 µL
- Sample temperature: 10 ˚C
- UV detection: λ = 280 nm
- MS: ACQUITY QDa™ II Mass Detector
- Scan range: 250-1500 m/z @ 5Hz
- ESI mode: positive
- Capillary voltage: 1.5 kV
- Cone voltage: 15 V
- Probe temperature: 600 ˚C

ACQUITY QDa II Mass Detector

BEH™ HILIC analysis

- Sample: 0.25 mg/mL in 50:50 ACN: pH 4.5 acetate buffer
- Column: ACQUITY Premier BEH HILIC Column, 130 Å, 1.7 µm, 2.1 mm X 100 mm (p/n 186010378)
- Column temperature: 40 ˚C
- Mobile phase
  A: 50 mM NH_4HCO_2, formic acid, pH 2.8, in water
  B: acetonitrile (ACN)

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.300	20	80	Initial
5	0.300	20	80	6
30	0.300	45	55	6
31	0.300	75	25	6
34	0.300	75	25	6
35	0.300	20	80	6
45	0.300	20	80	6

BEH amide HILIC analysis

- Sample: 0.25 mg/mL in 50:50 ACN: pH 4.5 acetate buffer
- Column: ACQUITY Premier BEH Amide Column, 130 Å, 1.7 µm, 2.1 mm X 150 mm (p/n 186009506)
- Column temperature: 40 ˚C
- Mobile phase
  A: 10 mM NH_4HCO_2, pH 3.1, in water
  B: 10 mM NH_4HCO_2 in 10:90 water:ACN

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.200	99	1	Initial
1	0.200	99	1	6
99	0.200	50	50	6
100	0.200	5	95	6
103	0.200	5	95	6
104	0.200	99	1	6
114	0.200	99	1	6

Reversed-phase (RP) analysis

- Sample: 0.25 mg/mL in pH 4.5 acetate buffer
- Column: ACQUITY Premier Peptide CSH™ C18 Column, 130Å, 1.7 µm, 2.1 x 100 mm (p/n 186009488)
- Column temperature: 60 ˚C
- Mobile phase
  A: 0.1% formic acid (v/v) in water
  B: 0.1% formic acid (v/v) in ACN

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.200	15	85	Initial
5	0.200	15	85	6
25	0.200	35	65	6
26	0.200	75	25	6
28	0.200	75	25	6
30	0.200	15	85	6
55	0.200	15	85	6

Results & Discussion

Orthogonal screening for impurity detection

GLP-1 analog: exenatide (exe)
Formula: $C_{184}H_{282}N_{50}O_{60}S$
MW = 4,186.6 Da

Impurity: exenatide + 1 proline (exe+pro)
Formula: $C_{189}H_{289}N_{51}O_{61}S$
MW = 4,283.7 Da, Δ +97.1 Da

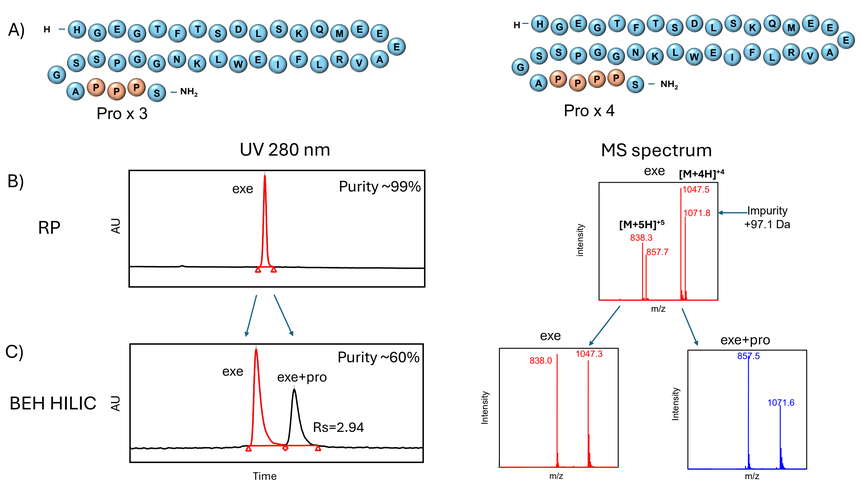
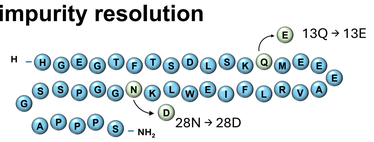


Figure 1 (A) One exenatide raw material contains an impurity that accounts for 40% of total mass. This impurity is confirmed to be an amino acid insertion event of an extra proline residue via sequence analysis enabled by Waters Xevo™ QTof G3 Mass Spectrometer configured with UNIFI™ Scientific Information System. Amino acid analysis via Waters AccQ·Tag™ Ultra Chemistry Kit confirmed that the proline residue exceeds USP monograph acceptance criteria. (B) Under RP analysis, this impurity coeluted with the native species; mass spectrum revealed this impurity that would be ignored by UV spectrum. Reducing column temperature to 30 ˚C achieved partial resolution, which is outside robust RP analysis operation range. (C) HILIC separation using BEH stationary phase readily achieved baseline resolution (Rs = 2.94), verified by mass spectrum. HILIC separation leverages the hydrophilicity and polarity difference to resolve impurities.

Improving efficiency of impurity resolution

Exenatide deamidation species:
exe-D: MW = 4,187.6 Da, Δ +1 Da
exe-E: MW = 4,187.6 Da, Δ +1 Da
exe-DE: MW = 4,188.6 Da, Δ +2 Da



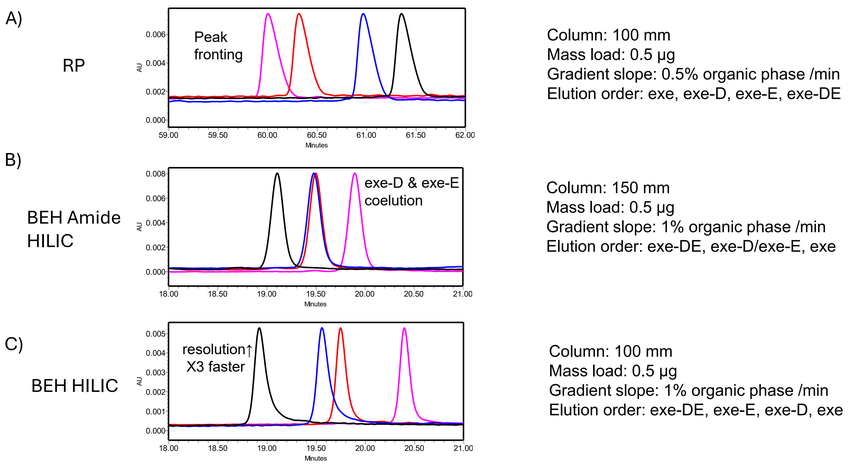
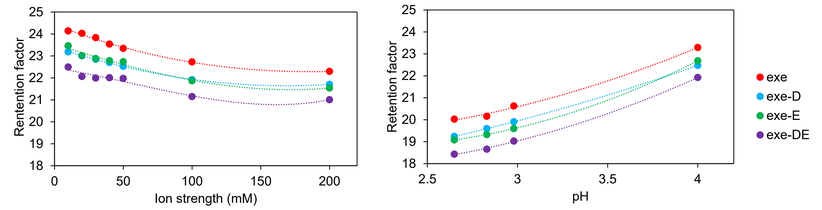


Figure 2 Exenatide contains two amino acids, position 13 glutamine and position 28 asparagine that are subjected to deamidation. Deamidation leads to one Dalton mass shift which is difficult to confirm unless high resolution mass spectrometer is used. Synthesized standards containing one or two deamidation events were custom-made. Deamidation species are challenged for separation under RP condition (A). BEH amide HILIC separation (B) improved peak symmetry and BEH HILIC separation (C) achieve improved resolution at steeper gradient and faster analysis.

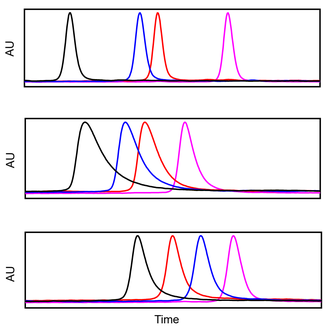
MP composition optimization to control resolution



- At constant pH (~4), retention decreased under higher ion strength
- Strong salt masking effect: MP ions compete for charged sites on stationary phase

- At constant ion strength (50mM), retention decreased with lower pH
- A lower pH introduces more positive charges on both analytes and stationary phases and weaker net electrostatic interaction which leads to earlier elution

Figure 3 In BEH HILIC separation, exenatide and impurity retention are sensitive to mobile phase composition: ion strength and pH.

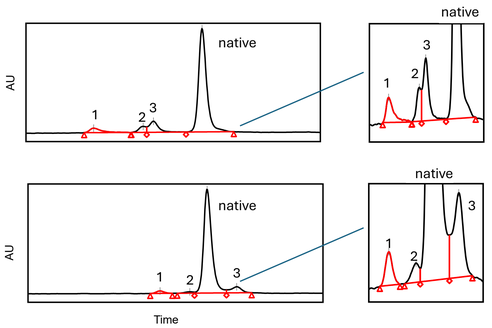


- Formic acid (FA) + 50 mM NH_4HCO_2, pH ~3
- Elution order: exe-DE (31% aqueous), exe-E, exe-D, exe

- Difluoroacetic acid (DFA) + 50 mM $NH_4CH_3O_2$, pH ~3
- Elution order: exe-DE (28% aqueous), exe-E, exe-D, exe

- Trifluoroacetic acid (TFA) + 50 mM $NH_4CH_3O_2$, pH ~3
- Elution order: exe-DE (20% aqueous), exe-D, exe-E, exe
- Lowest pKa, strongest ion pair strength, more hydrophobic anions positively charged peptide

Figure 4 In BEH HILIC separation, at constant pH (~3), ion pairing reagent affects elution order of deamidation impurities.



With spike-in impurity
Native, 1 µg
1: exe-DE, 0.1 µg
2: exe-E, 0.1 µg
3: exe-D, 0.1 µg

Tris-HCl, pH 8, 37°C, overnight
Native: 2 µg
1: exe-D, 2.2%
2: -16 Da, 1.6%
3: exe-isoD, 8.6%
Total impurity: 12.4%

Figure 5 In BEH HILIC separation, optimized condition achieved separation for samples with impurity spike-in and forced degradation. More than 12.4% of impurities were generated after overnight high pH degradation which presented risks on drug safety and efficacy.

Conclusions

- Leveraging orthogonal selectivity of RP and HILIC strengthens impurity detection, raw material screening, and overall QC strategies for increasingly complex peptide therapeutics
- Mass spectrum information enables detection of co-eluted impurity that is ignored by UV detection

References

D. Han et al. “Accelerating Method Development and Manufacturing of GLP-1 Analogs with LC-UV/MS” Application Note 720008800, May 2025, Waters Corporation

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