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Rapid High-Throughput Amino Acid Analysis of GLP-1 Analogs

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Abstract

Due to increased demand for glucagon-like-peptide-1 (GLP-1) receptor agonist-based drugs, manufacturers are continually looking for fast and reliable solutions to screen and evaluate products to ensure that they meet regulatory standards. Amino acid analysis is a quantitative approach to assess composition and optimize bioprocess parameters during drug production, but often require lengthy hydrolysis and derivatization steps. In this study, a microwave-assisted UPLC™ workflow is demonstrated, designed to rapidly hydrolyze, label, and identify amino acids in GLP-1 analogs using Waters™ AccQ·Tag™ Ultra Chemistry Kit as a turnkey solution to expedite amino acid analysis of GLP-1 analogs.

Benefits

- ACQUITY™ Premier System coupled with AccQ·Tag Ultra Chemistry Kit facilitates a turnkey solution of high-throughput and reproducible amino acid analysis
- Empower™ Chromatography Data System (CDS) reduces error with automated workflows for calibration quantitation, and analysis of results using custom fields
- Microwave-assisted acid hydrolysis provides rapid non-specific peptide digestion to amino acids within

minutes

Introduction

GLP-1 analogs are metabolic regulators initially approved for glycemic control in type 2 diabetes. Their subsequent approval for weight management has drawn significant public interest and media attention spurring research in their use to treat other diseases, including cardiovascular and liver disease. As a peptide therapeutic, GLP-1 analogs have been heavily modified compared to the native GLP-1 to increase resistance to *in vivo* degradation and extend system circulation time. These analogs can be readily made through chemical synthesis or recombinant DNA technology. While mass spectrometry (MS) remains the standard solution platform for peptide sequencing during drug discovery and characterization phases, manufacturing environments benefit from UV-based detection methods that are capable of rapidly and reliably providing information on composition to guide method development and bioprocess optimization.

Here, a QC-friendly microwave-assisted UV-based detection workflow is introduced, offering high-throughput amino acid analysis. The Waters AccQ·Tag Ultra Chemistry Kit provides a turnkey solution, including column, standards, eluents, and derivatization kit, that enables fast, reliable, and reproducible amino acid analysis in under 10 minutes, when using Waters ACQUITY Premier System.

Experimental

For the amino acid hydrolysate quantitation, calibration standards were prepared using Waters Amino Acid Standard (p/n: WAT088122 <https://www.waters.com/nextgen/global/shop/standards--reagents/wat088122-amino-acid-standard-accq-tag-pico-tag-accq-tag-ultra.html>) and 0.1N HCl as the diluent. The calibrant concentrations were 10, 20, 50, 80, 100, 200, 400, and 500 µM for all amino acids (except cysteine, which was 5, 10, 25, 40, 50, 100, 200, and 250 µM).

Microwave-assisted Acid Hydrolysis

Exenatide stock was prepared at 2.5 mg/mL in 6N HCl, and 200 µL of solution was added to a 10 mL borosilicate glass pressure vessel. Then it was hydrolyzed using a microwave hydrolysis device (Discover Prep™, CEM Corp,

North Carolina, USA). The hydrolysis step was performed using the dynamic mode with maximum power of 300 W. The hydrolysis temperature and time were set to 160 °C and 20 minutes. The hydrolyzed peptide was neutralized with NaOH, diluted with water, and derivatized using Waters AccQ·Tag Ultra Derivatization Kit (p/n: 186003836 <https://www.waters.com/nextgen/global/shop/application-kits/186003836-accq-tag-ultra-derivatization-kit.html>) following the user manual.

LC Conditions

LC system:	ACQUITY Premier Binary System equipped with a column heater (CH-A) and FTN sample manager
Detection:	TUV, λ=260 nm @ 10 Hz
Detector inlet tubing:	Tubing Assembly, Detector Inlet, PEEK 0.0025" x 8.5" (p/n: 700009971)
Column:	AccQ·Tag Ultra Column 130 Å, 1.7 µm, 2.1 x 100 mm (p/n: 186003837), with ACQUITY Column In-Line Filter (p/n: 205000343)
Column temperature:	45 °C
Sample temperature:	20 °C
Injection volume:	1 µL
Flow rate:	0.70 mL/min
Mobile phase:	A: 1:20 AccQ·Tag Ultra Eluent A Concentrate (p/n: 186003838): water (LC-MS grade) B: AccQ·Tag Ultra Eluent B (p/n: 186003839)
Wash:	Needle wash: 95:5 water: acetonitrile

	Seal wash: 50:50 water: acetonitrile
Chromatography software:	Empower Chromatography Data System (CDS)

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.70	99.9	0.1	Initial
0.54	0.70	99.9	0.1	6
5.74	0.70	90.9	9.1	7
7.74	0.70	78.8	21.2	6
8.04	0.70	40.4	59.6	6
8.05	0.70	10.0	90.0	6
8.64	0.70	10.0	90.0	6
8.73	0.70	99.9	0.1	6
9.50	0.70	99.9	0.1	6

Results and Discussion

Chromatograph Parameters

In manufacturing environments, analytical methods are driven by UV-based workflows with high-throughput and efficiency. Since amino acids have diverse chemical properties and low or absent UV absorbance, Waters AccQ·Tag Ultra Chemistry Kit provides consistent pre-column derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) to generate stable derivatized amino acids. When paired with the ACQUITY Premier System, precise and reliable quantitative analysis of amino acids can be performed as demonstrated in Figure 1.[1, 2, 3] A differentiating feature of the ACQUITY Premier System is the ability to deliver non-linear gradients to effectively utilize the analytical gradient space. This is demonstrated in the separation of critical pair serine and arginine in under 10 minutes when using a non-linear gradient (curve 7) while maintaining separation of the remaining amino acids of the hydrolysate standard.

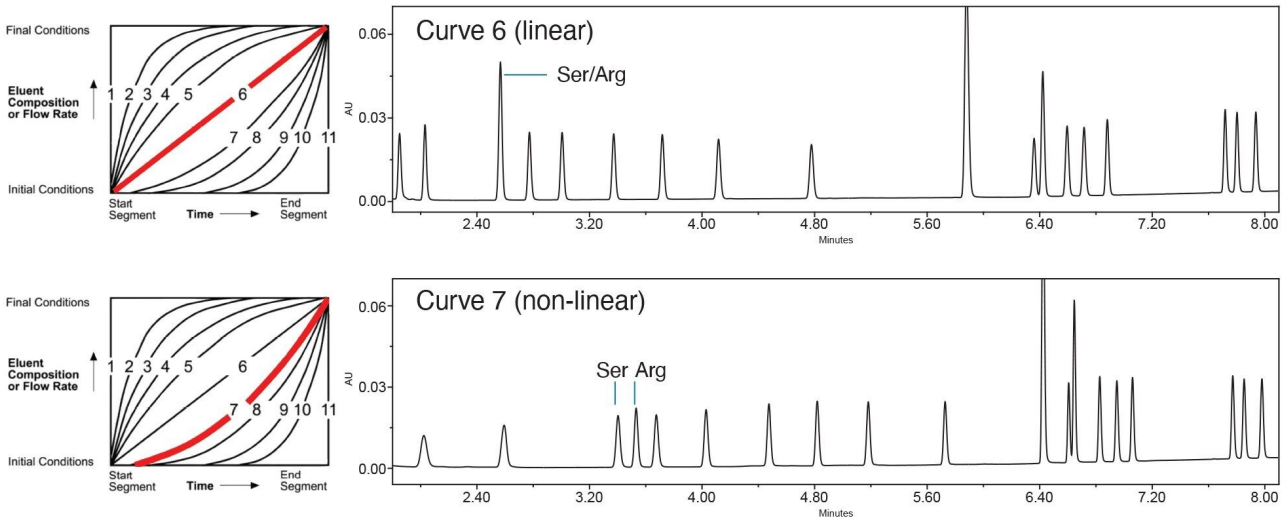


Figure 1. UV chromatogram of amino acid hydrolysate standard (100 µM) on the ACQUITY Premier UPLC System. (A) Linear gradient (curve 6). (B) Non-linear gradient (curve 7).

Microwave-assisted Acid Hydrolysis

To further increase the efficiency of amino acid analysis, microwave-assisted acid hydrolysis was used as a rapid alternative to conventional acid hydrolysis, which may require more than 24 hours of reaction time. Under microwave irradiation, peptides are non-specifically digested into amino acids within minutes. Like the chromatography of amino acids, optimizing hydrolysis condition is essential to ensure complete hydrolysis while minimizing degradation of labile amino acids. Hydrolysis temperature was set to 160 °C.[4] The optimized hydrolysis time was determined by monitoring intact exenatide peak in the chromatographs. As shown in Figure 2, the exenatide peak at 8.7 minutes decreased noticeably when the hydrolysis time increased from 10 minutes to 20 minutes but only improved marginally with hydrolysis times greater than 20 minutes. Given this, a 20-minute hydrolysis time was chosen as optimal since longer hydrolysis may degrade labile species.

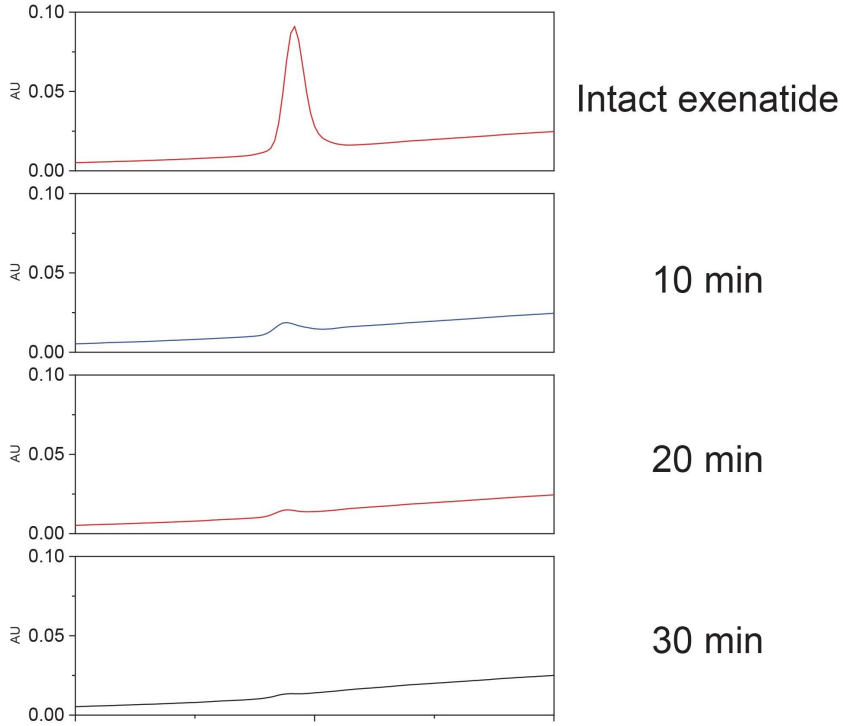


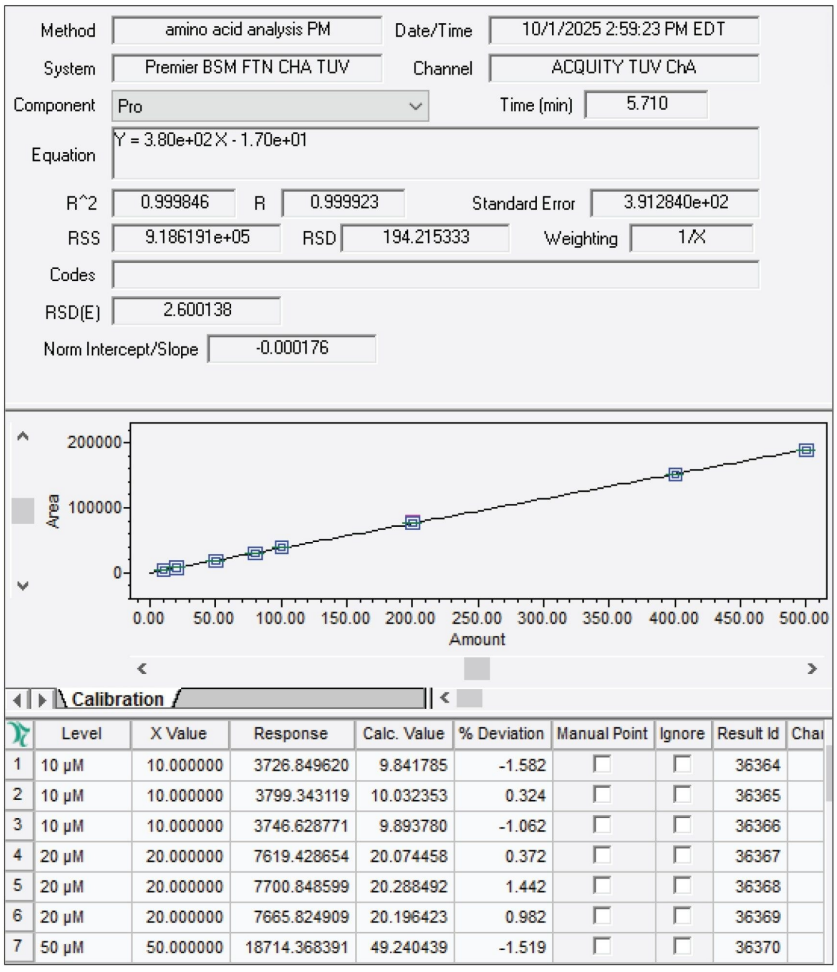
Figure 2. Microwave irradiation energy accelerates acid hydrolysis. A 20-minute reaction is able to provide adequate hydrolysis of exenatide and minimize degradation of labile species.

Automated Data Processing with Empower CDS

As part of the Empower CDS, users have the ability to generate integrated calibration plots to streamline the data analysis process. To demonstrate this, the amino acid hydrolysate standards were diluted and each calibrant was injected in triplicate. The concentration of each component was defined within the sample set at the time of acquisition. The average peak area from three injections was plotted against component concentration to generate a calibration curve within Empower CDS (Figure 3), which can then be used for processing sample data in an automated fashion.

As an example, exenatide from two vendors was subjected to microwave-assisted acid hydrolysis followed by derivatization in triplicate. The peak identities in hydrolyzed exenatide were assigned based on the elution order and retention time matching with the amino acid standards. Peak areas were used to quantify individual amino acids via the calibration curves, and the results were normalized to the intact peptide concentration prior to

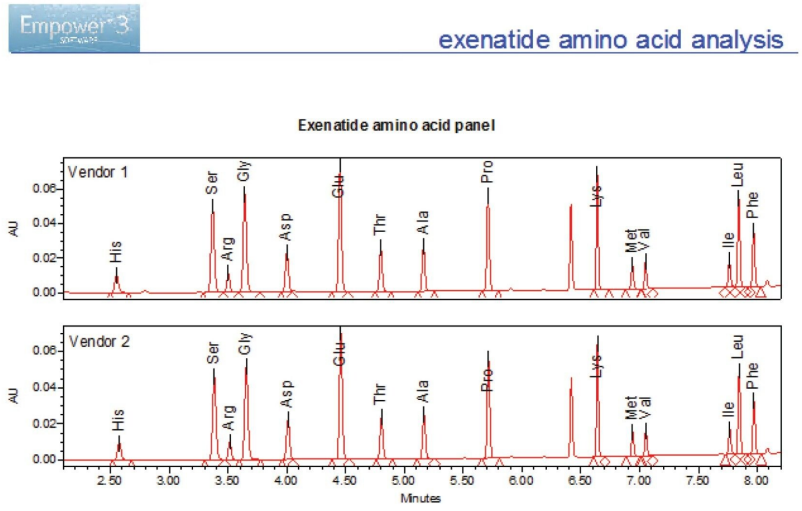
hydrolysis (custom field “peptide concentration”) to determine amino acids residue count. A custom field “observed residues” was generated using the formula “amount/peptide concentration”. For calculation purposes, tryptophan was excluded because it was degraded by acid hydrolysis. Asparagine and glutamine were quantified as aspartic acid and glutamic acid due to deamidation during acid analysis. The observed residues were compared using the USP monograph.[5, 6] Acceptance criteria ranges were entered within the Limit tab of the processing method so that any out of specification results would be automatically flagged.



	Level	X Value	Response	Calc. Value	% Deviation	Manual Point	Ignore	Result Id	Cha
1	10 µM	10.000000	3726.849620	9.841785	-1.582	☐	☐	36364	
2	10 µM	10.000000	3799.343119	10.032353	0.324	☐	☐	36365	
3	10 µM	10.000000	3746.628771	9.893780	-1.062	☐	☐	36366	
4	20 µM	20.000000	7619.428654	20.074458	0.372	☐	☐	36367	
5	20 µM	20.000000	7700.848599	20.288492	1.442	☐	☐	36368	
6	20 µM	20.000000	7665.824909	20.196423	0.982	☐	☐	36369	
7	50 µM	50.000000	18714.368391	49.240439	-1.519	☐	☐	36370	

Figure 3. Empower software automates the generation of calibration curves. The calibration curve is plotted as “Amount” (concentration of component) versus “Averaged Response” of three replicates of each calibrant.

The observed residue results of two samples were summarized in report (Figure 4). Repeated injections of 6 displayed excellent reproducibility of area RSD <0.5% and retention time RSD <0.6%. Vendor 1 sample met the acceptance criteria. Vendor 2 sample contained proline residues that exceeded the acceptance criteria. This out of specification event was confirmed as a proline insertion impurity as investigated via mass and sequencing analysis in previous work.[7] As shown in the report, the out of specification proline count is flagged in red for easy interpretation of process deviation. This integrated workflow eliminates the need for external software to generate calibration curves or calculate analyte concentrations, reducing the risk of errors from manual data entry and transfer.



Vendor 1 Peak Results

	Name	RT	Area	Observed_Residues	Acceptance_criteria
1	His	2.551	22961	1.03	0.5 - 1.5
2	Ser	3.368	101001	4.63	4.2 - 5.5
3	Arg	3.502	19930	0.90	0.5 - 1.5
4	Gly	3.642	113349	5.26	4.5 - 5.5
5	Asp	4.000	43642	2.05	1.5 - 2.5
6	Glu	4.452	126552	6.05	5.2 - 6.8
7	Thr	4.798	42532	1.90	1.5 - 2.5
8	Ala	5.162	44350	2.04	1.5 - 2.5
9	Pro	5.711	86367	4.23	3.5 - 4.5
10	Lys	6.639	75373	2.01	1.5 - 2.5
11	Met	6.937	19453	0.86	0.5 - 1.5
12	Val	7.049	20782	0.91	0.5 - 1.5
13	Ile	7.762	22443	0.97	0.5 - 1.5
14	Leu	7.843	69672	3.14	2.5 - 3.5
15	Phe	7.969	45711	2.00	1.5 - 2.5

Vendor 2 Peak Results

	Name	RT	Area	Observed_Residues	Acceptance_criteria
1	His	2.574	21150	1.07	0.5 - 1.5
2	Ser	3.382	93969	4.85	4.2 - 5.5
3	Arg	3.514	17932	0.91	0.5 - 1.5
4	Gly	3.654	103465	5.41	4.5 - 5.5
5	Asp	4.009	42650	2.25	1.5 - 2.5
6	Glu	4.458	122781	6.60	5.2 - 6.8
7	Thr	4.804	39471	1.98	1.5 - 2.5
8	Ala	5.166	42038	2.17	1.5 - 2.5
9	Pro	5.714	87748	4.84	3.5 - 4.5
10	Lys	6.640	71610	2.14	1.5 - 2.5
11	Met	6.940	18648	0.93	0.5 - 1.5
12	Val	7.052	19655	0.96	0.5 - 1.5
13	Ile	7.766	20049	0.97	0.5 - 1.5
14	Leu	7.846	64095	3.25	2.5 - 3.5
15	Phe	7.972	41603	2.05	1.5 - 2.5

Figure 4. Empower report comparing the observed residues and acceptance criteria of USP monograph. The proline residue in vendor 2 sample exceeded the acceptance criteria and was flagged in red.

Conclusion

This study demonstrated the effective use of AccQ·Tag Ultra Chemistry Kit coupled with ACQUITY Premier System to perform rapid and reliable analysis of amino acids in GLP-1 analogs. Microwave irradiation accelerates acid hydrolysis and completes the digestion within minutes. Empower CDS automates calibration curve

generation and analyte quantitation. Custom fields and Limit tab streamline result analysis, reduce errors, and flag out of specification results automatically.

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