The Microwave-Assisted Amino Acid Hydrolysis of Liraglutide

Introduction

Peptide therapeutics are increasingly being used for the treatment of diseases. New generations are being discovered at a rapid pace. These drugs often mimic the function of native proteins and thus are generally regarded as safe and well tolerated because they can be broken down by the human body.[1] Peptides for this purpose can be synthesized by bacterial, insect, or mammalian cells and then purified. Alternatively, they can be prepared chemically via solid-phase peptide synthesis (SPPS). Ideally, the synthesis should be accomplished in a rapid and controlled manner at a multi-kilogram scale. CEM's Liberty PRO™ automated peptide synthesizer allows for the high-throughput microwave-assisted SPPS of functional peptides at the production scale.

Unsurprisingly, peptide therapeutics are held to high regulatory standards. Whether biologically or synthetically produced, the amino acid composition and content of these peptides can serve as an important quality control check. Amino acid analysis can be used to verify a successful synthesis, identify isolated peptides, detect atypical amino acid residues, and support structural analysis. Typically, amino acid analysis of these macromolecules is accomplished via acidic or alkaline hydrolysis in a sealed vial using conventional heating for up to 24 hrs. Microwave power can be used to speed up this reaction, often requiring only 15 minutes to complete the hydrolysis.[2]

In this work, the CEM Discover Prep™ microwave-based sample preparation system was used to confirm the amino acid content of Liraglutide, a peptide synthesized using the Liberty PRO. In order to hydrolyze amide bonds and liberate the individual amino acid residues, acidic and alkaline hydrolysis of the peptide was performed using the Discover Prep. Alkaline hydrolysis is generally applied for the quantification of tryptophan, as its indole side chain is destroyed under acidic conditions. Following Discover Prep amino acid hydrolysis, the Waters AccQ-Tag™ Ultra Derivatization Kit was used in conjunction with a Waters ACQUITY H-Class™ UPLC system and a PDA detector to measure the amino acid content of the hydrolysates.[3] The Discover Prep successfully prepared the peptide for LC-PDA analysis for amino acid quantification. Upon comparison to the theoretical yield of each amino acid residue present in the synthesized peptide, the microwave-assisted hydrolysis resulted in comparable recoveries in a very rapid and efficient manner.

Materials and Methods

All reagents were purchased from commercial suppliers. All hydrolysis solutions were freshly prepared prior to use. Liraglutide was synthesized via Fmoc-SPPS on the Liberty PRO and purified via preparative HPLC, >99% purity was assumed. The peptide was hydrolyzed as a dry powder using the Discover Prep. The contents for His, Ser, Arg, Gly, Asp, Glu, Gln, Thr, Ala, Lys, Tyr, Val, Ile, Leu, and Phe were measured using acid hydrolysis, and the Trp content was determined following alkaline hydrolysis. The amino acid content of the hydrolysates was quantified via pre-column derivatization, followed by UPLC injection and PDA detection at 260 nm. The Waters AccQ-Tag Ultra Derivatization Kit was used for LC-PDA analysis of the hydrolysates. All samples were analyzed in triplicate.

Discover Prep Hydrolysis Methods

Acid-Hydrolysis Reaction Preparation: A 10 mg sample portion of the peptide was added to a pyrolyzed 10-mL Pyrex vial equipped with a micro stir bar. A 2 mL aliquot of 6 N HCl containing 1% phenol was then added to each microwave reaction vial. Vials were purged with N_2 for 1 minute, quickly sealed with a Teflon® lined silicon cap, and placed in a sample rack prior to being placed in the Discover Prep cavity.

Alkaline-Hydrolysis Reaction Preparation: A 10 mg sample portion of the peptide was added to a pyrolyzed 10-mL Pyrex vial equipped with a PFA liner and micro stir bar. A 2 mL aliquot of 4 N NaOH was then added to each microwave reaction vial. Vials were purged with N_2 for 1 minute, quickly sealed with a Teflon lined silicon cap, and placed in a sample rack prior to being placed in the Discover Prep cavity.

Discover Prep Method Programming: A one-step Dynamic method was programed for the amino acid hydrolysis of Liraglutide. The following method parameters were programmed into the Discover Prep:

Acid Hydrolysis
Vial Type: Pyrex
Control Type: Dynamic
Temperature: 155 °C
Time: 15 min
Pressure: 300 PSI
Power: 300 W
Stirring: High

Alkaline Hydrolysis
Vial Type: Pyrex with PFA Liner
Control Type: Dynamic
Temperature: 195 °C
Time: 30 min
Pressure: 250 PSI
Power: 300 W
Stirring: High

Sample Preparation for Analysis

All acidic and basic hydrolysates were filtered with 0.2 µm PTFE filters and then neutralized with equimolar NaOH or HCl, respectively, prior to derivatization with the Waters AccQ-Tag kit. A portion of 80 µL of borate buffer from the Waters AccQ-Tag Ultra Derivatization kit was added to a complete recovery vial. Then, 10 µL of the sample was added, and the sample was capped and vortexed. Next, 10 µL of prepared derivatization reagent was added to each sample reaction. Lastly, the reaction was vortexed for 10 s and then heated at 55 °C for 10 minutes prior to analysis.

Analysis

A 4 µL portion of each derivatized reaction was injected onto a Waters AccQ-Tag Ultra C18 column (1.7 µm, 2.1 x 100 mm) attached to a Waters ACQUITY H-Class UPLC with a Waters PDA detector. A flow rate of 0.4 mL/min was used for the separation. The column temperature was at 55 °C, and the absorbance was monitored at 260 nm. The separation gradient used is shown in **Table 1**. The mobile phases were A: Waters AccQ-Tag Eluent A diluted 10-fold in MilliQ water and B: Waters AccQ-Tag Eluent B. To create calibration curves for each amino acid to measure the concentration of each amino acid, Waters Amino Acid Standard (Waters Corporation, Part No. WAT088122) was derivatized at the final concentrations 1, 5, 10, 25, 50, 100, and 200 pmol/µL. Linear regression was also used to analyze the hydrolysates. All analyses were done using the Waters TargetLynx™ software.

Table 1. Gradient Used for Derivatized Amino Acid Separation

Time (min)	Flow (mL/min)	%A	%B
Initial	0.4	99.9	0.1
0.54	0.4	99.9	0.1
14.74	0.4	90.0	10.0
16.74	0.4	75.0	25.0
17.04	0.4	0.0	100.0
18.05	0.4	0.0	100.0
18.64	0.4	99.9	0.1
18.73	0.4	99.9	0.1

Results and Discussion

The results for the concentration of each amino acid were compared to the theoretical amino acid content based upon the peptide's unique sequence and >99% purity. The recoveries for experimental results are shown in **Table 2**. It was found that the microwave-assisted amino acid hydrolysis results were in the range of 85%-115%, indicating a comparable level of acid and alkaline hydrolysis to theoretical values in only 15 and 30 minutes, respectively. Also, the standard deviations for the recovery for each amino acid were determined, and the standard deviations were no greater than 4.7%, indicating good precision.

The Discover Prep microwave-based sample preparation system possesses several key benefits over conventional oven hydrolysis. Microwave energy offers a dielectric heating mechanism, resulting in volumetric heating via uniform and instantaneous heat transfer, in contrast to conventional heating mechanisms, which rely on the slow transfer of heat through vial walls and the reaction components. Also, the Discover Prep has a stirring feature, allowing for reaction homogeneity. Lastly, the system rapidly cools samples using compressed air, allowing for decreased sample analysis time. Thus, with its benefits, the Discover Prep is an excellent option for amino acid hydrolysis of purified peptides and proteins.

Table 2. Recovery Results for the Amino Acids Found in Liraglutide

Amino Acid	Average Recovery	Standard Deviation
His	102.1%	2.2%
Ser	85.8%	2.4%
Arg	89.1%	0.1%
Gly	115.1%	4.5%
Asp	111.4%	3.6%
Glu+Gln	109.3%	0.1%
Thr	86.4%	3.3%
Ala	113.8%	4.7%
Lys	104.5%	1.4%
Tyr	111.6%	3.0%
Val	103.8%	3.7%
Ile	90.2%	4.6%
Leu	107.3%	3.1%
Phe	104.0%	4.0%
Trp	106.2%	2.5%

Conclusion

In order to keep up with the rising demand surrounding peptide-based therapeutics, rapid and reliable methods for quality control are required. For example, amino acid analysis of these macromolecules is utilized to verify the successful synthesis of these drugs. Typically, this is accomplished using acidic or alkaline hydrolysis, requiring a multihour, manual reaction. Here, the Discover Prep was used for the microwave-assisted amino acid hydrolysis of Liraglutide that was synthesized using the CEM Liberty PRO. The resulting amino acid content was compared to the theoretical yield, and it was found that the Discover's recoveries for each amino acid were in the range of 85%-115%, indicating excellent recovery, with standard deviations less than 4.7%, also showing comparable precision to conventional hydrolysis methods. Ultimately, the Discover Prep can serve as a quality control option for peptide laboratories.

References

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