Size-Exclusion Ultra Performance Liquid Chromatography for the Analysis of Covalent High Molecular Weight Insulin

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APPLICATION BENEFITS
- Improved separation of insulin monomer and covalent high molecular weight forms
- Faster analysis times and high throughput SEC separation
- Increased resolution of low molecular weight insulin degradant
- Reduced acetonitrile containing waste-stream volumes

INTRODUCTION
According to the World Diabetes Foundation there were an estimated 285 million people worldwide with diabetes in 2010, which is predicted to grow to 438 million people by 2030 and insulin is the primary treatment for this affliction. In 1982, insulin was the first recombinant biopharmaceutical, and by 2009, worldwide insulin and insulin analog sales were $13.1 billion.1 As part of the US and European Pharmacopoeia (USP and EP), one of the critical quality control attributes for injectable insulin is the control of covalent high molecular weight (HMW) proteins.2, 3 The current USP 34 (p. 3134) and EP (Volume 5, p. 1800) monograph methods for this HMW determination are based on HPLC size-exclusion chromatography (SEC). The USP method prescribes an L20 packing (dihydroxypropylene groups chemically bonded to porous silica particles, 3 to 10 micro-m in diameter) in a 7.8-mm x 30-cm geometry and the EP method prescribes the use of a “hydrophilic silica gel for chromatography R (5-10 μm) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers” with a length of 30 cm and a minimum internal diameter of 7.5 mm.

Presented in this application are the advantages that may be realized using a 125Å pore size, sub-2-μm ethylene-bridged hybrid (BEH) silica packing material and Waters Ultra Performance Liquid Chromatography (UPLC®) instrumentation for this traditional analysis. Among these advantages are faster run times, higher sensitivity, and higher resolving separations of insulin and covalent insulin HMW, while at the same time greatly reducing acetonitrile containing waste-stream volumes.

WATERS SOLUTIONS
ACQUITY UPLC® H-Class Bio System
ACQUITY UPLC BEH125 SEC, 1.7 µm Column
Empower® 2 Software

KEY WORDS
Size-exclusion chromatography, UPLC, insulin, USP, EP, method development, aggregates
EXPERIMENTAL

LC Conditions

System: ACQUITY UPLC H-Class Bio with TUV and titanium flow cell
Wavelength: 276 nm
Column Temp.: 25 °C
Sample Temp.: 10 °C
Injection Volume: 10 µL (unless specified otherwise)
Flow Rate: 0.4 mL/min (unless specified otherwise)
Mobile Phase: L-arginine (1.0 g/L)/acetic acid (99%)/acetonitrile; 65/15/20 (v/v/v)
Wash and Injector Needle Purge: 10% acetonitrile
Seal Wash: 10% methanol
Sample Diluent: 0.01 N hydrogen chloride

Columns

ACQUITY UPLC BEH125 SEC, 1.7 µm, 4.6 x 150 mm (part number 186006505)
ACQUITY UPLC BEH125 SEC, 1.7 µm, 4.6 x 300 mm (part number 186006506)
ACQUITY UPLC BEH200 SEC, 1.7 µm, 4.6 x 300 mm (part number 186005226)
BioSuite™ 125 UHR SEC, 4 µm, 4.6 x 300 mm (part number 186002161)
Insulin HMWP HPLC, 10 µm, 7.8 x 300 mm (part number WAT201549)

Data Management
Software: Empower 2 with Auto•Blend Plus™

SAMPLE PREPARATION

The insulin control sample (Sigma, I2643) was reconstituted and diluted to 4.0 mg/mL in a 0.01 N hydrochloric acid solution. The injectable insulin product samples were analyzed past expiry.

RESULTS AND DISCUSSION

The focus of this application is an evaluation of the performance of the ACQUITY UPLC BEH125 SEC, 1.7 µm, 4.6 x 300 mm column (part number 186006506) under the conditions provided by the USP and EP monographs for the analysis of HMW protein in therapeutic insulin samples. The acidic mobile phase prescribed by both of these pharmacopeial methods is comprised of 0.65 g/L L-arginine, 15% acetic acid, and 20% (v/v) acetonitrile. This mobile phase provides an assessment of the levels of covalent HMW present in these preparations while disrupting non-covalent insulin self-association and column interactions. The column factors that were evaluated for this method include pore-size, particle diameter, and column length.

Selected columns, all of which possess a hydrophilic coating to minimize undesired secondary effects between analyte and particle surface chemistry, were evaluated based on resolution and HMW quantitation. All columns were configured on the same ACQUITY UPLC H-Class Bio System using Waters Empower 2 Software. The resolution results were determined based on the tangent peak widths USP determination and by peak-to-valley ratio (P/V) as directed by both the EP and USP monographs as part of the determination of system suitability. The P/V ratio is obtained by dividing \( H_p \) (height above the baseline of the peak due to the covalent dimer) by \( H_v \) (height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer). The requirement for this system suitability parameter is not less than (NLT) 2.0 for a sample containing more than 0.4% HMW in both the EP and USP monographs.

Pore Size

The insulin size-exclusion HMW determination method in the EP monograph prescribes the use of an SEC pore size of 120Å to 125Å (12 - 12.5 nm), however, the USP monograph does not specifically state a pore size requirement. The ACQUITY UPLC BEH125 SEC, 1.7 µm particles have a pore size of 125Å and, therefore, meet the EP monograph requirements. Figure 1 shows the comparison between the BEH125 SEC, 1.7 µm particle column and the 200Å pore diameter BEH200 SEC 1.7 µm particle column in order to demonstrate the importance of using the appropriate pore diameter for a SEC separation. The principal benefit that can be realized by selecting the optimal pore-size is increased resolution. The critical separation shown in these chromatograms is between the HMW species and the insulin monomer and an 8% improvement in that resolution is observed for 125Å pore-size particles as compared to the 200Å pore-size particles for the standard sample. The improvement observed in the two therapeutic samples, which were analyzed past expiry, is lessened due to the increased
extent of peptide degradation. In addition to an improvement in resolution, the average P/V determined for the two therapeutic samples, which had HMW peak area percentages above 0.4%, was higher for the 125Å pore-size particles (P/V=37) versus the 200Å pore-size particles (P/V=11). However, both columns were able to surpass the US and EP monograph system suitability criterion of a P/V of NLT 2.0 for a sample containing more than 0.4% HMW. Additional improvements in the separation between the covalent insulin dimer HMW form and the multimeric HMW forms, and between the insulin monomer and an insulin fragment, are also observed in the therapeutic samples analyzed using the 125Å pore-size particles that are not observed using the 200Å pore-size particles, thereby providing an additional assessment of sample quality.

ACQUITY UPLC BEH125, 1.7 µm (4.6 x 300 mm)

ACQUITY UPLC BEH200, 1.7 µm (4.6 x 300 mm)

<table>
<thead>
<tr>
<th>Fragment</th>
<th>HMW</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>Rs=3.37</td>
</tr>
<tr>
<td>Sample 1</td>
<td>Rs=2.63</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Rs=2.63</td>
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<table>
<thead>
<tr>
<th>Fragment</th>
<th>HMW</th>
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<tbody>
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<td>Sample 1</td>
<td>Rs=2.38</td>
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<tr>
<td>Sample 2</td>
<td>Rs=2.55</td>
</tr>
</tbody>
</table>

Figure 1. Zoomed view of insulin HMW, monomer, and low molecular weight fragment SEC separations using a 125Å pore-size ACQUITY UPLC BEH125 column and a 200Å pore-size ACQUITY UPLC BEH200 column. Samples 1 and 2 were therapeutic insulin samples analyzed past expiry.

Particle Size

The insulin SEC HMW determination method in the EP monograph prescribes an SEC particle size of 5 to 10 µm while the USP monograph specifies a particle size of 3 to 10 µm. As part of this study, a comparison was performed among three SEC columns with particle sizes of 1.7, 4, and 10 µm that are of comparable 125Å pore size and equivalent 300 mm length (Figure 2). The 1.7-µm column demonstrates a significant increase (>35%) in resolution as compared to the 4- and 10-µm columns. The average HMW P/V system suitability criterion for the two therapeutic samples was also higher for the 1.7-µm column (P/V=37) than for the 4-µm (P/V=9) and 10-µm (P/V=8) columns. All three columns met the EP and USP HMW P/V system suitability criterion of NLT 2. In addition to increased resolution, the 1.7-µm column also provides a smaller total elution volume (~5 mL) than the 4-µm (~6 mL) and 10-µm (~13 mL) columns. This decrease in total elution volume provides the analyst with increased sample throughput in addition to a decrease in mobile-phase use. Shown in Figure 3 is an expanded base-line view comparison of the chromatograms obtained from the 1.7- and 10-µm particle size columns that highlights the dramatic improvements in resolution that are observed for the sub-2-µm UPLC column configuration.
Figure 2. Zoomed view of insulin HMW, monomer, and low molecular weight fragment SEC separations using 125Å pore-size columns with particle sizes of 1.7, 4 and 10 µm. The sample load volume and flow rate for the 10-µm particle size column were 100 µL and 0.5 mL/minute in accordance with the EP monograph method. Samples 1 and 2 were therapeutic insulin samples analyzed past expiry.

Figure 3. Zoomed view of chromatograms from Figure 2 of a biotherapeutic insulin sample (analyzed past expiry) generated by the ACQUITY UPLC BEH125 and the Waters HMWP columns. The complete chromatographic profiles are presented in the insets. The sample load volume and flow rate for the Waters HMWP column were 100 µL and 0.5 mL/minute in accordance with the EP monograph method. The ACQUITY UPLC BEH column provides improved resolution, improved sensitivity, significantly shorter analysis time, and reduced mobile-phase use.
Column Length

The effect of column length was also evaluated by comparing the insulin separation on both a 4.6 x 150 mm and a 4.6 x 300 mm ACQUITY UPLC BEH125 column (Figure 4). Chromatographic principles predict that resolution should be linearly proportional to the square-root of column length and that is what was observed. The 300 mm column provided a 41% to 43% increase in resolution as compared to the 150 mm column, consistent with the predicted increase of 41% (\sqrt{2})\). The average HMW peak-to-valley ratio system suitability criterion for the two therapeutic insulin samples was also higher for the 300 mm column (P/V=37) than for the 150 mm column (P/V=5). Both columns met the EP and USP HMW peak-to-valley ratio system suitability criterion of NLT 2. The improved resolution is also apparent in the monomer peak tail, in which a small, lower molecular weight fragment peak is partially resolved on the 300 mm but not on the 150 mm column. However, it should also be noted that the improved resolution is accompanied by a two-fold increase in analysis time and mobile-phase use.

Figure 4. A comparison of two ACQUITY UPLC BEH125 columns with lengths of 300 mm and 150 mm. The flow rates for this study were equivalent (0.5 mL/minute). The injection volumes were 10 µL and 5 µL for the 300 mm and 150 mm columns, respectively.

Depending on the method requirements, column length can be chosen to either provide improved resolution or higher sample throughput. For example, for a registered quality test a longer column provides improved resolution that can result in better quantitative reliability. While in discovery, development, or during real-time process monitoring, a shorter column allows for faster analysis time and higher sample throughput.
CONCLUSIONS

Size exclusion chromatography is the USP and EP standard method for the analysis of covalent HMW insulin in therapeutic preparations. The chromatographic profiles demonstrating the performance of this method using SEC columns of different pore size, particle size, and length have been presented and these data are summarized in Figure 5. Based on these results the use of 125Å pore size, sub-2-µm ethylene-bridged hybrid (BEH) silica packing material and Waters Ultra Performance Liquid Chromatography (UPLC) instrumentation for this traditional SEC-based analysis provides significant improvements in resolution compared to traditional SE-HPLC methods while reducing analysis time and mobile-phase use.

REFERENCES

2. Insulin, Human, European Directorate for the Quality of Medicines (EDQM), 2001.

Figure 5. A summary of the performance with respect to HMW peak-to-valley ratio (P/V) and USP resolution (Rs) for the columns evaluated. Shown for reference is the minimum P/V required by the EP and USP monograph methods.