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Transfer of the USP Human Insulin Related Compounds HPLC Method to the ACQUITY UPLC System

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Abstract

The process of method transfer can be tedious and time-consuming, especially for a validated method. As a result, many LC methods developed using dated technology are still in use today. However, with the incentive of significant potential improvements in chromatographic separation speed and resolution, taking the necessary steps to transfer an existing protocol over to newer, better performing technology becomes less daunting.

The transfer of the USP (United States Pharmacopeia) method for the related compounds of human insulin is particularly challenging, as it often requires re-optimization for each analysis. In this application note, we outline the successful method transfer steps from the USP-recommended 4.6 x 250 mm, 5 µm L1 Column run on a traditional HPLC system to a Waters ACQUITY UPLC BEH 2.1 x 100 mm, 1.7 µm Column on a Waters ACQUITY UltraPerformance LC System. To optimize the new method, experimental van Deemter curves were generated to determine the optimal flow rate. Due to the high molecular weight of the human insulin molecule (~5800), diffusion into the pores of the packing material is slower. Therefore, the optimal flow rate for the separation is relatively slow and its impact on run time was considered for the final method. Instrument considerations, such as detection parameters for sensitivity and injector carryover performance, were optimized to maximize the benefits of the ACQUITY UPLC System for this method. The resulting UPLC method reduced the overall run time by 60%, with improved resolution. The data meets or exceeds acceptance criteria.

Introduction

Human insulin is a relatively small protein containing 51 amino acids in two polypeptide chains. The drug product is a biopharmaceutical synthesized through recombinant DNA technology. The insulin made by these cells is identical to the insulin made by the human pancreas. The USP method for the related compounds of human insulin is a 68-minute separation involving the isocratic elution of the main component (within a specified retention window of 15–25 minutes) and the major related compound (A21 desamido insulin), followed by the gradient elution of the high MW impurities.[1] The run is followed by a re-equilibration step which is typically 23 minutes (5x column and 3x system volume). The elution time of the main insulin peak is extremely sensitive to the amount of organic modifier in the mobile phase. Adjustments to the gradient table are necessary for each analysis to ensure that the main insulin peak is eluted within the

specified retention window to achieve sufficient resolution with A21 desamido inslulin peak. A change of less than 0.5% of the organic modifier can cause the peak to elute outside of this retention window, making the on-line mixing and gradient performance of the instrumentation critical for this application. The method also utilizes high salt concentrations at low pH. Small changes in either of these parameters impact peak shape and selectivity. Elution times are also sensitive to changes in temperature. A difference of 1–2 °C can push the retention of the main insulin peak outside of the specified window. Injector carryover is also an important consideration. Sample concentrations of up to 4 mg/mL are used to measure impurities and managing carryover can be challenging for HPLC injectors.

A method is desired which will reduce the run time. Time is crucial since samples and standards are only useable for up to 12 hours. The current USP method with a total analysis time of 91 minutes is long, considering the optimization time required before samples can be analyzed. However, any reduction of analysis time cannot sacrifice the performance of the method. Precision and carryover must still meet requirements.

Experimental

Method Scaling Equations

The HPLC to UPLC method transfer process can be streamlined by using a series of equations to geometrically scale the original method to the new column dimensions.[2] These equations account for changes in gradient times, flow rate, and injection volume. They do not compensate for changes in system volume, column selectivity or column load (mass or volume). To start, the gradient steps are scaled from the HPLC column to the UPLC column:

Geometrically Scaled Gradient Time =

(UPLC Column Length/HPLC Column Length) * HPLC Gradient Time

Next, the flow rate is scaled taking into account the difference in the internal diameters of the columns.

Geometrically Scaled Flow Rate =

$[(\text{UPLC Column Diameter})^2/(\text{HPLC Column Diameter})^2]$ * HPLC Gradient Time

The flow rate should then be further scaled to take into account the new optimal linear velocity of the separation using the smaller 1.7 µm particles.

For 2.1 mm i.d. columns, appropriate starting points are typically 650 µL/min for small molecules and 100 µL/min for high MW compounds. To keep the column volumes proportional, the gradient steps should be readjusted for the new flow rate.

UPLC Gradient Times =

(Scaled Flow Rate * Gradient Time)/UPLC Flow Rate

The injection volume is scaled taking into account the volume of the columns.

UPLC Injection Volume =

(UPLC Column Volume/HPLC Column Volume) * HPLC Injection Volume

Alternatively, the ACQUITY UPLC System Console Calculator (Figure 1) will scale the method automatically using the same scaling principles outlined here.

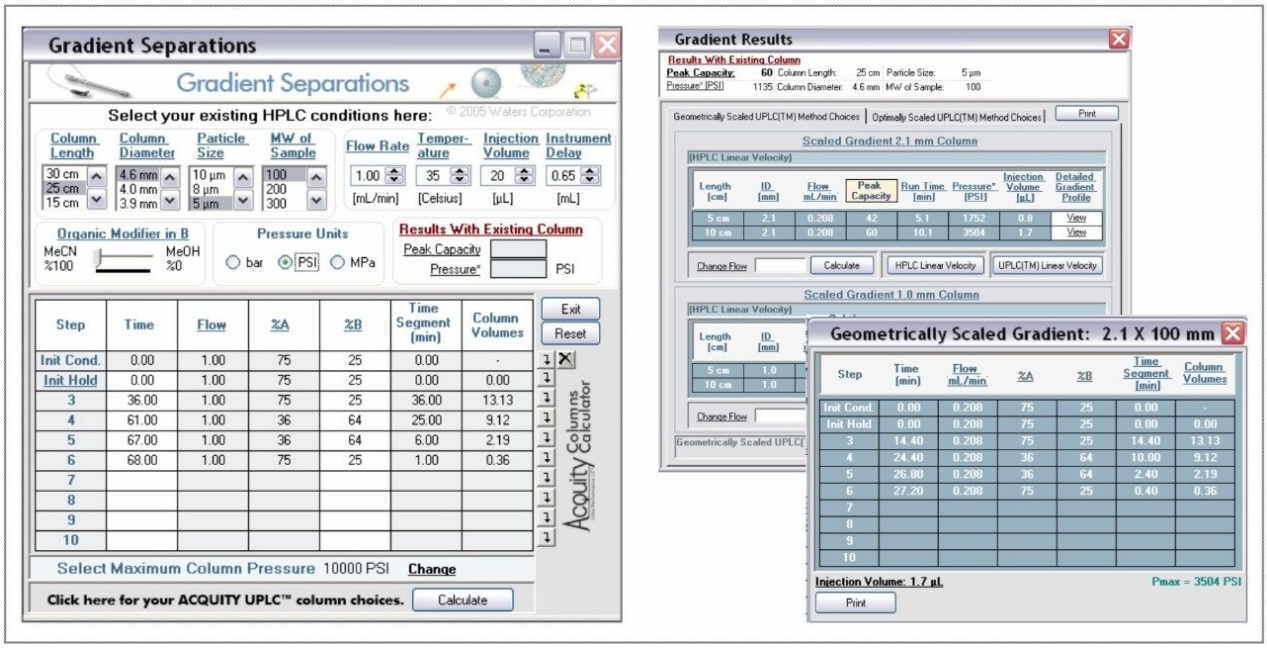

Figure 1. The ACQUITY UPLC System Console Calculator.

Original USP HPLC Method

System:	Alliance 2695 XC Separations Module
	2996 Photodiode Array Detector

	Empower Software
Column:	Symmetry 300 C_{18}, 4.6 mm x 250 mm, 5 µm
Detection:	214 nm, 1.2 nm digital bandwidth 1 pt/sec, 1.0 sec time constant
Eluents:	Buffer: 0.2 M Sodium sulphate, pH 2.3 A: 82/18 Buffer/Acetonitrile B: 50/50 Buffer/Acetonitrile
Gradient:	0–36 min isocratic at 24.5% B 36–61 min linear gradient to 64% B 61–67 min isocratic at 64% B 67–68 min linear gradient to 24.5% B
RT window:	15–25 min
Flow rate:	1 mL/min
Temp:	35 °C
Sample:	USP Human Insulin Reference Standard 3.75 mg/mL in 0.01 M Hydrochloric acid
Volume:	20 µL
Wash:	Extended Wash Cycle with 6:3:1 of 0.1% Phosphoric acid : Acetonitrile : Isopropyl alcohol

Final Transferred UPLC Method

System:	ACQUITY UPLC System with Photodiode Array

	Detector
	Empower Software
Column:	ACQUITY UPLC BEH C_{18}, 2.1 mm x 100 mm, 1.7 µm
Detection:	214 nm, 12.0 nm digital bandwidth
	5 pts/sec, 0.3 sec time constant
Eluents:	Buffer: 0.2 M Sodium sulphate, pH 2.3
	A: 82/18 Buffer/Acetonitrile
	B: 50/50 Buffer/Acetonitrile
Gradient:	0–14.4 min isocratic at 26% B
	14.4–24.4 min linear gradient to 64% B
	24.4–26.8 min isocratic at 64% B
	26.8–27.2 min linear gradient to 26% B
RT window:	6–10 min
Flow rate:	208 µL/min
Temp:	35 °C
Sample:	USP Human Insulin Reference Standard
	1.25 mg/mL in 0.01 M Hydrochloric acid
Volume:	1.8 µL characterized full loop
Strong wash:	200 µL of 6:3:1 of 0.1% Phosphoric acid : Acetonitrile : Isopropyl alcohol
Weak wash:	1200 µL of 0.01 M Hydrochloric acid

Results and Discussion

The USP method was scaled geometrically and then optimized, according to the USP specifications, to have the main insulin peak elute within the required retention window. This process involved increasing the organic modifier composition by 1.5% for the isocratic portion of the separation. The scaled injection volume and sample concentration were adjusted such that the height of the main insulin peak was within the linear range of the detector. The resulting UPLC method had a run time of 27.2 minutes (a 60% time reduction compared to the HPLC method), with a total analysis time of 37.5 minutes (compared to the 91-minute HPLC analysis –a time reduction of 54 minutes).

To determine the optimal flow rate for the UPLC column, a plot of the number of theoretical plates versus flow rate was generated (Figure 4). For small molecules, the optimal flow rate for a 2.1 mm i.d. column packed with 1.7 µm particles is typically around 650 µL/min. However, for larger molecules the optimal flow rate is lower, and for the human insulin method it was under 100 µL/min. Since the goal of this method transfer is to improve run time, the final method was chosen to operate above the optimal flow rate.

Even when operating above the optimum flow rate for the separation, improvements in resolution were observed. The relative peak widths for the UPLC separation (Figure 3) were narrower than those of the orginal HPLC method (Figure 2). Improved resolution was attained with an impurity in the tail of the main insulin peak, as well as with some of the other lower level impurities. The HPLC method had resolution between the main insulin peak and the A21 desamido insulin peak of 5.0, while the UPLC method had a resolution of 6.8.

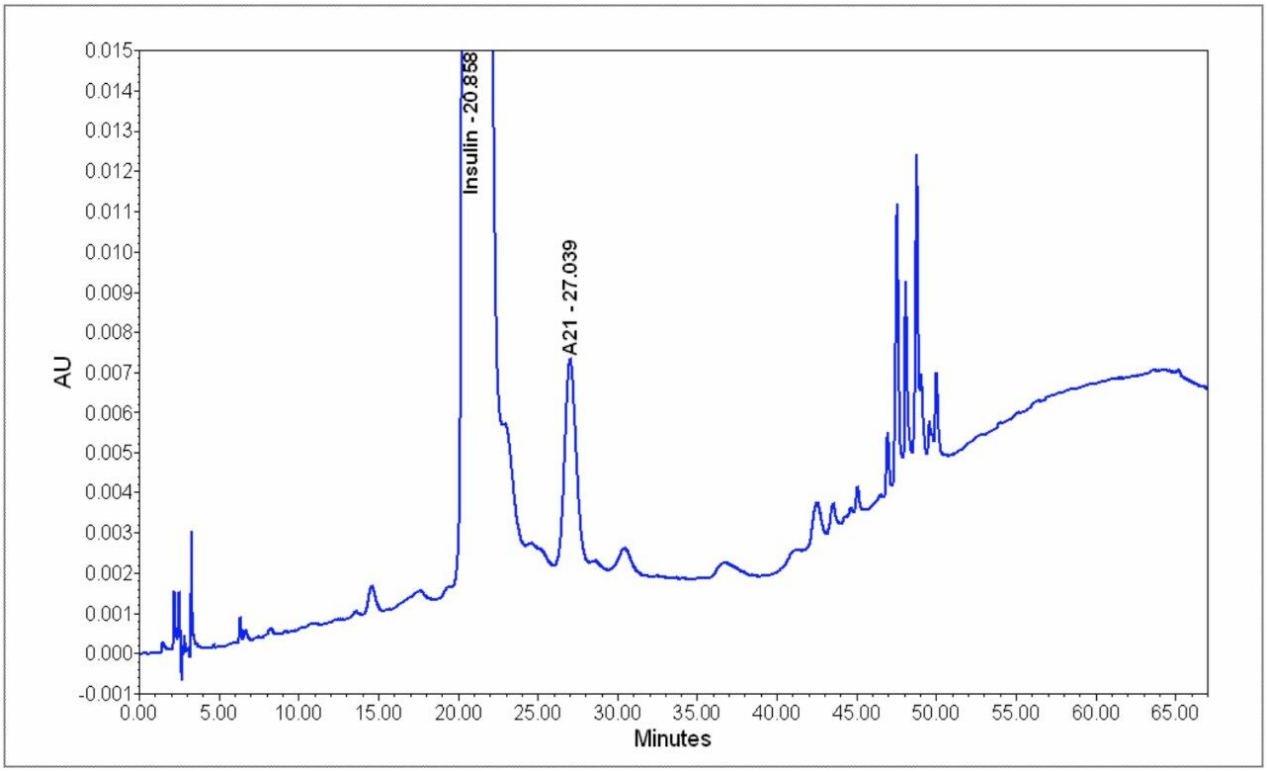

Figure 2. Chromatogram of the 68-minute USP HPLC method for the human insulin related compounds assay.

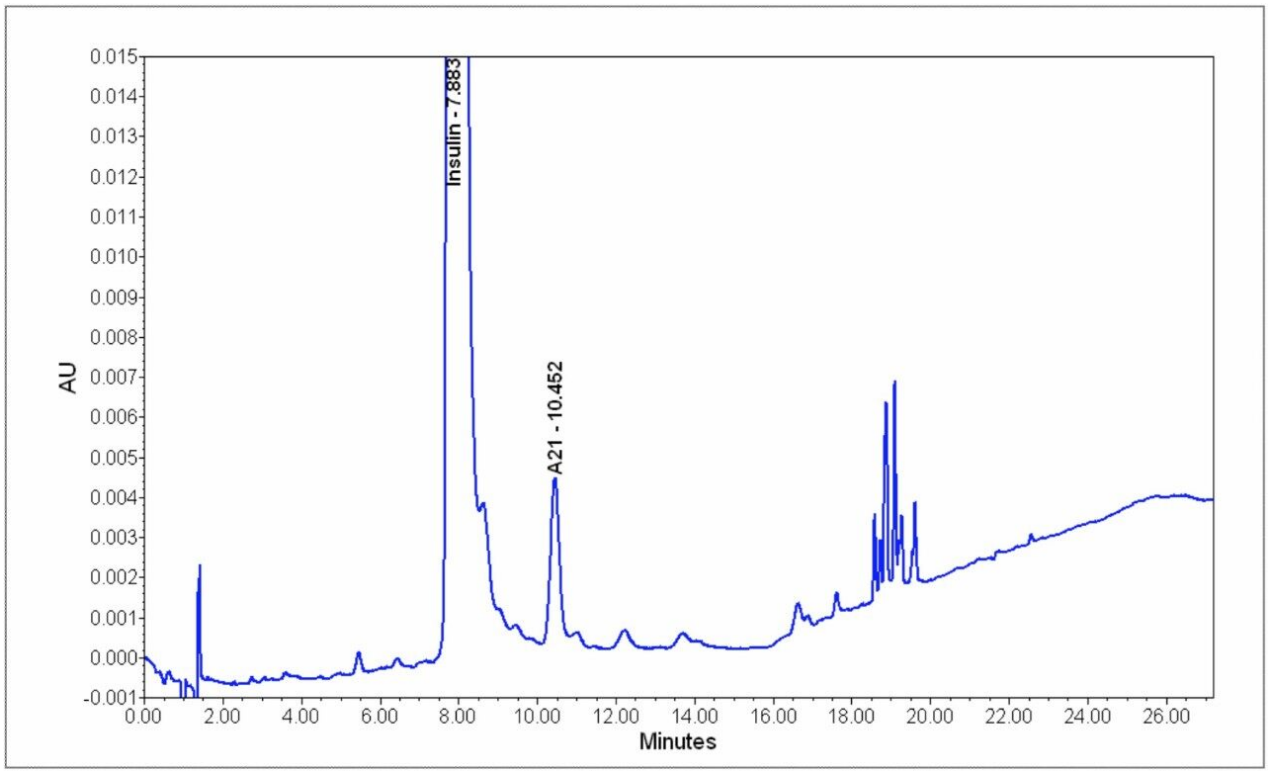

Figure 3. Chromatogram of the 27-minute UPLC method for the human insulin related compounds assay.

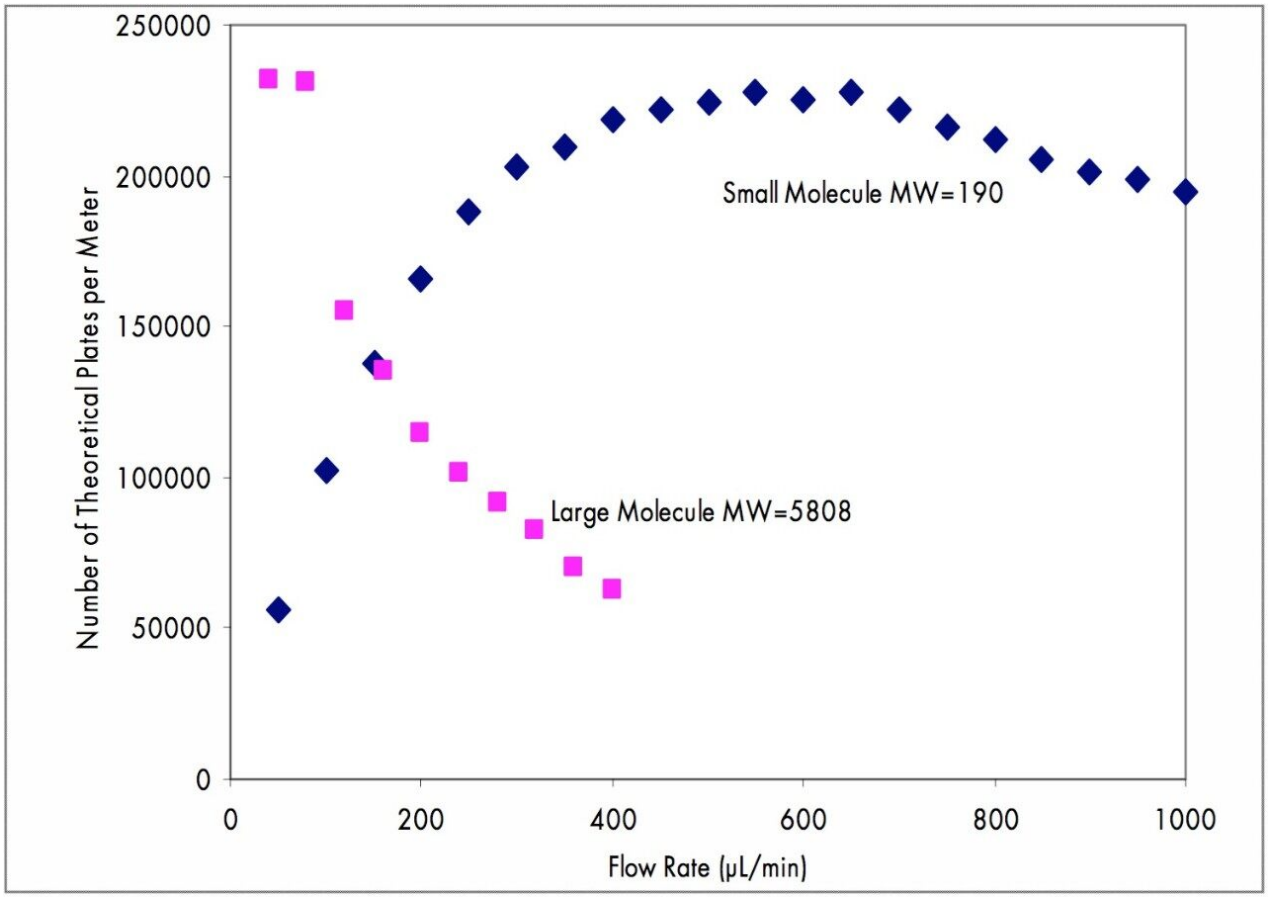

Figure 4. Dependence of the number of theoretical plates on the flow rate.

Signal-to-noise for the A21 desamido insulin peak was 100:1. The narrow peaks generated by the UPLC separation required the detection parameters to be re-optimized. The data rate was increased and the filtering constant decreased to be more compatible with the chromatography and to maximize sensitvity.

The performance of the human insulin method on the ACQUITY UPLC System was excellent (Table 1), with precision values far below typical requirements. Retention time reproducibility was under 0.5% at 0.23%. The area reproducibility of the main insulin peak was below 1% at 0.21% and the A21 desamido insulin peak was well below 10% at only 1.06%.

Peak	Retention Time %RSD	Peak Area %RSD
Human Insulin	0.23	0.21
A21 Desamido Insulin	0.23	1.06

Table 1. Reproducibility values for the human insulin UPLC method.

The dual wash capabilities of the ACQUITY UPLC System efficiently removes residual sample from the system, resulting in negligible carryover. The wash cycle does not contribute to injection cycle time as it occurs post-injection, while the sample is running.

To measure carryover, a set of high concentration standards (10 mg/mL) was used. The injection sequence was 2 blanks, 3 standards at 0.005% (carryover specification), 6 high concentration standards, and 3 blanks. Table 2 shows the first injection after the blank had a carryover of 0.004% and by the third blank injection, no insulin could be detected. Figure 5 shows the high concentration standard compared to the first blank injection

Injection	Average Peak Height mAU	%Carryover
Standard	1.552	0.0050%
Blank 1	1.109	0.0036%
Blank 2	0.334	0.0010%
Blank 3	none detected	none detected

Table 2. Carryover of human insulin in subsequent blank injections.

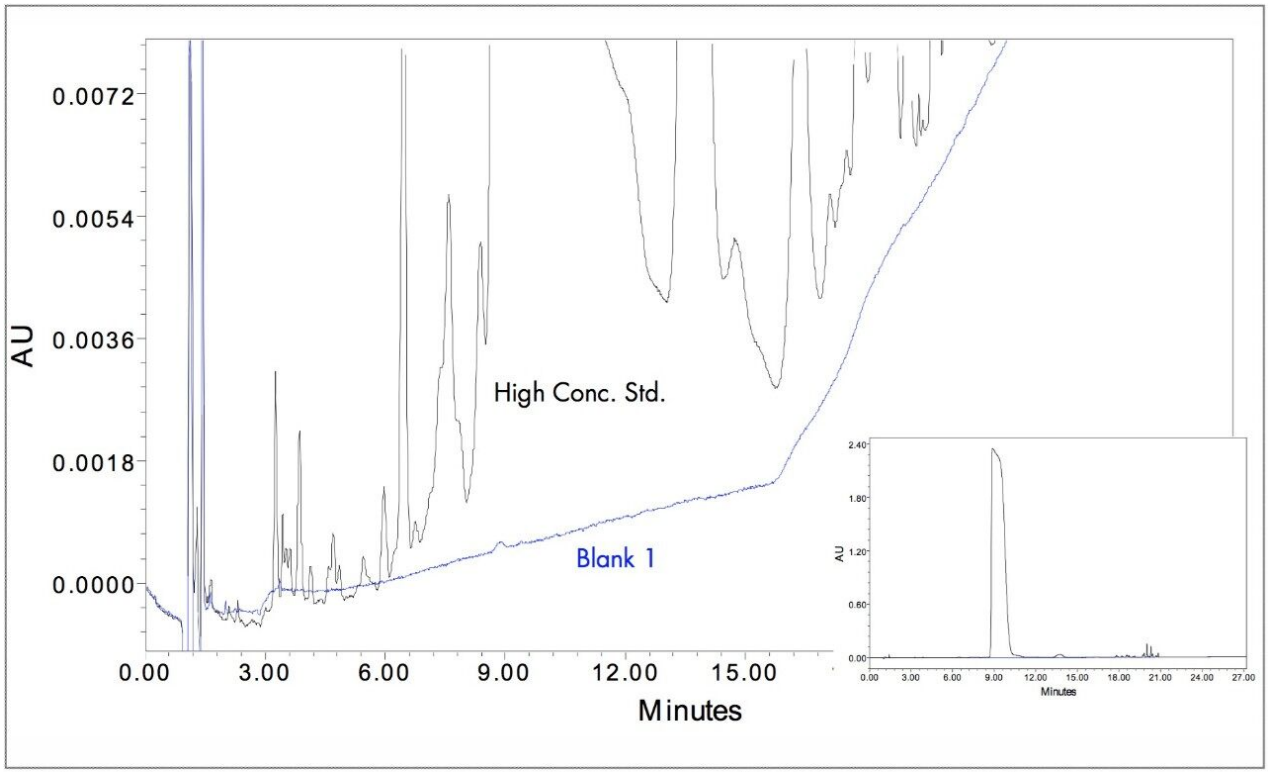

Figure 5. Carryover of human insulin on the ACQUITY UPLC System.

Conclusion

We have shown that challenging USP HPLC methods can be successfully transferred to the Waters ACQUITY UPLC System, with numerous chromatographic performance benefits.Transfer of the human insulin method was accomplished by simple scaling with an adjustment to the sample concentration and injection volume. The gradient table was adjusted for the new method according to the USP guidelines. The new UPLC method offered a 60% reduction in run time over the USP HPLC method (a 54-minute reduction in total analysis time) with improved resolution. UPLC reproducibility for both peak area and retention time was very good, especially for such a chromatographically sensitive sample. In addition, sample carryover was easily managed and minimized by the ACQUITY UPLC System. This exercise clearly demonstrates the advantages that can be realized by transferring from HPLC to UPLC methodology.

References

1. The United States Pharmacopeia USP28, The National Formulary NF23. United States Pharmacopeial Convention, Inc. 2004, pg. 1022.2.
2. Grumbach, E., Wheat, T., and Mazzeo, J. An Efficient HPLC to UPLC Method Transfer Protocol. Poster presented at HPLC 2005, Waters Literature Code WA41923.

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