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TRANSFER OF THE USP HUMAN INSULIN RELATED COMPOUNDS METHOD FROM HPLC TO UPLC™

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OVERVIEW

The process of method transfer can be very tedious and time consuming especially for a validated method and therefore many methods still exist that were developed using dated technology. However, significant improvements in separation speed and resolution can make it very advantageous to transfer an existing method to new technology. The USP human insulin related compounds method, with a run time of 67 minutes, can be relatively difficult, often requiring re-optimization for each analysis. Transfer of the method from the recommended 4.6 x 250mm, 5µm L1 column to a 2.1 x 100mm, 1.7µm ACQUITY BEH column on the ACQUITY UPLC™ system results in a 60% decrease in run time with improved resolution. To optimize the new method, experimental van Deemter curves were generated to determine the optimal linear velocity for the separation. Due to the high molecular weight of the human insulin molecule (~5800) the optimal linear velocity 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.

INTRODUCTION

Human insulin is a relatively small protein containing 51 amino acids in 2 polypeptide chains. The protein is manufactured 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 can be challenging to both the user and the instrumentation. It utilizes high salt concentrations at low pH and the peak shape and selectivity can be affected by very small changes in these parameters. The method is a 68 minute separation involving the isocratic elution of the main peak, the gradient elution of the high MW impurities followed by a 23 minute re-equilibration step (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. Optimization of the method is required for each analysis to ensure the elution of the main insulin peak is within the specified retention window to achieve sufficient resolution with the A21 desamido impurity. A change in less than 0.5% 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. Injector carryover is also an important consideration. Sample concentrations of up to 4mg/mL are used for looking at impurities and managing carryover can be challenging for HPLC injectors.

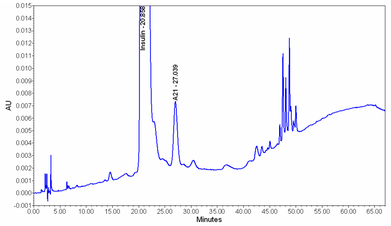

Figure 1 — HPLC USP Method for Human Insulin Related Compounds.

METHODS

Method Scaling Equations

The method transfer process can be a very challenging endeavor, however using a series of equation to geometrically scale the original method to the new column dimensions is a good starting point. These equations take into account the changes in the gradient times, the flow rate, and the injection volume but do not compensate for changes in system volume or column selectivity and load. The gradient steps are scaled from the HPLC column to the UPLC column using:

$$L_2/L_1 \times t_{g1} = t_{g2}$$

Where L_1 and L_2 are the lengths of the HPLC and UPLC columns and t_{g1} and t_{g2} are the times of each gradient step respectively. Flow rate is scaled taking into account the difference in the diameters of the two columns:

$$(d_2)^2/(d_1)^2 \times F_1 = F_2$$

Where d_1 and d_2 are the column diameters and F_1 and F_2 are the flow rates. The flow rate should be optimized for the smaller particles (for 2.1 mm i.d. columns, typically 650 µL/min for small molecules and 100 µL/min for high MW compounds are appropriate starting points). To keep the column volumes proportional, the gradient steps should be readjusted for the new flow rate:

$$(F_2 \times t_{g2})/F_3 = t_{g3}$$

Where F_2 and t_{g2} are the flow rate and gradient times are the geometrically scaled values and F_3 and t_{g3} are the optimized values. The injection volume is scaled taking into account the volume of the columns:

$$V_1 \times [(r_2^2 \times L_2) / (r_1^2 \times L_1)] = V_2$$

Where r_1 and r_2 are the radii of the columns, L_1 and L_2 are the lengths of the columns and V_1 and V_2 are the injection volumes.

Alternatively, the ACQUITY UPLC calculator will scale the method using the same scaling theory.

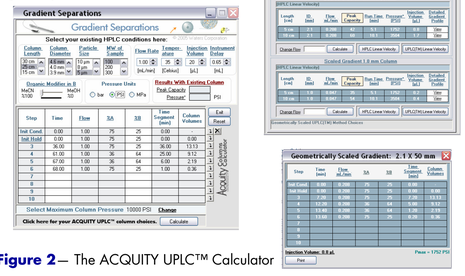

Figure 2— The ACQUITY UPLC™ Calculator

Original USP HPLC Method

System:	Alliance® 2695 XC Separations Module 2996 Photodiode Array Detector Empower™ Software
Column:	Symmetry® 300 C_{18}, 4.6mmx250mm, 5µm
Detection:	214nm, 1.2 nm bandwidth 1pt/s, 1.0s Time Constant
Eluents:	Buffer: 0.2M Sodium Sulphate, pH 2.3 A: 82/18 Buffer/ACN B: 50/50 Buffer/ACN
Gradient:	0-36 minutes isocratic at 24.5% B 36-61 minutes linear gradient to 64% B 61-67 minutes isocratic at 64% B 67-68 minutes linear gradient to 24.5% B
RT Window:	15-25 minutes
Flow Rate:	1 mL/min
Temp:	35°C
Sample:	USP Human Insulin Reference Standard 3.75 mg/mL in 0.01M HCl
Volume:	20µL
Wash:	Extended Wash Cycle

Converted UPLC™ Method

System:	ACQUITY UPLC™ System with PDA Empower™ Software
Column:	ACQUITY UPLC™ BEH C_{18},2.1mm x 100mm, 1.7µm

Detection:	214nm, 12.0nm bandwidth 5pts/s, 0.3s Time Constant
Eluents:	Buffer: 0.2M Sodium Sulphate, pH 2.3 A: 82/18 Buffer/ACN B: 50/50 Buffer/ACN
Gradient:	0-14.4 minutes isocratic at 26% B 14.4-24.4 minutes linear gradient to 64% B 24.4-26.8 minutes isocratic at 64% B 26.8-27.2 minutes linear gradient to 26% B
RT Window:	6-10 minutes
Flow Rate:	208 µL/min
Temp:	35°C
Sample:	USP Human Insulin Reference Standard 1.25 mg/mL in 0.01M HCl
Volume:	1.8 µL Characterized Full Loop
Strong Wash:	200 µL of 6:3:1 0.1% Phosphoric Acid:ACN:IPA
Weak Wash:	1200µL of 0.01M HCl

RESULTS AND DISCUSSION

The USP method was scaled geometrically and them optimized according to the specifications to have the main insulin peak elute within the required retention window. The concentration of the sample needed to be adjusted to have the intensity of the main insulin peak within in the linear range of the detector,. The resulting method had a run time of 27.2 minutes (60% reduction) with a total analysis time of 37.5 minutes (60% reduction or 54 minutes).

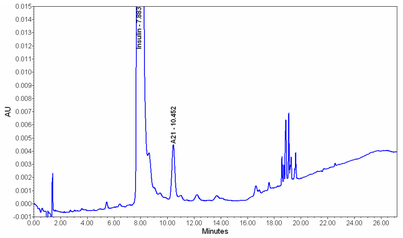

Figure 3—Chromatogram of the USP UPLC Method for the Human Insulin Related Compounds Assay.

For this separation the scaled flow rate was used but it may not necessarily be at the optimal linear velocity for the new smaller particles. To determine the optimal flow rate a plot of the number of theoretical plates versus the linear velocity was generated. For small molecules the optimal linear velocity typically is around 650 µL/min, however for larger molecules the optimal linear velocity is much lower, around 100 µL/min or less. However, for this transfer a reduction in run time was desired and therefore the method using the geometrically scaled flow rate was preferred due to the much shorter run time, especially since improvements in resolution were observed even though the linear velocity was not optimal.

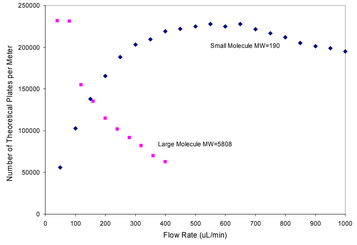

Figure 4—Dependence of the number of theoretical plates on the flow rate.

The improvements in resolution can be observed visually (narrower peaks and improved resolution in the tail of the main insulin peak). The HPLC method had resolution between the main insulin peak and the A21 impurity of 5.0, while the new ACQUITY UPLC method had a resolution of 6.8.

The dual wash capabilities of the ACQUITY UPLC system can efficiently remove residual sample from the system resulting in very low carryover. To measure carryover, a concentrated sample (10x) was injected. A standard of such high concentration was needed to be able to see the carryover. The injection sequence was 2 blanks, 3 standards @ 0.005% (carryover specification), 6 high concentration standards, and 3 blanks. The first injection after the blank displayed very low carryover and by the third blank injection no insulin could be detected.

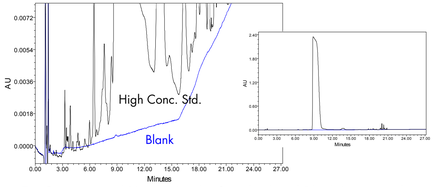

Figure 5—Concentrated Human Insulin Standard and Blank Injection showing the low carryover of the ACQUITY UPLC system.

Injection	Average Peak Height (mAU)
0.005% Standard	1.552
Blank 1	1.109
Blank 2	0.334
Blank 3	none detected

Table 1 — Carryover of human insulin in subsequent blank injections.

The reproducibility of the human insulin method on the ACQUITY UPLC system is extremely good. Both retention time and peak area reproducibilities for the main insulin peak and the A21 desamido peaks are well within method requirements.

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

Table 2—Reproducibility values for human insulin and its major impurity.

Signal-to-Noise levels for the main impurity peak were very good at 100:1, with an average peak-to-peak noise of 45µAU. When the method was transferred to UPLC from the original HPLC method, the data rate and filtering constants needed to be adjusted to be compatible with the narrower peak widths. These changes will result in an increase in noise and therefore the bandwidth was adjusted to compensate.

CONCLUSIONS

- Difficult USP related compounds assays can be successfully transferred to the ACQUITY UPLC system.
- Significant improvements in run time were achieved for the human insulin related compounds method (60% or 54 minutes reduction in analysis time).
- Reproducibility for both peak area and retention time is well below the required levels.
- Injector carryover is very low even for such a difficult analyte.

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