LIQUID CHROMATOGRAPHIC GRADIENT METHOD ALLOWANCES PROVIDED BY GENERAL CHAPTER, USP CHROMATOGRAPHY <621>

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INTRODUCTION

The U.S. Pharmacopeia (USP) portfolio of solutions addresses quality assurance, enhances regulatory predictability, and helps manufacturers distribute quality medicines, dietary supplements and foods. On Dec 1, 2022, a harmonized standard for General Chapter <621> Chromatography was released. This standard incorporates <621> Chromatography (USP), 2.2.46. Chromatographic Separation Techniques (EuPh) and 2.01 Liquid Chromatography (JP) texts. Additions provide limits of flexibility for liquid chromatographic gradient method separation parameters such particle size, flow rate, gradient slope, and injection volume. In this poster, we implement the gradient method adjustments described in U.S. Pharmacopeia (USP) General Chapter <621> to apply method modernization for an antiviral drug impurities monograph.

METHODS

Method Adjustment Guidance:	USP General Chapter <621>, Official Dec1, 2022
USP Monograph:	Abacavir Sulfate, Organic Impurities
Columns:	Symmetry™ C18 Column,3.9x150mm, 5 µm, 100Å, p/n:WAT046980 Symmetry C18 Column, 4.6x150mm, 5 µm, 100Å, p/n: WAT045905 Symmetry C18 Column, 4.6x100mm, 3.5 µm, 100Å p/n: WAT066220 XBridge™ C18 Column, 4.6x100mm, 3.5 µm,100Å, p/n: 1860033 XSelect™ HSS T3 Column, 4.6x150mm, 3.5 µm, 100Å, p/n: 186004786 Symmetry C18 Column, 2.1 x 100mm, 3.5um,130Å, p/n:186005256 XSelect HSS T3 Column, 2.1 x 75mm, 2.5um,100Å, p/n: 186005644 XBridge BEH C18 Column, 2.1 x 75mm, 2.5um, 130Å, p/n:186006030 Symmetry C18 Column, 3.0x150 mm, 3.5 µm, p/n:18600695
Software:	Empower™ 3 Chromatography Data System (CDS)
Sample:	Abacavir Sulfate System Suitability Test Mixture (SST) Mixture (p/n 1000500, USP)

The System Suitability Test Mixture (SST) was prepared according to the USP monograph for antiviral compound, abacavir sulfate. Specifications for column size and flow rate were recorded for the LC instrumentation shown in **Figure 1**.

Figure 1. *LC instrumentation employed when implementing the USP <621> gradient method adjustment guidance.*

RESULTS AND DISCUSSION

A systematic approach was implemented for modernization of the USP, abacavir sulfate monograph separation. First, the monograph column was identified as an L1 stationary phase substituent with a 5 µm particle size and 3.9 x 150 mm column hardware. Modern 4.6 mm and 2.1 diameter column hardware, equipped with L1 stationary phase substituent was selected in lengths (L) of 75 mm, 100 mm, and 150 mm to perform method modernization. The column stationary phase substituent was of 5 µm, 3.5 µm, or 2.5 µm particle size (d_p). In all instances, the USP <621> guidance L/d_p ratio allowance was met at -25% to +50% of the monograph ratio for all of the particle size and column dimension combinations employed.

The flow rate, injection volume, and gradient start times were mathematically adjusted for the modern dimension target columns, according to the equations provided in the USP <621> guidance. First, **Equation 1**, **(A)** was used to maintain the linear velocity of the monograph separation by adjusting the flow rate. Second, the injection volume was adjusted according to **Equation 1, (B)** to maintain the ratio of the analyte to column volume. Finally, gradient start times were adjusted in **Equation 2** according to the calculated target column flow rate, length, and particle size. This equation preserved the gradient slope to column volume ratio utilized in the monograph.

$$F_2 = F_1 X \left[\frac{(d_{c2}^2 \times dp_1)}{(d_{c1}^2 \times dp_2)}\right] = \frac{1.000\ mL}{min} X \left[\frac{(2.1\ mm^2 \times 5\ \mu m)}{(3.9\ mm^2 \times 2.5\ \mu m)}\right] = 0.580\ mL/min$$

$$V_{inj2} = V_{inj1} X \left[\frac{(L_2\ dc_2^2)}{(L_1\ dc_1^2)}\right] = 20\ \mu L\ X \left[\frac{(100\ mm \times 4.6\ mm^2)}{(150\ mm \times 3.9\ mm^2)}\right] = 18.5\ \mu L$$

F_1 = Monograph flow rate (mL/min)
F_2 = Adjusted flow rate (mL/min)
dc_1 = Internal diameter monograph column (mm)
dc_2 = Internal diameter target column (mm)
dp_1 = Particle size monograph column (µm)
dp_2 = Particle size target column (µm)

V_{inj1} = Monograph injection volume (µL)
V_{inj2} = Adjusted injection volume (µL)
L_1 = Length monograph column (mm)
L_2 = Length target column (mm)
dc_1 = Internal diameter monograph column (mm)
dc_2 = Internal diameter target column (mm)

Equation 1: (A) *Adjustment for flow rate and* ***(B)*** *injection volume when moving from the 3.9 x 150 mm, 5 µm monograph column to 2.1 x 75 mm, 2.5 µm column dimensions.*

$$t_{G2} = t_{G1} x \left(\frac{F_1}{F_2}\right) \left[\frac{(L_2 \times d_{c2}^2)}{(L_1 \times d_{c1}^2)}\right] = t_{G1} x \left(\frac{1.000\ mL/min}{0.580\ mL/min}\right) \left[\frac{(75 \times 2.1\ mm^2)}{(150 \times 3.9\ mm^2)}\right] = 0.250$$

t_{G1} = Time monograph gradient (min)
t_{G2} = Time adjusted gradient (min)
F_1 = Monograph flow rate (mL/min)
F_2 = Adjusted flow rate (mL/min)
d_{c1} = Diameter monograph column (mm)
d_{c2} = Diameter target column (mm)

Time (min)	% B
0 min	5
20 min	30
35 min	90
35.1 min	5
50 min	5

Target Column Adjusted Time	% B
0 min	5
0 min + (20 min *0.250) = 5.00 min	30
5.00 min + (15 min *0.250) = 8.75 min	90
8.75 min + (0.1 min *0.250) = 8.78 min	5
8.78 min + (14.9 min*0.250) = 12.50 min	5

Equation 2: *Adjustment of gradient time when moving from the monograph column to modern, 2.1 x 75 mm, 2.5 µm column dimensions.*

Although not defined un USP <621>, **Equation 3** was applied to the gradient (**Table 1**) to account for the 15 fold difference in instrument dwell volume, and over 6 fold difference in column void volume, when moving from a HPLC to UHPLC platform. The monograph did not provide an isocratic step before the start of the gradient, therefore the equation for gradient dwell volume provided in USP <621> was not employed in our study.

Manual gradient calculations for the target columns were confirmed using both the Waters Preparative OBD Column Calculator, and the Columns Calculator 2.0 at *www.waters.com*. Online calculators were especially important because they provided an estimated maximum gradient backpressure for the adjusted gradients. Although computed for 100% organic mobile phase composition, rather than 85% organic starting composition (monograph), the estimation added confidence that the adjusted methods were in range of the upper backpressure limit for the HPLC/UHPLC Systems.

$$\mathbf{Offset = Vd_{Target\ Instrument} - \{Vd_{Original\ Instrument} x \left[\frac{(V0_{Target\ Instrument})}{(V0_{Original\ Instrument})}\right]\}}$$

Vd = Dwell Volume
V0 = Column void volume = 0.66 x V Where $V = L\pi\left(\frac{D}{2}\right)^2$

V = Empty volume (mL)
L = Column length (cm)
D = Column diameter (cm)

$$\text{Offset} = 0.073\ mL - \{1.145\ mL\ x\ (\frac{0.171 mL}{1.182 mL})\} = \mathbf{0.0937\ mL}$$

Option 1:

Set Empower™ Software to hold 93.7 µL after injection (Waters' Columns Calculator)

0.0937 mL offset = 93.7 µL

Option 2:

Add 0.17 mins to all steps in the gradient
(as shown in Waters Preparative OBD Column Calculator)

0.0937 mL offset / 0.580 mL/min = 0.17 mins

Equation 3: *Offset adjustment of gradient time when moving from the 3.9 x 150 mm, 5 µm HPLC monograph platform to the 2.1 x 75 mm, 2.5 µm UHPLC platform. This step is not defined in USP <621>, therefore it is for information only.*

Adjust HPLC platform to Target UHPLC Platform (Column + Instrument)	
ACQUITY™UPLC™ I-Class System Backpressure Max 18,000 psi. Dwell: 0.073 mL	
2.1 x 75 mm, 2.5 µm	
Gradient Option 1:	**Gradient Option 2:**
0 min with 93.7 µL Hold after injection (**Empower Software**)	0 min
5.00 min	5.00 min + 0.17 min = 5.17 min
8.75 min	8.75 min + 0.17 min = 8.92 min
8.78 min	8.78 min + 0.17 min = 8.94 min
12.5 min	12.5 min + 0.17 min = 12.67 min
Predicted Scaled Backpressure ~12,000 psi	

Table 1: *HPLC to UHPLC platform gradient method adjustment.*

For all column dimensions employed, the monograph system suitability resolution requirement of Not Less Than (NLT) 1.5 for the impurity critical pair was successfully achieved for the system suitability impurities mixture (SST) after applying gradient method adjustments (**Figure 2**).

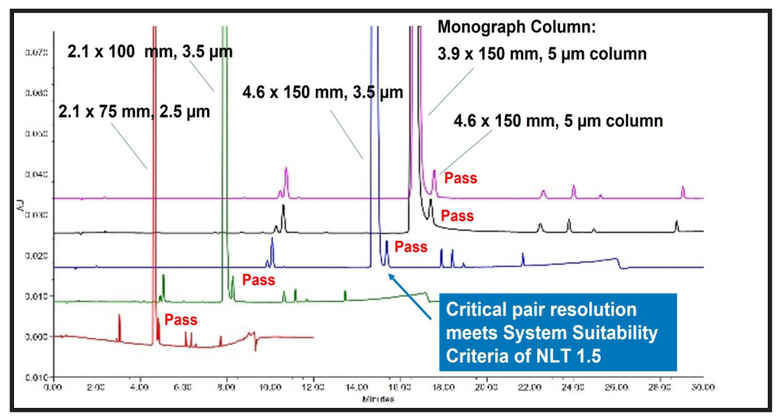

Figure 2. *Overlay of the chromatography for the various modernized column dimensions which fall within the USP guidance recommended* L/d_p *range.*

When the SST mixture relative retention times (RRTs) were compared to those generated with the stationary phase utilized for the initial monograph separation, RRTs were most similar for columns of the same L1 stationary phase substituents (**Figure 3).** As noted in USP <621>, peak deletions and/or inversions, may be observed between various L1 substituents, therefore chromatographic peak order was confirmed after method adjustment using PDA spectral analysis.

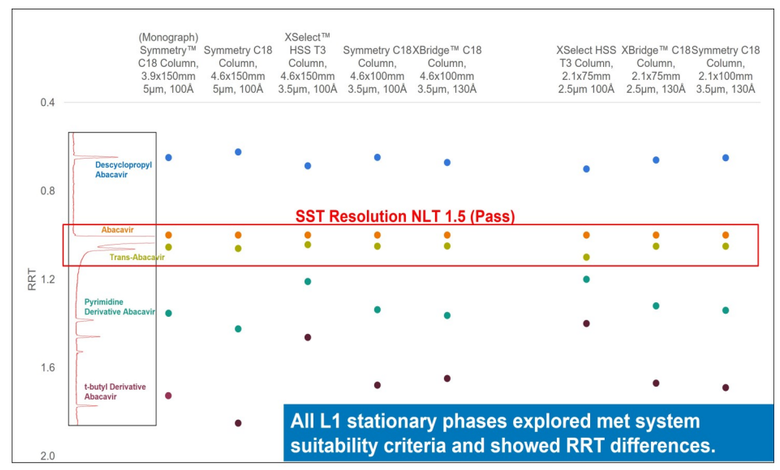

Figure 3. *Comparison of the RRTs for the system suitability impurities mixture analyzed by HPLC and UHPLC platforms.*

After implementing chromatographic method adjustments as per USP <621>, the chromatography passed the original monograph system suitability requirements of NLT 1.5, for the resolution of the abacavir and trans-abacavir sulfate. With the L1 column substituents employed for this study, peak inversions or co-elution did not occur. If peak interference was observed after column dimension modernization, an alternate L1 column stationary phase substituent, which passed SST criteria, would have been selected to proceed with sample testing. In this study, method adjustments provided flexibility for implementation of modern LC platforms (column dimensions and instrumentation) resulting in reduced run times, mobile phase and sample usage, with an overall increase in the number of sample runs per hour **(Table 2)**.

USP MONOGRAPH Abacavir Sulfate	Adjustment Savings	
Alliance HPLC System	**Alliance iS HPLC System**	**ACQUITY UPLC™ I-Class PLUS System**
3.9 x 150 mm, 5 µm, 100Å	4.6 X 100 mm, 3.5 µm, 100Å	2.1 x 75 mm, 2.5 µm 100Å
2,500 psi gradient	5,000 psi gradient	10,000 psi gradient
50 min run time	2x less run time	12x less run time
1 runs per hour	2x more runs / hr	5x more runs / hr
50 mL mobile phase used	Same volume mobile phase	6x less volume mobile phase
20 µL injection volume used	1.5 µL less injection volume	7x less injection volume
HPLC Platform	Modern HPLC Platform	UHPLC Platform
Monograph parameters	**Modern diameter Decreased run time Increased runs per hour Decreased mobile phase Decreased injection volume**	**Modern diameter Decreased run time Increased runs per hour Decreased mobile phase Decreased injection volume**

Table 2. *Example of chromatographic savings provided by gradient method adjustments allowed by the USP <621> Chapter guidance.*

CONCLUSIONS

- **With USP <621> (Dec 1, 2022), methods can be adjusted mathematically using manual calculations, or the Waters.com online calculators, in order to meet the original monograph system suitability criteria.**
- **Modern LC platforms (column dimensions and instruments) can be implemented successfully when adjusting a USP monograph.**
- **Significantly reduced run time, injection volume, and solvent consumption was observed while maintaining**

REFERENCES

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