CASE STUDY

Ph. Eur. Monograph 2217: Lamivudine Related Substances

Scaling from HPLC to UHPLC

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Introduction

Lamivudine is an antiretroviral medication that belongs to a class of drugs known as nucleoside reverse transcriptase inhibitors (NRTIs) and plays a crucial role in combination therapy for managing the human immunodeficiency virus (HIV) infection. Additionally, Lamivudine is also utilized in the treatment of chronic hepatitis B virus (HBV) infection. This Case Study for Lamivudine and its related substances is based on the Ph. Eur. monograph where an end-capped octadecylsilyl silica gel stationary phase is used under isocratic conditions. We demonstrate the potential method improvements that can be achieved within the defined allowable adjustments of chromatographic conditions per the Ph. Eur. Monograph 2217 for Lamivudine using Luna™ Omega C18.

The Ph. Eur. 2.2.46 chromatography chapter was updated, effective from July 2022. The adjustments of chromatographic conditions have suggested the extent to which the various parameters of a chromatographic analysis method may be adjusted without fundamentally modifying the pharmacopoeia analytical procedures. This Case Study will highlight the possibility to migrate existing HPLC monographs to UHPLC within the framework of the adjustments allowed. Based on the allowable adjustments several "Methods" were developed and listed in **Table 1**.

System suitability per Ph. Eur. Monograph 2217 for Lamivudine Related Substances requires a minimum resolution between Related Impurity F and Related Impurity A of 1.5, and a minimum resolution between Related Impurity B and Lamivudine of 1.5. An adjustment is only allowed when the system suitability requirements are met.

Key Concepts:

- Applying Allowable Adjustments, new column dimensions were utilized to explore method optimizations
- Scaling from 5 µm to 3 µm or 1.6 µm particle size columns can facilitate significant time and therefore cost savings

All the reference solutions were prepared as indicated in Ph. Eur. monograph 2217 for Lamivudine. Salicylic Acid R, Cytosine R, and Uracil R were purchased from SigmaAldrich®. The following certified reference standards (CRS) were purchased from the European Directorate for the Quality of Medicines & HealthCare (EDQM) – Council of Europe; Postal address: 7 Allee Kastner CS 30026 F - 67081 Strasbourg (France):

- Y0000425, Lamivudine CRS
- Y0000518, Lamivudine for System Suitability 1 CRS

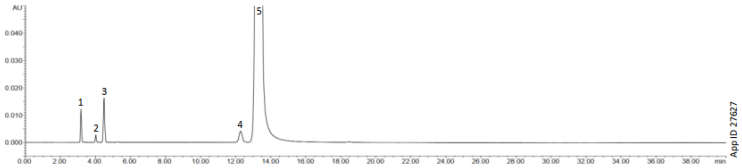
Table 1. Different Methods Within Allowable Adjustments of Chromatographic Conditions USP <621> and Ph. Eur. 2.2.46.

Method Parameters	Allowable Adjustments	Method 1 (Monograph Method)	Method 2	Method 3	Method 4	Method 5
Stationary Phase	No change of the identity of the substituent permitted	End-capped ocetecylsilylsilica gel per Ph. Eur. (As specified)	As specified	As specified	As specified	As specified
Column Dimension (particle size and length)	The particle size and/or length of the column may be modified provided that the ratio of the column length (L) to the particle size (dp) remains constant or in the range -25 % to +50 % of the prescribed L/dp ratio.	Length = 250 mm Particle Size = 5 µm L/dp = 50	Length = 150 mm Particle Size = 3 µm L/dp = 50(Deviation = 0.0 %) (Allowed)	Length = 150 mm Particle Size = 3 µm L/dp = 50(Deviation = 0.0 %) (Allowed)	Length = 100 mm Particle Size = 1.6 µm L/dp = 62.5(Deviation = +25 %) (Allowed)	Length = 100 mm Particle Size = 1.6 µm L/dp = 62.5(Deviation = +25 %) (Allowed)
Column Internal Diameter	In the absence of a change in particle size and/or length of the column, the internal diameter of the column may be adjusted.	ID = 4.6 mm (As specified)	ID = 4.6 mm (Allowed)	ID = 4.6 mm (Allowed)	ID = 2.1 mm (Allowed)	ID = 2.1 mm (Allowed)
Column Used		**Luna Omega 5 µm C18 250 x 4.6 mm** (00G-4785-E0)	**Luna Omega 3 µm C18 150 x 4.6 mm** (00F-4784-E0)	**Luna Omega 3 µm C18 150 x 4.6 mm** (00F-4784-E0)	**Luna Omega 1.6 µm C18 100 x 2.1 mm** (00D-4742-AN)	**Luna Omega 1.6 µm C18 100 x 2.1 mm** (00D-4742-AN)
Flow Rate	Flow rate is adjusted for changes in column diameter and particle size using the following equation: $F_2 = F_1 \times \frac{dc_2^2}{dc_1^2} \times \frac{dp_1}{dp_2}$ F_1 = flow rate indicated in the monograph, in mL/min F_2 = adjusted flow rate, in mL/mindc1 = internal diameter of the column indicated in the monograph, in mm dc_2 = internal diameter of the column used, in mm dp_1 = particle size indicated in the monograph, in µm dp_2 = particle size of the column used, in µm After an adjustment due to a change in column dimensions, an additional change in flow rate of ± 50 percent is permitted.	1.0 mL/min (As Specified)	1.67 mL/min(Deviation from linear flow rate after adjustment = 0.0 %) (Allowed)	1.0 mL/min(Deviation from linear flow rate after adjustment = -40 %) (Allowed)	0.65 mL/min(Deviation from linear flow rate after adjustment = 0.0 %) (Allowed)	0.4 mL/min(Deviation from Linear flow rate after adjustment = -38.5 %) (Allowed)
Column Temperature	± 10 °C	35 °C (As Specified)	35 °C (As Specified)	35 °C (As Specified)	35 °C (As Specified)	35 °C (As Specified)
Composition of Mobile Phase	The amount of the minor solvent components may be adjusted by ± 30 % relative; no component is altered by more than 10 % absolute.	As Specified	As Specified	As Specified	As Specified	As Specified
Detector Wavelength	No adjustment permitted	277 nm (As Specified)	As Specified	As Specified	As Specified	As Specified
Injection Volume	When column dimensions are changed, it may be adjusted with the equation: $V_{Inj2} = V_{Inj1} \times \frac{L_2}{L_1} \times \frac{dc_2^2}{dc_1^2}$ inj1 = injection volume indicated in the monograph, in mL inj2 = adjusted injection volume, in mL dc1 = internal diameter of the column indicated in the monograph, in mm dc2 = internal diameter of the column used, in mm L 1 = column length indicated in the monograph, in mm L2 = new column length, in mm	10 µL (As specified)	6 µL(Calculated injection volume as per the new column dimensions)	6 µL(Calculated injection volume as per the new column dimensions)	1 µL(Calculated injection volume as per the new column dimensions)	1 µL(Calculated injection volume as per the new column dimensions)
LC System Used		Waters® ACQUITY Arc® HPLC	Waters® ACQUITY Arc® HPLC	Waters® ACQUITY Arc® HPLC	Waters ACQUITY® H-Class UHPLC	Waters ACQUITY® H-Class UHPLC

Table 2. Preparation of Test and Reference Solutions

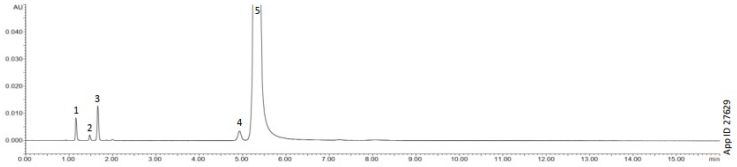
Solution	Composition
Mobile Phase	Mix 5 volumes of Methanol and 95 volumes of a 1.9 g/L solution of Ammonium Acetate R, previously adjusted to pH 3.8, with Glacial Acetic Acid R.
Test Solution	Dissolve 50 mg of Lamivudine CRS in Mobile Phase and dilute to 100 mL with Mobile Phase.
Reference solution (a)	Dilute 1 mL of the test solution to 100 mL with Mobile Phase. Dilute 1 mL of this solution to 10.0 mL with Mobile Phase.
Reference solution (b)	Dissolve 5 mg of Salicylic Acid R in Mobile Phase and dilute to 100 mL with Mobile Phase. Dilute 1 mL of the solution to 100 mL in Mobile Phase.
Reference solution (c)	Dissolve 50 mg of Lamivudine CRS in Mobile Phase and dilute to 100 mL in Mobile Phase.
Reference solution (d)	Dissolve 5mg of Cytosine R and 5 mg of Uracil R in Mobile Phase and dilute to 100 mL with Mobile Phase. Dilute 2 mL of the solution to 10 mL with Mobile Phase.
Reference solution (e)	Dissolve 5 mg of Lamivudine for System Suitability 1 CRS (containing impurities A and B) in 2 mL of Mobile Phase. Add 1 mL of reference solution (d) and dilute to 10 mL with Mobile Phase.

Figure 1. System Suitability Test for Related Substances using Reference Solution (e) for Method 1 on a Luna™ Omega 5 µm, 250 x 4.6 mm Column.



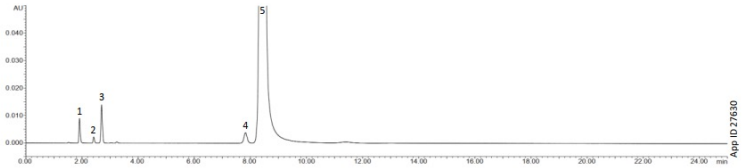
Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Impurity E	3.17	48970	12260	-	1.16
2	Impurity F	4.02	12416	2754	3.59	1.12
3	Impurity A	4.49	87249	16330		1.06
4	Impurity B	12.26	54297	4128	2.87	1.02
5	Lamivudine	13.29	12747943	890457		0.97
N=6 Injections						

Figure 2. System Suitability Test for Related Substances using Reference Solution (e) for Method 2 on a Luna Omega 3 µm, 150 x 4.6 mm Column.



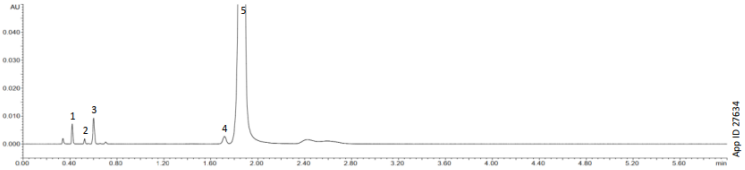
Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Impurity E	1.16	17318	8364	-	1.18
2	Impurity F	1.48	4435	2027	3.01	1.14
3	Impurity A	1.66	31017	12653		1.09
4	Impurity B	4.91	18402	3439	2.74	0.99
5	Lamivudine	5.32	4502183	755672		0.98
N=6 Injections						

Figure 3. System Suitability Test for Related Substances using Reference Solution (e) for Method 3 on a Luna Omega 3 µm, 150 x 4.6 mm Column.



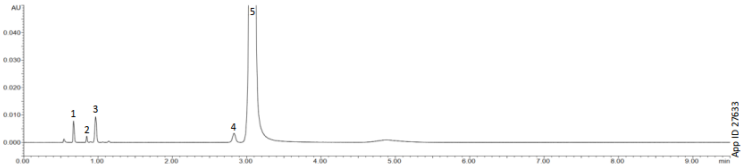
Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Impurity E	1.91	28433	9028	-	1.25
2	Impurity F	2.42	7361	2228	3.07	1.17
3	Impurity A	2.70	51080	13961		1.13
4	Impurity B	7.84	30722	3850	2.87	1.02
5	Lamivudine	8.46	7398722	841204		0.97
N=6 Injections						

Figure 4. System Suitability Test for Related Substances using Reference Solution (e) for Method 4 on a Luna Omega 1.6 µm, 100 x 2.1 mm Column.



Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Impurity E	0.42	5167	7143	-	1.15
2	Impurity F	0.52	1288	1876	3.49	1.16
3	Impurity A	0.60	8978	9073		1.07
4	Impurity B	1.72	5205	2636	2.65	0.93
5	Lamivudine	1.86	1318839	577333		0.96
N=6 Injections						

Figure 5. System Suitability Test for Related Substances using Reference Solution (e) for Method 5 on a Luna Omega 1.6 µm, 100 x 2.1 mm Column.



Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Impurity E	0.67	8782	7734	-	1.22
2	Impurity F	0.85	2214	2306	3.43	1.15
3	Impurity A	0.97	14910	8900		1.04
4	Impurity B	2.82	9594	3295	3.14	0.98
5	Lamivudine	3.07	2258695	702159		0.93
N=6 Injections						

Table 3. Summary of System Suitability Results per Method and Column with % Reduction in Run Time.

Method #	Column	L/dp	Resolution (Impurity F and A)	Resolution (Impurity B and Lamivudine)	System Suitability Pass	Run Time (min)	% Reduction	Column Backpressure (psi)
1	Luna™ Omega 5 µm C18 250 x 4.6 mm	50	3.59	2.87	Yes	40	-	2430
2	Luna Omega 3 µm C18 150 x 4.6 mm	50	3.01	2.74	Yes	16	60	5700
3	Luna Omega 3 µm C18 150 x 4.6 mm	50	3.07	2.87	Yes	26	35	3440
4	Luna Omega 1.6 µm C18 100 x 2.1 mm	62.5	3.49	2.65	Yes	6	85	14200
5	Luna Omega 1.6 µm C18 100 x 2.1 mm	62.5	3.43	3.14	Yes	9	78	9300

The column dimension parameter in pharmacopeia monograph methods is described as the ratio between the column length (L) and particle size (dp). Adjustments are allowed as long as the L/dp ratio remains constant or within -25% to +50% of the prescribed ratio. Maintaining a constant L/dp ratio allows adjustments in column length and particle size while keeping the number of theoretical plates (N) constant. Higher N values, or higher efficiency, indicates better separation. The formula for calculating N is:

$$N = 16 \times \left(\frac{R_t}{W}\right)^2$$

where *Rt* is the retention time and *W* is the peak width at the base.

Smaller particles enhance resolution by reducing band broadening. Adjusting column length and particle size proportionately maintains efficiency and separation.

Factors which influence efficiency are:

1. **System Volume:** Larger system volumes (including tubing, fittings, and detector cells) increase peak broadening and decrease efficiency. UHPLC systems, with optimized flow paths and lower volumes, offer higher efficiency.
2. **Particle Size:** Smaller particle sizes reduce diffusion paths and variability, leading to higher efficiency. They also decrease interstitial spacing and diffusion path length.
3. **Column Length:** Longer columns provide more theoretical plates, improving separation but increasing analysis time. There is a trade-off between efficiency and analysis speed.

When considering the quality of a separation it is important to remember that selectivity differentiates analytes based on their properties and is influenced by the stationary phase, mobile phase composition, pH, temperature, and other conditions. Efficiency and selectivity together determine resolution, as described by the Purnell equation:

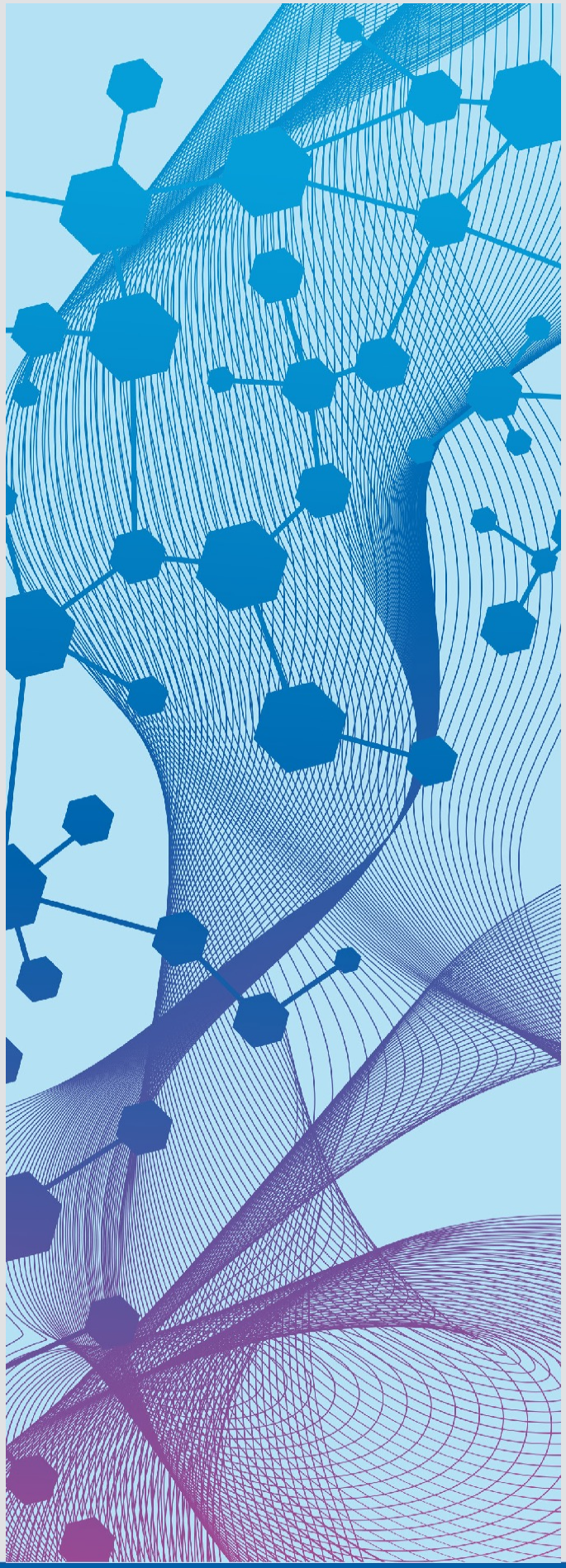
$$R = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{k}{k+1}\right)\left(\frac{\alpha-1}{\alpha}\right)$$

where *R* is resolution, *N* is the number of theoretical plates, *k* is the retention factor, and *α* is the selectivity factor. Higher efficiency leads to narrower peaks and better resolution, while higher selectivity increases differences in retention times and peak widths.

Flow rate (mL/min) affects efficiency through linear velocity (mm/sec). The van Deemter equation describes the contributions to band broadening, with efficiency inversely related to plate height (H). Key factors include:

- **Eddy Diffusion:** Movement of molecules perpendicular to the flow path, including within interstitial spaces and particles.
- **Longitudinal Diffusion:** Inversely related to linear velocity; higher flow rates reduce its impact.
- **Mass Transfer:** Equilibrium between stationary and mobile phases; higher velocities can lead to analyte smearing and reduced efficiency. Optimal velocity increases with decreasing particle size, allowing higher flow rates without significant efficiency loss, balanced against system back pressure.

When column particle size is reduced, increasing the linear velocity of the mobile phase allows efficiency to be maintained. Back pressure will increase as a result of the increase in linear flow velocity, therefore it is essential that the HPLC instrument utilised is capable of delivering the mobile phase flow rate at the backpressure generated by the column.



Conclusions

The results show that the system suitability criteria were met by all 3 columns. The use of a Luna Omega 3 µm C18, 150 x 4.6 mm column and a Luna Omega 1.6 µm C18, 100 x 2.1 mm column are allowed adjustments to the original column dimension with the flow rates scaled accordingly to accommodate the adjustment to column length (L), internal diameter (ID), and particle size (dp).

Utilizing the 3 µm 150 x 4.6 mm column at 1.67 mL/min provides a run time reduction of 60%, however the backpressure is 5700 psi meaning that a 600 bar system would be required. If a flow rate of 1 mL/min is used a corresponding 35% reduction in run time can be achieved while the method can be run on a regular 400 bar HPLC instrument.

When considering the 1.6 µm 100 x 2.1 mm column, when running at the scaled flow rate of 0.65 mL/min a run time reduction compared to the original method of 85% can be achieved, although the backpressure is high at 14200 psi. Working at a more conventional 0.4 mL/min it is possible to achieve a 78% reduction in run time while remaining comfortably within the pressure limits of a modern UHPLC instrument (9300 psi).

Key Takeaways:

- The use of smaller particle columns provides higher L/dp ratios, allowing for shorter columns to be used.
- Shorter columns can still meet system suitability requirements, while allow for shorter run times
- Achieving shorter run times will save both time and reduce solvent consumption, providing cost efficiencies in an analytical laboratory