WHITE PAPER

Demystifying L/d_p Ratio When Working with Allowable Adjustments

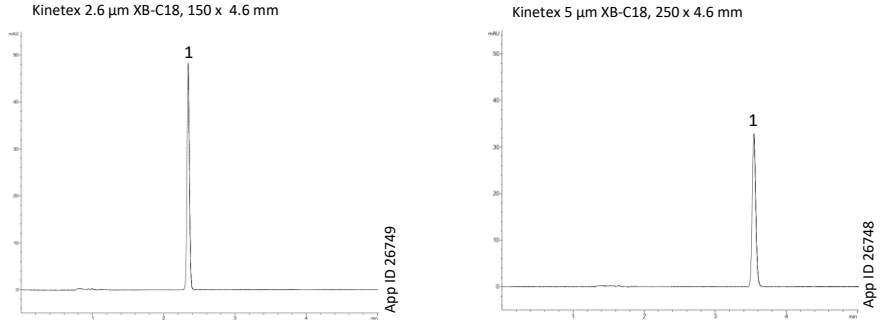
James Turner[1] and Bryan Tackett, PhD[2]
[1]Phenomenex Ltd., Queens Ave., Hurdsfield Ind. Est., Macclesfield, SK10 2BN, UK
[2]Phenomenex Inc., 411 Madrid Ave., Torrance, CA 90501, USA

Introduction

The most important aspect of a chromatographic system is the capability to separate and resolve sample components effectively and quickly. The term used to measure this capability is Efficiency. A more efficient HPLC system yields narrower and taller peaks, allowing for better separation and detection of individual analytes. Higher efficiency will result in increased resolution between peaks that will facilitate the identification and quantification of target compounds, even in complex samples. Narrower peaks result in greater peak heights and improved signal-to-noise ratio. This increased sensitivity allows for the detection of trace amounts of analytes, even in challenging matrices. Efficiency is also associated with reduced analysis time. A more efficient system enables faster separations, providing for higher sample throughput without compromising the quality of results.

System suitability per USP Monograph for the Desvenlafaxine Succinate Assay is a symmetry factor no more than (NMT) 1.5 and a percent relative standard deviation (%RSD) NMT 0.73 %. Both the Kinetex™ 2.6 µm XB-C18, 150 x 4.6 mm column and the Kinetex 5 µm XB-C18, 250 x 4.6 mm column met all system suitability requirements, with the shorter Kinetex 2.6 µm XB-C18 column providing a 34 % shorter run time and increased sensitivity (**Figure 1**).

Figure 1. Chromatograms of Standard Solution for Assay of Desvenlafaxine Succinate.



		Kinetex 2.6 µm XB-C18			Kinetex 5 µm XB-C18		
Peak No.	Analyte	Retention Time (min)	Symmetry Factor	Area %RSD	Retention Time (min)	Symmetry Factor	Area %RSD
1	Desvenlafaxine Succinate	2.34	1.072	0.079	3.54	1.142	0.062

N = 5 Injections

The USP Monograph for Desvenlafaxine Succinate Assay requires an L1 column with column dimensions 250 x 4.6 mm and a particle size of 5 µm. However, various parameters of a chromatographic test may be adjusted without modifying the fundamental pharmacopeial analytical procedures, including adjustments to column dimensions and particle size. These "allowable adjustments" are necessary to accommodate practical considerations and variations between available equipment while still ensuring consistency of analysis. **Table 1** outlines the adjustments that can be made for isocratic monograph methods.

The column dimension parameter of pharmacopeia monograph methods is described as a ratio between the length of the column (L) and the particle size (d_p). The allowable adjustment states that the particle size and/or length of the column may be modified provided that the ratio of the column length to the particle size remains constant or in the range −25 % to +50 % of the prescribed L/d_p ratio. In the case of the Desvenlafaxine Succinate Assay monograph, the L/d_p ratio that the monograph calls for is 50,000, using the 250 mm column length and 5 µm particle size. The shorter Kinetex column with length 150 mm and a particle size of 2.6 µm would give an L/d_p ratio of 57,692. This falls well within the −25 % to +50 % range of the L/d_p ratio prescribed in the allowable adjustments and allows for the shorter Kinetex column to be used for this monograph and results in a shorter overall run time. In this white paper, we will discuss how column efficiency based on column parameters can be maintained through the use of allowable adjustments and provide for alternative columns to be used for pharmacopeia monographs.

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

Method Parameter	Allowable Adjustments
Stationary Phase	No change of the identity of the substituent (e.g., no replacement of C18 by C8); the other Physico-chemical characteristics of the stationary phase (i.e. chromatographic support, surface modification and extent of chemical modification) must be similar; a change from totally porous particle (TPP) columns to superficially porous particle (SPP) columns is allowed provided the above-mentioned requirements are met.
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 (d_p) remains constant or in the range −25 % to +50 % of the prescribed L/d_p ratio.
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.
Flow Rate	When the particle size is changed, the flow rate requires adjustment. After an adjustment due to a change in column dimensions, an additional change in flow rate of ± 50 % is permitted.
Column Temperature	± 10 °C
Minor Solvent Composition	± 30 % relative
pH of the Aqueous Content of the Mobile Phase	± 0.2 units
Concentration of Salts in the Buffer Component of a Mobile Phase	± 10 %
Detector Wavelength	No adjustment permitted
Injection Volume	when the column dimensions are changed, the injection volume adjustment equation may be used for adjusting the injection volume.

Efficiency and How It Is Measured

Efficiency is commonly measured using the concept of theoretical plates (N). The higher the number of plates, the more efficient the separation. Efficiency can be calculated using the following equation:

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

N = Number of theoretical plates
R_t = Retention time of the solute
W = Peak width at the base

Factors Affecting Efficiency

There are several factors that can affect efficiency when choosing an appropriate column or system for analysis. The efficiency of an HPLC system can be affected by its total volume. This includes tubing, fittings, and detector cells, as well as column volume. Larger system volumes result in increased peak broadening due to extra dead volume, which leads to decreased efficiency. UHPLC systems offer lower dispersion due to optimized flow paths and lower volume, thereby increasing efficiency.

The particle size of the stationary phase chosen can greatly affect efficiency. Decreasing the particle size generally leads to higher efficiency because the diffusion with the porous particles is reduced, as well as a reduction in variability of diffusion pathways. Interstitial spacing between packing materials is also reduced, which again limits diffusion. The diffusion path length is also decreased with smaller particle sizes.

Longer columns increase efficiency by providing more theoretical plates for separation. As the solute moves through a longer column, there are more opportunities to interact with the stationary phase, resulting in better separation. However, longer columns also increase analysis time, so there is a trade-off between efficiency and analysis speed with column length alone.

Efficiency and L/d_p Ratio

As previously mentioned, the column dimension parameter of pharmacopeia monograph methods is defined as L/d_p, and as long as this term stays constant or falls within the allowable range, the length of the column and/or particle size can be adjusted. It is possible to change the L/d_p ratio while keeping the number of theoretical plates (N) constant by adjusting the column length and particle size proportionately. As we just discussed, increasing the length of the column generally increases the number of theoretical plates, leading to higher efficiency.

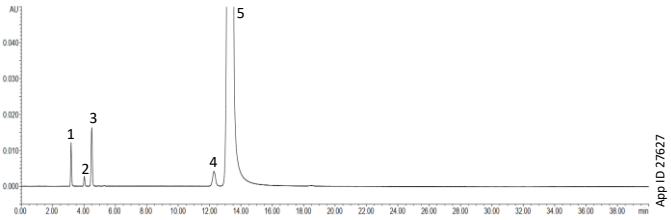
On the other hand, smaller particles provide a decreased diffusion path length, reducing band broadening and enhancing resolution. By adjusting column length and particle size proportionately to maintain a similar L/d_p ratio, the number of theoretical plates (efficiency) and overall separation will be maintained. If the particle size is decreased while proportionally decreasing the column length, the diffusion path length will be maintained, keeping the number of theoretical plates constant.

As an example of this concept, the European Pharmacopoeia Monograph 2217 for Lamivudine and its related substances was evaluated using two Luna™ Omega columns with different column lengths and particle sizes. The monograph was elucidated using a column that had the dimensions 250 x 4.6 mm and a 5 µm particle size. This gives an L/d_p ratio of 50,000.

Figure 2 shows the results of the System Suitability Test for Lamivudine and its related substances using a Luna Omega C18 column with the same dimensions and particle size called for in the monograph. System suitability per Ph. Eur. Monograph 2217 for Lamivudine related substances is 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. System suitability was easily met, and all peaks had a symmetry factor very close to 1.

Knowing that decreasing the column length can decrease analysis time, a shorter Luna Omega column, with dimensions 150 x 4.6 mm, was also used. Since the column length was adjusted, the particle size must also be adjusted to maintain the L/d_p ratio, or to fall within the acceptable range put forth in the allowable adjustments outlined in **Table 1**. Using a particle with a size of 3 µm, kept the L/d_p ratio at 50,000, and is therefore an allowable adjustment that can be made for this monograph. As can be seen in **Figure 3**, the shorter Luna Omega column also met the system suitability requirements for resolution, with all symmetry factors close to 1. This shows that maintaining the L/d_p ratio when moving to a shorter column, the efficiency is also maintained with the added benefit of a shorter analysis time.

Figure 2. System Suitability Test for Related Substances using Reference Solution (e) on a Luna™ Omega 5 µm C18, 250 x 4.6 mm Column.

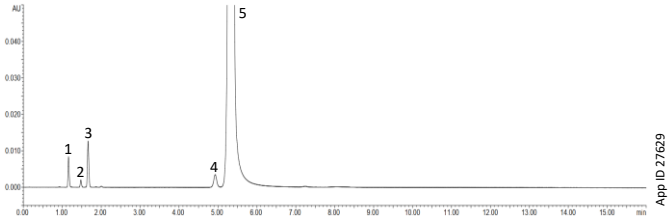


Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Impurity E	3.17	48970	12260	-	1.16
2	Impurity F	4.02	12416	2754	3.59	1.12
3	Impurity A	4.49	87249	16330		1.06
4	Impurity B	12.26	54297	4128	2.87	1.02
5	Lamivudine	13.29	12747943	890457		0.97
N=6 Injections						

LC Conditions

Column: Luna Omega 5 µm C18
Dimensions: 250 x 4.6 mm
Part No.: 00G-4785-E0
Mobile Phase: Mix 5 volumes of Methanol and 95 volumes of a 1.9g/L solution of Ammonium Acetate R, previously adjusted to pH 3.8, with Glacial Acetic Acid R.
Flow Rate: 1.0 mL/min
Injection Volume: 10 µL
Temperature: 35 °C
Detector: UV @ 277 nm
System: Waters® ACQUITY Arc® HPLC

Figure 3. System Suitability Test for Related Substances using Reference Solution (e) on a Luna Omega 3 µm C18, 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						

LC Conditions

Column: Luna Omega 3 µm C18
Dimensions: 150 x 4.6 mm
Part No.: 00F-4784-E0
Mobile Phase: Mix 5 volumes of Methanol and 95 volumes of a 1.9g/L solution of Ammonium Acetate R, previously adjusted to pH 3.8, with Glacial Acetic Acid R.
Flow Rate: 1.67 mL/min
Injection Volume: 6 µL
Temperature: 35 °C
Detector: UV @ 277 nm
System: Waters ACQUITY Arc HPLC

Efficiency and Selectivity

Selectivity refers to the ability of a chromatographic method to differentiate between analytes based on their chemical and physical properties. Parameters such as the nature of the stationary phase, the composition of the mobile phase, pH, temperature, and other experimental conditions influence selectivity. Specifically, selectivity determines how well the method separates analytes of interest from interfering substances or closely eluting peaks.

High efficiency generally leads to better peak resolution. This means that closely eluting peaks are better separated, which can improve specificity by reducing interference between peaks. However, efficiency does not affect selectivity directly. Efficiency and selectivity are related through the determination of resolution in the Purnell equation (aka resolution equation):

$$R = \underbrace{\left(\frac{\sqrt{N}}{4}\right)}_{\text{Efficiency}}\underbrace{\left(\frac{k}{k+1}\right)}_{\text{Retention}}\underbrace{\left(\frac{\alpha-1}{\alpha}\right)}_{\text{Selectivity}}$$

R = Resolution
N = Number of theoretical plates
k = Retention factor
α = Selectivity factor

Resolution is directly influenced by both efficiency and selectivity, but neither influence each other. As efficiency increases, the peaks become narrower, leading to improved resolution. Similarly, as selectivity increases, the differences in retention times and peak widths between analytes increase, also enhancing resolution.

Particle Morphology and Efficiency

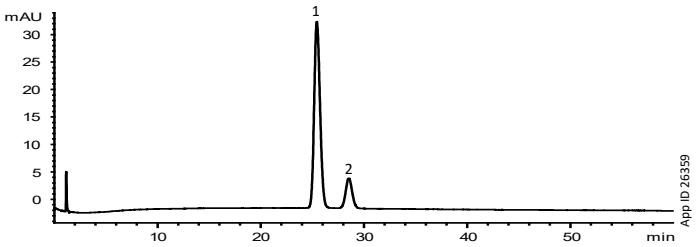
The morphology of the particles in the stationary phase can influence efficiency. The two most common types of particle morphologies used in HPLC are fully porous particles and core-shell particles. Fully porous particles are solid particles with a uniform porous structure throughout their entire volume and are typically made of silica or polymers. Core-shell particles, also known as superficially porous particles or fused-core particles, have a unique structure consisting of a solid core surrounded by a thin porous shell. The core-shell design aims to combine the advantages of both fully porous and non-porous particles.

Although fully porous particles are the traditional particle used for HPLC separations, core-shell particles offer some advantages. Core-shell particles offer higher efficiency than fully porous particles of the same diameter due to their smaller diffusion path length, enhancing separation while maintaining fast analysis times. Core-shell particles exhibit lower backpressure compared to fully porous particles with similar efficiency, thus allowing for the use of higher flow rates, reducing analysis time. The thin, porous shell of core-shell particles provides a more closely controlled diffusion environment, resulting in narrower analyte bands and enhanced resolution; generally, with lower overall retention times due to the lower surface area of core-shell materials.

For example, the USP monograph for Doxepin Hydrochloride Assay provides a method for the separation of the (E)- and (Z)-isomers of Doxepin Hydrochloride. A fully porous Luna C8(2) column and a core-shell Kinetex™ C8 column, both with the same column dimensions and particle size, were used to evaluate this monograph. The Luna C8(2) column was able to completely resolve the stereoisomers of Doxepin Hydrochloride with peak symmetry

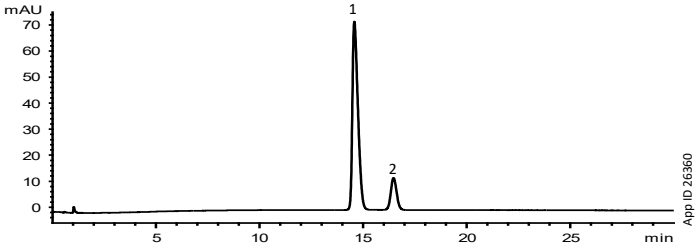
factors close to 1 (**Figure 4**). By using core-shell particles with the Kinetex™ C8 column, an increase in resolution and improved peak shape were observed, resulting in increased efficiency of separation of the Doxepin Hydrochloride stereoisomers. As expected, the analysis time was also reduced using the core-shell column (**Figure 5**).

Figure 4. Separation of Doxepin Hydrochloride Stereoisomers Using a Fully Porous Luna™ 5 µm C8(2), 150 x 4.6 mm Column.



Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Doxepin Hydrochloride (E)-isomer	25.4	1340	34	2.84	1.15
2	Doxepin Hydrochloride (Z)-isomer	28.5	238	5.5		1.05

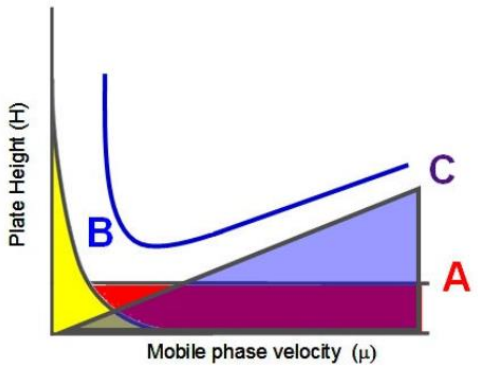
Figure 5. Separation of Doxepin Hydrochloride Stereoisomers Using a Core-Shell Kinetex 5 µm C8, 150 x 4.6 mm Column.



Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Doxepin Hydrochloride (E)-isomer	14.6	1346	72.4	3.78	1.49
2	Doxepin Hydrochloride (Z)-isomer	16.5	241	12.3		1.11

Efficiency and Flow Rate

Volumetric flow rate (mL/min) is the rate at which mobile phase is pumped through the column and is controlled by the user. It is more instructive to consider linear velocity (the speed at which the mobile phase passes through the column, mm/sec) which can be normalized to take account of different column dimensions, generally the column internal diameter. Linear velocity plays a crucial role in determining the efficiency of the separation. The effect of linear velocity on efficiency can be understood through the van Deemter equation (where H equals the plate height), which describes the different contributions to band broadening. Efficiency is inversely related to H; so smaller values of H yield higher efficiency.



$$H = (A \times d_p) + \frac{B}{\mu} + (C \times d_e^2 \times \mu)$$

Eddy Diffusion Multipath Effect | Longitudinal Diffusion | Mass Transfer

d_p = Diameter of particle
μ = Linear velocity, (mm/sec)
d_e = "Effective particle size"
In the case of fully porous particles this is d_p
For core-shell particles, this value is unique

Eddy diffusion is the movement of molecules perpendicular to the flow path. Most notably this involves diffusion within the interstitial spaces between particles, but also includes lateral movement within the particle itself.

Longitudinal diffusion is inversely related to linear velocity. The higher the flow rate, the more negligible the longitudinal diffusion becomes. This is independent of particle size and is related to the parallel plane to the flow of the mobile phase.

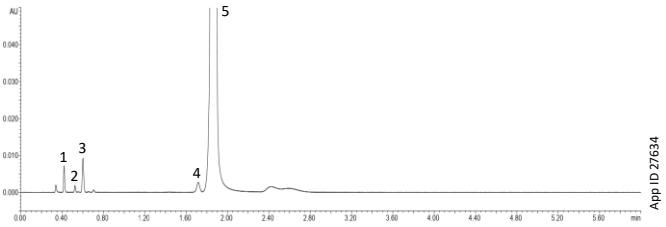
The Mass transfer term describes the ability of analytes to form a dynamic equilibrium between the stationary phase and the mobile phase. It is a function of the uniformity of the porous structure of the packing material and the velocity of the mobile phase passing through it. When mobile phase velocity exceeds the point where mass transfer can effectively take place, the analytes will start to smear through the column and efficiency is lost.
The van Deemter equation shows us what the optimal velocity is for a given particle size of packing material. In general, this optimum increases for decreasing particle size. Additionally, the rate of increase in plate height is slower (the curve is flatter) for smaller particle packing materials. A practical implication for the user is that flow rates can be increased without losing a significant amount of efficiency. This must of course be balanced by considering the operating back pressure of the system on which the column is being run.

As an example, the European Pharmacopoeia Monograph 2217 for Lamivudine was elucidated using a column that had the dimensions 250 x 4.6 mm and a 5 µm particle size. This gives an L/d_p ratio of 50,000. **Figures 2** and **3** showed that maintaining the L/d_p ratio when moving to a shorter column and smaller particle size, the efficiency is also maintained with the added benefit of a shorter analysis time due to increased flow rate. A Luna Omega 1.6 µm C18, 100 x 2.1 mm column was also used to evaluate Monograph 2217 for Lamivudine.

Figure 6 shows the results of the System Suitability Test for Lamivudine and its related substances using a LuMa Omega 1.6 µm C18, 100 x 2.1 mm column. System suitability per Ph. Eur. Monograph 2217 for Lamivudine related substances is 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. System suitability was easily met, and all peaks had a symmetry factor very close to 1.

The use of a Luna Omega 3 µm C18, 150 x 4.6 mm column (**Figure 3**) 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 (d_p). With the Luna Omega 1.6 µm C18, 100 x 2.1 mm column, we demonstrated a reduction in total analysis time by 60 % (from 40 minutes to 16 minutes with a 1.6 mL/min flow) over the Luna Omega 3 µm C18, 150 x 4.6 mm column, and by 85 % compared to the original column dimension (5 µm, 250 x 4.6 mm) specified in the monograph.

Figure 6. System Suitability Test for Related Substances using Reference Solution (e) on a Luna Omega 1.6 µm C18, 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						

LC Conditions

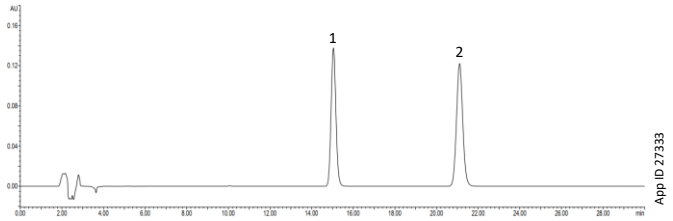
Column: Luna Omega 1.6 µm C18
Dimensions: 100 x 2.1 mm
Part No.: 00D-4742-AN
Mobile Phase: Mix 5 volumes of Methanol and 95 volumes of a 1.9g/L solution of Ammonium Acetate R, previously adjusted to pH 3.8, with Glacial Acetic Acid R.
Flow Rate: 0.65 mL/min
Injection Volume: 1 µL
Temperature: 35 °C
Detector: UV @ 277 nm
System: Waters® ACQUITY® H-Class HPLC

Another example can be seen in the evaluation of the European Pharmacopoeia Monograph 2465 for Irbesartan Related Substances. The original column used to elucidate the monograph was a 250 x 4.0 mm column with a particle size of 5 µm, meaning the L/d_p ratio was 50,000. By following the allowable adjustments, a Kinetex™ 5 µm C18, 250 x 4.6 mm column (L/d_p = 50,000) and a Kinetex 1.7 µm C18, 100 x 2.1 mm column (L/d_p = 58,824) were used to evaluate the monograph.

Figure 7 shows the results of the System Suitability Test for Irbesartan and its related substances using a Kinetex 5 µm C18, 250 x 4.6 mm column. The system suitability per European Pharmacopoeia Monograph 2465 for Irbesartan Related Substances is a minimum resolution between Related Impurity A and Irbesartan of 3.0. System suitability was met using this column.

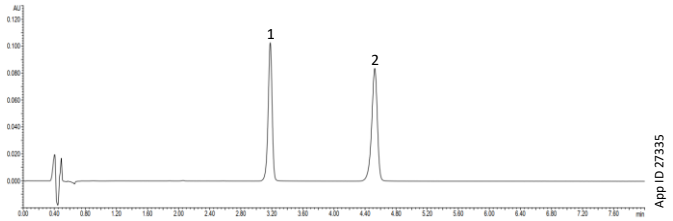
By decreasing the particle size and maintaining the L/d_p ratio within the allowable adjustments, using the Kinetex 1.7 µm C18, 100 x 2.1 mm column, the flow rate was scaled, and the analysis time was decreased. System suitability was also met using the smaller Kinetex column with smaller core-shell particles and the analysis time was reduced by a factor of 5 (**Figure 8**).

Figure 7. System Suitability Test for Related Substances using Reference Solution (b) on a Kinetex 5 µm C18, 250 x 4.6 mm Column.



Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Irbesartan Impurity A	15.05	2213344	137712	12.37	1.08
2	Irbesartan	21.11	2555501	122101		1.07
N=6 Injections						

Figure 8. System Suitability Test for Related Substances using Reference Solution (b) on a Kinetex 1.7 µm C18, 100 x 2.1 mm Column.

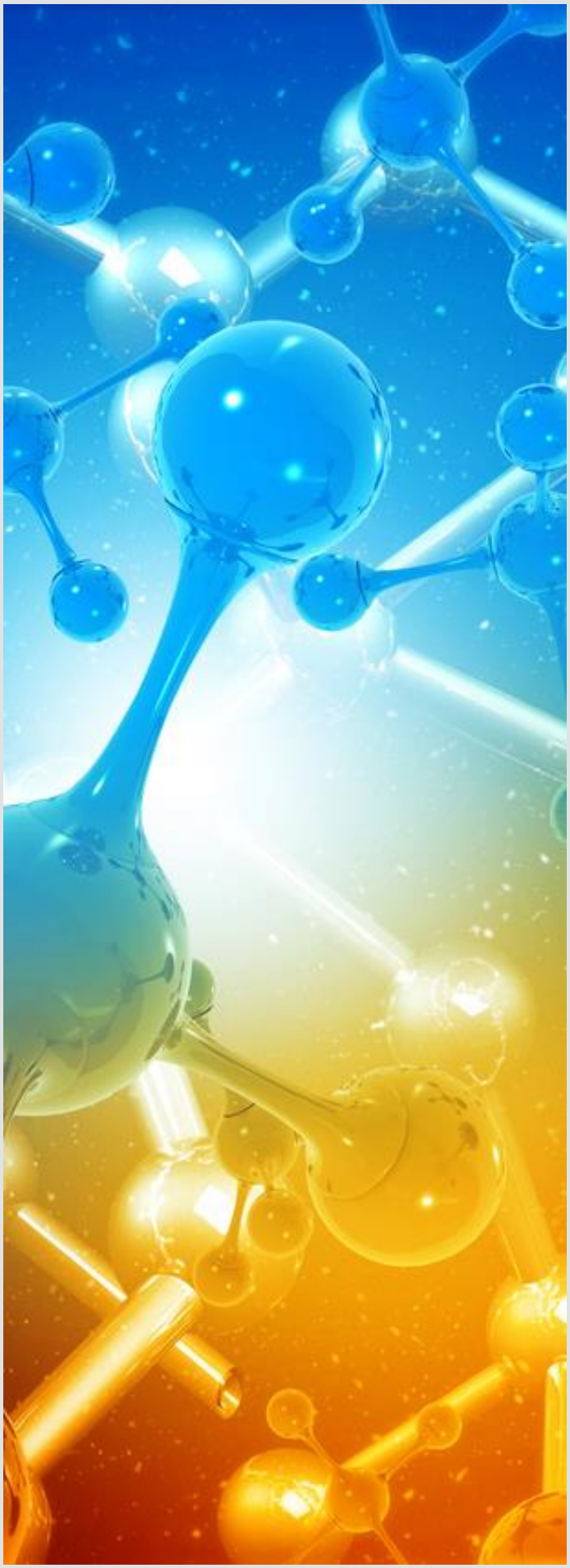


Peak No.	Analyte	Retention Time (min)	Area	Height	Resolution	Symmetry Factor
1	Irbesartan Impurity A	3.18	368368	102700	12.00	0.90
2	Irbesartan	4.53	424730	83744		0.87
N=6 Injections						

Summary

The allowable adjustments which are harmonized for the USP general chapter <621> and European Pharmacopoeia (2.2.46) allow for adjustments in L/d_p ratio, enabling the use of shorter columns packed with smaller particle size materials. As described in this white paper, this approach allows for the maintenance of efficiency levels for the separation, whilst allowing for the potential to save time, solvents, and ultimately money.

The allowable adjustments have opened the door to the use of UHPLC instruments and columns in existing monographs without the requirement for revalidation, providing that system suitability requirements are met. These changes acknowledge the fact that maintaining the L/d_p ratio does not impact the selectivity of the separation, as selectivity and efficiency are not directly related. The allowable adjustments also allow users to replace traditional fully porous columns with core-shell packing materials which can speed up methods without compromising on efficiency and therefore resolution of the separation. The L/d_p ratio allowable adjustments can potentially provide method improvements for both HPLC and UHPLC users.



Need a different column size or sample preparation format?
No problem! We have a majority of our available dimensions up on www.phenomenex.com, but if you can't find what you need right away, our super helpful Technical Specialists can guide you to the solution via our online chat portal www.phenomenex.com/Chat.

WP59440724_W