Chapter 1

UHPLC Method Development

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1.1 INTRODUCTION

UHPLC has been gradually adopted in industrial labs, especially the pharmaceutical industry due to its high resolution, high speed, and solvent saving, since its introduction in early 2004 (1). A UHPLC method using a sub-2 μ m column could reduce the analysis time by up to 80% and save the mobile phase consumption by at least 80% compared with an HPLC method using a conventional 3.5 μ m column without sacrificing separation performance (2). In addition, the much shorter run time significantly reduces UHPLC method development scouting time (1).

HPLC method development principles can be applied to UHPLC method development, although detailed procedures may differ. In addition, many existing HPLC methods used in the pharmaceutical industry can be converted to UHPLC methods. In practice, a UHPLC method may need to be converted to HPLC when a UHPLC system is not available.

This chapter provides an overview of the UHPLC method development process and the conversion process of an HPLC method to UHPLC or vice versa. It mainly focuses on analytical reversed phase UHPLC method development of small molecules. A general process and detailed steps are discussed as well as practical examples given.

1.2 METHOD DEVELOPMENT

There are many publications on HPLC method development strategies (3–10). These strategies can be applied to UHPLC method development, although instrumentation and columns are different. The UHPLC method development process includes gathering sample information and defining method goals, scouting columns and mobile phases, analyzing scouting results and selecting separation conditions, optimizing the method, and validating the method. Steps in common UHPLC method development

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processes are summarized in Figure 1.1. Each step is described and discussed in the following sections.

1.2.1 Gather Sample Information and Define Method Goals

1.2.1.1 Gather Sample Information

Sample information is very useful for method development. The sample information includes the process used to generate the sample, chemical structures and physical and chemical properties of sample components, and their toxicity.

Understanding the process is helpful to method development and is achieved by talking to a chemist or formulator who provides the sample. In the pharmaceutical industry, there is rarely a totally unknown sample. In process chemistry, active pharmaceutical ingredient (API), intermediate (IM), and starting material (SM) are made from synthetic schemes. Most impurities in the sample are known, although some impurities may be unpredictable. In the pharmaceutical process, drug products are manufactured by mixing API with excipients. The major components of the sample are known, although some degradation products may not be predictable.

In addition, knowledge of chemical structures and physicochemical properties of sample components is useful for method development. The chemical structures of possible sample components provide data on molecular weights and functional groups. Special attention should be paid to acidic, basic, aromatic, and other functional

groups from which pK_a , solubility, chromophore, and stability can be inferred if data of physical and chemical prosperities is not available. Based on pK_a , a suitable column and mobile phase buffer pH can be selected for a robust method. The solubility and stability of major components can be used to select a suitable diluent and mobile phase. The UV chromophore can be used to select a suitable detection wavelength.

It is very important to read material safety data sheet (MSDS) before working on samples. Some samples may be toxic and should be handled in a safe manner recommended by MSDS.

1.2.1.2 Define Method Goals

There are four major types of tests in the pharmaceutical industry. These are identification tests, quantitative tests for impurities' content, limit for control of impurities, and quantitative tests of active moiety (11). A quantitative method, for example, an assay method, can be used as an identification method. In addition, many methods can be used for quantitative tests for both assay and impurities' contents.

Different types of methods have different goals. The method goals are usually defined as specificity, accuracy, precision, sensitivity, and robustness, or more specifically as resolution, linearity, recovery, repeatability, and quantitation limit for an assay and impurity method. The method goal for an identification test is often defined as "specificity." This chapter mainly focuses on the assay and impurity method.

The same type of methods may have different goals for a different sample. For an API sample, its impurities must be separated from API and each other, and the limit of quantification (LOQ) for the impurities must be lower than the reporting threshold recommended in ICH Q3A(R2) guideline (12). For SM or IM, its impurities must be separated from SM or IM and from each other, and LOQs are determined by the criticality of the impurities. For a drug product, its impurities must be separated from its API, excipients, and each other, and LOQ must be lower than the reporting threshold recommended in ICH Q3B(R2) guideline (13).

In addition, method goals also change with the phase of each project. For an early-phase project, robustness and ruggedness are not required for an API method. However, for a late-phase project, robustness and ruggedness are required for an API method. The comparison of method goals between early- and late-phase projects is made in Table 1.1.

1.2.2 Scout Columns and Mobile Phases

1.2.2.1 Select a UHPLC Mode

After gathering sample information and defining goals, a suitable UHPLC mode can be selected. In HPLC, there are four major separation modes: reversed phase, normal phase, ion exchange, and size exclusion (14). However, there are only two UHPLC modes because of UHPLC column current availability. These are reversed-phase and normal-phase UHPLC (15, 16). Like HPLC, most pharmaceutical compounds can be separated with reversed-phase UHPLC, which is the main focus of this chapter.

Table 1.1 Companyon of M i memory Obaly Detween Larry- and Late-i mase i to	mparison of API Method Goals Between Early- and Late-Phase Projec
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	Early Phase	Late Phase
Specificity/resolution	Resolution ≥ 1.2	Resolution ≥ 1.5
	Discrimination factor ≥ 0.5	Discrimination factor ≥ 0.8
Accuracy/linearity	Quantitation limit (QL) to at	Quantitation limit (QL) to at
	least 120% of the analyte	least 120% of the analyte
	(main and specified	(main and specified
	impurities)	impurities)
Accuracy/recovery	No	Yes
Repeatability	$\leq 1.0\%$	$\leq 1.0\%$
Sensitivity	Depends on maximum daily	Depends on maximum daily
	dose	dose
Solution stability	Yes	Yes
Robustness and ruggedness	No	Yes

1.2.2.2 Select Columns, Mobile Phases, and Detection and Setting

1.2.2.2.1 Columns There are many commercial UHPLC columns, most of which are based on silica gel packing material. The major limitation of the silica gel packing is its instability at basic pH. Some UHPLC columns are packed with hybrid particles such as bridged ethylene hybrid (BEH) technology. The advantage of the hybrid packing material is its stability at alkaline pH and less peak tailing for basic compounds compared with the silica packing materials (17, 18). In addition, some UHPLC columns are packed with core-shell particles (19–21).

There are different bonded phases for each packing material. For example, there are C18, C8, C4, phenyl, and polar-embedded C18 bonded phases for BEH packing material. The selected UHPLC columns are listed in Table 1.2

Manufacturer	Туре	Column		
Waters Corp.	C18	BEH C18 1.7 μm		
-	Hydrophilic carbamate with C18	BEH Shield RP18 1.7 μm		
	C8	BEH C8 1.7 μm		
	Phenyl	BEH phenyl 1.7 μm BEH amide 1.7 μm		
	Amide			
	HILIC	BEH HILIC 1.7 μm		
	C18	HSS C18 1.7 μm		
	C18	HSS C18 SB 1.7 μm		
	T3	HSS T3 1.7 μm		
Agilent technologies	C18	Zorbax Eclipse Plus C18 RRHD 1.8 µm		
	C18	Zorbax SB-C18 RRHD 1.8 μm		
	C8	Zorbax SB-C8 RRHD 1.8 µm		

 Table 1.2
 Selected UHPLC Columns

Different bonded phases are used during method development because they provide different selectivity. Their different selectivity gives confidence in method specificity. Typically C18, C8, and phenyl are selected. In addition, mobile phase pH use range should be considered when selecting UHPLC columns. As mentioned, the silica-based packing columns are stable under acidic conditions but not at basic conditions. Therefore, the hybrid-based packing columns are selected under basic conditions.

Column stability needs to be checked before column selection or during method validation. A column must be stable for at least 200 injections for routine use (22). Column manufacturers may provide this information. In addition, studies on some column stability were published (23, 24). However, the information is limited at specific conditions and needs to be confirmed experimentally.

1.2.2.2 Mobile Phases Mobile phases mainly consist of organic solvent and buffer. Acetonitrile and methanol are commonly used organic solvents. However, the disadvantages of using methanol are its low sensitivity at low UV wavelength and high back pressure. Tetrahydrofuran (THF) may also be used as a UHPLC organic solvent. However, it may cause a detection problem and damage to a UHPLC instrument. Consult with the UHPLC instrument manufacturer beforehand.

The other component of mobile phase is aqueous buffer. The buffer pH may be critical to some separation. pKa values of compounds of interest should be considered when selecting mobile phase pH. The buffer pH of a mobile phase should be outside pKa \pm 2 to ensure method robustness. Table 1.3 lists the commonly used mobile phase buffers. The most commonly used mobile phase buffer is a trifluoroacetic acid (TFA) aqueous solution. One issue with TFA is its low sensitivity at wavelength 210 nm.

Different mobile phase organic solvents have different selectivity for neutral, acidic, and basic compounds. Therefore, different mobile phase organic solvents are tested as modifiers during method development. In addition, mobile phase buffer pH affects selectivity of acidic and basic compounds. Therefore, different buffer pHs are investigated during method development for acidic and basic compounds.

Ideal mobile phase provides excellent resolution as well as sensitivity (25–29). It needs to have a low UV cutoff to give sufficient sensitivity for UV detection. It is also preferable that it be compatible with mass spectrometry (MS).

Buffer	pKa	pH Range
Trifluoroacetic acid (TFA)	0.2	1.5–2.5
Formic acid	3.8	2.8-4.8
Acetic acid	4.8	3.8-5.8
Ammonium acetate	4.8 and 9.2	3.8–5.8 and 8.2–10.2
Ammonium hydroxide	9.2	8.2-10.2

 Table 1.3
 Commonly Used Mobile Phase Buffers



Figure 1.2 Example of a UV profile for wavelength selection.

1.2.2.2.3 Detection Most pharmaceutical compounds have UV chromophores. UV detector is the most commonly used detector. Wavelengths at λ_{max} or λ_{val} are usually selected for method robustness. For example, three wavelengths at 265 (λ_{max}), 226 (λ_{val}), and 210 nm (λ_{max}) can be potentially used for a compound with the UV profile shown in Figure 1.2. However, sensitivity is the highest at 265 nm, which is why 265 nm should be selected for best detection. In addition, UV profiles of impurities in the sample should be considered. A detection wavelength should be selected to ensure that all components have acceptable sensitivity, not just the major component.

Beside wavelength selection, UV detection setting is also critical (30). An HPLC peak is usually broad, and its peak width is typically on order of several seconds. However, a UHPLC peak is very narrow, and its peak width is usually on order of one second. For UHPLC, at least 20 Hz data acquisition rate should be used. Low data acquisition rates cause peak distortion and broadening as shown in Figure 1.3.

For analytes with no UV chromophore or weak UV chromophore, detection methods such as Corona charged aerosol detection (CAD), evaporative light scattering detection (ELSD), or MS can be used. These detectors require that analytes be nonvolatile and mobile phase be compatible with detectors.

1.2.2.3 Prepare Sample Solution

Sample diluent must be appropriately selected. It must be able to dissolve all components in a sample. In addition, all sample components must be stable in the selected diluent. The selected diluent also does not interfere with separation. Typically, aqueous mobile phase or a mixture of aqueous mobile phase and organic mobile phases is



1.40 1.42 1.44 1.46 1.48 1.50 1.52 1.54 1.56 1.58 1.60 1.62 1.64 1.66 1.68 1.70 1.72 1.74 1.76 1.78 1.80 Minutes

Figure 1.3 Example of the influence of the data acquisition rate on peak distortion. The filter time is 0.1 s. Conditions: Zorbax Bonus RP 100 \times 2.1 mm, 1.8 μ m; mobile phase A 0.1% TFA water; mobile phase B acetonitrile; gradient 20%–56% B for 2.06 min; flow rate 0.8 mL/min; wavelength 268 nm. Peaks 1 and 2 are impurities.

used as the sample diluent. A high percentage of organic solvent in the diluent may reduce resolution and sensitivity.

For drug products, sample preparation may involve grinding, extraction, and filtration (31–35). For samples with a more complicated matrix, solid phase extraction (SPE) and other sample preparation techniques can be used to remove interfering species and to increase sensitivity (36–41). The procedure needs to ensure sufficient recovery of target analytes for accuracy and sensitivity.

1.2.2.4 Run a Scouting Experiment

Once columns, mobile phase organic solvents and buffers, and method of detection are selected and the sample is prepared, a scouting experiment can be performed. There are many HPLC method strategies published (5–10, 42). However, these can be classified into two main approaches. One, called a trial-and-error approach, involves trying one mobile phase and column at a time. The other, called an automated screening approach, involves screening multiple columns and mobile phases using an automated system. These HPLC strategies can be applied to UHPLC.

1.2.2.4.1 Trial-and-Error Approach This strategy has often been used in the past and is still used widely today. A method can be developed very quickly by carefully selecting a column and mobile phase for a sample with simple composition. However, for a very complicated sample, an analyst needs to analyze the scouting results and decide what to do next, which usually is most effective when the analyst has extensive experience. In addition, the approach lacks efficiency due to manual column changing as well as good understanding due to limited information.

1.2.2.4.2 Automated Screening Approach The prerequisite for this strategy is to have an automated system. After selecting columns, mobile phases, and detection

and settings, it is necessary to build instrument methods and a sample set. All columns and mobile phases can be screened automatically. Typically a photo diode array (PDA) detector is used, and the automated system runs all conditions. This approach does not require an analyst to have a lot of experience. The analyst simply runs preselected columns and mobile phases on an automated system, called Toolkit. This approach is highly efficient. In addition, it may provide a lot of information on selectivity of different bonded phase columns, mobile phase organic solvents, and buffer pHs. It facilitates better understanding of the method and gives more insurance on method specificity. However, when a sample is not complicated, this approach takes longer to screen all columns and mobile phases than when using the trial-and-error approach.

In practice, both trial-and-error and automated screening approaches are used. Both have pros and cons. Analysts can choose an appropriate approach based on instrument availability and complexity of a sample.

1.2.3 Analyze Scouting Results and Select Separation Conditions

1.2.3.1 Analyze Scouting Results

After a sample set run is complete, an analyst needs to analyze the scouting results. Depending on the extent of sample complexity, it may have several conditions that provide desirable separations. However, it may turn out that no tried conditions can give desirable separation. The analyst needs to understand the results and find a clue as to what conditions, columns, or mobile phases may work for the separation. Then new conditions, columns, or mobile phases must be tried, until a desirable separation is achieved.

1.2.3.2 Select Separation Conditions

Once separation is achieved for all peaks of interest, a condition needs to be selected from several potential conditions. The goals of a method must be considered against candidate conditions. At this point, resolution and tailing factor must be evaluated for candidate conditions. For a small impurity peak on shoulder of a major peak shown in Figure 1.4, US pharmacopeia (USP) resolution at equal to or greater than 1.5 is not adequate to ensure accurate integration. The integration result of the small peak varies with a way of integration, which introduces uncertainty to the analytical results. In this case, a discrimination factor needs to be used (14). The discrimination factor is defined in the following:

$$d_o = \frac{h_p - h_v}{h_p} \tag{1.1}$$

where d_0 = discrimination factor, h_p = impurity peak height, and h_v = height of the valley by drop integration.



Figure 1.4 Illustration for a discrimination factor.

For an early-phase project, the discrimination factor is usually equal to or greater than 0.5. However, for a late-phase project, it needs to be equal to or greater than 0.8 for high accuracy.

1.2.4 **Optimize the Method**

Once separation conditions are selected, column, mobile phase organic solvent, and buffer are known. However, column dimension, flow rate, column temperature, mobile phase organic solvent gradient range and gradient time, and buffer pH need to be optimized for better resolution, robustness, and shorter run time. If necessary, buffer concentration needs to be optimized or triethylamine (TEA) needs to be added for better resolution or tailing factor.

There are several software and automated systems for HPLC method development and optimization, such as Drylab[®], Chromsword[®], and ACD/AutoChrom MDS, and others (43-47). Their principles can be applied to UHPLC. In addition, Waters Corp. (Milford, MA) has recently promoted Fusion Method DevelopmentTM software. Fusion Method DevelopmentTM software from S-Matrix integrates seamlessly with Water's ACQUITY UPLC and Empower 2 Chromatography software to automate method development. The software automatically generates instrument methods and sample sets. Another feature of this software is to visualize data by statistically fitting the results. However, it cannot generate simulated chromatograms at predicted conditions, like Drylab[®] can.

The optimization can be efficiently performed with method development software such as Drylab[®] using data generated from the scouting experiment or a few additional runs. This approach can effectively use the data generated during the scouting and necessitates the limited number of additional runs. Furthermore, Drylab[®] provides useful information on robustness and ruggedness based on the limited number of runs (48). This is discussed in Examples 1.1 and 1.2.

In addition, injection amount determined by sample concentration and injection volume may need to be optimized. Lower injection amount may result in less sensitivity and higher efficiency and resolution. However, higher injection amount may cause higher sensitivity and lower efficiency and resolution. Therefore, the injection amount needs to be optimized to meet requirements for both sensitivity and resolution.

1.2.5 Validate the Method

After carefully selecting separation conditions and optimizing the method, the method must be validated. However, if any parameter cannot meet the predefined method goals, the method must be re-optimized or even redeveloped.

1.2.6 Phase-Appropriate Method Development

Appropriate analytical methods are required for drug development at different phases, which span from drug discovery to new drug application (NDA) or marketing authorization application (MAA) filing to launch and manufacture, as shown in Figure 1.5.

API method development effort during drug discovery is limited. In phase I, all impurities must be separated from a major peak to ensure peak purity. In phase II, a synthetic route needs to be selected and optimized. The method needs to be challenged when the route or process is changed. In phase III, the route is selected and the process is finalized. Critical Quality Attribute (CQA) impurities are defined and their references are typically prepared for method development. Method robustness and ruggedness must be demonstrated, and relative response factors of all impurities CQAs are determined before regulatory filing such as NDA and MAA. The validated definitive methods are transferred to manufacturing sites.

The API method can be modified for pharmaceutical dosage forms that consist of API and excipients throughout the drug development. The modified method should be able to separate API, degradants, impurities, and excipients from each other to accurately determine assay and impurity in the pharmaceutical dosage forms. An appropriate sample preparation is needed to ensure method accuracy and sensitivity for the pharmaceutical dosage forms (31–35).



Figure 1.5 Drug development process.

1.2.7 Very High Pressure and Frictional Heating in UHPLC

Very high pressure in UHPLC alone can increase the retention factor compared with HPLC (49–53). For neutral compounds, a relatively small increase in the retention factor was observed over a pressure increase of 500 bar. However, for polar solute, or solutes with large molecular weight, acids, or bases, a much larger increase in the retention factor was observed. The differential increase in retention factors can provide significant selectivity effect for a mixture of different types of analytes in UHPLC.

Frictional heating becomes more pronounced in UHPLC, although smaller column diameters are used compared with HPLC. Both theoretical and experimental results indicate that significant radial and longitudinal temperature gradients are formed along the column (54). The frictional heating manifests itself in the retention factor reduction with pressure increase (2, 55). The change in the retention factor with pressure may provide an additional tool to manipulate selectivity in UHPLC.

Very high pressure and frictional heating in UHPLC may provide different selectivity than HPLC. Therefore, they may make HPLC method conversion to UHPLC or vice versa more complicated. Their effects on separation need to be considered when an HPLC or UHPLC method is converted.

1.2.8 Relevance of Various Instrumentation to Method Development

Currently, there are several UHPLC instrument vendors; Waters Corp and Agilent Technologies are two major suppliers. Ideally, a method developed in one instrument works in another instrument. However, these instruments may perform differently because their specifications or even their designs may be different. For example, Agilent 1290 uses a Jet weaver to mix mobile phases, whereas Waters Acquity uses a regular volume mixer to mix mobile phases. The dwell volume of the former may be smaller than that of the latter. In addition, the Jet Weaver has much better mixing efficiency than the regular volume mixer. The effect is pronounced when mobile phase contains high concentration TFA, e.g., 0.1%. Therefore, the difference in instrumentation needs to be considered when a method is developed on one instrument but validated or used on another instrument.

1.2.9 Method Resolution and Speed Requirements

For most methods, resolution is required for method specificity, and high speed is desirable for short turnaround time and high throughput. In addition, high speed also contributes to great solvent saving. However, for some methods, only a major component is required to be determined. For example, dissolution testing only determines API concentration in dissolution media at different time points. It is unnecessary to

determine impurities. In this case, high speed is required, especially for real-time monitoring.

There are different approaches to increasing UHPLC speed. These include using a short column of small particle sizes, high flow rate, and high temperature. The small particle sizes make it possible to use a short column and high flow rate without sacrificing efficiency or resolution. In addition, high temperature reduces mobile phase viscosity, resulting in high diffusion coefficient and flow rate without significant loss of efficiency and increase in column back pressure (23).

1.2.10 Example 1.1

Method development and simultaneous optimization of gradient program and column temperature for UHPLC separation

This example describes method development for an intermediate. Four columns, two organic solvents, and six buffers were scouted at UV wavelength 265 nm. Four columns were BEH C18 50 \times 2.1 mm, 1.7 μ m, BEH phenyl 50 \times 2.1 mm, 1.7 μ m, Zorbax SB-C8 50 \times 2.1 mm, 1.8 μ m, and Bonus RP 50 \times 2.1 mm, 1.8 μ m. Two organic solvents were acetonitrile and methanol. Six buffers were 0.1% TFA in water (pH \sim 2.0), 0.1% acetic acid (pH 3.3), and ammonium acetate (pH 4, 4.8, 5.8, and 6.8).

The scouting results indicate that the compound was unstable at pH close to 7, and it was difficult to elute the intermediate and one of its impurities out of the columns with methanol. Therefore, 0.1% TFA buffer, acetonitrile, and BEH phenyl column were selected. However, separation of its impurities needs to be further optimized.

Two basic gradients with different gradient times (2.5 and 7.5 min) were run at two different column temperatures (20 and 50°C) on BEH phenyl 100 × 2.1 mm, 1.7 μ m to optimize the separation of an API intermediate and its impurities. The data was input to Drylab[®], and a resolution map was obtained and shown in Figure 1.6, where the smallest value of resolution (R_s) of any two critical peaks in



Figure 1.6 Two-dimensional resolution map of the column temperature ($^{\circ}$ C) against gradient time (t_{G} , min) for the separation of an intermediate and its impurities 1–4.



Figure 1.7 Predicted (a) and experimental (b) chromatograms of an intermediate were optimized by 2.5 and 7.5 min gradient basic runs at two different column temperatures (20 and 50°C). Conditions: BEH Phenyl 100 \times 21 mm, 1.7 μ m; mobile phase A 0.1% TFA in water; mobile phase B acetonitrile; gradient program 32%–60% B for 6.9 min, 60%–100% B for 1.1 min and hold at 100% B for 1 min; flow rate 0.6 mL/min; column temperature 50°C; injection volume 0.7 μ L; detection wavelength 265 nm. Peaks 1–5 are impurities.

the chromatogram is plotted as a function of two simultaneously varied experimental parameters. In this case the parameters are gradient time and column temperature. Figure 1.6 indicates that higher temperature and longer gradient time can achieve better resolution. Gradient time (tG) of 6.9 min and column temperature of 50°C were selected with the consideration of resolution criterion, run time, and column life at high temperature.

The predicted optimum conditions were run, and the predicted and experimental chromatograms are shown in Figure 1.7. Only data of impurities 1–4 was input to Drylab[®] because the intermediate and impurity 5 eluted after the gradient. The experimental chromatogram is similar to the predicted chromatogram for impurities 1–4. In addition, retention times of the predicted and experimental chromatograms were listed and compared in Table 1.4. The results indicate that the predicted retention

Experimental (min)	Predicted (min)	Difference (min) ^a	% Error ^b
5.803	5.58	0.223	4.0
5.951	5.82	0.131	2.3
6.37	6.43	-0.06	-0.9
7.778	7.78	-0.002	0.0
	Experimental (min) 5.803 5.951 6.37 7.778	Experimental (min)Predicted (min)5.8035.585.9515.826.376.437.7787.78	Experimental (min)Predicted (min)Difference (min)^a5.8035.580.2235.9515.820.1316.376.43-0.067.7787.78-0.002

 Table 1.4
 Comparison of Experimental Retention Times with the Predicted by Drylab[®]

^aDifference = experimental - predicted by Drylab.

^b% error = [(experimental – predicted)/predicted] \times 100.

times were close to the experimental ones. The relative retention time error was not greater than 4.0%. DryLab[®] demonstrated reasonable prediction accuracy for retention time.

1.2.11 Example 1.2

Simultaneous optimization of gradient program and mobile phase pH for UHPLC separation of basic compounds

This example describes method development for basic API, its impurities, and degradation products (48). Zorbax SB C18 50 \times 2.1 mm, 1.8 μ m was selected. The flow rate was set at 0.5 mL/min. Methanol and buffer were used as a mobile phase. The mobile phase A consisted of 5% methanol and 95% buffer (10 mM phosphate + 0.1% triethylamine); the mobile phase B was 80% methanol and 20% buffer. Two basic gradients with different gradient times (7 and 21 min) were carried out at three different buffer pH values (6.2, 6.6, and 7.0).

The data was input to Drylab[®], and the resolution map has been obtained and is shown in Figure 1.8. In this case the critical resolution in the chromatogram is plotted as a function of gradient time (min) and mobile phase buffer pH. The resolution map



Figure 1.8 Two-dimensional resolution map of the gradient time (min) against mobile phase pH for the separation of basic API and its related impurities and degradation products. Reprinted from Fekete, S.; Fekete, J.; Molnar, I.; Ganzler, K.; *J. Chromatogr. A.* 2009, 1216: 7816–7823. Copyright (2009), with permission from Elsevier.



Figure 1.9 Predicted (a) and experimental (b) chromatograms. Column: Zorbax SB C18 50 × 2.1 mm, 1.8 μ m, mobile phase A: methanol–buffer 5–95 V/V% (buffer: 10 mM phosphate + 0.1% triethylamine, pH 6.7), mobile phase B: methanol–buffer 80–20 V/V% (buffer: 10 mM phosphate + 0.1% triethylamine, pH 6.7), gradient elution (initial 0% B, at 0.7 min 0% B, at 3.1 min 65% B and 100% B at 10 min), flow: 0.5 mL/min (p = 531 bar), column temperature: 30°C, injection volume: 3 μ L, detection: 230 nm, analytes: basic drug API and its related impurities and degradation products: (1) peak of light stress origin (unknown) (2) 1-naphtol (3) duloxetine (4) duloxetine-3-isomer impurity (5) dimethyl-duloxetine impurity, and (6) duloxetine impurity A. Reprinted from Fekete, S.; Fekete, J.; Molnar, I.; Ganzler, K.; *J. Chromatogr. A.* 2009, 1216: 7816–7823. Copyright (2009), with permission from Elsevier.

indicates buffer pH 6.7 would achieve enough resolution within 10 min. The predicted optimal condition was tested. The predicted and experimental chromatograms are shown in Figure 1.9. The experimental chromatogram was similar to the predicted chromatogram. The predicted retention times and resolution were in good agreement with the experimental ones. The average relative predicted retention time error was less than 2%, and the average relative predicted resolution (R_s) error was 6.5%.



1.3 CONVERSION OF AN HPLC METHOD TO UHPLC

There are many HPLC methods used for commercial drug products and new investigational drug products in the pharmaceutical industry. Some converted methods were reported in the literature (2, 56–62). HPLC methods can be converted to UHPLC systematically via the following steps: (i) select a UHPLC column with the same chemistry to maintain the same selectivity; (ii) measure instrument dwell volumes; (iii) calculate injection volume, flow rate, holding time, and gradient segment time for UHPLC; (iv) run an experiment to verify conditions; and (v) validate the method. Each step is described and discussed in detail and summarized in Figure 1.10.

1.3.1 Select a UHPLC Column with the Same Chemistry

There are a variety of HPLC columns used in the pharmaceutical industry. To convert HPLC methods to UHPLC, one should always contact the HPLC column vendors to see if they provide UHPLC columns with the same chemistry. The major UHPLC instrument manufacturers such as Waters Cor. (Milford, MA) and Agilent Technologies, Inc. (Santa Clara, CA) also provide some UHPLC columns with the same chemistry of their HPLC columns. Table 1.5 lists some examples.

If UHPLC columns with the same chemistry as HPLC columns are not available, UHPLC columns with a similar chemistry from other column vendors can be evaluated.

Once the column with the same or similar chemistry is selected, column dimensions such as length and diameter are chosen. The most common diameter of UHPLC

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Table 1.5	Examples of	HPLC and	UHPLC	Columns	with the	Same	Chemistry

	HPLC	UHPLC
Waters	XBridge C18 3.5 or 5 μm XBridge Phenyl 3.5 or 5 μm	BEH C18 1.7 μm BEH Phenyl 1.7 μm
Agilent	XBridge CN 3.5 or 5 μm Zorbax Eclipse Plus C18 3.5 or 5 μm Zorbax SB-C18 3.5 or 5 μm	BEH CN 1.7 μm Zorbax Eclipse Plus C18 RRHD 1.8 μm Zorbax SB-C18 RRHD 1.8 μm

columns is 2.1 mm, and the column lengths are 10 and 5 cm. Different column lengths can be selected, depending on the complexity of separation.

1.3.2 Measure Dwell Volumes of HPLC and UHPLC Systems

To calculate holding time, the dwell volumes of both the HPLC and UHPLC systems need to be determined. There are several ways to measure the dwell volume. The following is an example for an HPLC system.

Prepare 1% acetone in water as mobile phase B, and water alone as mobile phase A. A column under measurement is removed from the line, and the tubing is connected with a zero dead volume union. A gradient of 10 min is run from 100% A to 100% B at the flow rate of 2 mL/min and held for 10 min at 100% B with detection wavelength at 260 nm. The difference between 5 min and the time at half height between the initial baseline and the plateau times the flow rate is the dwell volume for the HPLC system. The typical dwell volume for most HPLC systems is approximately 1 mL.

For a UHPLC system, a similar procedure can be used. However, the flow rate needs to be adjusted according to maximum pressure of the UHPLC system. In addition, a restrictor may be needed if the back pressure is too low. The typical dwell volume for most UHPLC systems is 0.1 mL as shown in Figure 1.11. The approximate dwell volumes for Waters Acquity and Agilent 1290 are listed in Table 1.6.



Figure 1.11 Measurement of UHPLC dwell volume.

 Table 1.6
 Approximate Dwell Volumes of Waters Acquity and Agilent 1290

System Name	Mixer (µL)	Approximate Dwell Volume (μL)
Waters Acquity	50	100
	250	300
Agilent 1290	35	100
0	100	165

1.3.3 Calculate Injection Volume, Flow Rate, Holding Time, and Gradient Segment Time

A few calculation tools are available from instrument vendors and UHPLC practitioners. The basic principle is to keep the number of the elution column volume the same for HPLC as for UHPLC. In addition, appropriate flow rate and injection volume should be used (63,64).

1.3.3.1 Injection Volume

Injection volume is adjusted to be proportional to UHPLC column dimensions to maintain its resolution and sensitivity. Sample diluent and concentration are kept the same. The following is used to calculate the UHPLC injection volume:

UHPLC injection volume = HPLC injection volume

 $\times \frac{UHPLC \ column \ volume}{HPLC \ colume \ volume}$

Column volume =
$$\pi \times \left(\frac{d}{2}\right)^2 \times l \times \varepsilon$$
 (1.2)

where *d* is the column inner diameter, *l* is the column length, and ε is the packing material porosity (generally 0.4)(2).

1.3.3.2 Flow Rate

The UHPLC flow rate needs to be adjusted according to column diameter (d) and optimal linear velocity (μ_{opt}) as expressed in the following equation:

Flow rate(mL/min) =
$$60 \times \pi \left(\frac{d}{2}\right)^2 \times \mu_{opt}$$
 (1.3)

1.3 Conversion of an HPLC Method to UHPLC 19

The optimal linear velocity (μ_{opt}) depends on particle size (d_p) and the diffusion coefficient in the mobile phase of compounds to be separated (D_m) . The relationship is shown in the following equation (52):

$$\mu_{opt} = 3 D_m / d_p \tag{1.4}$$

For small molecules with a diffusion coefficient of 6×10^{-6} cm²/s, the optimal flow rate on a 2.1 mm i. d. 1.7 μ m UHPLC column is approximately 0.2 mL/min. However, higher flow rates can be used because the efficiency decreases little at higher flow rates (1). In practice, the flow rates of 0.4 mL/min or 0.6 mL/min or even higher are used.

1.3.3.3 Holding Time and Gradient Segment Time

When converting an HPLC method to UHPLC, one should express system dwell volumes and gradient segments in terms of column volume and maintain the same numbers of column volume for UHPLC and HPLC.

Number of column volume for system =
$$\frac{System \ dwell \ volume}{Column \ volume}$$
 (1.5)

Number of column volume for gradient segment =
$$\frac{Gradient \ volume}{Column \ volume}$$
 (1.6)

1.3.3.4 UHPLC Columns Calculator

Besides manual calculation, UHPLC conditions can be obtained with a UHPLC calculator, for example, the ACQUITY UPLC Columns Calculator, with the following procedure: (i) enter parameters and conditions such as column length, diameter, particle size, molecule weight of samples, flow rate, column temperature, injection volume, instrument dwell volume, and gradient as shown in Figure 1.12; (ii) click the Calculate button to open the Gradient Results window then check the Show Additional Options box as shown in the right of Figure 1.13; and (iii) click a gradient result, and the corresponding gradient will appear. Different gradient results are shown in Figure 1.13, Figure 1.14, Figure 1.15, and Figure 1.16. In addition, the ACQUITY UPLC Columns Calculator provides gradient results for different column dimensions as shown in Figure 1.17.

Usually, the UPLC conditions with scaled gradient (accounting for particle size) are selected for method verification; however, the UPLC conditions with scaled gradient (disregarding particle size) are not correct. The UPLC conditions for shortest analysis time at original peak capacity usually have column back pressure close to or over the instrument limit. The conditions with maximum peak capacity at original

ACI	QUITY UPLC Colour	ns Galcolator - Untitled *			0	
rie D	dit Halp					
An	alytical Factors					A
1	O Isocratic	Holecular Weight Colu	nn Temperature			9
3	③ Gradient	301-500 M Da 40	~C			Carcinos
HILC	(Imary) 💌	From HPLC	To UPL	с	UPLC (FSP()	*
Fro	IN HPLC	L/dg = 42.857	TO UPLC		Udp =	55.524
0	olumn Length (L):	150 M mm	Column Le	ngth (L):	00 <u>v</u> mm	
G	olumn Diameter:	4.6 ¥ mm	Column Di	ameter: 2	2.1 ¥ mm	
PI	article Diameter (dp)	1: 3.5 ¥ µm	Particle Di	ameter (dp):	.7 ¥ µm	
In	rjection Volume:	5 µL	Maximum	Pressure: 1	4997 pai	
D	well Volume:	1.00 mL	Dwell Volu	mes 0	.300 mL	
-	ateal Gradient		Organic Mix			_
on	ginai Grauieni		Acctonitri	ka: 100.0 %	Hethanol: 0.0	46
126	s por	1		1 80	1	
	Time (min)	 Flow (mL/min) 	(Aqueous)	(Organic)	Column Volum	86
Þ 1	Initial	1.00	80.0	20.0		
2	12.00	1.00	44.0	20.0	0.06	
4	20.00	1.00	80.0	20.0	1.80	
+ 5	5					
_	_		_	_		-
-						
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Figure 1.12 Enter parameters and conditions.



Figure 1.13 New UPLC conditions with scaled gradient (accounting for particle size).

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ACQ	UITY UPLC Colum	us Galculator - Untitled *				Crashent Results - Untitle	ed				
e til	t Malo					Column	Bun Tine (min)	Peak Capacity	Flow Rate (sL/nm)	Precoure	Injection Vol
	Adical Exclore					Original HPLC column c	onditions				
-	Transfer	Mate a deal Wate bit			6	150 mm x 4.6 mm 3.5 um	12.00	88	1.00	1263	5.0
	Gendent	101 500 Y Ba 40	vc	51	Calcula	New LIFE Consultance	all cooled are	And frameworking	for controls simply		
	Gradient					New OPEC conditions a	with scaled gra	dient (accounting	for particle size)	-	
						100 min x 2.1 min. 1.7 µm	6.43	104	0.43	7800	0.7
10	(tnary) 👻	From HPLC	To UP	LC	TMC (1340 ~	New UPLC conditions a	th scaled gra	dient (daregerdir	g particle size)		
	HPLC	L/da = 42.887	ToUPLC		1.14e = 55.424	100 mm is 2.1 mm, 1.7 µm	15:33	107	0.21 :	8718	07
col	larnn Length (L):	150 v mm	Column	ength (L):	100 v mm	New URLC conditions for	or elevations and	dama tama at estat	and much manager		
						THE OF DE CONDUCTOR	of all official series	iyata tante ta tanga	in ben cabaci	,	
	anno fissuetree	4.6 V mm	Colorna t	houseter	1 × mm						
	lumn Diameter:	4.6 Y mm	Column t	Nameter: 2	2.1 <u>~</u> mm	100 mm x 2.1 mm. 1.7 µm	2.87	88	0.99	16964	0.7
Pa	lumn Diameter: rticle Diameter (dp)	4.6 V mm 3.5 V pm	Column t Particle I	Nameter (dp): 1	L7 ⊻ μ⊪	100 nm x 2.1 nm . 1.7 µn New UPLC conditions 5 100 nm x 2.1 nm , 1.7 µn	2.87 or maximum per 20.00	88 alk capacity at or 150	0.99 Iginal run time 0.02	16964	0.7
Fa Fa Inj Dw	lumn Diameter rticle Diameter (dp) action Volume: rell Volumes	4.6 v mm 1 3.5 v pm 5 pL 1.00 mL	Columns Particle I Nacdmur Dwell Vol	Nameter: 2 Nameter (dp): 3 n Prossurs: 3 lumes 0	2.1 × mm 1.7 × μm 1.300 mt.	100 mm x 2.1 mm, 1.7 µm New UPLC conditions 5 100 mm x 2.1 mm, 1.7 µm	2.87 or maximum pe 20:00	88 alk capacity at or 150	0.59 Iginal run time 0.62	16964	a7 a7
Pa Inj Dw Orlig	lumn Diametee rticle Diameter (dp) action Volume: ell Volume: pinal Giradient pa	4.6 v mm 8 3.5 v pm 5 pL mL	Column t Particle I Naximur Dwell Vol Organic Pla Acctonit	Nemeter (dp) 1 Dismeter (dp) 1 n Prossars: 0 sens: 0 c c c c c c c c c c c c c c c c c c c	2.1 v mm 1.2 v pn 1.300 nt 1.300 t	100 mm x 2.1 mm, 1.7 µm New UPLC conditions 5 100 mm x 2.1 mm, 1.7 µm	2.87 or maximum pe 20.00	88 aik capacity at or 190	0.59 Iginal run time 0.62	16564	0.7
Col Pa Inj Dw	tome Unametee eticle Diametee (dp) action Volume: eti Volume: pinal Giradient psi Time (min)	4.6 v mm 1 3.5 v pm 5 pL 1.00 mL 4.6 v mL	Column C Particle I Plastimur Dwell Vol Crganic His Acctualt Sta	Nemeter (da): 1 Nameter (da): 1 In Pransura: 1 Ismes 0 In Pransura: 1 In Pransura	2.1 v mm 1.7 v μm 4950 psi 1.300 mt • Picthanob <u>0.0</u> 4 Columi Volumes	100 mm x 2.1 mm, 1.7 µm New UPLC conditions 5 100 mm x 2.1 mm, 1.7 µm	2.87 or maximum pe 20:00	88 ak capacity at or 150	0.59 Iginal run time 0.62	16564	a7 a7
Col Pa Inj Du Inj 263	umn Diameter: (de) Diameter (dp) action Volume: ell Volume: linal Gradient psi Time (min) Initial	4.6 v mm 3.5 v mm 1.00 mL 1.00 mL	Column C Particle I Naximur Dwell Vol Organic Na Acctonit SA (Aquecus) 90.0	Numeter: 2 Nameter (dp): 3 In Pranara: 1 Iumes 0 C Iumes 0 Iumes	2.1 v mm 1.7 v mm 4950 pri 1.300 ns Plethanek 0.0 4 Column Volume	100mm x 21mm 17um New UPLC conditions 9 100mm x 21mm 17um	2.87 20.00	88 ak capacity at or 150	0.59 Iginal run time 0.62	16964	a7 a7
Col Pa Inj Do Pa	unn Danetter rticle Diameter (dp) action Volume: ell Volume: pai Time (min) baltiat 12.00	4.6 W MMR 3.5 W MMR 5 pit. MK 1.00 mL MK 1.00 L.00 MK	Column C Particle I Naximum Dwell Viol Acctonit (Aquecus) 90.0 944.0	Nemeter: 2 Nameter (dp): 3 In Pranara: 0 In	2.1 v mm 1.7 v mm 4950 pn 1.300 mi • Picthasoli 0.0 i Colume Volume • 7.20	100 mmx 2.1 mmx 1.7 um New UPLC constitutes 1 100 mmx 2.1 mm, 1.7 um 1 diven 2.2 time, 1.7 um	2.87 or maximum per 20.00	88 ak capacity at or 150 mice UPLC column	0.59 Iginal run time 0.52	11185	0.7 0.7 Сор.
Col Pa Inj Du 1nj 263 1 263	umn Diameter: tticle Diameter (dp) action Volume: ell Volume: pinal Gradient pi Time (min) Jaittal 12.00 12.10 Dia Dia	4.6. W MMR 3.5. W yrm 5 pl. rnL 1.00 FORM (ML/M/M) rnL 1.00 1.00 1.00	Column L Particle J Naximue Dwell Vol Corgenic Mie Acctositi ShA (Aquecus) 90.0 44.0 90.0	Nemeter: 2 Nameter: (0): n Pressure: 0 same: 0 c 0 rsk: 100.0 %8 (Organic) 20.0 56.0 20.0 0	2.1 (v) mm 1.7 (v) µm 4950 pei 1.300 mt Ficthanob 0.0 4 Colume Volumes 7,20 0.00	100 mmx 2.1 mm, 1.7 um Here UPEC conditions 5 100 mmx 2.1 mm, 1.7 um C Shown 2.21 km, 1.7 um	2.87 or maximum per 20.00	88 ak capacity at or 150 more UPLC columns latery UPL	0.56 Sginal run time 0.02	16564 11185 scaled grav	0.7 0.7
Col Pa Inj Dw 11j 2345	unn Danetter ricle Diameter (dp) action Volame: ell Volames inal Gradient pi Time (mn) bittol 12,00 12,10 20,00	4.6. V mm 3.5. V ym 5 pL st. 1.00 st. st.	Column L Particle I Nassimue Dwell Vol Crganic Mic Acctualt (Aquecua) 90.0 44.0 90.0 53.0	Nemeter: 2 Nameter: (dp): 1 n Pressure: 0 ismes: 0 c 0 chic: 100.0 (Organic) 20.0 20.0 20.0	2.1 ≤ mm 1.7 ≤ μm 4050 psi 3.300 mi Tethandi 5.0 € Colums Volumes 	100mmx 21mmx 17um New UPLC conditions 1 100mmx 21mmx 17um 100mmx 21mmx 17um 101mmx 21mmx 17um 101mmx 21mmx 17um	2.87 or maximum per 20:00	83 ak capacity at or 190 more UPLC columns likew UPL 100 mm	0.59 iginal run time 0.62 C conditions with x 2.1 mm, 1.7 pm	16964 111185 scaled grav column	0.7 0.7 Elent (disreg
Col Pa Inj Dw 11j 234 5	inne Daniette: rtide Diameter (dp) action Volume: ell Volume: linal Gradient pi Time (min) bitist 12,00 12,10 20,00	6.6. V MBR 3.5. V yers 5. pt. mt. 1.00 MSR mt. 1.00 L00 L00 1.00 L00 L00	Column t Particle I Naximum Durell Vol Organis: Na Acctoall Stat (Aquesca) Stat Stat (Aquesca) Stat Stat Stat Stat Stat Stat Stat St	Numeter: 2 Nameter: (dg): 2 n Pressure: 0 kmme: 0 criss: 100.0 (Sgmic) 20.0 20.0 20.0	2.3 v nm 1.7 v pn 4950 pn .200 nt. Plathanck 2.0 v Colume Volumes 7,29 0,06 4,80	Internet 21 and 17 and Rev UPLC conditions 5 100 and 21 and 17 and and 100 and 21 and 19 and New UPLC Gradient New UPLC Gradient	2.87 or maximum per 20.03 	88 ak capacity at or 150 rote UPLC colores likew UPL likew UPL Flow (mL/m n)	0.59 iginal run time 0.02 C conditions with x 2.2 mm, 3.7 pm S& (Agencia	scaled grav column	0.7 0.7 Cop. Sient (disreg
Col Pa Inj Du Pa 12345	unn Danetter ride Diameter (dp) action Volume: eff Volume: eff Volume: final Gradient ps Trms (m n) bitbi 12.00 12.10 20.00	4.6. ann 9.7.5 ann 1.0.5 ann 5 ann 5 ann 5 ann 1.00 ann 1.00 1.00 1.00 1.00	Column t Particle J Nactimue Dwell Vol Acctosell Acctosell State S	Nameter: 2 Nameter: (do): 3 n Prosears: 0 tame: 100.0 % (Organic) 20.0 56.0 20.0 20.0	2.3 v nm 1.7 v pn 4550 pr 1.300 nt Tethand 0.0 n Column Values 7.79 0.066 4.80	100 rems 22 terms, 17 um 100 rems 22 terms, 17 um 100 rems 21 term, 17 um 100 rems 21 term, 17 um 100 rems 21 terms, 17 um 100 rems, 10	2.87 cr nashrun pe 20.03 Show t 0.21	83 sk capacity at or 150 rore UPLC columns Mere UPLC 100 mm Flow (mLmm)	0.59 iginal run time 0.00 C conditions with x 2.1 mm, 3.7 pm SA (Aqueou 80.0	scaled grat column column () %B(0 20.0	0.7 0.7 Elent (disreg
Col Pa Inj Dw Pa 1nj Dw 11j 263 1 263	unn Danetter rticle Dameter (dp) action Volume: ell Volume: pai Time (m m) Tatta 12,00 12,10 20,00	4.6. 2 ann 3.3. 2 pm 7 pd. sc. 1.00 sc. sc. 1.00 sc. sc.	Column I Particle I Nuestruce Organic Nu Acctoant Acctoant Acctoant Accession 441.0 800.0 5000	Nameter 2 2 33 30 meter (ds): 1 2 35 30 mete	2.3 v mm 1.2 v pm 4950 pm 3000 m6 Column Volumes 2.29 0.060 4.80 4.80	International Strength Strengt	2.87 20.03 20.03 Show t 0.21 0.21 0.22 0.21	88 ek capacity at or 199 nore UPLC column litere UPL 100 mm Flow (mL/m.n)	0.59 (ginal run time 0.62 C conditions with x 2.1 mm, 1.7 ym Sck. (Aquec: 90.0 44.0	scaled gras scaled gras column s) %8.00 20.0	0.7 0.7 Stent (disrep typenic)

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Figure 1.14 New UPLC conditions with scaled gradient (disregarding particle size).

 ACQL 	UTY UPI C Column	us Galculator - Untitled *				×	Gradient Results - Untitle	el .				
the fair	i Palo						Calumn	Bun Tine	Peak Capacity	Flow Rate	Precoure	Injection Volu
	half and first own						Original HPLC column ca	anditions			~	-
And	lytical Factors	Mala a desilitatable Color			(150 mm x 4.6 mm 3.5 um	12.00	88	1.00	1263	5.0
	Geodeset	301 500 Y Ba 40	vc		Cel	cuinte	New URLC conditions of	ally according to the	And free sting	des and the size		
							New OF CC conclusions w	en scaleo gra	alent (accounting	a for particle size		022
						-	100 min x 2.1 mil. 1.7 µn	6.43	104	9.43	7880	0.7
-9100	dmary) 😤	From HPLC	To UPLC		TAPE (834)	~	New UPLC conditions w	Ith scaled gra	dient (daregardir	ng particle size)		
From	HPLC	L/dg = 42.857	To UPLC		Udp = 587	524	100 mm x 2.1 mm, 1.7 µm	15.33	107	0.21	3768	0.7
Colt	urnn Length (L):	150 ~ mm	Column Len	gth (L): 1	00 ~ mm		New UPLC conditions for	r shortest and	lysta time at ong	inal peak capacit	,	
Coli	unn Dameter:	4.6 Y mm	Column Diar	meter: 2	.1 × mm		100 min x 2.1 min. 1.7 µm	2.87	88	0.99	16964	0.7
Par	ticle Diameter (dp)	3.5 ¥ µm	Particle Dia	meter (dp): 1	.7 v µm		New UPLC conditions for	c maximum pe	ak canadity at d	noinal nun time		
							100 mm a 21 mm 1.2 mm	50.00	160	A #2	11100	0.7
Inte	ection Volume:	5 at.	Naximum P	ressure: 1	4950 pst		Tourners 2. Lines, 1.2 Jan	20.00	130	1.14	11162	u.)
Day	ell Volumes	1.00 mL	Davell Volum	ars 0	.300 mL							
						-						
			- Organic Mis-									
Orig	inal Gradient		Acctonitrile	: 100.0 %	Hethanol: 0.0	44						
1263	psi					-						
	Time (min)	Flow (mLimin)	NA (Aqueous)	%8 (Organic)	Column Volumes							
1	Initial	1.00	80.0	20.0			Show additional options	Show	more UPLC columns			Deey 2
- 2	12.00	1.00	44.0	20.0	7.29	-						
F 4	20.00	1.00	80.0	20.0	4.80		New UPLC Gradient	t	New UPL capacity 100 mm	C conditions for s	hortest anal	lysis time at o
							Time (min)		Flow (mL/min)	SA (Acuecu	a) %B/On	cartic)
							▶ 1 Initial	0.99		80.0	20.0	
							2 1.72	0.99		44.0	56.0	7.48
							3 1.74	0.99		80.0	20.0	0.06
							4 4.67	0.99		00.0	12000	9.92
	_		_					_	_	_	_	
# st	tart 💊 u									8	16.0	2 11:45 dH

Figure 1.15 New UPLC conditions for shortest analysis time at original peak capacity.

ACQUITY UPLC Colu						Gradient Results - Untitle	el				
tia bin tala						Column	Run Time (nin)	Peak Capacity	Row Rate Pro (mL/mm) (po	scure inject ((4)	ion Volume
Amphibial Exclose						Original HPLC column o	anditions				
Isocratic	Holeoular Weight	Column Temperatures			C	150 mm x 4.8 mm, 3.5 µm	12.00	55	1.00 125	13 5.0	
Gradient	301-500 × Da	40 %			Calculate	New UPLC conditions w	th scaled grad	kent (accounting	for particle size)		
						100 nn x 2.1 nn. 1.7 µn	6.48	104	0.43 780	0.7	
HPLC (truny) 🔗	From HPLC	To UPL	с	UPLC (894)	~	New UPLC conditions v	ith scaled grad	lent (divegandin	g particle size)		
Leona Mill C		To UNIT				100 nm x 2.1 nm, 1.7 µm	13.33	107	0.21 335	88 0.7	
Column Length (L):	150 v mm	Column Lo	muth (L):	100 v mm		New UPLC combines for	a shartest and	and a large of estates	and much comparity		
Column Diameter:	4.6 V mm	Column Di	ameter:	2.1 × mm		100 nov 21 nov 17 up	on a row to view b	he comesponding g	ndient 155	n7	
Particle Diameter (d	(p): 3.5 v um	Particle Di	iameter (da)s	L7 V µm		New URL Constitues 6			and and the s	10 ⁻¹	
						New UPDC conditions to	or meaning in pee	ic capacity at or	iginal fun time		_
Intertion Volume:	5 ut.	Nasteurs	Pressure: D	4950 mt		100 mm x 2 1 mm 1 2 µm	2010	150	100 11	au 107	
Dwell Volumes	1.00 mL	Davell Volu	mes C	.300 mL							
					_						
		Organic Mix			_						
Original Gradient	5	Acctonitri	ke: 100.0 %	Hethanok 0.0	0 4						
1263 psi											
Time (min	Bow (miLimin)	MA (Aqueous)	%B (Organic)	Column Volum	mes						
1 Initial	1.00	80.0	20.0			Show additional options	E Shown	were UPLC columns		ſ	Copy Grad
2 12.00	1.00	44.0	56.0	7.29						-	
> 12.10	1.00	80.0	20.0	0.06	_	New LIPLC Gradien		New UPL	C conditions for may	dmun peak cap	acity at or
+ 5						New OF LC Gradien		100 1111	x all min, all pin of		
						Time (min)	1	low (mL/min)	%A (Aqueous)	%B (Organic)	Cal
						Initial	0.62		80.0	20.0	
						2 12.00	0.62		44.0	56.0	32.31
						3 12.10	0.62		80.0	20.0	0.27
						4 20.00	0.62		80.0	20.0	21.27
👍 start 👘 💊	ACQUITY UPLC Colu								6	26,00	11:49 44

Figure 1.16 New UPLC conditions for maximum peak capacity at original run time.

Part 10-10-10 Dorum 10-10 Dorum 10-10 <thdorum 10-10<="" th=""> <thdorum 10-10<="" th=""></thdorum></thdorum>	Pictor Count Bay Thm Description Incode Machada Count Bay Thm Description Description Incode Machada Count Bay Thm Description Description Incode Machada Description Description Description Description Incode Machada Description Description Description Description Incode Incode Description Description	m heads 01 02 02 02 03 04 03 10 04 04 04 04 04 04 04 04 04 0	mana ky N S 5 S 0 S 0 S 0 S 0 S 0 S 0 S 0 S 0 S 0 S 0	263 263 248 1121 280 127 200 1477 231 200 273	Piec (09) 126 stree) 744 111 2005 744 111 2005 740 1114	Ploy Rets (vrL/min) 1.00 1.00 0.00 0.10 0.10 0.43 0.43	Peak Capacity 73 diant (accounting 90 81 43	Plan Tine Initi Sanditiona 12.00 dth acceled grav ERC 6.48 5.71	Column Original HPLC column of 190 mm 4,5 mm, 3,5 m New UPLC conditions w 50 mm 1,1 (conditions 1,7 gr)			unin Temperaturei	ecular Weight Colu	t Hals Aytical Factors
Marketal factors Marketal factors Output Additional factors Output Additional factors The method factors <	ModyCoal Factors Operation	50 02 02 03 07 14 21 01 02		263 448 1121 249 1121 240 1127 250 251 251 251 251 251 251 251 251 251 251	126 stre) 744 111 2125 412 780 114	1.00 for particle 0.10 0.10 0.43 0.43	73 dieni (accounting 34 97 81 43	anditiona 12.00 fith scaled gra 5.43 9.71	Original HPLC column of 153 mm a 4,5 mm, 3,5 m New UPLC conditions w 53 mm a 1,3 mm, 1,7 m 153 mm, 1,7 m	0		unin Temperaturei	ecular Weights Colu	lytical Factors
Increase Chaine Temperature Control Temperature <	Income Column Temperature Colum Temperature Column Temperature </td <td>5.0 0.2 0.2 0.3 0.7 1.0 0.4 0.7 1.4 2.1 0.1 0.2</td> <td>5 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1</td> <td>263 248 1121 280 127 260 14.72 231 260 261 273</td> <td>126 stre) 744 111 235 412 780 114</td> <td>1.00 tor particle 0.10 0.10 0.40 0.43</td> <td>73 dient (accounting 93 93 81 45</td> <td>12.00 dth scaled gm 3234 6.43 9.71</td> <td>150 mm x 4.5 mm 3.5 ym New UPLC conditions w 50 mm x 1.0 mm 1.7 ym</td> <td>0</td> <td></td> <td>unin Temperaturei</td> <td>ecular Weight Colu</td> <td>Tescatic Hole</td>	5.0 0.2 0.2 0.3 0.7 1.0 0.4 0.7 1.4 2.1 0.1 0.2	5 0 1	263 248 1121 280 127 260 14.72 231 260 261 273	126 stre) 744 111 235 412 780 114	1.00 tor particle 0.10 0.10 0.40 0.43	73 dient (accounting 93 93 81 45	12.00 dth scaled gm 3234 6.43 9.71	150 mm x 4.5 mm 3.5 ym New UPLC conditions w 50 mm x 1.0 mm 1.7 ym	0		unin Temperaturei	ecular Weight Colu	Tescatic Hole
Die office Prom HPLC To UPLC Cuter status To UPLC To UPLC Cuter status To UPLC	Notice State State State State State State State State State State State State State State State State State State State State State State State State State State State State State	01 02 02 03 03 07 10 04 07 14 21 01 01		013 145 1121 227 200 1172 31 200	altre) 744 111 205 412 780 114	0.10 0.10 0.10 0.10 0.40 0.43	dient (accounting 93 93 81 45	4th scaled gra 548 5.48 9.71	New UPLC conditions w Stress 10 mm, 17 pr 100 mm s 10 mm, 17 pr	0		unin Temperaturei	ecular Weight Colu	Tencontric Nick
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The low of an interaction of the second s	Barton W From HPLC Description From HPLC East and the state of the	02 02 03 07 10 04 07 14 21 01 02		28 1121 27 20 127 20 127 20 127 20 275	764 111 205 412 780 114	0.10 0.10 0.43	90 81 45	6.48	100 mm x 1,0 mm, 1,7 ym					
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Original Gradient Original Gradient Original Gradient Office Office <t< td=""><td>Original Gradient Organic Nin Organic Nin 9 9 Test 2 (rs. 1) rin 4.61 63 2.21 100 1283 pic Automatelic Libb.0 % Techanik D.0 % 10.0 10.0 % 10.0 % 10.0 10.0 % 10.0 10.0 10.0 % 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0</td></t<> <td>0.2</td> <td>a</td> <td>102</td> <td>540</td> <td>0.05</td> <td>81</td> <td>20.00</td> <td>150 mm s 1.0 mm, 1.7 am</td> <td></td> <td></td> <td></td> <td>_</td> <td></td>	Original Gradient Organic Nin Organic Nin 9 9 Test 2 (rs. 1) rin 4.61 63 2.21 100 1283 pic Automatelic Libb.0 % Techanik D.0 % 10.0 10.0 % 10.0 % 10.0 10.0 % 10.0 10.0 10.0 % 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0 10.0	0.2	a	102	540	0.05	81	20.00	150 mm s 1.0 mm, 1.7 am				_	
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Links per Links per Stat	Table per la forme (men) / Filme (men) Filme (men) <th< td=""><td>0.7</td><td>a a</td><td>11</td><td>370</td><td>0.21</td><td>35</td><td>13.33</td><td>103 mm x 2.1 mm, 1.7 µm</td><td>/ Hethanot 0.0</td><td>le: 100.0</td><td>Acctonitri</td><td></td><td>inno conocian</td></th<>	0.7	a a	11	370	0.21	35	13.33	103 mm x 2.1 mm, 1.7 µm	/ Hethanot 0.0	le: 100.0	Acctonitri		inno conocian
Time (min) Flow (m1,min) PA State State <thstate< th=""></thstate<>	Time (min) Flow (mL/min) Stat. (Approxim) Stat. (Opamic) Stat. (Opa	0.1		72	957	0.21	197	20.99	150 mm x 2,1 mm, 1,7 µm					psi
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1 Instabil 1.00 90.0 20.0 20.0 2 112.00 1.00 64.0 56.0 7.29 21.3 20.0 20.0 21.2 21.2 1.00 60.0 20.0 20.0 20.0 20.0 20.0 20.0 21.2 20.0 2	1 I (bitta) 1.00 60.0 20.0 statement 20 are 1.2 µr 20.2 to 30 550 are 5.3 km r, 12 µr 20.2 to 30 550 are 5.3 km r, 12 µr 20.2 to 30 550 are 5.3 km r, 12 µr 20.2 to 30 550 are 5.3 km r, 12 µr 20.2 to 30 550 are 1.3 km r, 12 µr 20.2 to 30 550 are 1.3 km r, 12 µr 20.2 to 30 550 are 1.3 km r, 12 µr 20.2 to 30 550 are 1.3 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 550 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r, 12 µr 20.2 to 30 are 1.4 km r,	14		118	201	5.43	22	13.33	100 mm x 3.0 mm 1.7 mm	Column Volumee	(Organic)	(Aguesag)	Flow (mL/min)	Time (min)
I 1/100 1.00 44.0 56.0 7.29 Para Acclerations Para Acclerations <td>2 12.00 1.00 44.0 56.0 7.29 Image: State and State</td> <td>21</td> <td>2</td> <td>12</td> <td>580</td> <td>0.43</td> <td>538</td> <td>20.23</td> <td>150 mm x 3.0 mm, 1.7 µm</td> <td></td> <td>20.0</td> <td>80.0</td> <td>1.00</td> <td>Initial</td>	2 12.00 1.00 44.0 56.0 7.29 Image: State and State	21	2	12	580	0.43	538	20.23	150 mm x 3.0 mm, 1.7 µm		20.0	80.0	1.00	Initial
3 12.10 1.00 00.0 20.0 0.00 1.00 00.0 20.0 1.00 0.00	3 12.10 1.00 80.0 20.0 0.06 For a construction of the second data of t						mana 10 Cashanas	El oras	C Cana additional actions	7.29	56.0	44.0	1.00	12.00
4 (20.00) L60 90.0 20.0 1.80 New UPLC Gradient Brow URC analities with excite quantities (and the cold or quantities (and the cold or quantities) (and t		6					1012-0-00 000185	[0] 34M	El sina susces opens	0.06	20.0	80.0	1.00	12.10
▲ 5 New UPLC Gradient Stem x 1.0 mm 5.1 mm odum Image: State of the	4 20.00 1.00 80.0 20.0 1.80 Northly and the self of an	d are death in a	ad another	caled as		C conditions :	Manu 1101			1.80	20.0	80.0	1.00	20.00
Image Time (min) Time (min) </td <td>New UPLC Gradient S9 mm x 1/8 mm, 1/2 pm colum</td> <td>0</td> <td>mo</td> <td>lunn</td> <td>µm colu</td> <td>1/0 mm, 1.7</td> <td>50 mm x</td> <td>t</td> <td>New UPLC Gradien</td> <td></td> <td></td> <td></td> <td></td> <td></td>	New UPLC Gradient S9 mm x 1/8 mm, 1/2 pm colum	0	mo	lunn	µm colu	1/0 mm, 1.7	50 mm x	t	New UPLC Gradien					
P 1 1 1 0.10 80.0 20.0 - 2 1.94 0.10 44.0 56.0 2 -	Time (min) Flow (mL/min) 7cA (Agueous)	(Organic)	%B (Organ	1 %B	queous)	%A (Aq.	Flow (mL/min)	0	Time (min)					
2 1.944 0.10 44.0 56.0 2 3 1.969 0.10 100.0 20.0 4 4 3.24 0.10 80.0 20.0 4	▶ 1 Initial 0.10 80.0 2	0.0	20.0	20.0		80.0		0.10	I Initial					
2 1.56 0.10 00.0 20.0 0 4 3.24 0.30 80.0 20.0 0	2 1.94 0.10 44.0 5	6.0	56.0	56.0		44.0	0	0.10	2 1.94		_	-		
4 3.24 0.10 80.0 20.0 4	3 1.96 0.10 00.0 2	0.0	20.0	20.0		80.0		0.10	3 1.96					
	4 3.24 0.10 80.0 2	0.0	20.0	20.0		80.0		0.10	4 3.24					
				0.200.00										

Figure 1.17 Gradient results for different column dimensions.



1.3 Conversion of an HPLC Method to UHPLC 23

Figure 1.18 Flowchart for the use of the ACQUITY UPLC Columns Calculator.

run time have too long a run time. The flowchart for the use of the ACQUITY UPLC Columns Calculator is shown in Figure 1.18.

1.3.4 Perform Verification Experiment

Once a column is selected and the injection volume, flow rate, holding time, and gradient time are calculated, the UHPLC method conditions are known. The mobile phase, sample diluent and concentration, column temperature, and UV detection wavelength are the same as the HPLC method. However, the UV detection setting needs to be adjusted. The data acquisition rate must be at least 20 Hz to avoid peak distortion and broadening. Perform the verification experiment and compare the results with HPLC in resolution, tailing factor, sensitivity, and repeatability. Different results from HPLC may be obtained because of the effect of very high pressure and frictional heating in UHPLC as discussed in Section 1.2.7. If worse results are obtained, analyze the conditions and adjust accordingly. The conditions such as flow rate and gradient time can be optimized if necessary.

1.3.5 Validate the Method

Method validation is performed like HPLC. However, column lifetime needs to be carefully examined because the knowledge of UHPLC columns is very limited. In addition, an abridged validation protocol is possible if justified.

1.3.6 Example 1.3

This example converts an HPLC method in the left column of Table 1.7. The typical HPLC chromatogram is shown in Figure 1.19. The HPLC column is Zorbax Bonus-RP, $3.5 \mu m$, $150 \times 4.6 mm$, manufactured by Agilent Technologies, Inc. It provides the UHPLC column with the same chemistry. The dwell volume of an Agilent 1100 HPLC system is 1 mL, and the dwell volume of a Waters Acquity UPLC system with a TFA mixer is approximately 0.3 mL. The flow rate is 0.43 mL/min based on Figure 1.13. However, the flow rate of 0.6 mL/min was used, and the gradient was adjusted accordingly. The UHPLC conditions are shown in the right column of Table 1.7. The typical UHPLC chromatogram is shown in Figure 1.20. The run time was reduced from 20 to 4.65 min without sacrificing performance.

Column details (column type, particle size and column dimensions)	Zorbax Bonus 4.6 mm, or	-RP, 3.5 validated	μm, 150 × l equivalent	Zorbax Bonus-RP, 1.8 μm, 1 t 2.1 mm, or validated equiv			
Column temperature	40°C			40°C			
Mobile phase A	0.1% (v/v) TF	Δ in wat	er	0.1% (v/v) TEA in water			
Mobile phase R	Acetonitrile	7 1 111 wat	.01	A cetonitrile			
Flow rate	1.0 mI /min			0.6 mL/min			
Gradient profile	Time (min)	%Δ	%B	Time (min)	%Δ	%B	
P	0	80	20	0	80	20	
	12	44	56	2.78	44	20 56	
	12.1	80	20	2.80	80	20	
	20	80	20	4.65	80	20	
Detector wavelength	268 nm			268 nm			
Injection volume	5 µL			0.7 μL			
Data collection	12 min			2.78 min			
Run time	20 min			4.65 min			
UV detection data acquisition rate	5 Hz			20 Hz			
Autosampler wash solvent	Diluent if nee	ded		Weak washing solvent: mobile phase A			
				Strong washin phase B	ng solven	t: mobile	

Table 1.7 HPLC and UHPLC Method Conditions for API Assay and Impurities



1.3 Conversion of an HPLC Method to UHPLC 25

Figure 1.19 Typical HPLC chromatogram. Peaks 1–5 are impurities.

1.3.7 Example 1.4

This example converts an HPLC method for an intermediate to UHPLC. The HPLC conditions are listed in the left column of Table 1.8. The typical HPLC chromatogram is shown in Figure 1.21. The HPLC column is XBridge C18, 150×4.6 mm, 3.5μ m, manufactured by Waters Corp. It provided the UHPLC column with the same chemistry. The dwell volume of an Agilent 1100 system is 1 mL, and the dwell volume of a Waters Acquity UPLC system with a TFA mixer is 0.3 mL. The flow rate is 0.43 mL/min based on Figure 1.22. However, the flow rate of 0.6 mL/min was used, and the gradient was adjusted accordingly. The UHPLC conditions are shown in the right column of Table 1.8. The typical UHPLC chromatogram is shown in Figure 1.23. The run time was reduced from 25 to 5.80 min without sacrificing performance.



Figure 1.20 Typical UHPLC chromatogram. Peaks 1–5 are impurities.

Table 1.8	HPLC and	UHPLC	Method	Conditions	for an	Interme	ediate
Table Tio	III LC unu	UIII LC	mounou	Contaitions	ioi un	monne	Juliuce

Column details(column type, particle size and column dimensions)	XBridge C18, 3.5 μm, or v	150×4.6 validated e	ó mm, equivalent	BEH C18, 100 × 2.1 mm, t 1.7 μm, or validated equivale			
Column temperature	40°C			40°C			
Mobile phase A	10 mM NH ₄ O	Ac in wate	er	$10 \text{ mM NH}_4\text{OAc in water}$			
Flow rote	1.0 mL/min 0.6 mL/min						
Gradient profile	Time (min)	0% Λ	0% D	0.0 IIIL/IIIII Time (min)	0% A	0% P	
Gradient prome	0	84	70 D 16	$\frac{1}{0}$	84	ло Б 16	
	20	53	47	4 63	53	10 47	
	201	84	16	4 65	84	16	
	25	84	16	5.80	84	16	
Detector wavelength	242 nm	01	10	242 nm	01	10	
UV detection data acquisition rate	5 Hz			20 Hz			
Injection volume	5 µL			0.7 μL			
Data collection time/reporting time	20 min			4.63 min			
Run time	25 min			5.80 min			
Autosampler wash solvent	Not applicable			Weak washing solvent: mobile phase A			
				Strong washin phase B	g solvent: m	nobile	

1.4 CONVERSION OF A UHPLC METHOD TO HPLC

UHPLC has many advantages over HPLC. It is faster and has large solvent saving. However, it is much more expensive than HPLC. The high cost of UHPLC is one of major reasons that prohibits wide adoption of UHPLC. Because it takes much less solvent and time to develop a UHPLC method than an HPLC, one strategy is to develop a UHPLC method and then convert it to an HPLC method and validate the method.



Figure 1.21 Typical HPLC chromatogram. Peaks 1–5 are impurities.

Run (min Peak Capacit Orig Calcula 20.00 1.00 50 mm x 4.6 mm, 3.5 µm New UPLC of UPLC x 2.1 mi. 1.7 µn 16.67 0.21 3788 0.0 Fio 20.00 20.10 25.00 53.0 84.0 84.0 Copy Gra New UPLC conditions with 100 mm x 2.1 mm, 1.7 µm New UPLC Gradient Z# C 12.16 2 6.48 3 6.51 4 8.10 53.0 84.0 84.0 0.43 ब <u>°</u> ∢)⊎ di start

1.4 Conversion of a UHPLC Method to HPLC 27

Figure 1.22 New UPLC conditions with scaled gradient (accounting for particle size).

In addition, outsourcing becomes more and more common to reduce the cost of drug development in the pharmaceutical industry. UHPLC methods developed in a company with UHPLC capability need to be transferred to contract research organizations (CROs), which may not have UHPLC capability.

Either case described earlier needs to convert a UHPLC method to HPLC. The principle of the conversion from a UHPLC method to HPLC is the same as the



Figure 1.23 Typical UHPLC chromatogram. Peaks 1–5 are impurities.

conversion from an HPLC method to UHPLC. The examples in Section 1.3 can also serve as examples for conversion from a UHPLC method to HPLC.

1.5 SUMMARY

This chapter describes the method development process and strategies as well as the process of converting an HPLC method to UHPLC, or vice versa. The method development process includes gathering sample information, defining method goals, scouting columns and mobile phases, analyzing scouting results, selecting separation conditions, optimizing the method, and validating the method. Each step is discussed in detail, and the examples of simultaneous optimization of gradient and column temperature and gradient and mobile phase buffer pH are given. In addition, the process of converting an HPLC method to UHPLC or vice versa is described and discussed. It includes selecting a column with the same chemistry; measuring dwell volumes of both HPLC and UHPLC systems; calculating injection volume, flow rate, holding time, and gradient segment time; performing experiments for verification; and validating methods. API and intermediate HPLC methods are converted to UHPLC methods as examples using the ACQUITY UPLC Columns Calculator. The same principle, process, and examples can be used for conversion from a UHPLC method to HPLC.

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