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 μm 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 μm 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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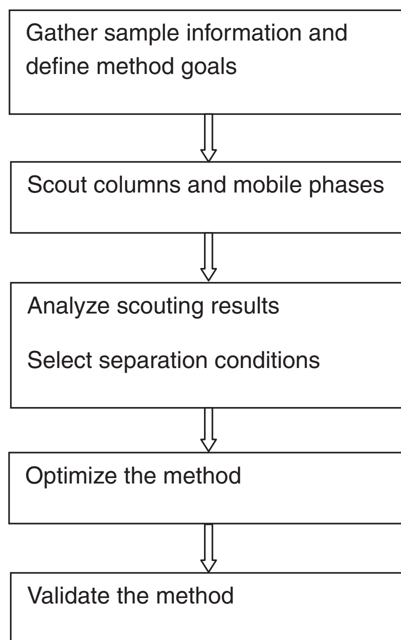

Figure 1.1 General process of UHPLC method development.

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 Comparison of API Method Goals Between Early- and Late-Phase Projects

	Early Phase	Late Phase
Specificity/resolution	Resolution ≥ 1.2 Discrimination factor ≥ 0.5	Resolution ≥ 1.5 Discrimination factor ≥ 0.8
Accuracy/linearity	Quantitation limit (QL) to at least 120% of the analyte (main and specified impurities)	Quantitation limit (QL) to at least 120% of the analyte (main and specified impurities)
Accuracy/recovery	No	Yes
Repeatability	≤ 1.0%	≤ 1.0%
Sensitivity	Depends on maximum daily dose	Depends on maximum daily 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

Table 1.2 Selected UHPLC Columns

Manufacturer	Type	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
	Amide	BEH amide 1.7 μm
	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

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.

1.2.2.2.2 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).

Table 1.3 Commonly Used Mobile Phase Buffers

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

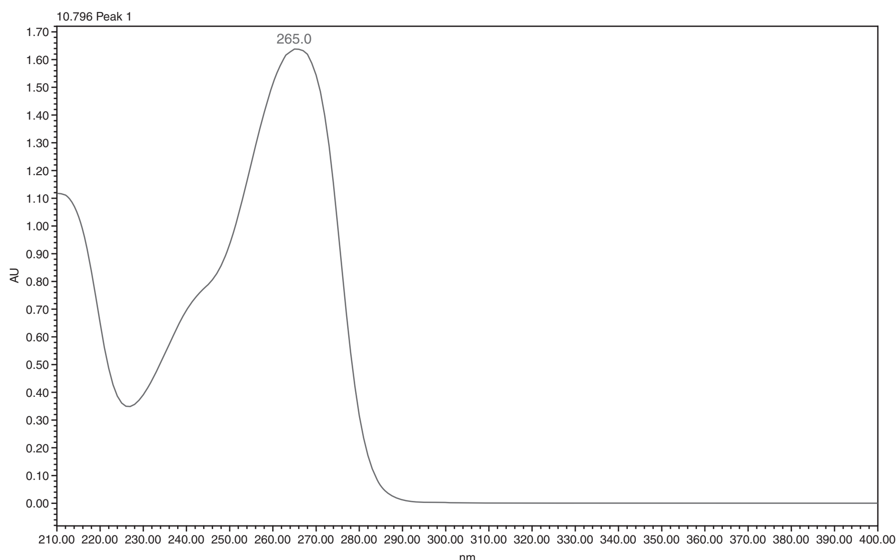

Figure 1.2 Example of a UV profile for wavelength selection.

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

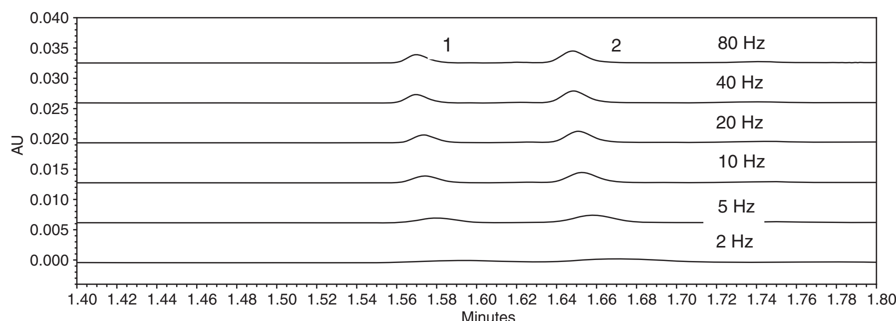

Figure 1.3 Example of the influence of the data acquisition rate on peak distortion. The filter time is 0.1 s. Conditions: Zorbax Bonus RP 100 × 2.1 mm, 1.8 μm; mobile phase A 0.1% TFA water; mobile phase B acetonitrile; gradient 20%–56% B for 2.06 min; flow rate 0.8 mL/min; wavelength 268 nm. Peaks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

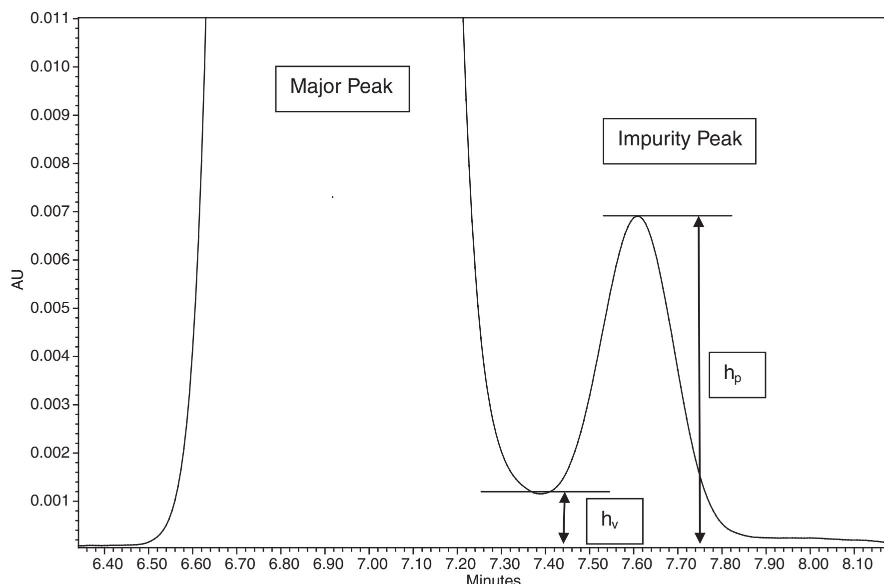

Figure 1.4 Illustration for a discrimination factor.

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 Development™ software. Fusion Method Development™ 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 × 2.1 mm, 1.7 μm, BEH phenyl 50 × 2.1 mm, 1.7 μm, Zorbax SB-C8 50 × 2.1 mm, 1.8 μm, and Bonus RP 50 × 2.1 mm, 1.8 μm. Two organic solvents were acetonitrile and methanol. Six buffers were 0.1% TFA in water (pH ~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 μm 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

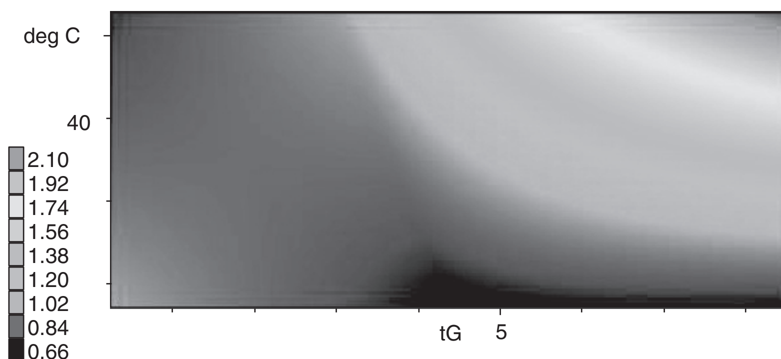

Figure 1.6 Two-dimensional resolution map of the column temperature (°C) against gradient time (t_G, min) for the separation of an intermediate and its impurities 1–4.

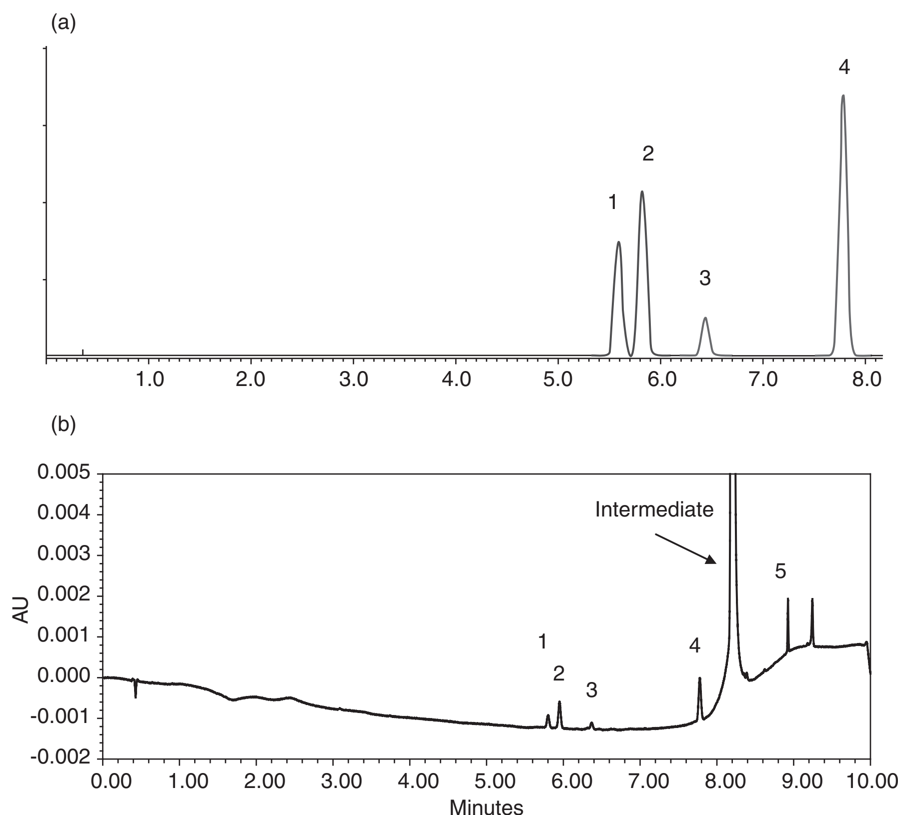

Figure 1.7 Predicted (a) and experimental (b) chromatograms of an intermediate were optimized by 2.5 and 7.5 min gradient basic runs at two different column temperatures (20 and 50°C). Conditions: BEH Phenyl 100 × 21 mm, 1.7 μm; mobile phase A 0.1% TFA in water; mobile phase B acetonitrile; gradient 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 μL; 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

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

Peak	Experimental (min)	Predicted (min)	Difference (min)[a]	% Error[b]
1	5.803	5.58	0.223	4.0
2	5.951	5.82	0.131	2.3
3	6.37	6.43	−0.06	−0.9
4	7.778	7.78	−0.002	0.0

[a]Difference = experimental − predicted by Drylab.
[b]% error = [(experimental − predicted)/predicted] × 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 × 2.1 mm, 1.8 μm 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

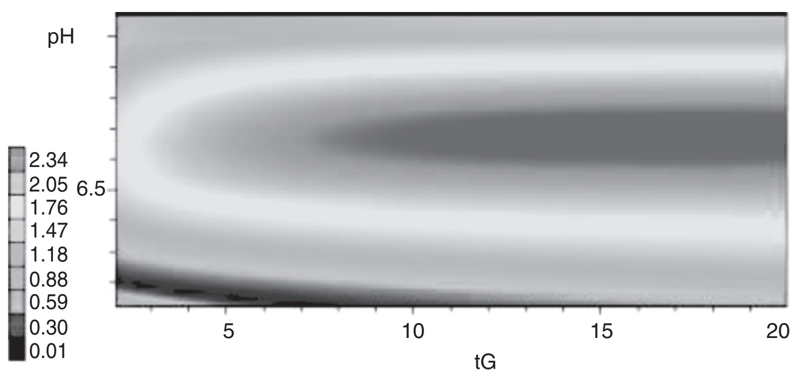

Figure 1.8 Two-dimensional resolution map of the gradient time (min) against mobile phase pH for the separation of basic API and its related impurities and degradation products. Reprinted from Fekete, S.; Fekete, J.; Molnar, I.; Ganzler, K.; *J. Chromatogr. A.* 2009, 1216: 7816–7823. Copyright (2009),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

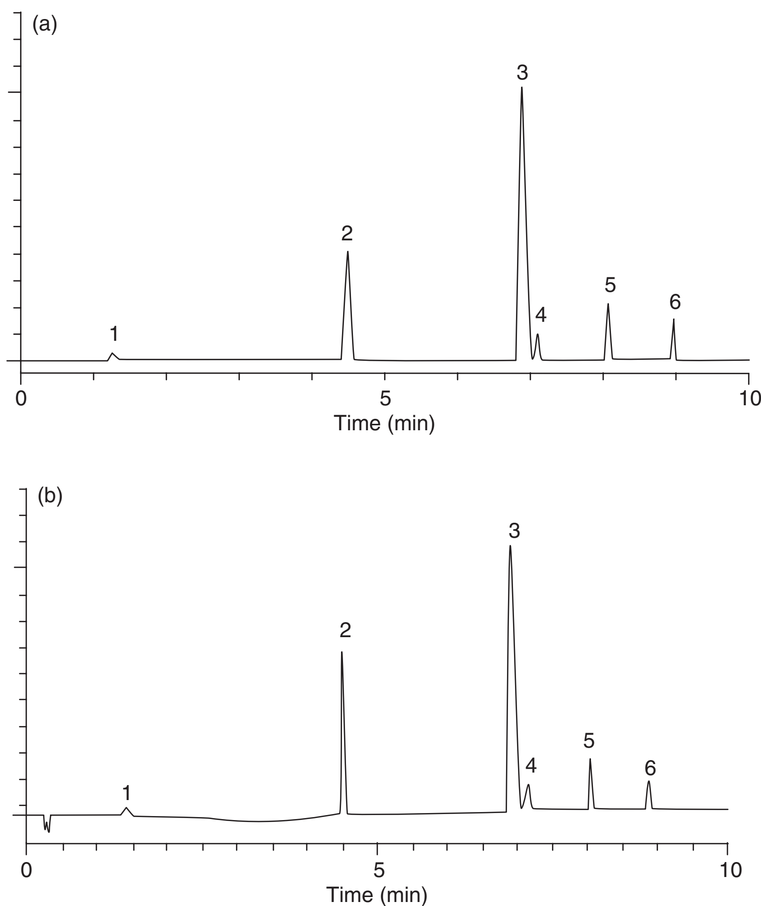

Figure 1.9 Predicted (a) and experimental (b) chromatograms. Column: Zorbax SB C18 50 × 2.1 mm, 1.8 μm, mobile phase A: methanol–buffer 5–95 V/V% (buffer: 10 mM phosphate + 0.1% triethylamine, pH 6.7), mobile phase B: methanol–buffer 80–20 V/V% (buffer: 10 mM phosphate + 0.1% triethylamine, pH 6.7), 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 μL, 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%.

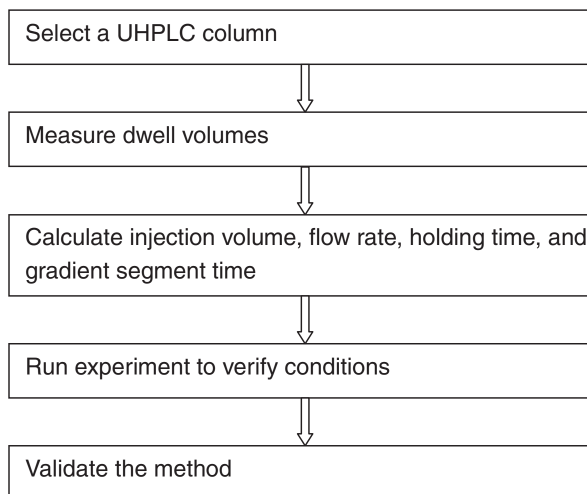

Figure 1.10 General process for conversion of an HPLC method to UHPLC.

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

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

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

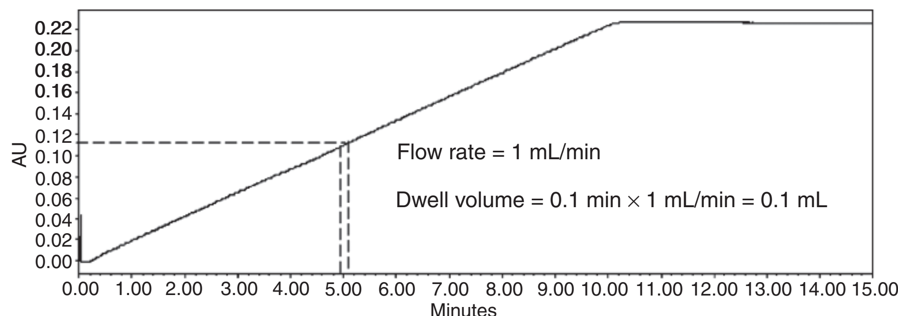

Figure 1.11 Measurement of UHPLC dwell volume.

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
	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:

$$\textit{UHPLC injection volume} = \textit{HPLC injection volume} \times \frac{\textit{UHPLC column volume}}{\textit{HPLC colume volume}}$$

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

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:

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

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} cm^2/s, the optimal flow rate on a 2.1 mm i. d. 1.7 μm 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.

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

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

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

Figure 1.12 Enter parameters and conditions.

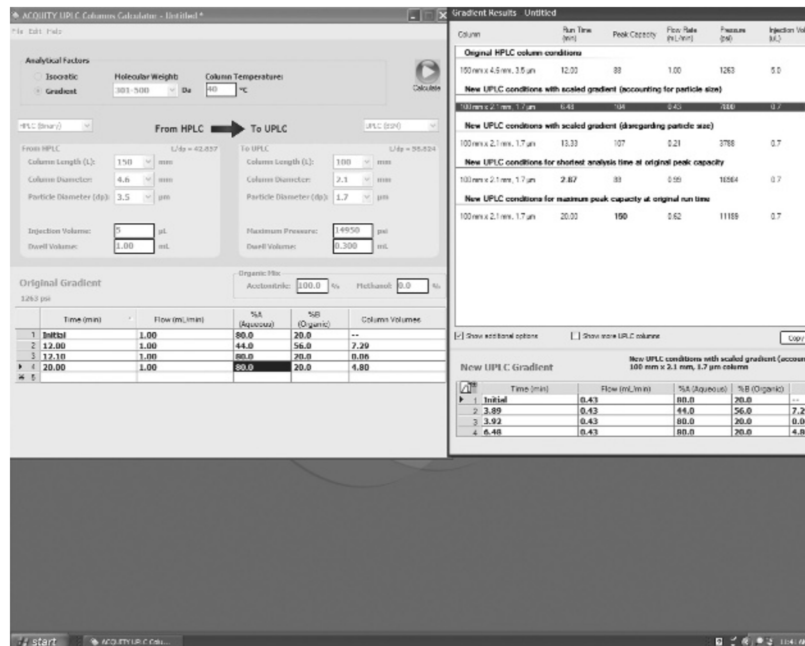

Figure 1.13 New UPLC conditions with scaled gradient (accounting for particle size).

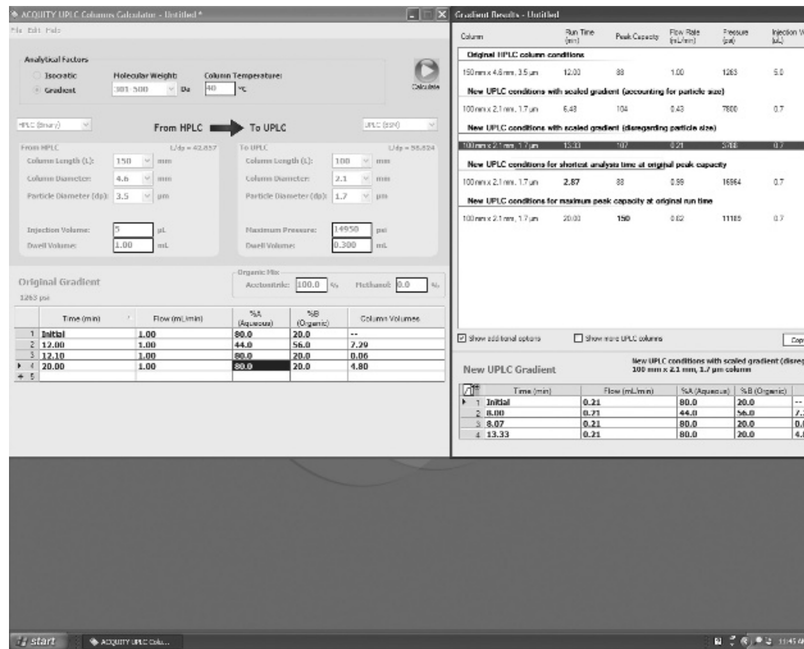

Figure 1.14 New UPLC conditions with scaled gradient (disregarding particle size).

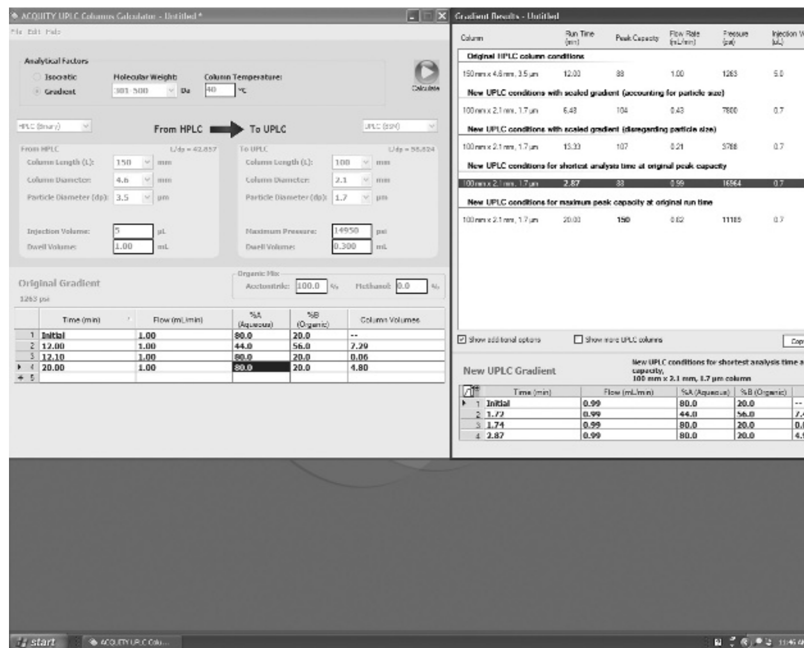

Figure 1.15 New UPLC conditions for shortest analysis time at original peak capacity.

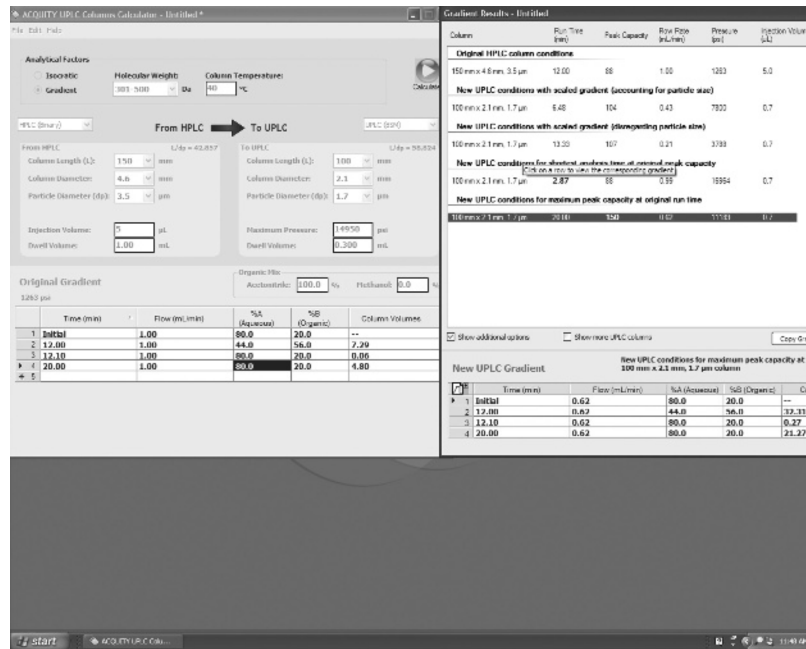

Figure 1.16 New UPLC conditions for maximum peak capacity at original run time.

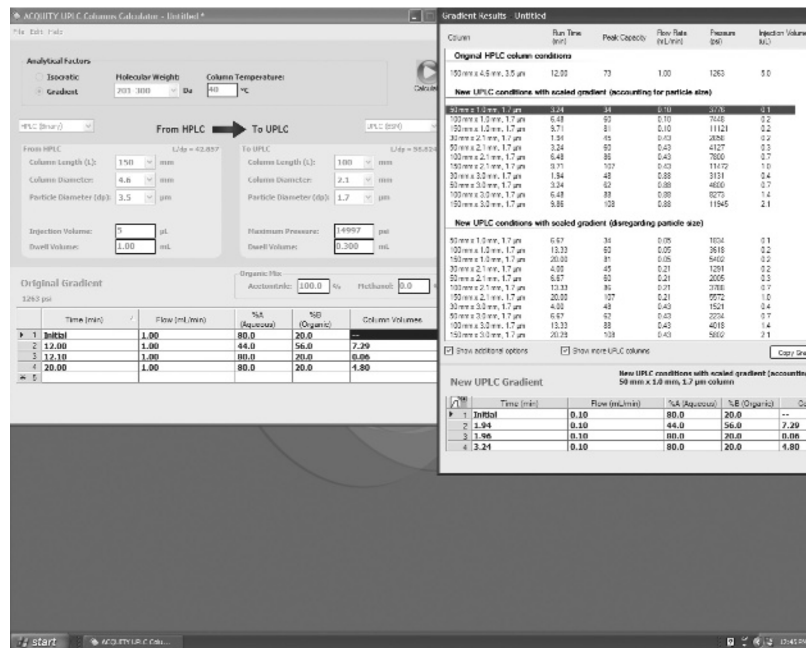

Figure 1.17 Gradient results for different column dimensions.

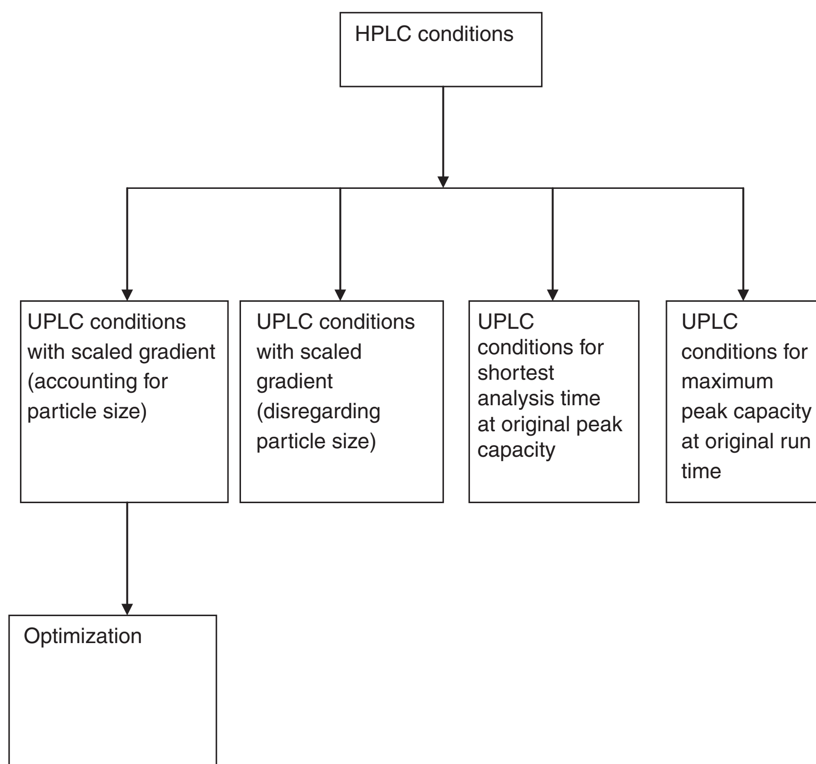

Figure 1.18 Flowchart for the use of the ACQUITY UPLC Columns Calculator.

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 μm, 150 × 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.

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

	HPLC			UHPLC		
Column details (column type, particle size and column dimensions)	Zorbax Bonus-RP, 3.5 μm, 150 × 4.6 mm, or validated equivalent			Zorbax Bonus-RP, 1.8 μm, 100 × 2.1 mm, or validated equivalent		
Column temperature	40°C			40°C		
Mobile phase A	0.1% (v/v) TFA in water			0.1% (v/v) TFA in water		
Mobile phase B	Acetonitrile			Acetonitrile		
Flow rate	1.0 mL/min			0.6 mL/min		
Gradient profile	Time (min)	%A	%B	Time (min)	%A	%B
	0	80	20	0	80	20
	12	44	56	2.78	44	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 time/reporting time	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 needed			Weak washing solvent: mobile phase A Strong washing solvent: mobile phase B		

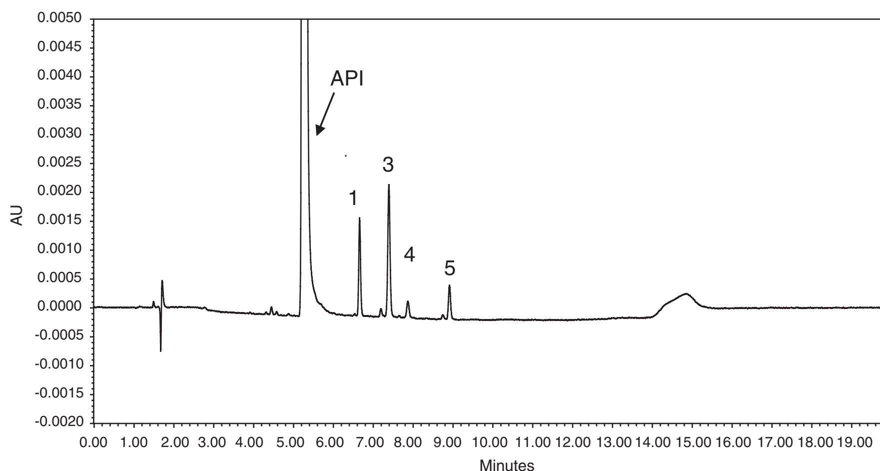

Figure 1.19 Typical HPLC chromatogram. Peaks 1–5 are impurities.

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, 3.5 μm, 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.

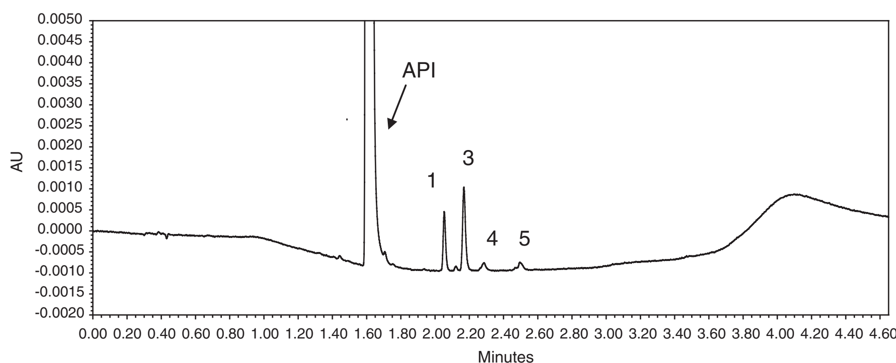

Figure 1.20 Typical UHPLC chromatogram. Peaks 1–5 are impurities.

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Table 1.8 HPLC and UHPLC Method Conditions for an Intermediate

Column details(column type, particle size and column dimensions)	XBridge C18, 150 × 4.6 mm, 3.5 μm, or validated equivalent			BEH C18, 100 × 2.1 mm, 1.7 μm, or validated equivalent		
Column temperature	40°C			40°C		
Mobile phase A	10 mM NH_4OAc in water			10 mM NH_4OAc in water		
Mobile phase B	Acetonitrile			Acetonitrile		
Flow rate	1.0 mL/min			0.6 mL/min		
Gradient profile	Time (min)	%A	%B	Time (min)	%A	%B
	0	84	16	0	84	16
	20	53	47	4.63	53	47
	20.1	84	16	4.65	84	16
	25	84	16	5.80	84	16
Detector wavelength	242 nm			242 nm		
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 washing solvent: mobile phase B		

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.

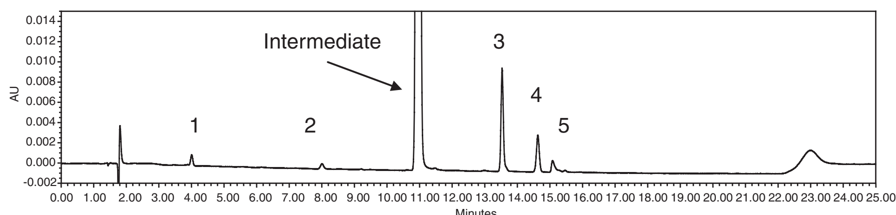

Figure 1.21 Typical HPLC chromatogram. Peaks 1–5 are impurities.

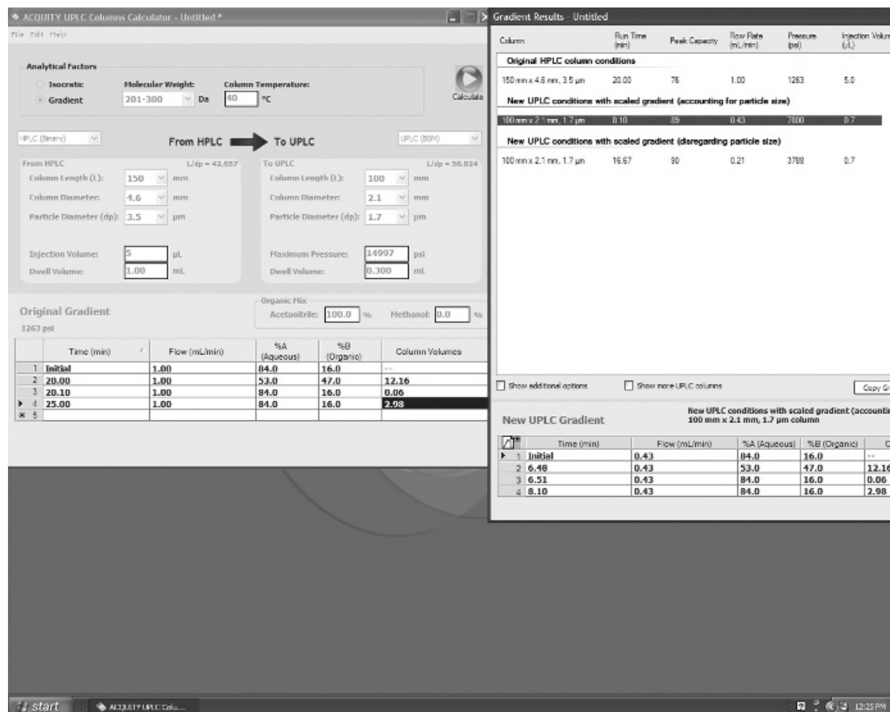

Figure 1.22 New UPLC conditions with scaled gradient (accounting for particle size).

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

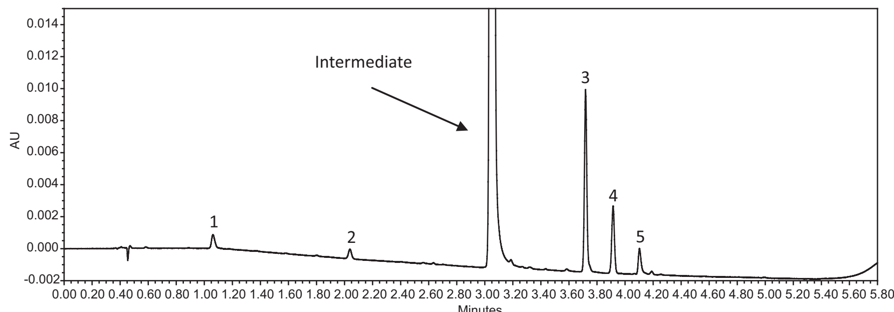

Figure 1.23 Typical UHPLC chromatogram. Peaks 1–5 are impurities.

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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