The use of ultra-high pressure liquid chromatography in high-throughput pharmaceutical separations

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THE USE OF ULTRA-HIGH PRESSURE LIQUID CHROMATOGRAPHY IN HIGH-THROUGHPUT PHARMACEUTICAL SEPARATIONS

by

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Abstract

Glenn Anthony Kresge
THE USE OF ULTRA-HIGH PRESSURE LIQUID CHROMATOGRAPHY IN HIGH-THROUGHPUT PHARMACEUTICAL SEPARATIONS
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The higher pressures and flow rates needed to increase throughput in ultra-high pressure liquid chromatography (UHPLC) can lead to thermal broadening due to viscous friction. The use of superficially porous particles and still-air thermal environments can help reduce this broadening, which is especially important in applications requiring high-throughput, isocratic separations, such as monograph methods for over-the-counter analgesics. In the first experiment discussed below, system suitability parameters (resolution and peak asymmetry) and temperature changes across the axial length of the column were monitored at conditions near column or system pressure limits. Results from this investigation indicated that shorter columns packed with 2.6 µm particles provide the best opportunity for increased throughput, which was demonstrated with a 20 s cycle time method for the separation of four compounds while maintaining a baseline resolution of 1.5 between all peaks. This was the basis for the idea of creating a method qualification protocol of an adapted ibuprofen method. Linearity, accuracy, recovery and repeatability measurements were completed in 16 minutes using this approach, a sequence that often requires a full day of analysis. These results demonstrate the capability of modern instrumentation to readily implement high-throughput LC methods into qualified pharmaceutical workflows.
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Chapter 1

Introduction

1.1 The Monograph Modernization Initiative

The United States Pharmacopeia (USP) is a nonprofit organization that sets standards for the identity, strength, quality, and purity of medicines, food ingredients, excipients, and supplements that are manufactured, distributed, and consumed worldwide. The standards set forth by the USP are used in many countries, are enforceable here in the United States by the Food and Drug Administration (FDA). Since 2005, the USP has identified over 2,600 monographs that need revision to meet the modern demands of the pharmaceutical industry.\(^1\) One key area in need of modernization is the analysis of over-the-counter (OTC) drugs.\(^2\) In a push for updates to these monographs, the USP has partnered with industry, academic, and government labs to replace older analytical methods that rely on either outdated technology or techniques.\(^3\)

A large majority of monographs use non-specific tests, including titrations and UV-Vis spectroscopy, that need to be reworked with more instrument-based, quantitative methods. Ultra-high pressure liquid chromatography is one of those methods that can significantly aid in the modernization process.\(^1\) If changes are sought to be made to monographs that already require LC, the guidelines for doing so are listed in Chapter 621 of the USP-NF.\(^4\) Here, all permitted modifications to mobile phase composition, column length, column diameter, and particle size are listed. The guidelines allow for methods to be updated while utilizing state-of-the-art LC columns and instrumentation, which will be demonstrated in this thesis. However, there are still limits set to how far the throughput and performance can be increased within these guidelines.\(^4,5\)
1.2 Modern Column Technology

There have been innovations in UHPLC over the past decade that have improved the speed and efficiency of analyses of pharmaceutical compounds.\(^6\)\(^7\) This can be attributed to the incorporation of higher pressure instrumentation and columns packed with smaller particles.\(^8\)\(^–\)\(^14\) During the same time period, the field has also seen increased use of superficially porous particles (SPPs) to improve the performance of pharmaceutical analyses by LC.\(^15\)\(^–\)\(^19\) SPPs are comprised of a nonporous silica core surrounded by a porous layer. The theoretical advantage of columns packed with these particles is the reduction of the analyte diffusion path length and reduction of the longitudinal diffusion (B) term of the van Deemter equation (Equation 1),\(^20\)

\[
HETP = A + \frac{B}{u} + (C_s + C_m)u
\]  

(1)

where the height equivalent to a theoretical plate (HETP) is equal to the sum of the multiple paths term (A), longitudinal diffusion term (B), and mass transfer term (C) at a given mobile phase linear velocity (u). Several theoretical and experimental studies have also showed that columns packed with SPPs exhibit lower velocity-dependent A-term contributions to band broadening than columns packed with fully porous particles (FPPs).\(^21\) When columns packed with 2.7-μm SPPs were first introduced, they exhibited efficiencies similar to columns packed with sub-2-μm fully porous particles at just 40% of the required pressure.\(^23\) This is especially useful for high-throughput LC where the remaining pressure could be used to operate at higher flow rates for faster analysis times.\(^24\)
1.3 High-Throughput LC

There will always be a need to improve productivity to keep up with the modern demands of the pharmaceutical industry where high-throughput methodologies are required to accelerate discovery, development and manufacturing of both new and existing chemical entities. Analytical scientists working in this sector can greatly benefit from increasingly fast chromatographic separations that can be used throughout the drug manufacturing process. Both the use of smaller particles and higher mobile phase velocities require higher pressures than are achievable with traditional HPLC instruments which possess maximum system pressure limits of approximately 400 bar. This facilitated the development of UHPLC instrumentation, increasing limits to the range of 1000–1500 bar allowing for the incorporation of more narrow bore columns packed with small particles. In some cases, high linear velocities achieved from smaller column dimensions can lead to shorter chromatographic runs while reducing the amount of chemical waste generated when compared to older methods utilizing large column dimensions. It should be noted that reducing individual sample analysis time is not the only means of increasing throughput; a reduction in the full method cycle time can be achieved by streamlining other parts of instrument operation, such as autosampler movement speed or by using combined analyses.

1.4 Viscous Friction

When increasing the flow rate of a chromatographic method to reduce the analysis time, heating due to viscous friction can actually diminish column performance. The friction from liquid flowing through the packed bed of a column generates heat. The rate
at which this heat is dissipated (Power), is equivalent to the product of the flow rate ($F$) and pressure drop ($\Delta P$) as described by Equation 2.\textsuperscript{31}

$$Power = F\Delta P$$

(2)

The heat resulting from viscous friction is dissipated from the column in two ways. First, it flows and exits the column outlet along with the mobile phase, thus creating an axial temperature gradient within the column. The mobile phase is cooler at the column inlet and warmer at the column outlet (Figure 1). Axial temperature gradients have been shown to affect the retention time repeatability of separations in rapidly sequential runs of high-throughput methods.\textsuperscript{32} Second, heat can be removed through the column walls, causing radial temperature gradient. This is categorized by having warmer temperatures at the center of the column but cooler temperatures at the column walls. Radial temperature gradients are predominantly detrimental to chromatographic performance.\textsuperscript{33}

The warmer, less viscous fluid at the center of the column flows faster than the cooler liquid at the walls and results in different radially localized flow velocities (Figure 1). Simultaneously, the partition coefficients of analytes tend to be lower in the higher temperature regions. These effects act in tandem and lead to reduced chromatographic efficiencies.
Figure 1. Visual representation of a theoretical adiabatic thermal environment, leading to an axial temperature gradient (top), and an isothermal environment creating a radial temperature gradient (bottom).

The column’s external environment also affects the formation of these thermal gradients: adiabatic conditions maximize the axial thermal gradient while isothermal conditions maximize the radial thermal gradient. An adiabatic environment can be created by using either insulation or a still air oven, whereas circulating water baths and forced air ovens lead to isothermal conditions. One way that the negative effects of the radial thermal gradient can be reduced is by increasing the thermal conductivity of the packed bed. By using SPPs, the nonporous core increases the total amount of silica in the bed when compared to FPPs. This raises the overall thermal conductivity of the column and can decrease thermally induced band broadening. At higher operating pressures (near 2500 bar), beyond the operating capacity of modern commercial instruments, a solid silica core is just one of the ways to increase conductivity, but may not be sufficient enough. For the operating pressures described in the following chapters,
an increase in thermal conductivity due to higher silica content is sufficient to reduce the magnitude of thermal broadening.\textsuperscript{44,45}

In the following chapters, experiments are discussed that explore the methodologies and challenges that surround the concept of improving upon more outdated methods for analyzing pharmaceutical drug substances by utilizing modern UHPLC instrumentation and column technologies.
Chapter 2

Pharmacopeial Monograph Modernization of Common Analgesics*

2.1 Introduction

In this chapter, a series of experiments are described that tested a series of modern LC columns packed with SPPs to determine which column dimensions and particle sizes provided the greatest increase in throughput for USP monograph methods for three OTC analgesics: ibuprofen, naproxen and acetaminophen. The USP monographs for these drug substances already utilize traditional HPLC as a means for analysis but require the use of large bore columns (4.6 mm inner diameter) that reach lengths of up to 300 mm. The modern LC columns selected for these experiments vary in length, diameter and particle size, but all are packed with SPPs. Since the developed methods required higher flow rates and pressures that lead to viscous friction effects, the potential impact of thermal broadening on the chromatographic performance of these methods was also investigated. The experiments outlined in this chapter focus on isocratic monograph methods, as the impact of the column’s thermal environment was also examined and its effects should be more prominent compared to gradient elution. Finally, the potential for developing ultrafast techniques for OTC analgesic analysis using UHPLC and SPPs is explored.

2.2 Experimental Design

2.2.1 Sample preparation. USP monographs for ibuprofen, naproxen, and acetaminophen were used to make all required stock and sample solutions. Unless

otherwise noted, all compounds were purchased from Alfa Aesar (Ward Hill, MA) and mobile phase solvents were acquired from Fisher Scientific (Fair Lawn, NJ). To study the USP monograph for naproxen, stock solutions of 0.11 mg/mL ethylparaben and 1.25 mg/mL naproxen were prepared in 0.1 µm filtered UHPLC grade methanol, then diluted 25-fold with 50:50 water:methanol containing 30 mM sodium acetate (with the 18.2 MΩ water used for this dilution and other dilutions listed below supplied by a Barnstead GenPure UV-TOC/UF xCAD Plus purifier, Thermo Electron, Germany). The mobile phase was 50:50 water:methanol (for mobile phases, water was 0.1 µm filtered UHPLC grade) with 30 mM sodium acetate, and was adjusted with glacial acetic acid (VWR International, Solon, OH) to a pH of 5.8 ± 0.2. Mobile phase was prepared fresh at least every two days to prevent any potential issues with buffer stability.

For the USP monograph of ibuprofen, stock solutions of 60 mg/mL ibuprofen, 7 mg/mL valerophenone, and 0.12 mg/mL ibuprofen-related compound C (4’-isobutylacetophenone, from Beantown Chemical, Hudson, NH) were prepared in 0.1 µm filtered UHPLC grade acetonitrile. Then, a mixture of these three stock solutions was made with a final concentration of 12 mg/mL ibuprofen, 0.35 mg/mL valerophenone, and 0.012 mg/mL ibuprofen-related compound C in 40:60 water:acetonitrile and 0.4% chloroacetic acid. The mobile phase for this method was 40:60 water:acetonitrile with 0.4% chloroacetic acid, adjusted with ammonium hydroxide (Pharmco-Aaper, Brookfield, CT) to to a pH value of 3.0 ± 0.2.

For the testing a high-throughput monograph-derived method for acetaminophen and aspirin, two current USP monographs were combined. Stock solutions of 8 mg/mL benzoic acid and 13 mg/mL of acetaminophen, aspirin, and salicylic acid each
were prepared in 78:20:2 chloroform:methanol:glacial acetic acid per the method described in the monograph for acetaminophen and aspirin tablets. These stock solutions were then diluted to 2 mg/mL and 3.25 mg/mL in this same solvent mixture for injection, respectively. The mobile phase for this method consisted of 25.2:2.7:62.1:10 methanol:glacial acetic acid:water:acetonitrile, combining aspects of the mobile phase constituents of each monograph. The monographs also listed the critical parameters that needed to be met to be considered a successful chromatographic separation, these parameters are listed in Table 1 along with the analytes of interest, mobile phases and method designations for clarity.
<table>
<thead>
<tr>
<th>Method designation</th>
<th>Analytes of interest</th>
<th>Other compounds</th>
<th>Minimum resolution</th>
<th>Maximum tailing factor</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>Naproxen</td>
<td>Ethylparaben</td>
<td>3.0</td>
<td>2</td>
<td>50:50 water:methanol with 30 mM sodium acetate (pH 5.8 ± 0.2)</td>
</tr>
<tr>
<td>Method 2</td>
<td>Ibuprofen</td>
<td>Valerophenone, ibuprofen related compound C</td>
<td>2.5</td>
<td>2.5</td>
<td>40:60 water:acetonitrile with 0.4% chloroacetic acid (pH 3.0 ± 0.2)</td>
</tr>
<tr>
<td>Method 3</td>
<td>Acetaminophen, aspirin</td>
<td>Benzoic acid, salicylic acid</td>
<td>N/A</td>
<td>N/A</td>
<td>75:12.5:12.5 water:methanol:acetonitrile with 0.1% glacial acetic acid and 1.24 mM tetramethylammonium hydroxide pentahydrate</td>
</tr>
<tr>
<td>Method 4</td>
<td>Acetaminophen, aspirin, caffeine</td>
<td>Benzoic acid, salicylic acid</td>
<td>1.4</td>
<td>1.2</td>
<td>28:3:69 methanol:glacial acetic acid:water</td>
</tr>
<tr>
<td>Method 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Acetaminophen, aspirin</td>
<td>Benzoic acid, salicylic acid</td>
<td>N/A</td>
<td>N/A</td>
<td>25.2:2.7:62.1:10 methanol:glacial acetic acid:water:acetonitrile</td>
</tr>
</tbody>
</table>

*Note.* <sup>a</sup>Combined method for high-throughput analysis based on Methods 3 and 4.
2.2.2 Chromatographic methods and instrumentation. All methods were run on a Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with a diode array detector (DAD). Five Accucore columns packed with superficially porous C18 particles manufactured by Thermo Fisher Scientific (Bellefonte, PA) were selected for this study: 2.1x50 mm and 2.1x100 mm columns containing 1.5 µm diameter particles and 2.1x50 mm, 2.1x100 mm, and 4.6x50 mm columns containing 2.6 µm diameter particles. Each column was connected to the injector with 0.1x380 mm Viper tubing with a volume of 3.0 µL and contained a mobile phase preheater. The column outlet was connected to the detector with 0.1x445 mm tubing with a volume of 3.5 µL and containing the post column cooler. Neither of these were used for temperature control. A 0.5 µm frit inline filter was also placed at the inlet of each column to ensure no large particulate could enter the column. The injection volume for each experiment was set to 0.1 µL to reduce broadening effects due to large injection volume, this was increased to 1.0 µL for the 4.6 mm inner diameter (i.d.) column to ensure sufficient signal for the impurity trace peak in Method 2. For each column and monograph method, the highest flow rate possible while maintaining a minimum resolution between peaks and limiting the asymmetry below a listed maximum USP tailing factor was used (listed in Tables 1, 2). All methods used in this study were isocratic as defined by the monograph, with column equilibration conducted at a low flow rate and pressure, and then increased to the method flow rate as the first injection began. The flow rate ramp for all methods was set to 6.0 mL min⁻². For each method, fifteen consecutive injections from a prepared sample were made, with relevant figures of merit averaged over the final ten runs after thermal equilibrium had been reached. The column
oven compartment was maintained at 303 K for Methods 1 and 2 and were tested in both the still air (no convective fan) and forced air oven modes (turned to maximum instrument fan speed). Method 5 was performed only in the still air mode, with the column compartment maintained at 318 K.
Table 2

*Five columns used in the modernization experiment, along with their selected flow rates and resulting back pressures*

<table>
<thead>
<tr>
<th>Column Designation</th>
<th>Diameter (mm)</th>
<th>Length (mm)</th>
<th>Particle size (µm)</th>
<th>Method 1 flow rate (mL min⁻¹)</th>
<th>Method 1 still air pressure (bar)</th>
<th>Method 2 flow rate (mL min⁻¹)</th>
<th>Method 2 still air pressure (bar)</th>
</tr>
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<tr>
<td>Column A</td>
<td>2.1</td>
<td>50</td>
<td>2.6</td>
<td>1.10</td>
<td>670</td>
<td>1.10</td>
<td>320</td>
</tr>
<tr>
<td>Column B</td>
<td>2.1</td>
<td>100</td>
<td>2.6</td>
<td>0.70</td>
<td>730</td>
<td>1.50</td>
<td>820</td>
</tr>
<tr>
<td>Column C</td>
<td>4.6</td>
<td>50</td>
<td>2.6</td>
<td>2.80</td>
<td>650</td>
<td>5.00</td>
<td>740</td>
</tr>
<tr>
<td>Column D</td>
<td>2.1</td>
<td>50</td>
<td>1.5</td>
<td>0.40</td>
<td>750</td>
<td>0.80</td>
<td>740</td>
</tr>
<tr>
<td>Column E</td>
<td>2.1</td>
<td>100</td>
<td>1.5</td>
<td>0.33</td>
<td>1160</td>
<td>0.75</td>
<td>1420</td>
</tr>
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Data acquisition and analysis of USP tailing factor \((A_{s,USP})\), USP resolution \((R_{s,USP})\), and USP plate count \((N_{USP})\) were completed using Chromelone 7.2 software (Thermo Fisher Scientific, Germering, Germany) based on the following equations listed in Chap. 621 of the USP-NF4:

\[
A_{s,USP} = \frac{w_{5\%}}{2f_{5\%}} \tag{3}
\]

\[
R_{s,USP} = \frac{2(t_{r2}-t_{r1})}{(w_{b1}+w_{b2})} \tag{4}
\]

\[
N_{USP} = 16 \left( \frac{t_r}{w_p} \right)^2 \tag{5}
\]

where \(w\) is the peak width at either the peak base \(b\) or 5% of the peak height, \(f_{5\%}\) is the distance between the leading edge of the peak and the peak maximum at 5% of the peak height, and \(t_r\) is the peak retention time. UV-DAD data acquisition was conducted at 254 nm for Methods 1 and 2, and 280 nm for Method 5, utilizing a 0.8 µL flow cell. All methods used an acquired data at a rate of 50 Hz to ensure that at least 40 data points were collected per peak. All chromatograms in figures were plotted using Igor Pro 6.0 (Wavemetrics, Inc., Lake Oswego, OR).

2.2.3 Temperature measurements. Recording the mobile phase temperature at the column outlet required the use of a Type-T (Copper–Constantan) HYP-0 hypodermic (0.008” diameter) thermocouple probe (Omega Engineering, Stamford, CT). The probe was inserted into a 1.5-cm piece of 0.015” inner diameter PEEK tubing (Idex Health and Science, Oak Harbor, WA) and inserted into the column outlet. The probe was then connected to a OM-EL-USB-TC Thermocouple Data Logger interfaced to Easy-Log.
USB software for temperature data acquisition at a rate of 1 Hz (Omega Engineering). For each chromatographic method and column, baseline readings were made at a low flow rate and pressure (under a maximum viscous friction level of 1 mW) for 30 min, followed by 30 min at the higher flow rate of each method described above, and then a final 30 min at the initial baseline level. Reported temperature values are described as a change in temperature from the initial level where negligible column heating occurred (303 K, the set oven temperature) to the final temperature at the method flow rate and pressure. Thermal friction levels were calculated using Equation 1 and then normalized by the column mobile phase volume, which was determined by averaging the elution times of an unretained marker (uracil) at four different flow rates (including the analysis flow rate) and correcting for extra-column volume by measuring its elution time with a zero-dead volume union in place of the column.

2.3 Results and Discussion

2.3.1 Increasing method speed. For common pharmaceutical drugs that are produced in very high volumes, like OTC analgesics, rapid chromatographic methods are useful to increase the throughput of required assay and impurity analyses. However, many current USP monographs for these drugs are longer methods that rely on older LC column technology and are in need of modernization. As an initial investigation into the use of SPPs and ultra-high pressures to increase throughput for USP methods, the naproxen oral solution monograph was studied. This method consists of the separation of naproxen and an ethylparaben internal standard, with a required minimum resolution between the peaks of 3.0 and a maximum peak tailing factor of 2.0 (Table 1). As described above, the goal for these studies was to determine the shortest time that could
be achieved when considering these parameter restrictions, column and instrument pressure limits, and potential repeatability issues that can arise due to thermal gradients caused by viscous friction. On each of the five columns, the naproxen sample was injected 15 consecutive times, with various instrument parameters tracked as both the flow rate and temperature increased in the column. Because of the initial instrument flow rate ramp required when first going to the higher flow rate levels, as well as the time needed for thermal gradients to form, results from the first five injections demonstrated lower efficiency and were not included in calculations, this is represented in Figure 2 using Column A as an example. A comparison of all relevant method parameters calculated over the final ten injections are shown in Figures 3-7 for Columns A-E respectively.\textsuperscript{50} Numerical data for these columns can be found in Table A1 of the Appendix.
Figure 2. Plot of the thermal gradient and plate count for Column A over time for Method 1, showing lower efficiency during the formation of the thermal gradients.
In the radar plots of Figures 3-7, the parameters listed clockwise from the top position (maximum value for each radial axis listed in parentheses) are: inverse plate count for ethylparaben peak (0.00036), inverse plate count for naproxen peak (0.00036), USP tailing factor for ethylparaben peak (1.5), USP tailing factor for naproxen peak (1.5), inverse resolution between ethylparaben and naproxen (0.2), magnitude of the axial thermal gradient across the column (11 K), system pressure (1200 bar), and the retention time of the last eluting peak (naproxen, 4 min). The radar plots in were designed to compare multiple method and system suitability parameters in a single compact visual. The plate count and resolution attributes are depicted using their reciprocal so that an overall smaller enclosed area demonstrates a faster, more efficient separation with lower pressure and frictional heating.

The 2.1x100 mm column packed with 1.5 µm particles (Column E) provides the highest chromatographic efficiency of all the columns tested. However, the higher efficiency comes at the cost of a much higher required pressure (1160 bar) and longer method time (3.6 min) when compared to the other four columns. The higher pressure also results in the magnitude of the axial thermal gradient being noticeably larger (10.5 K) for this column. Using the same particle type and reducing the column length in half (Column D) decreases the required pressure (760 bar) and method time (1.3 min), but resulted in higher peak asymmetry likely due to the extra-column dispersion affecting peak shape because of the lower volume of the column. With a larger particle diameter, the column efficiency is lower, which limited the maximum flow rate that could be used to increase speed since the resolution between peaks suffered from kinetic broadening effects. The 50 mm long columns with 2.6 µm particles (Columns A, C) both had the
shortest method times (0.45 and 0.73 min respectively) even when decreasing the flow rate to ensure that the minimum resolution between naproxen and ethylparaben was achieved for both oven modes. In general, the asymmetry factor was lower with the larger particles, as the 2.1x100 mm column with 2.6 µm particles (Column B) had the most symmetric peaks for these analytes. The larger asymmetry factors with the 1.5 µm particles are most likely a result of the greater impact of extra-column volumes on more efficient peaks, but could also arise from packing differences, surface silanol differences between the two particle sizes, or thermal mismatch between the mobile phase at the inlet and the higher temperatures at the outlet with these columns. As other drug monographs have different resolution and asymmetry requirements, choice of column for use in modernization should include consideration to what the limiting factor will be for increasing throughput.

A comparison of the magnitude of the axial thermal gradient across different columns and thermal environments provides another set of information that could potentially impact method performance at higher mobile phase velocities. As noted above, Column E had the highest measured increase in outlet mobile phase temperature (10.5 K) in the still air oven mode. Switching to a forced air oven decreased this amount by nearly 30%, (to a change of 7.5 K) with a comparable drop only seen in the other 100 mm long column (Column B). The higher magnitude of the axial thermal gradient is a combination of the higher viscous friction effects due to higher pressure from the longer column and the increased column length ensuring that a sufficient thermal entrance length is present for the gradient to develop. The greater length also means that the columns have a larger surface area in which temperature exchange with the surrounding
environment can occur causing the larger difference between still and forced air oven modes that is observed in these columns.\textsuperscript{52} The forced air oven mode does increase the magnitude of the radial thermal gradient, which lowers the efficiency and resolution for each method when compared to the still air mode. For Column E resolutions of 12.4 and 13.7 were observed under forced and still air modes respectively. This suggests the use of still air mode when resolution is the critical parameter limiting higher throughput in a method.

\textbf{2.3.2 Combining analyses.} In addition to decreasing the time for a single method, another aspect that can be explored in monograph modernization to increase throughput is the combination of multiple analysis steps into single runs. The monograph describing the analysis of ibuprofen requires two steps: the separation of ibuprofen and a valerophenone internal standard (as a content assay) and the separation of the same internal standard with ibuprofen-related compound C (for impurity analysis). However, modern column technology and UHPLC instrumentation makes the integration of these two separations into a single method a simpler task, one that effectively halves the original analysis time even before increasing the method speed itself. Again, the goal was to increase method speed while still maintaining the required resolution between each pair of peaks and ensure that the asymmetry was below a maximum threshold. Similar to the naproxen method, comparison of the figures of merit for the five columns tested are shown in Figures 8-12.\textsuperscript{50} Numerical data for these columns can be found in Table A2 of the Appendix.
Compared to the naproxen method, the biggest limitation in increasing the speed of the combined ibuprofen method was maintaining the minimum resolution between the analytes. This was made more difficult by the increased impact of extra-column broadening since these analytes are retained less than naproxen and ethylparaben. Here, both 50 mm columns packed with 2.6 µm particles (Columns A, C) were the fastest methods (retention time for ibuprofen-related compound C of 0.33 min for both columns), but further reductions in time were limited by the lower efficiency (achieving a resolution of at least 2.6 to exceed the minimum limit of 2.5) compared to the other columns rather than column or instrument pressure limits. With these two columns, the 2.1 mm diameter column had similar resolution between the critical pair (2.6-2.7, valerophenone and ibuprofen-related compound C) in both oven modes, but the 4.6 mm diameter column had more measurable effects from viscous friction and demonstrated a near 10% higher resolution for this pair in the still air oven mode (2.9). Doubling the column length required higher flow rates and pressures approaching the column limits (increasing the viscous friction), but the retention time of the last eluted analyte was only slightly higher (0.5 min) because the higher column efficiency ensured that the minimum resolution was still achieved (Column B). As this method utilized acetonitrile as the organic component rather than methanol (as in the naproxen method), the lower viscosity of the mobile phase meant that when column or instrument pressure limits were reached, a higher flow rate was achieved, and higher temperatures were measured at the outlet. Some preliminary results were then collected that indicate that increasing the mobile phase strength within USP guidelines can improve throughput by reducing analyte retention and raising the flow rate. But this may not work with every method since
composition changes can reduce resolution based on varying retention changes and an overall loss in selectivity due to reduced retention at higher temperatures. This is further discussed in Chapter 3.

As with the naproxen method, the 2.1x100 mm column packed with 1.5 µm particles (Column E) had the largest temperature increase across the column, with a measured magnitude of 18 K for this method. In a perfectly adiabatic system, the magnitude of this axial thermal gradient ($\Delta T_L$) is dependent on the physical properties of the mobile phase and the pressure drop across the column (Equation 6).53,54

$$\Delta T_L = \frac{1-\alpha T_{in}}{c_v} \Delta P$$

where $\alpha$ is the thermal expansion coefficient of the mobile phase, $T_{in}$ is the mobile phase temperature at the column inlet, $c_v$ is the volumetric specific heat capacity, and $\Delta P$ is the pressure drop across the column. In the still air oven mode, the magnitude of the thermal axial gradient was approximately half of that calculated for an adiabatic thermal environment for all five columns as seen in Table 3. This indicates that some of the heat is being lost from the column due to natural convection from the column walls and endfittings,53 with the forced air mode increasing the convection levels due to the fan and further reducing the measured temperature at the column outlet. Because neither oven mode is completely adiabatic, radial thermal gradients form in both column compartment environments, although they are magnified in the forced air mode as the measured temperature increases are diminished and the observed efficiencies are lower. In this study, the focus was on maximizing the throughput in each column until USP performance thresholds, column pressure limits, or instrument flow rate limits were exceeded. This led to various pressures, flow rates, and mobile phase linear velocities for
each column that may prevent a more direct comparison between each column in terms of the formation of each of the thermal gradients, especially as different column dimensions play a significant role in the magnitude of radial thermal gradients and the thermal entrance length needed for both gradients to develop.\textsuperscript{51,52}

Table 3

*Theoretical and experimentally measured magnitude of the axial thermal gradient*

<table>
<thead>
<tr>
<th>Column Designation</th>
<th>$\Delta T_{L,\text{calculated adiabatic}}$</th>
<th>$\Delta T_{L,\text{still air mode}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column A</td>
<td>8.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Column B</td>
<td>21.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Column C</td>
<td>19.4</td>
<td>10.5</td>
</tr>
<tr>
<td>Column D</td>
<td>19.4</td>
<td>10.0</td>
</tr>
<tr>
<td>Column E</td>
<td>37.3</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Although small variations occur between the naproxen and combined ibuprofen methods because of differences in the performance requirements and the conditions that were selected to achieve them, sub-2 $\mu$m SPPs and longer columns provided higher chromatographic efficiency at the expense of higher temperature changes across the column and longer method times in both monographs. Increasing the column diameter marginally improves tailing factors, but does not provide a large improvement in overall efficiency compared to lower diameter columns with the same particle diameter and length. The maximum speed when using larger column diameters can also be impacted by instrument flow rate limits, which can be reached before the actual instrument or
column pressure limit is exceeded, as was the case here for the ibuprofen method. Thus, shorter columns with larger SPPs can provide the best opportunity for higher throughput methods under these various monograph-related restraints. This potential was further explored in the following section to determine how far instrument operating limits can be pushed for monograph modernization.

2.3.3 Reducing autosampler cycle times. In the process of increasing the speed of the previous methods, the main focus relied on changing the column dimensions, particle size, and mobile phase flow rate to achieve the minimum performance parameter. However, some USP monograph methods lack requirements for asymmetry and resolution, permitting even higher throughput beyond what has been described above. Two monographs have previously been published for tablets containing both acetaminophen and aspirin: one contains caffeine and the other does not. Only the method for tablets that contain caffeine lists performance thresholds, so the focus here was to maximize the speed of the non-caffeine method that would not be limited by the lower efficiencies observed at high flow rates. For this purpose, the 2.1x50 mm column containing 2.6 μm particles (Column A) was selected since the usual limitation for this column with the other methods was due to chromatographic performance rather than flow rate or pressure. Because the still air oven mode provided higher plate counts than the forced air oven mode for both the naproxen and ibuprofen methods, it was the only thermal environment utilized here to try and limit further increases to plate height at high mobile phase velocities. Additionally, a hybrid mobile phase based on the composition listed in each separate monograph (Table 1) was used to help reduce viscosity so that higher flow rates could be obtained before the column pressure limit was reached, while
still maintaining some resolution between the four peaks. To further increase method speed, the injection preparation process was changed so that the sample to be injected for the next run in a sequence was prepared during the current analysis rather than in between each run as is standard in most instrument operating procedures. By maximizing the speed of the method and eliminating any time associated with the injection between consecutive runs, a complete cycle time of 20 s was achieved so that fifteen runs could be completed in 5 min (Figure 13).
The vertical dashed lines designate each injection point, while Panel B is an expanded view of the final minute (last three runs) of the experiment. The order of separation is: (1) acetaminophen, (2) aspirin, (3) benzoic acid, and (4) salicylic acid. In this set of fifteen consecutive 20 s runs, there was some performance loss in the first few injections due to the flow rate ramp and initial formation of thermal gradients, similar to the previous experiments. Across these final ten injections, the critical pair (benzoic acid and salicylic acid) maintained a resolution exceeding 1.5, indicating baseline resolution between all compounds. Efficiency was rather low for all compounds because of the high mobile phase velocity, especially for the least retained compound (acetaminophen) which had an average plate count of 1400, but this did not diminish the resolution between any two peaks below 1.5. Specialized autosampler and injection schemes can be implemented to increase the speed slightly more, but these 20 s cycle times are readily achievable with currently available commercial instrumentation. This concept is especially relevant to high-throughput experimentation, as it’s possible that the cycle time of the autosampler can be nearly as long as the separation itself, if not longer than some ultrafast separation methods.

2.4. Conclusion

In the modernization of USP monographs for widely used drugs, column selection plays an important role in the throughput that can be achieved. Because performance requirements vary for each method, the critical parameters (resolution, asymmetry, etc.) must be identified to determine which column properties are most necessary. Shorter columns enable faster methods but can be limited by the efficiency and peak symmetry that can be achieved. Longer columns improve these parameters but increase the analysis
time. Between particle sizes, the efficiency gained by reducing the particle diameter also results in a marked increase in the magnitude of the axial thermal gradient due to viscous friction, especially at longer column lengths. In these cases, with larger increases in the mobile phase temperature at the column outlet, the use of the still air oven mode provided much better chromatographic efficiency than more isothermal environment created by a forced air oven mode. For two of the methods examined here (naproxen and ibuprofen) with five different columns, a 2.1x50-mm column packed with 2.6 µm SPPs generally provided the highest throughput within the resolution and asymmetry thresholds set by the individual USP monographs. All five columns, however, were significantly faster and required less mobile phase than the listed column types in the monographs and can be used to improve method throughput (Table 4). When increasing throughput for isocratic monograph methods within these performance guidelines, the key aspect is determining whether performance criteria, column pressure limits, or instrument pressure/flow limits for a given column type. For shorter, narrower columns, the limit is typically performance, while longer and wider columns are usually limited by pressure and/or flow rate.

As increased focus is placed on achieving method times in the 1–30 s range for pharmaceutical methods, specialized instrument set-ups and injection schemes are being utilized. Here, as demonstrated in the acetaminophen method, a standard instrument flow path and readily available column were used to achieve a 20-s cycle time, demonstrating the capability of current technology to be implemented into high-throughput workflows. This demonstrates that monograph modernization goes beyond the chromatographic parameters detailed in Chapter 621 of the USP-NF (mobile phases, temperatures, etc.) as
a wider range of experimental conditions have been implemented that dramatically reduced method time. Specifically, isocratic methods were studied which eliminates any column equilibration time required between runs that would be necessary for gradient elution. Additional work in this area focused on qualifying these low cycle time methods (testing accuracy, robustness, linearity, etc.) is discussed in Chapter 3.
<table>
<thead>
<tr>
<th></th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
<th>Method 4</th>
<th>Method 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture components</td>
<td>Naproxen, Ethylparaben</td>
<td>Ibuprofen, Valerophenone, Ibuprofen Related Compound C</td>
<td>Acetaminophen, Aspirin, Benzoic Acid, Salicylic Acid</td>
<td>Acetaminophen, Aspirin, Caffeine, Benzoic Acid, Salicylic Acid</td>
<td>Acetaminophen, Aspirin, Benzoic Acid, Salicylic Acid</td>
</tr>
<tr>
<td>Column size and flow (Monograph)</td>
<td>4.6 x 100 mm, 1.5 mL/min</td>
<td>4.6 x 250 mm, 2 mL/min</td>
<td>4.6 x 250 mm, 1.6 mL/min</td>
<td>4.6 x 100 mm, 2 mL/min</td>
<td>N/A</td>
</tr>
<tr>
<td>Last eluted peak and volume (Monograph)</td>
<td>6.5 min, 9.8 mL</td>
<td>8 min, 16 mL</td>
<td>5.2 min, 8.3 mL</td>
<td>7.1 min, 14.2 mL</td>
<td>N/A</td>
</tr>
<tr>
<td>Last eluted peak and Volume (Column A)</td>
<td>0.45 min, 0.5 mL</td>
<td>0.34 min, 0.4 mL</td>
<td>N/A</td>
<td>N/A</td>
<td>0.26 min, 0.3 mL</td>
</tr>
<tr>
<td>Last eluted peak and Volume (Column B)</td>
<td>1.58 min, 1.1 mL</td>
<td>0.50 min, 0.8 mL</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Last eluted peak and Volume (Column C)</td>
<td>0.73 min, 2.0 mL</td>
<td>0.33 min, 1.7 mL</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Last eluted peak and Volume (Column D)</td>
<td>1.35 min, 0.5 mL</td>
<td>0.53 min, 0.4 mL</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Last eluted peak and Volume (Column E)</td>
<td>3.62 min, 1.2 mL</td>
<td>1.25 min, 0.9 mL</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Chapter 3

Developing a High-Throughput Protocol for Method Qualification†

3.1 Introduction

Two critical aspects of chemical analysis in pharmaceutical quality control are drug substance assays and impurity testing. Once initial method development screening is completed, a method is optimized and qualified prior to its use for routine chromatographic characterization. The International Conference on Harmonization (ICH) and various pharmacopeial compendia provide guidelines on this qualification process and what factors of the method must be considered.56–59 These factors typically include accuracy, precision, specificity, linearity, stability, and quantification limit. The USP-NF contain a large number of analytical methods for common pharmaceutical compounds that can be readily adapted for qualification and use without extensive method development steps. However, as mentioned in Chapter 1, many of these methods are merely qualitative, and for quantitative methods that require LC separations, many are dated and utilize older column technology and instrumentation. Whereas modernized monograph techniques previously mentioned have been successful in achieving higher throughput utilizing standard UHPLC instrumentation without modification, and it was concluded for that 2.6 µm core-shell particles provided the best compromise between speed and method performance. In this continuation of the previous experiment set, these techniques are used to demonstrate strategies for further method qualification for a

combined drug substance and impurity assay for ibuprofen based on the current USP monograph. The full set of runs required to complete a typical data collection sequence for qualification can be completed in under 16 minutes, which is similar to the time scale needed for only two ibuprofen monograph runs with published methodology. Such capabilities can be of great use in the pharmaceutical industry, as it would allow issues with instrumentation or the drug product to be identified much faster than with standard method workflows that can take an entire day. This work is not necessarily focused on re-validating a monograph method, but rather used as an exemplary study on how modern LC technology can be applied to better develop robust, high-throughput methods for pharmaceutical analysis.

3.2 Experimental Design

3.2.1 Effects of mobile phase composition changes. One of the major changes made to the ibuprofen method for this qualification study was the composition of mobile phase. In the USP-NF Chapter 621 Guidelines, the mobile phase composition can be shifted by up to 10%. When acetonitrile is used as the organic mobile phase component, increasing its amount not only reduces the viscosity of the solution (allowing higher flow rates at the instrument pressure limit), but also increases the elution strength of the solvent. For columns where backpressure is a limiting factor to method speed, this can make a noticeable difference in the overall method time. To demonstrate this concept, using 70% acetonitrile in the ibuprofen method in Chapter 2 instead of 60% (Table 1), leads to a 13% increase in flow rate at a similar pressure (Figure 14) when demonstrated on a 2.1x100mm column packed with 1.5µm particles. Due to the higher flow rate and increase in eluting strength of the mobile phase, the final peak (ibuprofen-related
compound C) elutes in nearly half the time of the published monograph method. The effects of mobile phase changes show that significant increases in throughput can be made even within the current guidelines, which is the basis for demonstrating the robustness of the qualification protocol over a range of method parameters, which is discussed further in Section 3.3.1.

Figure 14. Separation of ibuprofen sample using 70% acetonitrile and higher flow rate compared to 60% acetonitrile on a 2.1x100mm column packed with 1.5µm particles. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Chromatographia. Using Superficially Porous Particles and Ultrahigh Pressure Liquid Chromatography in Pharmacopeial Monograph Modernization of Common Analgesics. Kresge, G. A.; Wong, J.-M. T.; De Pra, M.; Steiner, F.; Grinias, J. P. Copyright Springer-Verlag GmbH Germany 2018. https://www.springer.com/journal/10337
3.2.2 Sample preparation. For robustness measurements, the mobile phase used was between 65–70% acetonitrile in water with 0.4% chloroacetic acid, adjusted with ammonium hydroxide to a pH value of 3.0 ± 0.2. For the high-throughput qualification sequences, 67.5% acetonitrile in water with 0.4% chloroacetic acid at a pH of 3.0 was used. Higher organic modifier than the published monograph was used because this change was previously demonstrated to reduce peak elution times without greatly reducing chromatographic resolution (Section 3.2.1).47,50

Stock solutions of 60 mg/mL ibuprofen, 7 mg/mL valerophenone, and 0.12 mg/mL ibuprofen-related compound C were prepared in acetonitrile based on guidelines from the published monograph. Then, five mixtures of these three stock solutions were made with varying concentrations of ibuprofen and ibuprofen-related compound C with the mobile phase as the diluent per the procedure listed in the monograph (Table 5).61
Table 5

*Description of samples utilized in the qualification study*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ibuprofen (mg/mL)</th>
<th>Valerophenone (mg/mL)</th>
<th>Ibuprofen-related compound C (mg/mL)</th>
<th>Consecutive Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>9.0 (75.0%)</td>
<td>0.35</td>
<td>0.006 (0.05%)</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>10.5 (87.5%)</td>
<td>0.35</td>
<td>0.009 (0.08%)</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>12.0 (100%)</td>
<td>0.35</td>
<td>0.012 (0.10%)</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>13.5 (113%)</td>
<td>0.35</td>
<td>0.018 (0.15%)</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>15.0 (125%)</td>
<td>0.35</td>
<td>0.024 (0.20%)</td>
<td>3</td>
</tr>
</tbody>
</table>

*Note.* Value in parentheses indicates percentage of nominal active pharmaceutical ingredient listed in monograph.

3.2.3 **Instrumentation and methodology.** This experiment utilized the chromatographic setup mentioned in Chapter 2: a Vanquish Horizon UHPLC system equipped with the same UV-DAD, column compartment, split autosampler, binary pump, and tubing dimensions. Four Accucore C18 columns with the dimensions of 2.1x100 mm packed with 2.6 µm diameter core-shell particles were used. An additional 2.1x100 mm Halo column packed with 2.7 µm diameter core-shell particles was also tested (Advanced Materials Technology, Wilmington, DE). The injection volume was 0.5 µL, which provided sufficient signal for the impurity trace peak at lower concentrations. Flow rates between 1.1 and 1.2 mL/min were selected and the column equilibrated for 20 min to achieve thermal equilibrium before the sequence of injections was initiated. For this
qualification demonstration, 24 consecutive injections with analysis times of 40 s (~16 min total) were programmed in the sequence list (3 blanks, 21 sample runs). To further increase throughput, the autosampler was programmed to begin preparing the next sample (27 s cycle time with needle wash) while the previous one injection was being analyzed to eliminate any delay time between injections, resulting in a total method cycle time of 40 s. The column compartment oven was maintained between 30 °C and 40 °C using the still air oven mode. The DAD detector was set to a 50 Hz acquisition rate (0.1 s response time for instrument digital filter) at 254 nm. This wavelength selection is based on the monograph method and ensures high absorbance for the impurity peak (ibuprofen related compound C), even at low concentrations relative to the ibuprofen peak. Five demonstrations of the qualified method were achieved: three Accucore columns on the Rowan University system, one was a previously used column (Column A, approximately 1000 injections), the other two were newer and from the same lot (Columns B and C). A fourth Accucore column was tested on a different UHPLC instrument with identical parameters and a different user in Germering, Germany as a demonstration of reproducibility (Column D). Finally, one Halo column was tested on the Rowan University system as a robustness demonstration of a similar column from a different vendor (Column E). Data was processed in Chromeleon 7.2.10 and worked up in Igor Pro 6.0.

3.3 Results and Discussion

3.3.1 Considerations for robustness in high-throughput LC methods. In the previous chapter, the potential for high-throughput approaches to LC-based monograph methods of OTC analgesic drugs was demonstrated. However, this was an optimized
method designed specifically to minimize assay cycle time and did not include method
robustness investigations that provide ranges for various experimental parameters. Rather
than complete a full statistical design of experiment (DoE) study that would be
recommended for full method validation, this investigation focused on developing
flexible ranges that still maintained high-throughput conditions. Column dimensions and
flow rates were selected beyond the allowable changes within USP-NF Chapter 621,\(^4,5\) as
the goal here was to determine conditions that provided the fastest separation times while
maintaining critical pair resolution above 2.5 and USP tailing factors below 2.5 for all
peaks, per the ibuprofen monograph.\(^{47}\) A summary of the final ranges is shown in Table
6. In terms of mobile phase flow rate, the maximum value that fulfilled these criteria was
1.2 mL/min, which was then set as the high end of the parameter range. Because the flow
rate is one of the critical aspects of method throughput, the minimum value was set only
slightly lower at 1.1 mL/min. In terms of mobile phase pH, no significant changes to
retention were observed within the standard range of 3.0 ± 0.2 described in the
monograph and Chapter 621, so these levels were maintained. The published monograph
describes use of a mobile phase containing 60% acetonitrile, although reduced elution
times and slightly higher resolution can be achieved for this separation with 67.5%
acetonitrile, so this solvent composition was used here. This minor variation was in
accordance with the allowable changes described in Chapter 621, which permit up to a
10% absolute (or 30% relative) maximum change in a given mobile phase component.
The increased organic content of the mobile phase changes the relative retention times to
valerophenone listed in the monograph (approximately 0.7 and 1.2) to 0.8 and 1.1 for
ibuprofen and ibuprofen related compound C, respectively, although the more critical
parameter of a minimum resolution between both peak pairs is still maintained. To
provide a range for method robustness, a lower limit of 65% acetonitrile and an upper
limit of 70% acetonitrile were selected, as both these endpoints were within the allowable
change range and provided faster method times than 60% acetonitrile. The ibuprofen
monograph lists 30 °C for the column oven temperature; as lower temperatures increase
analyte retention in this separation, this value was set as the lower end of the range.
Chapter 621 permits up to a 10 °C change in temperature from the published monograph,
so the higher end was set at 40 °C. For these compounds, the temperature change had a
much less dramatic effect on retention than the mobile phase composition. The minimum
value ranges that provided the slowest elution times were used to select the method time
of 40 s that ensures that all peaks elute on all columns tested in this study. With the
average conditions that were used to complete the full method qualification, seen below
in Section 3.3.2, 40 s was more than sufficient to complete each run in the sequence
before the next began. The final robustness parameter was injection volume, which did
not have a direct effect on method time. However, to ensure that the efficiency and peak
shape criteria were met, the maximum injection volume was set at 0.9 µL. At higher
injection volumes, both peak width and peak shape suffered due to larger extra-column
broadening effects. There is no limit to the amount that the injection volume can be
varied in Chapter 621, since the value is highly dependent on the instrument and column
that are used. Here, the range was extended down to a minimum value of 0.1 µL, which
was still sufficient to observe the impurity peak at its lowest tested concentration (Table
6).
Table 6

*Method parameter ranges based on robustness experiments*

<table>
<thead>
<tr>
<th>Method Parameter</th>
<th>Minimum Value</th>
<th>Average Value for Usage</th>
<th>Maximum Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate (mL/min)</td>
<td>1.10</td>
<td>1.15</td>
<td>1.20</td>
</tr>
<tr>
<td>Mobile Phase pH</td>
<td>2.8</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Mobile Phase Organic Content (%B)</td>
<td>65</td>
<td>67.5</td>
<td>70</td>
</tr>
<tr>
<td>Column Oven Temperature (°C)</td>
<td>30</td>
<td>35</td>
<td>40</td>
</tr>
<tr>
<td>Injection Volume (µL)</td>
<td>0.1</td>
<td>0.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

In general, the selection of robustness parameters when designing high-throughput methods for qualification must focus on ensuring that minimal separation time is required for the method. Thus, smaller ranges for flow rate and mobile phase composition close to the maximum values that achieve the desired performance criteria are required. Other parameters that have less effect on throughput, such as mobile phase pH and oven temperature in this demonstration, can have wider ranges that still maintain minimal time to complete the full chromatographic sequence. The use of this strategy to demonstrate a full method qualification is described in Section 3.3.2.

3.3.2 Demonstration of method qualification using high-throughput LC strategies. ICH and USP guidelines provide information on data that must be acquired for qualification of both drug substance and impurity assays, which are detailed in Table 7.56–59 While exact values that are required for a specific qualification depend on the
purpose of a given assay, these general guidelines represent the typical analytical figures of merit that must be determined. In this study, we utilized average method parameters from the robustness experiments in Section 3.3.1 to demonstrate a complete set of runs in 16 minutes (Figure 15). Because the monograph for ibuprofen contains well resolved drug substance and impurity (ibuprofen-related compound C) peaks, higher throughput can be achieved by combining the two assays into a single method, as outlined in Chapter 2, this concept was applied again here. By selecting appropriate concentration levels for a series of five samples and blanks (Table 5), the necessary data for method qualification can be achieved in 24 consecutive runs (Figure 15). With each sample injected in at least triplicate, measures for percent relative standard deviation (%RSD) can be calculated at multiple points as described in the ICH guidelines. Six injections were made at the lower impurity limit and six injections were made at the 100% drug substance level for specific repeatability criteria at these levels. Three additional concentration levels (injected in triplicate) were also used to test for linearity with recovery (accuracy), which is inferred from the linearity and repeatability data. To reduce the overall analysis time, the least number of samples was used that still met the requirements described in the ICH guidelines for this approach. Five sequences were performed on different days that met the set criteria limits, with one set in a different laboratory by a different analyst to further satisfy intermediate precision guidelines and another set using a similar column under the USP L1 designation (Table 7).
Table 7

*Summary of method qualification tests on five 2.1x100 mm columns packed with 2.6-2.7 μm core-shell particles*

<table>
<thead>
<tr>
<th></th>
<th>Column A</th>
<th>Column B</th>
<th>Column C</th>
<th>Column D</th>
<th>Column E</th>
<th>ICH Guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Resolution of Critical Pair</td>
<td>2.89</td>
<td>3.27</td>
<td>3.59</td>
<td>2.83</td>
<td>3.59</td>
<td>Minimum of 2.5</td>
</tr>
<tr>
<td>Average Drug Substance Recovery</td>
<td>99.83%</td>
<td>99.81%</td>
<td>99.78%</td>
<td>99.79%</td>
<td>99.83%</td>
<td>Recovery = 100 ± 2% compared to Certificate of Analysis</td>
</tr>
<tr>
<td>Drug Substance Repeatability (%RSD)</td>
<td>0.44%</td>
<td>0.24%</td>
<td>0.21%</td>
<td>0.95%</td>
<td>0.24%</td>
<td>%RSD ≤ 1% (n = 9)</td>
</tr>
<tr>
<td>Organic Impurity Recovery (Lower Limit)</td>
<td>98.58%</td>
<td>98.79%</td>
<td>98.91%</td>
<td>101.01%</td>
<td>98.98%</td>
<td>With sample spiked at lower limit (n = 6), recovery = 100 ± 20%</td>
</tr>
<tr>
<td>Organic Impurity Recovery (1/2 of Upper Limit)</td>
<td>100.10%</td>
<td>100.49%</td>
<td>100.40%</td>
<td>95.82%</td>
<td>100.40%</td>
<td>With sample spiked at half of upper limit (n = 3), recovery = 100 ± 10%</td>
</tr>
<tr>
<td>Organic Impurity Recovery (Upper Limit)</td>
<td>99.58%</td>
<td>99.45%</td>
<td>99.62%</td>
<td>100.33%</td>
<td>99.52%</td>
<td>With sample spiked at upper limit (n = 3), recovery = 100 ± 5%</td>
</tr>
</tbody>
</table>
Table 7 (continued)

<table>
<thead>
<tr>
<th></th>
<th>Column A</th>
<th>Column B</th>
<th>Column C</th>
<th>Column D</th>
<th>Column E</th>
<th>ICH Guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic Impurity</td>
<td>0.33%</td>
<td>0.25%</td>
<td>0.11%</td>
<td>0.20%</td>
<td>0.22%</td>
<td>With sample spiked at lower limit (n = 6), impurity %RSD ≤ 10%</td>
</tr>
<tr>
<td>Repeatability (%RSD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug Substance</td>
<td>0.9997</td>
<td>0.9996</td>
<td>0.9997</td>
<td>0.9995</td>
<td>0.9998</td>
<td>At least five points in range of 75% - 125% (R² ≥ 0.999)</td>
</tr>
<tr>
<td>Linearity (R²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic Impurity</td>
<td>0.9998</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9998</td>
<td>0.9999</td>
<td>At least five points in range of 0.05% - Upper Limit (R² ≥ 0.99)</td>
</tr>
<tr>
<td>Linearity (R²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. Columns A-D are the same column type and of the same manufacturer. Column D was tested with a different user on different system. Column E is the same dimensions as A-D from different vendor and packed with 2.7µm particles. Linearity values are based on peak area ratio to internal standard.
This 40 s cycle time method enables a complete chromatographic sequence of runs needed for method qualification in 16 min, while still maintaining robustness across a broad set of experimental parameters. This achievement is enabled through the use of a 2.1x100 mm column, which provides higher chromatographic resolution and improved peak shape compared to a 2.1x50 mm column while still being able to achieve high linear velocities that promote increased throughput. Another key aspect that provides reduced cycle times is the core concept of preparing the next sample injection during a chromatographic run. By eliminating autosampler needle movement and sample draw functions between runs and instead performing them simultaneously with a chromatographic separation, cycle time can be reduced by half when high-throughput LC methods are employed. Fast cycle times can also be achieved by injecting all samples within a single chromatogram as described in other studies, although this can complicate data analysis in typical chromatographic software systems as all peaks are in one single file rather than individually characterized using software algorithms for calculating USP parameters as in the technique demonstrated here. While there continues to be some hesitation to moving to UHPLC technology for routine pharmaceutical analysis if methods have previously been qualified and/or validated, this study shows how throughput can be increased by at least an order of magnitude without any modifications to current instrument platforms and suggests the potential for increased adoption of UHPLC in routine drug analysis.

3.4 Conclusion

In this study, strategies for designing high-throughput LC pharmaceutical monograph methods during the qualification process are described. With modern column
technology utilizing sub-3 μm core-shell particles, high efficiency separations can be achieved even at very high mobile phase velocities for specific methods. Although these approaches require exceeding some of the guidelines outlined in Chapter 621 of the USP-NF in terms of increased mobile phase velocity (greater than 50% increase), system suitability criteria related to analyte resolution and peak symmetry can still be achieved while demonstrating the required linearity and repeatability outlined by the ICH. Here, qualification of a combined method for drug substance and impurity analysis requiring 24 chromatographic runs was completed in under 16 min, which is a 24-fold increase in throughput compared to the original published monograph method that separated the two assays and took 8 min for each separation, not accounting for even higher sequence speed achieved by preparing injections during the preceding run. This approach utilizing shorter overall sequence times and smaller diameter columns can also reduce the environmental impact of the method as mentioned in Section 1.3, with further potential benefits gained through alternative “green” solvents or the use of capillary methods.

Even though some of these changes may go beyond Chapter 621 guidelines. As high-throughput LC becomes more commonplace for screening purposes, considerations for more robust methods used in routine analysis based on the strategies described here will also be important when more stringent analytical qualification is required. Although the maximum time required to ensure analyte elution can increase when considering parameters related to method robustness (flow rate, mobile phase composition, etc.), more limited ranges can be set to focus on conditions that promote higher throughput. The results from this experiment show how high-throughput methods can exceed the ICH requirements for method qualification and can be implemented in standard workflows for
specific methods. In future studies, further statistical design of experiment approaches required for more stringent validation criteria within this ready-to-implement high-throughput LC regime will be explored.
Chapter 4

Conclusions and Future Directions‡

The objectives of the above experiments are especially relevant to the area of pharmaceutical analysis where current methods and procedures are not always reflected in the existing drug monographs. The ever-increasing demand of the pharmaceutical industry requires newer, reliable testing methods utilizing modern technology that can improve upon the speed and efficiency of analysis. The concepts implemented in the experiments above included utilizing higher pressure instrumentation and columns, using columns packed with smaller particles of modern design, combining existing protocols to emphasize speed and throughput, and implementing methodologies to reduce system down time between separations. This provides a basis for future experiments that will utilize stricter validation criteria and will in turn create an optimal starting point in the method development process for faster separations on newer systems, and for method transfer between current setups. To further adopt the idea of ultrafast and high-throughput separations (not just limited to the pharmaceutical industry), the current needs of the field must be addressed along with any potential problems that may be encountered, some examples of this are discussed below.

Improvements can be made to separations to increase speed and efficiency by using approaches that are mentioned within established USP and ICH regulations, and going beyond them as well. Changes to mobile phase composition and velocity,

temperature, column dimensions, and injection volumes are available to be altered within regulations providing a decent amount of ways to increase separation speed. It has been shown that changes made that exceed these regulations can still aid in increased throughput and meet the critical parameters. There are also changes that were made that were not explicitly mentioned in the written guidelines such as the use of combining content assays and qualification tests, further reducing the total number of analyses to be performed. Changes to instrument modules also proved suitable when autosampler cycling was shifted to take place during analysis rather than before, further showing the relevance of such changes made outside the scope of written guidelines. Strategies like these can be employed for traditionally slower separations or even ultrafast separations that approach sub-second speeds.

One of the techniques employed in Chapters 2 and 3 to reduce overall method time was to overlapped sample injections, where a sample is prepared for injection between runs. This was essential to reducing the total sequence time as the autosampler cycle time can sometimes be larger than the separation time itself. In addition to this change that can be implemented by the operator, there are commercial systems that have dual needle injection paths to achieve this. Another unique LC application that benefits from high-throughput autosampling is described as “Multiple Injections in a Single Experimental Run” (MISER) chromatography. Here, samples are injected sequentially over time during a single chromatographic run, resulting in a chromatogram (misergram) that shows all analyte peaks from consecutive injections for a qualitative comparison of samples. It has proven useful in certain scenarios for reaction monitoring, pharmaceutical impurity and formulation assays, drug screening, and food and beverage samples. Since
the misergram details all analyte peaks from each sample, there is a lack of procedures for regenerating the individual chromatogram files for further data processing. This could lead to apprehension when considering a methodology like Miser, although this is not an issue in the techniques used here since each file is an individual chromatogram that can be still be processed using instrument data packages. It should be noted that as separations continue to increase in speed, the need for high throughput sampling processes will rise as well to keep up with the workload.

For high-throughput separations, smaller particles offer the ability to decrease analysis time while keeping decent efficiency. When used in shorter columns (around 50mm or less), higher mobile phase velocities can be reached at reasonable pressures. The use of 5-10 mm columns has recently shown the ability to achieve sub-second separations at extremely high flow rates. However, separations like this have not been implemented in routine analysis as it gives rise to potential problems including loss of efficiency from frits, tubing connections and other extra-column broadening sources. When conducting separations at high mobile phase velocities, using columns packed with SPP's allows for higher performance at speeds exceeding optimum column efficiency. The design of these columns can continue to be improved in terms of ordered pore structure of the outer shell, bed homogeneity and stability during high pressure cycles which will further advocate for their use in ultrafast separations. An alternative column packing demonstrated in ultrafast separations is the use of a monolithic column. The unique design consists of one single piece of either porous silica or organic polymer and offers lower flow resistance allowing for high flow rates to be employed, with lower backpressures. Since monolithic columns can operate over such a wide flow rate range,
a suitable way to reduce elution times of highly retained molecules is to increase the flow rate as analysis time continues resulting in a flow gradient. This theoretically makes them ideas to adopt for ultrafast separations, but poor kinetic performance at high mobile phase velocities has hindered their implementation in this area.

As mentioned previously, increasing the flow rate and back pressure while with narrower columns will cause thermal broadening to take effect. Solid core silica particles can increase the thermal conductivity of the packed bed, alleviating some of the effects. However it was shown recently, using simulated fluid dynamics at more extreme operating pressures (near 2500 bar), that the presence of a solid silica core may not have a great enough effect on the packed bed conductivity than previously thought. These simulations also showed that the use of core-shell particles with a superconducting core made of copper or gold, cannot be expected to give more than a 50-60% increase of the thermal bed conductivity. Meaning at these higher operating pressures, a solid silica core is not able to reduce the magnitude of a radial thermal gradient by the degree needed to reach the level shown by current operating pressures on commercial instruments (1000-1500 bar). Additional thermal conductivity can be achieved by using a monolithic support consisting of a solid core of highly conductive material creating an uninterrupted path of axial connectivity, which would further reduce radial heat transport. Other avenues of approach that would increase the overall thermal conductivity would be to use improve adiabatic insulation conditions or switch to a less conductive column wall material, though this would be less practical as it would likely reduce the mechanical strength of the column.
There is no single technological development that will satisfy the needs of every analyst in each field of chemical analysis when it comes to increasing throughput for LC methodologies. The many advancements in method development strategies, instrument performance and column design, provide selections that can be combined to suit the requirements of a separation to increase its throughput. An increase in the implementation of UHPLC over traditional instrumentation increases the demand for columns that can perform at higher pressure limits and faster linear velocities. However, some of the fastest separation speeds seen recently have used a modified or home-built system, which impacts the ease at which these advances could be implemented. Therefore, one of the current challenges faced in this area of separations is a lack of commercially available instrumentation that can utilize the high-performance potential of newer, low-volume, high-efficiency columns. To overcome this hurdle, a system will eventually need to be developed that can meet all the requirements of maintaining this efficiency. The difficulties of achieving high pressure limits while maintaining system robustness with minimal extra-column volumes in a new format will likely require re-engineering of how current LC instruments are developed. Such examples can include increasing the speed of autosamplers or combining valve technology with integrated separation mechanisms. These opportunities can provide separation scientists with the means to implement high-throughput separation methods into their workflows in situations where they would normally be limited with current setups.
References


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Appendix

Additional Tables

Table A1

*Full numerical data set from the column radar plots discussed in Chapter 2 for Method 1 (Naproxen, Figures 3-7)*

<table>
<thead>
<tr>
<th>Column Designation</th>
<th>Oven Mode</th>
<th>N&lt;sub&gt;Ethylparaben&lt;/sub&gt;</th>
<th>N&lt;sub&gt;Naproxen&lt;/sub&gt;</th>
<th>Ethylparaben Tailing Factor</th>
<th>Naproxen Tailing Factor</th>
<th>Rs</th>
<th>T&lt;sub&gt;Outlet&lt;/sub&gt; (K)</th>
<th>tr&lt;sub&gt;tr,Naproxen&lt;/sub&gt; (min)</th>
<th>P (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column A</td>
<td>Forced</td>
<td>2930</td>
<td>2840</td>
<td>1.215</td>
<td>1.150</td>
<td>5.02</td>
<td>8.0</td>
<td>0.438</td>
<td>660</td>
</tr>
<tr>
<td>Column A</td>
<td>Still</td>
<td>3030</td>
<td>2990</td>
<td>1.223</td>
<td>1.151</td>
<td>5.23</td>
<td>8.5</td>
<td>0.447</td>
<td>670</td>
</tr>
<tr>
<td>Column B</td>
<td>Forced</td>
<td>6550</td>
<td>6660</td>
<td>1.179</td>
<td>1.134</td>
<td>12.39</td>
<td>7.0</td>
<td>1.539</td>
<td>740</td>
</tr>
<tr>
<td>Column B</td>
<td>Still</td>
<td>7100</td>
<td>7240</td>
<td>1.154</td>
<td>1.106</td>
<td>13.13</td>
<td>8.5</td>
<td>1.582</td>
<td>730</td>
</tr>
<tr>
<td>Column C</td>
<td>Forced</td>
<td>4000</td>
<td>3770</td>
<td>1.249</td>
<td>1.237</td>
<td>5.77</td>
<td>6.5</td>
<td>0.713</td>
<td>630</td>
</tr>
<tr>
<td>Column C</td>
<td>Still</td>
<td>4380</td>
<td>4120</td>
<td>1.253</td>
<td>1.221</td>
<td>6.14</td>
<td>7.0</td>
<td>0.725</td>
<td>650</td>
</tr>
<tr>
<td>Column D</td>
<td>Forced</td>
<td>4680</td>
<td>5430</td>
<td>1.347</td>
<td>1.335</td>
<td>7.13</td>
<td>7.0</td>
<td>1.348</td>
<td>740</td>
</tr>
<tr>
<td>Column D</td>
<td>Still</td>
<td>5190</td>
<td>6240</td>
<td>1.326</td>
<td>1.285</td>
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<td>8.0</td>
<td>1.346</td>
<td>750</td>
</tr>
<tr>
<td>Column E</td>
<td>Forced</td>
<td>13800</td>
<td>13220</td>
<td>1.220</td>
<td>1.228</td>
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<td>16250</td>
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<td>1.184</td>
<td>13.68</td>
<td>10.5</td>
<td>3.619</td>
<td>1160</td>
</tr>
</tbody>
</table>
Table A2

*Full numerical data set from the column radar plots discussed in Chapter 2 for Method 2 (Ibuprofen, Figures 8-12)*

<table>
<thead>
<tr>
<th>Column Designation</th>
<th>Oven Mode</th>
<th>( N_{\text{Ibuprofen}} )</th>
<th>( N_{\text{Compound C}} )</th>
<th>Ibuprofen Tailing Factor</th>
<th>( R_s,1 )</th>
<th>( R_s,2 )</th>
<th>( T_{\text{Outlet (K)}} )</th>
<th>( t_{\text{r,Compound C (min)}} )</th>
<th>( P ) (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column A</td>
<td>Forced</td>
<td>3280</td>
<td>4900</td>
<td>1.338</td>
<td>3.161</td>
<td>2.694</td>
<td>3.5</td>
<td>0.338</td>
<td>350</td>
</tr>
<tr>
<td>Column A</td>
<td>Still</td>
<td>3200</td>
<td>4670</td>
<td>1.308</td>
<td>3.122</td>
<td>2.637</td>
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<td>0.339</td>
<td>320</td>
</tr>
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<td>Column B</td>
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<td>5610</td>
<td>7420</td>
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<td>3.703</td>
<td>3.539</td>
<td>10.5</td>
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<td>830</td>
</tr>
<tr>
<td>Column B</td>
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<td>5870</td>
<td>7920</td>
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<td>3.844</td>
<td>3.697</td>
<td>11.5</td>
<td>0.499</td>
<td>820</td>
</tr>
<tr>
<td>Column C</td>
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<td>4950</td>
<td>1.392</td>
<td>3.077</td>
<td>2.670</td>
<td>9.5</td>
<td>0.325</td>
<td>740</td>
</tr>
<tr>
<td>Column C</td>
<td>Still</td>
<td>3920</td>
<td>5770</td>
<td>1.366</td>
<td>3.347</td>
<td>2.909</td>
<td>10.5</td>
<td>0.328</td>
<td>740</td>
</tr>
<tr>
<td>Column D</td>
<td>Forced</td>
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<td>8770</td>
<td>1.635</td>
<td>4.531</td>
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<td>0.530</td>
<td>750</td>
</tr>
<tr>
<td>Column D</td>
<td>Still</td>
<td>6030</td>
<td>10160</td>
<td>1.719</td>
<td>4.830</td>
<td>3.812</td>
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<td>740</td>
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<tr>
<td>Column E</td>
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<td>19500</td>
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<td>Column E</td>
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<td>24460</td>
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<td>7.563</td>
<td>6.255</td>
<td>18.0</td>
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</tbody>
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