Ultrahigh-Pressure LC in Pharmaceutical Analysis: Performance and Practical Issues

M. W. Dong. Ultra-high-pressure LC in pharmaceutical analysis: Performance and practical issues, LC.GC 25(7), 656-666, 2007.

This article describes the use of ultrahigh-pressure liquid chromatography (UHPLC) in pharmaceutical analysis of drug substances and drug products with UV absorption detection. The fundamentals and benefits of UHPLC in increasing analysis speed and resolution are reviewed. Its performance (precision, extracolumn dispersion, sensitivity, and column life) in pharmaceutical analysis was assessed. Several case studies on method development of an over-the-counter product, a complex formulation, and a drug substance are presented to illustrate the utility and benefits of UHPLC. Practical issues on system compatibility to existing methods, injection precision, system carryover, and sensitivity for low-UV absorption detection, as well as regulatory aspects on method adjustment and modification, are discussed.

Michael W. Dong

Synomics Pharma, Wareham, Massachusetts

Please direct correspondence to Michael Dong at dong.michael_w@gene.com

High performance liquid chromatography (HPLC) is the predominant analytical technique for pharmaceutical analysis. A fundamental weakness of HPLC has been its moderate separation efficiency (or resolution) and speed, limiting its utility for very complex mixtures (1–5). One straightforward approach to enhancing HPLC performance is the use of columns packed with very small particle diameters (d_p). However, since the pressure drop of the column is inversely proportional to d_p^2, a very high system pressure will be required unless short columns are used (2,4,5). In the last four decades, HPLC performance has been constrained by a system pressure limit of 6000 psi in most systems — effectively limiting its separation performance to a column efficiency (N) of ~20,000 plates or a peak capacity (P_c or n, number of peaks that can be resolved in the chromatogram) of ~200 (1,3,5). One potential approach to reduce the pressure drop of small-particle columns is to use high column temperatures. However, for pharmaceutical analysis, column temperature is typically limited to <60 °C due to concern for on-column analyte degradation (1,3). Research in ultrahigh-pressure LC (UHPLC), pioneered by J. Jorgenson and colleagues (4,5) and joined later by Milton Lee and colleagues (6) and others, demonstrated that substantially higher efficiencies and ultrafast separations could be achieved using very high system pressures with capillary columns packed with sub-2-μm nonporous particles. For most HPLC practitioners, the dream of realizing such higher HPLC performance could not be possible without advances in commercial instrumentation (7–15). While these systems are excellent research tools well-suited to LC–mass spectrometry (MS) analysis (10), their current use in routine pharmaceutical analysis using UV absorption detection is just emerging and is not yet accepted universally (11–15). Their compatibility to existing methods based on columns with larger internal diameters (3–4.6 mm i.d) is also expected to be highly dependent on specific system design.

In this article, UHPLC refers to with the use of system pressures of ~15,000 psi rather than the original term used to describe research work using capillary columns at much higher pressure (4-6). Fast LC is a common term for the use of

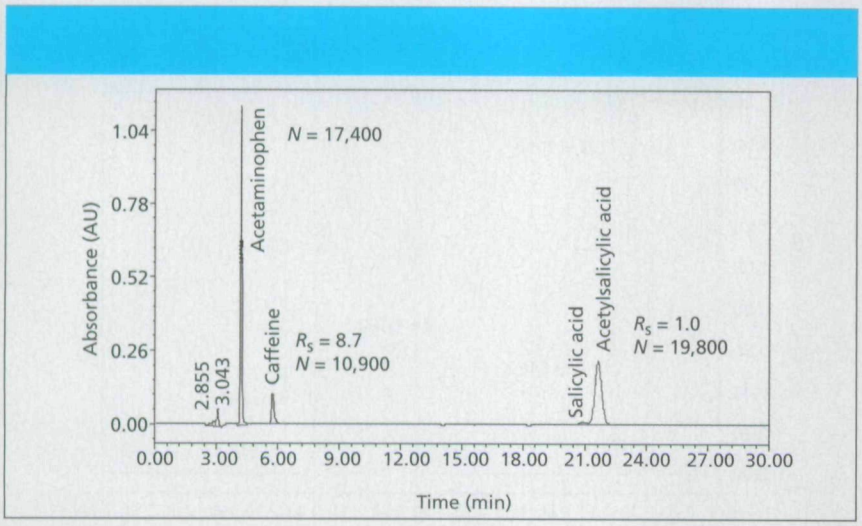

Figure 1: HPLC chromatogram of a stability indicating assay of an OTC analgesic tablet extract using an existing method with a 250 mm × 4.6 mm, 5-µm d_p column, showing the separation of three APIs and a degradant. Column: Agilent Zorbax StableBond C18; mobile phase: 15% acetonitrile in 0.1% acetic acid in water; flow rate: 1 mL/min; temperature: 30 °C; pressure: 1400 psi; detection: UV absorbance at 227 nm; sample: 5 µL of an analgesic tablet extract (one tablet in 500 mL of 0.1% acetic acid).

short 3-µm columns used with conventional or modified HPLC systems (1,3). Analysis time quoted here refers to total sample run time including column equilibration.

This article describes the use of UHPLC for pharmaceutical analysis with UV absorption detection. The concepts and benefits of UHPLC in high-speed and high-resolution applications are reviewed. Case studies involving fast method development and method conversion from HPLC to UHPLC are used to illustrate this new performance benchmark as well as potential issues for regulated assays such as precision, sensitivity, ease of use, carryover, and column life. The compatibility of this new technology to existing HPLC methods is discussed with regards to recent regulatory guidelines on method adjustments vs. modifications. While all experimental studies were conducted using a single manufacturer's system, the findings and discussion should be relevant to other UHPLC or enhanced-performance HPLC systems.

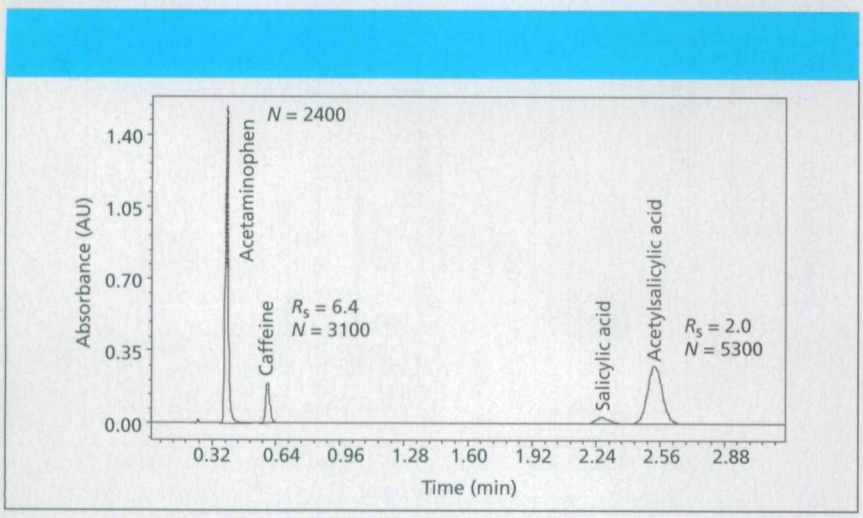

Figure 2: HPLC chromatogram on the analysis of the same OTC analgesic tablet extract using conventional HPLC equipment with a 50 mm × 4.6 mm, 3.5-µm fast LC column. Column: Waters X-Bridge C18; mobile phase: 11% acetonitrile in 0.1% acetic acid; flow rate: 3 mL/min; temperature: 30 °C; pressure: 3200 psi; detection: UV absorbance at 227 nm; sample: 2 µL of an analgesic tablet extract.

Experimental

Chemicals and reagents: ACS-grade reagents (ammonium hydroxide, phosphoric acid, potassium monobasic phosphate) and HPLC-grade solvents (acetonitrile, methanol) were obtained from J.T. Baker (Phillipsburg, New Jersey). Ammonium formate (99.999% purity), and formic acid (99%+) were obtained from Aldrich Chemicals (Milwaukee, Wisconsin).

Equipment: An ACQUITY UPLC system equipped with binary solvent manager (binary high-pressure mixing pump), sample manager (autosampler), column oven, and photodiode array detector (0.5-µL, 10-mm flow cell), and system control and data handling, was used in this study. Comparative studies were performed on a system equipped with a model 2695 quaternary pump/autosampler and a model 2996 photodiode array detector (8-µL, 10-mm flow cell). The 4-σ instrumental bandwidths (1,3) of the ACQUITY and the Alliance were found to be 10 and 45 µL, respectively. Their system dwell volumes (1,3) were found be 0.1 to 0.2 mL for UHPLC (depending on the mixer) and 1.0 mL for HPLC, respectively. Both systems were obtained from Waters (Milford, Massachusetts). Details on the system are available elsewhere (7).

The HPLC columns used included Waters ACQUITY C18 and ACQUITY Shield RP18 columns of various lengths (50, 100, and 150 mm, 2.1-mm i.d., 1.7-µm d_p) as well as Waters X-Bridge C18 and Shield RP18 (150 mm × 3.0 mm, 3.5-µm d_p), Agilent Technologies StableBond C18 (250 mm × 4.6 mm, 5 µm d_p), and Thermo Hypersil Gold C18 columns (150 mm × 2.1 mm, 3-µm d_p). Sample preparation and HPLC operating conditions as well as method performance are documented in each case study.

Results and Discussion

Fundamentals: concepts and history: The concept of performing fast and efficient chromatography using columns packed with very small particles is not new. In 1941, Martin and Synge (16) postulated that "the smallest plate height should be obtained by using very small particles and a high pressure difference across the length of the column." In the 1950s, the van Deemter equation was developed to explain chromatographic

band broadening by correlating plate height with linear flow velocity (17). It took several decades for the practical realization of these concepts as particle diameters (d_p) slowly decreased from >30 μm to sub-2 μm today (1,3). In the early 1980s, the advent of fast LC using short columns packed with 3-μm particles demonstrated the feasibility of fast separations of relatively simple mixtures (18–20). Since pressure drop across the column is inversely proportional to d_p^2, further performance enhancement by reducing d_p below 3 μm was hampered by the pressure limit of typical HPLC equipment at 6000 psi (42 MPa). In addition, the deleterious effect of instrumental band-broadening in most existing systems also deters the effective utilization of smaller (shorter) columns packed with small particles (19,20). In the late 1990s, research demonstrated the feasibility of improving separation efficiency (as much as 200,000 plates) or analysis speed using system pressures up to 100,000 psi with long capillary columns packed with sub-2-μm nonporous particles (4–7). Several instrument manufacturers responded by introducing low-dispersion systems with operating pressure to 15,000 psi. The technical challenges of designing pumps, columns, fittings, and injectors capable of reliable operations at 15,000 psi are many. In addition, UV absorption detectors must handle rapidly eluted and narrow peaks without loss of sensitivity or resolution. Currently, the focus of most commercial systems are for 1- or 2-mm i.d. columns, which are more compatible with routine LC or LC–MS analysis. While these systems allow substantial enhancements in analysis speed and resolution (~ two- to fourfold), many practitioners are concerned about their practicality in routine analysis and performance for regulated assays. This article addresses some of these issues using case studies in pharmaceutical analysis of small-molecule drug substances and products.

Case study 1: analysis of over-the-counter drug product illustrating method migration from HPLC to fast LC and UHPLC: This case study on method migration (conversion) of an existing stability-indicating HPLC method of an over-the-counter (OTC) drug product to fast LC and UHPLC illustrates the performance gain (speed and resolution) achievable and potential issues at each stage of method enhancement using small-particle columns.

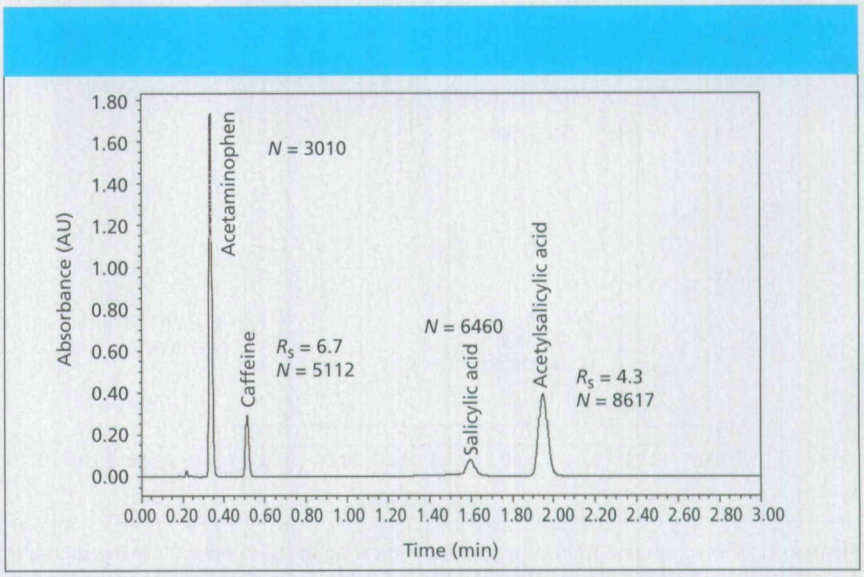

Figure 3: UHPLC chromatogram on the analysis of the same OTC analgesic tablet extract demonstrating the performance of a sub-2-μm particle column (50 mm × 2.1 mm, 1.7-μm d_p). Column: Waters ACQUITY C18; mobile phase: 11% acetonitrile in 0.1% acetic acid; flow rate 0.7 mL/min; temperature: 30 °C; pressure: 9000 psi; detection: UV absorbance at 227 nm; sample: 0.5 μL of an analgesic tablet extract.

Figure 1 shows an HPLC chromatogram of an OTC analgesic tablet (existing method using a 250 mm × 4.6 mm, 5-μm d_p column) showing peaks from three active pharmaceutical ingredients (API), acetaminophen, caffeine, and acetylsalicylic acid, and a degradant (salicylic acid) in 21 min. Column efficiency (N = 19,800) and USP resolution (R_s = 1.0) of the critical pair are also shown in the chromatogram. It can be observed that the column is well-matched to the HPLC system as the effect of instrument related dispersion is negligible (plate counts of acetaminophen, an early eluted peak, and acetylsalicylic acid are similar).

Figure 2 shows the separation of the same tablet extract achieved in 3 min using a fast LC column (50 mm × 4.6 mm, 3.5-μm d_p, N = 6000). A lower-solvent-strength mobile phase was used to

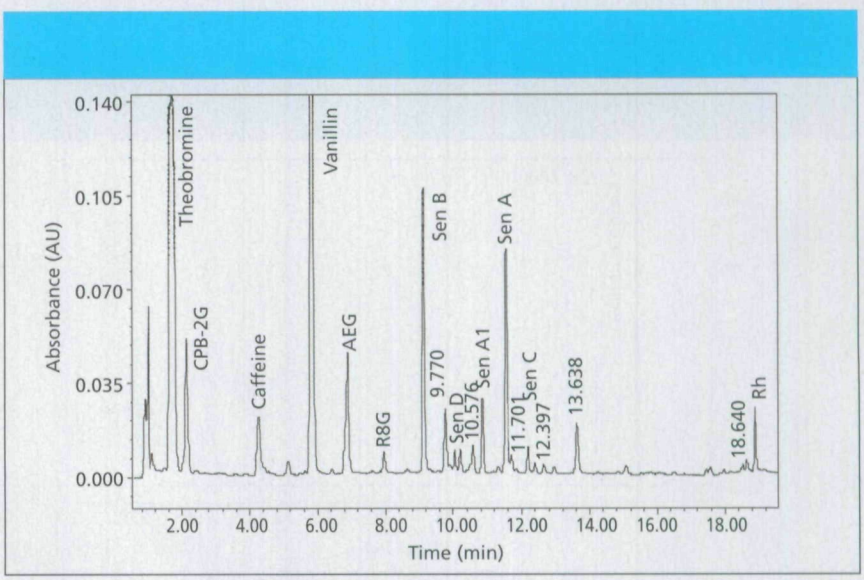

Figure 4: HPLC chromatogram of an OTC drug product extract from natural materials using a high-efficiency 150 mm × 2.1 mm, 3-μm d_p column (N = 18,000). Thermo Gold C18; mobile phase A: 20 mM ammonium formate (pH 3.75); mobile phase B: acetonitrile; gradient: 5–15% B in 15 min, 15–85% B in 10 min; flow rate: 0.5 mL/min; temperature: 50 °C; pressure: 2400 psi; detection: UV absorbance at 270 nm; sample: 5 μL of drug product extract.

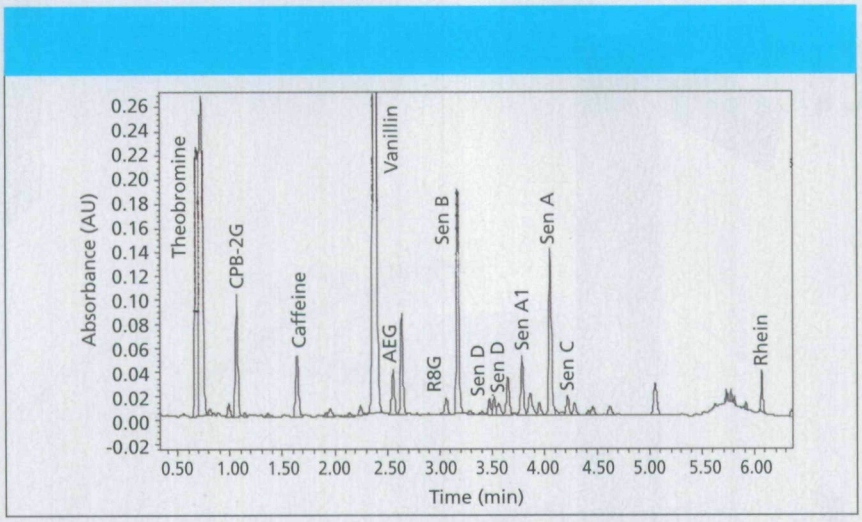

Figure 5: UHPLC chromatogram on the analysis of the same OTC drug product extract from natural materials using a 50 mm x 2.1 mm, 1.7-μm d_p column (N = 12,000), demonstrating similar resolution to that of Figure 4. Column: Waters ACQUITY C18; mobile phase A: 20 mM ammonium formate (pH 3.75); mobile phase B: acetonitrile; gradient: 5–15% B in 5 min, 15–85% B in 3 min; flow rate: 0.5 mL/min; temperature: 40 °C; pressure: 8200 psi; detection: UV absorbance at 270 nm; sample: 3 μL of extract.

provide better peak resolution and to compensate for the shorter and lower-efficiency column. Substantial efficiency losses were observed for early eluted peaks (N = ~3100 for the first peak vs. 5300 for the last peak) due to extracolumn dispersion from the HPLC system and the small column void volume (0.5 mL). Figure 2 represents the typical fast LC performance level achievable for relatively simple mixtures using conventional HPLC equipment (19).

Figure 3 illustrates the high performance achieved using a sub-2-μm particle column (50 mm × 2.1 mm, 1.7-μm d_p) with a low-dispersion UHPLC system using flow rate and injection volume scaled to suit the column dimension. Substantially higher efficiency (N = 8600) and resolution (R_s = 4.3) were achieved in 2 min. The operating pressure was ~9000 psi due to the smaller particles (1.7 μm) in the column. The effect of instrumental dispersion is also evident here by comparing plate counts of early and late eluted peaks (1,2). The efficiency loss for low retained peaks is in-line with the measured 4-σ instrumental bandwidth (IBW) of ~ 10 μL found in this study and by Jerkovich and colleagues (8–11 μL) (13). Details on how to measure IBW can be found elsewhere (1). It should be noted that this level of IBW would not be acceptable for isocratic separations with 1-mm i.d columns (13), though further IBW reduction can be realized by using injection loops and connection tubing with smaller internal diameters. However, the instrumental broadening effects are less problematic for gradient separations, when the contribution to dispersion from the injector becomes negligible due to gradient focusing effect of the sample solution.

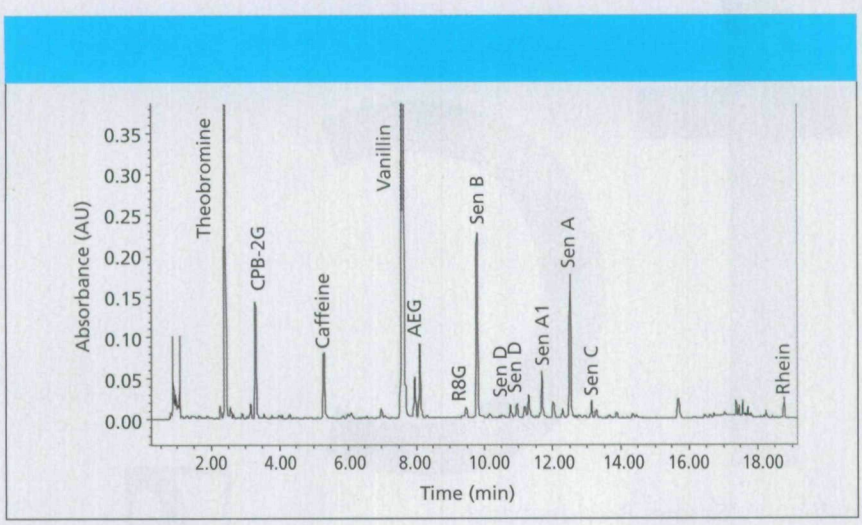

Figure 6: UHPLC chromatogram on the analysis of the same OTC drug product extract from natural materials using a high-efficiency 150 mm x 2.1 mm, 1.7-μm d_p column (N = ~35,000), demonstrating improved resolution to that in Figure 4. Column: Waters ACQUITY C18; mobile phase A: 20 mM ammonium formate (pH 3.75); mobile phase B: acetonitrile; gradient: 5–15% B in 15 min, 15–85% B in 10 min; flow rate: 0.4 mL/min; temperature: 40 °C; pressure: 10,200 psi; detection: UV absorbance at 270 nm; sample: 5 μL of extract.

This study demonstrates how substantially faster analyses can be achieved by using short 3-μm d_p columns with most existing HPLC systems (fast LC). To compensate for the lower efficiency of the short columns, peak resolution can often be enhanced by lowering the mobile phase strength. Fast LC is the most expedient approach to enhance laboratory productivity without substantial investment in new system purchases (19). It is useful for separating relatively simple mixtures or non-stability indicating assays such as dissolution testing or cleaning validation. Impurity testing by HPLC, in most cases, would require longer 3-μm d_p columns (100–150 mm with N = 10,000–20,000 plates) (3). The regulatory implications of improving existing validated methods (method modification) are covered in later sections. UHPLC performance shown in Figure 3 represents the next step in ultrafast LC separations: higher column efficiencies (1.7 μm) and reduced extracolumn dispersion. Solvent consumption per analysis is also reduced from 30 mL for HPLC to 9 mL for fast LC and again to 2 mL for UHPLC. Other examples of successful method conversion (sometimes referred to as "method transfer") of HPLC to UHPLC methods are documented elsewhere 11–15).

Table I lists useful comparative parameters for scaling method conversion from HPLC to Fast LC and UHPLC. Note values in the table are typical estimates from chromatographic theories (for example, flow proportional to square of column diameters, injection volume proportional to column void volume, gradient time proportional to column length). It is important to emphasize that for exact

Table I: Comparative parameters of HPLC, fast LC, and UHPLC

	HPLC		Fast LC		UHPLC	
	Hign-resolution	High-speed	Hign-resolution	High-speed	Hign-resolution	High-speed
Column						
Dimension (L × i.d., mm)	250 × 4.6	150 × 4.6	150 × 3.0	50 × 4.6	100 × 2.1	50 × 2.1
Particle diameter (d_p, m)	5	5	3.5	3.5	1.7	1.7
Efficiency (N = L/2.5 dp)	20,000	12,000	18,000	6000	24,000	12,000
Column void vol. (V_m, mL)	2.5	1.5	0.7	0.5	0.2	0.1
Peak vol (at k = 3, L)	318	246	94	116	23	16
Peak capacity (P_c)	200	150	200	100	220	150
Instrument						
Instrument bandwidth (µL)	50	50	30	30	10	10
Dwell volume (mL)	1.0	1.0	0.5	0.5	0.1	0.1
Flow cell, vol (µL), path (mm)	8, 10	8,10	2.5, 6	2.5, 6	0.5,10	0.5, 10
Operating conditions						
Flow (mL/min)	1.5	1.5	0.7	1.5	0.4	0.5
Injection volume (L)	20	10	5	5	2	1
Gradient time (t_G, min)	50	30	30	10	10	5
Analysis time (min)	60	40	40	15	15	10
Solv. consump. (mL/assay)	90	60	28	23	6	5
Pressure (psi)	2000	1200	3000	1500	10,000	6000

comparative studies, identical column chemistry should be used with the same mobile phase to eliminate selectivity changes. This might be difficult in practice. Thus, the case studies in this article should be interpreted as storyboards of one separation scientist's efforts to tackle method conversion with laboratory resources available at hand.

Case Study 2: high-resolution separations of complex formulations: A fundamental shortcoming of HPLC is the limited resolving power for analyzing very complex mixtures. For years, the maximum number of theoretical plates (N) achievable in a reasonable time (<1 h) was ~20,000 plates (for example, N for a 150-mm long, 3-µm d_p column), limiting the peak capacity (P_c) under gradient conditions to ~ 200 (1, 21). Martin and colleagues (21) calculated that the probability of achieving baseline separation of a random mixture of 30 components is ~3–5% for systems with peak capacity of 200. This current level of HPLC peak capacity may be inadequate for complex pharmaceutical formulations from natural materials or combination drug products (22). The advent of UHPLC with increased peak capacity (P_c > 400) is expected to have a strong impact on the analysis of these complex samples (23,24).

Figure 4 shows the separation of an extract of an OTC drug product containing a natural material and flavor components. This chromatogram exemplifies the best resolution using a conventional HPLC system after substantial method development efforts. A 150 mm × 2.1 mm column packed with 3-µm C18 particles was used (N = 18,000) with an analysis time of 38 min. Since the HPLC assay for potency requires the summation of eight identified APIs (Senna material from plant extracts) eluted between 7 to 13 min, adequate resolution of the APIs from interferences is important (3). Further resolution improvements in this critical region by extending the gradient time or by using selectivity tuning (1,2) (changing mobile phase composition or column temperature) were found almost impossible due to the large number of interfering components in the sample. Figures 5 and 6 illustrate UHPLC chromatograms of the same sample using 50-mm (N = ~12,000) and 150-mm long (N = 35,000) columns packed with 1.7-µm particles. Note that the 50-mm column achieves a separation similar to

Table II: Injection precision of UHPLC vs. injection volume

Injection Volume (mL)	Peak Area Precision (%RSD)	
	Sampling Syringe	
	100-mL	50-mL
1 µL	0.44 -1.45%	0.26 0.31%
2 µL	0.79 0.83%	0.14 0.15%
3 µL	0.48 - 0.48%	0.05 0.13%
5 µL	0.18 0.20%	0.14 0.18%
7 µL	0.11 0.18%	0.12 0.13%
10 µL (full-loop)	0.06 0.18%	N/A

HPLC in ~10 min while the 150-mm column achieves significantly improved separation in ~30 min as shown by comparative traces of the critical region in Figure 7.

HPLC method development for impurity analysis of complex formulations by the traditional approach of selectivity tuning (2) is often time-consuming and ineffective (22–24). This case study illustrates the benefits of UHPLC-type systems for complex samples. Compared with optimized HPLC separations, UHPLC should achieve similar separations in less time (2–5 times less using 50-mm columns) or substantially improved resolution (2–3 times) using 100–150 mm columns in comparable run times. Other examples of an increase in peak capacity and sensitivity using UHPLC are documented elsewhere (11,13). Further increase of peak capacity can be accomplished by increasing the column length (300 mm to N = 70,000) and gradient time (t_G) (1).

Case Study 3: method development for composite assay of a drug substance: This case study illustrates the method development (conversion or "transfer" from HPLC to UHPLC) of a composite assay (assay and impurity testing) of a drug substance. Figure 8 shows a chromatogram from the existing HPLC method, which is not mass spectrometry compatible due to the use of nonvolatile phosphate buffer in the mobile phase. A slight peak shoulder at 6.5 min of the API peak indicates a hidden impurity. The run time was 35 min. Figure 9 shows a UHPLC separation with similar resolution using an ACQUITY C18 column (50 mm × 2.1 mm, 1.7-µm d_p) with a run time of 8 min. Figure 10 shows the improved resolution of the critical region using an ACQUITY Shield RP18 column (100 mm × 2.1 mm, 1.7-µm d_p polar-embedded phase) with a run time of 13 min. This polar-embedded column provides different selectivity ("orthogonal" separation) than that of a C18 column and also resolves two additional impurities near the API.

In our laboratory, we found UHPLC to be very effective for mobile phase screening and column selection during early development for new chemical entities (1). The shorter run times (5–10 min) and equilibration times (1 min) coupled with excellent column efficiencies (N = 12,000–24,000) allow rapid mobile phase optimization. Frequently, we were able to perform initial sample assessment and method development, and obtain all analyte λ_{max}, mobile phase, and gradient conditions in addition to some column selection runs (C18, phenyl or polar-embedded phase) in a single day. However, as UHPLC systems are currently not widely used, most pharmaceutical companies appear to prefer "HPLC" assay methods that are "portable" and can be performed in most laboratories. Therefore, the final method development process in this case continued using a column packed with 3.5-µm particles using similar mobile phase and bonded phase defined by the initial UHPLC study. This backward "method transfer," using flow rates, t_G and injection volumes "scaled" to column dimensions, should be relatively straightforward if the same column chemistry is used (See Table I). However, some method fine-tuning may often be needed for specific samples (for example, it took two or three days for this case).

Figure 11 shows a chromatogram from

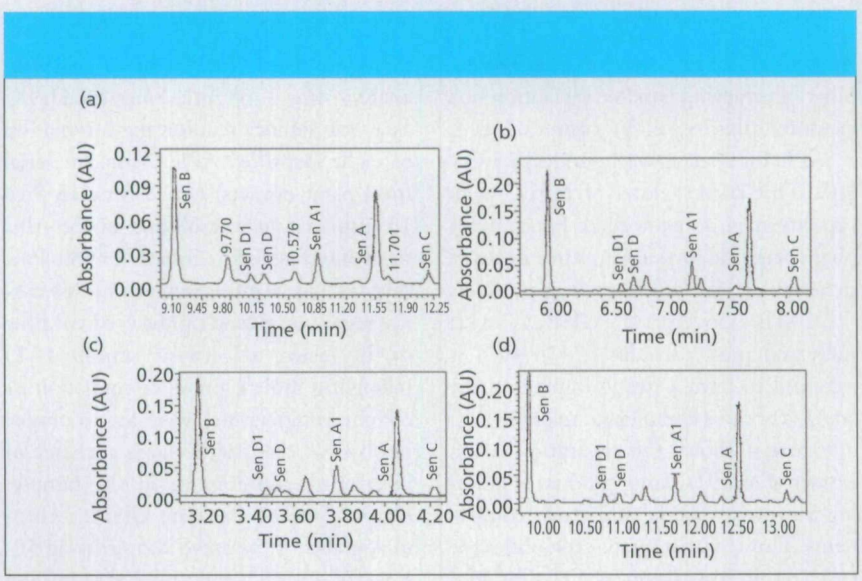

Figure 7: Comparative chromatograms of an OTC drug product extract from natural materials using (a) HPLC (150 mm x 2.1 mm, 3-µm d_p) and UHPLC with (b) 100 mm, (c) 50 mm, and (d) 150 mm columns packed with 1.7-µm d_p particles.

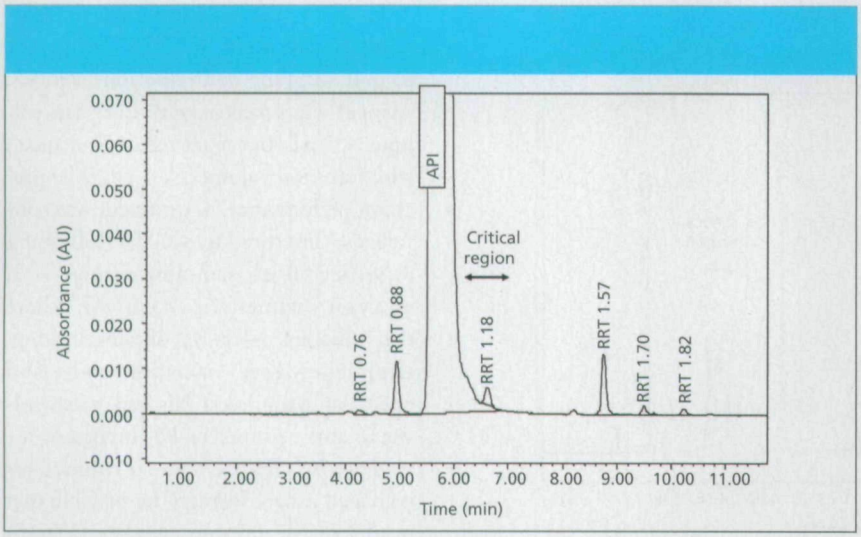

Figure 8: HPLC chromatogram of a composite assay of a drug substance solution using an existing method with a 250 mm × 4.6 mm, 5-µm d_p C18 column (N = 20,000). Waters Symmetry C8; mobile phase A: 20% acetonitrile in phosphate buffer (pH 3.1); mobile phase B: acetonitrile; gradient: 0–50% B in 25 min; flow rate: 0.8 mL/min; temperature: 30 °C; pressure: 1500 psi; detection: UV absorbance at 280 nm; sample: 10 µL of 0.68 mg/mL API.

the final method, utilizing a Waters X-Bridge Shield RP18 column (150 mm × 3.0 mm, 3.5-µm d_p polar-embedded phase), which is compatible with most existing HPLC systems. This HPLC method was then validated for regulated testing while the faster UHPLC method shown in Figure 10 was used for rapid in-process monitoring and experimental testing.

Practical issues of UHPLC in regulated pharmaceutical analysis: There is a strong consensus in the literature on the benefits of UHPLC for research projects, routine LC–MS analysis (using small-bore columns for bioanalytical assays and high-throughput screening) (8–10), and for expediting HPLC method development (7,11,13). However, there are mixed reviews concerning the practicality of UHPLC technologies for regulated pharmaceutical analysis using UV absorption detection (11–15), the dominant application for HPLC. Fundamental technical concerns about viscous heating effects of sub-2-µm particles columns and safety concerns of ultra-high-pressure systems have been addressed elsewhere (25,26).

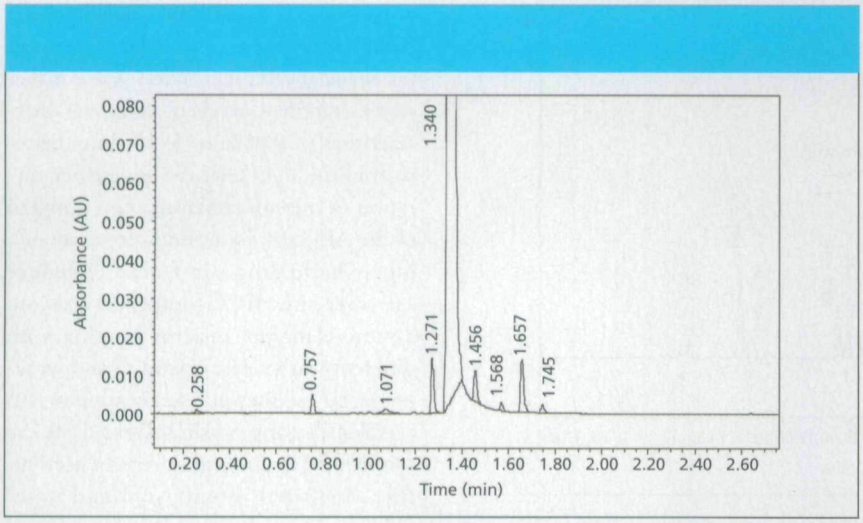

Figure 9: UHPLC chromatogram of same drug substance solution using a 50 mm × 2.1 mm, 1.7-µm d_p C18 column (N = 12,000) using an MS-compatible mobile phase, demonstrating similar resolution to that of Figure 8. Waters ACQUITY C18; mobile phase A: 20 mM ammonium formate (pH 3.75); mobile phase B: acetonitrile; gradient: 5–95% B in 5 min; flow rate: 0.5 mL/min; temperature: 30 °C; pressure: 5700 psi; detection: UV absorbance at 253 nm; sample: 0.5 µL of API at 0.5 mg/mL.

Frictional heating effects causing thermal gradients in the column and loss of efficiency appears to be acceptable for columns with internal diameters less than 2 mm (27). However, it was suggested that a lower column temperature setting be used for "transferring" HPLC methods to UHPLC to compensate for the self heating effects of these columns (27). The reliability of the columns and UHPLC system also seems to be acceptable from our studies and will certainly be continuously improved by manufacturers as the technology matures. King and colleagues (15) have documented excellent column life of at least 4000 injections for various dosing formulations.

One major concern for new users is the compatibility of UHPLC-type systems with existing HPLC methods using larger-internal-diameter analytical columns (3–4.6-mm i.d.). On paper, low-dispersion UHPLC systems should work well with conventional analytical columns, although practical issues such as connection fitting compatibility, column oven size and flow rate range might pose potential obstacles. Another common complaint is the lack of column chemistry diversity in UHPLC. Current UHPLC-compatible detectors include MS, photodiode array, UV–vis, and evaporative light scattering detectors. Other performance and regulatory issues are discussed below.

Injection precision and carryover: Injection precision is pertinent in drug assays that must pass system suitability testing criteria before any GMP testing (28,29). While peak area precision of <2.0 % RSD is cited in most analytical methods, a higher level of precision of <1.0 % is usually expected by regulatory experts and for drug substance testing with specifications of 98–102%. An injection precision of <0.5% RSD is required for most system operational qualification protocols (3). Currently, an injection precision of ~0.2–0.3% RSD is expected by pharmaceutical analysts and is routinely achieved in most high-end HPLC systems for injection volumes > 5 µL (1,3,28). Precise injection of small volumes (that is, < 5 µL) using a partial-loop fill mode under high pressure is inherently more difficult and is often limited by the volumetric sampling precision of the sampling syringe (1,28).

Previous studies using UHPLC

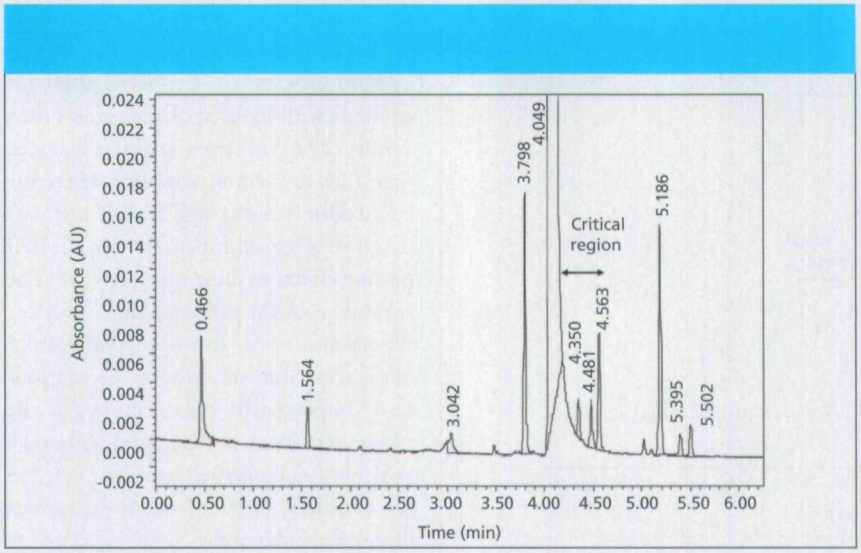

Figure 10: Waters ACQUITY Shield RP18; mobile phase A: 20 mM ammonium formate (pH 3.75); mobile phase B: acetonitrile; gradient: 5–50% B in 10 min; flow rate: 0.5 mL/min; temperature: 30 °C; pressure: 9700 psi; detection: UV absorbance at 253 nm; sample: 1.0 µL of API at 0.5 mg/mL.

reported excellent retention precision but found peak area precision to be highly variable depending on injection mode (full or partial loop), injection volume and flow rate (11–15). While the full-loop injection mode yields the best precision in UHPLC (<0.2% RSD), most pharmaceutical analysts prefer the convenience of the partial-loop mode. Using partial-loop injections, a marginal precision of <1% RSD was typically reported (11–15) with worse precision (1–3%) for smaller volumes (13) and high flow rates (15).

In our studies, the partial loop injection with needle overfill mode with air gaps to segment the sample plug was used with a 10-µL sampling loop and either a 100-µL sampling syringe (standard configuration) or a 50-µL syringe. Table II shows the injection precision performance for the first two peaks (acetaminophen and caffeine) using conditions shown in Figure 3.

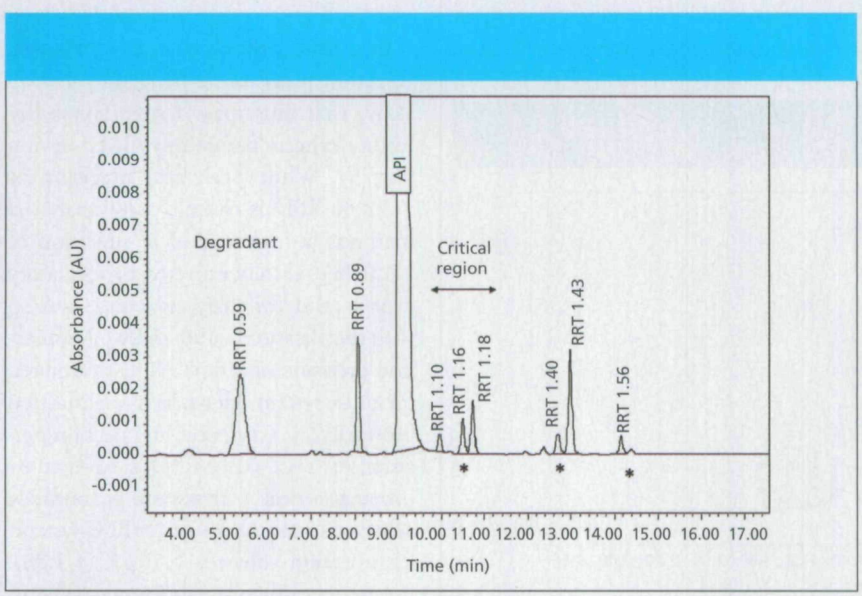

Figure 11: HPLC Chromatogram of the drug substance using a 150 mm × 3.0 mm, 3.5-µm d_p polar-embedded column (N = 18,000), demonstrating similar resolution near the API region as in the UHPLC chromatogram in Figure 10. Waters X-Bridge Shield RP18; mobile phase A: 20 mM ammonium formate (pH 3.7); mobile phase B: acetonitrile; gradient: 10-40% B in 25 min, 40-100% B in 2 min, 100% B for 3 min; flow rate: 0.8 mL/min; temperature: 30 °C; pressure: 3600 psi; detection: UV absorbance at 253 nm; sample: 5.0 µL of API at 0.2 mg/mL. The asterisks indicate light degradants

The data in Table II show that the sampling precision of UHPLC using the default shipping configuration (100-µL syringe) was excellent with injection volume > 5 µL, but marginal or inadequate with injection volumes < 3 µL. The precision performance, as expected, was considerably improved to <0.2% RSD using a smaller 50-µL sampling syringe with injection volumes of 2–7 µL. We believe the judicious selection of sample loop, sampling syringe, injection mode, and operating parameters (air gap, wash solvents, and volume) to be important for very precise UHPLC analysis required for regulated assays. It might be possible that results of the previous studies (11–15) were based on earlier injector designs, inadequate data sampling rates or nonoptimized injector configurations.

Injector carryover is a potential problem for HPLC trace analysis (bioanalytical and impurity assays) and is commonly caused by adsorbed or residual samples in the injector or the column. It is particularly troublesome for basic analytes and protein samples (30). Many high-end HPLC systems use integrated-loop autosamplers that have low carryover characteristics because the sampling loops are washed constantly by the mobile phase (3). UHPLC systems tend to have *x-y-z* push-loop type autosamplers (3) fitted with microsampling valves whose rotor seals are in direct contact with sample components. The use of higher operating pressures and smaller-internal-diameter columns may also accentuate carryover problems of certain analytes. While earlier studies reported excellent carryover characteristics (<0.01% or 5–10 times better than HPLC) (14), we did experience carryover or injector contamination problem of the API peak in an impurity assay of a highly-basic drug substance. To reduce carryover in UHPLC for specific analytes, a more elaborate injector purging with different wash solvents (strong and weak) might be required for some samples. An excellent strong wash solvent is 0.2% formic acid in a mixed solvent of acetonitrile, methanol, isopropanol and water (25% each). A typical weak solvent is acetonitrile–water (1:9). The injection of a wash sample after a high-concentration sample or at the end of the sample sequence may also reduce possible contamination of the injection valve with

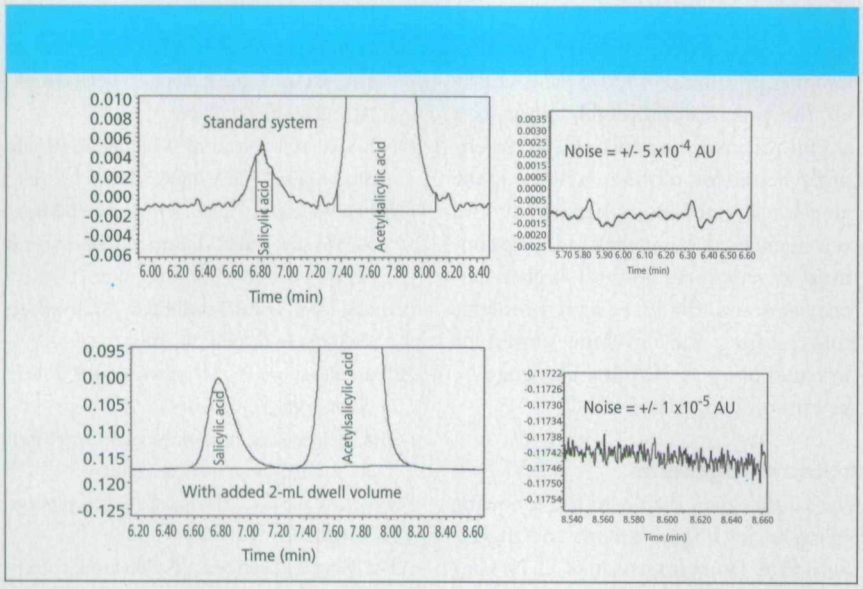

Figure 12: Comparative UHPLC chromatograms illustrating potential issue of increased detector noise using pump-blended mobile phase with low UV absorbance detection and a mobile phase with absorbance in far UV (0.1% acetic acid). The lower chromatogram demonstrates the elimination of the blending noise by increasing the mixing volume with an additional 2-mL coil placed after the 100-µL static mixer to increase mixing efficiency. Column: 50 mm × 4.6 mm, 3.5-µm d_p Waters X-Bridge C18; mobile phase A: acetonitrile; mobile phase B: 0.1% acetic acid in water; flow rate: 1.0 mL/min, 11% mobile phase B blended by pump; temperature: 30 °C; pressure: 1360 psi; detection: UV absorbance at 227 nm.

problematic analytes.

Detection sensitivity at low-UV wavelengths: The sensitivity of the PDA detector in our UHPLC system, measured by short-term detector noise using a premixed mobile phase, was found to be similar to the published specification of ±1.0 × 10^{-5} AU. In general, the mass sensitivity of UHPLC is significantly higher than that of HPLC due to the lower peak dilution (1), while the concentration sensitivity was found to be equal or better (11–15) (~ 2–3 times). Sensitivity can be further increased by a factor of 2 using the high-sensitivity flow cell (25-mm pathlength, 2.4 µL).

During our studies, we found a problem when pump blending was used with low-UV absorption detection. For instance, the noise was found to be considerably higher at low UV absorption detection (227 nm) under operating conditions similar to those shown in Figure 3 for the analgesic sample using a pump-blended mobile phase. Investigation showed that the higher detector noise was attributable to inadequate blending of mobile phase A (0.1% acetic acid which has absorbance at low UV) and acetonitrile (mobile phase B) using the standard 100-µL static mixer. The severity of this problem is expected to be a function of the flow rate, the piston stroke, the volume and efficiency of the mixer, and the relative absorbance of the two blended solvents. The blending noise, which was synchronous with the piston stroke of pump B, can be eliminated by using premixed mobile phases or by adding mixing volumes to the system as shown in Figure 12. Since pump blending and low-UV absorption detection are common HPLC applications, this potential issue can be addressed by larger-volume mixers, which also increase system dwell volumes (1) leading to higher gradient delays. A more elegant solution might be a system design with selectable mixers of different volumes allowing good performance for both high-throughput screening applications (small mixers to reduce dwell volumes to enhance gradient throughput) and pharmaceutical analysis with low-UV absorption detection (larger or more-efficient mixer to enhance mixing efficiency).

Regulatory aspects: method adjustment vs. modifications: A potential regulatory issue for method improvement of a previously validated method is what method change is considered acceptable as adjustment before re-validation is required. A detailed discussion on the topic is available elsewhere (31).

For improvements of existing HPLC method by adopting fast LC or UHPLC, pharmaceutical scientists could perform a partial method validation because both are based on the same separation principle. The validation parameters to consider are specificity, intermediate precision, linearity and robustness. Demonstration of method equivalency between the two methods should be included. Additional discussion on the topic is available elsewhere (31).

With the regulatory climate changing more towards a science-based and risk-based approach, each company should weigh the benefit/risk ratio or return on investment scenario before adopting any new technologies or method changes.

Operating precautions of UHPLC: Our experience with UHPLC indicated that effective use of UHPLC systems might require better understanding of chromatographic concepts such as column void volumes, peak volumes, extracolumn dispersion, and dwell volumes (1–3). A higher level of "chromatographic hygiene" — using highly purified reagents and better solvent and sample filtration should be exercised as higher-performance systems and columns and very small internal diameter connection tubing (0.002–0.004 in.) are less forgiving than traditional HPLC systems. Understanding peak volume, which is dependent on column void volume and retention factors (k) is particularly important for isocratic analysis (1). For example, a typical HPLC column (150 mm × 4.6 mm, 5-µm d_p) generates 15,000 plates and has a void volume of 1.5 mL, while a high-speed UHPLC type column (for example, 50 mm × 2.1 mm, 1.7 µm d_p) yields 12,000 plates and has a void volume of 0.1 mL, resulting in peak volumes which are 15 times smaller and, thus, more affected by extracolumn dispersion. Along these lines, UHPLC systems might be less accommodating of "dirty samples" or applications requiring large-volume injections.

In our studies, we found the UHPLC pump and the detector to be relatively easier to use than the autosampler with its myriad injection modes, more elaborate cleaning cycles with weak and strong solvents and user-settable air gaps. The ACQUITY column life appeared to be excellent in our studies that are in agreement with an earlier report of at least 4000 injections (15).

Summary and Conclusions

This paper describes the use of UHPLC in pharmaceutical analysis with UV absorption detection. Case studies on method development illustrate the utility and benefits of UHPLC in delivering significantly higher speed and efficiency. Our results indicate that UHPLC, as exemplified by a current UHPLC system, can increase analysis speed by two- to five-fold vs. conventional HPLC and enhance resolution 2–3 times, for impurity analysis of complex formulations. Typical reduction of solvent consumption is 3–10 times, while sensitivity is enhanced by 2–3 times. In our studies, injection precision was found to be excellent for partial-loop injection with proper selection of sampling syringe, injection parameters and data sampling rates. Potential issues include injection carryover of basic analytes, blending noise at low UV, and system compatibility with existing methods using larger-internal-diameter columns. These issues, however, may be reduced with appropriate configuration changes. Column longevity appeared excellent. UHPLC was found to be very effective for rapid method development for initial screening of columns and mobile phases. However, a "method transfer" back to HPLC may be necessary until UHPLC-type systems are standardized in most laboratories. The effective use of UHPLC in routine pharmaceutical analysis may also require more operators training and a deeper understanding of fundamental concepts and instrumental nuances. Method improvements (conversion) of validated HPLC methods to fast LC and UHPLC would probably require at least a partial validation to demonstrate method equivalency.

With a preponderance of positive feedback from early adapters, the trend toward higher-pressure, lower-dispersion UHPLC systems will no doubt continue and eventually turn mainstream for routine analysis. Until then, UHPLC method "portability" will remain a concern. The beginning user in UHPLC should be cognizant of the performance caveats and practical issues discussed here, since some of them might be minimized by changes in system configuration or operation. Other issues will be addressed by the manufacturers as UHPLC matures. UHPLC, parallel analysis, 2-D HPLC, and nano-LC are modern definitive trends for enhancing productivity and separation power. A pressure of 15,000 psi is clearly not the practical achievable limit as a 30,000-psi instrument would be significantly better for routine analysis, if the same level of reliability, sensitivity and convenience can be maintained. The continual development towards higher performance and the associated problems from pushing the envelope should be viewed as progress with new challenges in the ever-evolving world of LC.

Acknowledgments

The author gives thanks to the following colleagues for helpful reviews and suggestions: Prof. James Jorgenson of U. N. Carolina, Prof. Milton Lee of Brigham Young U, Dr. John Dolan of LC Resources, Dr. Linda Ng of the U.S. FDA, Drs. Michael Swartz, Mary Cassis, Marianna Kele, and Uwe Neue of Waters, Dr. Nanjun Wu of Merck, Dr. Anton Jerkovich of Novartis, Dr. Chris Foti of Pfizer, Dr. Jenny Wang of Parr Pharmaceuticals, Drs. Jeff Dai and Anne-François Aubrey of Bristol-Myers Squibb, Dr. Joseph Lepore of Lohrmann Therapeutic Systems, Dr. Nik Chetwyn from Genentech, and Michael Hochschwender, Dr. Kirk Smith, Kathy Cornell, and Mark Emanuele of Synomics Pharmaceutical Services, LLC. The author also acknowledges the technical assistance of Dr. Michael Swartz, Michael D. Jones, Peter Ruiz, Scott Fernandez, Susan Ryan and Robin Andreotti of Waters Corporation in the operation and maintenance of the UHPLC system.

References

(1) M.W. Dong, *Modern HPLC for Practicing Scientists* (Wiley-Interscience, Hoboken, New Jersey, 2006).

(2) L.R. Snyder, J.J. Kirkland, and J. L. Glajch, *Practical HPLC Method Development*, 2nd Edition (Wiley-Interscience, New York, 1997).

(3) S. Ahuja and M.W. Dong, Eds., *Handbook of Pharmaceutical Analysis by HPLC* (Elsevier/Academic Press, Amsterdam, 2005).

(4) J.E. MacNair, K.E. Lewis, and J.W. Jorgenson, *Anal. Chem.* **69**, 983 (1997).

(5) A.D. Jerkovich, J.S. Mellors, and J. W. Jorgenson, *LCGC* **21**(7), 600 (2003).

(6) N. Wu, J.A. Lippert, and M.L. Lee, *J. Chromatogr., A* **911**, 1 (2001).

(7) M.E. Swartz, *J. Liq. Chromatogr.* **28** (7–8), 1253 (2005).

(8) A. de. Villers, F. Lestremau R. Szucs, S. Gelebart, F. David, and P. Sandra, *J. Chromatogr., A*, **1127**(1–2), 60 (2006).

(9) J. Mazzeo, U. Neue, M. Kele, and R. Plumb, *Anal. Chem.* 77(23), 460A (2005).

(10) I.D. Wilson, R. Plumb, J. Granger, H. Major, R. Williams, and E.M. Lenz, *J. Chromatogr. B* **817**(11), 67 (2005).

(11) S.A. Wren and P. Tchelitcheff, *J. Chromatogr A.* **1119** (1–2), 140 (2006).

(12) L. Nováková, L. Matysová, and P. Solich, *Talanta* **68**(3), 908 (2006).

(13) A.D. Jerkovich, R. LoBrutto, and R. Vivilecchia, *LCGC Supplement*, 15 (2005).

(14) Y. Yang and C.C. Hodges, *LCGC Supplement*, 31 (2005).

(15) S. King, P.J. Stoffolano, E. Robinson, T. Eichhold, S.H. Hoke, T.R. Baker, E.C. Richardson, and K.R. Wehmeyer, *LCGC Supplement*, 36 (2005).

(16) A.J.P. Martin and R.L.M. Synge, *Biochem. J.* **35**, 1358 (1941).

(17) J.J. Van Deemter, F.J. Zuidenweg, and A. Klinkenberg, *Chem. Eng. Sci.* **5**, 271 (1956).

(18) M.W. Dong, *Today's Chemist at Work* **9**(2), 46 (2000).

(19) M.W. Dong and J.R. Gant, *LCGC* **2**, 294 (1984).

(20) U.D. Neue, *HPLC Columns: Theory, Technology, and Practice* (Wiley-VCH, New York, 1997).

(21) M. Martin, D.P. Hermann, and G. Guiochon, *Anal. Chem.* **58**, 2200 (1986).

(22) U.D. Neue, *J. Chromatogr., A* **1079** (1–2), 153 (2005).

(23) R.S. Plumb, R. Rainville, B.W. Smith, K.A. Johnson, D. Castro-Perez, I.D. Wilson, and J.K. Nicholson, *Anal. Chem.* **78**(20), 7278 (2006).

(24) M.W. Dong, G. Miller, and R. Paul, *J. Chromatog.* **987**, 283 (2003).

(25) A. de Villiers A, H. Lauer, R. Szucs, S. Goodall, and P. Sandra, *J. Chromatogr., A.* **1113**(1–2), 84 (2006).

(26) Y. Xiang, D. Maynes, and M.L. Lee, *J. Chromatogr. A* **991**, 189 (2003).

(27) U. Neue, Waters Corp., private communication.

(28) M.W. Dong, Today's Chemist at Work 9(8), 28 (2000).

(29) M.W. Dong, R. Paul, and L. Gershanov. *Today's Chemist at Work* **10**(9), 38 (2001).

(30) J. Dolan, *LCGC* **19**(4), 389 (2001).

(31) M. Swartz and I. Krull, *LCGC* **24**(8), 480 (2006). ■