Assay Transfer from HPLC to UPLC for Higher Analysis Throughput

A typical HPLC assay was transferred and optimized for a Waters ACQUITY UPLC™ system to achieve both higher sample analysis throughput and better assay sensitivity. Strategies to expedite future method transfers were compiled. Analysis of operation costs and sample throughput found UPLC cost advantageous over HPLC.

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Increasing demand for greater pharmaceutical analysis throughput prompted the testing of the Waters ACQUITY Ultra Performance LC (UPLC™). This system claims to provide faster analyses through the use of a novel separation material of very fine particle size (1.7 μm) and unique core chemistry (1–5).

To effect fast separations on this material, the column hardware and instrument have significant design modifications from typical HPLC. The UPLC operates at higher pressures (up to 15,000 psi.), injects samples into a smaller system dwell volume, and captures detector signals at high data rates for fast eluting peaks. A new needle design has been claimed to substantially reduce carryover which can aid in the lowering of limits of quantitation (LOQ).

In this work, an HPLC method for quality control (QC) was optimized for UPLC. Strategies to reduce total runtime, lower cost per assay, and promote instrument uptime were considered.

Method Development

The original 10-min HPLC QC assay was developed to quantify the content of a heterocyclic drug (Cpd A) in organic solvent extracts. An internal standard (IS) was used to compensate for sample preparation losses and a terminal washing gradient was necessary to remove late eluting interferences.

Initial transfer of the HPLC assay to UPLC was accomplished by simply applying a scaling factor to the mobile phase flow rate and the sample injection volume. This scaling factor was derived from the ratio of the column cross sectional areas in order to retain the mobile phase linear velocity.

Chromatograms from this UPLC method had very narrow peaks, and the excessive resolution indicated opportunity for method improvement. The mobile phase flow rate was increased until limited by column backpressure. However, subsequent column lifetime studies indicated that reducing total run time by increasing organic solvent content was more economical. A dramatic decrease in solvent consumption was also obtained. Chromatograms in Figure 1 compare the original HPLC method to those of the initial scaling and the final UPLC conditions. Parameters of the HPLC and final UPLC methods are listed in Table I.

Method Optimization Guidelines and Observations

During the course of optimizing the UPLC method, considerations to expedite future method transfers were developed, and the following recommendations were made:

- Increase elution solvent strength to reduce run times taking advantage of the high resolution potential of UPLC columns (see Table II).
- Increase mobile phase flow rate secondarily to solvent strength in order to promote longer column lifetimes. While high mobile phase linear velocities with good resolution are possible (Figure 2), as with any column, routine operation at 80% maximum rated pressure led to shortened lifetimes. In our experience, UPLC operation around 8000 psi or less provided comparable or lower column cost per assay than HPLC. Maintaining low flows as much as possible also reduces solvent and waste disposal costs, although these are already an order of

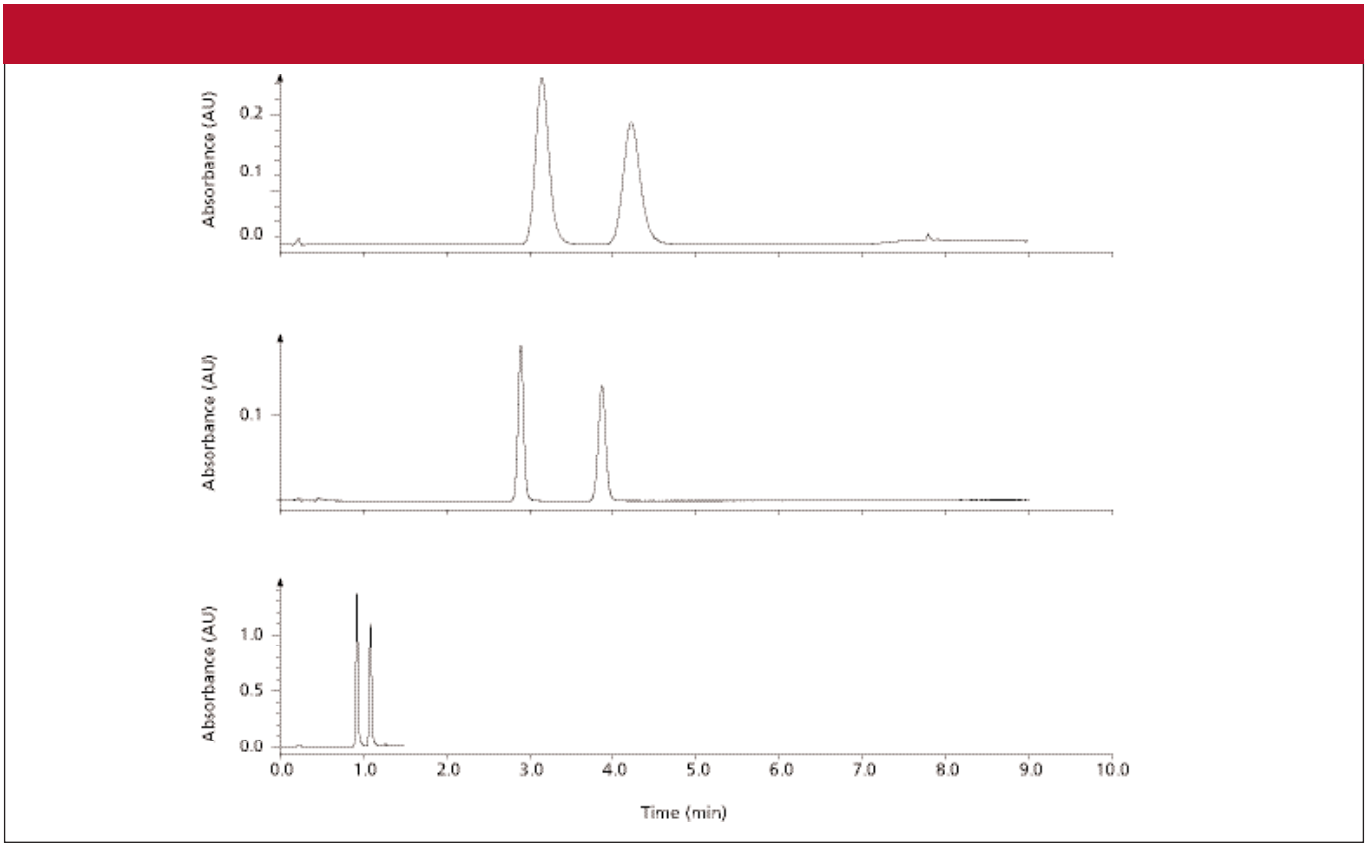

Figure 1: Chromatograms (from top to bottom): original HPLC, initial scaling to UPLC showing peak shape improvement and possibility for further method optimization, and final UPLC method. Order of peak elution: internal standard (IS) then Cpd A.

magnitude less than HPLC.

- Reduce column re-equilibration times by taking advantage of the low system dwell volume. Programmed changes in the mobile phase take time to reach the column. The small UPLC dwell volume (measured as 110 µL, 15% of that of the HPLC) allowed in part the abbreviation of the original assay. Column re-equilibration was accomplished during next sample loading in the UPLC, further increasing throughput.

Table I: Original HPLC versus optimized UPLC assay parameters

	HPLC Assay	UPLC Assay
Column	XTerra C18, 50 × 4.6 mm, 4 µm particles	ACQUITY UPLC BEH C18, , 50 × 2.1mm, 1.7 µm particles
Flow Rate	3.0 mL/min	0.6 mL/min
Needle Wash	Methanol	Strong Needle Wash: 200 µL Methanol; Weak Needle Wash: 600 µL ACN:H_2O 10:90
Injection Volume	20 µL	3 µL partial loop fill or 5 µL full loop fill with automatic overfill
Gradient (time in min) (ACN:H_2O)	T0 (25:75), T6.5 (25:75), T7.5(95:5), T9 (25:75), T10 (25:75)	T0 (36:64), T1.1 (95:05), T1.3 (36:64)
Flow Rate	3.0 mL/min	0.6 mL/min
Total Run Time	10 min	1.5 min
Total Solvent Consumption (including 0.5 min of delay time in between injections)	Acetontrile:10.5 mL Water: 21.0 mL	Acetonitrile: 0.53 mL Water: 0.66 mL
Plate Count for Cpd A	2000	7500
USP Resolution	3.2	3.4
LOQ	~0.2 µg/mL	0.054 µg/mL
Carry-over	< 0.05% with needle wash	0.01%
Delay Volume	~720 µL	~110 µL

- Reduce injection volumes appropriately for the column diameter to achieve good peak shapes. Peak splitting can occur when too large of a strong sample solvent bolus overwhelms the packing at the column head. While this assay method tolerated 5 µL injections, volumes of 1–3 µL are more typical starting points in our experience. Note that smaller injection volumes may be compensated by enhanced peak height from use of the high resolution columns and by the low carryover from the UPLC injector (measured as 10% of the HPLC carryover for this analyte) to achieve an equivalent or even lower LOQ). An alternative to smaller injection volumes might be to lower sample solvent strength to accomplish sample focusing on the head of the column.
- Utilize partial loop-fill injections in preference to full loop-fill. Partial loop-fill precision was good even at volumes up to 80% of the loop total volume (Figure 3). Typical laboratory practice is to limit sample volume injections to roughly 50% of the total loop volume. The UPLC injection system, which utilizes air-gap sandwiching of the sample, allows better utilization of the sample loop and

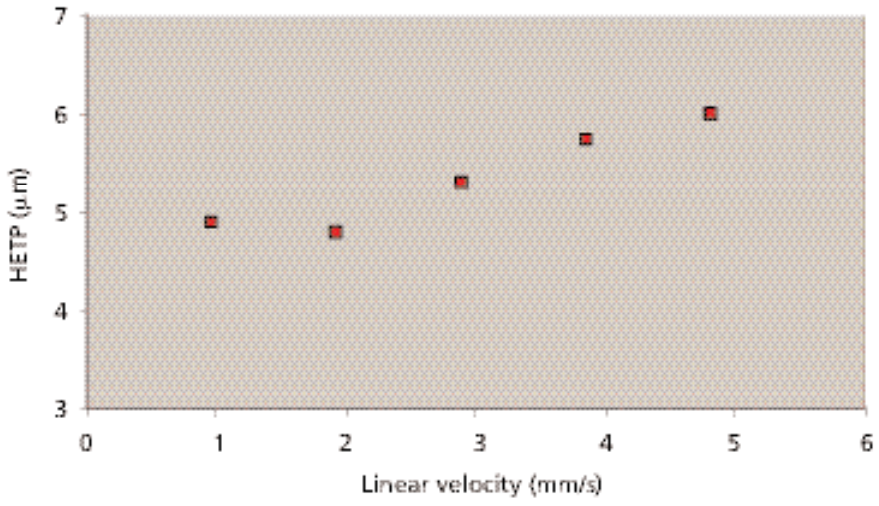

Figure 2: A van Deemter plot derived from UPLC experimental data indicates that use of high flow rates is a plausible strategy to decreasing overall runtime. This should be balanced with backpressure effects on overall column lifetime (see text).

Table II: Adjusting mobile phase parameters utilizing resolution potential of UPLC

	HPLC Original	UPLC Initial Scaling	UPLC Final
Flow Rate (mL/min)	3.0	0.63	0.60
% ACN in Mobile Phase	25	25	36
Plate Count for Cpd A	2000	9100	7500
USP Resolution Between A and IS	3.2	6.7	3.4

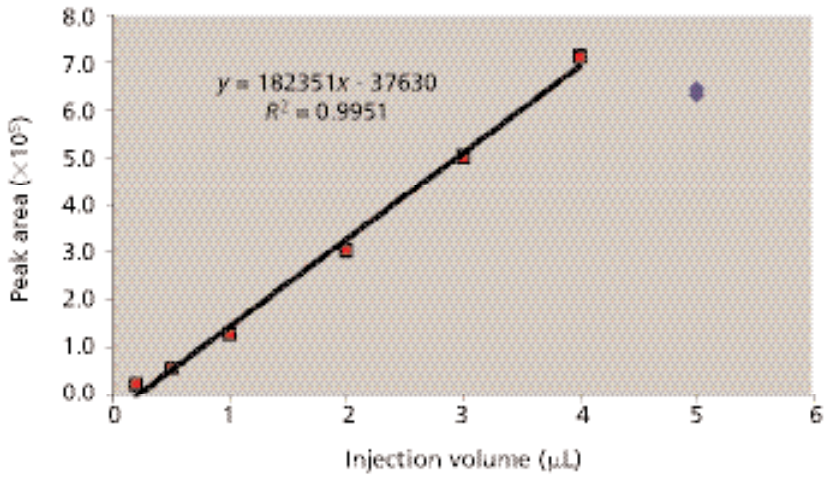

Figure 3: Peak area data generated by partial loop-fill mode of a 5 µL nominal (4.8 µL actual) sample loop in the UPLC injector. For standard loop injectors, the deviation from linear injection volume, as seen above in the 5-µL injection, occurs at much lower loop utilization so the general rule is to only load 40–50% of the loop capacity.

higher injection precision, reducing the need for use of the full loop-fill mode. From a practical point of view, full loop fill requires substantially greater sample movement considering overfill functions. This likely increases subsequent needle washing, which may impact sample throughput and increase wear of the washing hardware. Larger sample volume transfers also increases exposure to sample particulates, lowering long-term instrument reliability.

- If full loop-fill mode is utilized, perhaps for very high precision requirements, ensure adequate loop overfilling. A significant laminar flow velocity differential in the loading sample between its wall interface and center is created in the very narrow bore tubing of the UPLC injector. Overfilling the sample loop by at least four loop volumes was found necessary to fully displace wash solvent from the 5 µL injector loop. For this instrument, the manufacturer has determined and set as the default the optimum overfill volume with typical sample solvents for each sample loop size. Operators can specify other overfill volumes for unusual sample compositions.
- Choose the proper composition and volume of weak sample wash to obtain good peak shape. A portion of the weak sample wash solvent will be co-injected with partial-loop filled samples. The weak solvent wash should therefore mimic the initial conditions mobile phase in solvent

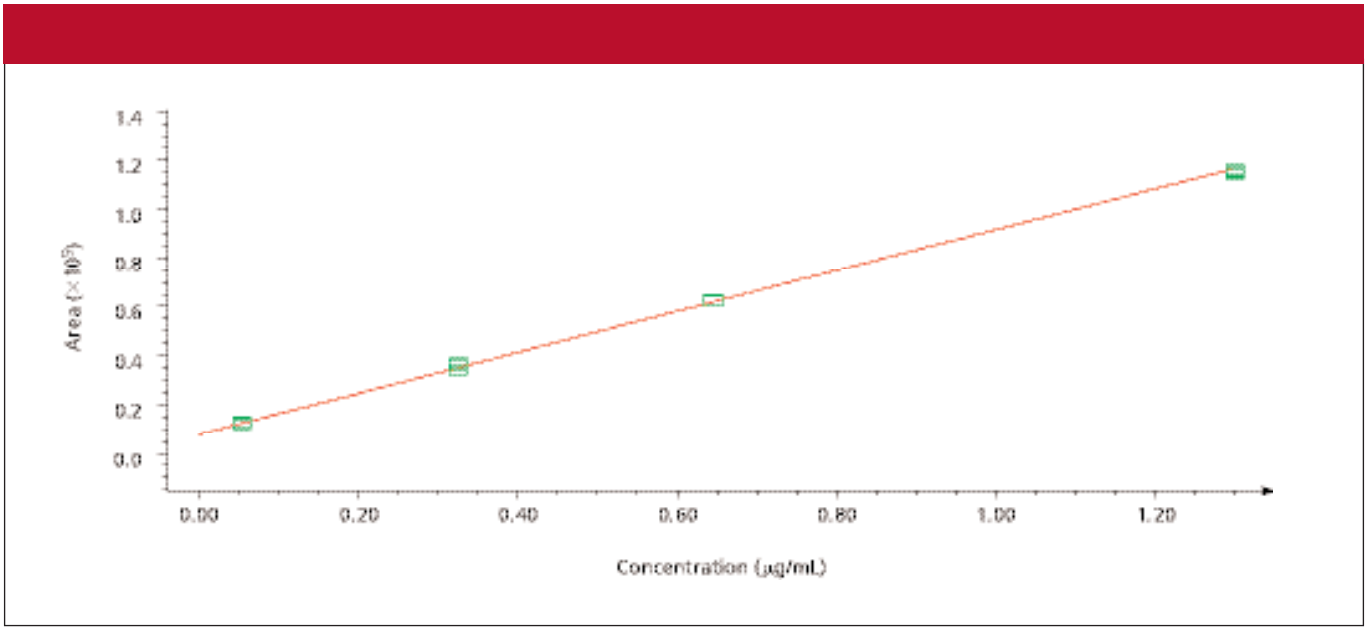

Figure 4: Linear correlation between concentration and peak area at lower concentrations from 0.054 to 1.30 µg/mL (R2 = 0.996 with 1/X2 weighting).

Table III: Evaluation of precision and accuracy for low range calibration

Theoretical Conc. (µg/mL)	Peak Area	Precision[1] (% RSD)/Result	Calculated Conc. (µg/mL)	Accuracy[2] (% Deviation)
0.054	11834	4.5	0.0484	−10.3
	12127		0.0519	−3.8
	12897		0.0611	13.2
0.325	35647	3.3	0.332	2.3
	34344		0.316	−2.5
	36703		0.345	6.2
0.645	62518	0.3	0.652	1.2
	62173		0.648	0.6
	62264		0.649	0.8
1.3	115988	0.1	1.290	−0.7
	114863		1.277	−1.8
	115826		1.288	−0.9

[1] Acceptance criterion: < 5.0%, all passed.
[2] Acceptance criteria: ±5.0%, except lowest concentration ±15.0%, all passed.

Table IV: Evaluation of precision and accuracy for low range calibration

Theoretical Conc. (µg/mL)	Peak Area	Precision[1] (% RSD)/Result	Calculated Conc. (µg/mL)	Accuracy[2] (% Deviation)
0.645	62518	0.3	0.646	0.3
	62173		0.642	−0.4
	62264		0.643	0.2
1.30	115988	0.1	1.308	0.7
	114863		1.294	−0.4
	115826		1.306	0.5
5.18	428428	0.1	5.174	−0.1
	428756		5.178	−0.03
	429553		5.188	0.2
25.8	2094015	0.2	25.78	−0.07
	2088395		25.71	−0.3
	2097868		25.83	0.1

[1] Acceptance criterion: < 5.0%, all passed.
[2] Acceptance criteria: ±5.0%, all passed.

strength. Utilizing the weak wash solvent as a sample diluent in the sample loop may enhance sample focusing onto the column. The volume of the weak wash must be sufficient to purge the former strong wash solvent from the loop.

Preliminary Method Validation

Preliminary assessment was made of the new assay and the instrument for linearity and linear range, precision, accuracy, system suitability, and sample carry over.

Linearity and Lower Limit of Quantification (LLOQ)

With the potential greater sensitivity of UPLC, the scope of the assay application was broadened to address samples which could differ in concentration by 500-fold. The same UPLC separation method was calibrated and found acceptably linear for two assay ranges (Figures 4 and 5). With an LLOQ of 54 ng/ml, the low range UPLC assay allowed analyses more typically addressed by liquid chromatography–mass spectrometry. Notably, this particular UPLC system is configured with a photodiode array detector. Use of a wavelength-specific detector could provide an even lower limit of quantification.

Precision and Accuracy

Triplicate injections were made at specified concentrations to assess precision (repeatability) and accuracy. Precision was evaluated

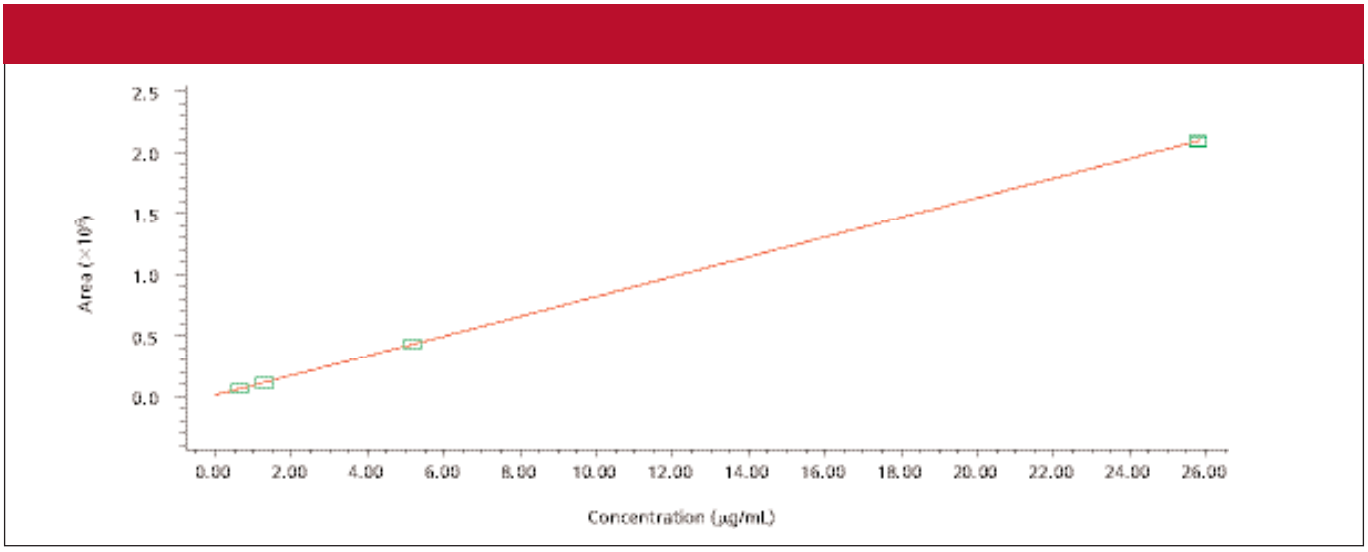

Figure 5: Linear correlation between concentration and peak area at higher concentrations from 0.325 to 25.8 µg/mL (R2 = 0.999967 with 1/X2 weighting).

by the peak area relative standard deviation (RSD). Accuracy was assessed by back-calculation of the injection peak areas using the calibration curve to give the calculated concentration for each injection. These values were compared to the theoretical value and reported in terms of % deviation from the theoretical value. The results for both low and high range assays passed acceptance criteria (Tables III and IV).

System Suitability

Five replicate injections were made to evaluate system suitability. The results passed all the common *USP* acceptance criteria (Table V).

Table V: Evaluation of system suitability[1]

Injection	Peak Area	Plate Count	USP Resolution
1	115988	7490	3.4
2	114864	7650	3.4
3	115827	7510	3.4
4	115896	7520	3.4
5	115104	7530	3.4
Average	115536	7540	3.4
%RSD	0.4	0.8	0.4
Acceptance Criteria/Result	%RSD < 2.0% /Pass	Plate Count > 2000/Pass	Resolution > 2.0/Pass

[1] Replicate injections of 1.30 µg/mL standard.

Injection–to-Injection Sample Carry-Over

Contamination of a sample injection by residues of the previous sample in the instrument (carry-over) can set the boundary for an assay's LLOQ. Carry-over frequently leads to failure of tests for precision, accuracy, and system suitability. However, depending on the protocol details of these studies, significant carry-over effects may not be revealed. Direct measurement of carry-over was performed here to anticipate inaccuracies arising in potentially mixed sets of concentrated and dilute samples.

The UPLC instrument had design features to reduce sample carry-over: a novel needle-in-needle injector design as well as two separate injector wash solvents. In this assay, 200 µL methanol were used as the first wash to remove the bulk of organic residues, followed by 600 µL water:ACN (90:10) to displace the strong solvent and bring the remaining sample loop, needle, and valve solutions to a composition compatible with initial method conditions.

Carry-over was evaluated here by analyzing a solvent blank sample after each of the calibration standards and measuring the area of any peak appearing at the analyte retention times. No interference peak was detected in the blanks run after the five lower concentration standards. For blanks run after injections of the highest concentration standard, faint peaks slightly above noise were measured at 0.01% of the analyte peak in the previous injection. This was acceptable for this assay, although carry-over may have been reduced further by optimizing the wash solvent parameters. In comparison, carry-over on the HPLC system was 5 to 10 fold higher.

Summary

A QC HPLC assay to quantitate a heterocyclic pharmaceutical in organic solvent extracts has been successfully transferred and optimized for UPLC. Preliminary assessment indicates that the assay can be validated. Guidelines to expedite the development of future UPLC assays were compiled. The application of UPLC will be cost advantageous. While UPLC column expense per analysis will be comparable to or slightly less than HPLC, solvent consumption and waste disposal charges should decrease better than an order of magnitude. Reduction of assay time by five-fold dramatically improves instrument return on investment and reduces the total number of instruments needed if only HPLC were employed.

References

(1) A.D. Jerkovich, J.S. Mellors, and J.W. Jorgenson, *LCGC* **21**(7), 660–611 (2003).

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

(3) K. K. Unger, D. Kumar, M. Grun, G. Buchel, S. Ludtke, Th. Adam, K. Scumacher, and S. Renker, *J. Chromatogr., A* **892**(47) (2000).

(4) M. E. Swartz and B. Murphy, *Lab Plus Int.*, **18**(6) (2004).

(5) M. E. Swartz and B. Murphy, *Pharm. Formulation Quality* **6**(5), p. 40 (2004). ■