

ENHANCED SPEED OF GRADIENT CAPILLARY HPLC ANALYSIS OF COMPOUNDS OF PHARMACEUTICAL INTEREST BY ULTRAFAST STATIONARY/MOBILE PHASE RE-EQUILIBRATION

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Abstract

While conventional bore and microbore LC analyses have made great strides in shortening chromatographic analysis times through the use of shorter columns, innovative stationary phases, and higher flow rates, most of the published applications have been isocratic. Using isocratic procedures for increased analysis speed avoids the introduction of a gradient that requires mobile phase re-equilibration between runs. In rapid gradient HPLC, the lengthy re-equilibration often results in a chromatographic separation that is much shorter than the stationary/mobile phase re-equilibration. The result yields only a modest improvement in total analysis time. The studies presented here will demonstrate that high-speed, high-precision gradient capillary LC data can be generated on samples of pharmaceutical interest by severely truncating the gradient re-equilibration time such that the chromatographic run time is essentially equal to the total run time. Chromatographic run time, total run time, and precision data on actual pharmaceutical samples will be presented.

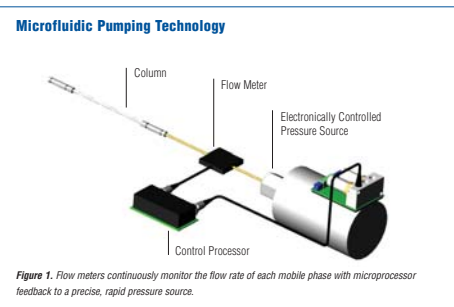
Experimental

Instrument: Eksigent ExpressLC™-100 Single-channel binary gradient HPLC System
Column: Zorbax SB-C18, 5 μ particle, 35 x 0.3 mm
Column Temperature: Ambient
Mobile Phase: linear gradients, A=5% aqueous acetonitrile; B=95% acetonitrile/5% water; both A and B containing 0.1% TFA
Flow Rate: 6–10 μ L/minute
Injection Volume: 80–120 nL
Detection Wavelength: 220–260 nm
Programmed Gradient Re-Equilibration Time: 0 minutes, unless otherwise noted

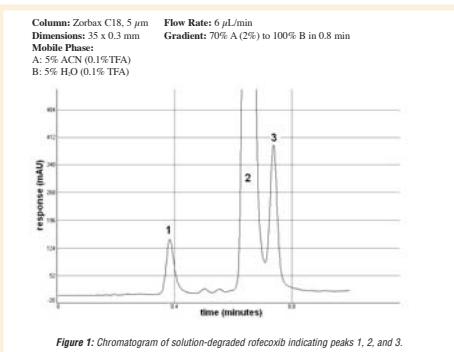
Results

The Sensitivity of Rapid HPLC Gradient Methods to Initial Conditions

While it is well known that the retention times of peaks in conventional high performance liquid chromatography (HPLC) are reasonably constant when the initial gradient composition is varied slightly, the effect in rapid gradient HPLC has not been well characterized. To investigate the effect of later-retained species as a function of initial gradient composition in rapid HPLC, formulated anti-inflammatory COX-2 inhibitor rofecoxib (Vioxx) was subjected to aqueous solution degradation over the period of four weeks. Using this sample (Figure 1), the initial gradient conditions for the separation (see the Experimental section for details) were varied from 68–72% mobile phase A. The gradient proceeded from these initial points to 100% mobile phase B in 0.8 minutes. The data appears in Table 1.



The ExpressLC system utilizes a novel pumping technology called Microfluidic Flow Control (MFC). MFC consists of an electronically controlled pressure source, an in-line flow meter, and a control processor. The flow meters continuously measure the actual flow rate of each mobile phase. This flow rate information is passed to an embedded processor that controls a rapidly responding, electronically adjusted variable pressure source. MFC provides extremely rapid, precise gradient control.



Initial Gradient Value	RT, Peak 1 (min)	RT, Peak 2 (min)	RT, Peak 3 (min)
72% A	0.430	0.689	0.773
	0.428	0.688	0.772
	0.428	0.687	0.771
	X=0.428, RSD=0.27%	X=0.688, RSD=0.15%	X=0.772, RSD=0.13%
71% A	0.419	0.677	0.761
	0.416	0.676	0.760
	0.415	0.675	0.760
	X=0.417, RSD=0.50%	X=0.676, RSD=0.15%	X=0.760, RSD=0.08%
70% A	0.402	0.662	0.745
	0.403	0.663	0.747
	0.401	0.662	0.746
	X=0.402, RSD=0.25%	X=0.662, RSD=0.09%	X=0.746, RSD=0.13%
69% A	0.393	0.652	0.739
	0.392	0.652	0.738
	0.391	0.652	0.737
	X=0.392, RSD=0.26	X=0.652, RSD=0.0%	X=0.738, RSD=0.14%
68% A	0.379	0.639	0.724
	0.382	0.640	0.725
	0.379	0.638	0.724
	X=0.380, RSD=0.46%	X=0.639, RSD=0.16%	X=0.724, RSD=0.08%

Table 1: Effect of initial gradient composition on retention times of 3 peaks in the separation of a room temperature solution-degraded sample of rofecoxib.

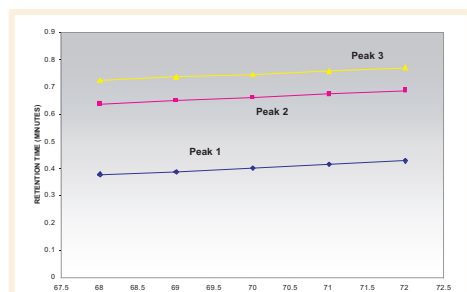


Figure 2: Effect of initial gradient composition on retention times of three peaks in the separation of a room temperature solution-degraded sample of rofecoxib.

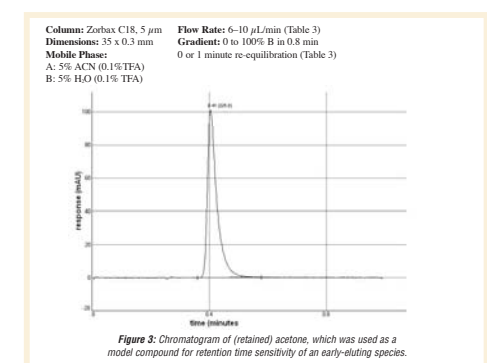
Determination of the Gradient Re-equilibration Time for a High Speed Capillary System

Even with an optimized pumping and mixing system, the data from the previous section suggest that the re-equilibration time necessary to reach precise initial composition might be so long as to negate the obvious gain in actual separation time. As described in the Introduction, the pumping system used for these studies uses a microfluidic flow controller to closely regulate gradient composition and allow the system to reach rapid stationary phase/mobile phase equilibrium. To investigate empirical re-equilibration times on this system, two studies were performed. In the first, acetone (Figure 3, Table 3) was used to measure how long the system required for chromatographic (as opposed to compositional) re-equilibrium. For the second study, a degraded drug formulation was chosen and a calibration of the retention times of four peaks (Figure 4, Table 4) versus initial mobile phase composition was tabulated. In both studies re-equilibration times of 1 minute and 0 minutes were compared. The chromatographic re-equilibration time under the given conditions, for both studies, from 100% mobile phase B to 0% mobile phase A, was determined to be zero; no programmed re-equilibration time was required.

Due to the very precise retention time data, a meaningful correlation between the retention times of peaks 1–3 with initial gradient composition could be demonstrated. The retention time versus initial percent of A mobile phase is plotted in Figure 2. The results are surprising. Even an initial gradient compositional variation of 0.25–0.5% may be expected to generate variation not only in the retention times of early-retained species, but in all species. The clear implication is that for rapid gradient chromatography, establishment of reproducible initial conditions is required for precise analysis. In a study in which the initial mobile phase gradient composition was subjected to a slight perturbation by increasing the B concentration by 3% for 3 seconds and then returning to the standard gradient, the effect on peak retention times was still evident (Table 2), even on the last peak eluted. The change in retention times of the peaks in the gradient-perturbed separation was as would be predicted: the peaks eluted earlier than in the non-perturbed case. The one-sigma variation for the retention times was approximately 0.001 minutes in all cases.

Initial Gradient Value	RT, Peak 1 (min)	RT, Peak 2 (min)	RT, Peak 3 (min)
70% A	0.402	0.662	0.745
	0.403	0.663	0.747
	0.401	0.662	0.746
	X=0.402, RSD=0.25%	X=0.662, RSD=0.09%	X=0.746, RSD=0.13%
3 second disturbance followed by above	0.384	0.653	0.738
gradient	0.382	0.651	0.736
	X=0.383, RSD=0.30%	X=0.652, RSD=0.15%	X=0.737, RSD=0.16%

Table 2: Comparison of retention times for three species in a degraded rofecoxib solution with and without a 3 second, 3% initial perturbation of the gradient.



Injection	6 μ L/min Re-equilibration time = 1 minute	6 μ L/min Re-equilibration time = 0 minutes	10 μ L/min Re-equilibration time = 1 minute	10 μ L/min Re-equilibration time = 0 minutes
1	0.690	0.684	0.406	0.407
2	0.683	0.687	0.407	0.406
3	0.685	0.685	0.408	0.406
	X=686, RSD=0.53	X=685, RSD=0.22	X=407, RSD=0.25	X=407, RSD=0.20

Table 3: Determination of programmed re-equilibration from 100% B to 100% A mobile phase by comparison of the retention time of acetone with and without a re-equilibration time. A calibration table of the retention time of acetone versus initial %A was performed prior to the study above.

Initial %B	Re-equilibration time (min)	RT, Peak 1 (min)	RT, Peak 2 (min)	RT, Peak 3 (min)	RT, Peak 4 (min)
Calibration					
5	1	0.451	0.504	0.547	0.596
2	1	0.461	0.522	0.568	0.610
0	1	0.473	0.527	0.573	0.614
Sample					
0	1	0.491	0.532	0.578	0.617
0	0	0.491	0.532	0.578	0.617

Table 4: Retention time of four species in a degraded drug formulation as a function of initial B% and re-equilibration time demonstrating that no programmed re-equilibration time was required for accurate and consistent retention time data.

Quantitative Precision Data for Retention Times and Peak Areas for a High-Speed Analysis of Degraded Alprazolam

The data presented would suggest that high-speed capillary HPLC separations can be performed with the ExpressLC system with very precise retention times and, under at least some chromatographic conditions, with no programmed re-equilibration time. Another important question is the quality of quantitative concentration data that can be generated under such conditions. To address this issue, solution-degraded alprazolam was injected nine times and the resulting quantitative data for both the retention times and the peak areas appears in Table 5. Peak 1 was generated by degradation of the parent alprazolam species (peak 2). The gradient method incorporated no programmed re-equilibration time. Both retention time and peak area for this high speed separation exhibit repeatability comparable to conventional HPLC.

Injection	RT, Peak 1 (min)	Peak 1 Area (mAU)	RT, Peak 2 (min)	Peak 2 Area (mAU)
1	0.439	357.99	0.525	1703.07
2	0.439	350.34	0.525	1742.52
3	0.438	352.60	0.525	1700.28
4	0.440	350.82	0.525	1721.14
5	0.439	348.79	0.525	1706.07
6	0.440	349.30	0.526	1761.71
7	0.440	349.41	0.525	1725.78
8	0.440	353.92	0.527	1723.11
9	0.442	350.64	0.527	1716.26
	X=440, RSD=0.25%	X=352, RSD=0.85%	X=525, RSD=0.17%	X=1722, RSD=1.1%

Table 5: Repeatability data for alprazolam and a major solution degradation product (peaks 2 and 1, respectively, in Figure 5) no re-equilibration time between gradient runs.

Conclusion

- Rapid gradient HPLC requires exceedingly reproducible initial mobile phase compositions to yield consistent retention time data.
- Chromatographic conditions using the high speed ExpressLC were found that did not require long gradient re-equilibration times; in some cases, the analyses required no programmed re-equilibration time, even for re-equilibration from 100% mobile phase B to 100% A.
- High-speed capillary analysis (in the case cited, a total run time of 36 seconds) of pharmaceutical compounds can be performed with the precision of conventional HPLC analysis.

