

Scaling Reverse-Phase and Hydrophilic Interaction Liquid Chromatography (HILIC) Methods to HPLC

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INTRODUCTION

Achieving identical chromatographic separation and equivalent results are critical when scaling and migrating methods between different liquid chromatography (LC) instruments.

In this work, two different methods developed on 1.7 µm particle size columns using a low dispersion system were scaled to HPLC. In first example, a reverse-phase method for naphazoline hydrochloride, pheniramine maleate and associated related substances was scaled from 1.7 µm to a 2.5 µm particle column. A second method for the analysis of ribavirin and related compounds, operated under HILIC conditions, was scaled from 1.7 µm to 2.5 and 3.5 µm particle columns and run on different HPLC platforms. The scaled methods generated equivalent chromatographic performance and assay results as the original method.

METHODS

EXPERIMENTAL

Reverse-phase method

Standard solutions

Individual stock solutions were prepared in methanol at 4.0 mg/mL. Stocks were diluted with 80:20 water/methanol diluent to make a mixture standard solution with 0.1 mg/mL of APIs and related substances at 10 µg/mL.

Compound	Name	Formula
Pheniramine maleate	PHEN API	C ₂₀ H ₂₃ N ₃ O ₄
2-benzylpyridine	PHEN Imp. A	C ₁₂ H ₁₁ N
4-benzylpyridine	PHEN Imp. B	C ₁₂ H ₁₁ N
Naphazoline HCl	NAPH API	C ₁₄ H ₁₅ ClN ₂
N-(2-Aminoethyl)-2-(naphthalen-1-yl) acetamide	NAPH Imp. A	C ₁₇ H ₁₉ N ₃ O
1-naphthylacetic acid	NAPH Imp. B	C ₁₂ H ₁₀ O ₂
1-Naphthylacetoneitrile	NAPH Imp. C	C ₁₀ H ₇ CH ₃ CN
β-naphazoline	NAPH Imp. D	C ₁₂ H ₁₁ N ₂

Table 1. Compounds for reverse-method scaling.

Ophthalmic solutions

Ophthalmic sample solutions were diluted with 80:20 water/methanol diluent to the working concentrations of 500 µg/mL pheniramine maleate and 40 µg/mL naphazoline HCl.

Parameter	Conditions for 1.7 µm column	Scaled conditions to 2.5 µm column																																																																						
Systems	ACQUITY™ UPLC™ H-Class Plus System, ACQUITY PDA Detector, active pre-heating	ACQUITY Arc™ System, 2998 PDA, passive pre-heater																																																																						
Column	ACQUITY UPLC CSH™ C ₁₈ Column, 2.1 x 100 mm, 1.7 µm (P/N: 186005297)	XSELECT™ CSH C ₁₈ Column, 4.6 x 150 mm, 2.5 µm (P/N: 186006729)																																																																						
L/dp	58,824	60,000																																																																						
Flow rate	0.3 mL/min	1.2 mL/min																																																																						
Gradient	<table><tr><th>Step</th><th>Time</th><th>%A</th><th>%B</th><th>Curve</th></tr><tr><td>1</td><td>Initial</td><td>90.0</td><td>10.0</td><td>6</td></tr><tr><td>2</td><td>0.10</td><td>90.0</td><td>10.0</td><td>6</td></tr><tr><td>3</td><td>8.00</td><td>10.0</td><td>90.0</td><td>6</td></tr><tr><td>4</td><td>8.50</td><td>10.0</td><td>90.0</td><td>6</td></tr><tr><td>5</td><td>8.60</td><td>90.0</td><td>10.0</td><td>6</td></tr><tr><td>6</td><td>13.00</td><td>90.0</td><td>10.0</td><td>6</td></tr></table>	Step	Time	%A	%B	Curve	1	Initial	90.0	10.0	6	2	0.10	90.0	10.0	6	3	8.00	10.0	90.0	6	4	8.50	10.0	90.0	6	5	8.60	90.0	10.0	6	6	13.00	90.0	10.0	6	<table><tr><th>Step</th><th>Time</th><th>%A</th><th>%B</th><th>Curve</th></tr><tr><td>1</td><td>Initial</td><td>90.0</td><td>10.0</td><td>6</td></tr><tr><td>2</td><td>0.18</td><td>90.0</td><td>10.0</td><td>6</td></tr><tr><td>3</td><td>14.40</td><td>10.0</td><td>90.0</td><td>6</td></tr><tr><td>4</td><td>15.30</td><td>10.0</td><td>90.0</td><td>6</td></tr><tr><td>5</td><td>15.50</td><td>90.0</td><td>10.0</td><td>6</td></tr><tr><td>6</td><td>21.00</td><td>90.0</td><td>10.0</td><td>6</td></tr></table>	Step	Time	%A	%B	Curve	1	Initial	90.0	10.0	6	2	0.18	90.0	10.0	6	3	14.40	10.0	90.0	6	4	15.30	10.0	90.0	6	5	15.50	90.0	10.0	6	6	21.00	90.0	10.0	6
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Gradient start 1.2 min after injection																																																																								
Inj. volume	1.0 µL	7.2 µL																																																																						

Table 2. Operating conditions for reverse-phase method scaling.

HILIC method

Standard solutions

Individual stock solutions were prepared in 50:50 acetonitrile/water at 1.0 mg/mL. Stock solutions were diluted with 90:10 acetonitrile/water diluent to make a mixture standard solution containing ribavirin API at 0.1 mg/mL and related compounds at 10 µg/mL.

Compound	Name	Formula
Ribavirin API	Ribavirin	C ₈ H ₁₂ N ₄ O ₅
Ribavirin related compound A	RC-A	C ₈ H ₁₁ N ₃ O ₅
Ribavirin related compound D	RC-D	C ₃ H ₄ N ₄ O
Uridine	Uridine	C ₉ H ₁₂ N ₂ O ₆
Uralic	Uralic	C ₄ H ₈ N ₂ O ₂

Table 3. Compounds for HILIC method scaling.

Parameter	Conditions for 1.7 µm column	Scaled conditions for 2.5 µm column	Scaled conditions for 3.5 µm column																																																																																																																														
Systems	ACQUITY UPLC H-Class Plus System, ACQUITY PDA Detector, active pre-heating	ACQUITY Arc System, 2998 PDA, passive pre-heater	• Arc HPLC System, 2998 PDA, passive pre-heater • Alliance™ IS HPLC System with TUV Detector, passive pre-heating																																																																																																																														
Column	ACQUITY UPLC BEH™ Amide Column, 2.1 x 50 mm, 1.7 µm (P/N: 186004800)	XBridge™ BEH Amide Column, 3.0 x 75 mm, 2.5 µm (P/N: 186006094)	XBridge BEH Amide Column, 4.6 x 100 mm, 3.5 µm (P/N: 186004868)																																																																																																																														
L/dp	28,412	30,000	28,571																																																																																																																														
Flow rate	0.5 mL/min	1.0 mL/min	2.0 mL/min																																																																																																																														
Gradient	<table><tr><th>Step</th><th>Time</th><th>%A</th><th>%B</th><th>%C</th><th>Curve</th></tr><tr><td>1</td><td>Initial</td><td>90.0</td><td>5.0</td><td>5.0</td><td>Initial</td></tr><tr><td>2</td><td>1.50</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr><tr><td>3</td><td>3.00</td><td>30.0</td><td>65.0</td><td>5.0</td><td>6</td></tr><tr><td>4</td><td>4.00</td><td>30.0</td><td>65.0</td><td>5.0</td><td>6</td></tr><tr><td>5</td><td>4.10</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr><tr><td>6</td><td>7.00</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr></table>	Step	Time	%A	%B	%C	Curve	1	Initial	90.0	5.0	5.0	Initial	2	1.50	90.0	5.0	5.0	6	3	3.00	30.0	65.0	5.0	6	4	4.00	30.0	65.0	5.0	6	5	4.10	90.0	5.0	5.0	6	6	7.00	90.0	5.0	5.0	6	<table><tr><th>Step</th><th>Time</th><th>%A</th><th>%B</th><th>%C</th><th>Curve</th></tr><tr><td>1</td><td>Initial</td><td>90.0</td><td>5.0</td><td>5.0</td><td>Initial</td></tr><tr><td>2</td><td>2.30</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr><tr><td>3</td><td>4.60</td><td>30.0</td><td>65.0</td><td>5.0</td><td>6</td></tr><tr><td>4</td><td>6.10</td><td>30.0</td><td>65.0</td><td>5.0</td><td>6</td></tr><tr><td>5</td><td>6.20</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr><tr><td>6</td><td>9.50</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr></table>	Step	Time	%A	%B	%C	Curve	1	Initial	90.0	5.0	5.0	Initial	2	2.30	90.0	5.0	5.0	6	3	4.60	30.0	65.0	5.0	6	4	6.10	30.0	65.0	5.0	6	5	6.20	90.0	5.0	5.0	6	6	9.50	90.0	5.0	5.0	6	<table><tr><th>Step</th><th>Time</th><th>%A</th><th>%B</th><th>%C</th><th>Curve</th></tr><tr><td>1</td><td>Initial</td><td>90.0</td><td>5.0</td><td>5.0</td><td>Initial</td></tr><tr><td>2</td><td>3.60</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr><tr><td>3</td><td>7.20</td><td>30.0</td><td>65.0</td><td>5.0</td><td>6</td></tr><tr><td>4</td><td>9.60</td><td>30.0</td><td>65.0</td><td>5.0</td><td>6</td></tr><tr><td>5</td><td>9.70</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr><tr><td>6</td><td>14.00</td><td>90.0</td><td>5.0</td><td>5.0</td><td>6</td></tr></table>	Step	Time	%A	%B	%C	Curve	1	Initial	90.0	5.0	5.0	Initial	2	3.60	90.0	5.0	5.0	6	3	7.20	30.0	65.0	5.0	6	4	9.60	30.0	65.0	5.0	6	5	9.70	90.0	5.0	5.0	6	6	14.00	90.0	5.0	5.0	6
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Inj. volume	1.0 µL	3.1 µL	9.6 µL																																																																																																																														

Table 4. Operating conditions for HILIC method scaling.

RESULTS

Numerous factors must be considered when scaling and migrating methods across different LC systems.

Maintaining ratio of column length (L) to the particle size (dp) preserves the resolving power of the method.

HPLC systems generally have larger dwell volume and extra-column dispersion compared to systems used with sub-2 µm particles. These differences will impact chromatographic separation and must be considered when scaling and migrating methods¹.

In this work, the operating conditions for the target methods were calculated using a columns calculator². The Gradient SmartStart Technology was used to account for the differences in system dwell volumes.

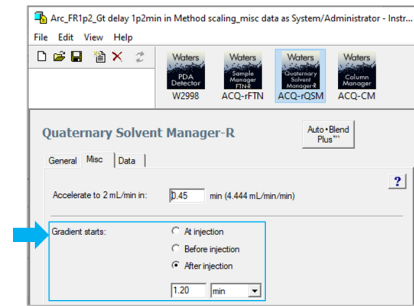


Figure 1. Gradient SmartStart Technology in the instrument method. Gradient started 1.2 minutes after the injection to compensate for larger system dwell volume of the ACQUITY Arc System compared to UPLC System.

Reverse-phase method scaling

A method for related substances of pheniramine maleate and naphazoline HCl APIs operated on a 1.7 µm particle column with an ACQUITY UPLC H-Class Plus System was scaled to 2.5 µm and run on an ACQUITY Arc System³.

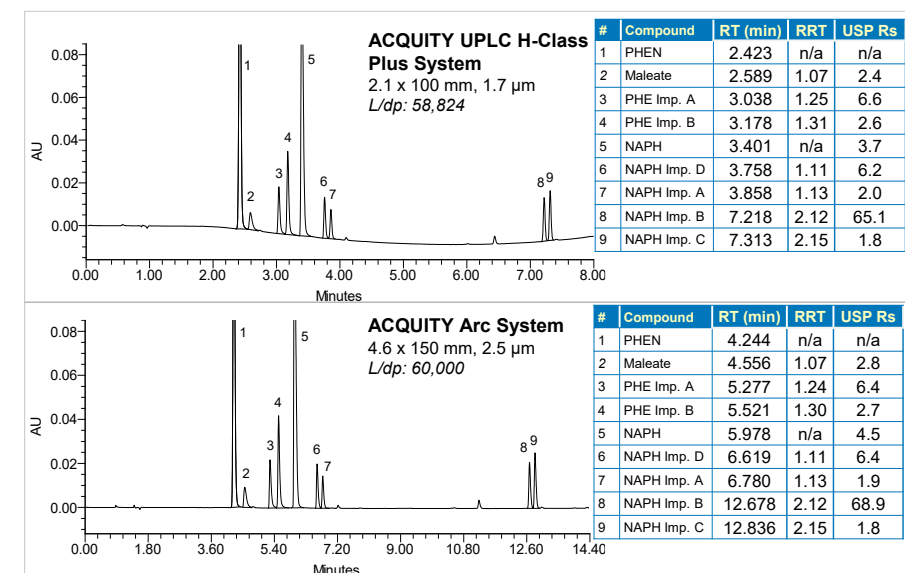


Figure 2. Reverse-phase method scaling from 1.7 to 2.5 µm particle size columns produced comparable separation on both systems. UV at 260 nm. RT: retention time, RRT: relative retention time, Rs: resolution between peaks.

The assay results for APIs and related substances content in the ophthalmic sample generated by the ACQUITY Arc System were equivalent to the data obtained on the ACQUITY UPLC H-Class Plus System (Figure 3).

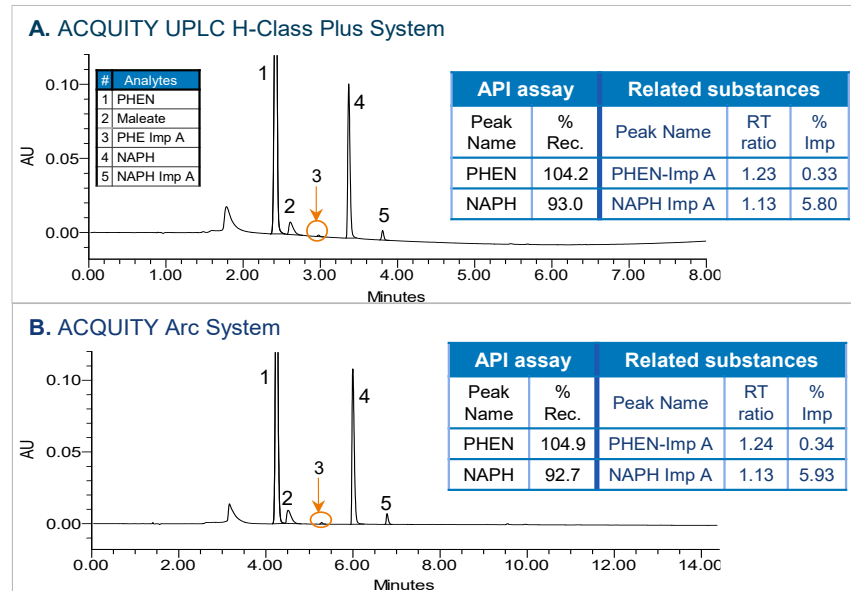


Figure 3. Analysis of ophthalmic sample with 500 µg/mL of pheniramine maleate and 40 µg/mL of naphazoline HCl acquired using 1.7 µm column with an ACQUITY UPLC H-Class Plus System (A) and 2.5 µm column with an ACQUITY Arc System (B) produced comparable assay results. UV at 280 nm.

HILIC method scaling

A HILIC method for ribavirin and related compounds was scaled from 1.7 µm to 2.5 and 3.5 µm particle columns⁴. The scaled methods were run on the ACQUITY Arc, Arc HPLC and Alliance iS HPLC Systems (Figure 4).

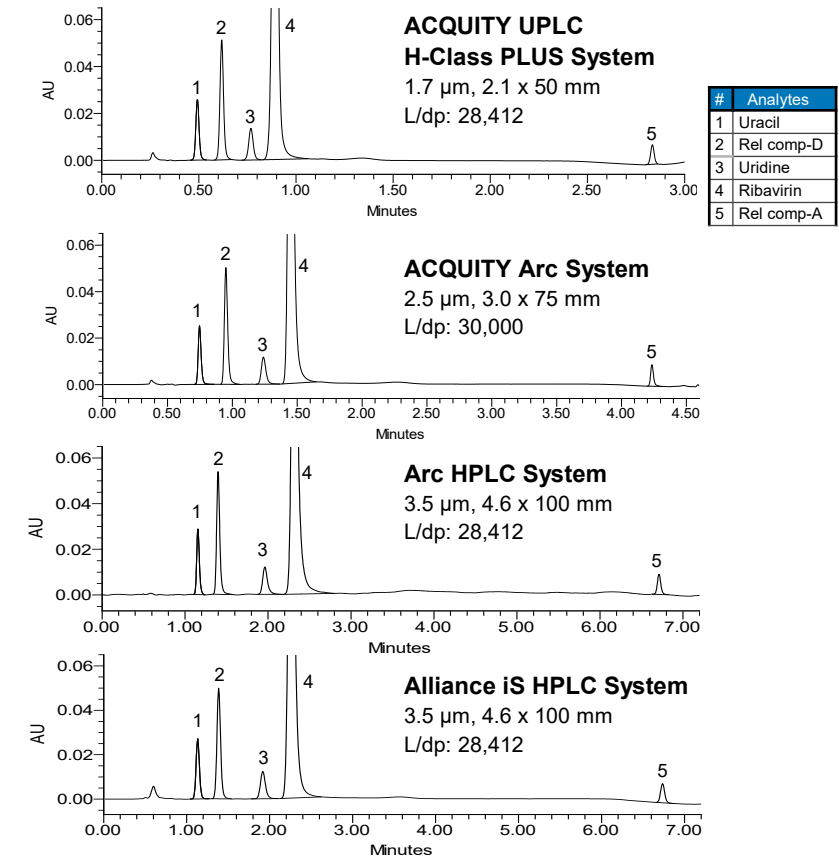


Figure 4. HILIC method scaling and migration between systems. Standard solution with ribavirin API at 0.1 mg/mL and related compounds at 10 µg/mL. UV at 220 nm.

The separation performance including the retention times, retentivity factor (k*), and USP Resolution (Rs) between peaks were comparable across all four systems (Table 5).

Compound	Retention Time (min)				Retentivity factor (k*)				USP Resolution (Rs)			
	UPLC H-Class System (1.7 µm)	Arc System (2.5 µm)	Arc HPLC System (3.5 µm)	Alliance iS HPLC System (3.5 µm)	UPLC H-Class System (1.7 µm)	Arc System (2.5 µm)	Arc HPLC System (3.5 µm)	Alliance iS HPLC System (3.5 µm)	UPLC H-Class System (1.7 µm)	Arc System (2.5 µm)	Arc HPLC System (3.5 µm)	Alliance iS HPLC System (3.5 µm)
Uracil	0.513	0.747	1.141	1.134	0.9	1.0	1.0	1.0	n/a	n/a	n/a	n/a
Rel comp-D	0.627	0.955	1.407	1.385	1.3	1.5	1.5	1.4	3.3	4.6	4.2	3.1
Uridine	0.802	1.242	1.921	1.919	2.0	2.3	2.4	2.4	4.4	5.2	6.2	5.5
Ribavirin	0.934	1.455	2.265	2.261	2.5	2.8	3.0	3.0	2.7	3.1	3.2	2.9
Rel comp-A	2.840	4.269	6.766	6.735	9.5	10.1	10.9	10.8	41.0	49.2	48.5	40.4

Table 5. Separation performance for HILIC method scaling from 1.7 µm to 2.5 and 3.5 µm particle size columns.

The assay results for related substances content generated by on the target systems were comparable to the results produced on the ACQUITY UPLC H-Class Plus System (Figures 5).

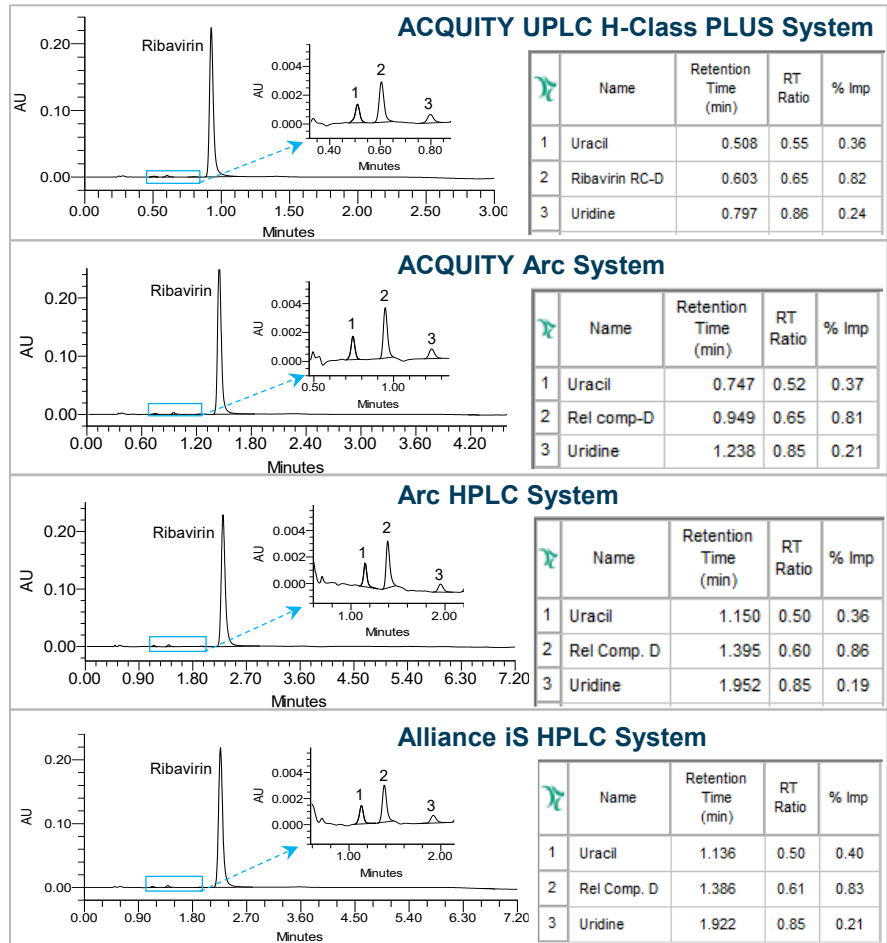


Figure 5. Assay for related compounds (% impurity) for HILIC method scaling. Ribavirin drug sample solution spiked with related compounds produced comparable results across systems. UV at 220 nm.

CONCLUSION

- Reverse-phase and HILIC methods were successfully scaled from 1.7 µm particle size columns to HPLC.
- The target LC systems including ACQUITY Arc, Arc HPLC and Alliance iS HPLC Systems generated equivalent performance as the original method run on an ACQUITY UPLC H-Class Plus System.
- Dwell and extra-column volumes were accounted for to ensure quality of the chromatographic data for method scaling and migration across different LC platforms.

References

- Hong H, McConville PR. Dwell Volume and Extra-Column Volume: What Are They and How Do They Impact Method Transfer?. Waters White Paper, 720005723En, 2018.
- Waters Columns Calculator. <https://www.waters.com/waters/support.htm?lid=134891632&type=DWNL>
- Maziarz M. Scaling and Migration of a Method for Naphazoline Hydrochloride, Pheniramine Maleate and Associated Related Substances to an ACQUITY™ Arc™ System. Waters Application Note 720008189, 2024.
- Maziarz M. Scaling and Migration of a Hydrophilic Interaction Liquid Chromatography (HILIC) Method for Related Compounds of Ribavirin to Modern HPLC Systems. Waters Application Note 720008255, 2024.

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