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Assessing the Impact of Increased Pre-Column System Volume on Peak Shape for High Organic Diluent Samples Using UHPLC

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APPLICATION BENEFITS

Ability to successfully run compendial methods on an ACQUITY™ UPLC™ H-Class System utilizing high percentage organic solvent as diluent without peak distortion.

WATERS SOLUTIONS

ACQUITY UPLC H-Class System coupled with a Photodiode Array Detector

Empower™ 3 Software

KEYWORDS

USP, compendial methods, UPLC, HPLC, Empower, regulatory, peak symmetry

INTRODUCTION

Ideally when running a chromatographic method, the sample diluent composition should be as close to the method starting conditions as possible. This is done in order to minimize the possibility of band spreading and peak distortion from sample solvent effects, which can lead to poor peak symmetry, peak splitting, or unusable data.

The cause for these effects is a difference in elutropic strength between the diluent and the mobile phase. Peak broadening and shape abnormalities generally get worse as the diluent becomes stronger than the mobile phase.[1-2]

In fact, it is widely recognized that the sample injected should ideally be dissolved in the starting mobile phase conditions. However the pre-treatment of a given sample often ends in the analytes dissolved in a solvent composition very different from that used in the mobile phase. In order to prevent solubility and poor peak shape problems, many protocols require evaporation of the sample solvent in pre-treatment and reconstitution in mobile phase. However, this added step is a time consuming process that often takes longer than the HPLC analysis.[1]

The practice widely recommended is to avoid stronger solvents than the mobile phase to dissolve samples and standards. The underlying assumption is that an injection solvent stronger than the mobile phase can interfere with the adsorption of the sample at the column head, especially when large injection volumes are used.[2] Unfortunately, in practice this is not always possible as sample solubility often dictates the amount of organic content needed to ensure complete dissolution.

With older, higher dispersion volume LC systems, this phenomenon is less problematic due to sufficient pre-column sample/solvent/mobile phase mixing which mitigated peak problems brought about by solvent effects.

However, for modern lower dispersion U(H)PLC systems, high organic diluents can be problematic when injected in larger volumes and can result in poor peak symmetry or splitting.

To understand this phenomenon and investigate the means for a simple solution to overcome the effect of strong solvents in the sample diluent, five United States Pharmacopeia (USP) methods were selected (i.e. acetaminophen, itraconazole, ketoconazole, loratidine, and bicalutamide) which require sample diluent organic levels ranging between 67%–100% organic.

All methods were conducted on the ACQUITY UPLC H-Class System with dispersion volumes calculated for every configuration used. These were combined with structured and iterative modifications to increase pre-column volume to assess the impact of additional pre-column volume on peak symmetry problems brought on as a result of high organic diluents.

EXPERIMENTAL

The methods were selected from the USP and the standards were analysed six times (n=6). The standards were prepared as per USP methods (Table 1).

The methods were run on an ACQUITY UPLC H-Class System with and without a 50 µL loop inserted between position six of the injector pod located in the sample manager and the column inlet tubing as shown in (Figure 1). Under both conditions, the chromatography was appraised visually, the mean of the peak asymmetry at 4.4% peak height and the % RSD of the peak area were compared as indicators of chromatographic performance in this study.

All methods with the exception of loratidine utilized the column manager (CM-A). Loratidine, required the CH30-A ACQUITY Column Heater to accommodate the 25 cm HPLC column detailed in the USP method.

Table 1. Diluent content of standards analyzed.

Compound	Diluent
Acetaminophen	100% methanol
Itraconazole	0.4% HCl in methanol
Haloperidol	100% methanol
Loratidine	100% methanol
Bicalutamide	67% methanol (0.01% TFA)
Ketoconazole	100% methanol

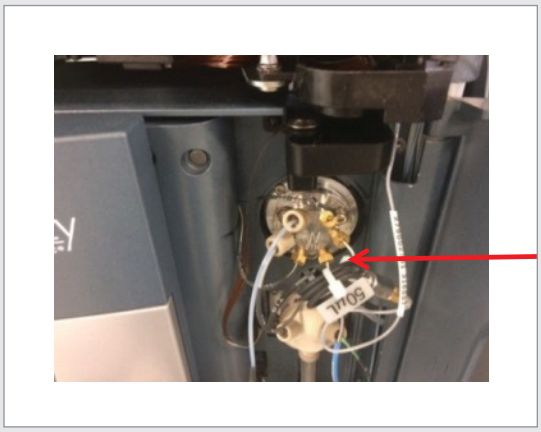


Figure 1. Additional 50 µL pre-column loop.

The materials used for analysis include (analytical standards in bold):

- **Ketoconazole: Sigma-Aldrich Lot SLBR1290V**
- **Itraconazole: EP reference standard Y0001100 Batch 2.0**
- **Bicalutamide: USP reference standard – Lot G01298**
- **Loratidine: Sigma-Aldrich – Lot LRAA9165**
- **Haloperidol: Sigma-Aldrich – Lot LRAA7399**
- **Acetaminophen: Sigma-Aldrich – Lot SLBM5923V**
- Water: ELGA Purelab
- Acetonitrile: Fisher Chemicals Optima LC-MS grade – Lot 1731013
- Methanol: Honeywell LC-MS Chromasolv – Lot SZBG246C
- Trifluoroacetic acid: Sigma-Aldrich – Lot 6942V
- Monobasic sodium phosphate: Sigma-Aldrich – Lot BCBV1183
- Dibasic sodium phosphate (anhydrous): Fisher Chemicals – Lot 164693
- Tetrabutylammonium hydrogen sulfate: ACROS Organics – Lot A0375G05
- Pentane sulphonic acid: ACROS Organics – Lot A0378319

Prior to analysis, the dispersion volume for all configurations were calculated using a blank union (P/N 700002636) under the following conditions (Table 2).

Table 2. Method for calculating extra-column volume in LC systems.

Parameter	Value
Sample	0.16 mg/L caffeine in 90:10 Water:ACN P/N 700002642, Solution7
Mobile phase A (MPA)	Water (30%)
Mobile phase B (MPB)	Acetonitrile (70%)
Flow rate	0.3 mL/min
Injection volume	1 µL
PDA λ (±1 NM)	273 NM

Extra column dispersion (µL) was calculated as the peak width (minutes) at 4.4% peak height (5σ) multiplied by the flow rate (µL per minute).

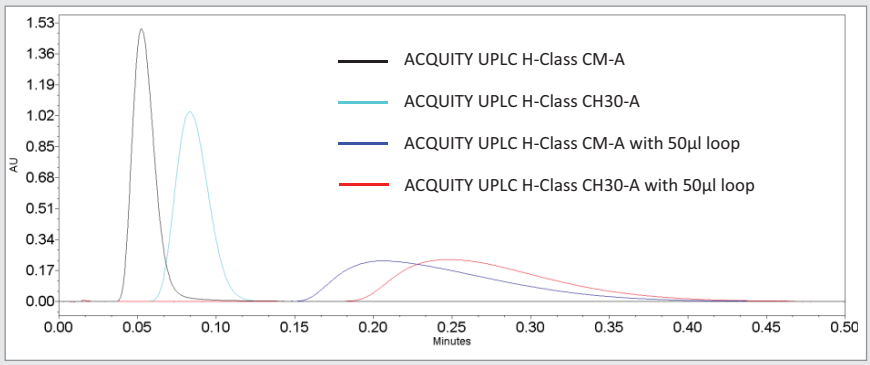


Figure 2. Overlay of caffeine standard using all configurations.

Table 3. Calculated peak width and dispersion for all configurations using caffeine standard.

Configuration	Peak width @4.4% Peak height (5σ)	Peak dispersion (Flow rate x peak width @ 4.4% Peak height – 5σ)
ACQUITY UPLC H-Class CM-A	0.033	9.9
ACQUITY UPLC H-Class CH30-A	0.048	14.4
ACQUITY UPLC H-Class CM-A with 50 µL loop	0.230	69.0
ACQUITY UPLC H-Class CH30-A with 50 µL loop	0.220	62.1

RESULTS AND DISCUSSION

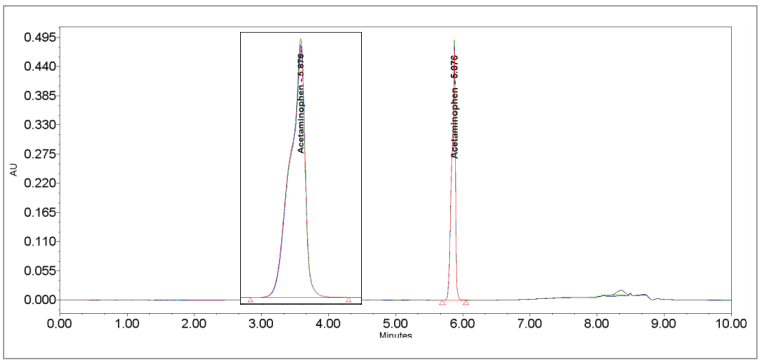
The inclusion of additional pre-column volume, i.e. a 50 µL loop on the ACQUITY UPLC H-Class System, has significantly improved the peak shape of acetaminophen (Figures 3–4/Tables 5–6), itraconazole (Figures 5–6/Tables 8–9), haloperidol (Figures 7–8/Tables 11–12) and loratidine (Figures 9–10/Tables 15–16). Peak symmetry improved noticeably for acetaminophen (0.2 to 1.0) and haloperidol (0.1 to 1.0). These compounds also exhibited an improvement in peak area % RSD (Table 22).

USP METHODS IMPROVED USING ADDITIONAL PRE-COLUMN VOLUME

Example 1: Acetaminophen (assay) – Diluent: 100% MeOH

Table 4. Acetaminophen USP method.

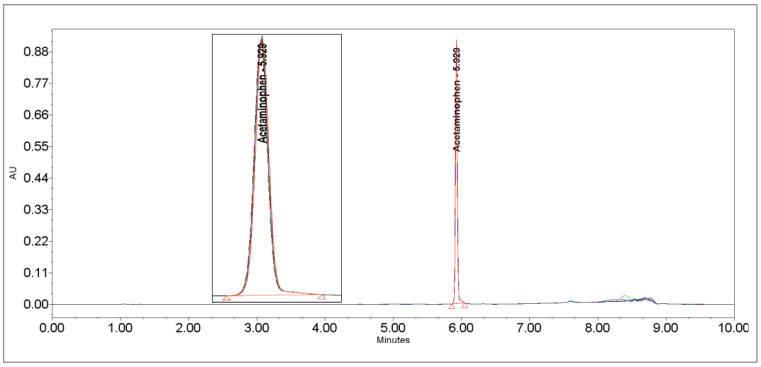
Parameter	Value		
Mobile phase A (MPA)	1.7 g/L monobasic sodium phosphate 1.8 g/L dibasic sodium phosphate, anhydrous		
Mobile phase B (MPB)	Methanol		
Flow rate	1.0 mL/min		
Injection volume	5 µL		
Gradient conditions	Time (min)	MPA	MPB
	0	99	1
	3	99	1
	7	19	81
	7.1	99	1
	10	99	1
Column	X-Bridge™ BEH C_8 100 x 4.6 mm, 3.5 µm, p/n 186003054		
Column temp.	35 °C		
PDA λ (± 1 nm)	230 nm		



Peak symmetry – no loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Acetaminophen	1	5.876	0.198	1785445
2	Acetaminophen	2	5.876	0.202	1794213
3	Acetaminophen	3	5.875	0.201	1791885
4	Acetaminophen	4	5.877	0.204	1764207
5	Acetaminophen	5	5.876	0.202	1787212
6	Acetaminophen	6	5.877	0.199	1768038
Mean				0.2	1781833.4
Std. dev.				0.0	12627.6
% RSD				1.0	0.7

Figure 3/Table 5. Acetaminophen USP method without additional pre-injection volume. Acetaminophen overlay (n=6) chromatogram and result table without loop fitted.



Peak symmetry – with loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Acetaminophen	1	5.929	1.050	1812525
2	Acetaminophen	2	5.927	1.047	1820560
3	Acetaminophen	3	5.927	1.050	1805403
4	Acetaminophen	4	5.927	1.049	1802224
5	Acetaminophen	5	5.927	1.050	1804972
6	Acetaminophen	6	5.926	1.047	1796794
Mean				1.0	1807079.9
Std. dev.				0.0	8342.7
% RSD				0.1	0.5

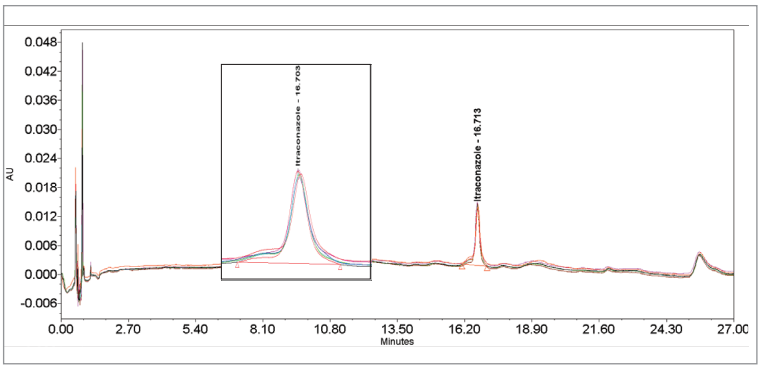
Figure 4/Table 6. Acetaminophen USP method with additional pre-injection volume. Acetaminophen overlay (n=6) chromatogram and result table with loop fitted.

EXAMPLE 2: ITRACONAZOLE

Itraconazole (imps) – Diluent: 0.4% HCl in MeOH

Table 7. Itraconazole USP method.

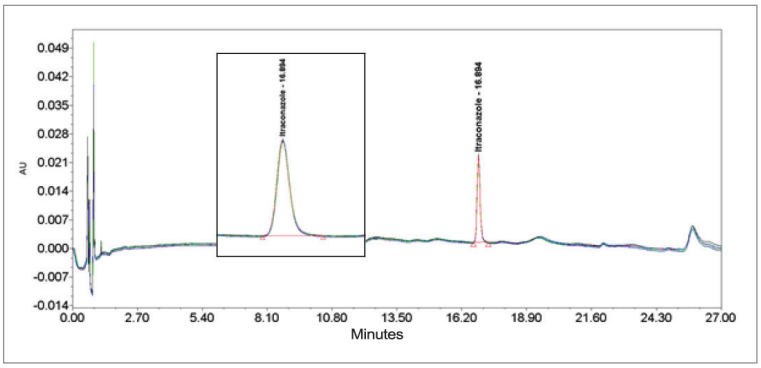
Parameter		Value	
Mobile phase A (MPA)		0.08 M Tetrabutyl ammonium hydrogen sulfate solution	
Mobile phase B (MPB)		Acetonitrile	
Flow rate		1.5 mL/min	
Injection volume		5 µL	
Gradient conditions	Time (min)	MPA	MPB
	0	80	20
	20	80	20
	25	50	50
	26	50	50
Column		Spherisorb ODS2 100 x 4.6 mm, 3 µm, p/n PSS832112	
Column temp.		30 °C	
PDA λ (± 1 nm)		225 nm	



Peak symmetry – no loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Itraconazole	1	16.730	0.381	168567
2	Itraconazole	2	16.713	0.432	170807
3	Itraconazole	3	16.716	0.386	162289
4	Itraconazole	4	16.712	0.505	165506
5	Itraconazole	5	16.708	0.675	168937
6	Itraconazole	6	16.703	0.507	172088
Mean				0.5	168032.4
Std. dev.				0.1	3595.0
% RSD				22.8	2.1

Figure 5/Table 8. Itraconazole USP method without additional pre-injection volume. Itraconazole overlay (n=6) chromatogram and result table without loop fitted.



Peak symmetry – with loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Itraconazole	1	16.984	2.028	193662
2	Itraconazole	2	16.984	2.232	198378
3	Itraconazole	3	16.984	2.237	196008
4	Itraconazole	4	16.988	2.051	196581
5	Itraconazole	5	16.989	1.938	193329
6	Itraconazole	6	16.984	2.008	195142
Mean				2.1	195516.5
Std. dev.				0.1	1893.4
% RSD				6.0	1.0

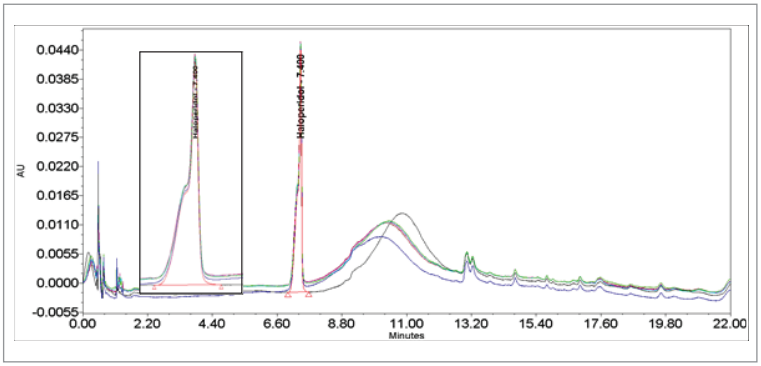
Figure 6/Table 9. Itraconazole USP method with additional pre-injection volume. Itraconazole overlay (n=6) chromatogram and result table with loop fitted.

EXAMPLE 3: HALOPERIDOL

Haloperidol USP method (imps) – Diluent 100% MeOH

Table 10. Haloperidol USP method.

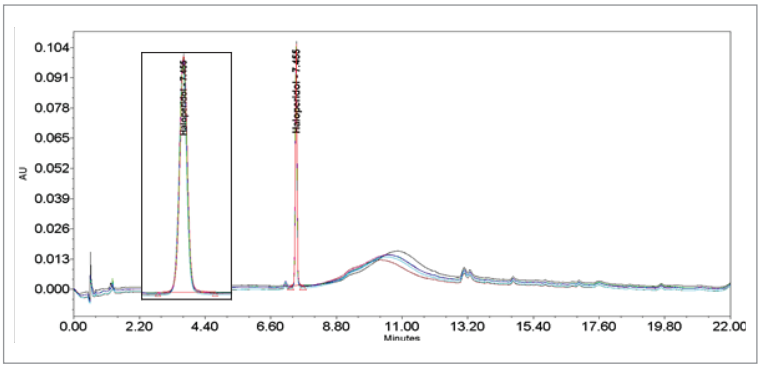
Parameter	Value		
Mobile phase A (MPA)	17 g/L pentane sulphonic acid (pH 3.0)		
Mobile phase B (MPB)	Acetonitrile		
Flow rate	1.2 mL/min		
Injection volume	10 µL		
Gradient conditions	Time (min)	MPA	MPB
	0	90	10
	2	90	10
	17	50	50
	22	50	50
Column	Spherisorb ODS2 100 x 4.6 mm, 3 µm, p/n PSS832112		
Column temp.	30 °C		
PDA λ (± 1 nm)	230 nm		



Peak symmetry – no loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Haloperidol	1	7.400	0.066	406034
2	Haloperidol	2	7.401	0.060	399908
3	Haloperidol	3	7.401	0.061	402802
4	Haloperidol	4	7.399	0.064	402121
5	Haloperidol	5	7.398	0.061	402155
6	Haloperidol	6	7.396	0.062	401866
Mean				0.1	402481.0
Std. dev.				0.0	1998.3
% RSD				3.9	0.5

Figure 7/Table 11. Haloperidol USP method without additional pre-injection volume. Ketoconazole overlay (n=6) chromatogram and result table with loop.



Peak symmetry – with loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Haloperidol	1	7.455	0.975	400839
2	Haloperidol	2	7.454	0.967	400511
3	Haloperidol	3	7.452	0.963	399617
4	Haloperidol	4	7.450	0.970	397566
5	Haloperidol	5	7.450	0.963	400581
6	Haloperidol	6	7.452	0.962	398898
Mean				1.0	399668.6
Std. dev.				0.0	1259.3
% RSD				0.6	0.3

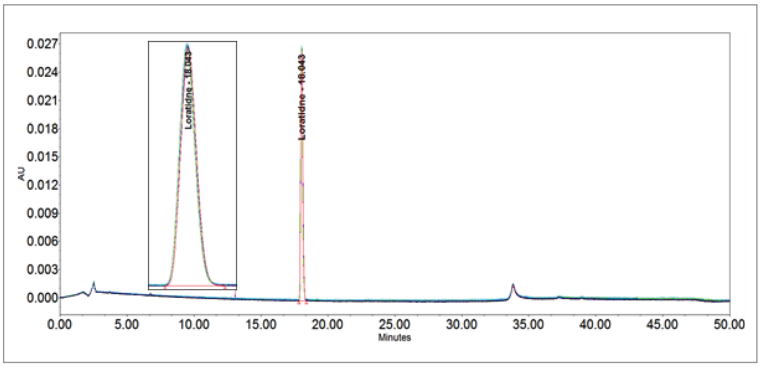
Figure 8/Table 12. Haloperidol USP method with additional pre-injection volume. Ketoconazole overlay (n=6) chromatogram and result table with loop.

EXAMPLE 4: LORATIDINE

Loratidine USP method (imps) – Diluent 100% MeOH

Table 13. Loratidine USP method.

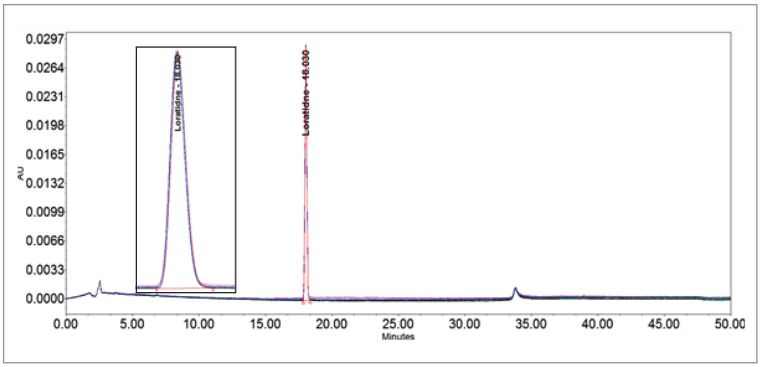
Parameter	Value		
Mobile phase A (MPA)	0.96 g/L pentane sulphonic acid (pH 3.0)		
Mobile phase B (MPB)	Acetonitrile		
Flow rate	1.2 mL/min		
Injection volume	20 µL		
Gradient conditions	Time (min)	MPA	MPB
	0	75	25
	20	50	50
	30	40	60
	35	30	70
	45	30	70
	50	75	25
Column	XBridge 25 cm x 4.6 mm, 5 µm, p/n 186003117		
Column temp.	30 °C		
PDA λ (± 1 nm)	254 nm		



Peak symmetry – no loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Loratidine	1	17.792	2.788	231395
2	Loratidine	2	17.792	2.864	229278
3	Loratidine	3	17.788	2.766	228530
4	Loratidine	4	17.787	2.769	227747
5	Loratidine	5	17.788	2.861	227574
6	Loratidine	6	17.781	2.851	226664
Mean				2.8	228531.3
Std. dev.				0.0	1660.0
% RSD				1.7	0.7

Figure 9/Table 14. Loratidine USP method without additional pre-injection volume. Loratidine overlay (n=6) chromatogram and result table without loop fitted.



Peak symmetry – with loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Loratidine	1	18.030	1.811	282785
2	Loratidine	2	18.025	1.803	279985
3	Loratidine	3	18.024	1.817	279396
4	Loratidine	4	18.019	1.834	279641
5	Loratidine	5	18.019	1.838	279970
6	Loratidine	6	18.022	1.786	279908
Mean				1.8	280280.6
Std. dev.				0.0	1247.9
% RSD				1.1	0.4

Figure 10/Table 15. Loratidine USP method with additional pre-injection volume. Loratidine overlay (n=6) chromatogram and result table with loop fitted.

Bicalutamide (Figures 11–12/Tables 17–18) showed no significant peak distortion without the loop and no significant impact (positive or negative) with the use of the additional pre-column volume. This may be due to the relatively low concentration of organic diluent (i.e. 67%).

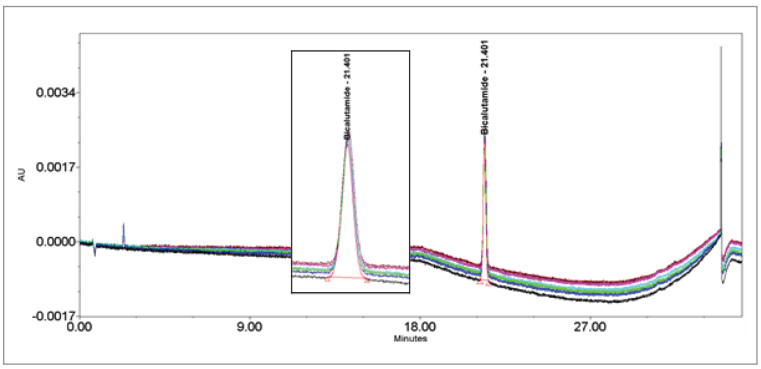
USP METHODS UNAFFECTED USING ADDITIONAL PRE-COLUMN VOLUME

EXAMPLE 5: BICALUTAMIDE

Bicalutamide USP method (imps) – Diluent 67% MeOH/0.01%TFA

Table 16. Loratidine USP method.

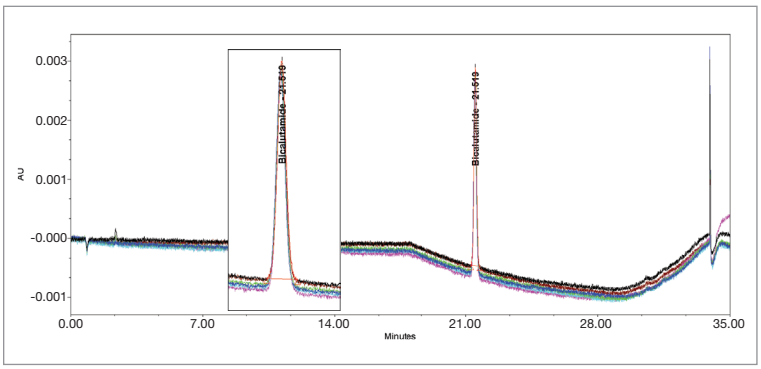
Parameter		Value	
Mobile phase A (MPA)		Water/0.01% TFA	
Mobile phase B (MPB)		Acetonitrile/0.01% TFA	
Flow rate		1.0 mL/min	
Injection volume		10 µL	
Gradient conditions	Time (min)	MPA	MPB
	0	75	25
	16.5	50	50
	26.5	40	60
	32.5	30	70
	32.6	30	70
	35	75	25
Column		Phenomenex ODS3 C_{18}, 100 x 4.0 mm, 3 µm, p/n 00D-4222-D0	
Column temp.		30 °C	
PDA λ (± 1 nm)		270 nm	



Peak symmetry – no loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Bicalutamide	1	21.401	0.947	31747
2	Bicalutamide	2	21.402	0.999	32346
3	Bicalutamide	3	21.419	1.039	32001
4	Bicalutamide	4	21.420	0.960	32552
5	Bicalutamide	5	21.425	0.824	31797
6	Bicalutamide	6	21.419	0.911	32027
Mean				0.9	32078.4
Std. dev.				0.1	314.2
% RSD				7.9	1.0

Figure 11/Table 17. Bicalutamide impurities USP method without additional pre-injection volume. Bicalutamide overlay (n=6) chromatogram and result table without loop fitted.



Peak symmetry – with loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Bicalutamide	1	21.490	1.160	31970
2	Bicalutamide	2	21.502	1.013	32503
3	Bicalutamide	3	21.499	1.065	32736
4	Bicalutamide	4	21.506	0.934	32287
5	Bicalutamide	5	21.506	1.084	32519
6	Bicalutamide	6	21.506	0.951	31920
Mean				1.0	32322.3
Std. dev.				0.1	325.5
% RSD				8.3	1.0

Figure 12/Table 18. Bicalutamide impurities USP method with additional pre-injection volume. Bicalutamide overlay (n=6) chromatogram and result table with loop fitted.

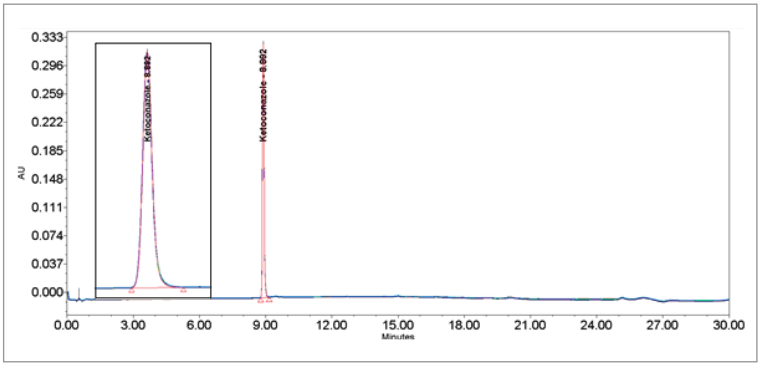
EXAMPLE 6: KETOCONAZOLE

Ketoconazole USP method (imps) – Diluent 100% MeOH

Table 19. Ketoconazole USP method.

Parameter		Value	
Mobile phase A (MPA)		3.4 mg/mL Tetrabutyl ammonium hydrogen sulphate solution: ACN (50:50)	
Mobile phase B (MPB)		3.4 mg/mL Tetrabutyl ammonium hydrogen sulphate solution: ACN (50:50)	
Flow rate		2.0 mL/min	
Injection volume		10 µL	
Gradient conditions	Time (min)	MPA	MPB
	0	100	0
	20	0	100
	25	0	100
	26	100	0
	30	100	0
Column		Spherisorb ODS2 100 x 4.6 mm, 3 µm, p/n PSS832112	
Column temp.		30 °C	
PDA λ (± 1 nm)		225 nm	

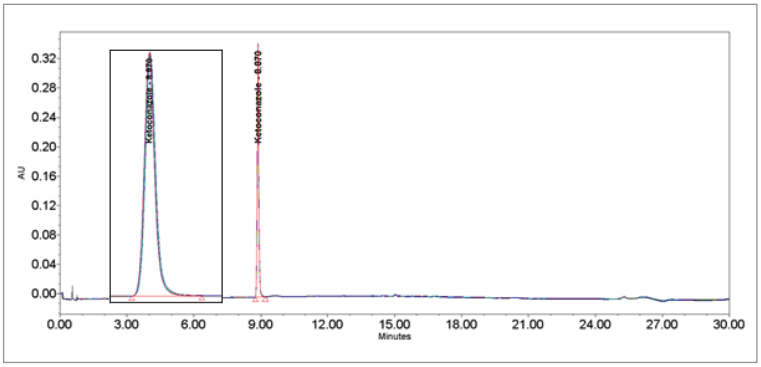
Ketoconazole (Figures 13–14/Tables 20–21) also showed no significant peak distortion without the loop and no significant impact (positive or negative) w ith the use of the additional pre-column volume despite having 100% methanol as diluent. This may be explained by the presence of organic component in both Mobile phase A and B, facilitating sufficient mixing prior to injection onto the column. Further work would be required to verify this theory.



Peak symmetry – no loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Ketoconazole	1	8.892	1.669	1596714
2	Ketoconazole	2	8.890	1.773	1611969
3	Ketoconazole	3	8.891	1.799	1608891
4	Ketoconazole	4	8.892	1.818	1607669
5	Ketoconazole	5	8.892	1.837	1609027
6	Ketoconazole	6	8.892	1.839	1609477
Mean				1.8	1607291.1
Std. dev.				0.1	5371.4
% RSD				3.6	0.3

Figure 13/Table 20. Ketoconazole USP method without additional pre-injection volume. Ketoconazole overlay (n=6) chromatogram and result table without loop fitted.



Peak symmetry – with loop

	Sample name	Inj.	RT	Asym @ (4.4)²	Area (µV* sec)
1	Ketoconazole	1	8.870	1.794	1586698
2	Ketoconazole	2	8.872	1.794	1571862
3	Ketoconazole	3	8.874	1.857	1581520
4	Ketoconazole	4	8.876	1.907	1588168
5	Ketoconazole	5	8.877	1.908	1605917
6	Ketoconazole	6	8.876	1.932	1608888
Mean				1.9	1590508.9
Std. dev.				0.1	14308.8
% RSD				3.2	0.9

Figure 14/Table 21. Ketoconazole USP method with additional pre-injection volume. Ketoconazole overlay (n=6) chromatogram and result table with loop.

All results are summarized in Table 22.

Table 22. Result summary of all compounds test with and without 50 µL increase in pre-column volume.

Compound	H-Class		H-Class (with 50 µL loop)	
	Mean asymmetry @ 4.4 (n=6)	Peak area %RSD (n=6)	Mean asymmetry @ 4.4 (n=6)	Peak area %RSD (n=6)
Acetaminophen	0.2	0.7	1.0	0.5
Itraconazole	0.5	2.1	2.1	1.0
Haloperidol	0.1	0.5	1.0	0.3
Loratidine	2.8	0.7	1.8	0.4
Bicalutamide	0.9	1.0	1.0	1.0
Ketoconazole	1.8	0.3	1.9	0.9

CONCLUSIONS

The addition of pre-column volume by utilizing a 50 µL loop has had a significant impact on the peak shape and peak area %RSD for acetaminophen, itraconazole, haloperidol, and loratidine indicating that it is a valid strategy for overcoming peak distortions when working with high organic diluent on a low dispersion LC system.

This solution would enable customers bound by compendial method parameters to overcome unsatisfactory/non-usable data with a simple fix that would be fully supported in a regulatory compliant laboratory environment.

References

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