

# UPLC™/MS/MS BIOANALYTICAL METHOD VALIDATION OF ACEBUTOLOL AND PINDOLOL USING AN ANALOGUE INTERNAL STANDARD

Ed Sprake and Iain Gibb  
Waters Corporation, Manchester, UK

## INTRODUCTION

Beta-blockers are a common class of drugs used to treat conditions such as high blood pressure, tachycardia and cardiac arrhythmia. In this application note, we show the partial validation of a bioanalytical method for Acebutolol and Pindolol in human plasma using Nadolol as an analogue internal standard (Figure 1). The validation was carried out according to the guidelines in the FDA Guidance for Industry on Bioanalytical Method Validation.

Through this experiment, we aim to show that the Waters® Ultra Performance LC™ System combined with the Waters Micromass® Quattro Premier™ XE Mass Spectrometer (UPLC™/MS/MS) operating in MRM mode is an accurate, precise, and robust technique which will also yield the benefits of greater speed, sensitivity and resolution over HPLC/MS/MS.

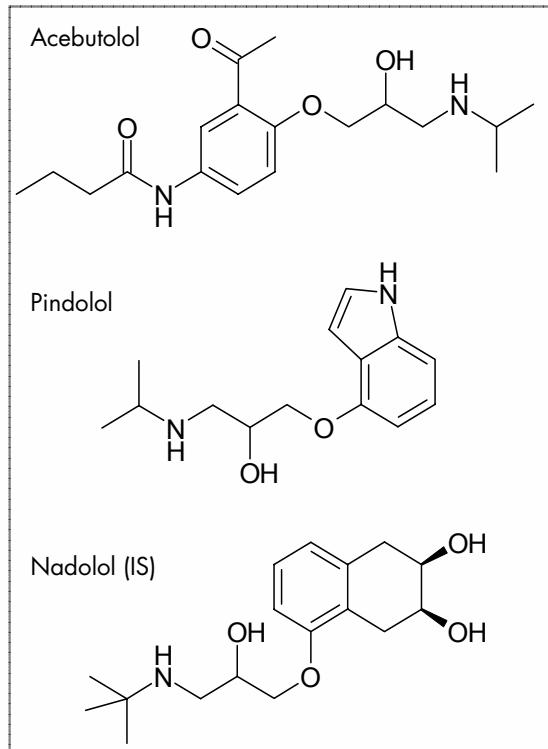


Figure 1. Chemical structures of acebutolol, pindolol, and nadolol.

## EXPERIMENTAL

During this experiment we performed a comparison between HPLC and UPLC using a protein precipitation (PPT) sample preparation method.

### Protein Precipitation Method

- 200 µL plasma was spiked with:
  - 50 µL IS (1.0 µg/mL in water)
  - 50 µL spike solution (from 0.8 ng/mL – 600 ng/mL in water)
  - When the IS and/or spike solution was not required, the appropriate volume of water was added
- 600 µL acetonitrile was added to crash proteins
- Centrifuged at 13,000 rpm for 5 minutes
- 200 µL of supernatant diluted with 800 µL water prior to injection

Spike Conc. (ng/mL)	Actual Conc. in Plasma (ng/mL)	Sample Type
0.8	0.2	Standard
2	0.5	
4	1	
20	5	
40	10	
200	50	
320	80	
400	100	
600	150	
0.8	0.2	
3	0.75	QC
80	20	
300	75	
360	90	
600	150	

Table 1. Spike concentrations and their equivalent concentrations in human plasma.

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Standard curves and QC samples were prepared as described and shown in Table 1. Three separately prepared validation batches were prepared by protein precipitation and run using UPLC/MS/MS. A standard curve prepared by protein precipitation in human plasma was run using HPLC/MS/MS for comparison.

A validation batch consisted of the following:

- 2 separately prepared calibration curves
- 6 individually prepared replicates of each QC concentration point
- A blank and double blank before each curve
- 2 carryover blanks after each curve

The HPLC, UPLC and MS conditions used are as follows:

## HPLC Conditions

LC System: Waters Alliance® HT System  
Column: XBridge™ C<sub>18</sub>  
Eluents:  
A: 2mM ammonium acetate  
+ 0.1% formic acid in water  
B: 0.1% formic acid in acetonitrile  
Column Temp: 40 °C  
Sample Temp: 4 °C  
Flow Rate: 0.3 mL/min  
Gradient: 

Time	%A	%B	Curve
0.0	85	15	–
1.6	5	95	8
2.0	85	15	11

  
Run Time: 3.2 min  
Injection Volume: 20 µL  
Pressure: 1800 psi

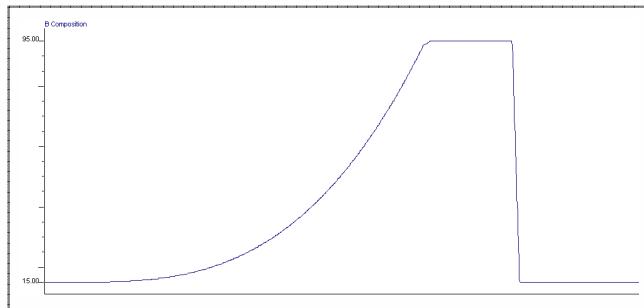


Figure 2. Curve 8 gradient profile.

## UPLC Conditions

LC System: ACQUITY UPLC system  
Column: ACQUITY UPLC BEH C<sub>18</sub>  
Eluents:  
A: 2 mM ammonium acetate  
+ 0.1% formic acid in water  
B: 0.1% formic acid in acetonitrile  
Column Temp: 40 °C  
Sample Temp: 4 °C  
Flow Rate: 0.6 mL/min  
Gradient: 

Time	%A	%B	Curve
0.0	85	15	–
0.8	5	95	8
1.0	85	15	11

  
Run Time: 1.6 min  
Injection Volume: 20 µL  
Pressure: 10500 psi

## MS Conditions

MS System: Quattro Premier XE tandem quadrupole mass spectrometer  
Ion Mode: Electrospray positive  
Capillary Voltage: 3.00 kV  
Source Temp: 120 °C  
Desolvation Temp: 380 °C  
Desolvation Gas: 1000 L/hour  
Cone Gas Flow: 50 L/hour  
Dwell Time: 0.02 seconds  
Inter-scan Delay: 0.01 seconds  
Collision Gas: Argon (3.45x10<sup>-3</sup> mbar)  
Detection Mode: MRM (see below)  

Compound	Transition	Cone Voltage (V)	Collision Energy (eV)
Acebutolol	337.25>116.00	35	22
Pindolol	249.15>116.00	35	18
Nadolol (IS)	310.30>201.20	25	20

The “Curve” setting in the above gradient tables refers to the gradient profile; adjusting the method to a non-linear curve setting can help separate close running peaks under some circumstances. A graphical representation of the gradient used for this analysis is shown in Figure 2.

## RESULTS AND DISCUSSION

All of the calibration standards run by UPLC/MS/MS generated calibration curves with a coefficient of calibration ( $R^2$ ) greater than 0.996. The HPLC/MS/MS run generated calibration curves where  $R^2$  was greater than 0.997. Typical examples of calibration curves for pindolol and acebutolol (using UPLC/MS/MS) are shown in Figure 3.

Inter-batch calibration statistics are shown in Tables 2 and 3. The statistics for the standard injections are based on 2 replicate injections of the 9 calibration points for each of the 3 inter-day batches. All calibration points show <8% CV with accuracy values between 93.6% – 103.7% for both pindolol and acebutolol.

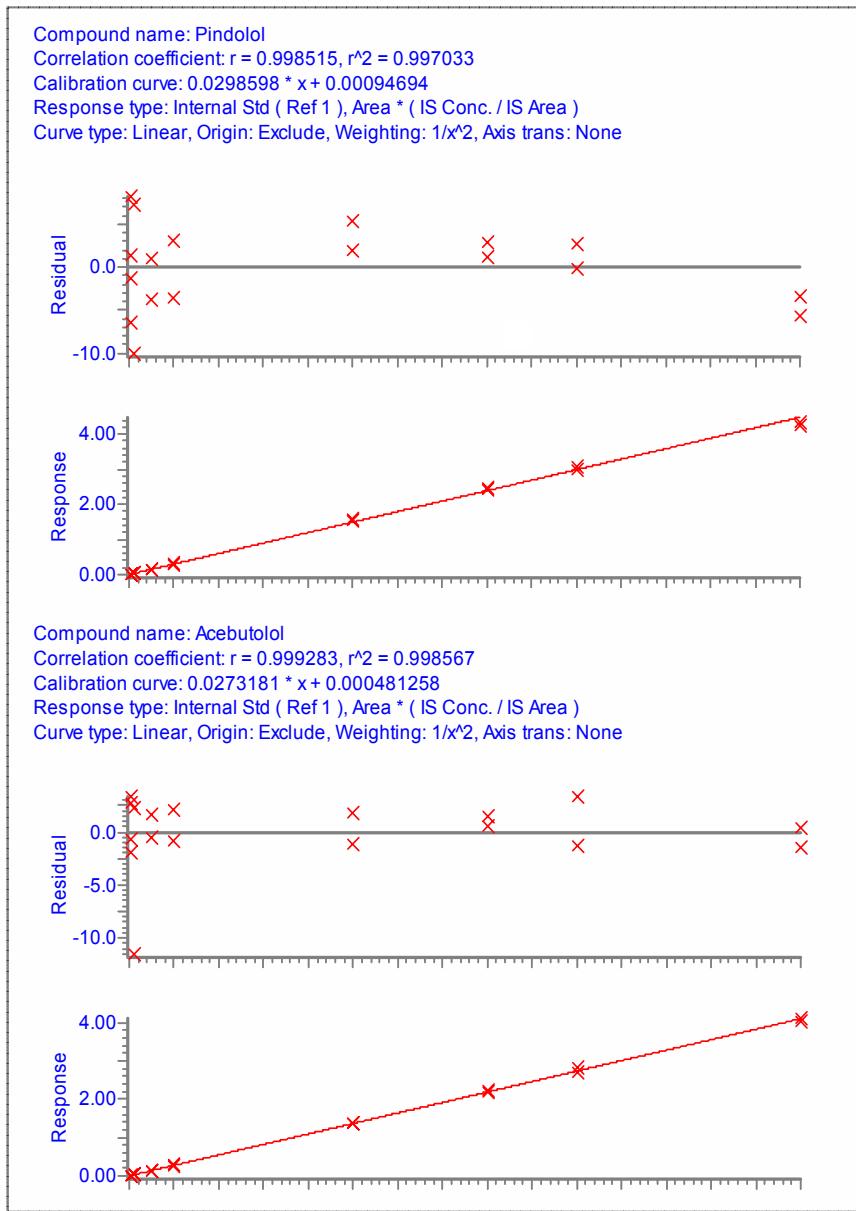


Figure 3. Typical calibration curves for pindolol and acebutolol in protein precipitated human plasma by UPLC/MS/MS.

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<b>Conc. Of Pindolol (ng/mL)</b>	<b>Batch A</b>	<b>Batch B</b>	<b>Batch C</b>	<b>Mean</b>	<b>SD</b>	<b>%CV</b>	<b>%Accuracy</b>
0.2	0.199 0.203	0.197 0.203	0.198 0.200	0.20	0.00	1.16	100.0
0.5	0.473 0.498	0.467 0.541	0.491 0.537	0.50	0.03	6.27	100.3
1	0.967 1.077	0.899 1.072	0.946 0.982	0.99	0.07	7.15	99.1
5	4.893 5.429	4.806 5.049	4.852 5.294	5.05	0.26	5.05	101.1
10	10.134 10.611	9.649 10.309	9.868 10.429	10.17	0.36	3.53	101.7
50	50.858 51.224	50.960 52.640	52.674 52.708	51.84	0.92	1.77	103.7
80	78.223 79.895	80.932 82.281	77.135 81.736	80.03	2.02	2.53	100.0
100	97.434 99.123	99.749 102.674	102.057 102.530	100.59	2.15	2.14	100.6
150	135.588 146.390	141.371 144.943	134.605 139.356	140.38	4.80	3.42	93.6
Gradient	0.031	0.030	0.031	0.031	0.001	2.68	N/A
Correlation	0.997	0.997	0.997				
Intercept	0.0001	0.0009	0.0006				

Table 2. Inter-batch statistics for pindolol - 9 calibration standard concentrations over 3 days by UPLC/MS/MS.

<b>Conc. Of Acebutolol (ng/mL)</b>	<b>Batch A</b>	<b>Batch B</b>	<b>Batch C</b>	<b>Mean</b>	<b>SD</b>	<b>%CV</b>	<b>%Accuracy</b>
0.2	0.189 0.200	0.196 0.206	0.200 0.214	0.20	0.01	4.21	100.4
0.5	0.524 0.545	0.496 0.516	0.469 0.476	0.50	0.03	5.82	100.9
1	0.935 1.071	0.885 1.022	0.885 0.971	0.96	0.08	7.81	96.1
5	4.687 4.939	4.971 5.085	5.144 5.233	5.01	0.19	3.83	100.2
10	9.642 9.783	9.912 10.211	10.028 10.144	9.95	0.22	2.18	99.5
50	51.553 52.211	49.417 50.877	52.168 53.900	51.69	1.50	2.90	103.4
80	81.351 82.154	80.481 81.233	77.581 82.070	80.81	1.70	2.10	101.0
100	100.269 100.689	98.684 103.338	103.105 106.025	102.02	2.64	2.59	102.0
150	139.407 147.382	147.795 150.527	140.968 141.673	144.63	4.51	3.12	96.4
Gradient	0.028	0.027	0.028	0.028	0.0006	2.14	N/A
Correlation	0.997	0.999	0.996				
Intercept	0.0003	0.00051	0.0002				

Table 3. Inter-batch statistics for acebutolol - 9 calibration standard concentrations over 3 days by UPLC/MS/MS.

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Statistics for the QC injections, shown in Tables 4 and 5, are based on single injections of 6 individually spiked QC solutions at each concentration, for each of the 3 inter-day batches. Both pindolol and acebutolol show <15% CV for the lower limit of quantitation (LLOQ) with <10% CV for the remainder of the quality control standards. Inter-batch accuracy values were observed between 93.2% – 111.99% for both pindolol and acebutolol.

FDA guidelines recommend that samples at the LLOQ should have less than 20% CV and deviation from the standard curve. All other unknowns, calibration standards, and QC standards should be within 15%, accuracy values should be within 80 – 120% at LLOQ, and 85 – 115% for other standards.

All of the results generated during the validation of this method comply with and exceed the guidelines set forth by the FDA.

		Intra-Batch			Inter-Batch
Conc. Of Pindolol (ng/mL)		Batch A n=6	Batch B n=6	Batch C n=6	n=18
0.2	Mean	0.21	0.20	0.19	0.20
	SD	0.03	0.01	0.03	0.02
	%CV	12.21	6.90	14.63	11.53
	%Accuracy	102.8	99.2	94.1	98.7
0.75	Mean	0.77	0.77	0.73	0.76
	SD	0.06	0.05	0.07	0.06
	%CV	7.36	6.25	9.52	7.82
	%Accuracy	102.3	103.3	97.0	100.9
20	Mean	19.6	20.6	19.8	20.0
	SD	0.80	0.85	0.81	0.88
	%CV	4.08	4.14	4.07	4.42
	%Accuracy	98.2	103.0	99.0	100.0
75	Mean	75.5	78.6	76.7	76.9
	SD	2.22	2.53	2.18	2.54
	%CV	2.94	3.22	2.85	3.30
	%Accuracy	100.6	104.8	102.3	102.6
90	Mean	85.1	88.4	86.6	86.6
	SD	1.64	2.75	3.37	2.80
	%CV	1.93	3.11	3.89	3.24
	%Accuracy	94.6	98.2	96.2	96.2
150	Mean	140.8	150.7	147.3	146.3
	SD	2.53	3.63	3.18	5.16
	%CV	1.80	2.41	2.16	3.53
	%Accuracy	93.9	107.7	101.0	97.5

Table 4. Intra- and inter-batch QC statistics for pindolol by UPLC/MS/MS.

		Intra-Batch			Inter-Batch
Conc. Of Acebutolol (ng/mL)		Batch A n=6	Batch B n=6	Batch C n=6	n=18
0.2	Mean	0.21	0.19	0.19	0.20
	SD	0.02	0.02	0.02	0.02
	%CV	9.02	9.84	10.60	10.98
	%Accuracy	105.9	93.2	94.5	97.9
0.75	Mean	0.76	0.75	0.76	0.76
	SD	0.05	0.05	0.06	0.05
	%CV	6.05	6.97	7.57	6.52
	%Accuracy	101.7	99.9	101.2	100.9
20	Mean	19.3	20.3	19.9	19.8
	SD	0.85	1.05	0.46	0.87
	%CV	4.42	5.19	2.30	4.41
	%Accuracy	96.5	101.3	99.6	99.1
75	Mean	76.8	80.2	78.1	78.3
	SD	2.66	3.33	2.59	3.06
	%CV	3.47	4.16	3.32	3.91
	%Accuracy	102.4	106.9	104.1	104.4
90	Mean	87.3	92.1	90.9	89.9
	SD	2.44	1.90	3.04	3.18
	%CV	2.80	2.07	3.35	3.53
	%Accuracy	97.0	102.3	101.0	99.9
150	Mean	148.54	154.47	154.67	152.56
	SD	5.95	3.91	4.59	5.45
	%CV	4.00	2.53	2.97	3.57
	%Accuracy	99.0	112.0	101.8	101.8

Table 5. Intra- and inter-batch QC statistics for acebutolol by UPLC/MS/MS.

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## HPLC vs. UPLC

In Figure 4, we can see that we get a 3.8-fold increase in signal-to-noise by using UPLC vs. HPLC methodology. As well as increases in signal-to-noise and limit of detection, there is also an increase in resolution, giving a better chance of separating the analyte from endogenous peaks. A 2-fold decrease in run time was also observed, meaning that a validation batch was run in only 2 hours by UPLC compared to 4 hours when run by HPLC. An example of both an HPLC and a UPLC chromatogram are shown below for comparison.

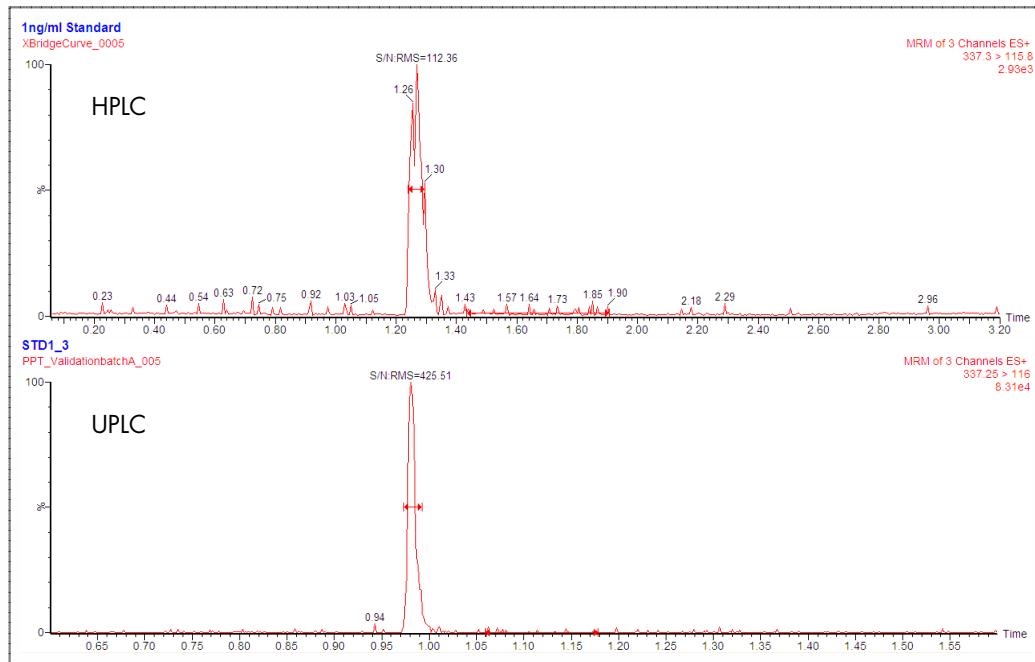


Figure 4. Signal-to-noise comparison using the 1 ng/mL calibration standard, HPLC vs. UPLC.

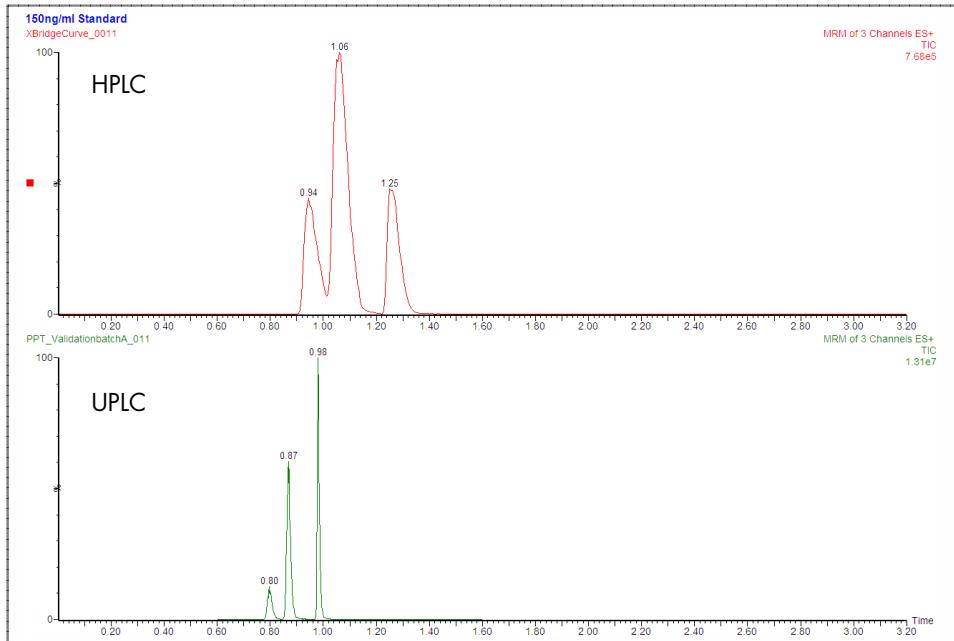


Figure 5. Chromatographic comparison, HPLC vs. UPLC.

## CONCLUSIONS

We have successfully produced a validated UPLC/MS/MS method for the analysis of Pindolol and Acebutolol in human plasma over the range of 0.2 – 150 ng/mL. Statistics for accuracy and precision were within the FDA guidelines for bioanalytical method validation. The data generated by UPLC/MS/MS were comparable to that generated by HPLC/MS/MS, however, it was shown that by using UPLC, a 4-fold increase in signal-to-noise ratio for the LLOQ, a 2-fold decrease in run time, and an increase in resolution was achieved. This equates to doubling the throughput of this method, as well as enabling the acquisition of meaningful data for lower sample concentrations. This has several benefits, for example, as it would allow more accurate measurement of the lower part of the PK curve.

WATERS CORPORATION  
34 Maple St.  
Milford, MA 01757 U.S.A.  
T: 508 478 2000  
F: 508 872 1990  
[www.waters.com](http://www.waters.com)

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