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Nota de aplicación

UPLC-MS/MS Bioanalytical Method Validation of Acebutolol and Pindolol using an Analogue Internal Standard

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Abstract

This application note shows the partial validation of a bioanalytical method for acebutolol and pindolol in human plasma using nadolol as an analogue internal standard.

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.

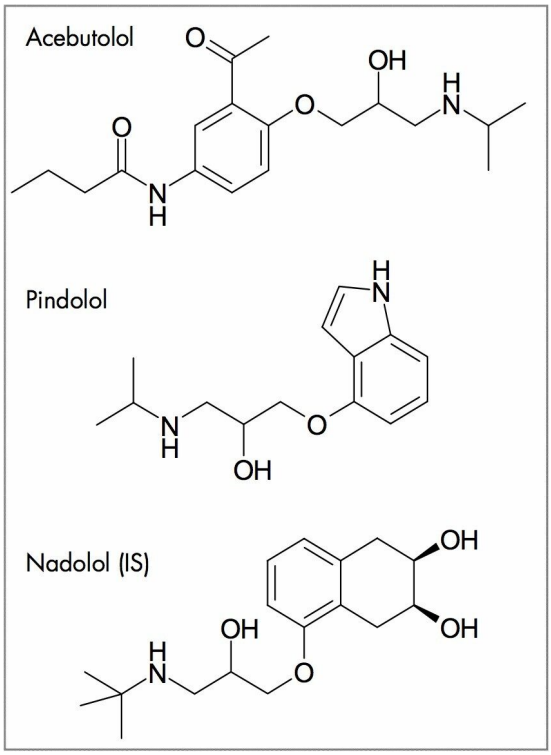

Figure 1. Chemical structures of acebutolol, pindolol, and nadolol.

Through this experiment, we aim to show that the Waters UltraPerformance 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.

Experimental

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

Protein Precipitation Method

1. 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
2. 600 µL acetonitrile was added to crash proteins
3. Centrifuged at 13,000 rpm for 5 minutes
4. 200 µL of supernatant diluted with 800 µL water prior to injection

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.

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	QC
3	0.75	
80	20	
300	75	
360	90	
600	150	

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

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_{18}, 2.1 x 50 mm, 3.5 µm
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
Run time:	3.2 min
Injection volume:	20 µL
Pressure:	1800 psi

Gradient

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

UPLC Conditions

LC system:	ACQUITY UPLC System

Column:	ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm, 1.7 µm
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:	0.6 mL/min
Run time:	1.6 min
Injection volume:	20 µL
Pressure:	10500 psi

Gradient

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

MS Conditions

MS system:	Quattro Premier XE Tandem Quadrupole Mass Spectrometer
Ionization mode:	ES+

Capillary voltage:	3.00 kV
Source temp.:	120 °C
Desolvation temp.:	380 °C
Cone gas flow:	50 L/hr
Dwell time:	0.02 seconds
Inter-scan delay:	0.01 seconds
Collision gas:	Argon (3.45 x 10^{-3} mbar)
Detection mode:	MRM (see below)

MRM

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.

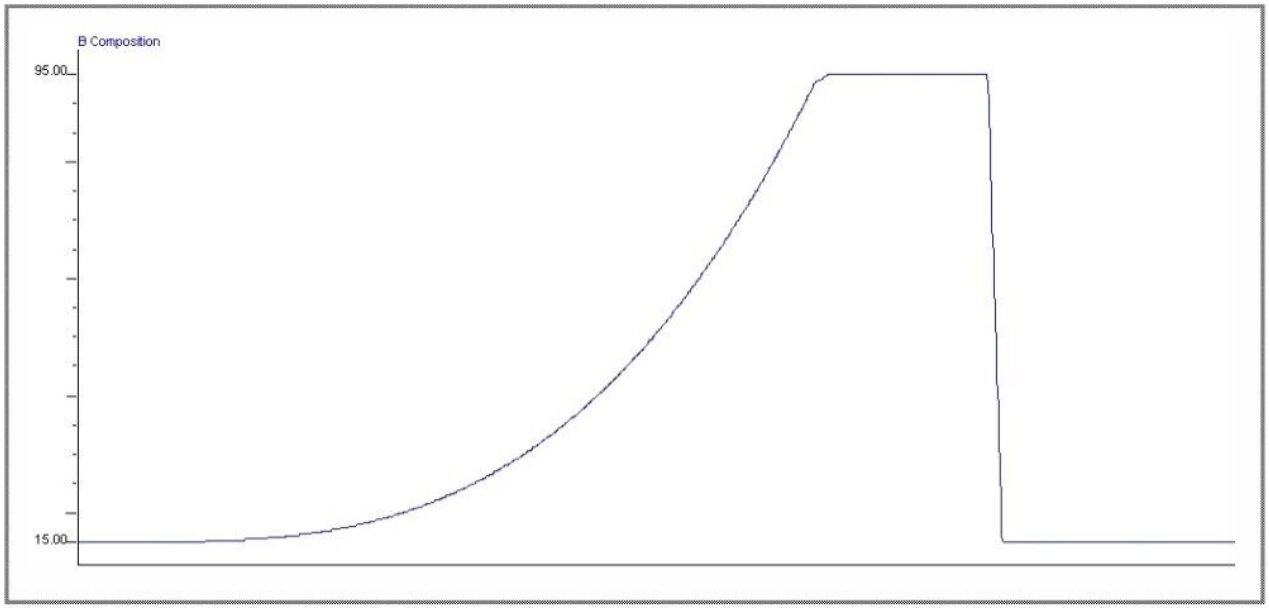

Figure 2. Curve 8 gradient profile.

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.

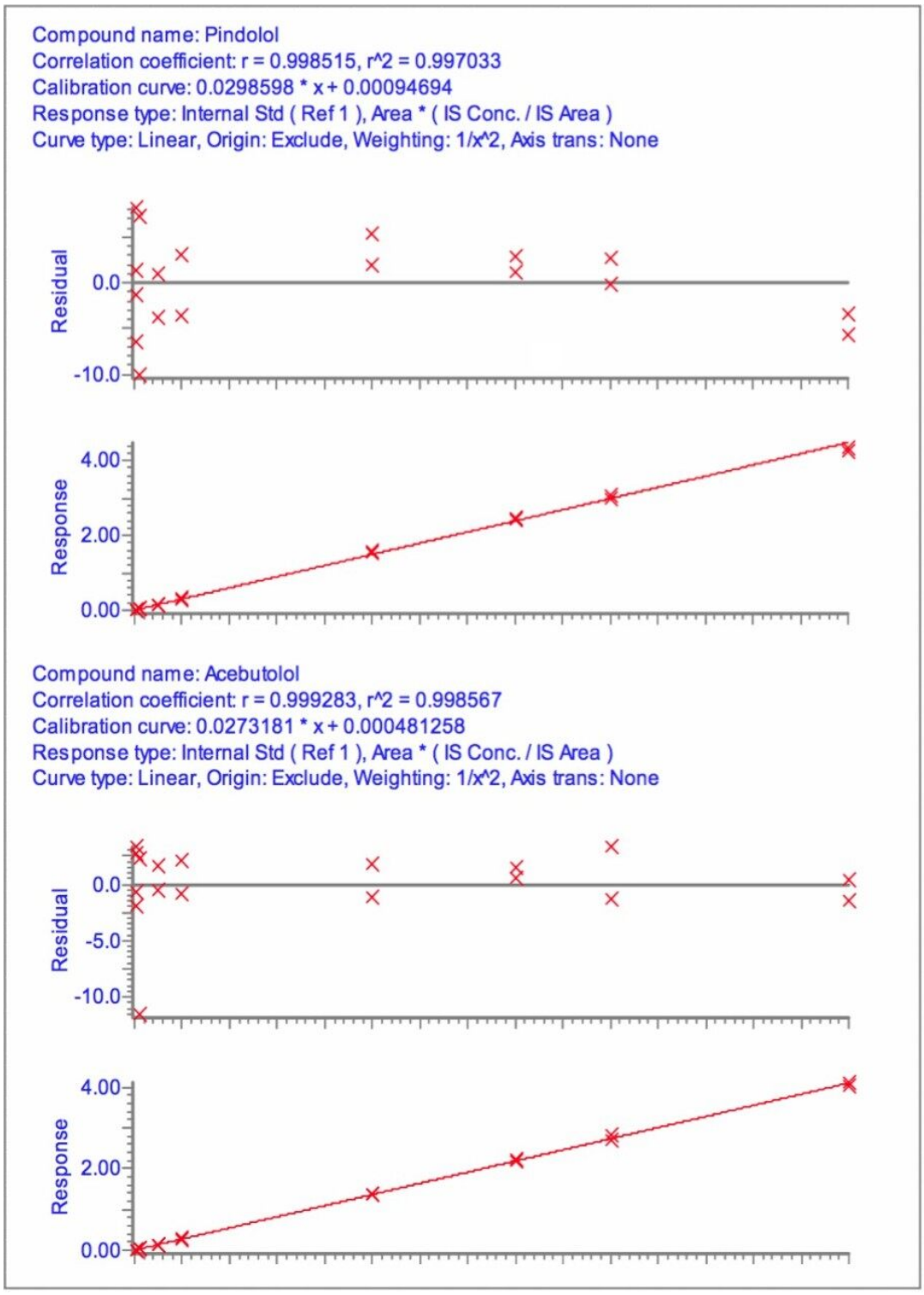

Figure 3. Typical calibration curves for pindolol and acebutololin protein precipitated human plasma by UPLC-MS/MS.

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.

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

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

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.

		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.

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.

HPLC versus UPLC

In Figure 4, we can see that we get a 3.8 fold increase in signal-to-noise by using UPLC versus 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.

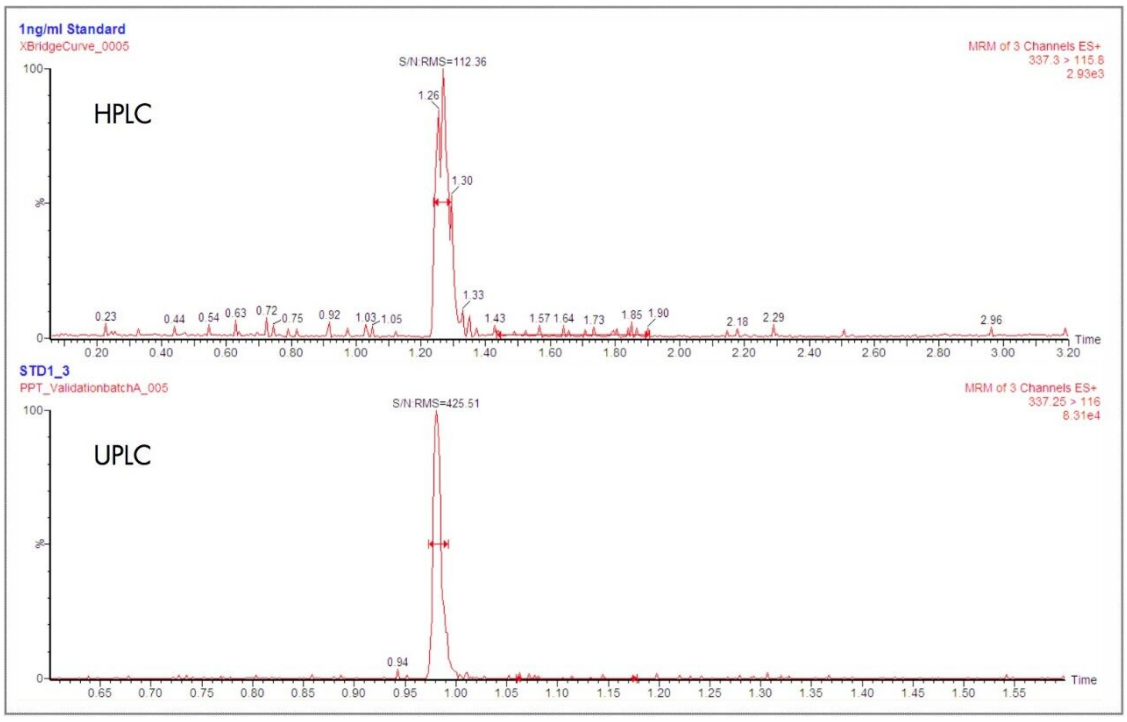

Figure 4. Signal-to-noise comparison using the 1 ng/mL calibration standard, HPLC versus UPLC.

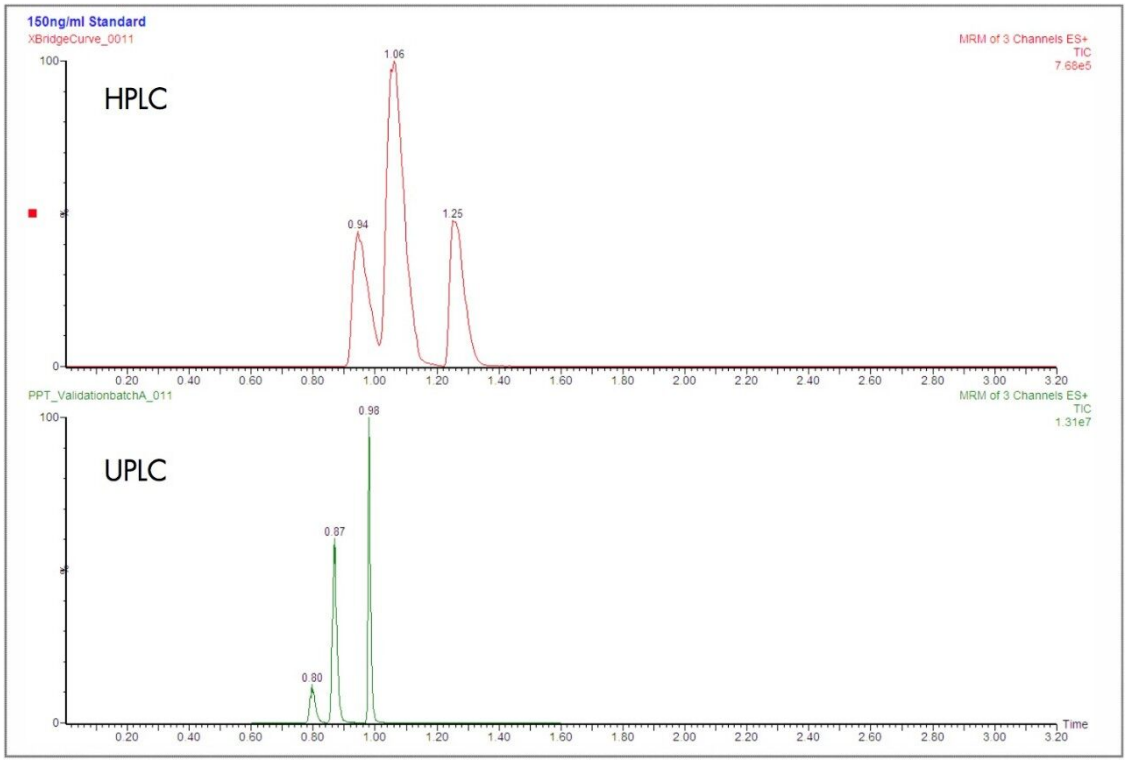

Figure 5. Chromatographic comparison, HPLC versus UPLC.

Conclusion

We have successfully produced a validated UPLC-MS/MS method for the analysis of pindolol and acebutololin 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.

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