



# UPLC-MS/MS Analysis of Bimatoprost and its Free Acid Metabolite from Minipig Skin

## Authors

**Lisa Ford, Kevin Wilkinson<sup>1</sup>, Mike Allen, Lisa Borbridge<sup>2</sup>**

<sup>1</sup>Enthalpy Analytical, Durham, NC, <sup>2</sup>Allergan Inc., Irvine CA

## Overview

Development of a method for the successful UPLC/MS/MS analysis of an unstable parent analyte and its free acid metabolite from minipig skin punches:

A variety of homogenization techniques were attempted until homogenization conditions were optimized

Several liquid-liquid extraction techniques were surveyed

Stability of the parent and free acid metabolite was determined for all successful homogenizations

A reverse-phase UPLC method was developed to replace the normal-phase chromatography method

## Introduction

Quantitation of compounds from skin matrices is a complex process, further complicated in this case by the enzymatic degradation and chemical instability of the parent compound under certain conditions, in both cases forming the acid metabolite. Not only is minipig skin difficult to homogenize, but the amount of hydrophobic interferences that can be co-extracted is much greater than in other liquid biological matrices or homogenates. Traditional homogenization techniques prove ineffective against the tough, fibrous tissue found in minipig skin. It was necessary to develop a method which utilized non-traditional homogenization, extraction, and analysis techniques in order to successfully quantitate the parent compound bimatoprost and its free acid metabolite from pigskin.



## Methods

### HOMOGENIZATION

FastPrep™-24  
15-mL tube adapter



### ANALYSIS

LC-MS/MS System

Waters ACQUITY UPLC®

Column: Waters Acquity BEH C18 2.1x100 mm

Mobile Phases: A = 1 mM ammonium bicarbonate buffer

B = methanol

API-5000™ triple quadrupole mass spectrometer

Period 1 (negative ionization, 0 min-3.3 min)

- Bimatoprost free acid 387.1-343.1, 387.1-192.8
- d4-Bimatoprost free acid 391.1-347.1, 391.1-196.8

Period 2 (positive ionization, 3.3 min-5.9 min)

- Bimatoprost 398.3-131.2, 398.3-117.2
- d4-bimatoprost 402.3-131.2, 402.3-117.2



## Methods (continued)

### HOMOGENIZATION TECHNIQUES AND EFFECTIVENESS

Incubation technique	No Homogenization	Some Homogenization	Highly Homogenized	Fully Homogenized
Liquid nitrogen freeze followed by immediate homogenization	X			
Liquid nitrogen freeze followed by mechanical stress	X			
Dimethyl sulfoxide followed by homogenization	X			
Corn oil followed by homogenization	X			
Reagent alcohol followed by sonication	X			
Incubate dry pigskin at 60 °C for 60 minutes followed by homogenization	X			
Incubate dry pigskin at 60 °C overnight followed by homogenization	X			
0.1M NaOH, 45°C overnight			X	
0.1M NaOH, 60°C overnight				X
Buffer pH containing 8MUrea/1%SDS, 60 °C, 2 hr followed by homogenization		X		
Buffer containing 8MUrea/1%SDS, 60 °C, overnight followed by homogenization		X		
Buffer pH 10 containing 8MUrea/1%SDS, 100 °C intermittent homogenization over 5 hours				X
NaHCO <sub>3</sub> buffer pH 10, 60 °C, intermittent homogenization over 5 hours			X	
Liquid Nitrogen freeze followed by NaHCO <sub>3</sub> buffer pH 10, then immediate homogenization		X		
NaHCO <sub>3</sub> buffer pH 10, 95 °C, 1% SDS, intermittent homogenization over 5 hours				X
NaHCO <sub>3</sub> buffer pH 10, homogenize, incubate at 95 °C 1hr, homogenize again				X
NaHCO <sub>3</sub> buffer pH 10, 95 °C overnight followed by homogenization				X

### SPECIAL CONSIDERATIONS

Basic or neutral conditions necessary to prevent the chemical degradation of bimatoprost to the free acid metabolite.

Must be able to inhibit enzymatic conversion of bimatoprost to the free acid metabolite.

Conditions used (heat, buffers) must also not introduce any chemical conversion.



## Methods (continued)

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### EXTRACTION

Extraction was difficult since the parent compound is relatively hydrophobic and the free acid metabolite polar.

Any solvents/conditions used must prevent degradation of bimatoprost to the free acid metabolite.

Due to high amount of interferants in the pigskin homogenate, protein precipitation was not an option.

Traditional LLE solvents MTBE, EtOAc, Hexane were attempted but co-extracted excessive amounts of hydrophobic material. Extracts could not be completely dried down.

Salt-assisted acetonitrile extraction (SALLE) was utilized in which high salt concentration was introduced, followed by addition of acetonitrile to form two layers. Acetonitrile layer is sufficiently polar to avoid co-extraction of excessive hydrophobic material from the homogenate. Acetonitrile layer is removed, then evaporated and reconstituted to initial mobile phase conditions.

### STABILITY

Stability of the analytes under successful homogenization conditions was tested with solution standards.

- Degradation of 2.5% observed for bimatoprost under the 0.1M NaOH, 60 °C overnight condition.
- Degradation of 0% observed for the pH 10, 60 °C and 95 °C overnight condition.
- Degradation of 0% observed for the pH10 with urea/SDS at 100 °C.

Stability of the analytes in pigskin at 37 °C was tested

- A 2.5% conversion of bimatoprost to the free acid was observed over 180 minutes.

The stability issue was solved by holding pigskin on dry ice during reagent addition until immersion in water bath at 95 °C, at which point hydrolysis of the amide is inhibited.

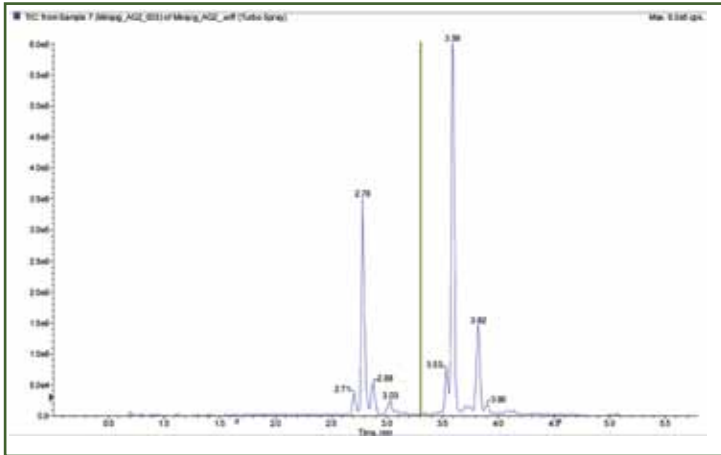
Stability of the final procedure (pH 10, 95 °C followed by homogenization and SALLE) was tested and less than 0.5% conversion of bimatoprost to the free acid was observed.



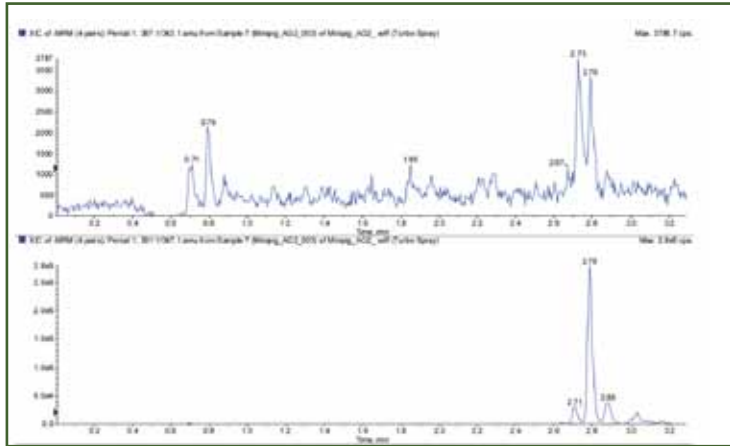
## Results

### LLOQ, 0.1 NG/SAMPLE

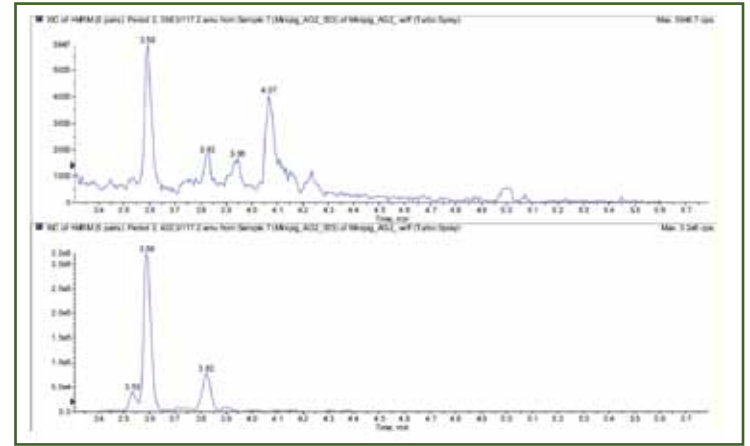
Chromatography, multiperiod



Period 1, bumato prost free acid and *d4* internal standard  
RT 2.8 min



Period 2, bimatoprost and *d4* internal standard  
RT 3.6 min





## Results (continued)

FREE ACID QC PERFORMANCE						
Run Number	QC 1 2.00 ng/sample	%Bias	QC 2 100 ng/sample	%Bias	QC 3 2500 ng/sample	%Bias
1	2.17	8.5	108	8.0	2460	-1.6
	2.01	0.5	110	10.0	2450	-2.0
	2.16	8.0	104	4.0	2640	5.6
	2.11	5.5	105	5.0	2630	5.2
Intraran Mean	2.11		107		2550	
Intraran SD	0.0732		2.75		104	
Intraran %CV	3.5		2.6		4.1	
Intraran %Bias	5.5		7.0		2.0	
n	4		4		4	
2	2.21	10.5	108	8.0	2310	-7.6
	2.31	15.5	108	8.0	2490	-0.4
	2.20	10.0	103	3.0	2540	1.6
	2.09	4.5	105	5.0	2380	-4.8
Intraran Mean	2.20		106		2430	
Intraran SD	0.0900		2.45		104	
Intraran %CV	4.1		2.3		4.3	
Intraran %Bias	10.0		6.0		-2.8	
n	4		4		4	
3	2.00	0.0	95.6	-4.4	2590	3.6
	2.05	2.5	101	1.0	2580	3.2
	1.93	-3.5	102	2.0	2420	-3.2
	1.99	-0.5	106	6.0	2470	-1.2
Intraran Mean	1.99		101		2520	
Intraran SD	0.0492		4.28		83.5	
Intraran %CV	2.5		4.2		3.3	
Intraran %Bias	-0.5		1.0		0.8	
n	4		4		4	
4	1.99	-0.5	105	5.0	2400	-4.0
	2.06	3.0	103	3.0	2240	-10.4
	2.00	0.0	93.8	-6.2	2300	-8.0
	2.14	7.0	90.6	-9.4	2750	10.0
Intraran Mean	2.05		98.1		2420	
Intraran SD	0.0690		6.98		228	
Intraran %CV	3.4		7.1		9.4	
Intraran %Bias	2.5		-1.9		-3.2	
n	4		4		4	
5	2.01	0.5	104	4.0	2390	-4.4
	1.95	-2.5	97.5	-2.5	2430	-2.8
	2.03	1.5	107	7.0	2340	-6.4
	1.97	-1.5	102	2.0	2470	-1.2
Intraran Mean	1.99		103		2410	
Intraran SD	0.0365		3.99		55.6	
Intraran %CV	1.8		3.9		2.3	
Intraran %Bias	-0.5		3.0		-3.6	
n	4		4		4	
Mean Concentration (ng/sample)	2.07		103		2460	
Inter-run SD	0.101		5.09		128	
Inter-run %CV	4.9		4.9		5.2	
Inter-run %Bias	3.5		3.0		-1.6	
n	20		20		20	



## Results (continued)

### BIMATOPROST STANDARD CURVE PERFORMANCE

Run Number	Standard 1 0.100 ng/sample	Standard 2 0.250 ng/sample	Standard 3 0.500 ng/sample	Standard 4 1.00 ng/sample	Standard 5 5.00 ng/sample	Standard 6 10.0 ng/sample	Standard 7 25.0 ng/sample	Standard 8 50.0 ng/sample	Standard 9 100 ng/sample	Standard 10 250 ng/sample	R-Squared
1	0.0984	0.253	0.512	1.08	4.65	NA	26.0	52.3	97.9	226	0.9961
2	NA	NA	0.493	1.01	5.42	10.3	26.5	46.5	97.3	232	0.9960
3	0.101	0.240	0.499	0.981	5.70	9.98	27.0	51.4	95.1	213	0.9929
n	2	2	3	3	3	2	3	3	3	3	
Overall Mean	0.0997	0.247	0.501	1.02	5.26	10.1	26.5	50.1	96.8	224	
S.D.			0.00971	0.0509	0.544		0.500	3.12	1.47	9.71	
%CV			1.9	5.0	10.3		1.9	6.2	1.5	4.3	
%Bias	-0.3	-1.2	0.2	2.0	5.2	1.0	6.0	0.2	-3.2	-10.4	

### FREE ACID STANDARD CURVE PERFORMANCE

Run Number	Standard 1 0.100 ng/sample	Standard 2 0.250 ng/sample	Standard 3 0.500 ng/sample	Standard 4 1.00 ng/sample	Standard 5 5.00 ng/sample	Standard 6 10.0 ng/sample	Standard 7 25.0 ng/sample	Standard 8 50.0 ng/sample	Standard 9 100 ng/sample	Standard 10 250 ng/sample	R-Squared
1	0.0994	0.244	0.548	0.980	4.56	10.3	26.5	51.6	99.1	232	0.9961
2	0.100	0.263	0.431	1.06	5.24	9.65	25.6	48.8	102	249	0.9960
3	0.0979	0.259	0.520	0.991	4.92	9.20	26.5	53.2	99.3	233	0.9970
4	0.0943	0.289	0.484	1.02	5.02	9.35	25.4	52.3	97.3	234	0.9946
5	0.0966	0.287	0.421	1.07	4.97	9.83	25.1	48.9	102	248	0.9929
n	5	5	5	5	5	5	5	5	5	5	
Overall Mean	0.0976	0.268	0.481	1.02	4.94	9.67	25.8	51.0	99.9	239	
S.D.	0.00229	0.0193	0.0550	0.0402	0.246	0.432	0.646	2.01	2.04	8.53	
%CV	2.3	7.2	11.4	3.9	5.0	4.5	2.5	3.9	2.0	3.6	
%Bias	-2.4	7.2	-3.8	2.0	-1.2	-3.3	3.2	2.0	-0.1	-4.4	



## Results (continued)

BIMATOPROST QC PERFORMANCE						
Run Number	QC 1 2.00 ng/sample	%Bias	QC 2 100 ng/sample	%Bias	QC 3 2500 ng/sample	%Bias
1	2.00	0.0	102	2.0	2250	-10.0
	2.06	3.0	109	9.0	2330	-6.8
	2.30	15.0	100	0.0	2710	8.4
	2.13	6.5	103	3.0	2570	2.8
Intraran Mean	2.12		104		2470	
Intraran SD	0.130		3.87		213	
Intraran %CV	6.1		3.7		8.6	
Intraran %Bias	6.0		4.0		-1.2	
n	4		4		4	
2	2.07	3.5	109	9.0	2420	-3.2
	2.30	15.0	110	10.0	2440	-2.4
	2.14	7.0	99.1	-0.9	2550	2.0
	2.33	16.5	106	6.0	2540	1.6
Intraran Mean	2.21		106		2490	
Intraran SD	0.125		4.92		67.0	
Intraran %CV	5.7		4.6		2.7	
Intraran %Bias	10.5		6.0		-0.4	
n	4		4		4	
3	2.27	13.5	94.5	-5.5	2650	6.0
	2.23	11.5	101	1.0	2930	17.2
	2.13	6.5	107	7.0	2680	7.2
	2.19	9.5	99.5	-0.5	2780	11.2
Intraran Mean	2.21		101		2760	
Intraran SD	0.0597		5.15		126	
Intraran %CV	2.7		5.1		4.6	
Intraran %Bias	10.5		1.0		10.4	
n	4		4		4	
Mean Concentration (ng/sample)	2.18		103		2570	
Inter-run SD	0.108		4.85		194	
Inter-run %CV	5.0		4.7		7.5	
Inter-run %Bias	9.0		3.0		2.8	
n	12		12		12	



## Conclusions

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The best homogenization was obtained using heat and high-pH to incubate the pigskin prior to homogenization on the FastPrep® bead-beating homogenization apparatus.

A salt-assisted liquid-liquid extraction was used to selectively extract the parent and the free acid from the non-polar matrix materials with 99% overall recovery of bimatoprost and 84% recovery of the free acid metabolite.

Less than 0.5% degradation of the parent was observed under the incubation and extraction conditions utilized.

Reverse-phase UPLC proved to have similar sensitivity to the normal-phase chromatography previously utilized, but fit better within the workflow of a high-throughput bioanalytical laboratory.

Standard and QC performance show this method is accurate and precise.