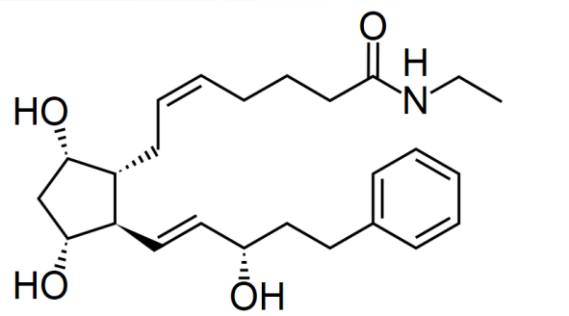
Case Study II: Small Molecule Quantitation

InVision Biomedical Group, Inc.
16590 Aston
Irvine, CA 92606
Phone: 949-825-7556
www.invisionbiomedical.com
Email: info@invisionbiomedical.com

Bimatoprost UPLC-MS Method Development and Quantitation in Test Samples and Latisse Control

CHALLENGE: Bimatoprost Prostaglandin Analogue Quantitation
SOLUTION: UPLC – UV – High Resolution Accurate Mass MS Analysis and Quantitation Workflow

Bimatoprost



Elemental composition: $C_{25}H_{37}NO_4$

Exact monoisotopic neutral mass: 415.2723 g/mol

Exact monoisotopic mass of sodium $[P+Na^+]^+$ adduct: 438.2620 g/mol

Exact monoisotopic mass of potassium $[P+K^+]^+$ adduct: 454.2360 g/mol

Method Development Target Goals

Sample Stability – sample analysis was performed after 1:2 dilution to methanol to avoid sample instability after thawing due to enzymatic activity

Maximum Sensitivity – built an MS method to achieve the lowest possible limit of detection of Bimatoprost signal based on detection of dominant sodium and potassium adduct ions

Linearity commensurate with expected range of sample Bimatoprost concentration - evaluated expected sample concentrations by randomized analysis of four samples, and confirmed that expected sample concentrations fall within the range of concentrations with linear MS response.

Minimal Matrix Effects – developed UPLC method with chromatographic separation of Bimatoprost signal from eluting matrix interferences such as hyaluronic acid.

No Carryover – autosampler optimization and blank

High Accuracy – demonstrated with replicate Bimatoprost primary standards (from Sigma-Aldrich) and spike recovery

Reproducibility – demonstrated with method analysis of 24 samples repeated 3 different days

Chromatography and Mass Spectrometry Method Summaries

UPLC Conditions

1. Column: Acquity UPLC BEH C18 1.7µm (2.1mm x 50mm)
2. Column Temperature: 50°C
3. Flowrate: 0.6 mL/minute
4. Solvent A: LCMS Water with 0.1% formic acid
5. Solvent B: LCMS Acetonitrile with 0.1% formic acid
6. Injection Volume: 1 microliter
7. Gradient:
   Initial 90% Solvent A/10% Solvent B
   0.21 minutes 90% Solvent A/10% Solvent B
   Ramp to 2% Solvent A/98% Solvent B (@3.61 min
   Hold 2% Solvent A/98% Solvent B to 4.8 minutes
   Ramp to 90%Solvent A/10% Solvent B to 5.31 min

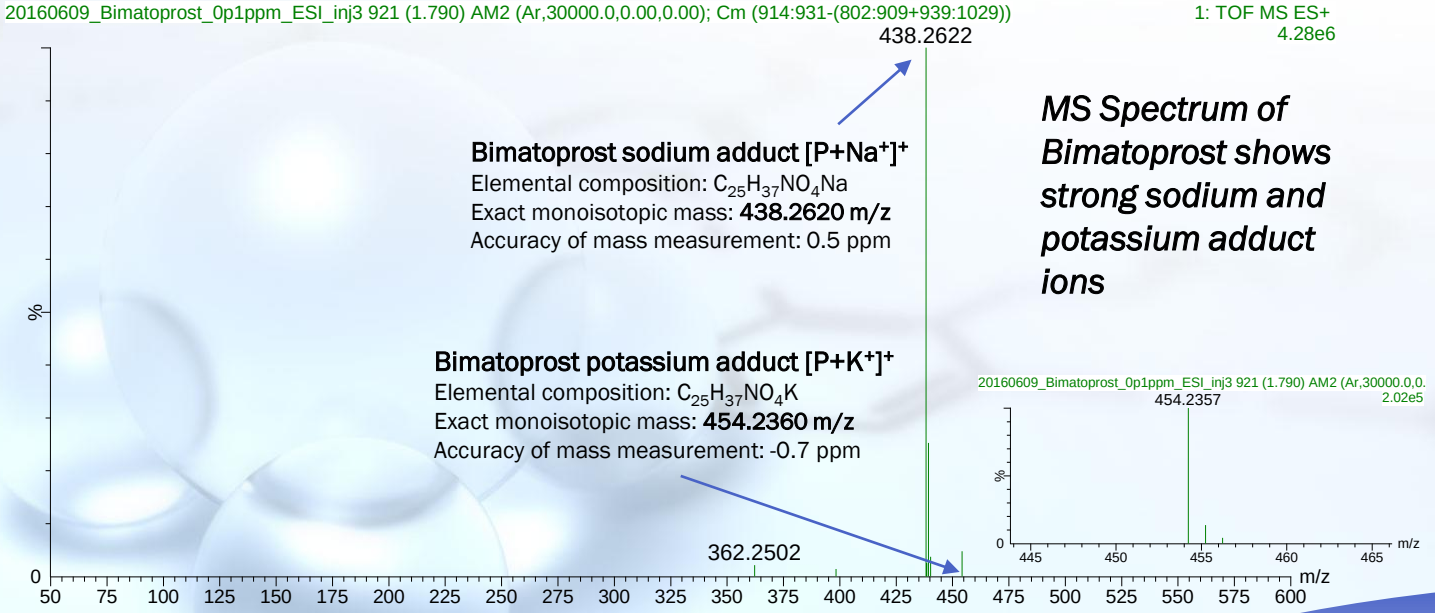
Detectors

1. Acquity PDA; 210-350 nm wavelength scan range
2. Xevo QTOF Mass Spectrometer
   a. TOF MS Mode with targeted enhancement (TE) of Bimatoprost 438.26 m/z signal
   b. Data Format: Continuum
   c. Mass Range 50-600amu
   d. Positive Polarity Resolution Mode
   e. Mass Calibrant: Sodium Formate Solution
   f. Internal Lock Mass: 5 microliter/min of 0.3ng/ µL Leucine enkephalin solution
   g. Tune File: uplchighflow (Capillary voltage: 2.75kV, Desolvation gas: 1000L/hr)
   h. Ionization: ESI

"7Mix" Performance and QC Standard Composition*

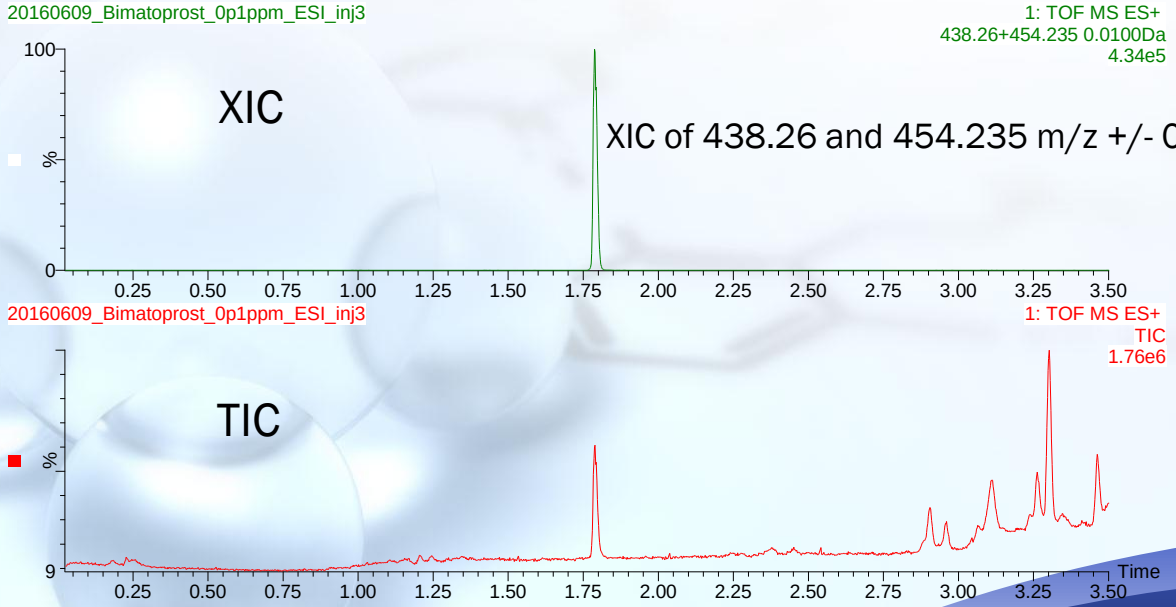
Component	Chemical Formula	Monoisotopic Mass (amu)	CAS #	10X Dilution Sample Concentration (ppm w/v)
Acetaminophen	$C_8H_9NO_2$	152.0711	103-228-1	4
Caffeine	$C_8H_{10}N_4O_2$	195.0882	58-08-2	4
Sulfadoxine	$C_{12}H_{14}N_4O_4S$	311.0814	2447-56-6	1
Chloramphenicol	$C_{11}H_{12}C_{12}N_2O_5$	321.0045	56-75-7	1
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	311.0814	122-11-2	1
Verapamil	$C_{27}H_{38}N_2O_4$	455.2910	152-11-4	1
17-α-hydroxyprogesterone	$C_{21}H_{30}O_3$	331.2273	68-96-2	4

Bimatoprost Standard 1ppm MS Spectrum



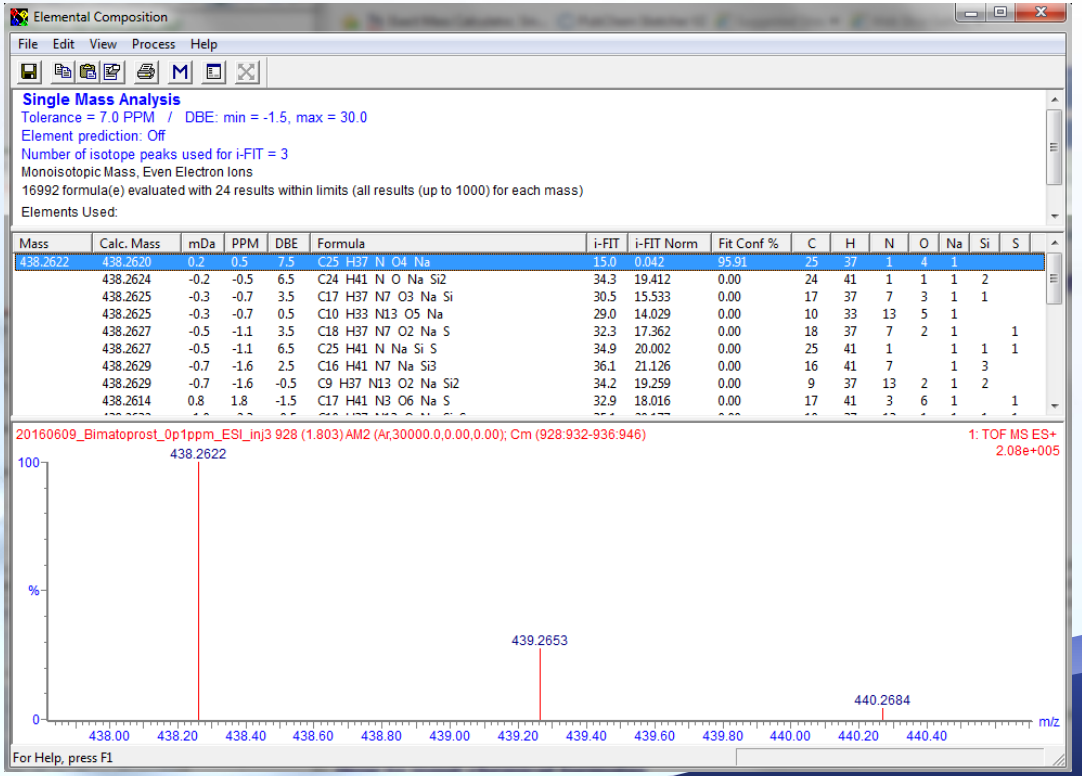
Bimatoprost Standard 1ppm Total Ion Chromatogram (TIC) and Extracted Ion Chromatogram (XIC) of 438.26 and 454.235 m/z

MS response of sodium (438.26 m/z) and potassium (454.24 m/z) adduct ions was used for quantitation of Bimatoprost in samples #1-24 and in Latisse control.



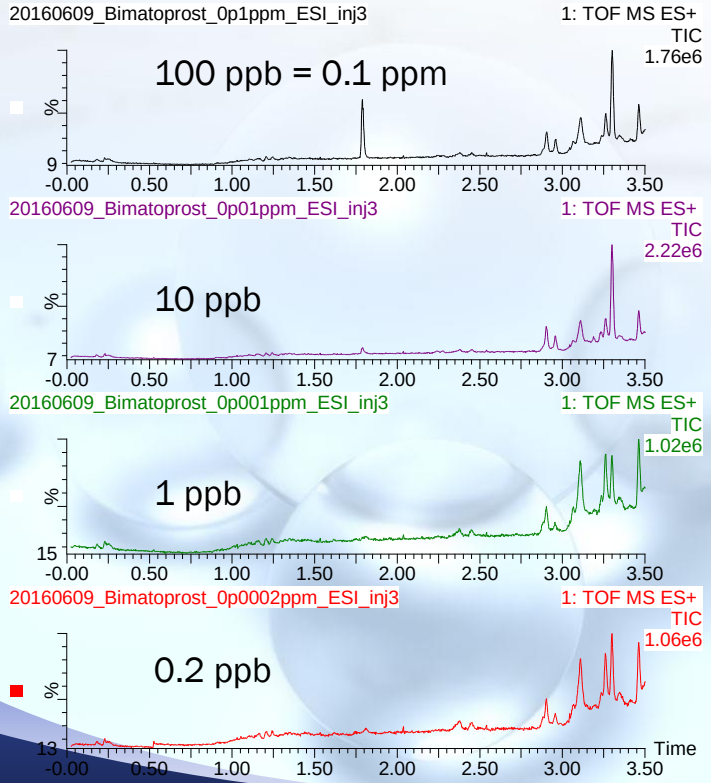
Elemental Composition ($C_{25}H_{37}NO_4Na$) Confirmation of 438.2622 m/z

Expected elemental composition of the sodium adduct ion of Bimatoprost was confirmed using accurate mass and isotopic pattern matching of experimental MS data.

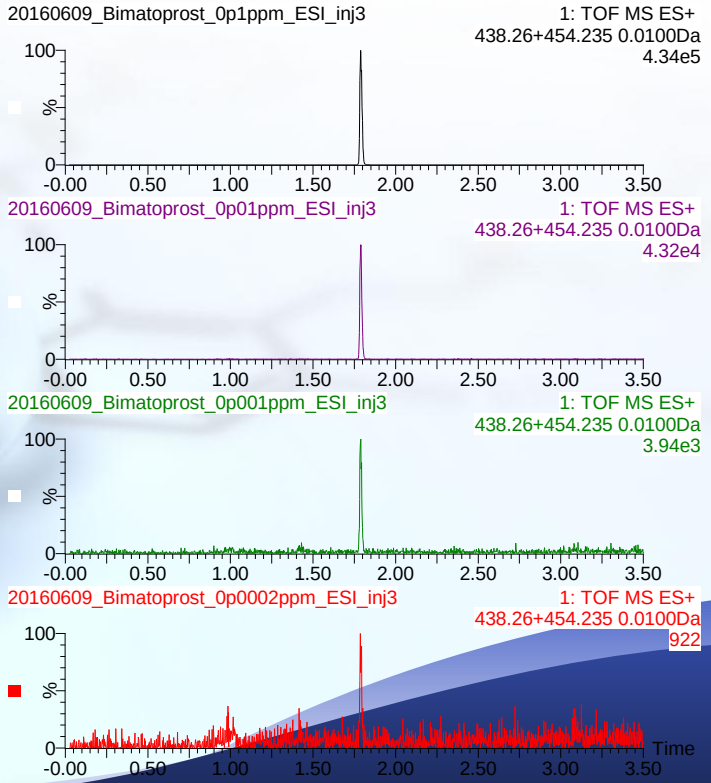


Bimatoprost Calibration Standards TICs and XICs

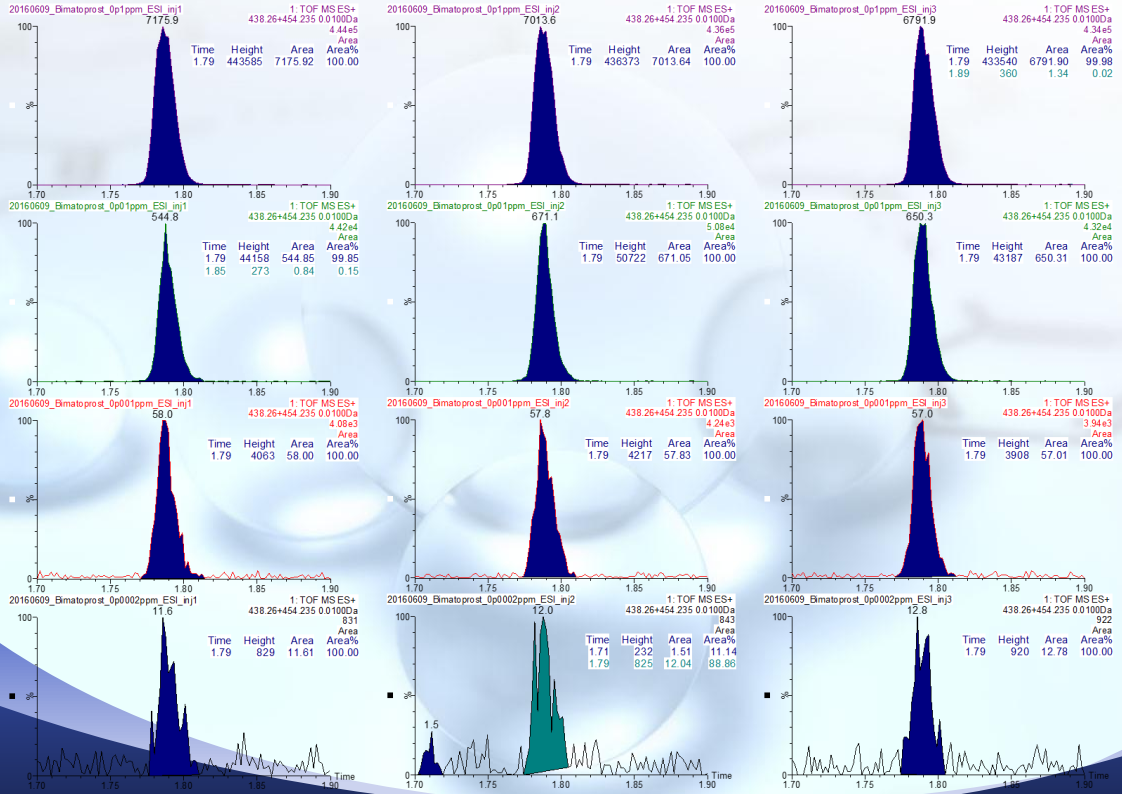
TICs

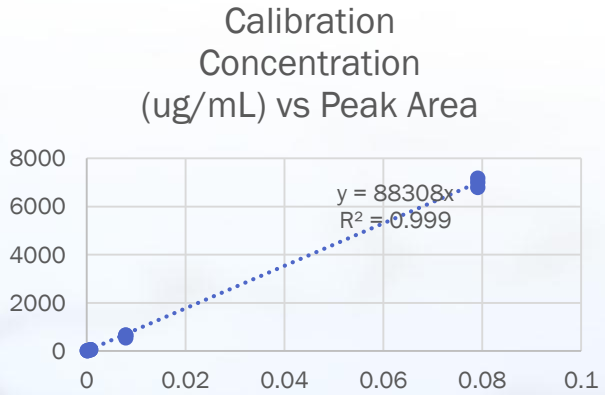


XICs of 438.26 and 454.235 m/z +/- 0.01 m/z



Day 3 Calibration Curve from XIC Data



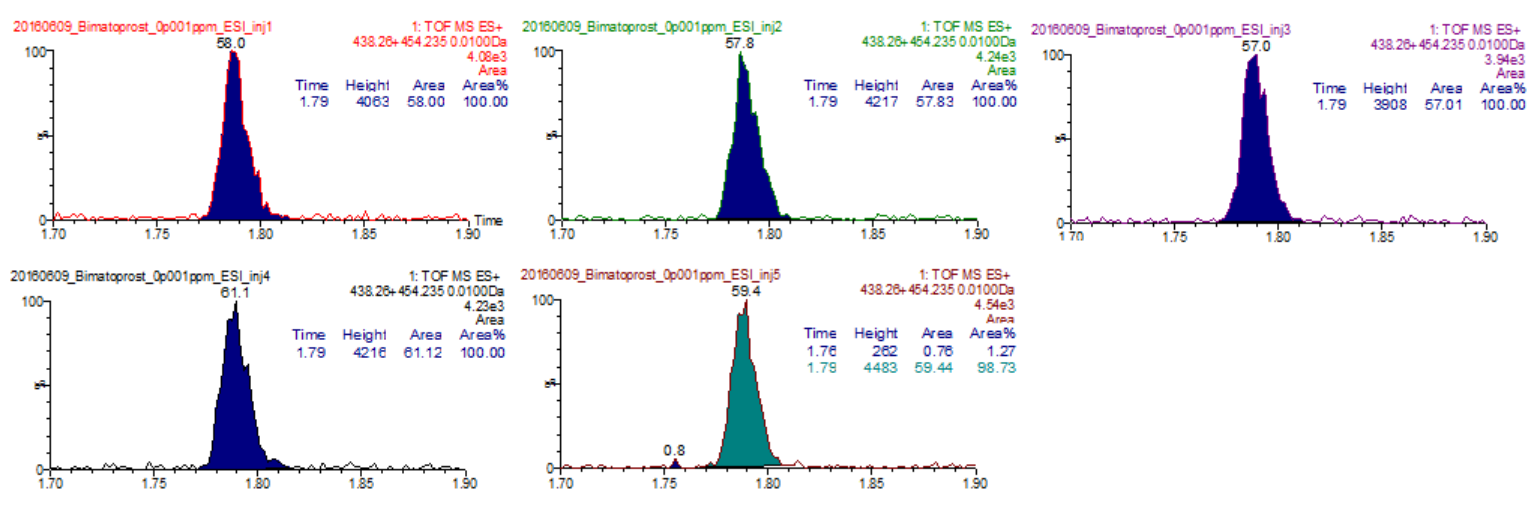


linear response over nearly four orders of magnitude of Bimatoprost concentration

Day 3 Limit of Detection (LOD) from XIC Data

	0.00079 ug/mL injection number								
	1	2	3	4	5				
Major Peak	Area (counts-sec)						st dev	IDL=t*stdev	**LOD (ug/mL)**
	58	57.8	57	61.1	59.4	58.66	1.61493	6.05114398	**0.00008**

0.08 ng/mL limit of detection was calculated with 99% probability of detected value being greater than zero.



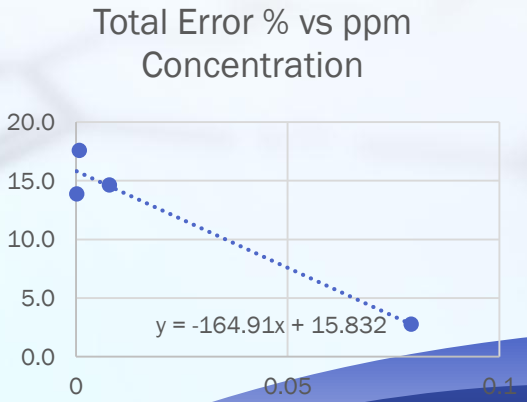
Day 3 Limit of Quantitation (LOQ) from XIC Data

Summary: LOQ is established based on the value of Total Error (TE) rising above 20%. In this data, the LOQ is below 20% at all standard concentrations, thus all values above LOD are quantitative.

LOQ Calculation Based on Scatter Plot Calibration Curve

ppm	area	ppm (calc)			
0.07911	7175.9	0.08125991			
0.07911	7013.6	0.07942202			
0.07911	6791.9	0.07691149	**0.1 ppm Bias**	**Total Error**	**Total Error %**
Average	6993.8	0.07919781	8.78077E-05	0.00218463	2.8
Stdev		0.00218286			
0.007911	544.8	0.00616932			
0.007911	671.1	0.00759954			
0.007911	650.3	0.007364	**0.01 ppm Bias**	**Total Error**	**Total Error %**
Average	622.0667	0.00704428	-0.000866716	0.00115726	14.6
Stdev		0.00076684			
0.0007911	58	0.00065679			
0.0007911	57.8	0.00065453			
0.0007911	57	0.00064547	**1ppb Bias**	**Total Error**	**Total Error %**
Average	57.6	0.00065226	-0.000138837	0.00013897	17.6
Stdev		5.9921E-06			
0.00015822	11.6	0.00013136			
0.00015822	12	0.00013589			
0.00015822	12.8	0.00014495	**0.2ppb Bias**	**Total Error**	**Total Error %**
Average	12.13333	0.0001374	-2.08221E-05	2.1942E-05	13.9
Stdev		6.9191E-06			

ug/mL	TE%
0.0791	2.8
0.0079	14.6
0.0008	17.6
0.0002	13.9



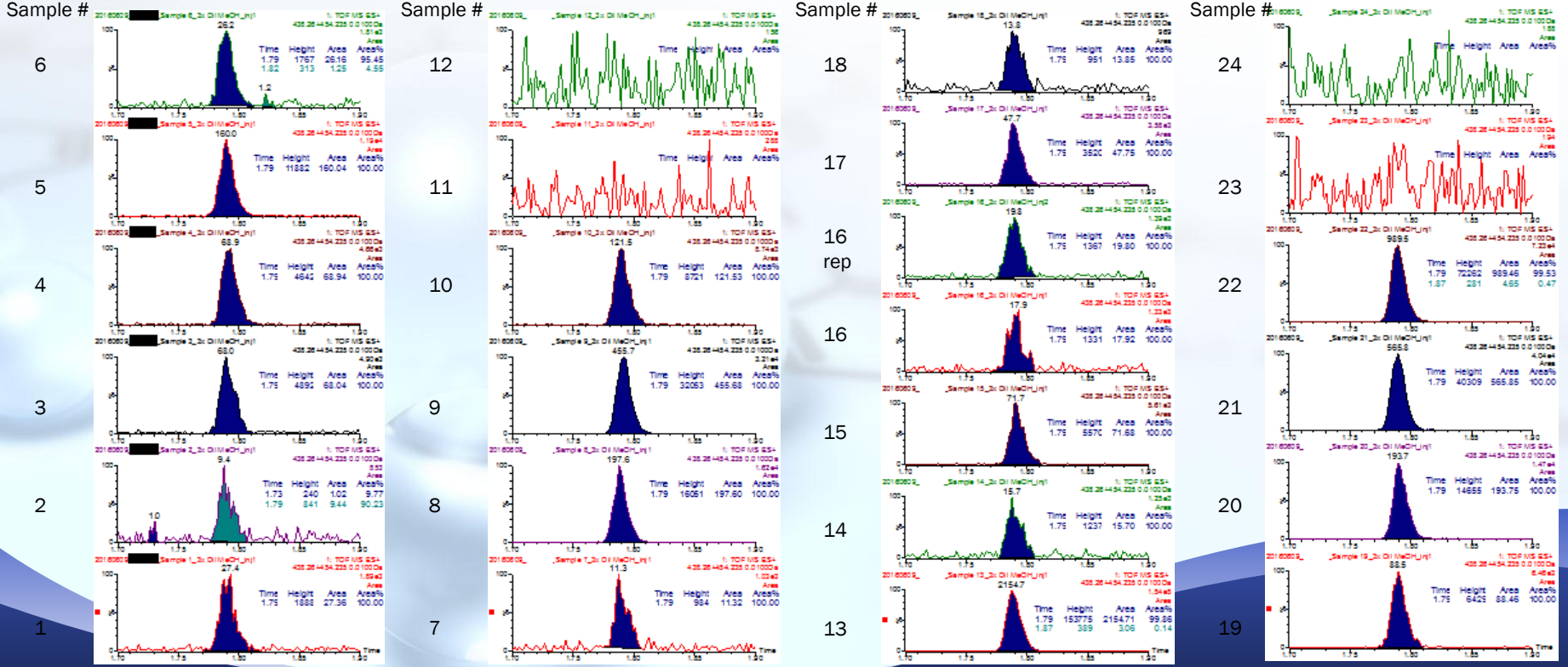
Analysis Reproducibility – Average CV Calculation

Test Samples Bimatoprost Quantitation (n=3)* in ug/mL or ppm With Average CVs

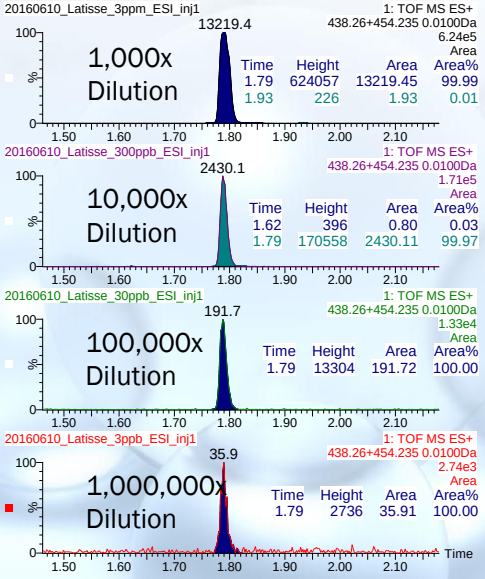
Sample	Average (n=3, ug/mL)	Stdev (n=3)	CV (%)	Sample	Average (n=3, ug/mL)	Stdev (n=3)	CV (%)
1	0.0011	0.0002	23	**13**	0.0865	0.0117	14
2	0.0004	0.0001	32	**14**	0.0006	0.0001	18
3	0.0029	0.0005	18	**15**	0.0029	0.0004	14
4	0.0027	0.0004	15	**16**	0.0009	0.0005	54
5	0.0063	0.0007	12	**17**	0.0019	0.0002	9
6	0.0011	0.0002	17	**18**	0.0004	0.0001	12
7	0.0004	0.0000	10	**19**	0.0035	0.0005	13
8	0.0078	0.0009	12	**20**	0.0078	0.0011	14
9	0.0167	0.0018	11	**21**	0.0208	0.0024	12
10	0.0044	0.0008	18	**22**	0.0376	0.0045	12
11	<LOD	<LOD		**23**	<LOD	<LOD	
12	<LOD	<LOD		**24**	<LOD	<LOD	
	Average CVs	**Average (1-10)**	**17**		**Average CVs**	**Average (13-22)**	**17**
		Average (>2ng/mL)	**14**			**Average (>2ng/mL)**	**13**

*Notes:
1) Individual replicates were analyzed over a period of three days, each sample analysis was preceded by analysis of calibration, QC, LOD, and LOQ standards in triplicates.
2) Internal Bimatoprost (Sigma Aldrich) 1000 ppm (w/w) in MeOH standard was used and dilution concentrations were confirmed by a second internal Bimatoprost standard.

Day 3 Raw Integrated XIC Chromatograms



Latisse Control Sample Results*



Latisse Control	Expected Concentration (ug/mL)	Integrated Area	Detected Concentration (ug/mL)
1000x Dilution	3	13219.4	0.1559
10,000x Dilution	0.3	2430	0.0287
100,000x Dilution	0.03	191.7	0.0023
1,000,000x Dilution	0.003	35.9	0.0004

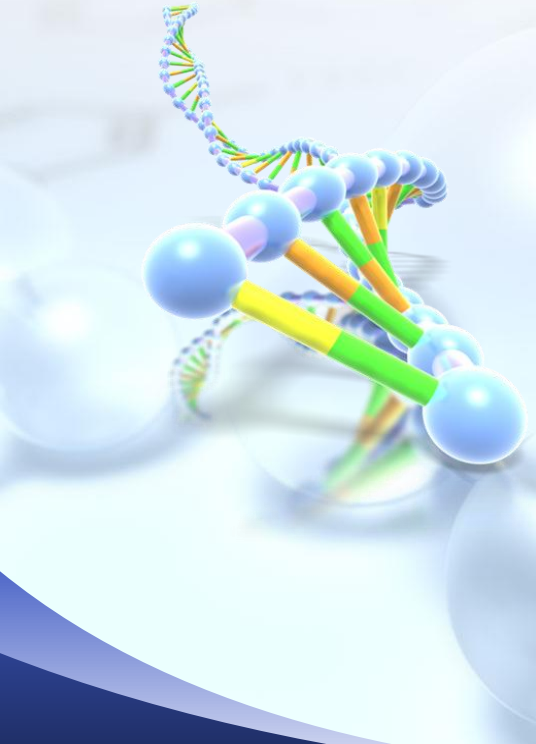
*Note: Latisse Control vial labeled 0.3% (3,000 ppm) was found to contain 0.03% (300 ppm) of Bimatoprost

Conclusions:

- Sample concentrations were analyzed in triplicate ranging in concentration from <0.1ng/mL to 37.6ng/mL.
- Latisse Control sample concentration was determined to be approximately 300,000 ng/mL (0.03%).
- Linearity of MS response was verified for the range of analyzed sample concentrations, i.e., 0.1ng/mL-100ng/mL (0.0001-0.10µg/mL). Run 3 Calibration curve (y=88308x with x in µg/mL and y in XIC area units) demonstrated excellent linearity with correlation coefficient r=0.9995 .
- UPLC and MS methods were optimized for chromatographic resolution and MS response, yielding Bimatoprost LOD and LOQ of 0.1 ng/mL. Refinement of LOD and LOQ would require further work and focusing on method performance at 0.1 ng/mL concentration level.
- Sample analysis demonstrated high reproducibility over 3 independent analytical runs with average CV for all samples of 17%, . and an average CV of 14% for samples with greater than mid range (>2ng/mL) sample concentration.
- Spike recovery experiments demonstrated good method accuracy in sample matrices (samples 2 and 18). Average spike recoveries of 84.2% and 97.2% at 8 and 80ng/mL Bimatoprost concentration levels were achieved.
- Sample dilution in methanol prevented degradation of Bimatoprost in refrigerated sample vials over the course of the quantitation experiment (3 days).
- Bimatoprost ionized predominately as sodium and potassium adduct ions in samples with concentrations <0.1ug/mL, and thus did not yield fragment ions in MSMS collision induced dissociation. Lack of Bimatoprost fragment ions might eliminate any sensitivity advantage the more typical unit mass resolution triple quad (TQ) might have especially in complex samples with high background.

Questions?

More Information?



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