Determination and Confirmation of Lubabegron in Bovine Liver Tissue by LC-MS/MS

Laboratory Procedure for Method G1886

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Analyte(s): Lubabegron (LY488756 Hemifumarate)
Matrix: Bovine Liver Tissue

1. SCOPE AND FIELD OF APPLICATION

Lubabegron is extracted 2x from bovine liver tissue with a solution of acidified methanol: acetonitrile. The extract is then diluted with a solution of acidified methanol:acetonitrile and subsequently analyzed by LC-MS/MS. Concentrations are determined using a matrix-matched standard curve with LSN543100 as the internal standard added to the control tissue extract after extraction.

The method was previously validated at Covance for the analysis of lubabegron in bovine liver from 1.7 ng/g to 40 ng/g for matrix matched calibration. Recovery from fortified tissue was in the range of 90.1 – 107% and the estimated limit of detection (LOD) and limit of quantitation (LOQ) are 0.4 ng/g and 1.0 ng/g, respectively. Supplemental validation demonstrated the suitability of a narrower calibration range using un-weighted regression and additional robustness testing with alternate instrumentation. Confirmation of identity is accomplished by comparing the product ions measured in the samples to those present in the standard injections in both mass and relative intensity.

2. PRINCIPLE

Residues are analyzed by extraction into acidified methanol:acetonitrile, dilution in acidified methanol:acetonitrile:water, and subsequent analysis by LC-MS/MS. There are no chemical reactions involved.

3. DEFINITIONS

gram	=	g
milligram	=	mg
microgram	=	µg
milliliter	=	mL
microliter	=	µL
millimeter	=	mm
micrometer	=	µm
parts per billion	=	ppb
seconds	=	s
revolutions per minute	=	rpm
relative centrifugal force	=	Rcf
minute	=	min
Celsius	=	C

atomic mass unit	=	amu
collision energy	=	CE
collision cell-exit potential	=	CxP
declustering potential	=	DP
nebulizer gas	=	GS1
heater gas	=	GS2
curtain gas	=	CUR
ionspray voltage	=	IS
temperature	=	Tem
collisionally activated dissociation	=	CAD
milliseconds	=	msec
entrance potential	=	EP
electrospray ionization	=	ESI
selected reaction monitoring	=	SRM
multiple reaction monitoring	=	MRM
Methanol	=	MeOH
Acetonitrile	=	ACN
Glacial acetic acid	=	GAA

4. PRECAUTIONS

Detailed hazard information for the item to be tested should be obtained from the Safety Data Sheets (SDS). It is also recommended that the analyst refer to hazard statements from the manufacturer(s) for handling all chemicals and reagents associated with this method.

5. EQUIPMENT AND MATERIALS

Unless otherwise stated, use only analytical-grade reagents and chemicals, and only de-ionized water (e.g., Milli-Q) or chromatographic grade water. Reference to a company is for information and identification only and does not imply a recommendation unless so stated.

Note: Equivalent hardware and materials may be substituted for those specified below.

5.1. Equipment

1) Analytical balance (± 0.00001 g)
2) Top loading balance (± 0.01 g)
3) Magnetic stirrer and Teflon coated stir bars
4) Multi-tube vortexer
5) Centrifuge: Beckman Coulter Allegra 6
6) Ultrasonic bath
7) Branson Digital Sonifier Sonic Probe (or equivalent cell disrupter, blender, or tissuemizer)
8) LC-MS/MS: Sciex API4000, TurboIonSpray® probe, Analyst® software
9) HPLC pump and autosampler: Shimadzu or Agilent
10) Chromatographic column: Phenomenex Luna Phenyl-Hexyl, 5 µm, 2.0 x 50 mm
    Alternate columns that have been tested include Phenomenex Synergi Fusion-RP C18

(2.5 µm, 2.0 x 50mm), ThermoScientific Accucore phenyl-hexyl (2.6 µm, 2.1 x 50mm).

5.2. Materials

1) Eppendorf Repeater® stream pipette with assorted tips (i.e. 1, 5, 50 mL)
2) Volumetric flasks: Class A, glass, assorted sizes (10 mL to 100 mL)
3) Glass bottles: Corning (Corning, NY), 1 or 2 liter
4) Graduated cylinders: glass, assorted sizes (100 mL to 2 L)
5) Mixing cylinders: glass, 100-mL
6) 50-mL conical tubes
7) 15-mL conical tubes
8) Transfer pipettes: disposable
9) Whatman 0.45 µm PTFE syringe filters, i.e. Puradisc 25 TF
10) Autosampler vials

6. REAGENTS AND CHEMICALS

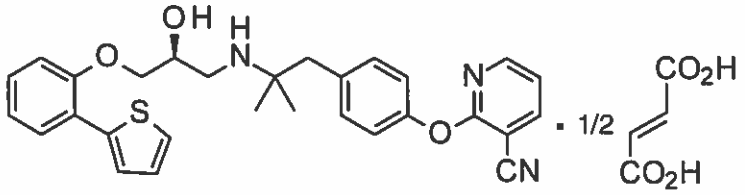
Unless otherwise stated, use only reagents and chemicals of analytical grade quality and only deionized water or chromatographic grade water. Reference to a company is for information and identification only and does not imply a recommendation unless so stated.

Reagents	Source	Product Code
Acetonitrile, HPLC grade	Fisher	A998-4
Methanol, HPLC grade	Fisher	A452-4
Water, HPLC grade or distilled, deionized	Fisher	W5-4
Glacial Acetic Acid (GAA), ACS certified	Fisher	A38-500

6.1. Analytical reference standard

Analyte name:	Lubabegron fumarate (LY488756 hemifumarate, LSN591281)
Molecular formula:	$C_{29}H_{29}N_3O_3S \cdot ½ (C_4H_4O_4)$
Molecular mass:	557.20 Da
IUPAC name:	Bis(2-{4-[2-({(2S)-2-hydroxy-3-[2-(thiophen-2 yl)phenoxy]propyl}amino)-2-methylpropyl]phenoxy}pyridine-3-carbonitrile) (2E)-but-2-enedioate
Supplier:	Eli Lilly and Company, Indianapolis
COA and SDS:	When ordered from Eli Lilly and Company, the order will be accompanied by a certificate of analysis that gives details on the purity of LSN591281. Consult the SDS for storage, safety and handling information.

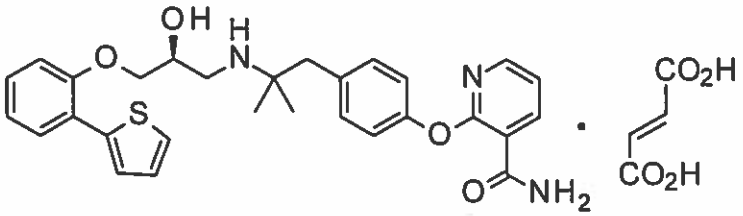
Molecular structure:



6.2. Analytical Internal standard

Analyte name:	LSN543100 Internal Standard
Molecular formula:	$C_{29}H_{32}N_3O_4S$
Molecular mass:	518.21 Da
IUPAC name:	2-[4-(2-{[(2S)-2-hydroxy-3-(2-thiophen-2-ylphenoxy)propyl]amino}-2-methylpropyl)phenoxy]pyridine-3-carboxamide(2E)-but-2-enedioate
Supplier:	Eli Lilly and Company, Indianapolis
COA and SDS:	Assume 100% potency of LSN543100. Consult the SDS for storage, safety and handling information.

Molecular structure:



7. SOLUTIONS

Note: Larger or smaller volumes of the solutions may be prepared using appropriate scaling factors provided they are documented accordingly and equivalent concentrations are maintained.

7.1. Mobile Phase A

Prepare 1 L by mixing 1000 mL of de-ionized H_2O with 1 mL glacial acetic acid.

7.2. Mobile Phase B

Prepare 1 L by mixing 500 mL of acetonitrile, 500 mL of methanol, and 1 mL of glacial acetic acid.

7.3. Tissue Extraction Solution

Prepare by mixing 500 mL acetonitrile, 500 mL methanol, and 1 mL of glacial acetic acid. Use this solution as Tissue Extraction Solution and for preparation of Working Standard and Standard Curve solutions.

Note: Higher grade reagents may be substituted for the specified grade. Larger or smaller volumes of solutions may be prepared provided the concentrations remain the same.

8. STANDARD PREPARATION

Note: Larger or smaller weights and volumes may be substituted when necessary as long as they are documented, and equivalent concentrations are maintained.

Stocks, Primaries, Intermediates, Working ISTD, and WS are stable for 30 days at 5 ± 3°C, sample extracts are stable for 7 days at 5 ± 3°C, and calibration standards are stable for 3 days at ambient temperature.

The same LSN543100 Working ISTD solution should be used to prepare calibration standards and tissue sample extracts for each analytical batch.

8.1. Lubabegron Primary Stock Solution (1000 µg/mL):

1) Accurately weigh approximately 50 ± 0.5 mg of lubabegron reference standard (corrected for potency; for example, with potency of 88.1%, weigh 56.82 ± 0.5 mg).
2) Record the exact weight to at least four significant digits.
3) Transfer into a 50-mL volumetric flask and dissolve with 20-30 mL of MeOH. Sonicate until particles are no longer visibly present. Allow solution to equilibrate to ambient temperature. Bring to volume with MeOH and mix thoroughly.

8.2. Lubabegron Primary QC Stock Solution (1000 µg/mL):

Prepare this solution the same as for Lubabegron Primary Stock Solution.

8.3. LSN543100 Internal Standard Stock Solution (100 µg/mL):

1) Accurately weigh approximately 10 ± 0.1 mg of LSN543100 reference material.
2) Record the exact weight to at least four significant digits.
3) Transfer into a 100-mL volumetric flask and dissolve in MeOH. Sonicate until particles are no longer visibly present. Allow solution to equilibrate to ambient temperature. Bring to volume with MeOH and mix thoroughly.

8.4. LSN543100 Working ISTD Solution (1 µg/mL):

1) Transfer 200 µL of LSN543100 Internal Stock Standard Solution (100 µg/mL) into a 20-mL volumetric flask.
2) Dilute to volume with MeOH and mix thoroughly.

8.5. Stock Comparison Diluent (containing LSN543100):

1) Add 200 µL of Working Internal Standard to a mixing cylinder.
2) Dilute to 60 mL with Tissue Extraction Solution and mix well.

8.6. Comparison of Stock Solutions

A stock solution comparison is required when new stock solutions are prepared.

1) Prepare comparison working solutions (0.1 µg/mL) from each of the two stock solutions as described for WS 3 in step 8.10.
2) To prepare the final stock comparison standards, quantitatively transfer 50 µL of each comparison working standard into suitable vessels containing 4950 µL of Stock Comparison Diluent (step 8.5). Mix thoroughly. Transfer into HPLC vials for analysis.
3) Analyze the solutions using 5 replicate injections of each solution using a fully equilibrated instrument.
4) Calculate the area ratios for each injection and calculate the mean area ratio for each solution.
5) Calculate the corrected mean by dividing the mean area ratio by the actual weight of reference standard used for each preparation.
6) The corrected mean percent difference of the solutions must be ≤ 5% and the %RSD of the area ratios of each stock must be ≤ 5%.

$$\text{mean percent difference} = \frac{|[\text{mean corrected ratio Soln A}] - [\text{mean corrected ratio Soln B}]|}{[\text{mean corrected ratio Soln A} + \text{mean corrected ratio Soln B}]/_2} \times 100$$

8.7. Lubabegron Intermediate Standard Solution 1 (50 µg/mL):

1) Quantitatively transfer 2.5 mL of the Lubabegron Primary Stock Solution (1000 µg/mL) into a 50-mL volumetric flask.
2) Dilute to volume with Tissue Extraction Solution and mix well.

8.8. Lubabegron Intermediate Standard Solution 2 (10 µg/mL):

1) Quantitatively transfer 0.25 mL of the Lubabegron Primary Stock Solution (1000 µg/mL) into a 25-mL volumetric flask.
2) Dilute to volume with Tissue Extraction Solution and mix well.

8.9. Lubabegron Intermediate QC Solution (10 µg/mL):

1) Quantitatively transfer 0.25 mL of the Lubabegron Primary QC Stock Solution (1000 µg/mL) into a 25-mL volumetric flask.
2) Dilute to volume with Tissue Extraction Solution and mix well.

8.10. Working Standard Solutions

Use Lubabegron Intermediate Solutions (1) 50 µg/mL and (2) 10 µg/mL to prepare working standard solutions of 0.5, 0.3, 0.2, 0.1, 0.075, and 0.05 µg/mL of lubabegron in Tissue Extraction Solution. Table 1 displays suggested dilution steps and nominal concentrations of STD solutions.

Table 1. Lubabegron Working Standard Solutions

Intermediate Standard Solution Concentration (µg/mL)	Volume of Intermediate Standard Solution Used (mL)	Dilute to Volume with Tissue Extraction Solution (mL)	Resulting Solution Concentration (µg/mL)	
50	0.5	50	0.5	WS6
50	0.3	50	0.3	WS5
50	0.2	50	0.2	WS4
10	0.5	50	0.1	WS3
10	0.375	50	0.075	WS2
10	0.25	50	0.05	WS1

8.11. Preparation of Matrix Matched Standards

1) Prepare a Control Matrix Extract by weighing 10.0 ± 0.5 g of control tissue and following the extraction procedures as outlined in Section 11.1 steps 3-12.
2) Decant the supernatant into the same 100-mL mixing cylinder.
3) Fortify Control Matrix Extract with 200 µL of the 1 µg/mL Working ISTD Solution.
4) Bring volume to 60 mL with Tissue Extraction Solution and mix well.
1) Take four aliquots of approximately 10 mL each of Control Matrix Extract and place into four 15-mL centrifuge tubes.
2) Centrifuge for 5 minutes at approximately 3700 rpm (1750 rcf) at approximately 20 °C.
3) Pool the centrifuged extract into a single vessel and mix well.
4) Quantitatively transfer 50 µL of each standard solution (WS1 through WS6) into suitable vessels containing 4950 µL of pooled Control Matrix Extract. Mix thoroughly.
5) Filter aliquots of the standards using 0.45 µm PTFE filters into HPLC vials for analysis.

Table 2. Table of Matrix Matched Calibration Concentrations

Calibration Solution ID	Concentration (ng/mL)	Tissue Equivalent (ng/g)
Cal 6	5	30
Cal 5	3	18
Cal 4	2	12
Cal 3	1	6
Cal 2	0.75	4.5
Cal 1	0.5	3

Note: Calibration Standards are stable for 3 days at ambient conditions.

9. QC SAMPLE PREPARATION

9.1. Use the Lubabegron Intermediate QC Solution (10 µg/mL) to prepare quality control fortification solutions of 0.5, 1, and 2 µg/mL of lubabegron in Tissue Extraction Solution. Table 4 displays dilution steps and concentrations of QC fortification solutions.

9.2. Duplicate QC samples at each of three different concentrations are prepared by fortifying 10.0 ± 0.5 g of control liver tissue with 100 µL of each of the resulting QC solutions shown in Table 4. After fortification, allow QC samples to sit for approximately 10 minutes at ambient temperature. QC samples are extracted following the procedures in Section 11.1 beginning with step 2.

Table 3. Lubabegron Quality Control Solutions

Volume of Lubabegron Intermediate QC Solution Used (mL)	Dilute to Volume with Tissue Extraction Solution (mL)	Resulting QC Solution Concentration (µg/mL)	QC Sample Tissue Equivalent Concentration (ng/g)
2	10	2	20
1	10	1	10
0.5	10	0.5	5

10. TISSUE PREPARATION

10.1. Tissue Cryopreparation Procedure

1) Following receipt of the samples, allow the tissue to warm just enough to allow it to be broken, chopped, or divided into manageable pieces (e.g. 1 to 2 inch cubes) while still remaining very cold.
2) Transfer sample into a chilled stainless steel pot or bowl and add sufficient liquid nitrogen to completely cover the tissue until thoroughly frozen.
3) If necessary, further break the tissue into smaller pieces using a mortar or similar tool to create a sample size that will make them easier to pulverize.
4) Pre-chill the blender cup with liquid nitrogen to ensure tissues will remain frozen during preparation.
5) Slowly transfer the sample, without adding the liquid nitrogen, into the pre-chilled blender and blend until the tissue is reduced to powder.
6) Transfer the powdered sample into a pre-cooled container(s) (e.g. Nalgene bottle) and keep on dry ice or transfer into a freezer -20 ± 10 °C or below.

11. TISSUE EXTRACTION

11.1. Tissue Extraction Procedure

1) Remove samples from the frozen storage condition and while still partially frozen, accurately weigh 10.0 ± 0.5 g of control (or blank) bovine liver or incurred liver samples into a sufficient number of 50-mL conical tubes.
2) Fortify all samples (including QC and control samples) with 200 µL of the 1 µg/mL Working ISTD Solution.
3) Add 25 mL of Tissue Extraction Solution to each sample. Shake or vortex the tube to disrupt the tissue.
4) Homogenize the sample for ≥ 20 s using a cell disrupter, blender, or tissuemizer.
5) Vortex sample for approximately 10-15 s.
6) Centrifuge the sample for 5 minutes at approximately 3700 rpm (1750 rcf) at approximately 20 °C.
7) Decant the supernatant from the centrifuged sample into a 100-mL mixing cylinder while retaining the tissue pellet in the conical centrifuge tube.
8) Add 25 mL of Tissue Extraction Solution to the original 50-mL conical tube containing the tissue pellet.
9) Vortex the conical tube to re-suspend the pellet. Alternatively, use a spatula to break up the tissue pellet and then vortex.
10) Homogenize the sample again for ≥ 20 s using a cell disrupter, blender, or tissuemizer or equivalent.
11) Vortex for 10-15 s.
12) Centrifuge the sample for 5 minutes at approximately 3700 rpm (1750 rcf) at approximately 20 °C.
13) Decant the supernatant into the same 100-mL mixing cylinder and bring volume to 60 mL with Tissue Extraction Solution and mix well.

    Note: This is an appropriate daily stopping point. Tissue extracts are stable for 7 days when stored under ambient conditions protected from light or under refrigerated conditions (5 ± 3 °C).

14) Transfer approximately 10 mL of the combined extract into a 15-mL conical tube and centrifuge for 5 min at 3700 rpm (1750 rcf) at approximately 20 °C.

    Note: The control sample is obtained from the pooled control matrix extract described in Section 8.11.

15) Filter aliquots of final extracts using 0.45 µm PTFE filters into HPLC vials for analysis.

12. SAMPLE ANALYSIS

Note: These guidelines may be modified to obtain desired chromatography. Any modification should be documented in the raw data.

Note: 50:50 acetonitrile: water (v/v) was used during method validation as a suitable needle wash fluid. Injection volume may be varied as long as linear calibration is maintained.

Note: A divert valve may be used as appropriate to ensure the detector source remains clean.

12.1. LC-MS/MS Operating Conditions:

Column:	Phenomenex Luna Phenyl-Hexyl, 5 µm, 2 x 50 mm			
Column Temperature:	40°C			
Mobile Phase Gradient:	**Step**	**Total Time (min)**	**% A**	**% B**
	1	0	90	10
	2	1	90	10
	3	4	20	80
	4	4.1	0	100
	5	6	0	100
	6	6.1	90	10
	7	10	90	10
Flow Rate:	0.5 mL/min			
Injection Volume:	1 – 30 µL			
Autosampler Temperature:	Ambient			
Run Time:	10 min			
Operating Mode:	Positive ion, multiple reaction monitoring (MRM)			
Determinative:	500>250			
Confirmatory:	500>209 500>187			
ISTD:	518>250			

Note: Optimize the parameters for the system used and establish the peak mass centers for the analytes of interest.

12.2. Compound Specific Parameters (API4000):

Compound	Q1 Mass (amu)	Q3 Mass (amu)	CE	CxP	DP
Lubabegron	500	250	30	15	120
Lubabegron	500	209	55	15	120
Lubabegron	500	187	55	15	120
LSN543100	518	250	30	15	120

12.3. Non-compound Specific Parameters (Sciex API 4000):

Instrument Parameter	Parameter Value
Ion Source	TurboIonspray
Resolution (Q1 and Q3)	Unit
GS1	50
GS2	50
CUR	20
IS	5500
TEM	650
CAD	6
Dwell Time (msec)	250
EP	10

12.4. Analysis Procedure

1) Interface the HPLC system with the ESI source and set the mobile phase flow rate into the source at 0.5 mL/min.
2) Equilibrate the system with 5-10 injections of standard level 3 (Cal 3) prior to beginning an analytical run.
3) To demonstrate system suitability, inject the lubabegron standard level 3 (Cal 3) at n=5 and observe that the analyte peak is present at the expected retention time. Additional system suitability criteria are provided in Section 15 below.
   A typical analysis sequence may include: single injections of the standard solutions, matrix blank (or control) sample, QC samples, each sample extract solution followed again by the standard solutions.

Table 4. Recommended test sequence

Sample Name
Solvent Blank – Tissue Extract Solution
Cal 1 (0.5 ng/mL)
Cal 2 (0.75 ng/mL)
Cal 3 (1 ng/mL)
Cal 4 (2 ng/mL)
Cal 5 (3 ng/mL)
Cal 6 (5 ng/mL)
Solvent Blank- Tissue Extract Solution
Control Matrix Extract
Low QC(s)
Unknown samples
Mid-level QC(s)
Unknown samples
High level QC(s)
Solvent Blank- Tissue Extract Solution
Cal 1 (0.5 ng/mL)
Cal 2 (0.75 ng/mL)
Cal 3 (1 ng/mL)
Cal 4 (2 ng/mL)
Cal 5 (3 ng/mL)
Cal 6 (5 ng/mL)
Solvent Blank- Tissue Extract Solution

13. CALCULATIONS

1) Measure the peak area for lubabegron and the ISTD in the standard and sample solutions. Construct a non-weighted linear standard curve using all of the standard responses ((ratio of 500>250 to 518>250 (lubabegron area/ISTD area)) vs. concentration). From the standard curve, calculate the concentration (ng/mL) of lubabegron in each of the extract solutions.
2) Using volume, dilutions and concentrations, calculate the concentration of lubabegron in the samples.

$$\text{ppb Lubabegron} = \text{ng/g} = [(A \times B)/C] \times D$$

A = ng/mL sample from standard curve
B = extract volume (mL)
C = weight of tissue sample (g)
D = dilution factor

3) If the concentration of lubabegron exceeds the concentration of the highest calibration standard, the sample extract should be diluted with Control Matrix Extract A and re-injected along with the standard curve.

14. CONFIRMATORY PROCEDURE

1) Confirmation of identity is accomplished by comparing the product ions (500>250; 500>209; 500>187) measured in the samples to those present in the standard injections in both mass and relative intensity.
2) Obtain the individual ion chromatograms for the product ions and ensure that the chromatographic retention times for the analytes are ±5% relative to the mean retention time of the analyte in the standard. Signal to noise should be ≥ 10:1.
3) Integrate the area of the lubabegron peak for each SRM trace for the standards (0.5 to 5 ng/mL) and all samples. The abundance ratio of each confirmatory ion relative to the most abundant ion (reference ion, 500>250) are calculated for both standards and samples. For each confirmatory ion, the abundance ratio for samples should be within ± 20% arithmetic difference compared to the standard. For example, at 30% relative abundance the acceptance range would be 10-50%.

15. SYSTEM SUITABILTY AND DATA ACCEPTABILITY CRITERIA

Note: The following criteria will be used for determining curve acceptability:

1) For system suitability, retention times of lubabegron and the internal standard, and the area ratio precision after equilibration must have RSD ≤ 5%.
2) The non-weighted linear regression coefficient of determination (r^2) must be ≥0.990, or the correlation coefficient (r) must be ≥0.995.
3) Back-calculated accuracy for standard curve points from 0.75 ng/mL to 5 ng/mL must be within ± 15% of the theoretical value, except the lowest point (0.5 ng/mL) must be within ± 20% of the theoretical value.
4) All sample results must be within the calibration range.
5) Acceptance criteria for the QC samples is 60 – 110% of their theoretical concentration.
6) Carryover is a known issue with lubabegron. Carryover should not exceed 20% of the area observed for the lowest standard of the calibration curve. If carryover greater than 20% of the area observed for the lowest standard is observed, then the system should be investigated to determine the cause of the carryover. This can possibly be corrected by increasing the needle wash volume, cleaning the mass spectrometer face plate, or by thoroughly cleaning all glassware that would be used to prepare any solutions used in the analysis.

16. FIGURES

Figure 1: Proposed Ion Fragments for Lubabegron

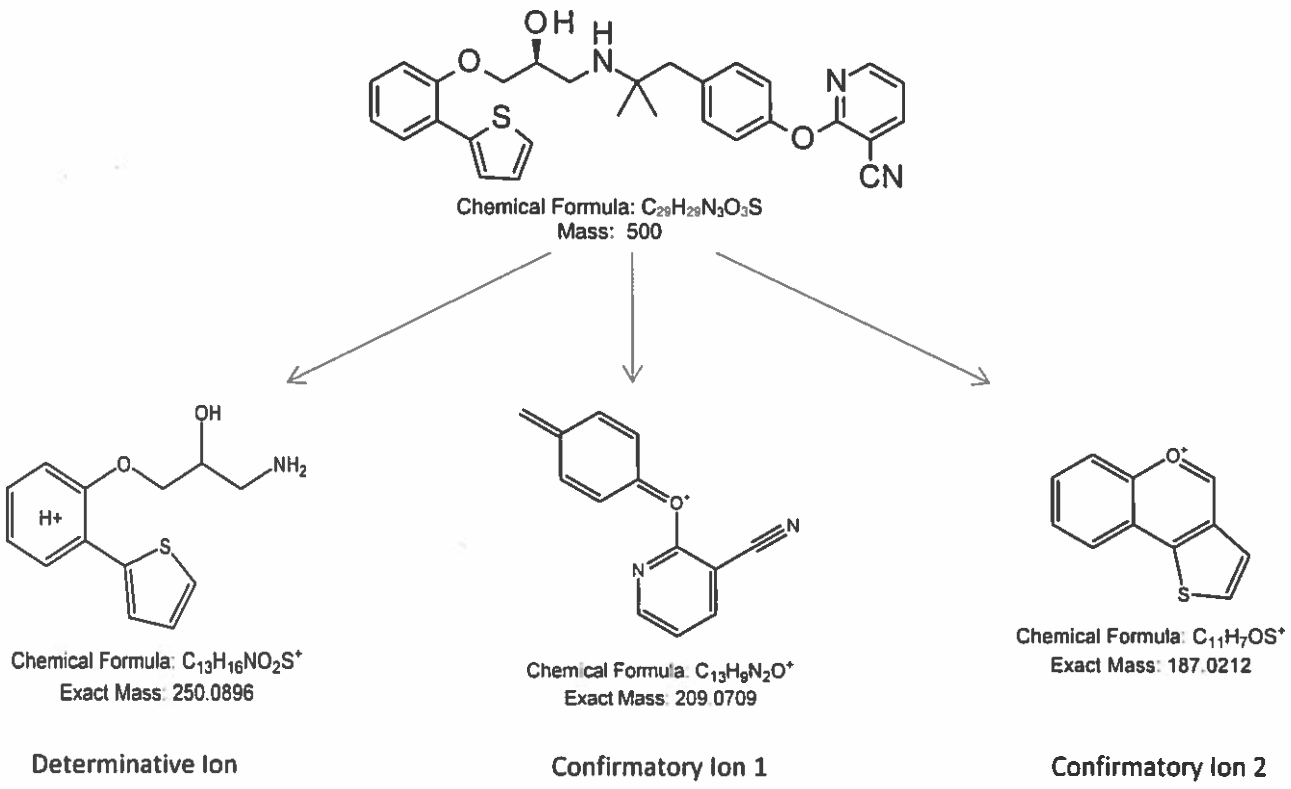


Figure 2: Sample Extraction Flow Chart

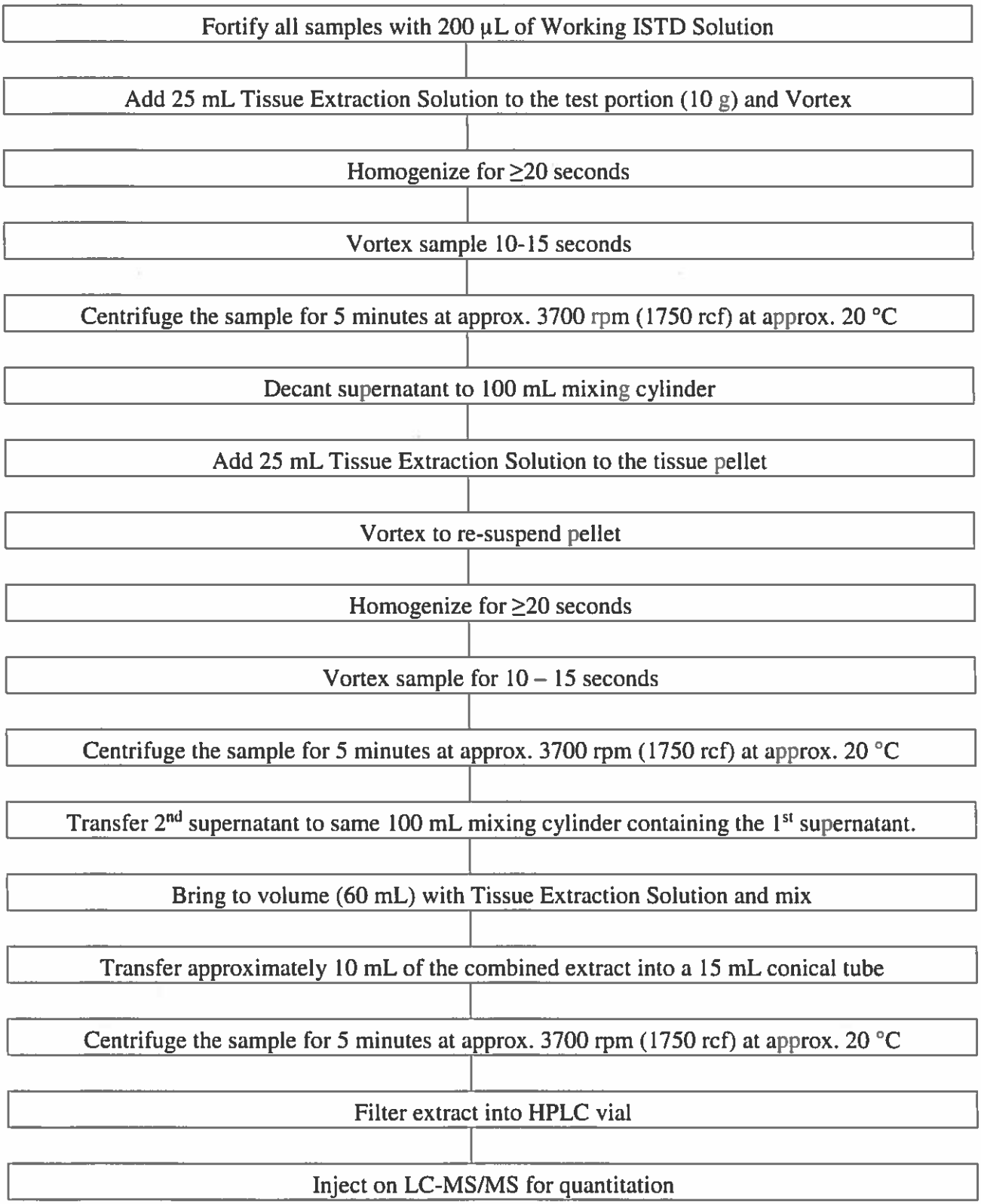


Figure 3: Typical standard curve

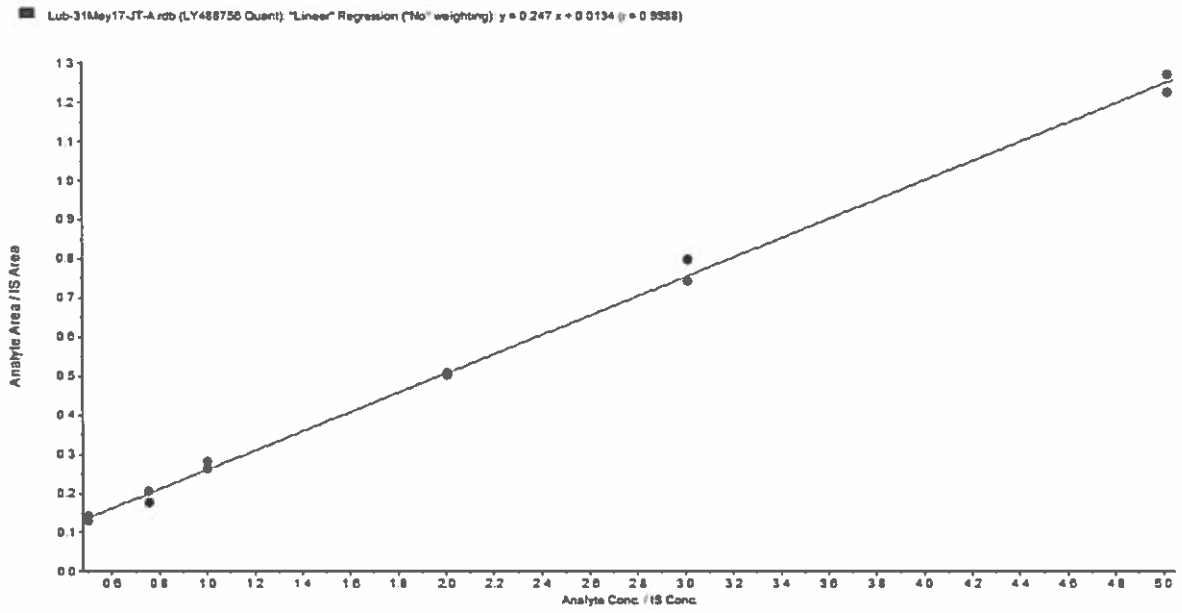


Figure 4: Typical chromatogram of a standard solution at 5 ng/mL

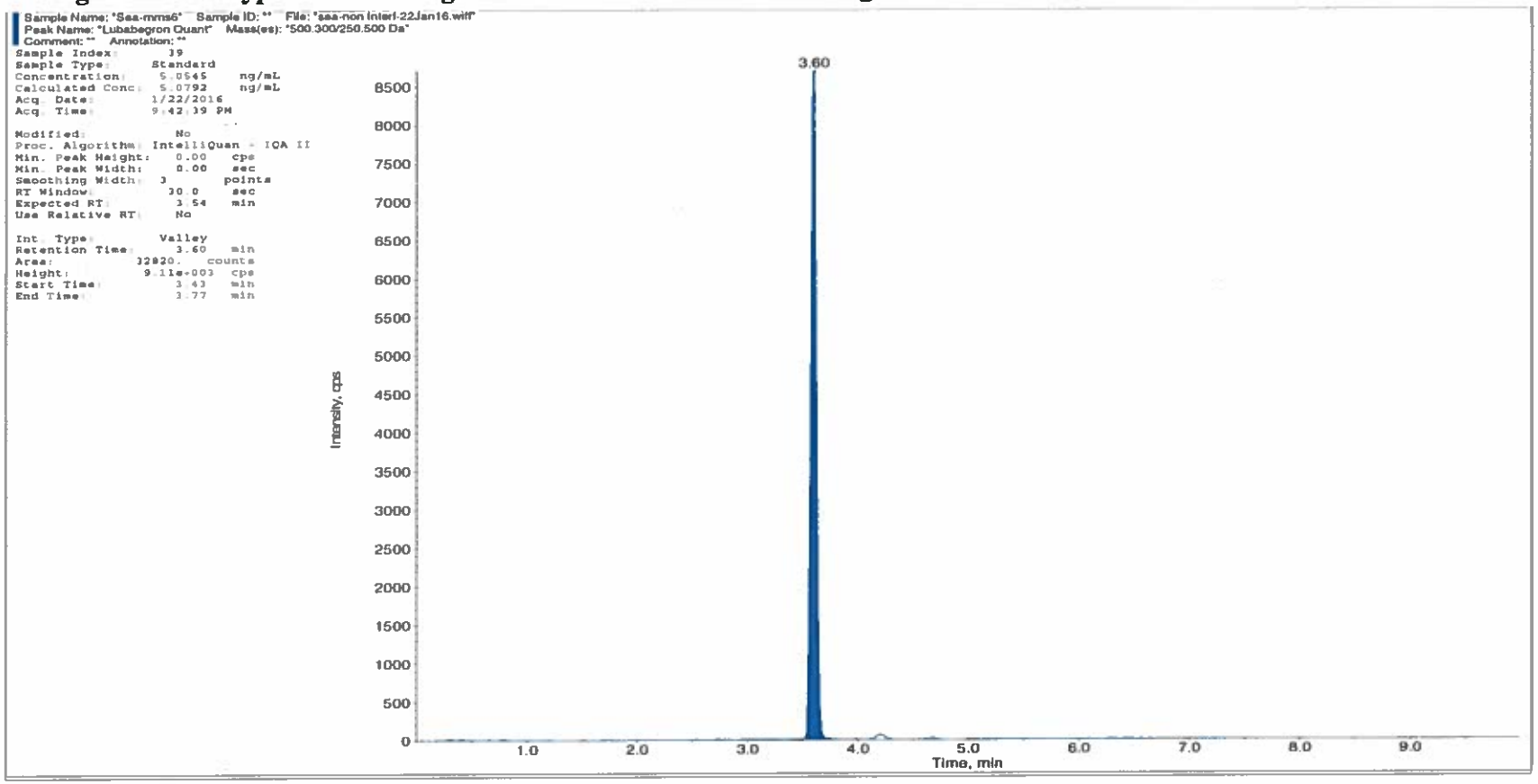


Figure 5: Typical Confirmatory Transition 1 chromatogram of a standard solution at 5 ng/mL

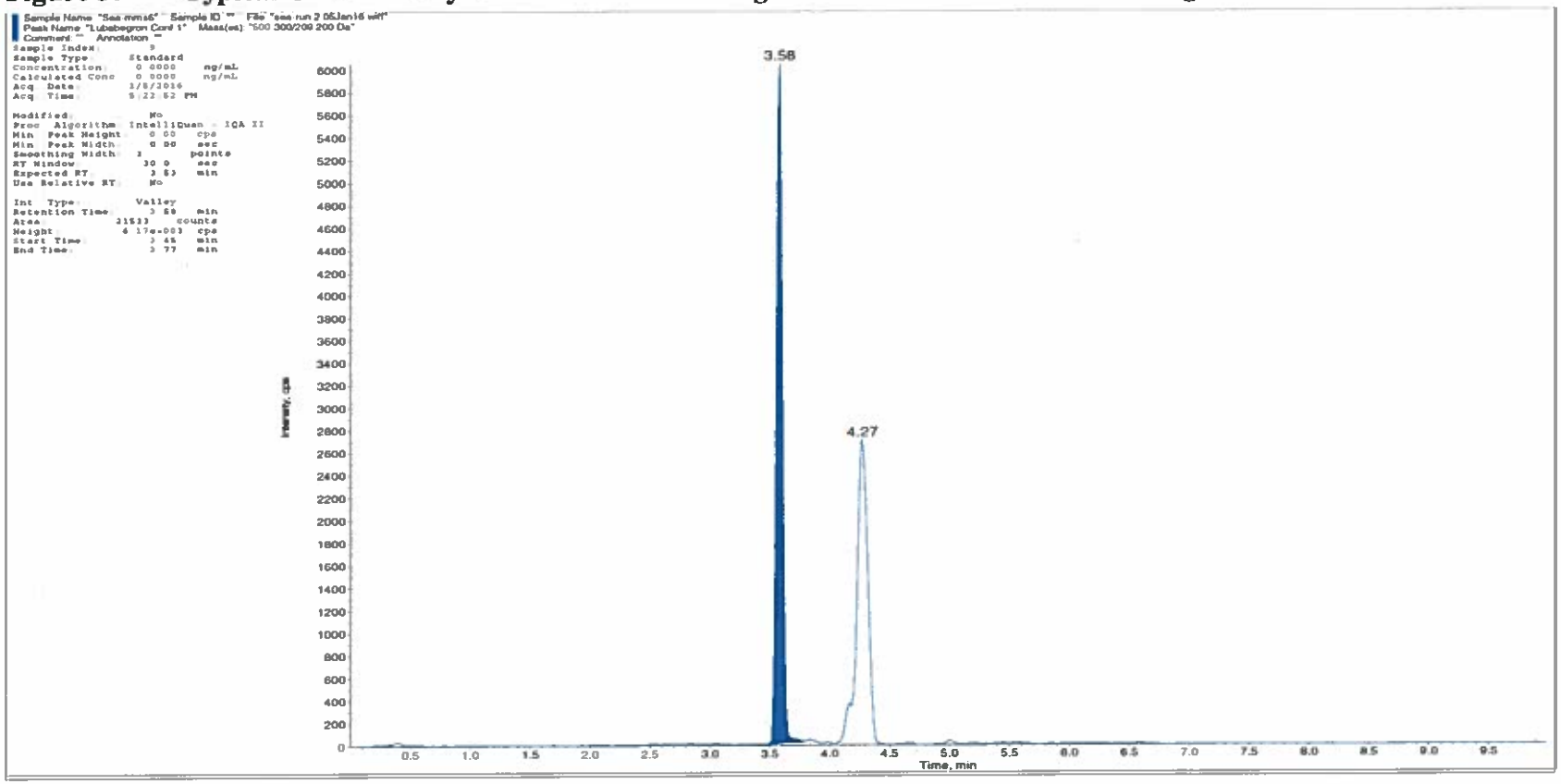


Figure 6: Typical Confirmatory Transition 2 chromatogram of a standard solution at 5 ng/mL

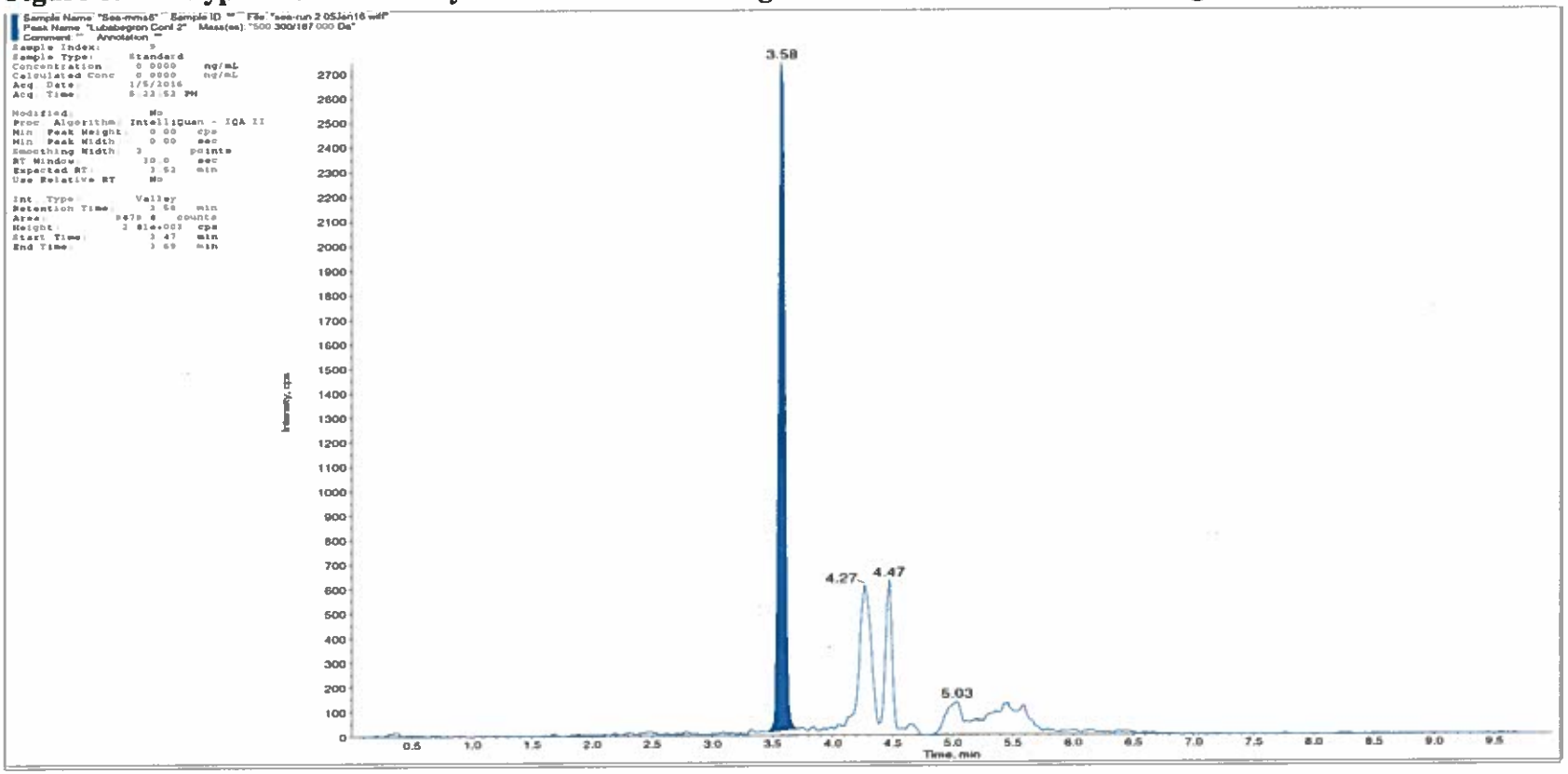


Figure 7: Typical chromatogram of the internal standard

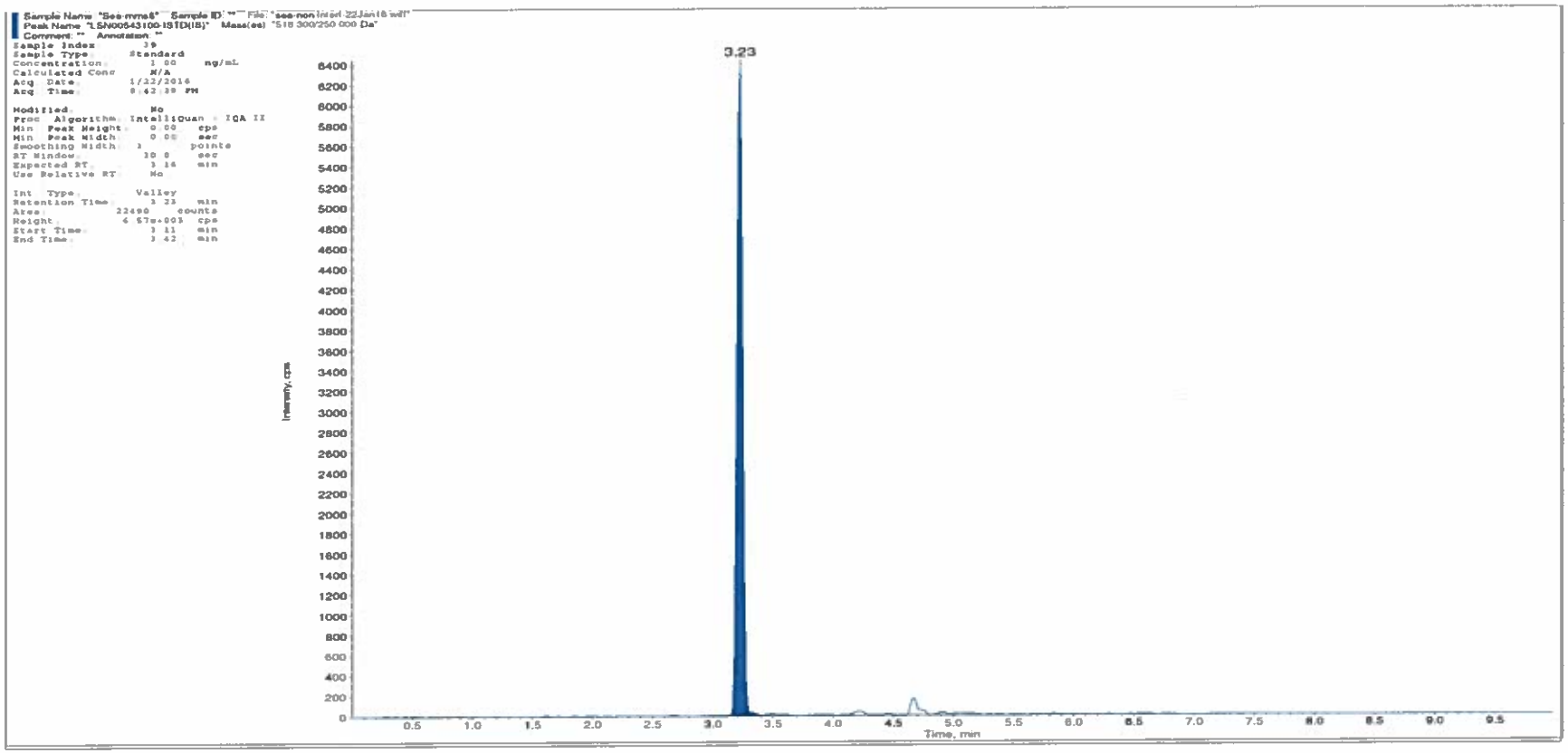


Figure 8: Typical chromatogram of a control extract

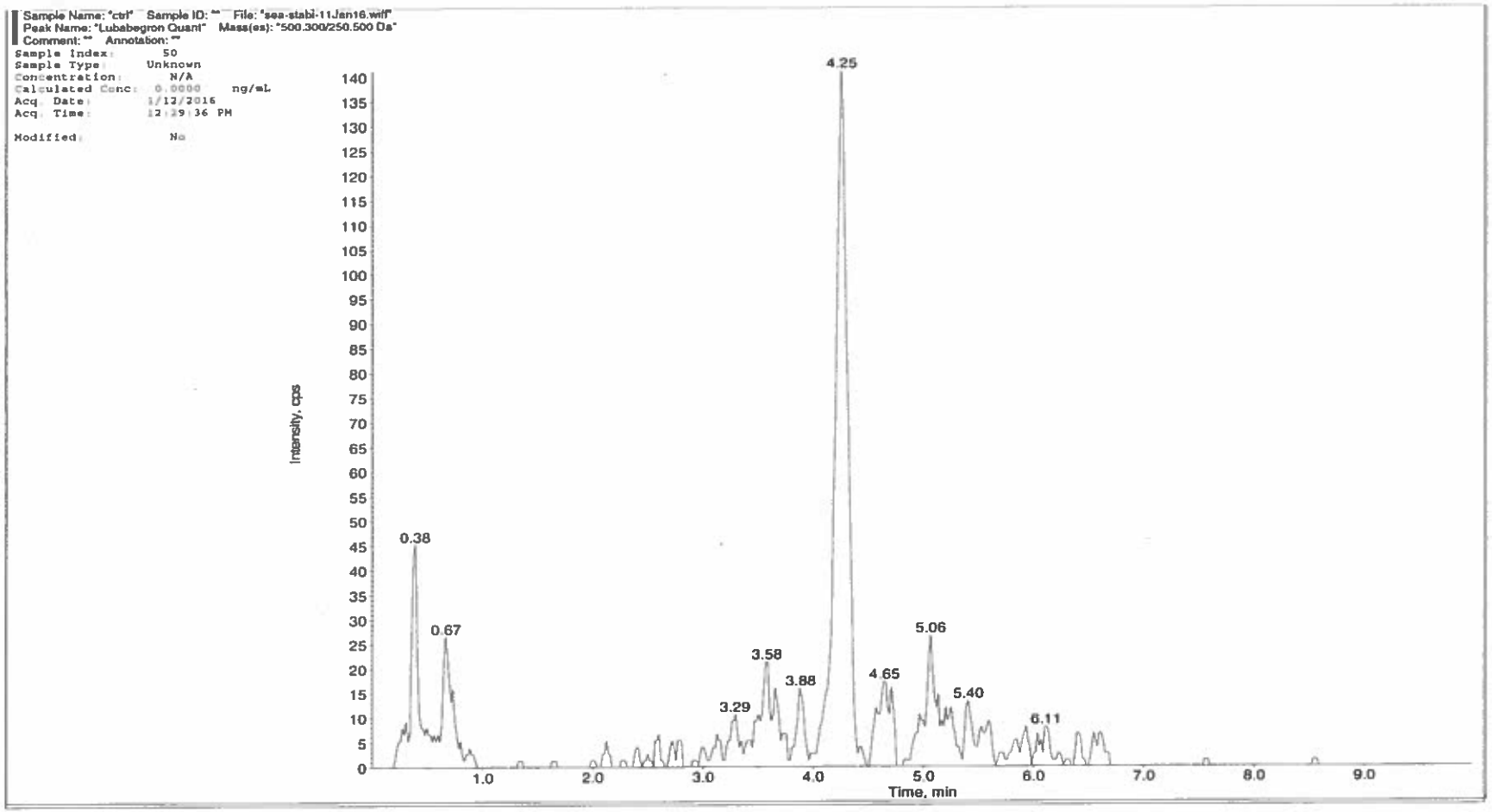


Figure 9: Typical chromatogram of a quality control sample at 10 ng/g

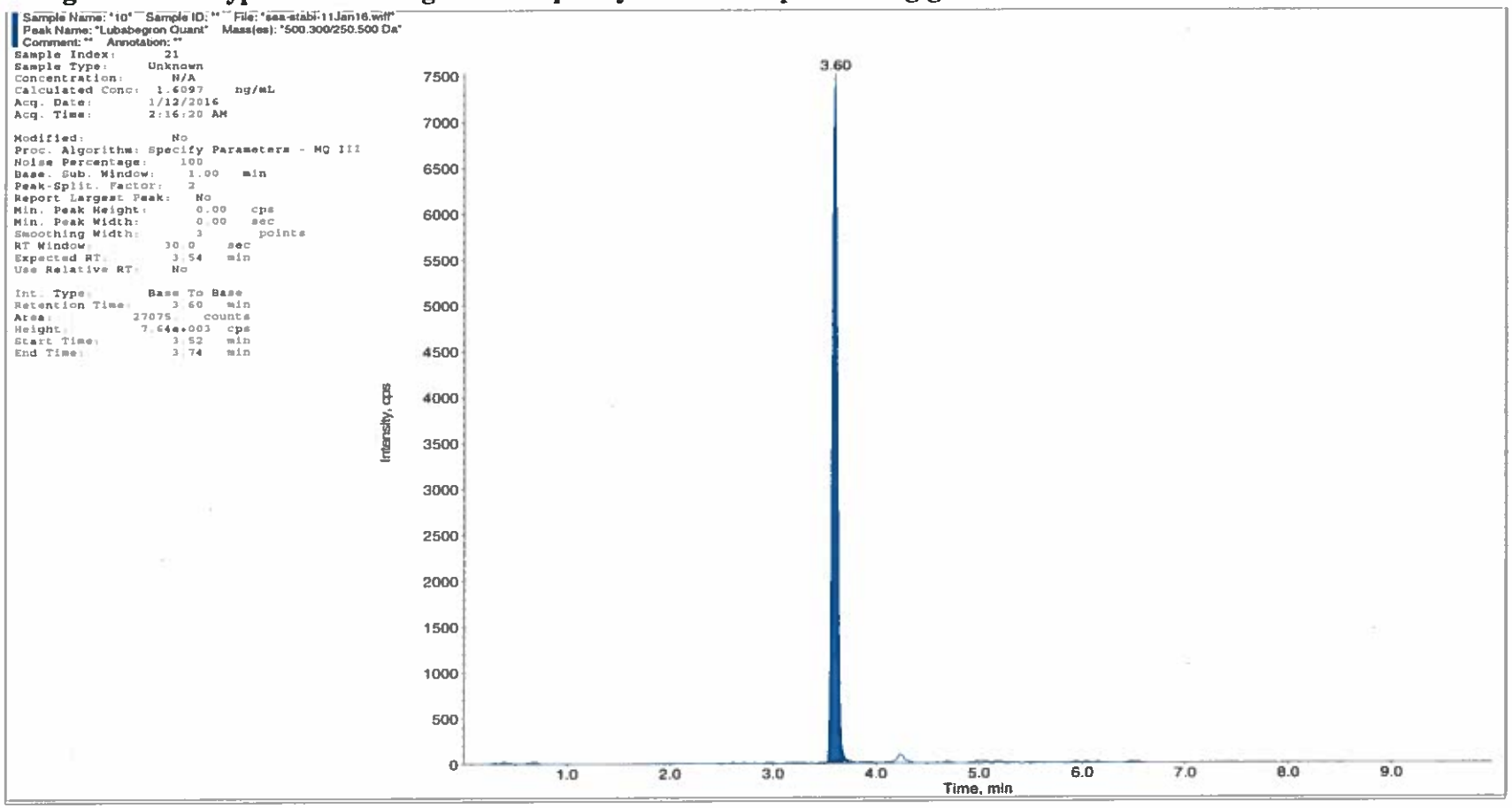
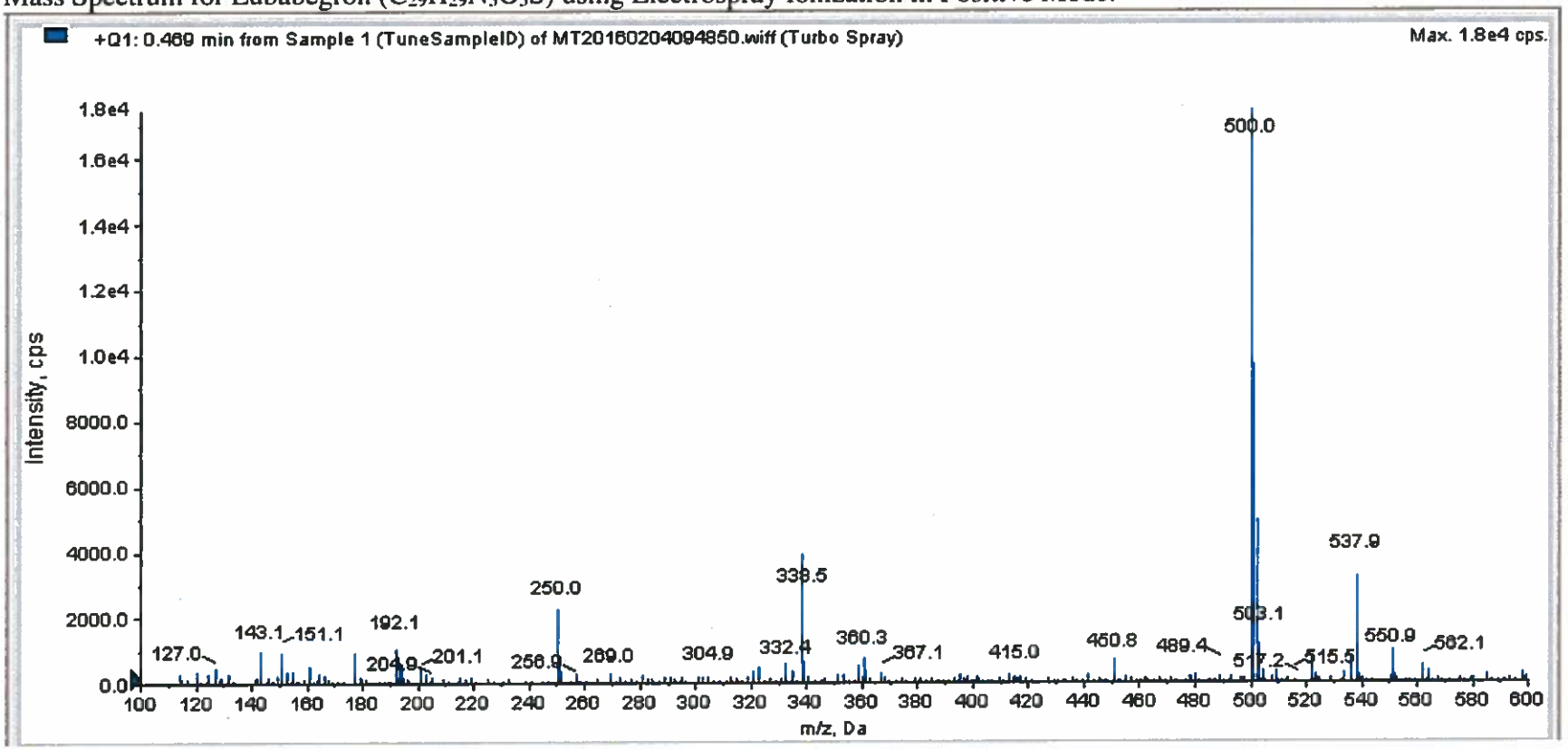


Figure 10: Typical mass spectrum of lubabegron

Mass Spectrum for Lubabegron ($C_{29}H_{29}N_3O_3S$) using Electrospray Ionization in Positive Mode.



17. METHOD TRANSFER DATA AND STUDY SUMMARY

The three naïve laboratories that participated in the method trial obtained acceptable within laboratory accuracy and precision for the determinative procedure for lubabegron in cattle liver.

The three naïve laboratories all obtained acceptable within laboratory accuracy for the analysis of lubabegron in incurred liver tissue having lubabegron concentrations of ~5 and 20 ppb.

There was no statistically significant difference between the set of all the concentration data obtained by the reference laboratory and the set of all the concentration data obtained by the respective test laboratories.

The confirmatory procedure performed acceptably with all control samples failing to confirm, and all fortified and incurred samples containing lubabegron confirming.

The difference in results between calibration Procedures A and B was not statistically significant. It was concluded that standard preparation Procedure B (Method G1886 Version 3.4) is the appropriate post-method trial SOP.

Table 5. Inter-day accuracy and precision of lubabegron concentration in fortified liver tissue samples (QC) as measured at the Reference Laboratory and two additional naïve test sites.

Test Site	Day	Raw Data File Reference	Replicate	Fortified QC Nominal Concentration (ng/g)		
				5	10	20
				Accuracy (%)		
Reference Laboratory	1	Lub-31May17-JT-B	1	109	105	106
			2	103	102	97.3
	2	Lub-01Jun17-JT-B-RI	1	99.9	97.2	103
			2	102	103	95.8
	3	Lub-02Jun17-JT-B	1	101	105	95.6
			2	105	98.4	97.2
			Mean	103.3	101.8	99.2
			%RSD	3.2	3.2	4.3
Site 1	1	2017_05_25_Run02	1	*124	101	110
			2	108	*112	107
	2	2017_05_26_Run01 2017_05_26_Run02	1	*113	108	104
			2	108	103	103
	3	2017_05_29_Run02_Rerun	1	106	106	106
			2	108	107	108
			Mean	107.5	105.0	106.3
			%RSD	0.9	2.8	2.4
Site 2	1	C150218516105182017C	1	97.3	98.5	98.5
			2	98.4	98.0	96.3
	2	C150218516105192017C	1	98.6	96.2	97.9
			2	95.8	97.3	97.8
	3	C150218516105222017C	1	102	102	101
			2	99.5	101	102
			Mean	98.6	98.8	98.9
			%RSD	2.1	2.3	2.2

*Did not meet acceptance criteria; excluded from calculation of mean and CV%

Table 6. Accuracy and precision of lubabegron concentration in blinded incurred liver tissue samples as measured at the Reference Laboratory and two additional naïve test sites.

Test Site	Run Day	Blinded Sample Code	Reported Concentration (ng/g)	Mean	Accuracy (%)	%RSD
Reference Laboratory	1	8079P	BLOQ	BLOQ	NA	NA
	2	8474P	BLOQ			
	2	5995P	BLOQ			
	2	7615P	BLOQ			
	3	4122B	BLOQ			
Reference Laboratory	1	5842P	6.58	6.3	115.2	5.8
	1	5134P	5.98			
	1	8676P	6.31			
	2	6819P	5.98			
	3	2928B	6.82			
Reference Laboratory	1	8199P	23.1	20.9	120.7	9.0
	2	1515P	22.1			
	3	5341B	19.2			
	3	2379B	18.7			
	3	1678B	21.3			
Site 1	1	6862P	BLOQ	BLOQ	NA	NA
	1	4693P	BLOQ			
	2	8355P	BLOQ			
	2	5442P	BLOQ			
	3	2022P	BLOQ			
Site 1	1	1279P	6.85	6.2	113.6	10.0
	2	1648P	5.86			
	3	8280P	6.42			
	3	5120P	6.74			
	3	1455P	5.36			
Site 1	1	8682P	24.6	21.7	125.2	11.8
	1	7177P	20.9			
	2	3584P	20.5			
	2	8130P	18.4			
	3	7673P	23.9			

Test Site	Run Day	Blinded Sample Code	Reported Concentration (ng/g)	Mean	Accuracy (%)	%RSD
Site 2	1	8560P	BLOQ	BLOQ	NA	NA
	1	7054P	BLOQ			
	2	5825P	BLOQ			
	3	8401P	BLOQ			
	3	6118P	BLOQ			
Site 2	1	7946P	5.90	6.2	112.4	3.0
	2	6657P	6.21			
	2	2868P	6.12			
	3	2655P	6.39			
	3	5321P	6.30			
Site 2	1	1019P	21.3	21.1	122.0	1.2
	1	7149P	20.8			
	2	5327P	21.4			
	2	7256P	20.9			
	3	7525P	21.1			

Note: Values for incurred bulk liver tissue used for blinded samples at 5 and 20 ng/g measured 5.50 and 17.3 ng/g for n =5 replicates at the Reference Laboratory using procedure A calibration (*Note: Procedure A was a pre-extraction internal standard addition versus Procedure B which is a post-extraction internal standard addition to the calibration standards*).

18. REFERENCES

Covance Report 8334-607. Validation of a Method for the Determination and Confirmation of LY488756 in Bovine Liver by LC-MS/MS. 22-Mar-2016.

Covance Report 8354-498. Supplemental Validation of a Method for the Determination and Confirmation of LY488756 in Bovine Liver by LC MS/MS. 23-Nov-2016.

Covance Report 8358-135. Inter-Laboratory Method Trial for Determination of Lubabegron in Cattle Liver Tissue. June 2017.