

**IDENTIFICATION, QUANTIFICATION AND CONFIRMATION OF
LAMOTRIGINE IN EQUINE PLASMA BY HIGH PERFORMANCE
LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY**

DEVELOPED BY

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INTRODUCTION

Lamotrigine (LAMICTAL[®]; 3,5, diamino-6-(2,3-dichlorophenyl)-as-triazine (C₉H₇N₅Cl₂, by GlaxoSmith Kline) is an anti-convulsant or anti-epileptic drug (AED) of the phenyltriazine family. Lamotrigine was cited as the 85th of the top and most prescribed 200 drugs in the United States in 2002 (Pharmacy Times, p. 20, April 2003). Lamotrigine is slightly soluble in water and 0.1 M HCl. The mechanism of action by which lamotrigine prevents seizures is not known. However, it has been shown to have some effect on sensitive sodium channel resulting in modulation of neuronal stability and pre-synaptic transmitter release of excitatory amino acids (glutamate and aspartate). Lamotrigine exhibits from weak to non-inhibitory effect on almost all neurotransmitter receptors for example adenosine A₁ and A₂, adrenergic α₁ and α₂ and β; dopamine D₁ and D₂, GABA_A and B receptors, histamine H₁, kappa opioid, muscarinic acetylcholine, serotonergic (5-HT₂) and lamotrigine does not affect dihydropyridine-sensitive calcium channel. Lamotrigine does not inhibit the re-uptake of norepinephrine, dopamine, serotonin or aspartic acid and thus, the ability of the pre-synaptic nerve terminal to increase storage of these neuro-transmitters is not altered by lamotrigine. N-methyl-d-aspartate (NMDA)-mediated depolarization in rat cortical brain slices or NMDA-induced cyclic AMP formation in undeveloped rat cerebellum is not known to be inhibited by lamotrigine, and it does not displace competitive or non-competitive ligands at the glutamate receptor complex. Lamotrigine is known to inhibit dihydrofolate reductase responsible for catalytic reduction of dihydrofolate to tetrahydrofolate; inhibition of which interferes with biosynthesis of nucleic acids and protein. Lamotrigine accumulates in the kidney of male rats and in melanin-containing tissues (eye and pigmented skin. In the dog, lamotrigine is extensively metabolized to an inactive moiety; 2-N-methyl metabolite (less than 1 % of the total dose is metabolized) has been detected in human urine. It is rapidly and completely absorbed (98 %) following oral administration with peak plasma concentration attained between 1.4 and 5 hours. Lamotrigine is moderately (50-55 %) bound to plasma proteins. It does not act in competition with other AED's such as phenytoin, carbamazepine and phenobarbital for binding sites. The mean apparent volume of distribution (Vd/F) of lamotrigine post oral administration is 0.9 to 1.3 L/kg. Vd/F is independent of dose. Lamotrigine is similar to phenytoin in its therapeutic indication and so it would be similarly classified by the Association of Racing Commissioners International. Thus, any concentration of lamotrigine detected and confirmed in plasma will be reported as a positive finding to the Racing Commission. Phenytoin is an AED but is used in horses for the management of "tying-up", condition known in veterinary medical practice as intermittent rhabdomyolysis (CIR). The condition can produce various degrees of discomfort and impairment of performance. In 1988, Dr. Beech of the University of Pennsylvania School of Veterinary Medicine first described the use of phenytoin for the management of CIR. Based on the seriousness and extent of lameness that CIR can imposed upon equine athletes, the use of phenytoin under the established guidelines of the Racing Commission, is approved for use in racehorses in PA. The use of any other medication with or without proven beneficial effect for CIR is unauthorized. It would be ill-advised to think that because lamotrigine is an AED as is phenytoin, its use in racehorses rises to the same guidelines as phenytoin. It is important to emphasize that there is no tolerance concentration for lamotrigine in plasma or urine at the time of participation of any racehorse in a sanctioned race by the Racing Commission in Pennsylvania. The purpose of this study was to demonstrate that if a sample of equine plasma contained lamotrigine, and was analyzed by this method there would be sufficient defensible and analytical data to demonstrate the presence of lamotrigine, and to quantify and confirm the presence of

lamotrigine in the sample. This SOP, therefore, describes a simple, rapid, sensitive and reliable LC/TSQ-MS-MS method to identify, quantify and confirm the presence or absence of lamotrigine in equine plasma samples.

SCOPE

This standard operating procedure describes the identification, quantification and confirmation of lamotrigine in equine plasma. Liquid-liquid extraction of equine plasma is employed with subsequent analysis by LC-MS (Thermo-Finnigan, San Jose, CA), consisting of a Surveyor MS pump with an on-line degasser, a Surveyor autosampler, and a TSQ Quantum mass spectrometer equipped with an electrospray ionization (ESI) probe. This method can be directly used as an instrumental screening method by appropriate alteration of the sample injection sequence. Clonixin is used as the internal standard (IS; Figure 1). The scope of this work covers procedures to be used in isolating from plasma, as the matrix, detecting, quantifying and confirming the presence or absence of lamotrigine (Figure 1) in equine plasma samples collected from racehorses. Reporting of a positive finding to the Racing Commission will be based solely on the results obtained by the LC/TSQ-MS/MS method described in this SOP. Any concentration of lamotrigine in equine plasma that does not meet the criteria presented by this SOP for reporting such a positive finding to the Racing Commissions, will be considered a negative finding and will be so reported to the appropriate PA Racing Commission.

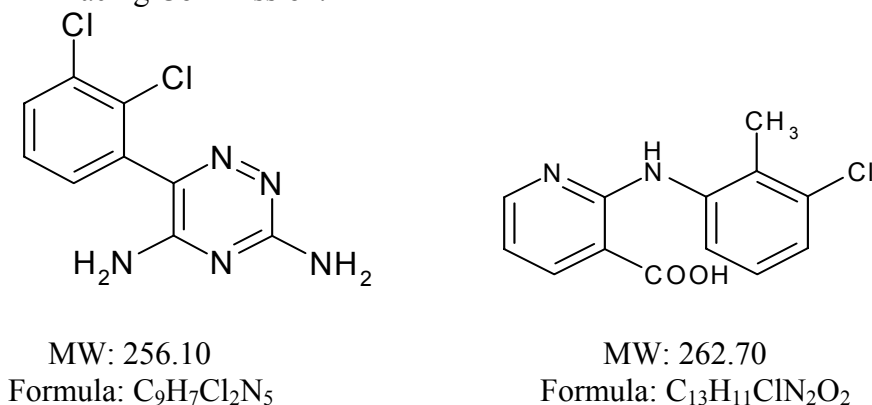


Figure 1. Chemical structures of Lamotrigine and Clonixin (IS)

PRINCIPLE OF METHOD

A sensitive method for rapid separation, identification, quantification and confirmation of lamotrigine in equine plasma by liquid chromatography coupled on-line with triple quadrupole Quantum tandem mass spectrometry (LC/TSQ-MS/MS; Thermo-Finnigan) is described in this SOP. Analysis is performed by positive electrospray ionization. Efficiency for analyte recovery using various solvents for extraction and pH (2-12) is determined. Methyl-tert butyl ether (MTBE) is the best solvent for extraction and cleanliness of the extract. Plasma sample (0.5 mL) is augmented with lamotrigine (0.1-100 ng/0.5mL), Clonixin is used as the internal standard. Plasma sample is directly extracted by using 5 mL MTBE, and then the extract is concentrated at 45 °C (Techne Dri-Block Concentrator). The residue is reconstituted in 100 µL of mobile phase solvent (2 mM ammonium formate (pH 3.4): ACN, 50:50, v/v) and 10 µL is used for analysis. The retention time of lamotrigine under the described LC-MS conditions is 2.98 min and 3.35 minutes for clonixin (IS). Gradient LC programming procedure is performed. An Ace 5 C₈ column (2.1 x 50 mm; Mac-Mod, Chads Ford, PA, USA, 1-800-) with its guard column is used for analyte

separation. Confirmation for the presence or absence of analyte is achieved by operating in multiple reaction monitoring (MRM) mode. The selected ions monitored are m/z 211 (BP), 166, 159 and 145 (please see Appendix A-3 on page 30). The limit of detection (LOD) is 50 pg/0.5mL ($S/N \geq 5$), limit of quantification (LOQ) is 0.1 ng/0.5mL with linear range of 0.1-50 ng/0.5mL ($r^2 > 0.995$), and limit of confirmation (LOC) is 0.5 ng/0.5mL. Entire analysis is completed in 5 minutes. The method accuracy and precision are also determined using three concentrations: low (0.5 ng/0.5 mL), medium (5.0 ng/0.5 mL) and high (50.0 ng/0.5 mL). The precision and accuracy at the selected concentrations for intra-day assay are 0.92% - 4.35% and 99.55% - 102.58%, respectively whereas those for inter-day assay are 1.29 - 2.11% and 97.92 - 101.96%, respectively. This method is fast, simple, sensitive and reliably reproducible.

SAFETY REQUIREMENTS:

A laboratory coat, protective goggles and gloves **MUST BE WORN** as the first line of protective measures taken while working in the laboratory at all times. All chemicals and reagents **MUST** be used in a fume hood. Transportation of reagents from the storage room to the workbench **MUST** be accomplished with the use of a reagent rubber bucket. Always observe the “STOP” signs and angle mirrors when coming out of the laboratory into the hallway. Foods and/or drinks are not allowed in the laboratory. All chemical wastes are to be properly transferred into waste containers pending waste removal by the contractor. Any situation or condition that compromises the safety of the laboratory personnel **MUST** be reported to the Safety Officer who must in turn inform the Lab Manager and Laboratory Director. Remember that the door of safety hinges on common sense.

PRIMARY REFERENCE STOCK SOLUTIONS

Primary Analytical Standard Reference Material

Lamotrigine (Sigma, Lot# 044K0720, Cat # L-3791, R-LAMO-1)

Chemical name: (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine)

Formula Weight: 256.10; Accurate Mass: 256.0151; Theoretical Mass: 256.0152

Primary Analytical Internal Standard Reference Material

Clonixin (Schering Research Division, Cat. # Sch 10304).

Formula Weight: 262.70

Obtain these materials from the QAO or Safe. Record accession of these materials on the pharmacy and Safe log sheets.

I. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

Lamotrigine: 1 mg/mL stock solution in methanol.

Materials

Lamotrigine free base

Methanol

Procedure

Weigh between 5 to 10 mg (X.xx mg) of lamotrigine standard powder into a glass bottle.

Dilute to volume using HPLC grade (or better) methanol (Volume Y.yy mL = X.xx).

Cap and mix until lamotrigine is completely dissolved in methanol.

The resulting concentration of lamotrigine is 1 mg/mL
Label, date, initial and store the resulting solution at approximately 4 °C (refrigerator).

Complete Balance Use Log and Record preparation for this process in the QA Reference Standard Preparation Log. Label the primary reference stock solutions with QA Primary Reference Log SR# (SR# 781) and Primary Reference Powder Designation (i.e. R-LAMO-1).

Clonixin (IS): 1 mg/mL of stock solution in methanol

Materials

Clonixin free base
Methanol

Procedure

Weigh between 5 and 10 mg (X.xx mg) Clonixin into a glass bottle.
Dilute to volume using HPLC grade (or better) methanol (Volume Y.yy mL = X.xx).
Cap and mix until Clonixin is completely dissolved in methanol.
The resulting concentration of Clonixin is 1 mg/mL
Label, date, initial and store the resulting solution at approximately 4 °C (refrigerator).

Complete Balance Use Log and Record preparation for this process in the QA Reference Standard Preparation Log. Label the primary reference stock solutions with QA Primary Reference Log SR# (SR# 63 and Lot #) and Primary Reference Powder Designation (i.e. R-Clon-1).

II. PREPARATION OF SECONDARY REFERENCE STOCK SOLUTIONS

Materials Needed: Lamotrigine Primary Reference Stock (1 mg/mL)
ACN:H₂O:FA (50:50:0.1, v/v/v): ACN = Acetonitrile, FA = Formic Acid
and H₂O = HPLC Water or better

Prepare Lamotrigine Secondary Reference Stock solutions according to Table 1 below:

Table 1. Standard working solution preparation of Lamotrigine

Target Conc. (µg/mL)	Made from stock solution (µg/mL)	Vol. Added (µL)	Vol. ACN/H ₂ O/FA (50:50:0.1) (µL)	Conc. Added 10 uL in 1 mL of plasma
10.0	1000.00	10	990	100
5.0	1000.00	5	995	50
1.0	10.00	100	900	10
0.5	5.00	100	900	5
0.1	1.00	100	900	1
0.05	0.50	100	900	0.5
0.01	0.10	100	900	0.1
0.005	0.05	100	900	0.05

Label secondary reference stock solutions with the Label designated in Table 1 and record preparation and labeling in the secondary preparation logbook in the appropriate Unit of the Laboratory.

III. PREPARATION OF LAMOTRIGINE QC WORKING SOLUTIONS

Materials Needed: Lamotrigine Primary Reference Stock Solution (1 mg/mL)
 ACN:H₂O:FA (50:50:0.1,v/v/v).

Prepare Lamotrigine QC Working solutions according to Table 2 below:

Table 2. Preparation of QC Working Solution of Lamotrigine

Target Conc. (µg/mL)	Made from stock solution (µg/mL)	Vol. Added (µL)	Vol. ACN/H ₂ O/FA (50:50:0.1) (µL)	Conc. Added 10 µL in 1 mL of plasma (ng/mL)
5.00	1000.00	5.0	995.0	50.0
0.50	5.00	100.0	900.0	5.0
0.05	0.50	100.0	900.0	0.5

Label Lamotrigine QC working solutions with the Label designated in Table 2. Record the preparation and labeling in the secondary preparation logbook in the appropriate Unit of the Laboratory.

IV. PREPARATION OF PLASMA CALIBRATORS AND QUALITY CONTROLS

The following calibrators are prepared in negative pooled equine plasma previously demonstrated by this SOP to be negative for lamotrigine. All lamotrigine plasma calibrators and QCs are freshly prepared the same day as the sample analysis.

Table 3. Preparation of Lamotrigine Calibrators in Equine Plasma

Calibrator Code #	Target Conc. (ng/0.5mL)	Working Solution (µg/mL)	Spike Working Solution (µL)	Volume of Plasma (mL)
MMDDYYLamotrigine0.1	0.10	0.01	10	0.5
MMDDYYLamotrigine0.5	0.50	0.05	10	0.5
MMDDYYLamotrigine1.0	1.00	0.1	10	0.5
MMDDYYLamotrigine5.0	5.00	0.5	10	0.5
MMDDYYLamotrigine10.0	10.00	1	10	0.5
MMDDYYLamotrigine50.0	50.00	5	10	0.5
MMDDYYLamotrigine100.0	100.00	10	10	0.5

Table 4. Preparation of Lamotrigine Equine Plasma Quality Control (QC) Sample

QC Code#	Target Conc. (ng/0.5mL)	Working Solution (µg/mL)	Spike Working Solution (µL)	Volume of Plasma (mL)
Plasma NQP XX MMY*	0.0	0.0	0.0	0.5
MMDDYYLamotrigine 0.5 QC	0.5	0.05	10	0.5
MMDDYYLamotrigine 5 QC	5.0	0.50	10	0.5
MMDDYYLamotrigine 50 QC	50.0	5.00	10	0.5

*: XX represents the batch number of lamotrigine free plasma, MMY represents the month and year the negative plasma was collected.

Record the preparation and labeling in the secondary preparation logbook in the appropriate Unit of the Laboratory.

Prepare 16x125 mm screw cap culture tubes (22 of 11 types from Table 3 and 4, for duplicate each calibrator and QC, respectively).

Dispensing of lamotrigine negative plasma, adding 0.5 ml plasma into each labeled tube.

Spiked 10 µL of each calibrators and QCs in Table 3 and 4 into individual tube, respectively.

Vortex and label each tube.

LABEL (format MMDDYYLamotrigineNG/MLCAL) and (format MMDDYYLamotrigine-NG/MLQC). Print label using AVERY Template 5267 in MS Word) 12 of each

V. PREPARATION OF CLONIXIN INTERNAL STANDARD WORKING SOLUTION

Materials 1 mg/mL of Clonixin primary reference stock
ACN:H₂O:FA (50:50:0.1, v/v/v)

Procedure

Dilute 10 µL of 1.0 mg/mL of Clonixin standard stock solution in a glass vial to volume using 990 µL of 2 mM ammonium formate, pH 3.4:acetonitrile (NH₄FA:ACN (50:50, v/v)). Mix.

The final concentration of Clonixin is 10 µg/mL.

Storage Requirements

Store at approximately 4 °C (refrigerator).

VI. SAMPLE REQUIREMENTS FOR ANALYSIS

Prepare Calibrators, Quality Control samples (including negative control), and suspect samples (in triplicate) for each analysis performed.

VII. SAMPLE PREPARATION BY LIQUID-LIQUID EXTRACTION

Remove one set of previously prepared 0.5 mL calibrators and quality control samples from freezer storage(-70 °C) and allow them to thaw at room temperature or in warm water bath.

1. Dispense 0.5 mL suspect sample (in triplicate) into individual clean, labeled 16x125 mm screw cap culture tubes.
2. Add 10 µL of 10 µg/mL clonixin into each tube, mix.

3. Add 5 mL of methyl-tert butyl ether (MTBE) into each tube (calibrators, controls, and samples) and extract for 5 minutes by mixing on a rotorack. Centrifuge at 3000 rpm for 5 minutes.
4. Transfer the organic phase (top layer) into a clean, labeled culture tube.
5. Evaporate to dryness at 45 °C under steady stream of nitrogen or air.
6. Reconstitute the residues with 100 uL pH 3.4 of 2 mM NH₄FA:ACN (50:50, v/v).
7. Transfer the above solution into labeled auto sampler vials fitted with limited volume inserts, and then cap. All the samples are now ready for LC/MS/MS analysis.

VIII. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF LAMOTRIGINE

Liquid Chromatography and Mass Spectrometer Operating Parameters

Instrumentation: Thermo-Finnigan TSQ Quantum mass spectrometer LC-MS system equipped with an electrospray ionization (ESI) probe. a Surveyor MS pump with an on-line degasser, a Surveyor autosampler. Xcalibur software is used for system control, acquisition, and data processing.

LC Column: MAC-MOD Ace C₈ column with guard column (Mac Modd, Chadds Ford, PA, USA, 1-800-441-7508)
Length: 50 mm
i.d. 2.1 mm
Particle size: 5 micron
Temperature: 27 ° C

Mobile Phase: A: 2 mM Ammonium formate (pH 3.40)
B: Acetonitrile

Gradient for Lamotrigine:

Table 5. HPLC Condition for the Separation of Lamotrigine

Time (min)	A	B	FlowRate (ul/min)
0	90	10	250
1.00	90	10	250
1.01	10	90	250
4	10	90	250
4.01	90	10	250
5.00	90	10	250

Injection Volume: 10 µL

Mass Spectrometric Parameters:

Table 6. Mass spectrometric parameters for Analysis of Lamotrigine and Clonixin (IS)

Compound	Retention time (min)	[M+H] ⁺	BP Product ion	Tube Lense Offset	CE	Spray Voltage	Sheath gas pressure	Aux gas pressure	Q1 Source CID
Lamotrigine	2.99	256.0	211.0	113/68	10	4100	41	18	20
Clonixin	3.36	263.0	245.0	109/94	18	4800	21	15	18

For both Lamotrigine and Clonixin capillary temperature was set at 285 °C and collision gas pressure was at 1.5 mTorr.

IX. SAMPLE LIST SETUP FOR LAMOTRIGINE ANALYSIS

1. Mobile Phase Blank
2. Mobile Phase Blank
3. Column test1
4. Mobile Phase Blank
5. Mobile Phase Blank
6. Column test2
7. Mobile Phase Blank
8. Mobile Phase Blank
9. Column test3
10. Mobile Phase Blank
11. Mobile Phase Blank
12. Blank Plasma (QC Negative Control)
13. Calibrator Series 1
14. Mobile Phase Blank
15. Mobile Phase Blank
16. Quality Control Series 1 (Positive Controls)
17. Mobile Phase Blank
18. Mobile Phase Blank
19. Sample 1, Replicate 1
20. Sample 1, Replicate 2
21. Sample 1, Replicate 3
22. Repeat steps 17 through 21 for each additional sample
23. Mobile Phase Blank
24. Mobile Phase Blank
25. Quality Control Series 2 (Positive Controls)
26. Mobile Phase Blank
27. Mobile Phase Blank
28. Calibrator Series 2
29. Mobile Phase Blank;
30. Mobile Phase Blank

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20 ng/ml Lamotrigine in 2 mM NH₄FA : MeOH (60:40)

RT: 0.00 - 6.00 SM: 3B

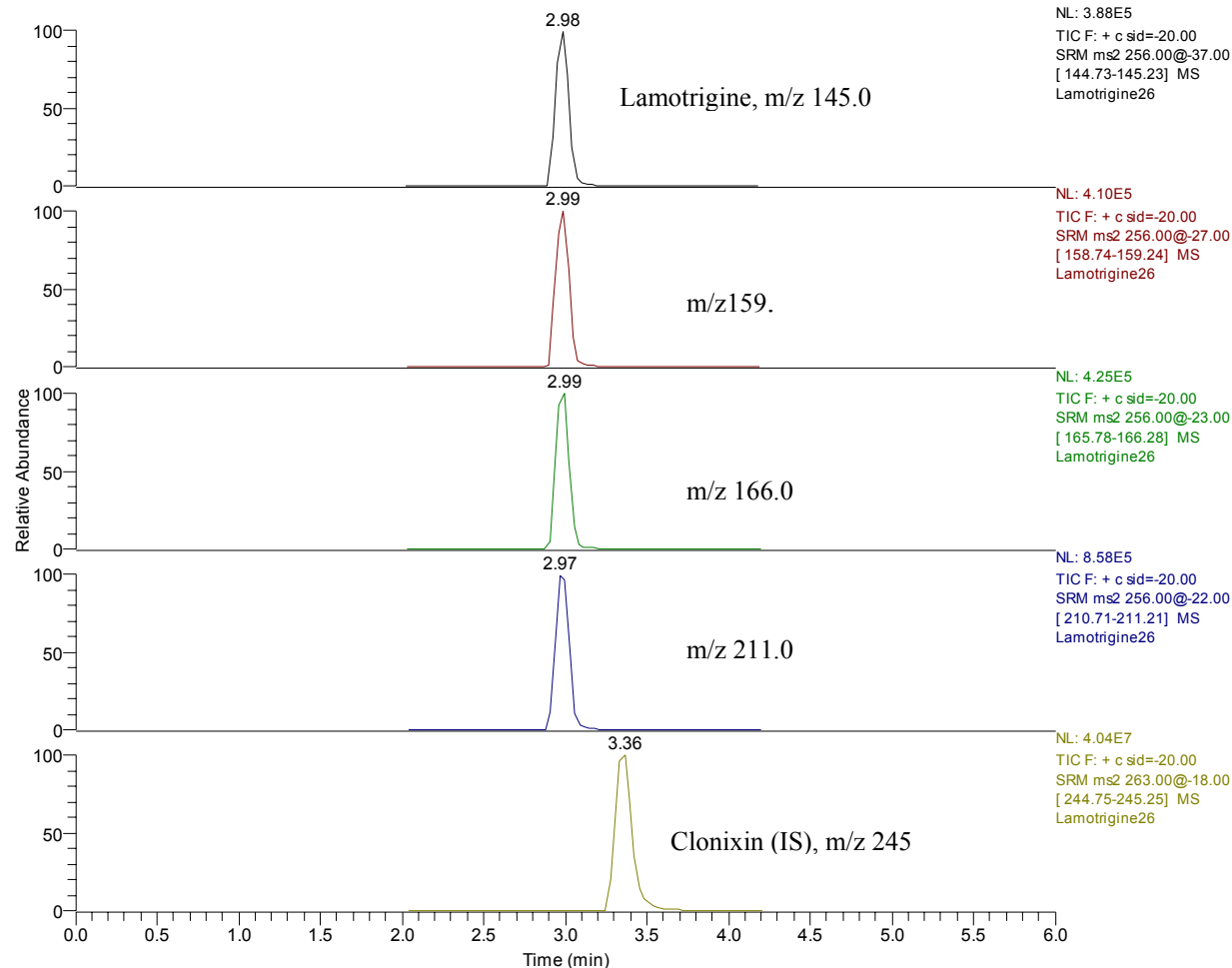


Figure 5. MRM chromatograms of lamotrigine in equine plasma extract

X. CRITERIA FOR IDENTIFICATION OF LAMOTRIGINE FROM EQUINE PLASMA EXTRACT

Identification of Lamotrigine

The qualifying diagnostic ions for identification of lamotrigine are m/z 256 [M+H]⁺, 211(BP), 166, 159 and 145 (Figure 4) at the same retention time of the MRM chromatograms.

All qualifying ions for lamotrigine are present in the MRM chromatogram, the relative coefficient variation mass ratios should be less than 5% (Table 7), and the retention time for the suspect sample, 5 ng/mL calibrator, and 5 ng/mL QC control should agree to $t_R \pm 0.15$ minutes for each individual analyte QC control, respectively (Figure 5).

Table 7. Mass ratio determination of Lamotrigine MRM confirmation in Equine Plasma (n=45)

Product ions	Mass Ratio determined ^a	C.V.(%)
145	0.482 ± 0.0158	3.28
159	0.453 ± 0.0124	2.74
166	0.477 ± 0.0101	2.12
211	1.000	0.00

a: Mass Ratio was determined in the concentration range from 0.1 ng/ml to 50 ng/ml.

Four product ions, m/z 145, 159, 166 and 211 are selected as confirmation ions in MRM determination. The ratios of m/z 145/211, 159/211, 166/211 and 211/211 are calculated based on peak area.

Identification of Clonixin (IS):

The qualifying and quantifying ions for the internal standard, Clonixin, are m/z 263 [M+H]⁺ and 245 (BP). Under LC-MS/MS analytical conditions described in this SOP, all the above diagnostic ions should be recognized at retention time of ~2.85 min.

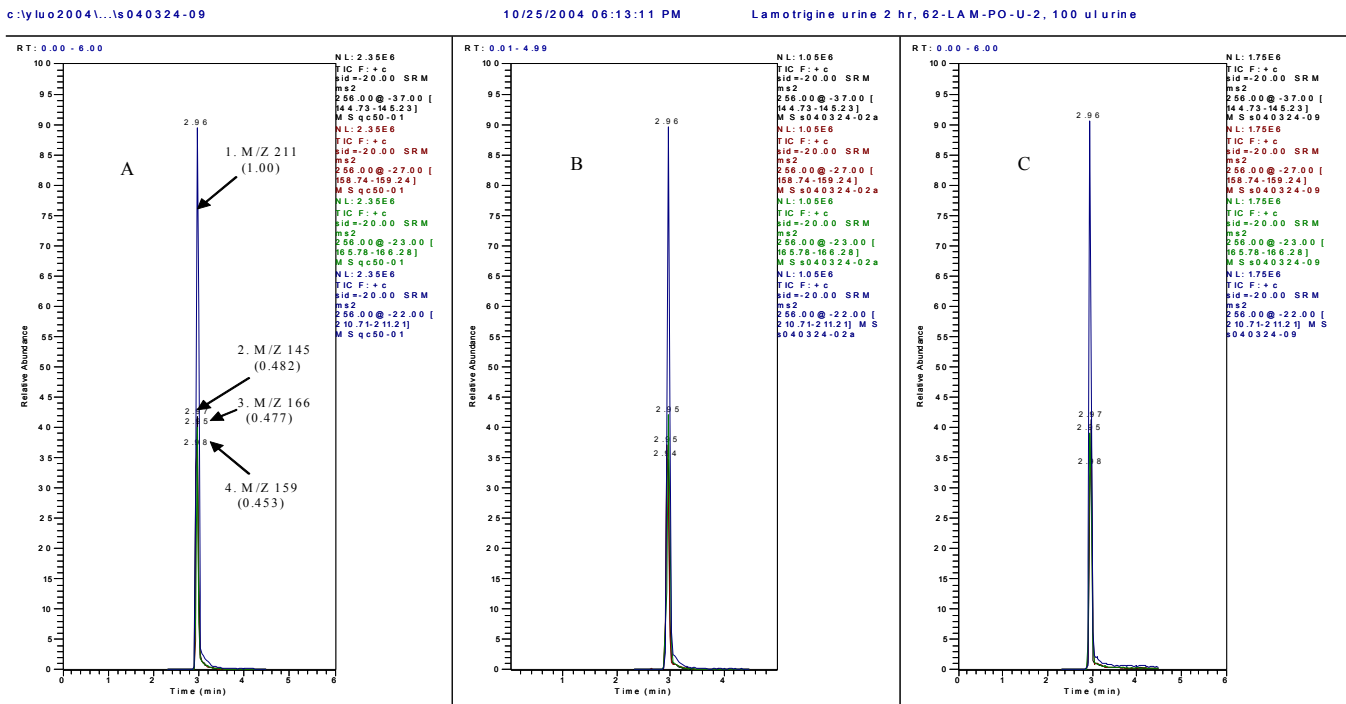


Figure 6. Comparison of Lamotrigine MRM chromatograms of QC with lamotrigine Administration (130 mg, po). A = 50 ng/mL QC;

B = Lamotrigine 2 hours plasma sample post administration; C = horse urine sample 2 hr post lamotrigine po administration

XI. CRITERIA FOR LAMOTRIGINE QUANTIFICATION IN EQUINE PLASMA

Determination of Lamotrigine

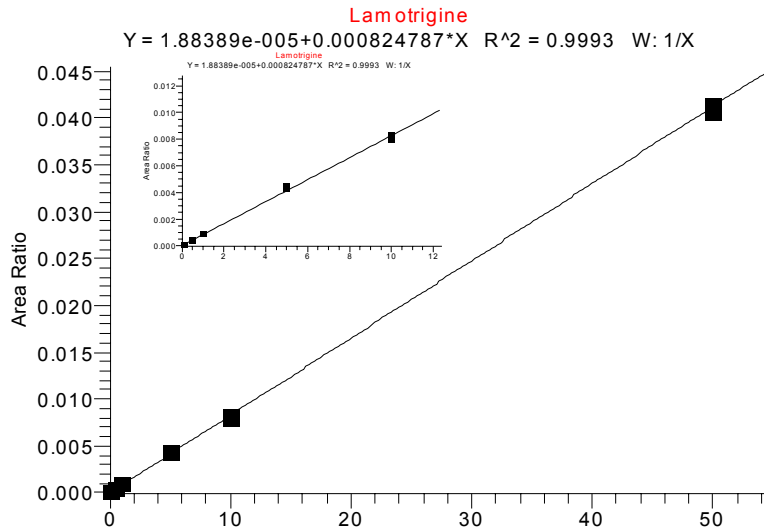


Figure 7. Calibration Curve of Lamotrigine in Equine Plasma.

The graph insert represents calibration curve for the lower concentrations of lamotrigine. Using the Xcalibur Quantification software, execute quantification method\Lamotrigine\LC MSmethod\LamotrigineMRM. Print the compound summary quantification report and calibration curve. The correlation should be greater than 0.995%.

Examine the reported concentration for all samples. The accuracy of concentration of QC samples should be 80% - 120% for lamotrigine.

The selected product ion of lamotrigine for quantification is m/z 211.

XII. CRITERIA FOR REPORTING A POSITIVE SAMPLE FOR LAMOTRIGINE

Report a test sample as positive per this standard operating procedure for lamotrigine if ALL of the following criteria are met:

The test sample contains lamotrigine according to the chromatographic and mass ratios of MRM peak areas (Figure 7), the difference in mass ratios should be less than 10%.

All confirmable suspect samples for MRM chromatograms should contain four selected ions of lamotrigine (m/z 211, 166, 159 and 145; Appendix A-2 on page 27).

The LC retention times of the quantifying ion for lamotrigine in the sample, 5 ng/mL QC control and the 5 ng/mL calibrators should be within +/- 0.15 minutes. This is determined by inspection of the extracted ion chromatogram comparisons that are included in the analysis data packet. These chromatograms may be subtracted and/or smoothed.

Table 8. Comparison of mass ratios of Lamotrigine in administration sample with Calibrator and QC Samples

m/z	Ratios of mass Intensity			Difference of mass ratio (%) ^a	
	Mean ^b ± SD	Plasma (n=14)	Urine (n=14)	Plasma	Urine
145	0.482 ± 0.0158	0.482 ± 0.00537	0.484 ± 0.00639	0.00	0.41
159	0.453 ± 0.0124	0.450 ± 0.00595	0.452 ± 0.00546	-0.67	-0.22
166	0.477 ± 0.0101	0.473 ± 0.00659	0.481 ± 0.0387	-0.85	0.83
211	1.000	1.000	1.000	0.00	0.00

^a. Mass ratio difference was determined by (mass ratio of analyte's product ion – mean mass ratio)/mean mass ratio x 100

^b. Mean mass ratios were determined by 45 calibrators and QCs of lamotrigine in equine plasma in the concentration range of 0.1 ng/mL - 50 ng/mL.

The signal to noise ratio of the quantifying ions for lamotrigine and internal standard (Clonixin) is greater than 5. This is determined by inspection of the data dependent scan mass spectra comparisons, which are included in the analysis data packet. These spectra should be averaged across the chromatographic peak at 20 % peak height. These spectra may be subtracted and/or smoothed.

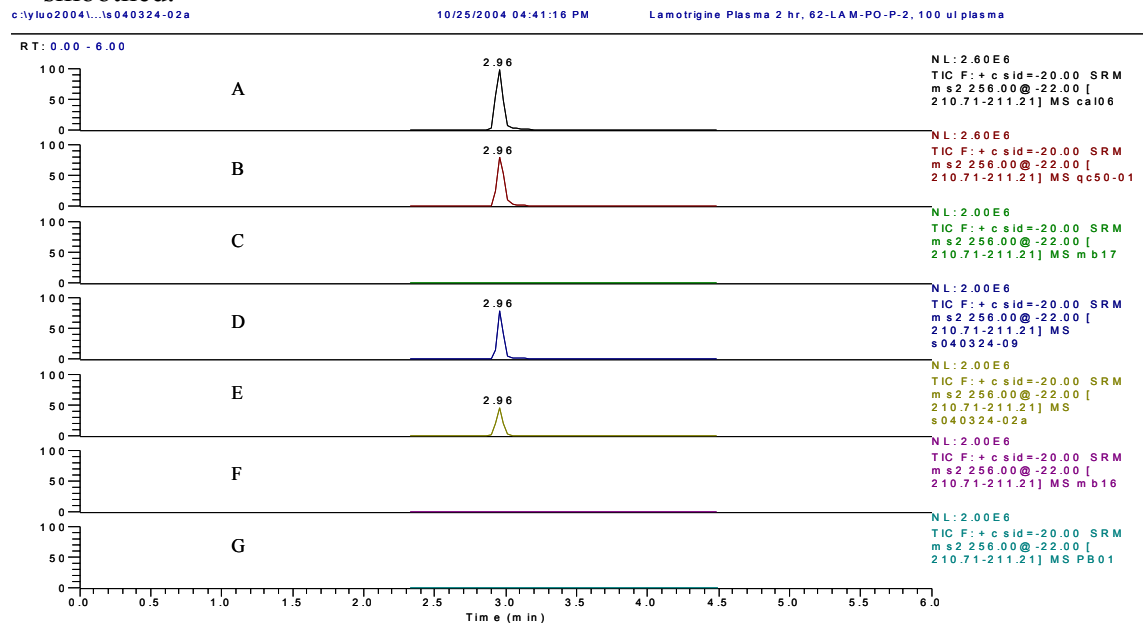


Figure 8. Comparison of chromatograms of po lamotrigine administration sample with lamotrigine calibrator, QC and negative control samples.

A = plasma calibrator, 50 ng/mL; B = plasma QC, 50 ng/mL; C = mobile phase blank;

D = urine sample post lamotrigine administration; E = plasma sample post lamotrigine administration; F = mobile phase blank; G: negative control plasma.

All blank and Negative Plasma Control Samples should not contain quantifiable and/or confirmable lamotrigine concentration greater than 0.1 ng/mL.

XIII. INTEGRATION

The integration parameters of the quantifying method (\Lamotrigine\LC-MS\ have been set to produce consistent and reproducible integration from run-to-run and from day-to-day. However, samples and conditions vary; therefore each chromatogram in the analysis panel must be individually inspected for proper integration. If improper integration is found, it may be manually corrected. With excessive manual corrections, the supervisor must be consulted.

XIV. METHOD VALIDATION

Extraction Efficiency of Lamotrigine Using Various Organic Solvents

The extraction efficiency of lamotrigine by different organic solvents is experimentally compared at pH 7.0. The results are listed in Figure 8. Of all the evaluated organic solvents used, MTBE is the most efficient extraction solvent for lamotrigine extraction from equine plasma.

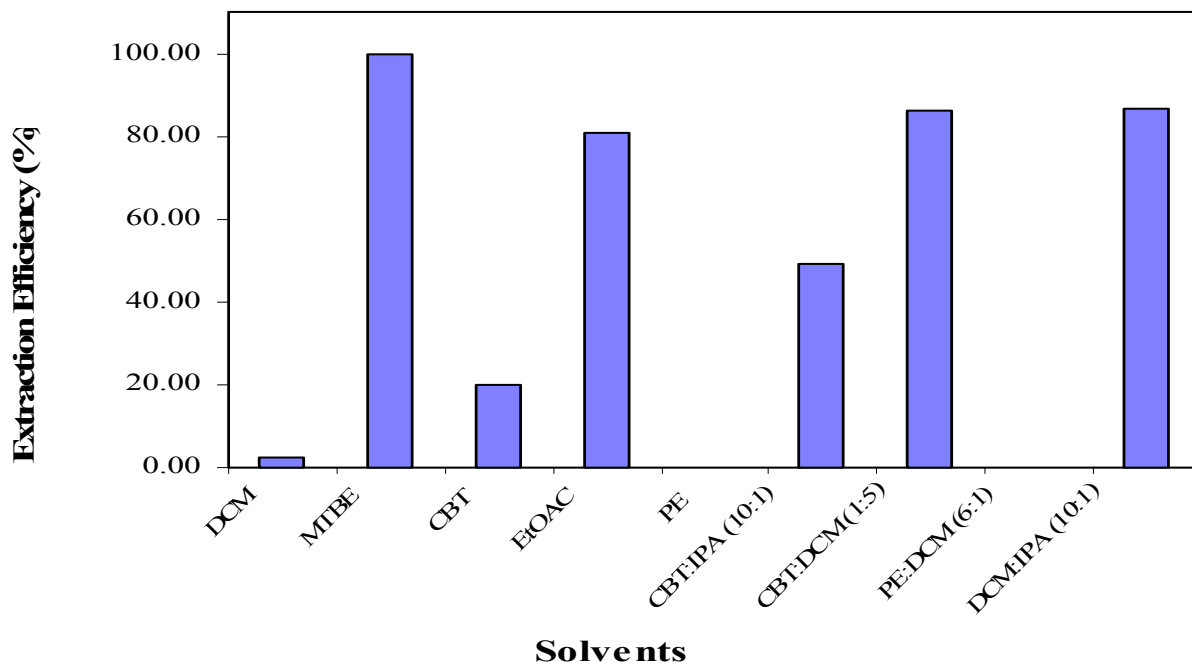


Figure 8. Extraction efficiency of various organic solvents for Lamotrigine (5 ng/mL) spiked into blank equine plasma (n=5). CBT=Chlorobutane; PE=Petroleum Ether.

Since MTBE provided the best extraction efficiency than any other evaluated solvents, it was chosen as the extraction solvent for use in this SOP.

Effect of Various pH Values on the Extraction of Lamotrigine from Equine Plasma

The effect of various pH values on the extraction of lamotrigine from equine plasma was compared. Figure 9 shows the influence of pH values on the recovery of lamotrigine from plasma matrix. Sample at pH 6 to pH 7.5 provided the best recovery of lamotrigine from plasma matrix and thus all samples are extracted by MTBE at neutral condition (see extraction procedure for lamotrigine on pages 7 and 8).

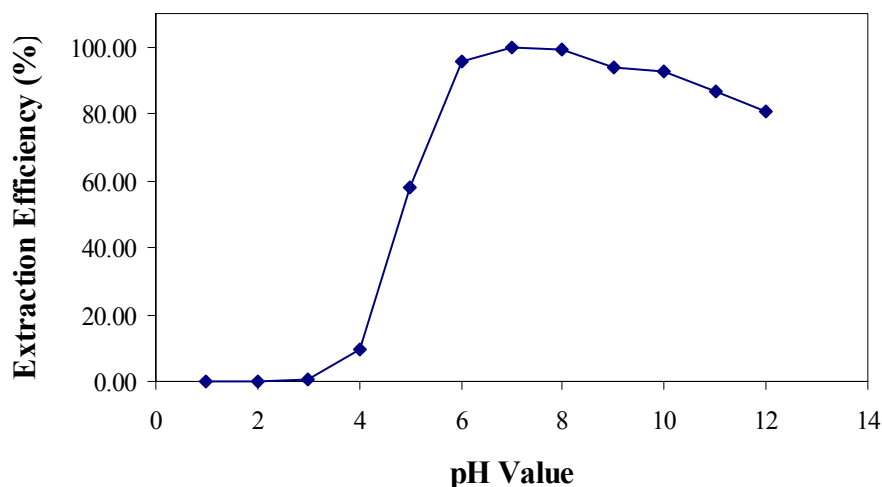


Figure 9. Influence of pH on the Extraction of Lamotrigine (5 ng/mL spiked; n=5).

Recovery of Lamotrigine from Equine Plasma

All samples are extracted with MTBE at neutral condition. MTBE extraction provided the highest extraction efficiency for lamotrigine from equine plasma than any other solvent evaluated. The recovery of lamotrigine by MTBE from equine plasma at the concentration range of 0.5 ng/mL to 50 ng/mL is greater than 91% with coefficient of variation (CV %) of less than 6.0%. The recovery of varying concentrations of lamotrigine and IS (100 ng/mL) from equine plasma is shown in Table 9.

Table 9. Recovery of Lamotrigine from Equine Plasma (n=6)

Concentration spiked (ng/mL)	Concentration determined (ng/mL)	Recoveries (%)	C.V*. (%)
0.5	0.463 ± 0.027	92.60	5.83
5.0	4.549 ± 0.171	90.98	3.76
50.0	45.803 ± 1.253	91.61	2.74
Clonixin (IS) 100 ng/mL	95.64 ± 0.0715	95.64	0.07

*C.V. (%) = standard deviation of the concentration detected/mean concentration detected x 100.

METHOD VALIDATION

The method is validated under the guidelines presented by Shah et al². Twelve assays for validation are performed; six for within-run (intra-day assay) and six for between-run (inter-day) to assess precision, accuracy and specificity of the method.

Intra-day and Inter-day Precision and Accuracy

Intra-day and inter-day assays for accuracy and precision are determined by analyzing two separate sets of eighteen validation samples at three separate lamotrigine concentrations (0.5, 5 and 50 ng/mL equine plasma) in six separate experiments, respectively. The concentrations of lamotrigine correspond to low, medium and high for constructing the calibration curves. Intra-day assay accuracy and precision are determined by analyzing six replicates of the three concentrations in each experiment. Inter-day assay accuracy and precision are determined by analyzing triplicates of the three concentrations in six different days, respectively. Accuracy is determined as the agreement between the concentration of the target analytes detected and that spiked into blank plasma. Precision of the assay is determined as the relative standard deviation expressed as a percentage of the standard deviation divided by the mean of observed concentration and is reported as percent coefficient of variation.

Table 10. Intra-day and Inter-days Precision and Accuracy of Lamotrigine in Equine Plasma (n= 6)

Lamotrigine added (ng/ml)	Intra-day			Inter-day		
	Lamotrigine determined	C.V. ^a (%)	AR ^b (%)	Lamotrigine determined (ng/ml)	C.V. ^a (%)	AR ^b (%)
0.5	0.499 ± 0.0217	4.35	99.80	0.497 ± 0.0105	2.11	99.40
5.0	5.129 ± 0.112	2.18	102.58	5.098 ± 0.0886	2.19	101.96
50.0	49.777 ± 0.458	0.92	99.55	48.961 ± 0.633	1.29	97.92

a: Coefficient of variation (C.V. %) = standard deviation of the quantity detected/mean quantity detected x 100.

b: Accuracy (AR %) = mean of detected quantity/spiked quantity x 100.

EVALUATION OF STABILITY OF LAMOTRIGINE IN EQUINE PLASMA

Since the official race samples are not delivered to the laboratory for analysis on the same day that the race is conducted, plasma samples are usually stored at -20 °C for a day or two after the blood

is collected at the racetrack. For this reason, the effect of temperature on the stability of lamotrigine in plasma is critical for maintaining the integrity of the samples so that accurate information can be obtained during analysis. Considering the fact that the samples may encounter different temperature conditions during transit and thus take longer to reach the laboratory during the sample delivery process, the stability experiment is designed to accommodate or reflect the possible conditions the samples are likely to be exposed to from collection to delivery to the laboratory for analysis. In this SOP, lamotrigine in three different concentrations (0.5 ng, 5.0 ng and 50 ng) are examined. Each concentration is spiked into 0.5 mL negative equine plasma. The stability of the analyte is evaluated at different storage temperatures (25 °C, 4 °C, – 20 °C and -70 °C). At room temperature (25 °C), 24 hr stability (0, 2, 4, 6, 8, 10 and 24hr) for the spiked plasma samples is evaluated. For short-term storage (4 °C), stability of the analyte is evaluated following storage for 10 days, and for long-term storage (– 20 °C and – 70 °C) stability of the analyte is evaluated after storage at these temperatures for five weeks (35 days). It should be noted that at each time period, samples are thawed, the desired aliquots are taken for analysis and the original samples are returned for storage at the previous temperature. This process allows evaluation of the effect of freeze-thaw cycles on the stability of the analyte (lamotrigine). The peak areas averaged from triplicate samples are compared with those of the relevant 0 hr sample and other samples at different times and different temperatures from which the percent change of the analyte at the specified temperature is calculated.

Table 11. Stability of Lamotrigine in Equine Plasma at Various Temperatures (n=3)*

Concentration added (ng/mL)	Concentration percentage determined at different temperature and time				
	Hour 0	25 °C, 24 hours	4 °C, 10 Day	– 20 °C, 35 Days	– 70 °C, 35Days
0.5	100	91.90 ± 7.65	92.08 ± 2.58	95.00 ± 4.83	96.90 ± 4.56
5	100	104.73 ± 2.76	95.05 ± 6.77	98.67 ± 3.67	100.20 ± 7.45
50	100	100.22 ± 1.40	96.74 ± 1.55	100.09 ± 4.81	100.00 ± 2.29

*Stability is expressed as percent concentration (concentration detected divided by concentration added x 100).

Demonstration of the Absence of Ionization Suppression or Enhancement

Co-eluting substances with parent ions differing from the target parent ion may exert either enhancement or suppression of the ionization process, thus posing a severe challenge to the validity of quantitative results. Therefore, ionization stability is determined for the chromatographic and mass spectrometric conditions described in this SOP.

Lamotrigine is examined at three different concentrations (0.5 ng/mL, 5 ng/mL and 50 ng/mL). Each concentration is added to each dried extract of 0.5 mL negative equine plasma followed by

drying and dissolution in 100 µL LC sample solvent. The same concentration of each analyte in methanol is dried and dissolved in the same volume of the LC sample solvent. An aliquot of 10 µL is injected into LC-MS and analyzed. The chromatographic peak areas averaged from six sample duplicates for the standards in sample solvent and for the standards added to plasma extracts and reconstituted as described above are used for estimation of the contribution of the sample matrix, plasma in this case, to ion suppression or enhancement.

Endogenous compounds extracted from a sample matrix might suppress or enhance ionization of an analyte(s) recovered from that matrix resulting in the change of signal intensities of the analyte(s), especially when ESI mode is applied. Matrix effect may occur in any biological samples, including plasma and urine, etc. In this experiment, matrix effect on the analysis of lamotrigine in plasma is evaluated. As mentioned in the experimental section of this SOP, matrix effect is determined by comparing the chromatographic peak areas of each drug standard with those of the drug standard added to the extracts of 0.5 mL negative plasma, according to the following equation below:

Table 12. Matrix Effect of Equine Plasma on Lamotrigine LC-MS Analysis (n=6)

Lamotrigine spiked (ng/ml)	Blank plasma	Determined standard only (ng/ml)	Determined with blank plasma extract (ng/ml)	Matrix effect ^a (%)
0.5	0.00	0.42 ± 0.006	0.40 ± 0.053	-4.76
5.0		5.77 ± 0.094	5.51 ± 0.35	-4.51
50.0		48.94 ± 6.392	47.18 ± 1.57	-3.60

a. Matrix effect was determined by (determined with blank plasma extract – Lamotrigine spiked)/determined standard only x 100%

Ion suppression or enhancement (%) = $(1 - A_{\text{extract}} / A_{\text{standard}}) \times 100$:

where A_{standard} is the concentration of a drug standard, and A_{extract} is the concentration of the same quantity of the drug standard added to the extract of a given plasma sample. As shown in Table 12, ion suppression or enhancement by plasma is less than 5% for all lamotrigine results evaluated. The results show that the contribution by matrix effect on the analysis of lamotrigine in plasma is insignificant under the experimental conditions described.

MEASUREMENT UNCERTAINTY

The following statements define the estimation of Measurement Uncertainty (MU):

Table 13. Estimation of Measurement Uncertainty in the Quantification of Lamotrigine in Plasma

Symbol	Source of Uncertainty	Value Units (%)	Distribution	Divisor	Standard Uncertainty	Degrees of Freedom (n-1)	Other
U ₁	Intermediate precision	3.04	N	1	3.04	10	Lamotrigine 0.5 ng/ml
U ₂	Intermediate precision	2.58	N	1	2.58	10	Lamotrigine 5 ng/ml
U ₃	Intermediate precision	1.18	N	1	1.18	10	Lamotrigine 50 ng/ml
Combined Uncertainty		1: (U ₁ ²) ^{1/2} = 3.04; 1: (U ₂ ²) ^{1/2} = 2.58; 1: (U ₃ ²) ^{1/2} = 1.18					
Expanded Uncertainty (k=2.3)		1: (3.04 x 2.3) = 6.99%; 1: (2.58 x 2.3) = 5.93%; 1: (1.18 x 2.3) = 2.71%					

Method Uncertainty is initially established based on method validation.

1. The 95% confidence interval is expressed as +/- Standard Deviation x Coverage factor (k) (SD x k) for both unknown determinations as well as control values.
2. In using Laboratory Control Samples (LCS) or Quality Control Samples (QCS) for estimating MU, k= 2.3 (Coverage Factor) is used.
3. Control records and charts (5 ng/mL n=4) for method development and all analyses are created and maintained.

An Excel template is provided to perform several quality control functions. For every analysis, all samples will be entered into the template.

Automatic building and charting of historical control database with run and historical 95% confidence interval plotting will be maintained.

Each entry is saved to the project (\\Xcalibur\Lamotrigine\Data) folder and is maintained as part of the analysis record archive.

Demonstration of Specificity of the Method:

Specificity of method is defined as the ability to measure the analyte of interest accurately and specifically in the presence of other components that could be expected to be present in the sample matrix. It is a measure of the degree of interference from active ingredients, impurities and unknown products, for example. Specificity as an identification test ensures the identity of the analyte of interest by the method described. For this purpose, an administration of Lamotrigine (130 mg; po) to a research horse was performed at the University of Pennsylvania School of Veterinary Medicine, New Bolton Center Campus, to evaluate the applicability of the method described in this SOP. Pre and post urine and plasma samples were collected. Post administration

samples were collected at 2, 6, 8, 10, 24 and 48hrs. The concentration of Lamotrigine in plasma and urine are shown in the Table (14) and Figure (11) below. The concentrations of lamotrigine in plasma (22.94 ng/mL) and urine (33.07 ng/mL) were the lowest at 48 hrs compared to other time periods post lamotrigine administration. The MRM confirmation results are shown in Table 15 and Figure 12.

Table 14. Plasma and urine concentrations of Lamotrigine

Time(hr)	Plamsa (ng/0.5ml)	Urine (ng/0.5ml)
0	0.00	0.00
2	53.42	84.19
6	61.00	95.83
8	50.04	93.19
24	45.53	82.81
48	22.94	33.07

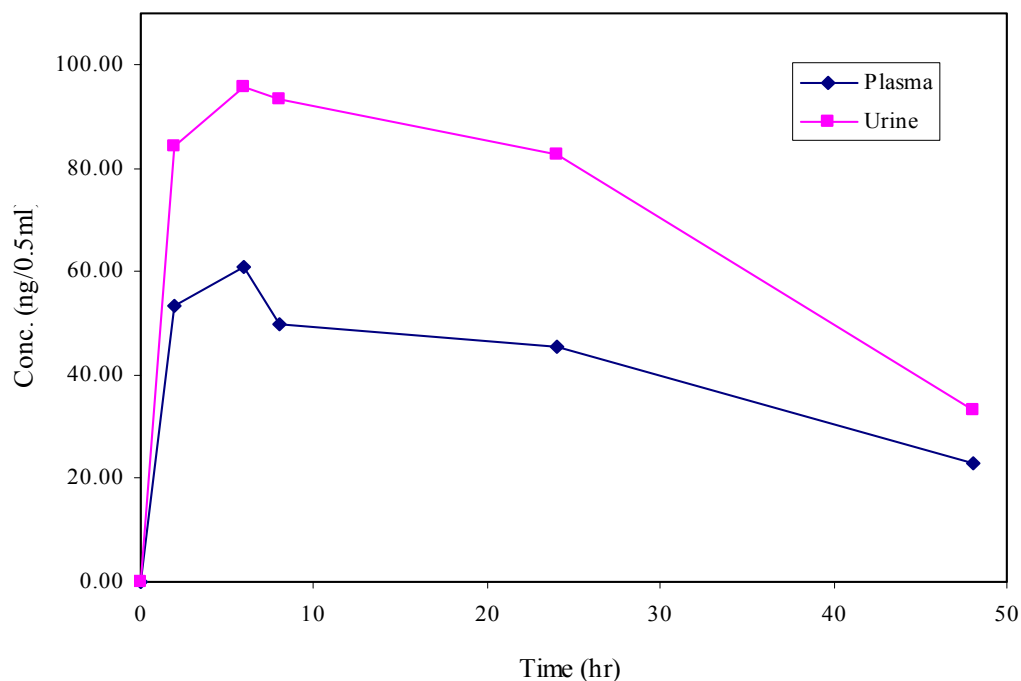


Figure 11. Concentration-time curve of lamotrigine in equine plasma (blue line) and urine (pink line) after 130 mg administration (po).

Figure 11 shows the plot of concentration of lamotrigine in plasma (blue diamond line) and urine (pink square line) versus time. It is obvious that with a single oral dose of lamotrigine (130 mg, po) administration to a horse, the concentration of lamotrigine in urine is significantly higher than that in plasma and remains high into 48 hr post lamotrigine administration. Figures 12 shows MRM chromatograms of lamotrigine in equine plasma and urine 48 hours post the administration of lamotrigine. The numbers in parentheses show the ratios of the most intense product ion (211 m/z) to other product ions at the same retention time. These results indicate that by using the method described in this SOP, the presence of lamotrigine in both plasma and urine samples can be confirmed. The method is, therefore, specific for the detection, identification, quantification and confirmation of lamotrigine in equine plasma and is equally applicable to urine.

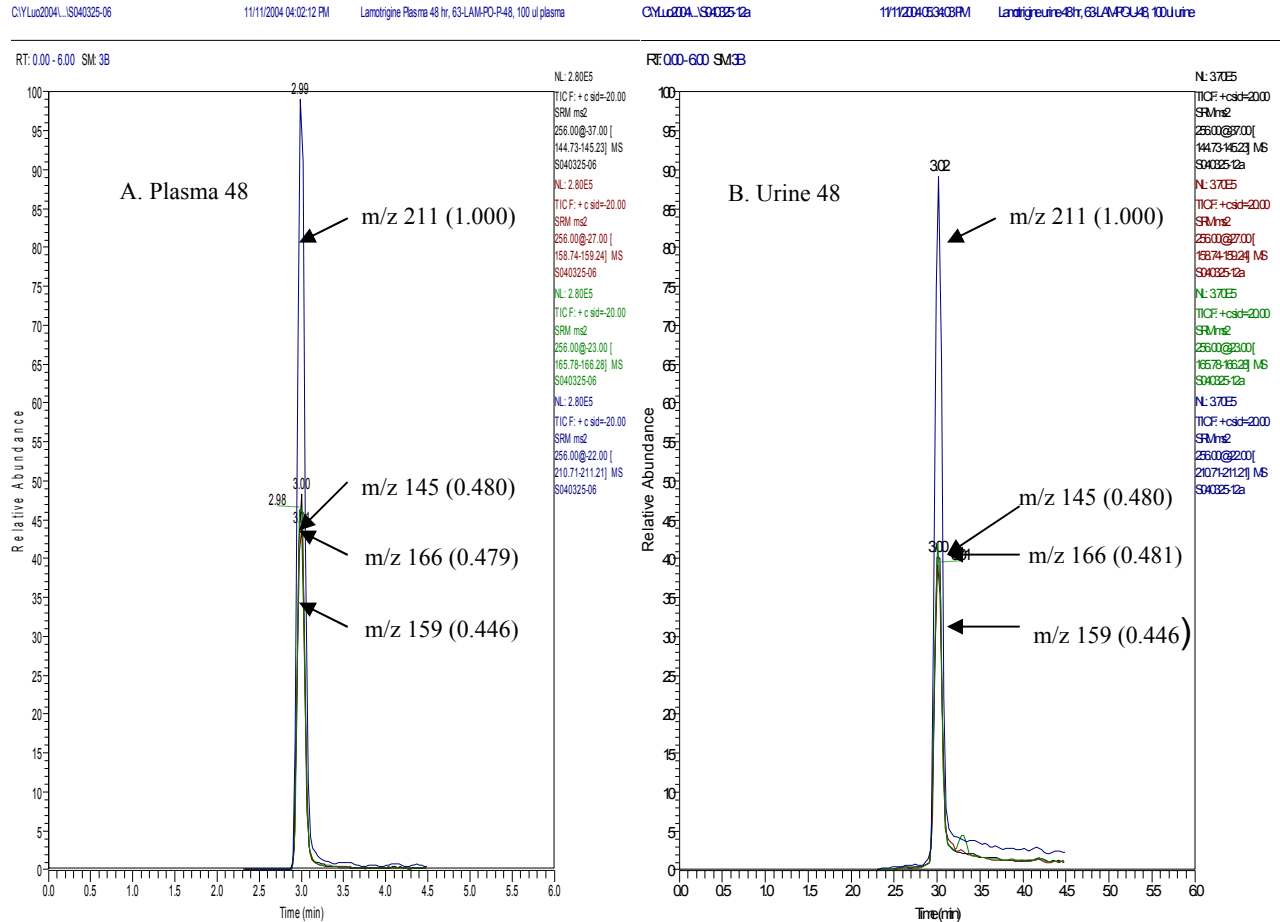


Figure 12. MRM chromatograms of Lamotrigine at 48 hours collection following 130 mg Administration, po. A = plasma sample 48 hr; B = urine sample 48 hr collection.

Table 15. Mass ratio Comparison of Lamotrigine in post administration samples with Calibrator and QC used for confirmation (n=40)

m/z	Ratios of Mass Intensity			Difference of mass ratio determined (%) ^a	
	Mean ^b ± SD	Plasma samples (n=14)	Urine Samples (n=14)	PO Plasma	PO Urine
145	0.478 ± 0.0066	0.480 ± 0.0043	0.480 ± 0.0036	0.41	0.41
159	0.446 ± 0.0059	0.446 ± 0.0052	0.447 ± 0.0038	0.00	0.22
166	0.479 ± 0.0084	0.479 ± 0.0045	0.481 ± 0.0031	0.00	0.42
211	1.000	1.000	1.000	0.00	0.00

^a: Mass ratio difference was determined by (mass ratio of analyte's product ion - mean mass ratio)

/mean mass ratio x100

^b: Mean mass ratio was determined by 45 calibrators and QC's of Lamotrigine in the concentration range from 0.1 ng/mL to 50 ng/mL.

XV. POSITIVE SAMPLE DATA PACKET ASSEMBLY ORDER

1. SAMPLE TRANSFER SHEET (WS#32)
2. SAMPLE USAGE SHEET (FORM #7)
3. CONFIDENCE DETERMINATION REPORT
4. SAMPLE LIST
5. MASS CALIBRATION REPORT
6. TUNE PAGE SETTINGS
7. LC METHOD
8. MS METHOD
9. QUANTIFICATION REPORT
10. QUANTIFICATION CALIBRATION CURVE
11. COLUMN TEST CHROMATOGRAM
12. COLUMN TEST SPECTRUM
13. EXTRACTED ION CHROMATOGRAM COMPARISON
14. SPECTRA COMPARISON

Other required Documentation

In addition to the positive data packet, the following documentation is required:

Sample list print-out that is maintained in the Quantum TSQ of three ring-binder
Routine usage checklist completion (and maintenance log as applicable)
Sample Analysis logbook, indicating date, project, operator initials, and description of official samples

Data packets for samples determined to be negative will contain the follow elements:

1. SAMPLE TRANSFER SHEET (WS#32)
2. SAMPLE USAGE SHEET (FORM #7)
3. CONFIDENCE DETERMINATION REPORT
4. QUANTIFICATION REPORT

XVI. DATA ARCHIVING

After successful assembly of required paper documentation for official analyses, whether positive or negative the data will be archived in the following manner:

On the Xcalibur workstation \\C: hard drive, create the following folder, if not already present:

\\C:\Project name (Drug name) for instance

C:\Yluo2004\Lamotrigine

Under this folder, create a file with the following format:

DRUGmmddy for instance:

C:\YLuo2004\Lamotrigine\data\LamotrigineSample120104

Now have ALL data placed there automatically.

This manner of archiving is required by the Xcalibur software to allow the easiest and most complete reconstruction of all analysis.

In addition to this local archive, the hard drive is backed up on a weekly basis by our site Information Technologist.

Materials, Reagents, and Formulae

I. REAGENTS

- Methanol, HPLC grade (Cat. No. A 452-4, Fisher Scientific.)
- Acetonitrile, HPLC grade (Cat. No. A 452-4, Fisher Scientific.)
- Water, HPLC grade (Cat. No. W5-4, Fisher Scientific.)
- Methyl-tert Butyl Ether (MTBE), HPLC grade. No. e127-4, Fisher Scientific)
- Ammonium Hydroxide, Certified A.C.S. PLUS No.A669C-21, Fisher Scientific.)
- Phosphoric Acid, meets A.C.S. Specification (Cat. No. 0260-3, J.T. Baker Chemicals)
- Formic Acid, ACS reagent (EEC No. 200-5791, Sigma)
- Monobasic Potassium Phosphate, ACS reagent (Cat. No. P-0662, Sigma)

II. SOLUTIONS

Record preparation of solutions in the Reagent Preparation Log for the corresponding section of the laboratory.

Acetonitrile:Water:Formic Acid (50:50:0.1)

Procedure

- a) Add 25 mL of acetonitrile to a liter glass container.
- b) Add 25 mL of water. Mix.
- c) Add 50 uL of formic acid. Mix.

Storage Requirements

Store at room temperature in a glass container.

Prepare fresh daily.

1 M Ammonium Formate (pH 3.4)

Procedure

Weigh 6.3 g of ammonium formate (6.3g)

- a) Add 80 mL of water to dissolve
- b) Transfer to 100 mL volumetric flask
- c) Add 4.3 mL of Formic acid
- d) Add distilled water to 100 mL
- e) The final pH of the formate buffer was 3.4.

Storage Requirements

a) Store at 4 °C (refrigerator) in a glass container.

HPLC Solvent A (2 mM Ammonium Formate, pH 3.4)

Procedure

Add 2 mL of 1 M ammonium formate solution to 998 mL water. Mix.

Storage Requirements

Store at room temperature in a glass bottle.

HPLC Solvent B (Acetonitrile)

HPLC grade acetonitrile in mobile phase B glass bottle

Storage Requirements

Store at room temperature in a glass bottle.

VII. MATERIALS

16 x 100mm culture tubes.

16 x 125mm screw cap test tubes.

Pipettes and tips.

Vortex mixer (Scientific Industries, Inc.)

Branson Ultrasonic Water Bath, 8510 (Fisher Scientific or equivalent)

pH meter (IQ Scientific Instruments)

Sample Concentrator (Dri-Block DB-3, Techne)

IEC HN-SII Centrifuge (International Equipment Company)

Rotorack (Specie-Mix, Thermolyne)

Kim wipes

2 mL auto sampler vials

200 uL Insert (Target PP Polyspring, National Scientific Company)

15 x 45 mm, 1 x 35 mm and 28 x 57 mm VWR brand vials

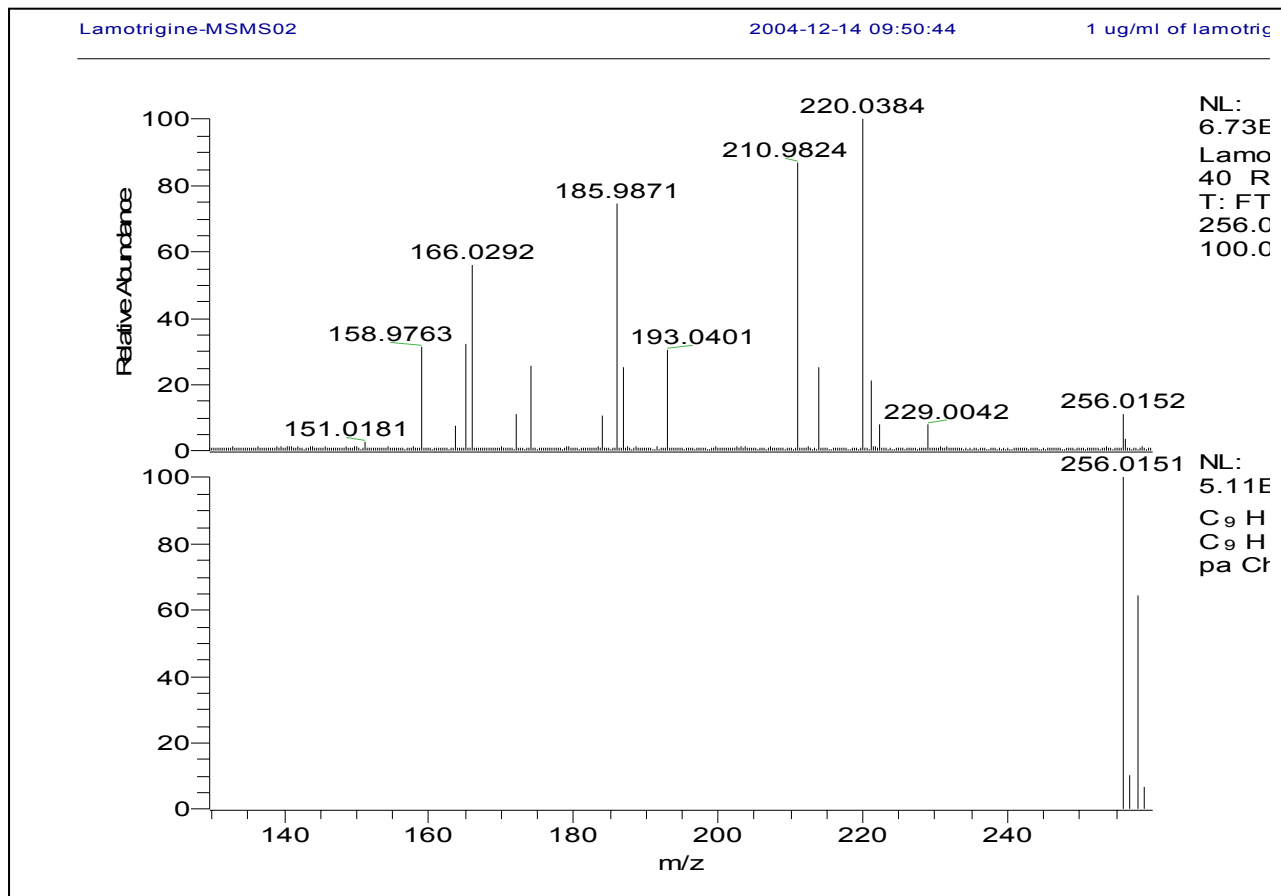
Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc

REFERENCES

Beech J., Fletcher JE, Lizzo F, Johnston J.: Effect of phenytoin on the clinical signs and in vitro muscle twitch characteristics in horses with chronic intermittent rhabdomyolysis and myotonia. American J Vet Res. 49 (12): 2130-2133, 1988.

David Vaczek: Top 200 Drugs of 2002. Pharmacy Times, page 20-24, April 2003

APPENDIX (A-1 TO A-6)



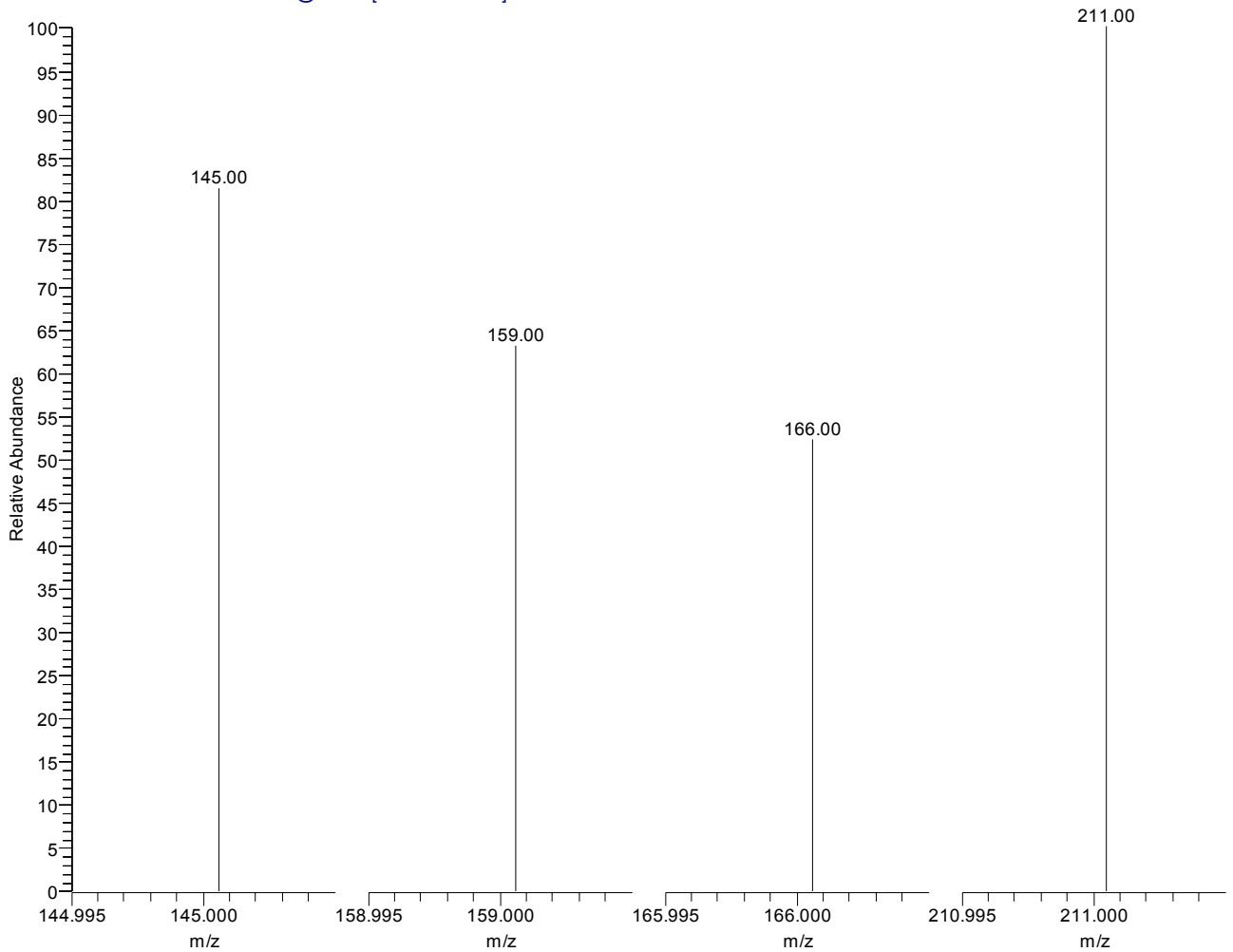
Appendix A-1: Comparison of practical mass (top panel; 256.0152 m/z) with theoretical mass (bottom panel; C₉H₇Cl₂N₅, 256.0151 m/z) determination by FT-MS (Thermo-Finnigan) with a Δ of 1×10^{-4} m/z.

C:\YLUo2005\...\LamotrigineMSMS012405
2 mM NH4FA:ACN (50:50)

01/24/2005 03:31:09 PM

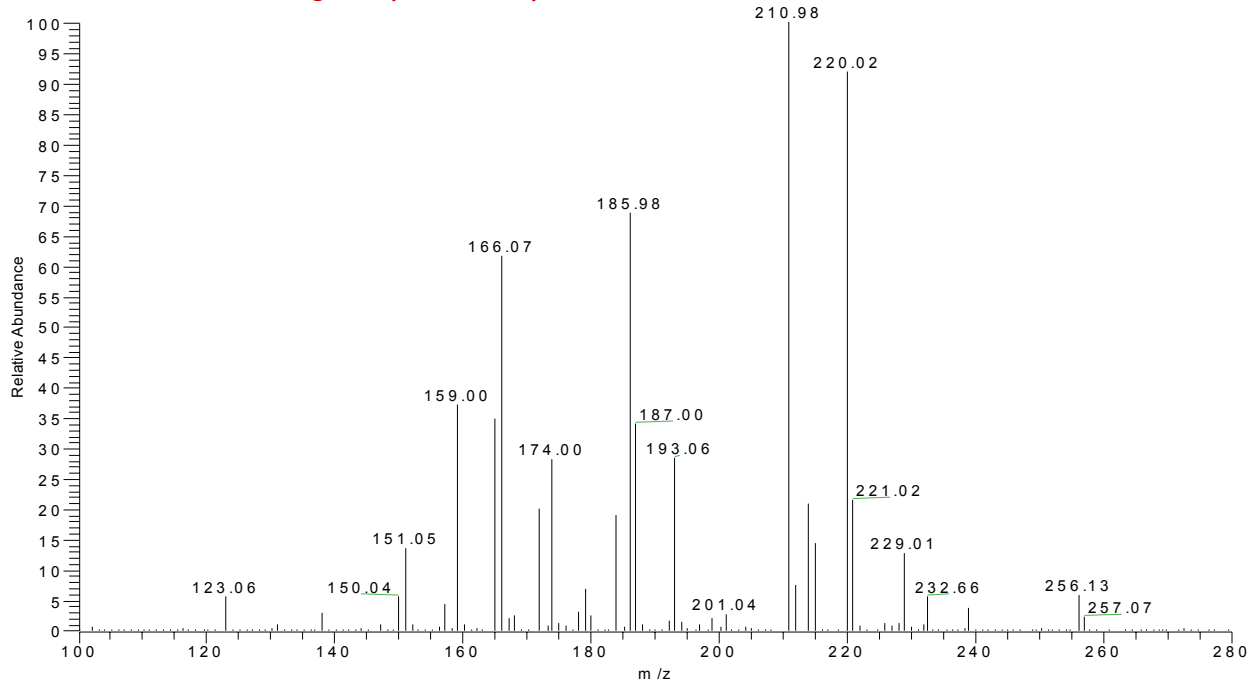
1ug/ml Lamotrigine infusion, flow rate:

LamotrigineMSMS012405 #43 RT: 0.31 AV: 1 NL: 1.90E5
T: + c sid=-18.00 SRMms2 256.00@-27.00 [144.99-211.00]



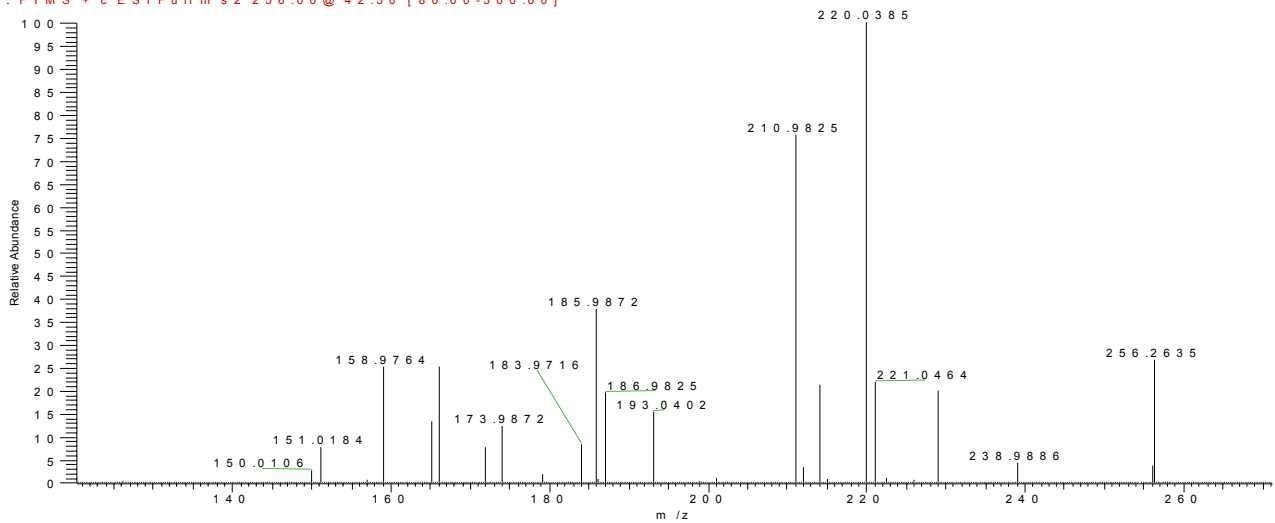
Appendix A-2: SRM MS/MS of lamotrigine (256 m/z) at varying collision energy (22, 23, 27 and 37 eV for 211, 166, 159 and 145 m/z, showing the 4 product ions that form part of the criteria for lamotrigine identification, respectively.

Lamotrigine-MSMS-LTQ #22-33 RT: 0.32-0.49 AV: 12 NL: 3.95E4
F: ITMS + c ESI Full m s 2 256.00 @ 34.00 [80.00-300.00]



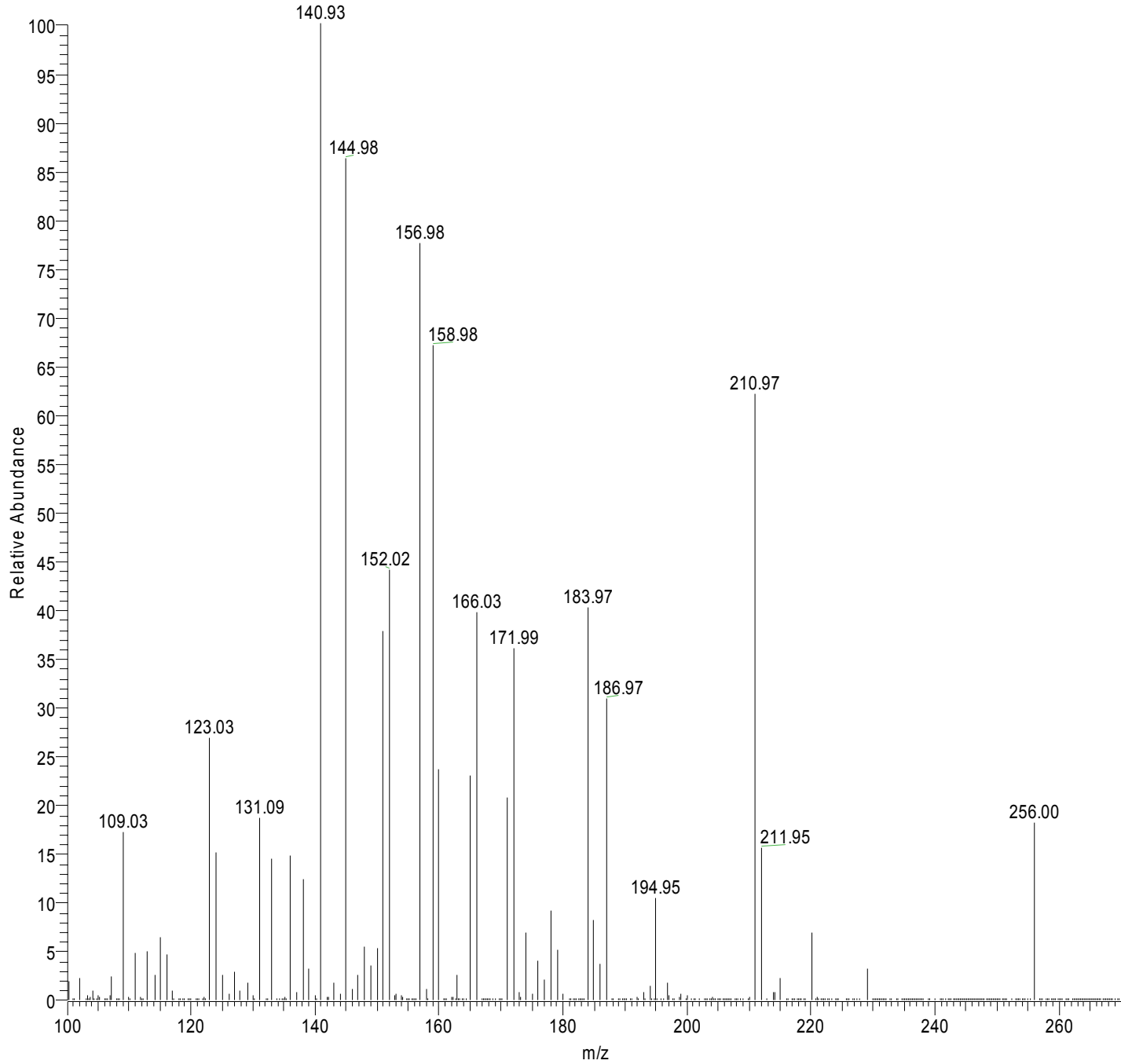
Appendix A-3: MSMS mass spectrum of lamotrigine by LTQ Linear Ion Trap (Thermo-Finnigan)

Lamotrigine-MSMS-FT03 #8-27 RT: 0.20-0.73 AV: 20 NL: 7.48E4
F: FTMS + c ESI Full m s 2 256.00 @ 42.50 [80.00-300.00]



Appendix A-4: MSMS mass spectrum of lamotrigine by LTQ-FTMS (Thermo-Finnigan)

LamotrigineProductScan012405 #6-56 RT: 0.09-0.94 AV: 51 NL: 3.81E5
T: + c sid=-18.00 Full ms2 256.00@-30.00 [100.00-270.00]



Appendix A-5: Full MS/MS spectrum of lamotrigine and product ion scan at collision energy of 30 eV and mass range of 100 – 270 m/z.