

TIP Approved SOP:

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

DEVELOPED BY

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INTRODUCTION

Reserpine is a potent alkaloid derived from *rauwolfia serpentina*. It was once popular in the treatment of hypertension and psychosis in humans. This drug was one of the first and effective agents used on a large scale in the treatment of hypertension. With the advent of new and more effective pharmaceutical preparations, the use of reserpine in human medicine has been relegated to research endeavors. However, this drug is still very popular as a tranquilizer in show horses, dressage and racehorses. Due to the apparent greater sensitivity of horses to the effects of this drug, it is administered in very low doses. The onset of action is delayed and following an intravenous administration effects were not noted for at least 2 hours and actions may persist for up to 48 hours. Its effects are due to the depletion of CNS catecholamines such as norepinephrine, dopamine and serotonin [1,2]. Plasma samples are targeted in the analysis for reserpine, as the parent drug has not been detected in urine. In the development of this SOP, we have, therefore, placed emphasis on plasma samples collected from racehorses. Generally, the presence of reserpine in test samples is predicted by immunoassay (ELISA, Neogen Corporation, Lexington, Kentucky, USA) but such a presumptive positive result requires confirmation by other acceptable analytical methods. A number of methods for the determination of reserpine in plasma have been reported, including thin-layer chromatography (TLC) [2-3], gas chromatography (GC) [4] and high-performance liquid chromatography (HPLC) with ultraviolet and fluorescence detection [5-14]. The use of traditional gas-chromatography-mass spectrometry or high-performance liquid chromatography techniques for confirmation of the presence of low concentrations of reserpine in urine or plasma has not been very successful. The technique is tedious, time-consuming and lacks repeatability in confirming the presence of reserpine in post-race samples. When the method involving tandem mass spectrometric determination was used, the limit of quantification of reserpine in plasma was 0.02 ng/mL [15] and 50 ng/mL [16]. However, none of these studies reported the confirmation of reserpine at a lower concentration than presented by this method. In addressing this problem, we have developed a method that takes advantage of the sensitivity and selectivity provided by tandem mass spectrometry, and focuses on plasma samples. The purpose of this study was to develop a method that can simultaneously determine and confirm reserpine in equine plasma at a very low concentration by using Q-TOF tandem mass spectrometry. This method can also be used for the purpose of screening for reserpine in equine plasma since the sensitivity of this methodology is much higher than that of Enzyme-Linked Immunosorbent Assay (ELISA).

The method involves quantification and confirmation of reserpine in equine plasma. Liquid-liquid extraction of reserpine from equine plasma is used with subsequent analysis on a Quadrupole Time-of-Flight tandem mass spectrometer (Micromass, Manchester, UK). The method covers procedures for screening, quantification and confirmation of the presence of reserpine in equine plasma. Plasma sample (0.5 or 1 mL) is subjected to liquid-liquid extraction prior to analysis by LC/Q-TOF-MS/MS.

SCOPE

This standard operating procedure describes the quantification and confirmation of Reserpine in equine plasma. Liquid-liquid extraction of equine plasma is employed with subsequent analysis on a MicroMass Q-TOF (Series 1) tandem mass spectrometer. The scope of this work covers procedures to be used in screening, quantifying and confirming the presence of reserpine (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/MS/MS method described in this SOP and the concomitant presence of reserpine in the test sample. Any concentration of reserpine 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. There is no tolerance concentration of reserpine in either urine or plasma of race horses competing in PA. Therefore any confirmable concentration of reserpine by this method is considered a positive finding.

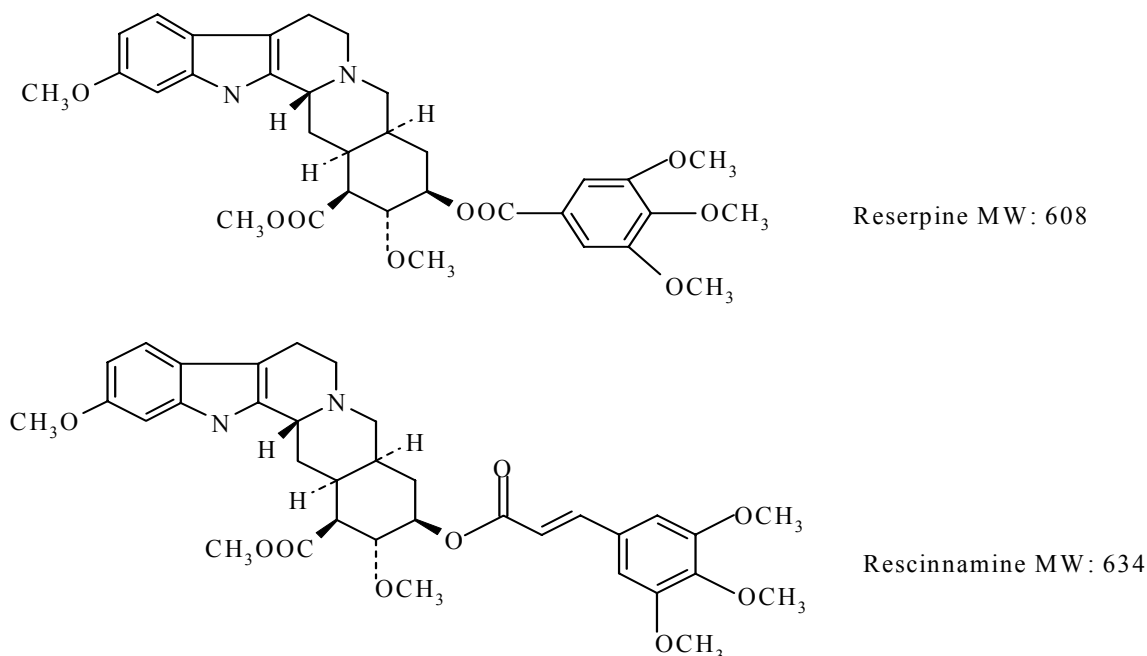


Figure 1. Structure of Reserpine and Rescinnamine (IS).

PRINCIPLE OF METHOD

Plasma sample is subjected to liquid-liquid extraction under basic conditions. The residue is dissolved in LC mobile phase and the analyte is identified, quantified and confirmed by liquid chromatography/ tandem mass spectrometry operated under positive mode electro-spray ionization conditions. The limit of quantification of Reserpine in equine plasma by this method is 50 pg/ml. The confirmable concentration for Reserpine in equine plasma is 0.5 ng/ml. The molecular ion $[M+1]^+$ for reserpine is 609 m/z with diagnostic ions at 195 (BP), 397, 174 and 448 m/z. Rescinnamine will be used as the internal standard (IS). The molecular ion $[M+H]^+$ for rescinnamine (IS) is 635 and the diagnostic ions are 221 (BP), 397 and 174. The mass spectra of reserpine and rescinnamine (IS) are shown in Figure 2.

Reserpine Plasma Calibrator 50

0113-01 1303Ca17 30 (1.125) Cm (29:31-(24+39)x2.000)

1: TOF MSMS 609.30ES+
2.94e3

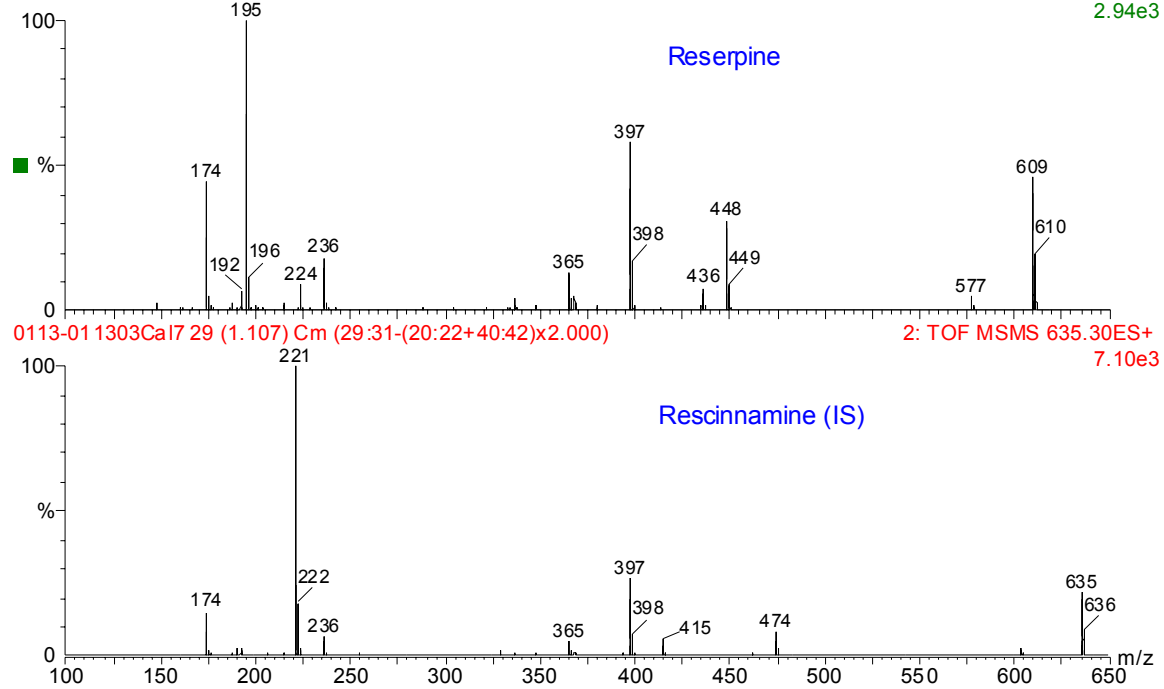


Figure 2. Mass Spectra of Reserpine and Rescinnamine (IS)

PRIMARY REFERENCE MATERIALS

Primary Analytical Standard Reference Material

Reserpine, FW: 608.7 (Cat. No. R-0875, Sigma)

Primary Analytical Internal Standard Reference Material

Rescinnamine, FW: 634.7 (Cat. No. R-0750, Sigma)

Obtain these materials from the pharmacy. Record accession of these materials on the pharmacy log sheet.

I. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

(A). 1 mg/ml solution of Reserpine Standard

Materials

Reserpine

Methanol

Procedure

Weigh between 5 to 10mg of Reserpine into a glass bottle.

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

Cap and mix until reserpine is completely dissolved in methanol.

The resulting concentration of Reserpine is 1 mg/mL.

Storage Requirements: Store at approximately 4 °C.

Complete Balance Use Log and QA Primary Reference Standard Log for this process.

Label the primary reference stock solutions with QA Primary Reference Log SR# (i.e. SR# 690.)

(B). 1 mg/ml solution of Rescinnamine (Internal Standard)

Materials

Rescinnamine

Methanol

Procedure

Weigh between 5 and 10 mg (**X.xx** mg) of Rescinnamine into a glass bottle.

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

Cap and mix until rescinnamine is completely dissolved in methanol.

The resulting concentration of rescinnamine is 1 mg/mL.

Storage Requirements: Store at approximately 4 °C .

Discard any unused portion of rescinnamine stock solution after 30 days from original date.

Complete Balance Use Log and QA Primary Reference Standard Log for this process.

Label the primary reference stock solutions with QA Primary Reference Log SR# (i.e. SR# 723.)

II. PREPARATION OF SECONDARY REFERENCE STOCK SOLUTIONS

Reserpine Standard Working Solutions

A. Materials

1. 1mg/mL of Reserpine primary reference stock solution
2. Acetonitrile: Water: Formic Acid (50:50:1).

B. Procedure

1. Reserpine standard working solutions of different concentrations are prepared as follows (see Table 1):

Table 1. Preparation of Reserpine Standard Working Solution

Target Con.(ug/mL)	Made From Stock Solution ug/mL	Vol.Added(uL)	Vol. ACN/H2O/FA (50:50:0.1) (uL)	Used For 10 uL + 1 mL Plasma (ng/mL)
25	1000	25	975	250
10	1000	10	990	100
5	1000	5	995	50
1	10	100	900	10
0.5	5	100	900	5
0.1	1	100	900	1
0.05	0.5	100	900	0.5
0.01	0.1	100	900	0.1
0.005	0.05	100	900	0.05

Storage Requirements: Store at approximately 4 °C.

Label Reserpine working solutions and record the preparation and labeling in the secondary preparation logbook in the Q-TOF Unit of the Laboratory (Room 117).

Rescinnamine Internal Standard (IS) Working Solution

A. Materials

1. 1mg/mL of Rescinnamine stock solution
2. Acetonitrile: Water: Formic Acid (50:50:0.1, v/v/v)

B. Procedure

1. Take 10 µL aliquot of 1 mg/ml rescinnamine stock solution and add 990 µL of Acetonitrile: Water: Formic Acid (50:50:0.1, v/v/v).
2. The final concentration is 10 µg/mL of Rescinnamine.

Storage Requirements: Store at approximately 4 °C. Prepare it fresh on day of use and discard any unused portion of the solution.

III. PREPARATION OF RESERPINE QC WORKING SOLUTIONS

Reserpine Quality Control Working Solutions

A. Materials

1. 1mg/mL reserpine QC stock solution
2. Acetonitrile: Water: Formic Acid (50:50:0.1, v/v/v)

B. Procedure

1. Reserpine QC working solution of different concentrations are prepared as summarized in Table 2.

Table 2. Preparation of Reserpine Standard QC Working Solution

Target Con.(ug/mL)	Made From Stock Solution ug/mL	Vol.Added(uL)	Vol. ACN/H2O/FA (50:50:0.1) (uL)	Used For 10 uL + 1 mL Plasma (ng/mL)
5.00	1000.0	5.0	995.0	50.0
0.50	5.0	100.0	900.0	5.0
0.10	5.0	20.0	980.0	1.0
0.05	0.5	100.0	900.0	0.5

Storage Requirements: Store at approximately 4 °C.

Label Reserpine QC working solutions and record the preparation and labeling in the secondary preparation logbook in the Q-TOF Unit of the Laboratory (Room 117).

IV. PREPARATION OF PLASMA CALIBRATORS AND QUALITY CONTROLS

The following calibrators are prepared in pooled negative equine plasma sample that was previously demonstrated by this SOP to be negative for the presence of reserpine.

A. Materials for Reserpine Calibrators

1. Reserpine standard working solution
2. Negative equine plasma

B. Procedure for Preparing Reserpine Calibrators

Reserpine plasma calibrators of different concentrations are prepared as summarized in Table 3.

Table 3. Preparation of Reserpine Plasma Calibrators

Calibrator Code #	Target Conc. (ng/mL)	Working Solution (ug/mL)	Spike Working Solution (uL)	Volume of Plasma (mL)
011303Reserpine0.05	0.05	0.005	150	15.0
011303Reserpine0.1	0.10	0.01	150	15.0
011303Reserpine0.5	0.50	0.05	150	15.0
011303Reserpine1.0	1.00	0.1	150	15.0
011303Reserpine5.0	5.00	0.5	150	15.0
011303Reserpine10.0	10.00	1	150	15.0
011303Reserpine50.0	50.00	5	150	15.0
011303Reserpine100.0	100.00	10	150	15.0

C. Materials for Preparing Reserpine Plasma QC Samples

1. Reserpine QC working solutions
2. Negative equine plasma

D. Procedure for Preparing Reserpine Plasma QC Samples

Reserpine plasma QC samples of different concentrations are prepared as summarized in Table 4.

Table 4. Preparation of Reserpine Plasma QC samples

QC Code#	Target Conc. (ng/mL)	Working Solution (ug/mL)	Spike Working Solution (uL)	Volume of Plasma (mL)
Plasma NQP 25	0.0	0.0	0.0	15.0
011303Reserpine 0.5 QC	0.5	0.05	150	15.0
011303Reserpine 1.0 QC	1.0	0.10	150	15.0
011303Reserpine 5 QC	5.0	0.50	150	15.0
011303Reserpine 50 QC	50.0	5.00	150	15.0

Record the preparation and labeling in the secondary preparation logbook in the Q-TOF Unit of the Laboratory (Room 117).

Label 16x125 mm screw cap culture tubes (14 replicate tubes of 12 types from Table 3 and 4.) for dispensing of batch calibrators and control samples.

Print 14 labels for each identifying category of Table 3 and Table 4, respectively.

***LABEL (format MMDDYYReserpine ng/ml) and (format M MDDYY Reserpine QC ng/mL)
 Print using AVERY Template 5267 in MS Word) 14 of each***

Aliquot 1 ml of the appropriate calibrators and QC samples from Tables 3 and 4 into each respective tube. Cap and store at -70°C .

V. SAMPLE REQUIREMENTS FOR ANALYSIS

Prepare Calibrators, Quality Control samples in duplicate, and “unknown” samples in triplicate for each analysis performed.

VI. EXTRACTION OF RESERPINE FROM PLASMA BY LIQUID-LIQUID EXTRACTION

Remove two sets of previously prepared 1 mL calibrators and quality control samples from freezer storage and thaw at room temperature or in lukewarm water.

Calibrator and Sample Preparation for Reserpine

1. Label 16 x 125 mm test tubes accordingly.
2. Transfer 1.0 mL of each unknown sample into the labeled tubes. (see Table 3 and Table 4 for the preparation of calibrators and QC samples)
3. Add 10 μl of 10 $\mu\text{g/mL}$ rescinnamine (IS) working solution into each calibrator, QC and unknown samples except the negative control sample.

4. Vortex the contents of each tube for 5 – 10 seconds to mix.
5. Add 0.4 mL of 2% disodium EDTA followed by 2 mL 0.1 M phosphate buffer (of pH 10.50) to adjust the final pH to 10, mix.
6. Add 5 mL of 1-Chlorobutane into each tube, rotor rack for 10 minutes.
7. Centrifuge x 10 minutes at 3000 rpm.
8. Transfer the organic phase (top layer) into labeled clean 16 x 100 mm culture tubes.
9. Repeat Steps 6 to 8. Combine the second organic extract with the first for each individual sample.
10. Evaporate to dryness under steady N₂ stream at 50 °C.
11. Dissolve the residue in 100 uL mobile phase mixture (5 mM NH₄AC:ACN 25:75, v/v).
12. Perform ultrasonic treatment for 5 minutes at 50 °C to dissolve the residue completely.
13. Transfer the above solution into 200 ul of insert then load in the auto sampler vials.
All the samples are now ready for analysis by LC/Q-TOF-MS/MS.

VII. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF RESERPINE

Liquid Chromatographic and Mass Spectrometric Operating Parameters

A. Liquid Chromatographic and Mass Spectrometric Operating Parameters

1. Instrumentation

Micromass Q-TOF Mass Spectrometer and Agilent Technologies Model 1100 HPLC pump, auto sampler, column compartment and degasser. Masslynx software is used for system control and data processing.

2. LC column

- a) Type: Zorbax XDB-C18 Analytical Column, Part No. 960967-902 (Agilent).
- b) Length: 50 mm, i.d. 2.1 mm
- c) Particle size: 5 micron
- d) Temperature: 27 ° C

3. Mobile Phase

A: 2 mM NH₄AC:AcN: NH₄OH (95:5:0.01, v/v/v)

B: 2mM NH₄AC: AcN (5;95, v/v)

Gradient for Reserpine

Time (min)	A	B	Flow Rate (mL/min)
0	15	85	0.2
3.0	15	85	0.2
3.01	0	100	0.2
4.0	0	100	0.2
4.01	15	85	0.2

5.0	15	85	0.2
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3. **Injection Volume:** 20 µL.

4. Mass Spectrometric Parameters

Parameters	Reserpine	Rescinnamine (IS)
TOF MSMS	609.2	635.2
Scan Range (Daltons)	100-650	100.-650
Collision Energy (eV)	30	45
Cone (Volts)	30	40
Scan time (sec)	1.00	1.00
Inter Scan Time (sec)	0.1	0.1
Start Time (min)	0.0	0.0
End Time (min)	3.0	3.0

VIII. SAMPLE LIST SETUP FOR RESERPINE ANALYSIS

1. Mobile Phase Blank
2. Mobile Phase Blank
3. Blank Plasma (QC Negative Control)
4. Calibrator Series 1
5. Mobile Phase Blank
6. Mobile Phase Blank
7. Quality Control Series 1 (Positive Controls)
8. Mobile Phase Blank
9. Mobile Phase Blank
10. Sample 1, Replicate 1
11. Sample 1, Replicate 2
12. Sample 1, Replicate 3
13. Repeat steps 8 through 12 for each additional sample
14. Mobile Phase Blank
15. Mobile Phase Blank
16. Quality Control Series 2 (Positive Controls)
17. Mobile Phase Blank
18. Mobile Phase Blank
19. Calibrator Series 2
20. Mobile Phase Blank
21. Mobile Phase Blank

IX. CRITERIA FOR IDENTIFICATION OF RESERPINE IN EQUINE PLASMA EXTRACTS

Identification of Reserpine

The qualifying ion for the identification of Reserpine is m/z 609 $[M+H]^+$, with diagnostic ions at m/z 174, 195 (BP), 397, 448 and 609. The quantifying ion is m/z 195+397+448. Under LC-MS/MS analytical conditions described in this SOP, all the above diagnostic ions should be recognized at retention time of ~ 1.15 minutes.

All described ions **for Reserpine** must be present in the full scan MSMS spectrum (averaged spectrum at 20% peak height); and, the retention time for the suspect sample, 0.5 ng/ml calibrator, and 0.5 ng/ml QC sample must agree to ± 0.15 minutes.

Identification of Rescinnamine (IS)

The qualifying ion for the identification of the internal standard, Rescinnamine, is m/z 635 $[M+H]^+$, and its diagnostic with quantifying ion is m/z 174, 221 (BP), 397, 474 and 635. Its quantifying ion is 221. Under LC-MS/MS analytical conditions described in this SOP, all the above diagnostic ions should be recognized at retention time ~ 1.15 min.

All described ions **for Rescinnamine** must be present in the full scan MSMS spectrum (averaged spectrum at 20% peak height), and the retention time for the “unknown” sample, 0.5 ng/mL calibrator, and 0.5 ng/mL QC sample must agree to ± 0.15 minutes.

X. CRITERIA FOR RESERPINE QUANTIFICATION IN EQUINE PLASMA

Determination of Reserpine Concentration

The product ion used for quantification of reserpine is m/z 195+397+448.

The product ion used for quantification for rescinnamine (IS) is m/z 221.

Using the MassLynx Quantification software, execute quantification method and print the compound summary quantification report and calibration curve (Figure 3). The correlation should be greater than 0.995%.

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

There is no allowable or tolerable concentration of reserpine in the horse at race time in PA. Thus, any concentration of reserpine in plasma collected from a horse that had competed in a race in PA will be reported as positive finding to the Racing Commission.

Compound 2 name: Reserpine Method File: Reserpine
Coefficient of Determination: 0.999963
Calibration curve: $0.00782474 * x + 0.000780214$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

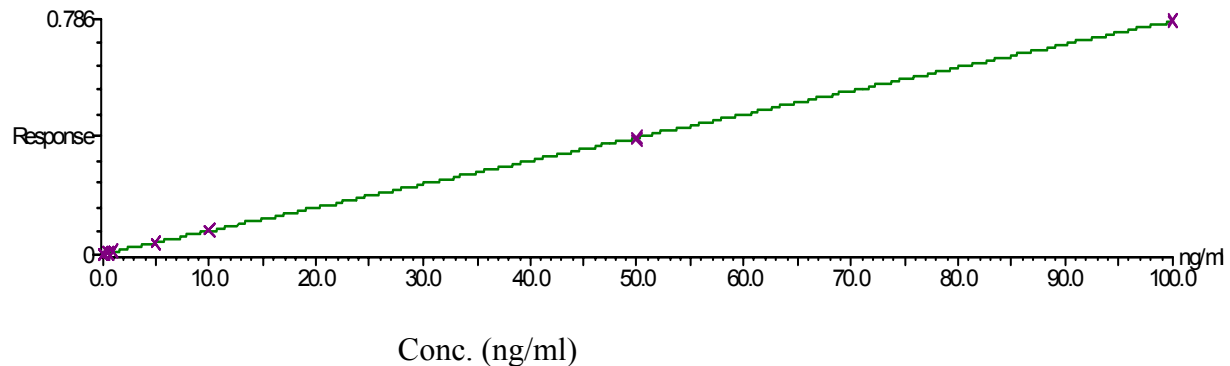


Figure 3. Calibration Curve of Reserpine in Equine Plasma

XI. CRITERIA FOR REPORTING A SAMPLE POSITIVE FOR RESERPINE

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

The test sample contains Reserpine according to the chromatographic and spectral criteria described above.

The LC retention times of the quantifying ion for Reserpine in the sample, 0.5 ng/ml QC control and the 0.5 ng/ml calibrators are 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.

The signal to noise ratio of the quantifying ions for Reserpine and internal standard (Rescinnamine) is greater than 10 (Figure 4). This is determined by inspection of the full scan MSMS 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 5).

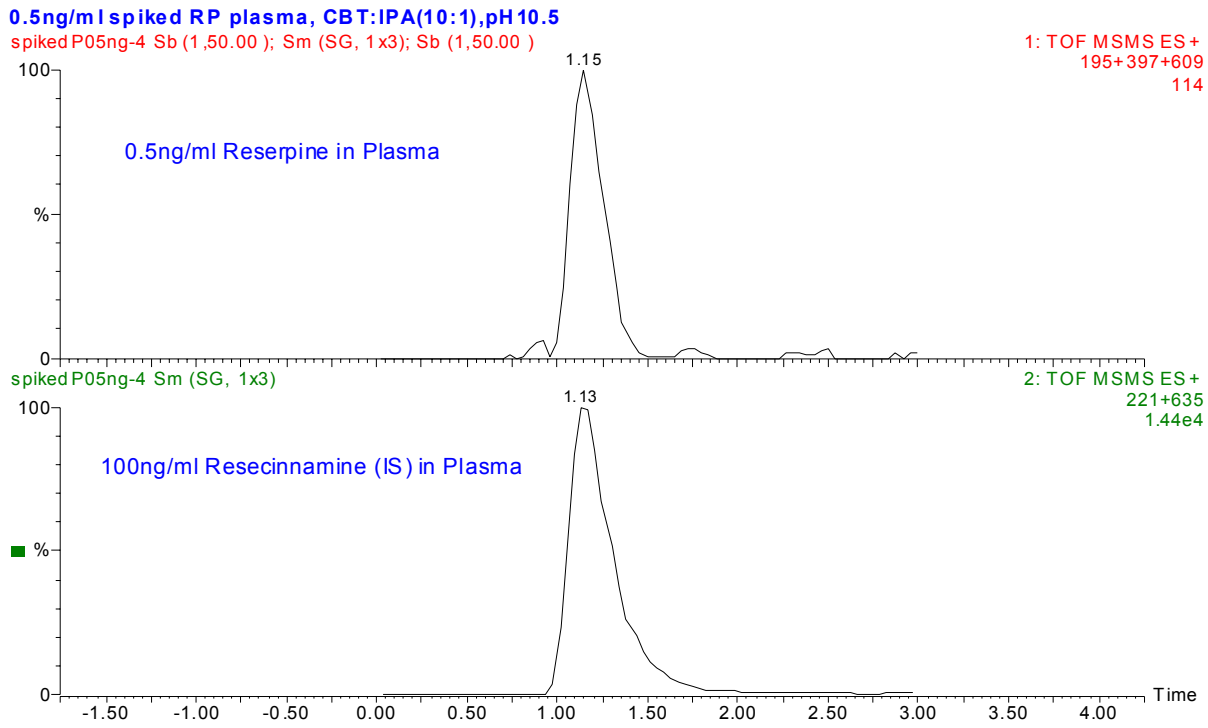


Figure 4. Mass Chromatograms of Reserpine (top pane) and Rescinnamine extracted from Equine Plasma

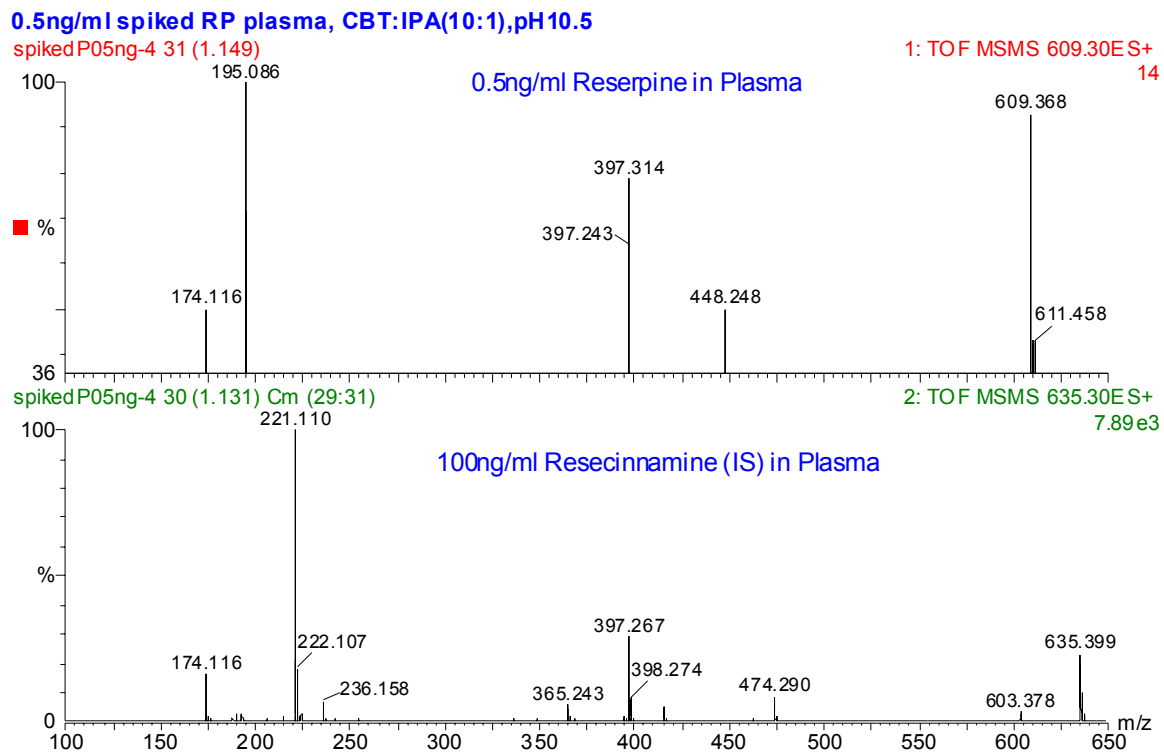


Figure 5. Mass Spectra of Reserpine (top pane) and Rescinnamine (bottom pane) Extracted from Equine Plasma

The MSMS full scan spectra comparison (as defined in the previous paragraph), contains no mass spectral peaks greater than 25% of the quantifying ion for Reserpine. This is determined by the mass spectrum comparison of the sample, standard, and control as described in the previous section. The presence of such peaks indicates the possibility that the values determined for the integration of the quantifying ion chromatograms may be skewed due to the presence of unknown co-eluting substances.

All Blanks and Negative controls contain no quantified and reported Reserpine concentration greater than 50 pg/ml.

XII. Integration

The integration parameters of the quantifying method have been set to produce consistent and reproducible integration from run-to-run and 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 is advisable to:

Adjust the integration program parameters

Investigate deteriorating conditions of:

The samples and standards
Chromatographic system
Mass spectrometer system

These are the only conditions that allow manual integration. Reproducible and systematic identification of deviations are an absolute requirement of a quantitative method to determine method and result precisions and confidence limits. Manual integration may produce slightly better precision, but all deviations then fall into the domain of “random error” instead of “random” or “systematic” error. By using a defined set of automatic instrumental integration parameters, results fall into the domain of “system error”, which allows for user control, intervention, and correction.

XIII. METHOD VALIDATION

The method was validated under the guidelines presented by Shah et al¹⁴. Ten assays for validation were performed; five for within-run (intra-day assay) and five for between-run (inter-day) to assess precision, accuracy and specificity.

Inter-day assay accuracy and precision were determined by analyzing twenty validation samples at three concentrations of reserpine (0.5, 5 and 50 ng/mL equine plasma) in five separate experiments. These concentration of reserpine corresponded to low, medium and high for constructing the calibration curve. Intra-day assay accuracy and precision were determined by analyzing five replicates of the three concentrations in each experiment. Accuracy was determined as the agreement between the concentration of the target analyte detected and that spiked into blank plasma. Precision of the assay was determined as the relative standard deviation expressed as a

percentage of the standard deviation divided by the mean of observed concentration and was reported as percent coefficient of variation (CV %).

Method Recovery

Table 6. Recoveries of Reserpine from Equine Plasma (n=6)

Spiked amount (ng/mL)	Determined amount (ng/mL)	Recoveries (%)
0.5	0.343 ± 0.003	68.52 ± 5.55
5	4.747 ± 0.87	94.93 ± 17.37
50	45.685 ± 4.49	92.82 ± 8.79

Precision, Reproducibility, and Accuracy

Table 7. Precision and Accuracy of Reserpine in Equine Plasma (n=8)

Reserpine added (ng/mL)	Intra-day			Inter-day		
	Reserpine Determined (ng/mL)	C.V ^a (%)	AR ^b (%)	Reserpine Determined (ng/mL)	C.V ^a (%)	AR ^b (%)
0.5	0.5 ± 0.011	2.14	100.00	0.51 ± 0.010	2.06	101.15
1	0.97 ± 0.047	4.81	97.13	0.99 ± 0.023	2.32	98.58
5	5.05 ± 0.189	3.73	101.05	5.09 ± 0.092	1.80	101.72
50	50.73 ± 0.863	1.70	101.46	51.55 ± 1.322	2.56	103.10

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

^b Accuracy (AR%) = mean detected concentration/spiked conc. x 100

Measurement Uncertainty

The following statements define the handling of measurement uncertainty and definition of the coverage factor (k) used for analysis and method uncertainty calculations:

1. Method measurement uncertainty is initially established based on method validation.
2. The **analysis** 95% confidence interval is expressed as +/- Standard Deviation x Coverage factor (k) (SD x k) for both unknown determinations as well as threshold control values.

Table 8. Measurement of Uncertainty of Reserpine in Equine Plasma

Symbol	Source of Uncertainty	Value Units (%)	Distribution	Divisor	Standard Uncertainty	Degrees of Freedom (n-1)	Other
U ₁	Intermediate precision	1.75	N	1	1.75	13	Reserpine 0.5 ng/mL
U ₂	Intermediate precision	1.87	N	1	1.87	13	Reserpine 5.0 ng/mL
U ₃	Intermediate precision	2.06	N	1	2.06	13	Reserpine 50 ng/mL
Combined Uncertainty			1: (U ₁ ²) ^{1/2} = 1.75; 2: (U ₂ ²) ^{1/2} = 1.87 3:(U ₃ ²) ^{1/2} = 2.06				
Expanded Uncertainty (k=2.3)		U ₁ : (1.75 x 2.3) = 4.03% U ₂ : (1.87 x 2.3) = 4.30% U ₃ : (2.06 x 2.3) = 4.74%					

3. The number of calibrators used for the individual analysis determines this k factor (n=14, k=2.3).
4. The **method** measurement uncertainty (reproducibility) coverage factor is expressed as k=2.3 for single laboratory determinations in the absence of inter laboratory comparative studies or certified “true value” calibrators. This is based on the reproducibility of the 0.5 ng/mL, 5 ng/mL and 50 ng/mL control mean for each analytical run (n=4). Greater than 10 independent runs must be acquired prior to validation acceptance.
5. Threshold control records and charts (0.5 ng/ml n=4) for method development and all subsequent analyses are created and maintained. The use of laboratory control samples (LCS) in calculating MU only provides an estimate of the Measurement Uncertainty (MU) in which case a k factor of 2.3 is used for 95 % CI.

Method Uncertainty Control Charting

An Excel template is provided to perform several quality control functions. For every analysis, all samples will be entered through this template. The template provides:

1. Drop-down boxes for Track Name and User Name
2. Automatic =NOW() function to track date and time
3. Built in IF statement logic to determine positive or negative
4. Automatic building and charting of historical control database with run and historical 95% confidence interval plotting
5. Each entry is saved to the project (\\Masslynx\Reserpine.Pro\Data) folder and is maintained as part of the analysis record archive.

Demonstration of Ionization Suppression Effects

Since parent-product ion LCMSMS is target compound specific, the determination of interfering substances can be only partially based on the purity of the product ion full scan mass spectrum.

Co-eluting substances with parent ions differing from the target parent ion may still exert either enhancement or suppression of the ionization process, thus posing a severe challenge to the validity of quantitative results. Thus, using the method described by Bonfiglio [17] ionization stability was determined for the chromatographic and mass spectrometric conditions described by this SOP. This determination was made for both the target compound Reserpine, as well as the internal standard, Rescinnamine.

Briefly, the test compound (Reserpine or Rescinnamine) is infused at a constant rate into the LC effluent prior to entering the mass spectrometer. Blank plasma samples (n=5) are then analyzed by the SOP operating conditions to measure effects, not only from one analysis, but also from late-eluting compounds that may not be detected until after several sequential analyses. The results of these experiments are presented below in Figure 6 and Figure 7 demonstrating the absence of ionization enhancement or suppression in the retention time ranges of the compounds (Reserpine and Rescinnamine) relevant to this standard operating procedure.

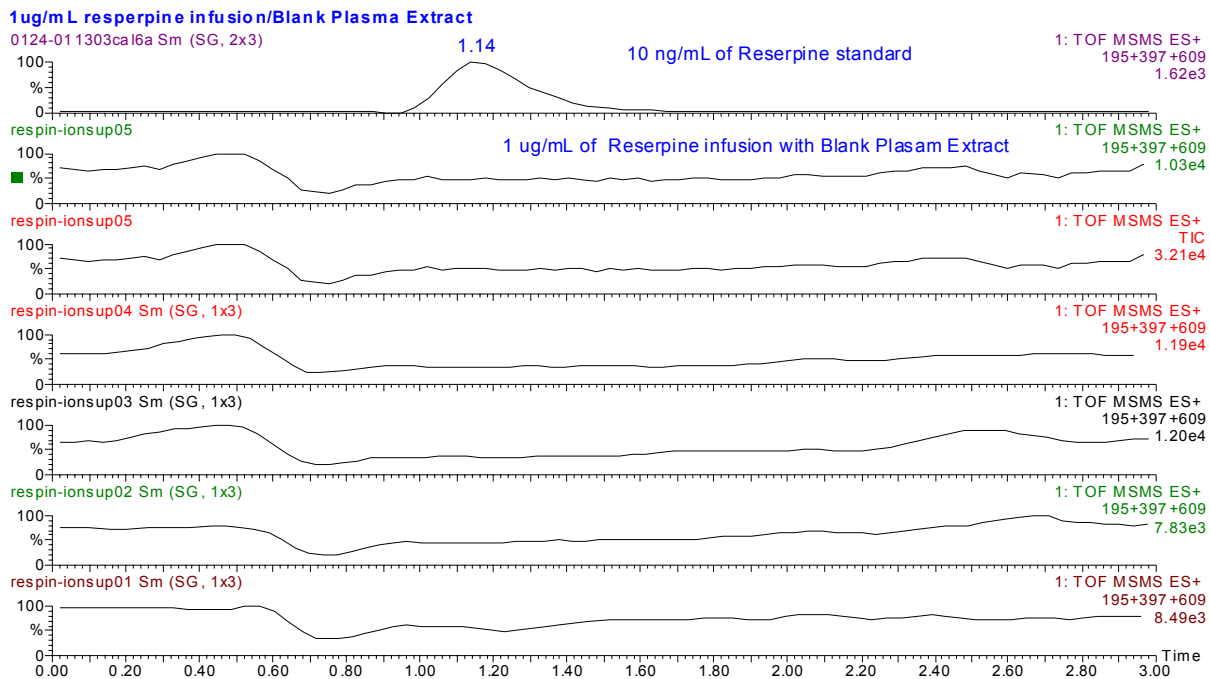


Figure 6. Effect of Blank Plasma extract on Reserpine LC-MS Analysis

Figure 6 demonstrates the absence of matrix ionization enhancement or inhibition at the retention time of Reserpine.

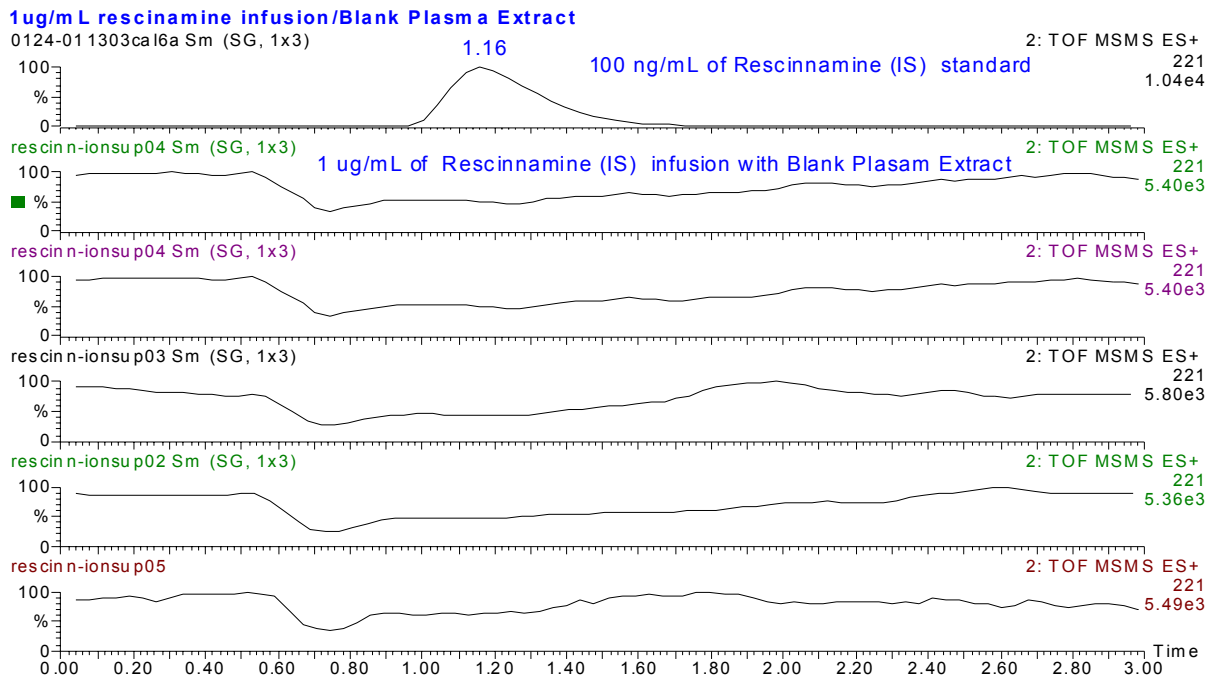


Figure 7. Effect of Blank Plasma extract on Rescinnamine (IS) LC-MS Analysis

Figure 7 also demonstrates the absence of matrix ionization enhancement or inhibition at the retention time of Rescinnamine.

Stability of Reserpine in Equine Plasma at Room Temperature

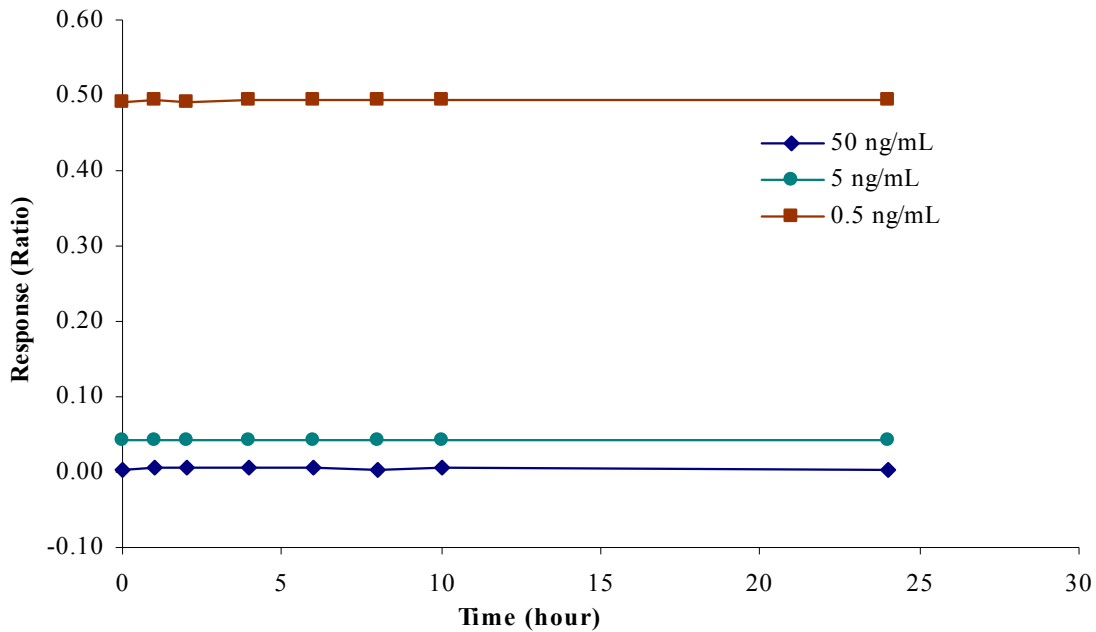


Figure 8. Stability of Reserpine in Equine Plasma at Room Temperature

Stability of Reserpine in Equine Plasma Stored at -70 °C

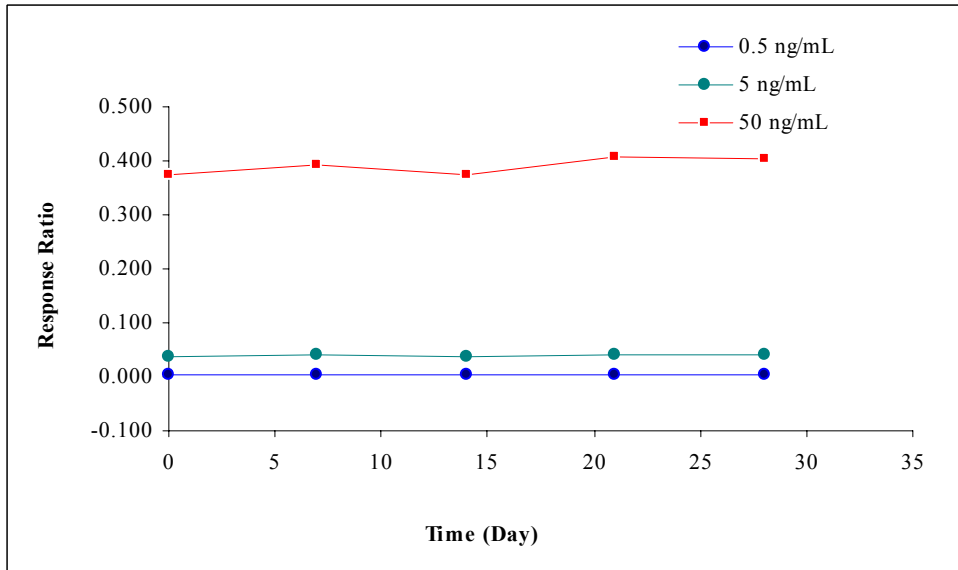


Figure 9. Stability of Reserpine in Equine Plasma Stored at -70 °C

Optimization of Extraction Efficiency of Reserpine from Equine Plasma

Optimization pH Value for Extraction

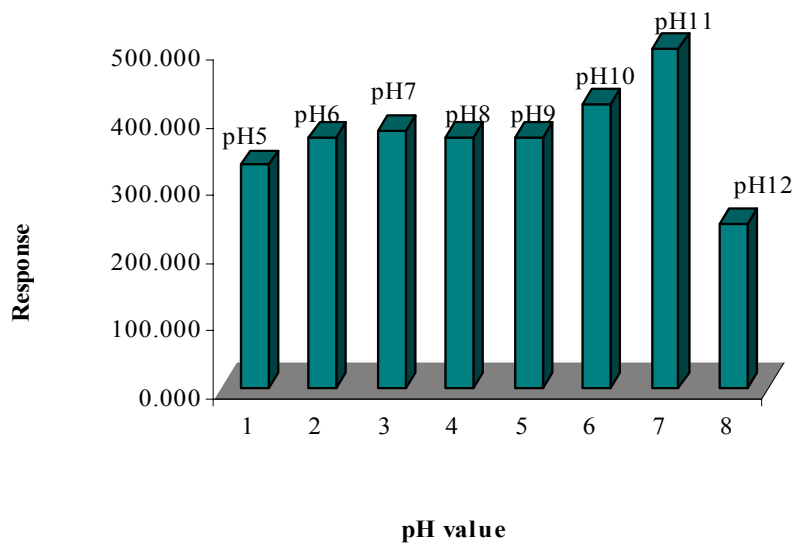


Figure 10. Effect of pH Value on Extraction Efficiency of Reserpine in Equine Plasma

Optimization of Solvents for Extraction

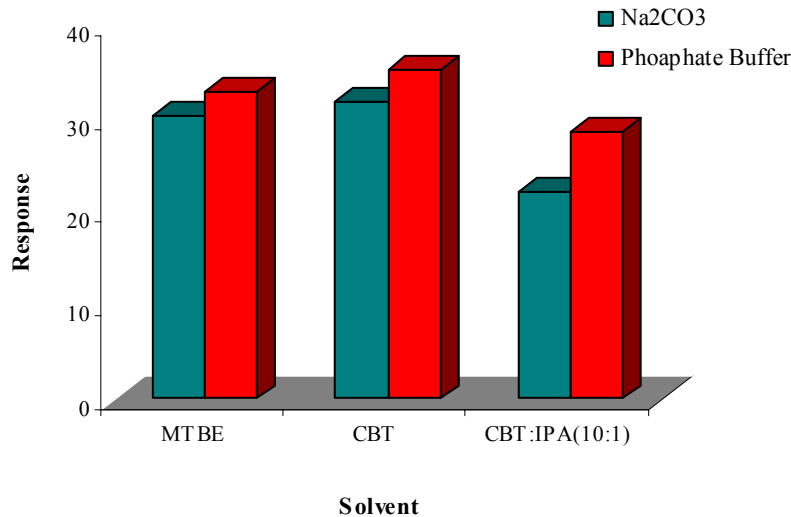


Figure 11. Effect of Solvent System on Extraction Efficiency of Reserpine from Equine Plasma

IVX. 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 AXIS 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 Q-ToF three ring binder

Routine usage checklist completion (and maintenance log if needed)

Sample Analysis logbook, indicating date, project, operator initials, and listing 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

X. 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 Masslynx workstation \\F: hard drive, create the following folder, if not already present:

\\F:\Project name (Drug name) for instance F:\Reserpine

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

DRUGmmdyy.pro for instance:

\\F:\Reserpine\Respine020303.pro

Copy ALL files from C:\Masslynx\Reserpine.pro project to the newly created archive folder.

Once all folders and files are successfully copied and verified, the files in C:\Masslynx\Reserpine.pro\Data only, may be deleted. DO NOT PERFORM THIS OPERATION UNLESS YOU HAVE BEEN SPECIFICALLY TRAINED AND CHECKED-OFF AS BEING ABLE TO PERFORM THIS FUNCTION!!!!

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

In addition to this local archive, our site Information Technologist performs back-up of this hard drive once a week.

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.)
Ammonium Acetate, HPLC grade (Cat. No.A639-500, Fisher Scientific.)
Ammonium Hydroxide, Certified A.C.S. PLUS (Cat. No.A669C-212, Fisher Scientific.)
Phosphoric Acid, meets A.C.S. Specification (Cat. No. 0260-3, J.T. Baker Chemicals)
Formic Acid, A.C.S reagent (EEC No. 200-5791, Sigma)
1-Chlorbutane, HPLC grade (Cat. No.CX0914-1, EM Science)
Monobasic Potassium Phosphate, HPLC grade (Cat. No. P-0662, Sigma)
Ethylenediaminetetraacetic acid (EDTA) Disodium Salt (Cat. No. E-5134, Sigma)

II. SOLUTIONS

0.1 M Phosphate Buffer (pH 10.5)

Reagents

Sodium phosphate dibasic anhydrous
Phosphoric Acid (85%, 14.7 M)
Sodium Hydroxide
HPLC grade Water

Procedure

Weigh 14.2 g of sodium phosphate dibasic anhydrous.
Add 800 mL water to dissolve it under stirring.
Adjust pH to 10.5 using sodium hydroxide or phosphoric acid.
Add water to bring final volume to 1 liter (1000 mL) and thoroughly mix.

Storage Requirements

Store at room temperature in a glass container.
Discard any unused portion after 3 months from the original date of preparation

2% Disodium EDTA solution

Reagent

Ethylenediaminetetraacetic acid (EDTA) Disodium Salt
HPLC grade water

Procedure

Weigh 2 g of EDTA disodium salt.
Add 100 ml of HPLC grade water to dissolve under stirring.

Storage Requirements

Store at refrigerator.

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

Procedure

Add 25 mL of methanol to a liter glass container.
Add 25 mL of water. Mix.
Add 50 uL of formic acid. Mix.

Storage Requirements

Store at room temperature in a glass container.

Prepare fresh daily.

2.0 M Ammonium Acetate (pH 5.0)

Reagents

Ammonium Acetate (HPLC grade)
Water (HPLC grade)

Procedure

Weigh 126.12 g of ammonium acetate. Dissolve in 800 mL of water, adjust pH to 5.0 with phosphoric acid or ammonium Hydroxide.
Dilute to 1000 mL with water and mix.

Storage Requirements

Store at 4 °C in a glass container

HPLC Solvent A: (2 mM Ammonium Acetate:Acetonitrile:Ammonium Hydroxide; 95:5:0.01, v/v/v)

Reagents

2 mM Ammonium Acetate (HPLC grade)
Acetonitrile (HPLC grade)
Ammonium Hydroxide

Procedure

Add 1 mL of 2 M ammonium acetate solution to 999 mL of water. Mix
Transfer a 950 mL aliquot into a 1-liter glass bottle and mix with 50 mL of acetonitrile and
add 0.1 mL of ammonium hydroxide. Mix thoroughly before placing on HPLC.

Storage Requirements

Store at room temperature in a glass bottle.

HPLC Solvent B: (2 mM Ammonium Acetate:Acetonitrile, 5:95, v/v)

Reagents

2 M Ammonium Acetate (HPLC grade)
Ammonium Hydroxide (ACS reagent)
Water (HPLC grade)
Acetonitrile (HPLC grade)

Procedure

Put 950 mL of acetonitrile in a glass bottle (a liter) and mix with 50 mL of 2 mM of
ammonium acetate.
Mix the solvents thoroughly before placing on HPLC.

Storage Requirements

Store at room temperature in a glass bottle.

III. MATERIALS

16 x 125mm screw cap culture tubes
16 x 100mm culture 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, NJ, USA)
IEC HN-SII Centrifuge (International Equipment Company)
Rotorack (Speci-Mix, Thermolyne)
Kimwipes
2 mL autosampler vials, caps
200 uL Insert (Target PP Polyspring, National Scientific Company)
Glass pasteur pipettes (disposable)
15 x 45 mm, 12 x 35 mm and 28 x 57 mm VWR brand vials
Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc.)

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