

SCREENING AND CONFIRMATION OF BROMOCRIPTINE IN EQUINE PLASMA AND URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ION TRAP MASS SPECTROMETRY

DEVELOPED BY

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INTRODUCTION

Bromocriptine [(5 α)-2-Bromo-12 β -hydroxy-2 β -(1-methylethyl)-5 β -(2-methylpropyl) ergotaman-3 β ,6 β ,18 Trione] mesylate (Parlodel ®) is a dopamine receptor agonist, which activates post-synaptic dopamine receptors. Bromocriptine is the 2-brominated analog of ergocryptine. Bromocriptine is indicated for the treatment of Parkinson's disease, a clinical condition characterized by progressive imbalance between cholinergic and dopaminergic stimulation caused by deficiency in dopamine synthesis in the *substantia nigra*, thus tilting the imbalance in favor of cholinergic receptor stimulation.

Bromocriptine has been available for clinical use for many years and is classified by the Association of Racing Commissioners International as a Class 2 Agent, having little therapeutic value but a high potential for performance enhancement in the racing equine. Interest in ergoid-based drugs has been rekindled over the past several years following the discovery and identification of these types of compounds in illicit unlabelled pre-race treatments confiscated by enforcement officials at various race tracks. These confiscated formulations were clearly being used with the intention of enhancing performance in racehorses or affecting the out-come of a race

Bromocriptine has vasoconstrictive properties similar to ergotamine and ergonovine, by virtue of an unsaturated bond in the D ring of the lysergide ring system. This is in contrast to the vasodilating properties of ergoline-based compounds such as pergolide and dihydroergotoxine alkaloids which have a saturated bond in the same position in the D ring. This class of compounds, including bromocriptine, is extremely lipophilic. Thus, bromocriptine is almost completely metabolized prior to excretion. The major route of excretion of absorbed drug is via the bile. Because traditional screening methods such as enzyme linked immunosorbent assay (ELISA) and gas chromatography-mass spectrometry (GC/MS) lack the sensitivity to screen blood or plasma at the very low concentrations that this agent is likely to present following intake, a sensitive and specific liquid chromatographic-mass spectrometric (LCMS) method is needed.

SCOPE

This standard operating procedure describes the analysis of bromocriptine and its major metabolite, hydroxy bromocriptine in equine plasma by liquid-liquid extraction and liquid chromatography tandem mass spectrometry utilizing an ion trap detection system operated in positive ion electrospray mode. Hydroxy bromocriptine reference standard is not commercially available, thus its retention and spectral properties must be acquired from microsomal incubations or equine administration samples of bromocriptine mesylate. Literature reports (1) indicate that hydroxylation occurs at the 8, and/or 9 position of the fused peptide ring system. This is consistent with the positive ion electrospray spectra described in this standard operating procedure. Results obtained from initial equine administration studies of bromocriptine indicated that hydroxy bromocriptine might be present in plasma at concentrations greater than bromocriptine. Neither bromocriptine nor hydroxy bromocriptine was detected in equine administration urine samples at concentrations greater than those in plasma. Screening and confirmation are described for bromocriptine and hydroxy bromocriptine in equine plasma. Any confirmed finding of bromocriptine or hydroxy bromocriptine in plasma will be reported, as there is no tolerance level of either bromocriptine or hydroxyl bromocriptine for a horse racing in Pennsylvania.

Bromocriptine

Molecular Formula = $C_{32}H_{40}BrN_5O_5$
 Formula Weight = 654.5945
 Monoisotopic Mass = 653.221274 Da

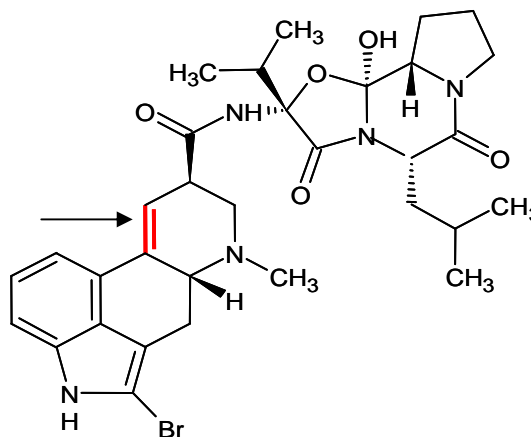


Figure 1. Structure, formula, and molecular weights of bromocriptine. The arrow indicates a primary pharmacophore modification site. Single bonds in this location result in vasodilation, while double bonds result in vasoconstriction.

8-hydroxy Bromocriptine

Molecular Formula = $C_{32}H_{40}BrN_5O_6$
 Formula Weight = 670.5939
 Monoisotopic Mass = 669.216189 Da

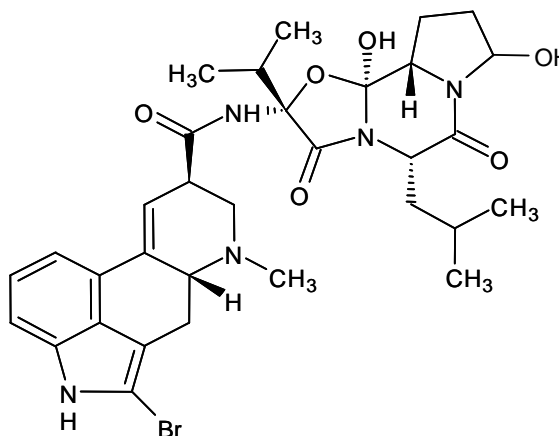


Figure 2. Chemical structure, formula, and molecular weights of 8-hydroxy bromocriptine. Mass spectra obtained from the equine plasma metabolite of bromocriptine are consistent with hydroxylation at either the 8th or 9th position.

MATERIALS AND METHODS

Bromocriptine was obtained from U.S. Pharmacopeia (Rockville, MD). The analytical instrumentation consisted of a Finnigan LTQ linear ion trap, with ThermoFisher Surveyor auto sampler and quaternary liquid chromatograph. The ionization inlet was a ThermoFisher Ion Max electro spray probe fitted with steel needle and operated in positive ion mode. The chromatographic column was a Thermo Hypersil Gold (2.1 x 50 mm) 3 micron reversed phase column. All solvents were LCMS Optima grade or equivalent.

REFERENCE SOLUTION PREPARATION

Stock and working reference solutions were prepared by serial dilution according to the following tables:

QA Ref Designation		Source	Product Code	Lot Number
R-BCRI-1	Bromocriptine Mesylate	USP	7650	Lot I

QA Ref Designation	Weight mg	mL MeOH	LABEL	Concentration
R-BCRI-1	Bromocriptine	7.2	042308-Bromocript-1	1 mg/mL

100 uL of:	mL 50:50:1	LABEL	Concentration
042308-Bromocript-1	9.9	FIA042308Bromcript	10 ug/mL
FIA042308Bromcript	9.9	LC042308Bromcript	100 ng/mL

CALIBRATOR STOCK SOLUTION PREPARATION

Calibrator stock solutions were prepared by dilution from working stock solutions according to the following tables:

uL added	using	mL 0.1 % Formic	ng/mL	Used for:	LABEL
1000	042308Bromcript10	9	1	0.1	042308Bromcript1
1000	042308Bromcript25	9	2.5	0.25	042308Bromcript2.5
1000	042308Bromcript50	9	5	0.5	042308Bromcript5
1000	042308Bromcript100	9	10	1	042308Bromcript10
25	FIA042308Bromcript	9.975	25	2.5	042308Bromcript25
50	FIA042308Bromcript	9.95	50	5	042308Bromcript50
100	FIA042308Bromcript	9.9	100	10	042308Bromcript100

SAMPLE PREPARATION

Analysis calibrators were prepared by addition of the appropriate bromocriptine calibrator stock solution to 0.8 mL of negative equine plasma (control). The primary matrix for instrumental screening and confirmation of bromocriptine in Pennsylvania is plasma. Bromocriptine and hydroxy bromocriptine are method analytes. Calibrators were prepared for the concentrations of 0.1, 0.25, 0.5, 0.75, 1.0, 5.0, and 10.0 ng/mL. Additional positive control samples were prepared for 0.5 and 5.0 ng/mL. Two additional negative plasma control samples of 1 mL each were prepared. Internal standard (d_9 -clenbuterol, 100 ng/mL) was added (100 uL) to all tubes except one negative control sample. Ammonium hydroxide:water (1:1), 3 mL, was added to all tubes, followed by 5 mL methyl tert-butyl ether. The tubes were capped, mixed for 10 minutes (Roto-Rack) and centrifuged for 10 minutes at 2500 rpm. The upper organic layer was transferred to clean culture tubes and evaporated under air at 65°C (Techne Dri Block). The dried extracts were reconstituted with 100 uL 0.1% aqueous formic acid and transferred to 2 mL autosampler vials fitted with 200 uL limited volume inserts. The vials were capped and transferred to the instrument autosampler.

SEQUENCE ORDER FOR CONFIRMATION ANALYSIS

Screening samples are sequentially ordered by track and sample, with blanks bracketing the individual track sets with no calibrators. Low positive controls are run pre and post sequence to verify instrument and method operation. The sequence order for confirmation is outlined below. Confirmation is independent, repeat preparation of all suspect samples in triplicate, with QC and calibrators.

1. Blank
2. Column Test
3. Blank
4. Negative Control
5. Negative Control +Internal Standard
6. Blank
7. C1
8. C2
9. C3
10. C4
11. C5
12. C6
13. C7
14. Blank
15. QC1
16. QC2
17. QC3 (optional)
18. Blank

19. Blank
20. Track A, Samples 1 thru N
21. Blank

22. Repeat 19 thru 21 as needed

23. Blank
24. QC1
25. QC2
26. QC3 (optional)
27. Blank
28. C1
29. C2
30. C3
31. C4
32. C5
33. C6
34. C7
35. Blank
36. Blank-Standby Method

- Blank
 Track A, Sample X₁
 Track A, Sample X₂
 Track A, Sample X₃
 Blank

Repeat as needed for the number of samples for confirmation

Screening analysis uses no waste injection, to allow for repeat analysis in case of power failure, sequence error, retention drift, or other unforeseen need for reanalysis. Confirmation uses partial loop injection due to lower sample throughput and better repeatability statistics at these concentrations.

LCMS ANALYSIS

Analysis was conducted on a Finnigan LTQ linear ion trap operated on positive ion electrospray mode. The auto sampler and liquid chromatograph pump were ThermoFisher Surveyor. The analytical column was reversed phase Thermo Hypersil Gold 3 μ, 2.1 x 50 mm. The liquid chromatograph was operated in a binary reversed phase linear gradient from 95% aqueous to 100 % organic at a flow rate of 0.2 milliliters per minute. Solvent A was 2.33 mM formic acid (pH 5.) and Solvent C was acetonitrile (0.1% formic acid). The analytical conditions for the mass spectrometer and liquid chromatograph are given below:

Scan Event Details:

- 1: ITMS + c norm .(654.30)->oW(180.0-670.0)
MS/MS: AT CID CE 16.0% Q 0.250 Time 30.000 IsoW 1.7
- 2: ITMS + c norm .(655.30)->oW(180.0-670.0)
MS/MS: AT CID CE 16.0% Q 0.250 Time 30.000 IsoW 5.0
- 3: ITMS + c norm .(286.10)->oW(150.0-300.0)
MS/MS: AT CID CE 15.0% Q 0.250 Time 30.000 IsoW 1.7
- 4: ITMS + c norm .(670.30)->oW(180.0-680.0)
MS/MS: AT CID CE 16.0% Q 0.250 Time 30.000 IsoW 1.7
- 5: ITMS + c norm .(671.30)->oW(180.0-680.0)
MS/MS: AT CID CE 16.0% Q 0.250 Time 30.000 IsoW 5.0

Step	Time	A%	B%	C%	D%	uL/min
0	0.00	95.00	0.00	5.00	0.00	20.00
1	0.20	95.00	0.00	5.00	0.00	20.00
2	0.21	95.00	0.00	5.00	0.00	200.00
3	2.00	0.00	0.00	100.00	0.00	200.00
4	4.99	0.00	0.00	100.00	0.00	200.00
5	5.00	95.00	0.00	5.00	0.00	200.00
6	5.01	95.00	0.00	5.00	0.00	400.00
7	5.90	95.00	0.00	5.00	0.00	400.00
8	5.91	95.00	0.00	5.00	0.00	20.00
9	6.20	95.00	0.00	5.00	0.00	20.00

To improve linearity and reduce carryover, auto sampler needle washes and flush were incorporated. These conditions are shown below.

Surveyor AS Method	Sample Preparation	Reservoir Content	Timed Events
Reservoir 1: Acetonitrile Reservoir 2: Acetonitrile, 10% Acetone Reservoir 3: 50:50:1 ACN:Water:Formic Acid Reservoir 4: Wash Bottle: Solvent A			

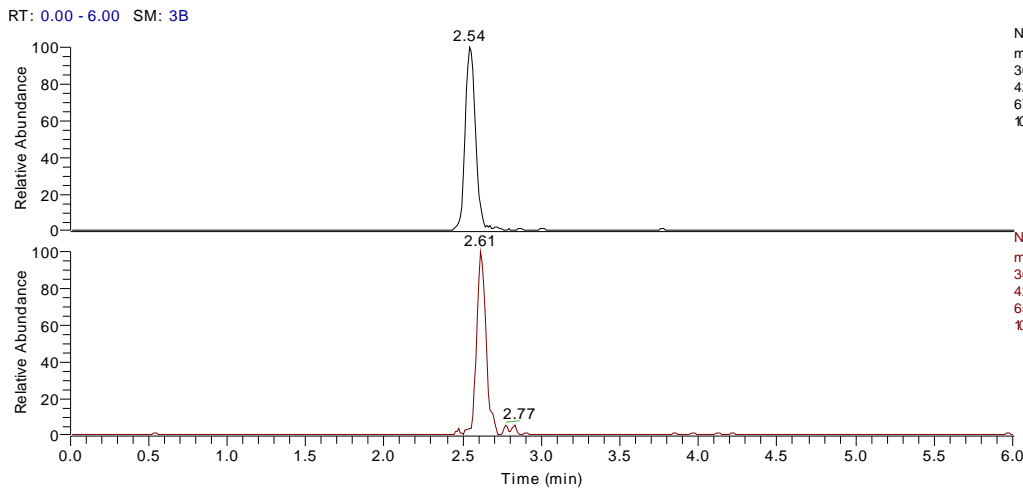
- Sample Preparation
- Wash needle
 - Volume (ul) is 100.0
 - Reservoir is RV1.
 - Wash needle
 - Volume (ul) is 100.0
 - Reservoir is RV2.
 - Wash needle
 - Volume (ul) is 100.0
 - Reservoir is RV3.
 - Flush to waste
 - Volume (ul) is 400.0
 - Reservoir is bottle.
 - Syringe speed (ul/s) is 250.00

Typical chromatograms and spectra are shown below. Note MS² spectra comparisons to bromocriptine are required for hydroxy bromocriptine confirmation.

102908_Bromocriptine_Admin_022

10/29/2008 5:55:55 PM

Cat_89_BRO_PO_plasma 6 hr

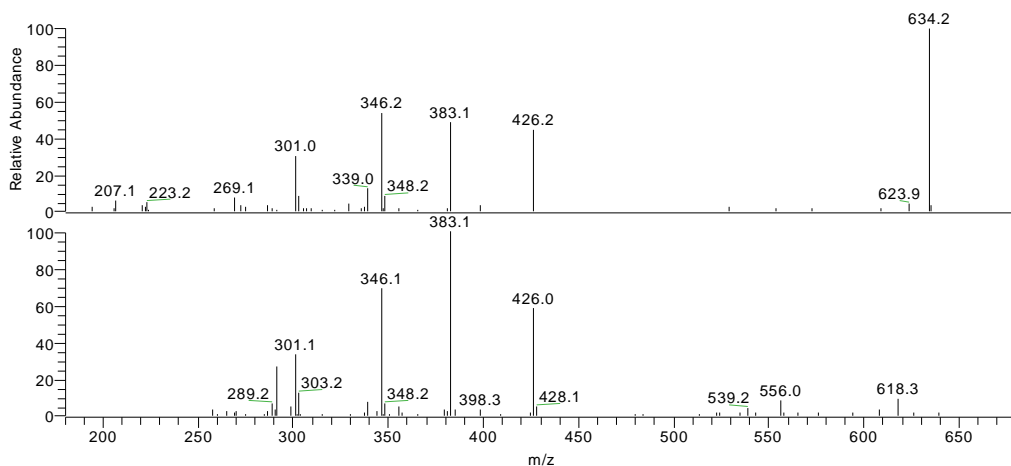


NL: 6.44E3
 m/z=
 300.50-301.50+345.50-346.50+382.50-383.50+
 425.50-426.50 F: ITM S +c ESI w Full ms2
 670.30@cid16.00 [180.00-680.00] M S
 102908_Bromocriptine_Admin_022

Hydroxy Bromocriptine

NL: 1.80E3
 m/z=
 300.50-301.50+345.50-346.50+382.50-383.50+
 425.50-426.50 F: ITM S +c ESI w Full ms2
 654.30@cid16.00 [250.00-670.00] M S
 102908_Bromocriptine_Admin_022

Bromocriptine



NL: 3.27E3
 102908_Bromocriptine_Admin_022#952
 RT: 2.57 AV: 1F: ITM S +c ESI w Full ms2
 670.30@cid16.00 [180.00-680.00]

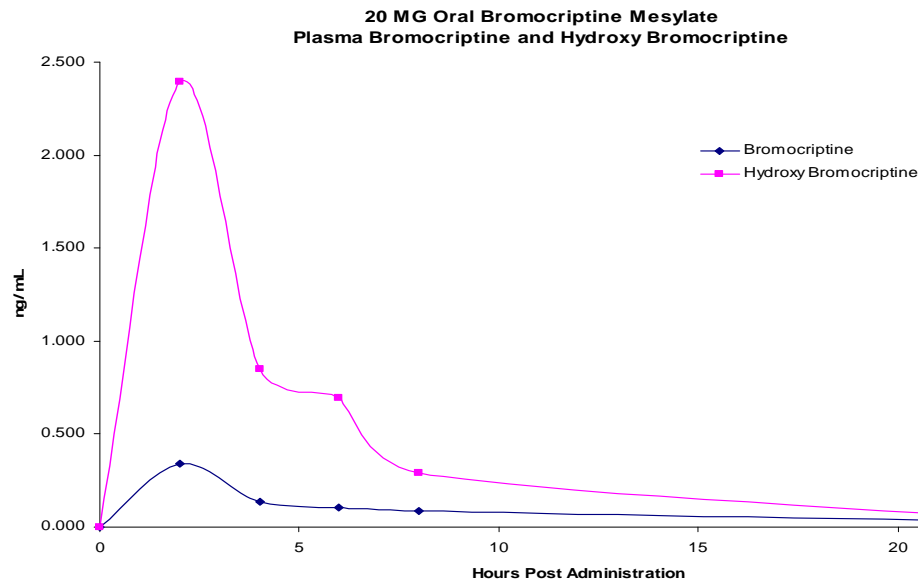
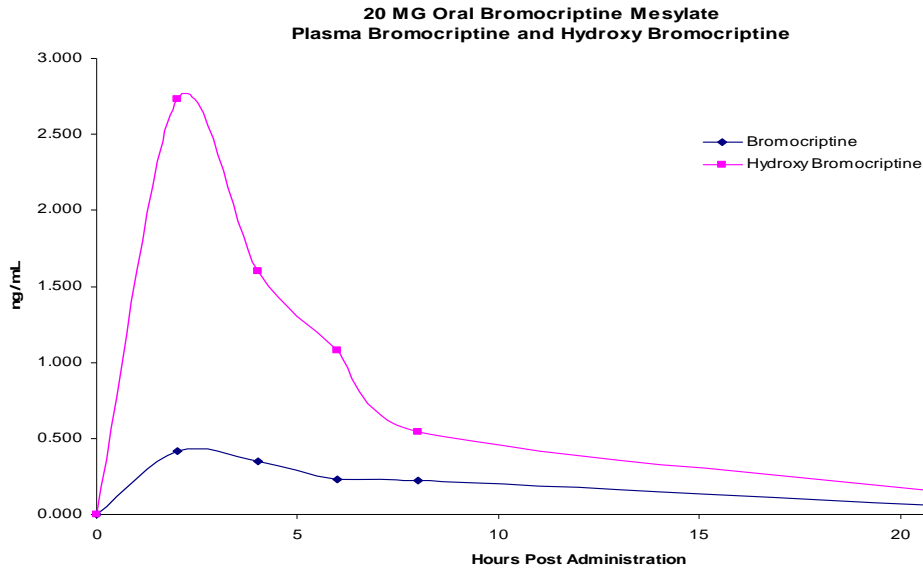
Hydroxy Bromocriptine

NL: 4.34E2
 102908_Bromocriptine_Admin_022#958-
 994 RT: 2.58-2.68 AV: 10 SB: 6 3.05-3.11F:
 ITM S +c ESI w Full ms2 654.30@cid16.00
 [250.00-670.00]

Bromocriptine

EQUINE ADMINISTRATION RESULTS

Equine plasma and urine samples were analyzed following oral administration of 20 milligrams of bromocriptine mesylate to the horse. Bromocriptine was detected in plasma but only barely in urine. Hydroxy bromocriptine was detected in plasma but not in urine following equine administration of bromocriptine mesylate. Independent results of analyses performed two weeks apart are shown below:



CRITERIA FOR IDENTIFICATION OF BROMOCRIPTINE AND HYDROXY BROMOCRIPTINE

This SOP is intended strictly for screening and confirmation purposes, but quantitative considerations are included to characterize method performance, deterioration of system responsiveness, and as value added estimates for reported findings. The positive threshold for this SOP is the limit of confirmation.

Identification of Bromocriptine

The qualifying ion for bromocriptine is 618 m/z. The diagnostic ions are 301, 346, 383, and 426 m/z and the retention time for the suspect sample, calibrator, and QC control must agree to +/- 0.15 minutes.

Confirmation of Bromocriptine

All diagnostic ions for bromocriptine are present in the full scan MSMS spectrum of the suspect sample, +/- 15% relative, of that ion compared with bromocriptine calibrator and control spectra, with no interfering ions > than 15% in relative abundance. Spectra may be averaged and/or subtracted.

Identification of Hydroxy Bromocriptine

The qualifying ion for hydroxy bromocriptine is 634 m/z. The diagnostic ions are 301, 346, 383, and 426 m/z. The retention time for the suspect sample must be within 0.5 minutes and less than that of bromocriptine calibrator and control retention times.

Confirmation of Hydroxy Bromocriptine

All diagnostic ions for hydroxy bromocriptine must be present in the full scan MS² spectrum of the suspect sample with abundances greater than 20%. The qualifying ion (634 m/z) must be present with abundance greater than 20%. The spectrum from the suspect sample must be graphically compared to bromocriptine calibrator and control spectra to demonstrate homology.

In addition, the spectra for bromocriptine and hydroxy bromocriptine acquired with the 5 dalton isolation widths must be compared. The presence of bromine must be clearly established for the qualifying and diagnostic ions of the unknown compared to the reference bromocriptine calibrator and control (pages 13 and 14). Spectra may be averaged and/or subtracted.

POSITIVE SAMPLE DATA PACKET

- A. SAMPLE TRANSFER SHEET (WS#32)
- B. SAMPLE USAGE SHEET (FORM #7)
- C. SAMPLE SEQUENCE LIST
- D. LC METHOD
- E. MS METHOD
- F. EXTRACTED ION CHROMATOGRAM COMPARISON
- G. SPECTRA COMPARISON
- H. QUANTIFICATION REPORT
- I. QUANTIFICATION CALIBRATION CURVE
- J. CONFIDENCE REPORT
- K. COLUMN TEST CHROMATOGRAM AND SPECTRA

Other Required Documentation

In addition to the positive data packet, the following documentations are required:
 Sequence Sample list print-outs are maintained in the instrument three ring binders.
 Instrument usage logbook is completed (and maintenance log if needed), indicating date, initials, and project.

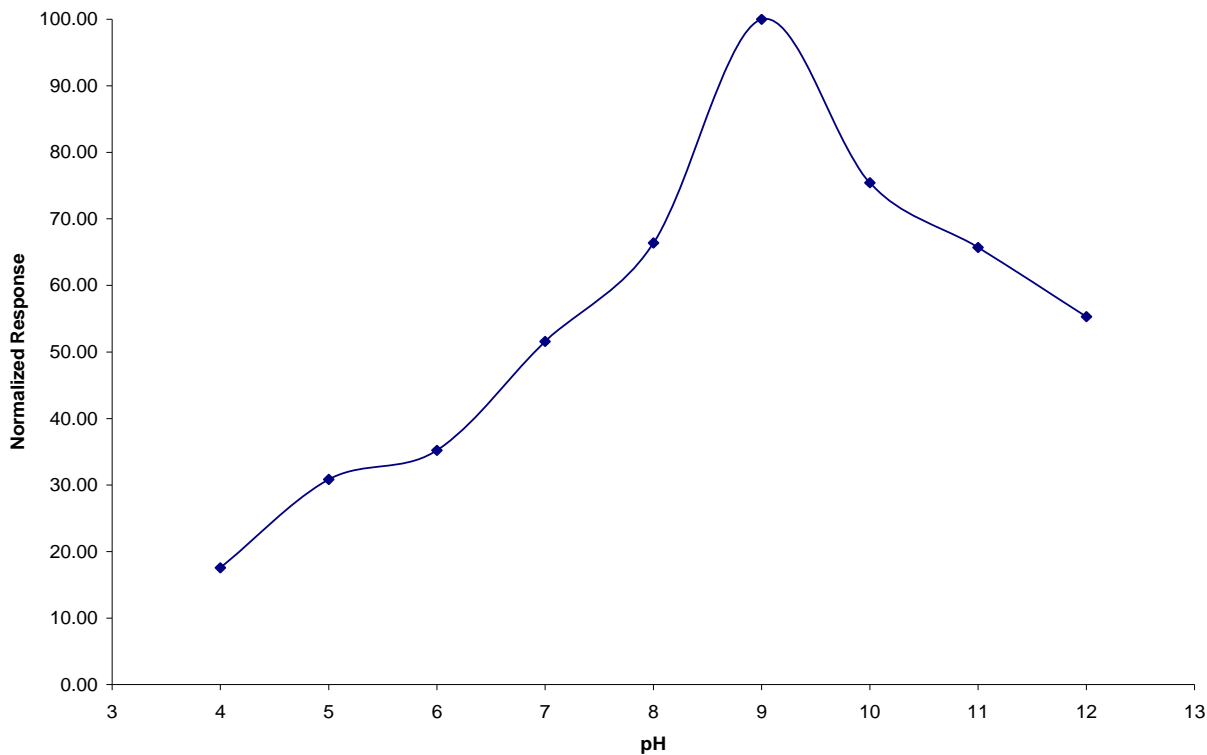
Data packets for samples determined to be negative will contain the following elements:

- a. Sample Transfer Sheet (WS # 32)
- b. Sample Usage Sheet (Form #7)
- c. Confidence Determination Report
- d. Quantification Report

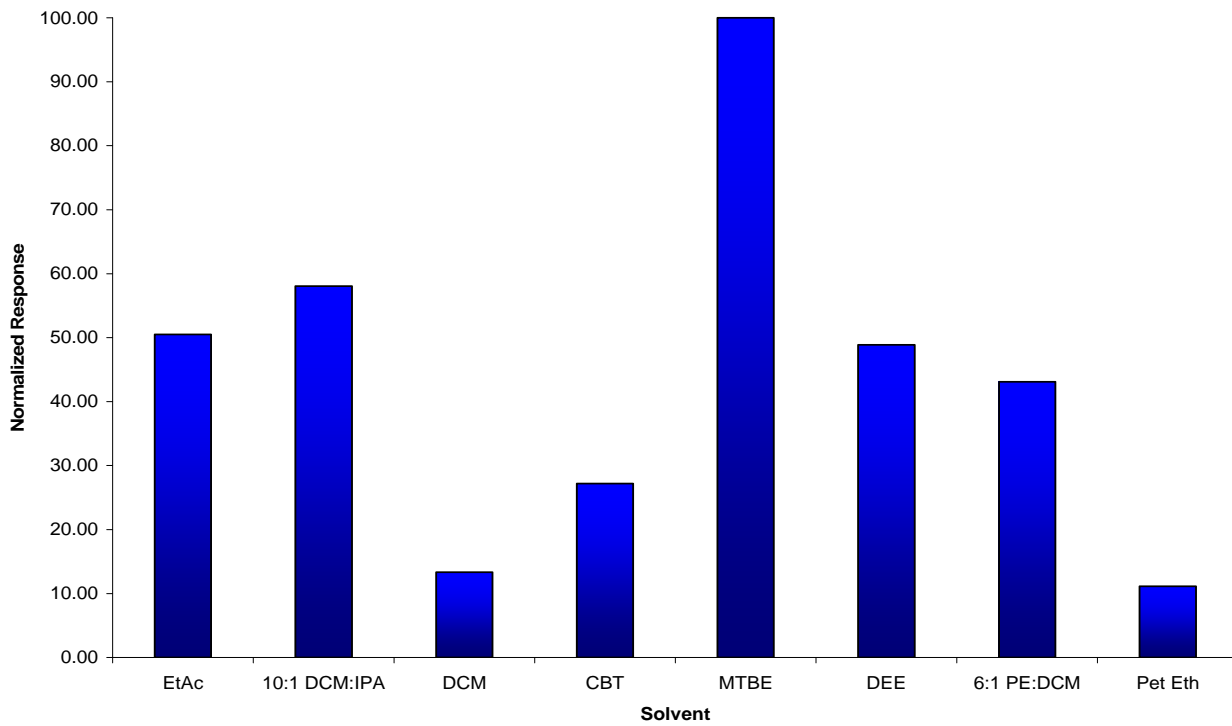
VALIDATION DATA

The liquid-liquid extraction conditions were determined from data sets depicted by the graphs below:

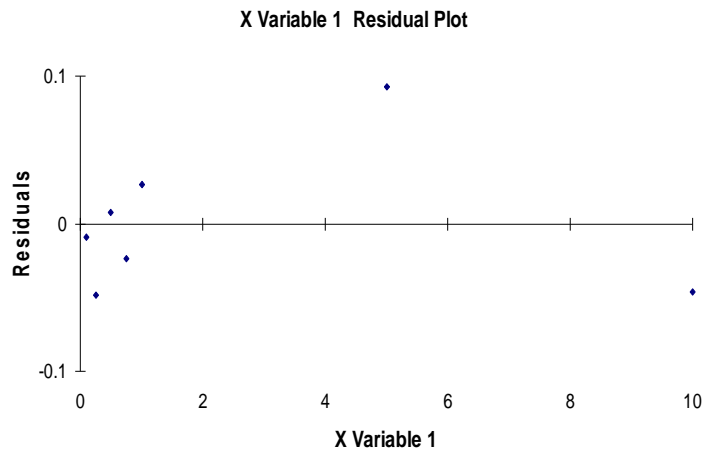
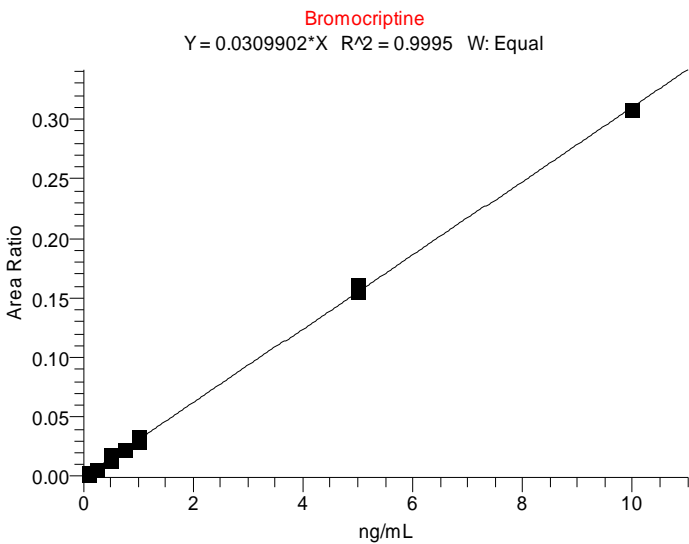
Bromocriptine pH Extraction Efficiency into methyl tert-butyl ether



Bromocriptine Solvent Extraction Efficiency at pH 10



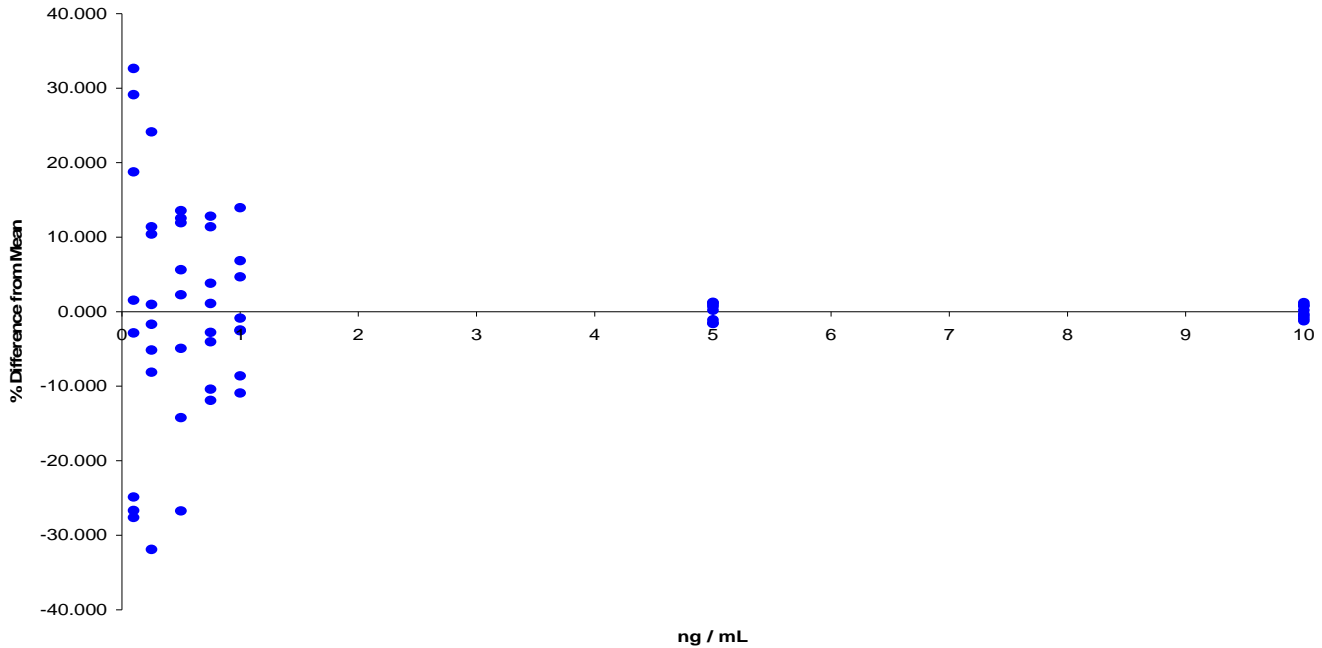
Typical calibration data for Bromocriptine is shown below:



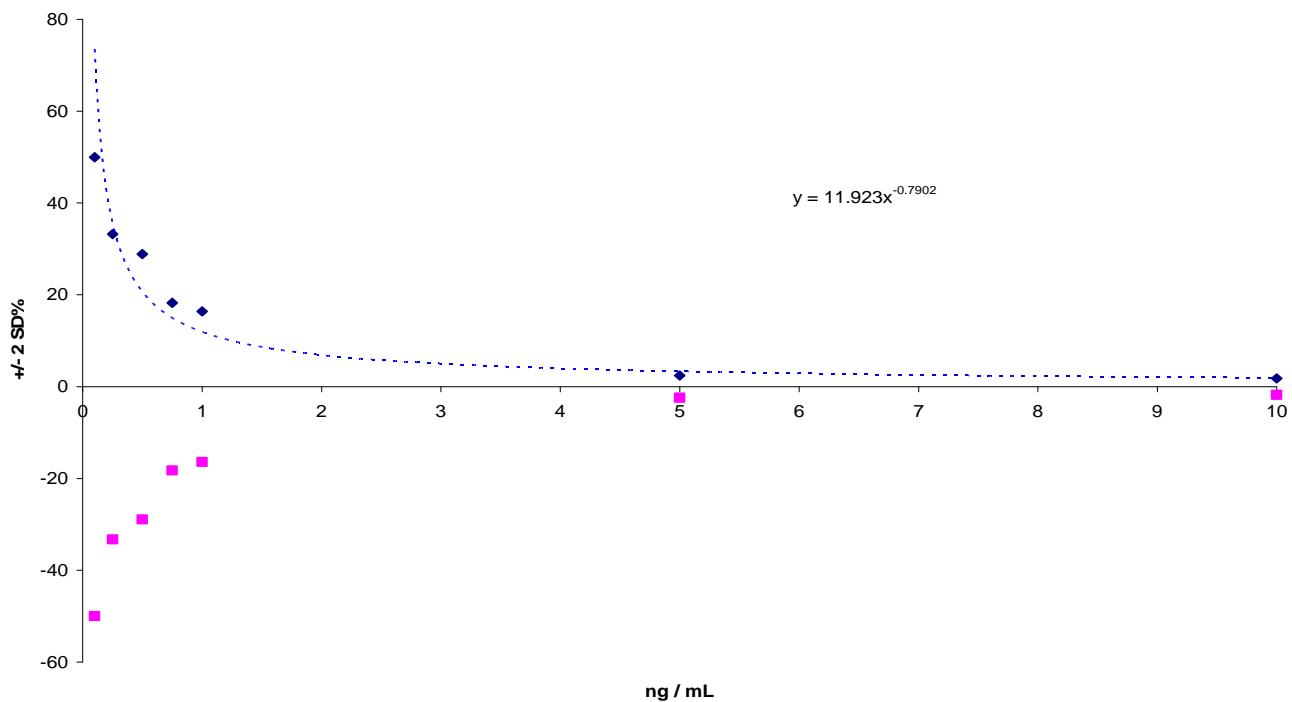
Measurement uncertainty for reporting of confirmed findings is treated as type A uncertainty, and is reported as the replicate mean plus/minus two times the replicate standard deviation with no expansion factor.

Method uncertainty is charted for the 0.5 ng/mL calibrators and controls for all analyses performed. Total method uncertainty intervals can be estimated by the following two graphics. The Bland-Altman plot shows the calibrator percent difference from the mean for the entire calibration series. The plus/minus two standard deviations from the mean plot is useful for determining the power function which describes the 95% confidence interval for the calibration series. This can be useful for estimating measurement uncertainty for type B situations (single measurements).

Bromocriptine Method Calibrator Uncertainty

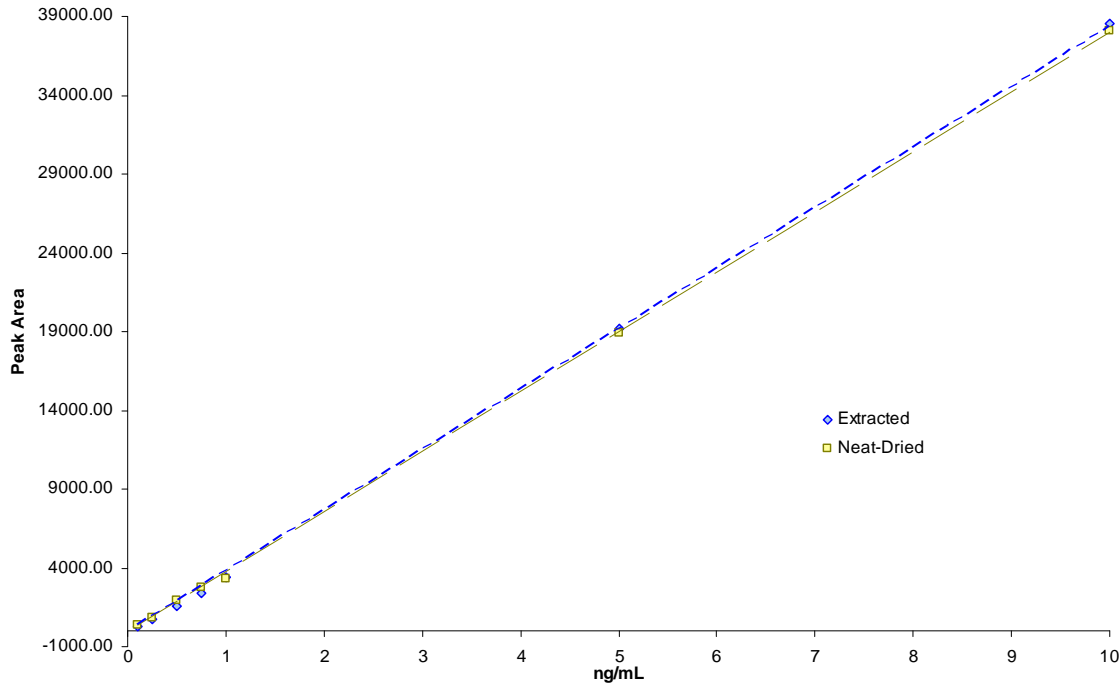


Bromocriptine Calibrators 95% Confidence Limits



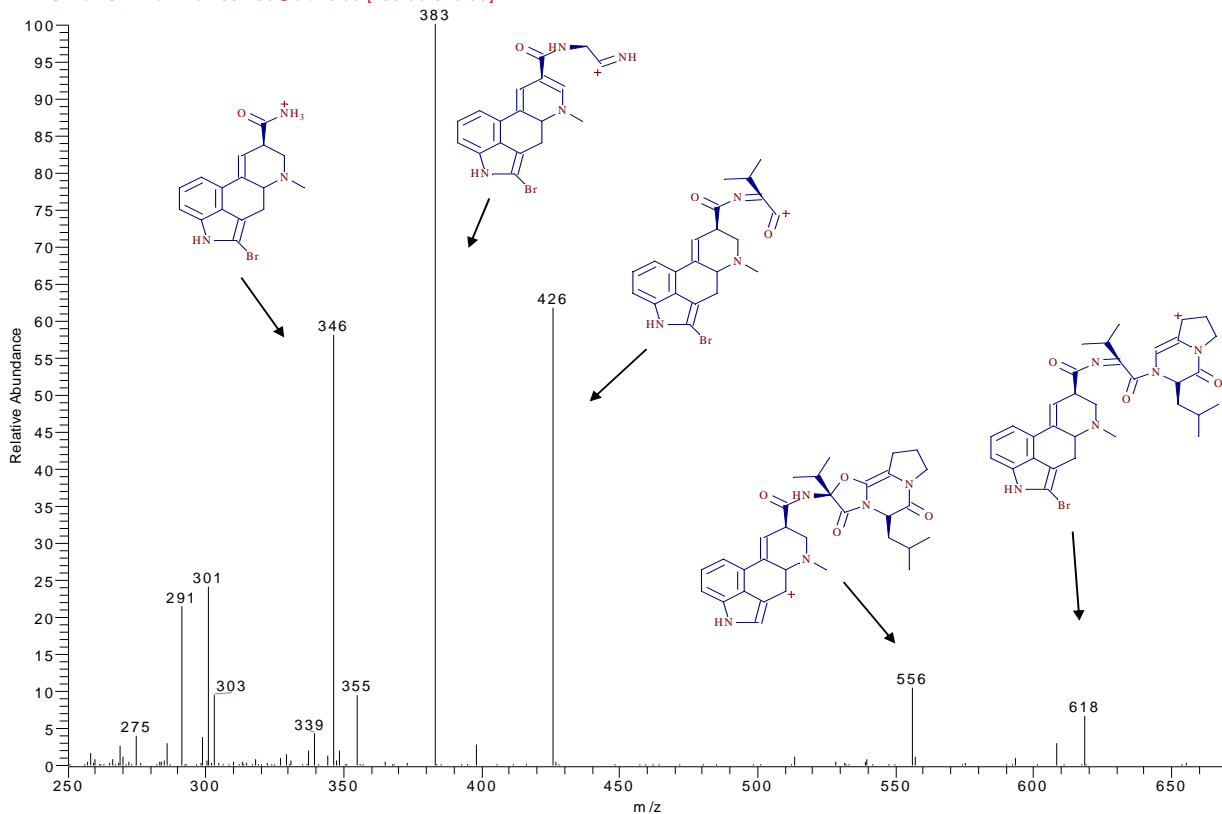
Method extraction recovery was estimated by comparing the slopes of neat calibrators and method extracted spiked plasma calibrators. Method recovery was approximately 100%.

Bromocriptine Calibrator Recovery

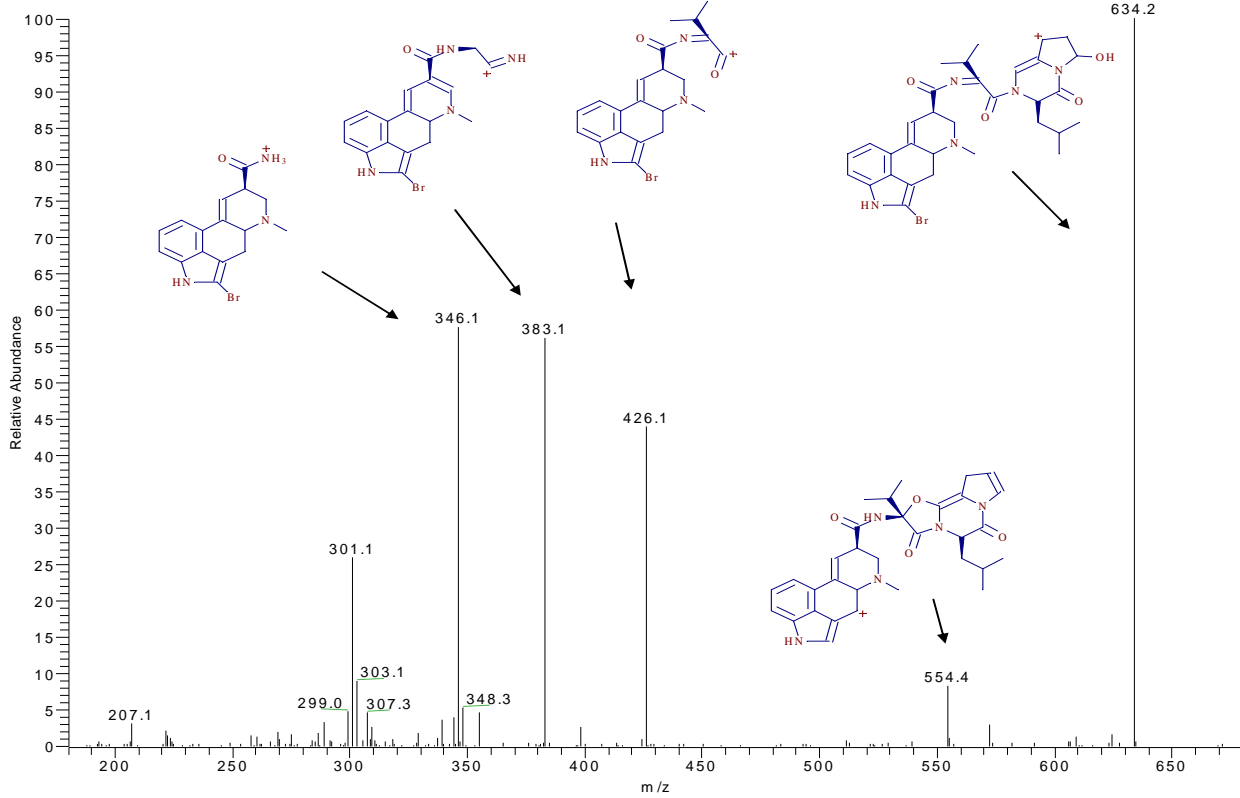


Mass Spectral fragment assignment for bromocriptine (below) and hydroxy bromocriptine (next page):

102908_Bromocriptine_Admin_013 #954-993 RT: 2.57-2.67 AV: 10 SB: 10 2.67-2.77 NL: 1.19E4
 F: ITMS + c ESI w Full m s 2 654.30@cid16.00 [250.00-670.00]



102908_bromocriptine_admin_022 #920-981 RT: 2.48-2.64 AV: 16 SB: 38 2.04-2.44 NL: 1.64E3
 F: ITMS + c ESI w Full m s 2 670.30 @ cid16.00 [180.00-680.00]

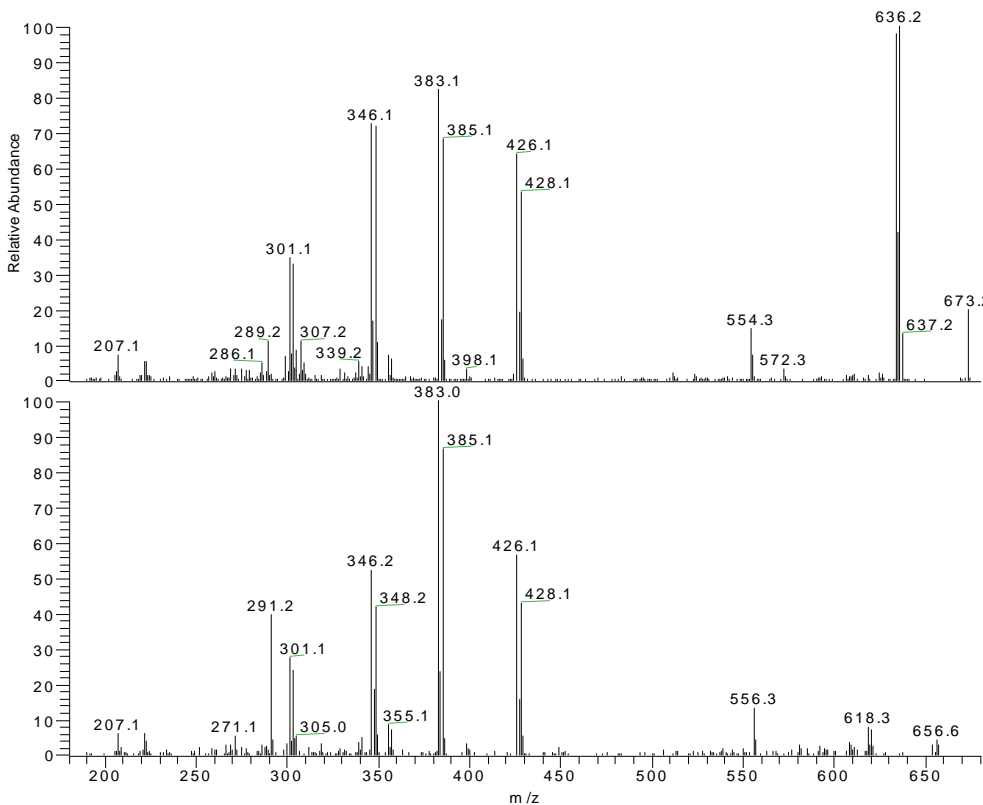


The structural assignments can be verified by comparison of MS² spectra acquired with a 5 dalton isolation width and observing the bromine isotope patterns (below, next page panel was zoomed):

102908_Bromocriptine_Admin_M021

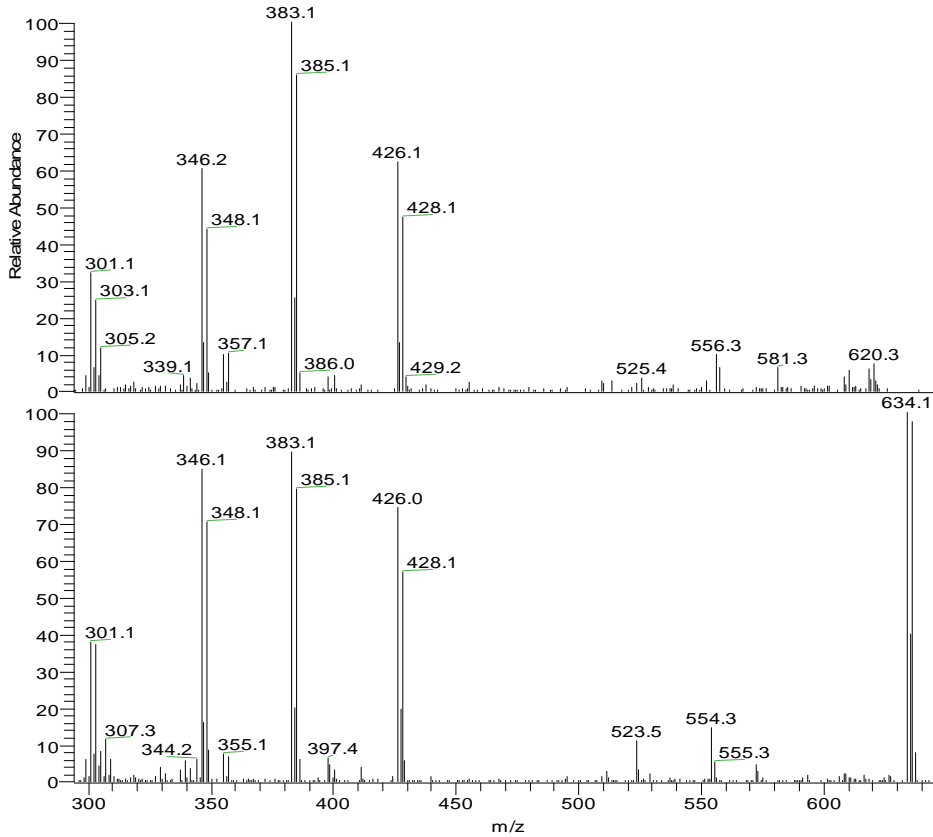
10/30/2008 9:02:35 AM

Cat_89_BRO_PO_plasma 4 hr



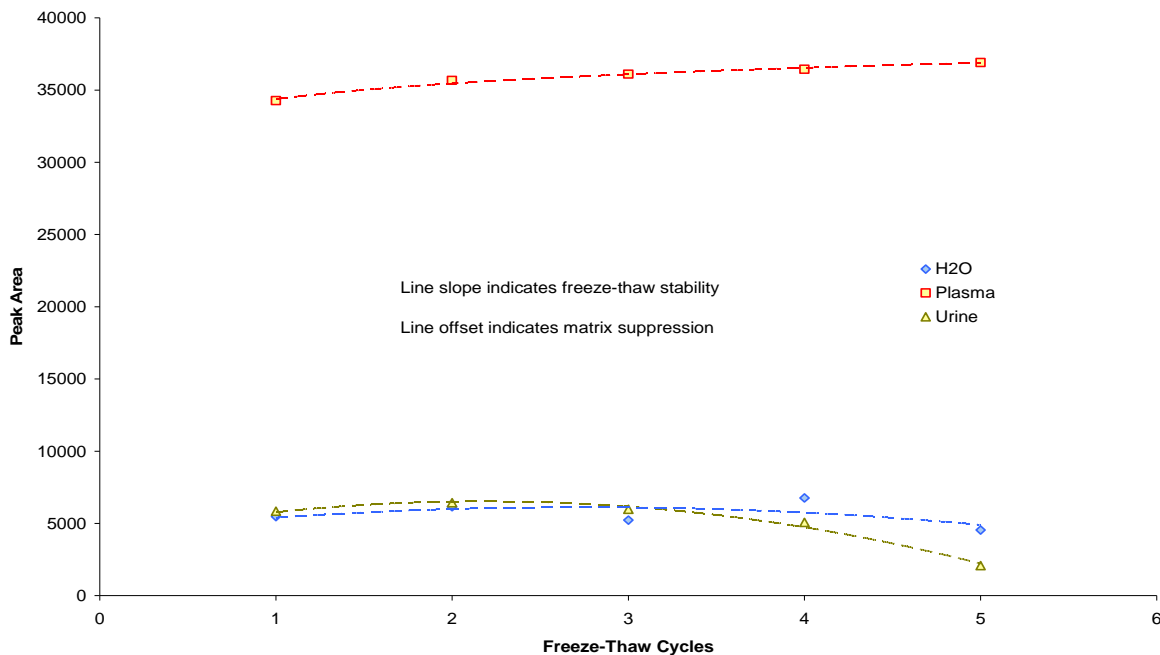
NL: 4.34E3
 102908_Bromocriptine_Admin_M0
 21#926-940 RT: 2.50-2.62 AV: 12
 F: ITMS + c ESI w Full m s 2
 671.30 @ cid16.00 [180.00-680.00]

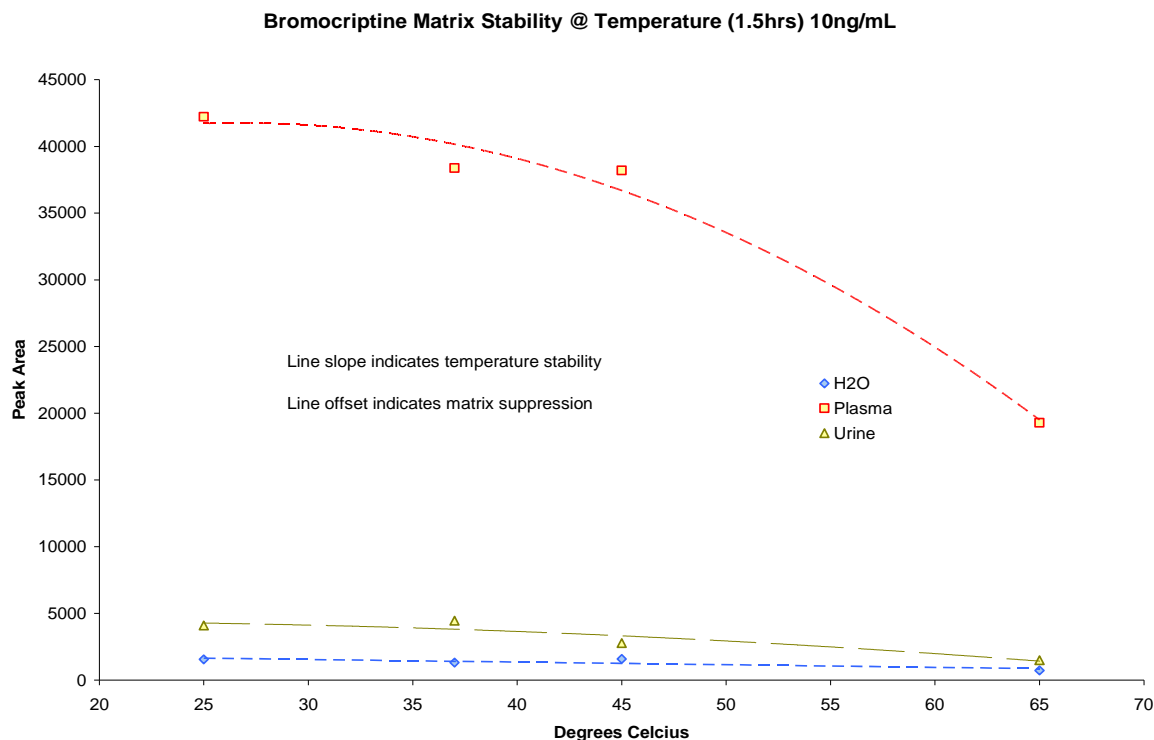
NL: 1.33E3
 102908_Bromocriptine_Admin_M0
 21#926-963 RT: 2.58-2.68 AV: 10
 SB: 28 2.19-2.50 F: ITMS + c ESI w
 Full m s 2 655.30 @ cid16.00
 [180.00-670.00]



The stability of bromocriptine was assessed by spiking water, equine negative control plasma, and equine negative control urine with 10 ng/mL bromocriptine. Freeze-thaw cycle stability was assessed by freezing a series of spiked matrix controls at -70°C, thawing the set at 37°C, removing one set of controls, and then refreezing the remaining sets of controls. This process was repeated until all sets were thawed. Temperature stability was assessed by subjecting spiked matrix control sets to temperatures of 25, 37, 45, and 65 degrees Celsius for 90 minutes. These sets were then extracted and analyzed using the sample preparation and instrumental conditions described above.

Bromocriptine Matrix Freeze-Thaw Stability 10ng/mL





DISCUSSION

Several methods have been described for multi-target analyte screens which include bromocriptine (2,3,4). These methods utilized intelligent data acquisition (IDA) triggered enhanced product ion scans (EPI) from fast duty cycle multiple reaction monitoring (MRM) scan events. Two references (2, 3) reported limits of detection (LOD) greater than 0.1 ng/mL, while another (4) did not report LODs. A specific targeted assay for bromocriptine in human plasma reported LODs of 2 pg/mL utilizing a triple quadrupole, tandem mass spectrometer. None of these multi-target drug analysis methods included hydroxy bromocriptine, because an authentic reference standard of hydroxy bromocriptine was not commercially available. However, hydroxy bromocriptine has been detected in equine plasma at concentrations 10-fold higher than bromocriptine.

Several factors must be considered in the analysis of bromocriptine. The presence of bromine immediately reduces the ion count sensitivity by 50% for both the precursor ion, and any resultant product ions. The cited multi-analyte methods employed solid phase extraction (SPE) best targeted for polar or amphoteric target compounds. While these preparative methods have the benefit of being semi- to fully automated, they all suffer from analyte recoveries less than 90%, with many analytes having even lower recoveries. While these methods have the advantage of extracting a wide range of target drugs with usable recoveries, they also suffer from extracting a wide range of matrix components from biological matrices. Thus, matrix suppression effects become more dramatic at the limits of detection, and expected concentrations of bromocriptine in plasma are predicted to occur at these challenging limits. Tune files for multi-analyte screening methods covering a wide range of molecular weights are at best a compromise, with many analytes having less than optimal response compared to

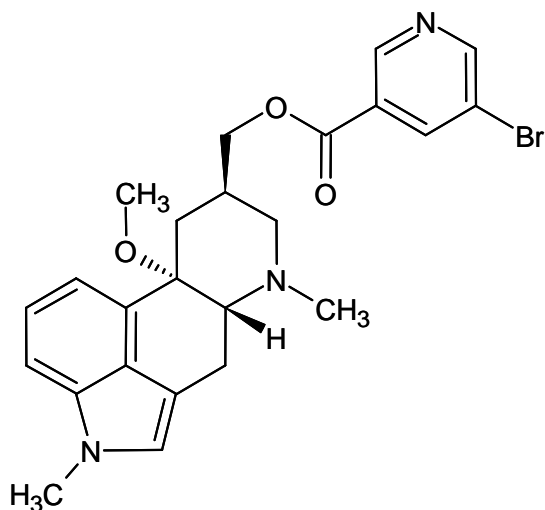
a tune file specific for that compound. One solution is to group screening target compounds by similar molecular weight ranges and preparative chemistry. Hydroxy bromocriptine reference standard is not commercially available, thus, multi-analyte methods may have excluded it as a target compound. Conversely, specific targeted metabolite information is difficult to generate or obtain for every compound included in large multi-analyte screening strategies.

Ion-trap screening methods are limited by either relatively low numbers (8-20) of specific targeted product ion scan events (MS^2) per chromatographic segment, or diminishing sensitivity (and triggering) when employing data dependant-based MS^2 acquisition from precursor ion scan events close to the limits of detection. The targeted ion trap method for bromocriptine described in this SOP can be modified to include wide isolation widths (5 amu) to capture the entire ion current for product ions extracted from the MS^2 total ion chromatogram. The inclusion of hydroxy bromocriptine as a method analyte in the absence of a reference standard is possible due to the homology of its MS^2 spectrum to that of bromocriptine. This targeted ion trap standard operating procedure represents an order of magnitude greater sensitivity for bromocriptine than the multi-analyte methods cited (2,3,4), and two orders of magnitude greater sensitivity with the inclusion of hydroxy bromocriptine as a target analyte.

Other ergoline-based compounds which may be included in screening strategies are shown below:

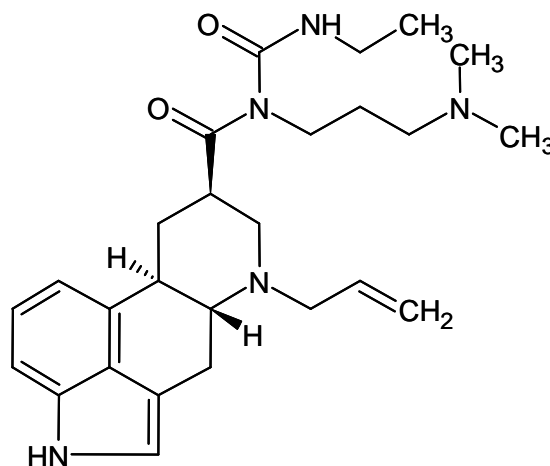
Nicergoline

Molecular Formula = $C_{24}H_{26}BrN_3O_3$
 Formula Weight = 484.38554
 Monoisotopic Mass = 483.115747 Da



Cabergoline

Molecular Formula = $C_{26}H_{37}N_5O_2$
 Formula Weight = 451.60428
 Monoisotopic Mass = 451.294725 Da



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