

QUANTIFICATION AND CONFIRMATION OF FLUPHENAZINE AND ITS METABOLITES IN EQUINE PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

DEVELOPED BY

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I. INTRODUCTION

Fluphenazine (FLU) is an antipsychotic drug in the phenothiazine class of compounds. The N-alkyl side chain is modified from a base phenothiazine structure (acepromazine, propionylpromazine) and thus, confers greater pharmacological activity. This class of compound is used in the equine athlete for controlling unruly behavior. Fluphenazine decanoate dose forms are intended for slow release from intermuscular injection depot. The effective dose coupled with this mode of administration results in plasma and urinary drug concentrations, that are both pharmacologically relevant but analytically challenging. To address the analytical problem, this method was, therefore, developed to monitor and confirm the presence of Fluphenazine and/or its metabolites in plasma (and urine) following the administration of this compound to racehorses in competition.

FLU may be detected by gas-chromatography mass spectrometry (GC/MS) in full scan mode following administration. However this technique is not sufficiently sensitive to provide full-scan electron impact spectra, that satisfy criteria for a positive finding for the presence of fluphenazine and/or its metabolites in a test sample. Described herein is the standard operating procedures involving the use of liquid chromatography mass spectrometry (LCMSMS) for the quantification and confirmation of FLU and/or its metabolites in equine urine and plasma samples following screening by enzyme-linked immunosorbent assay (ELISA with a presumptive presence of the analytes in the test sample).

II. SCOPE

This standard operating procedure describes the quantification and confirmation of Fluphenazine and its metabolites (Fluphenazine Sulfoxide and 7-Hydroxyl Fluphenazine) in equine plasma previously screened and flagged as a presumptive FLU positive by Enzyme-Linked Immunosorbent Assay. The method involves liquid-liquid extraction (LLE) of equine plasma with subsequent analysis on a Micromass Q-TOF tandem mass spectrometer. The scope of this work covers procedures to be used in quantifying and confirming the presence of Fluphenazine and its metabolites (Figure 1) in equine plasma (or urine) samples obtained from racehorses. Since there is no tolerance level of FLU or metabolites in racing equine athletes, reporting of the results to the Racing Commissions will be determined by the limit of confirmation of any detected and confirmed FLU and/or related analytes in either plasma or urine.

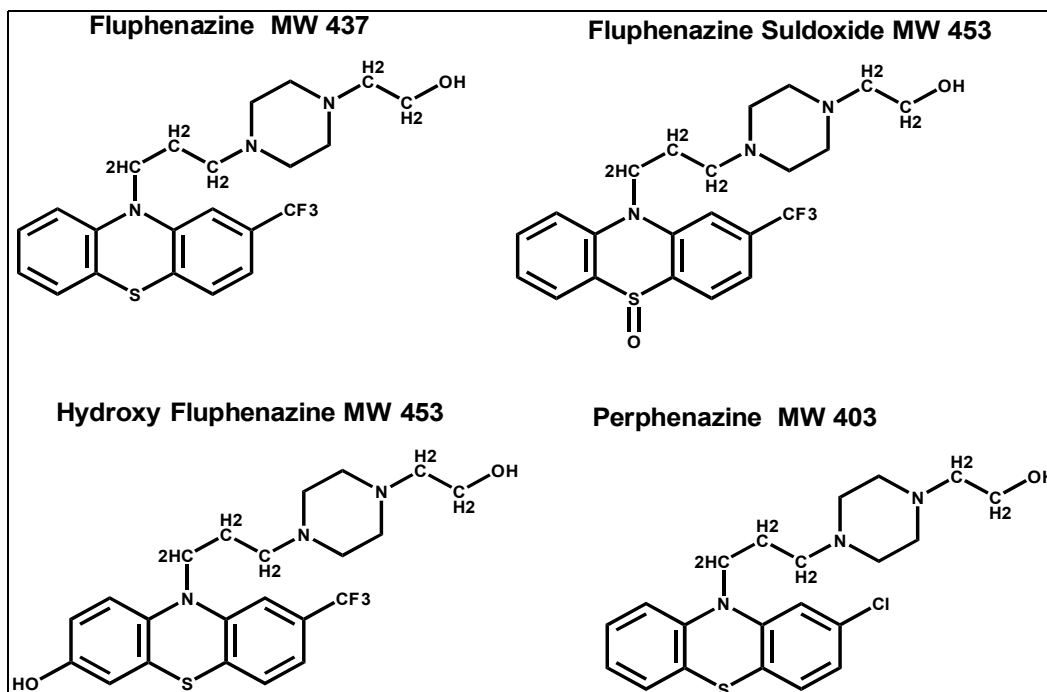
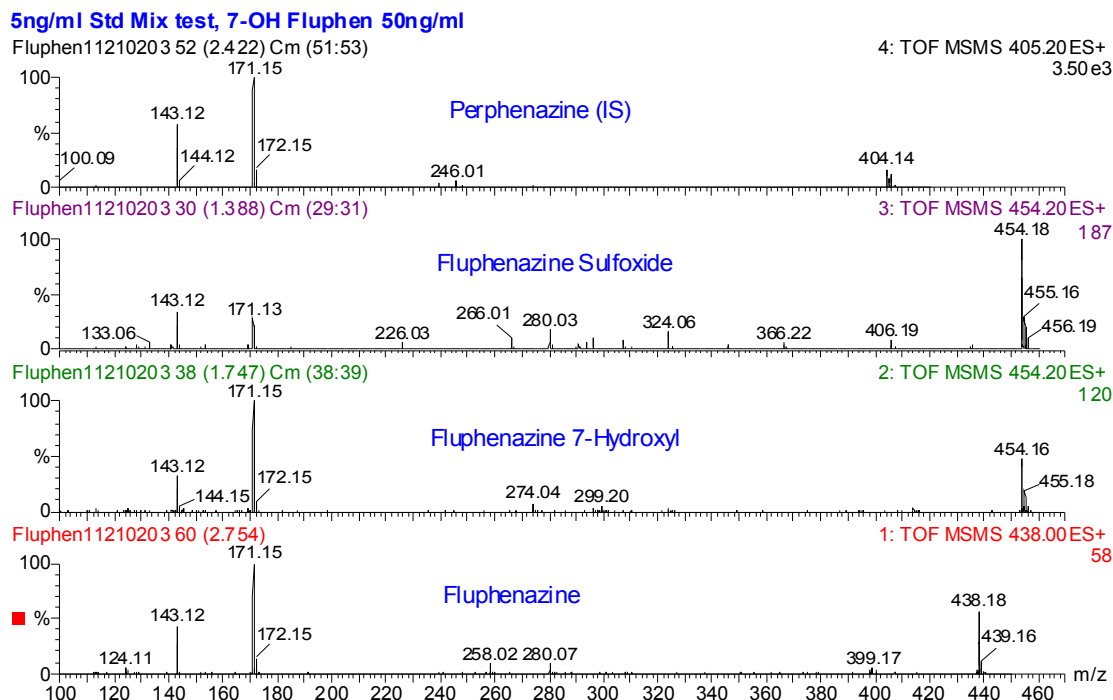


Figure 1: Chemical structures of fluphenazine, fluphenazine sulfoxide, 7-hydroxyl fluphenazine and perphenazine (IS).

III. PRINCIPLE OF METHOD

Plasma sample is subjected to LLE at pH 9.0 [1]. The residue is dissolved and the analyte is identified, quantified and confirmed by liquid chromatography/ Q-TOF mass spectrometry operated under electrospray ionization mode. For equine plasma, the primary analyte is parent Fluphenazine. Data for metabolites is included for possible application to other matrices. The upper limit of quantification of fluphenazine and its metabolites in equine plasma by this method is approximately 100 ng/mL. The lower limit of quantification and detection of fluphenazine and its metabolites in equine plasma is 0.1 ng/ml.



(NOTE: Precursor ion for perphenazine (IS) should be 404.14 NOT 405.20 m/z in Figs. 2 and 10)

Figure 2. Mass spectra of Fluphenazine (bottom panel), 7-Hydroxyl Fluphenazine (the Second panel from bottom) and Fluphenazine Sulfoxide (the third panel from bottom) and Perphenazine (as the internal standard, IS;the top panel).

IV. PRIMARY REFERENCE MATERIALS

1. Primary Analytical Standard Reference Material

- a) Fluphenazine dihydrochloride, FW 510.4, Salt Eq: 510.4/437.53=1.167 (Cat. No. F-4765, Lot No.79F0400, Sigma)
- b) Fluphenazine Sulfoxide, FW: 453.5 (Cat. No. BRTCH SQ-021488, Bristol-Meyers Squibb)
- c) 7-Hydroxyl Fluphenazine, FW: 453.52 (Donated by Dr. Tobin, University of Kentucky)

2. Primary Analytical Internal Standard Reference Material

Perphenazine, FW 403.0 (Cat. No. P-6402, Lot No. 64H0447, Sigma)

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

3. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

a) 1 mg/mL solution of Fluphenazine standard stock solution

Materials

Fluphenazine

Methanol

Procedure

Weigh about 3 mg (**X.xx** mg) Fluphenazine into a 4-mL glass bottle.

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

Cap and mix until Fluphenazine is completely dissolved in methanol. The resulting concentration of Fluphenazine 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# 659).

b) 1 mg/mL of Fluphenazine Sulfoxide standard stock solution

Materials

Fluphenazine Sulfoxide

Methanol

Procedure

Weigh about 3 mg (**X.xx** mg) Fluphenazine Sulfoxide into a glass bottle.

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

Cap and mix until Fluphenazine Sulfoxide is completely dissolved in methanol. The resulting concentration 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# 660).

c) 1 mg/mL of 7-Hydroxy Fluphenazine standard stock solution

Materials

Hydroxy Fluphenazine

Methanol

Procedure

Weigh about 3 mg (**X.xx** mg) Hydroxy Fluphenazine into a glass bottle.

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

Cap and mix until Hydroxy Fluphenazine is completely dissolved in methanol. The resulting concentration 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# 661).

d) 1 mg/mL of Perphenazine standard stock solution (Internal Standard)

Materials

Perphenazine

Methanol

Procedure

Weigh about 3 mg (**X.xx** mg) of Perphenazine into a glass bottle.
 Dilute to volume using HPLC grade (or better) methanol (Volume **Y.yy** mL = **X.xx** mg).

Cap and mix until it is completely dissolved in methanol.
 The resulting concentration of Perphenazine 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# 662).

4. PREPARATION OF SECONDARY REFERENCE STOCK SOLUTIONS (WORKING SOLUTION)

A. Materials

- 1.1mg/mL of Fluphenazine, Fluphenazine Sulfoxide and 7-Hydroxyl Fluphenazine primary reference stock solutions
- 2.Methanol: Water: Formic Acid (50:50:0.1, v/v/v)

B. Procedure

Fluphenazine and its metabolite standard working solutions of different concentrations are prepared as follows (see Table 1):

Table 1. Preparation of Fluphenazine and its Metabolite Standard Working Solutions

Target Con.(ug/mL)	Made From Stock Solution (ug/mL)	Vol.Added(uL)	Vol.(uL) MeOH/H2O/FA (50:50:0.1)	Used For 10 uL + 1 mL Plasma (ng/mL)
10	1000	10	990	100
5	50	100	900	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

Storage Requirements: Store at approximately 4 °C.

Label Fluphenazine and its metabolite working solutions and record the preparation and labeling in the secondary preparation logbook in the Q-TOF Unit of the Laboratory.

C. Preparation of 20 µg/mL of Perphenazine Internal Standard (IS) Working Solution

A. Materials

- 1.1mg/mL of Perphenazine stock solution
- 2.Methanol: Water: Formic Acid (50:50:0.1, v/v/v)

B. Procedure

Take 100 uL of 1 mg/mL Perphenazine stock solution following by adding 900 uL of Methanol: Water: Formic Acid (50:50:0.1, v/v/v), vortex.

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

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5. PREPARATION OF FLUPHENAZINE AND METABOLITES, QUALITY CONTROL WORKING SOLUTIONS

A. Materials

- 1mg/mL Fluphenazine and its metabolites QC secondary stock solution
- Methanol: Water: Formic Acid (50:50:0.1, v/v/v)

B. Procedure

- Fluphenazine and its metabolites QC working solution of different concentrations are prepared as summarized in Table 2.

Table 2. Preparation of Fluphenazine and its Metabolites QC Working Solutions

Target Con.(ug/mL)	Made From Stock Solution (ug/mL)	Vol.Added(uL)	Vol. (uL) MeOH/H2O/FA (50:50:0.1)	Used For 10 uL + 1 mL Plasma (ng/mL)
5.0	50	100	900	50
0.5	5	100	900	5
0.05	0.5	100	900	0.5

Storage Requirements: Store at approximately 4 °C.

Label Fluphenazine and its metabolites QC working solutions and record the preparation and labeling in the secondary preparation logbook in the Q-TOF Unit of the Laboratory.

V. PREPARATION OF PLASMA CALIBRATORS AND QUALITY CONTROLS

The following calibrators are prepared in negative pooled equine plasma samples, previously demonstrated by Enzyme Linked Immunosorbent Assay (ELISA) and this SOP to be negative for the presence of Fluphenazine and/or its metabolites.

A. Materials

- Fluphenazine and its metabolites standard working solutions
- Negative equine plasma

B. Procedure

Fluphenazine and its metabolites plasma calibrators of different concentrations are prepared as summarized in Table 3.

Table 3. Preparation of Fluphenazine and its Metabolites Calibrators in Plasma

Calibrator Code #	Target Conc. (ng/mL)	Working Solution (ug/mL)	Spike Working Solution (uL/mL)	Volume of Plasma (mL)
102202 FLUPHENMIX 0.1	0.1	0.01	150	15.0
102202 FLUPHENMIX 0.5	0.5	0.05	150	15.0
102202 FLUPHENMIX 1	1.0	0.1	150	15.0
102202 FLUPHENMIX 5	5.0	0.5	150	15.0
102202 FLUPHENMIX 10	10.0	1	150	15.0
102202 FLUPHENMIX 50	50.0	5	150	15.0
102202 FLUPHENMIX 100	100.0	10	150	15.0

C. Materials for preparing plasma QC samples

- Fluphenazine and its metabolites QC working solutions
- Negative equine plasma

D. Procedure for preparing plasma QC samples

Fluphenazine and its metabolites calibrators in plasma at different concentrations prepared as summarized in Table 4.

Table 4. Preparation of Fluphenazine and its Metabolites Plasma QC Samples

QC Code#	Target Conc. (ng/mL)	Working Solution (µg/mL)	Spike Working Solution (µL/mL)	Volume of Plasma (mL)
102202 QNP 23 PLASMA	0.0	0.0	0.0	15.0
102202 FLUPHENMIX 0.5 QC	0.5	0.05	150	15.0
102202 FLUPHENMIX 5 QC	5.0	0.50	150	15.0
102202 FLUPHENMIX 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.

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 label category of Table 3 and Table 4.

LABEL (format MMDDYY_FLUPHENMIX_ ng /mL) and (format MDDYYFLUPHENMIX_QC ng/mL)

Print using AVERY Template 5267 in MS Word) 14 of each

Label screw cap culture tubes (16x125 mm) in 14 x 14 format, and aliquot 1 mL of the appropriate calibrator from Tables 3 and 4 into each respective tube. Cap and store at -70°C.

VI. SAMPLE REQUIREMENTS FOR ANALYSIS

Prepare Calibrators, Quality Control samples in duplicate, and suspect samples in triplicate for each analysis performed.

VII. FLUPHENAZINE AND ITS METABOLITES SAMPLE EXTRACTION 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 warm water at 37 °C. Follow the procedure below to extract the Calibrators, QCs and samples for Fluphenazine and its Metabolites

- Label 16 x 125 mm test tubes accordingly.
- Prepare standards, QC and unknown samples into the labeled tubes.
- Add 10 µL of 10 µg/mL Perphenazine (IS) into each individual standard, QC and unknown samples except negative control plasma (blank).
- Vortex the contents of each tube for 5 – 10 seconds to mix.
- Add 3 mL of 0.1 M pH 9.0 phosphate buffer to adjust pH to 9.
- Add 5 mL of Methyl-tert Butyl Ether (MTBE) into each tube, rotorack for 10 minutes.
- Centrifuge 10 minutes at 3000 rpm.
- Transfer the organic phase (top layer) into labeled clean 16 x 100 mm culture tubes.
- Add another 5 mL of MTBE into the sample, followed by rotoracking for 10 minutes and centrifuge 10 minutes as described above.
- Combine the second organic extract with the first for each individual sample.
- Evaporate to dryness under steady N₂ stream at 45 °C.
- Dissolve the residue in 150 µL MeOH: H₂O:FA (50:50:1, v/v/v).
- Perform ultrasonic treatment for 5 minutes at 50 °C to dissolve the residue completely.
- Transfer the above solution into 200 µL inserts, then put in the auto sampler vials. All the samples are now ready for LC/MS/MS analysis.

VIII. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF FLUPHENAZINE AND ITS METABOLITES AND ITS METABOLITES

A. Liquid Chromatographic and Mass Spectrometer 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) HPLC Conditions

1). LC column

- a) Type: Zorbax Eclipse XDB Phenyl Cartridge Column, Part No. 820950-927 (Agilent Technologies)
- b) Length: 12.5 mm, i.d. 4.6 mm
- c) Particle size: 5 micron
- d) Temperature: 27 ° C

2) Mobile Phase

A: 2mM NH₄AC:ACN:NH₄OH (95:5:0.01, v/v/v, pH 4.70)

B: 2mM NH₄AC:ACN (5:95, v/v)

Gradient for Fluphenazine and its Metabolites

Time (min)	A	B	Flow Rate (mL/min)
0	50	50	0.3
0.6	50	50	0.3
0.61	85	15	0.3
2.50	85	15	0.3
2.51	90	10	0.3
3.50	90	10	0.3
3.51	50	50	0.3
5.00	50	50	0.3

3) Injection Volume: 20 µL.

4) Mass Spectrometric Parameters

Parameters	Fluphenazine	Fluphenazine Sulfoxide	7-Hydroxyl Fluphenazine	Perphenazine (IS)
TOF MSMS	438.0	454.2	454.2	404.2
Scan Range (Daltons)	100.0-450.0	100.0-460.0	100.0-460.0	100.0-420.0
Collision Energy (eV)	25	25	27	25
Cone (Volts)	30	35	40	30

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Scan time (sec)	0.5	0.5	0.5	0.5
Inter Scan Time (sec)	0.05	0.05	0.05	0.05
Start Time (min)	0.00	0.00	0.00	0.00
End Time (min)	4.5	4.5	4.5	4.5

IX. SAMPLE LIST SETUP FOR ANALYSIS OF FLUPHENAZINE AND ITS METABOLITES

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

X. CRITERIA FOR IDENTIFICATION OF FLUPHENAZINE AND ITS METABOLITES IN EQUINE PLASMA EXTRACTS

Identification of Fluphenazine

The qualifying ion for the identification of Fluphenazine is m/z 438 [M+H], with diagnostic ions of m/z 171, and 143. The quantifying ion is m/z 171. Under LC-MS/MS analytical conditions described, all the above diagnostic ions should be recognized at retention time of ~3.10 minutes.

All described ions for Fluphenazine must be present in the full scan MSMS spectrum (averaged spectrum at 20% peak height); and the retention time for the suspect sample, 5 ng/mL calibrator, and 5 ng/mL QC control must agree to +/- 0.15 minutes.

Identification of Fluphenazine Sulfoxide

The qualifying ion for the identification of Fluphenazine Sulfoxide is m/z 454 [M+H], with diagnostic ions of m/z 171 and 143. The quantifying ion is m/z 454. Under LC-MS/MS analytical conditions described, all the above diagnostic ions should be recognized at retention time of ~1.43 minutes.

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All described ions for Fluphenazine Sulfoxide must be present in the full scan MSMS spectrum (averaged spectrum at 20% peak height); and the retention time for the suspect sample, 5 ng/mL calibrator, and 5 ng/mL QC control must agree to +/- 0.15 minutes.

Identification of 7-Hydroxyl Fluphenazine

The qualifying ion for the identification of 7-Hydroxyl Fluphenazine is m/z 454 [M+H], with diagnostic ions of m/z 171 and 143. The quantifying ion is m/z 171. Under LC-MS/MS analytical conditions described, all the above diagnostic ions should be recognized at retention time of ~1.92 minutes.

All described ions for 7-Hydroxyl Fluphenazine must be present in the full scan MSMS spectrum (averaged spectrum at 20% peak height); and the retention time for the suspect sample, 5 ng/mL calibrator, and 5 ng/mL QC control must agree to +/- 0.15 minutes.

Identification of Perphenazine (IS)

The qualifying ion for the identification of the internal standard, perphenazine, is m/z 404 [M+H], and its diagnostic ions of m/z 171 and 143. The quantifying ion is m/z 171 (BP). Under LC-MS/MS analytical conditions described, all the above diagnostic ions should be recognized at retention time of ~2.70 min.

All described ions for the target analyte(s) must be present in the full scan MSMS spectrum (averaged spectrum at 20% peak height), and the retention time for the suspect sample, 5 ng/mL calibrator, and 5 ng/mL QC control must agree to +/- 0.15 minutes.

XI. CRITERIA FOR QUANTIFICATION OF FLUPHENAZINE AND ITS METABOLITES IN EQUINE PLASMA

Determination of Fluphenazine and Its Metabolites

The product ion used for quantification of Fluphenazine is m/z 171.

The product ions used for quantification of Fluphenazine metabolites, Fluphenazine Sulfoxide and 7-Hydroxyl Fluphenazine were m/z 454 and 171, respectively.

The product ion used for quantification for Perphenazine (IS) is m/z 171.

Using the MassLynx Quantification software, execute quantification method and 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 Fluphenazine and its metabolites.

The analysis confidence interval for the sample and 5 ng/mL calibrators and controls must be determined and logged for method control purposes. Since there is no tolerance concentration of Fluphenazine or metabolites allowed in the racing equine in PA, the reporting limit for these analytes is the limit of confirmation as described in this SOP. An Excel template on the MicroMass workstation desktop allows this report to be generated.

XII. CRITERIA FOR REPORTING A SAMPLE AS POSITIVE FOR FLUPHENAZINE AND ITS METABOLITES

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

The test sample contains Fluphenazine and/or its metabolites according to the chromatographic and spectral criteria described above.

Any confirmable test sample for Fluphenazine or its metabolites should be considered as positive.

The LC retention times of the quantifying ion for Fluphenazine and its metabolites 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 Fluphenazine and its metabolites and internal standard (Perphenazine) should be greater than 5. This is determined by inspection of the full scan MSMS extracted ion chromatogram comparisons, which are included in the analysis data packet. These will include the Negative Control, suspect sample, solvent blank following the sample, 5 ng/mL control, and 5 ng/mL calibrator.

The MSMS full scan spectra comparison contains no mass spectral peaks greater than 25% of the quantifying ion for Fluphenazine and its metabolites. This is determined by the average across the chromatographic peak at 10% peak height. These spectra may be subtracted and/or smoothed. 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. The mass spectrum comparison of the sample, control, and standard must be included with each report.

All Blanks and Negative control samples must not contain quantifiable and confirmable concentration of Fluphenazine and its metabolites.

XIII. 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 may be manually corrected. If an excessive number of manual corrections are found, the supervisor must be consulted to either:

- Adjust the integration program parameters or 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 confidences. Manual integration may produce slightly better precision..

XIV. METHOD VALIDATION

Analyte Recovery

The results of recoveries for Fluphenazine and its metabolites are listed in Table 5. The upper limit of quantification of fluphenazine and its metabolites in equine plasma by this method is approximately 100 ng/mL. The lower limit of quantification and detection of fluphenazine and its metabolites in equine plasma is 0.1 ng/ml.

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Table 5. Extraction efficiency of fluphenazine and its metabolites spiked in equine plasma (n=5)

Amount spiked (ng/mL)	Recoveries (%)			C.V. (%)		
	Fluphenazine	Fluphenazine Sulfoxide	7-Hydroxyl Fluphenazine	Fluphenazine	Fluphenazine Sulfoxide	7-Hydroxyl Fluphenazine
1	98.88 ± 11.68	94.13 ± 7.109	98.48 ± 9.26	11.82	7.54	9.4
10	93.65 ± 3.70	109.94 ± 5.77	98.16 ± 4.26	3.95	5.25	4.34
100	93.02 ± 6.33	99.55 ± 6.55	100.75 ± 7.52	6.8	6.58	7.46

Precision, Reproducibility, and Accuracy

Table 6. Intra-day Precision and Accuracy of Fluphenazine and its Metabolite Equine Plasma (n=18)

Amount added (ng/mL)	Intra-day								
	Fluphenazine Detected (ng/mL)	CVa (%)	Arb (%)	Fluphenazine Sulfoxide Detected (ng/mL)	CVa (%)	Arb (%)	7-Hydroxyl Fluphenazine Detected (ng/mL)	CVa (%)	Arb (%)
0.5	0.501 ± 0.025	4.91	100.14	0.517 ± 0.020	3.90	103.47	0.525 ± 0.028	5.24	104.91
5	4.924 ± 0.210	4.20	98.48	4.724 ± 0.204	4.32	94.48	4.772 ± 0.133	2.78	95.44
50	50.069 ± 1.720	3.44	100.14	50.174 ± 1.834	3.66	100.51	50.245 ± 2.230	4.78	100.49

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

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

Table 7. Inter-day Precision and Accuracy of Fluphenazine and its Metabolites in Equine Plasma (n=5)

Amount added (ng/mL)	Inter-day								
	Fluphenazine Detected (ng/mL)	CVa (%)	Arb (%)	Fluphenazine Sulfoxide Detected (ng/mL)	CVa (%)	Arb (%)	7-Hydroxyl Fluphenazine Detected (ng/mL)	CVa (%)	Arb (%)
0.5	0.506 ± 0.058	11.39	101.27	0.519 ± 0.027	5.28	103.80	0.523 ± 0.038	7.3	104.55
5	4.992 ± 0.192	3.84	99.85	4.791 ± 0.221	4.61	95.82	4.730 ± 0.163	3.44	94.60
50	49.874 ± 1.754	3.52	99.75	50.047 ± 1.398	2.79	99.81	50.228 ± 2.484	4.95	100.46

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

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

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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 established initially 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. The number of calibrators used for the individual analysis determines this k factor (n=6, k=2.5).
3. The **method** measurement uncertainty (reproducibility) coverage factor is expressed as k=2.5 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 5 ng/mL control mean for each analytical run (n=4). Greater than 5 independent runs must be acquired prior to validation acceptance.

Table 8. Measurement of Uncertainty for Fluphenazine and its Metabolites in Equine Plasma

Symbol	Source of Uncertainty	Value Units (%)	Distribution	Divisor	Standard Uncertainty	Degrees of Freedom (n-1)	Other
U ₁	Intermediate precision	2.16	N	1	2.16	5	Fluphenazine 5 ng/mL
U ₂	Intermediate precision	3.88	N	1	3.88	5	Fluphenazine Sulfoxide 5 ng/mL
U ₃	Intermediate precision	5.52	N	1	5.52	5	7-Hydroxyl Fluphenazine 5 ng/mL
Combined Uncertainty	1: (U ₁ ²) ^{1/2} = 2.16; 2. (U ₂ ²) ^{1/2} = 3.88; 3. (U ₃ ²) ^{1/2} = 5.52						
Expanded Uncertainty (k=2.5)	1: (2.16 x 2.5) = 5.40%; 2: (3.88 x 2.5) = 9.70%; 3: (5.52 x 2.5) = 13.80%						

Application of MU to Reported Results: Fluphenazine result of 5 ng/mL with an MU of 5.40 % will be reported as 5 ng/mL ± 0.27 ng/mL, for example (5 x 5.40 divided by 100 ng/mL = 0.27 ng/mL)

4. Threshold control records and charts (5 ng/mL n=4) for method development and all subsequent analyses are created and maintained.

Demonstration of Ionization Suppression or Enhancement 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. Therefore, using the method described by Bonfiglio, et al[2], ionization stability was determined for the chromatographic and mass spectrometric conditions described by this SOP. This determination was made for both the target compound, Fluphenazine and its metabolites, as well as the internal standard, Perphenazine.

Briefly, the test compound (Fluphenazine or its metabolites or Perphenazine) 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 run, but also from late-eluting compounds that may not be detected until after several sequential analyses had been performed. The results of these

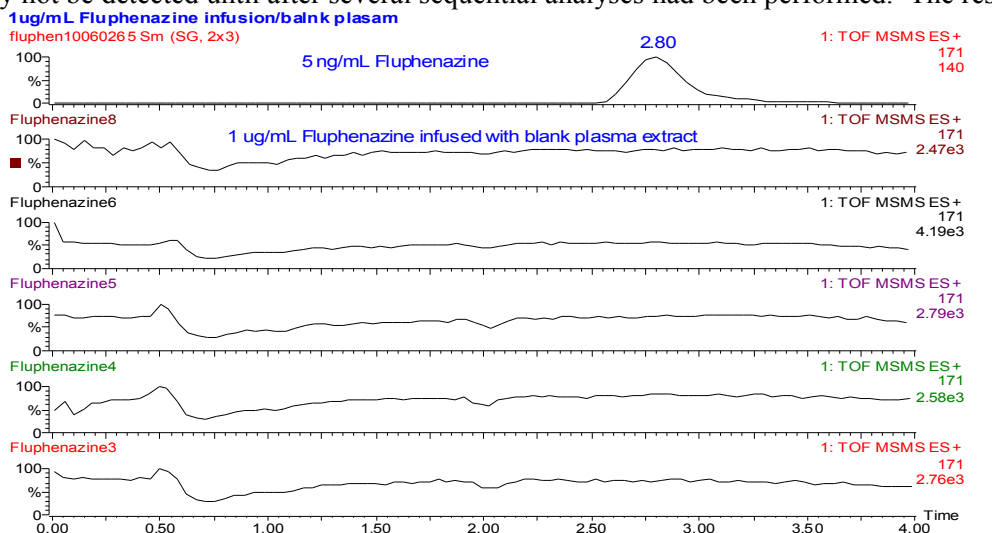


Figure 3. Fluphenazine infusion experiment demonstrating the absence of method and matrix ionization enhancement or suppression at the retention time of Fluphenazine (2.80 min.)

experiments are presented below demonstrating the absence of ionization enhancement or suppression in the retention time ranges of the target compounds (Fluphenazine and its metabolites and Perphenazine) relevant to this standard operating procedure.

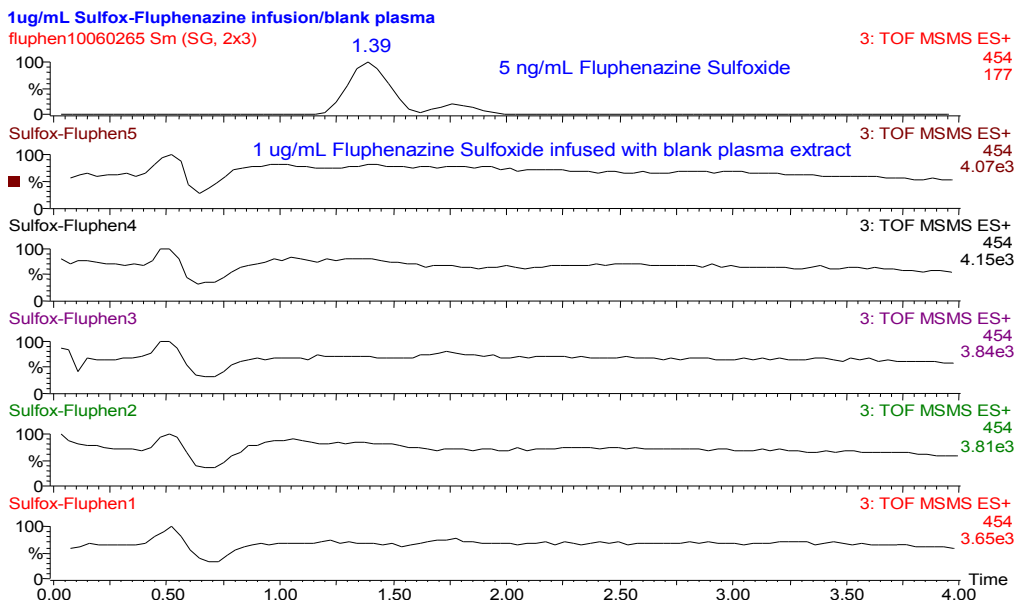


Figure 4. Fluphenazine Sulfoxide infusion experiment demonstrating the absence of method and matrix

ionization enhancement or suppression at the retention time of Fluphenazine Sulfoxide (1.39 min.)

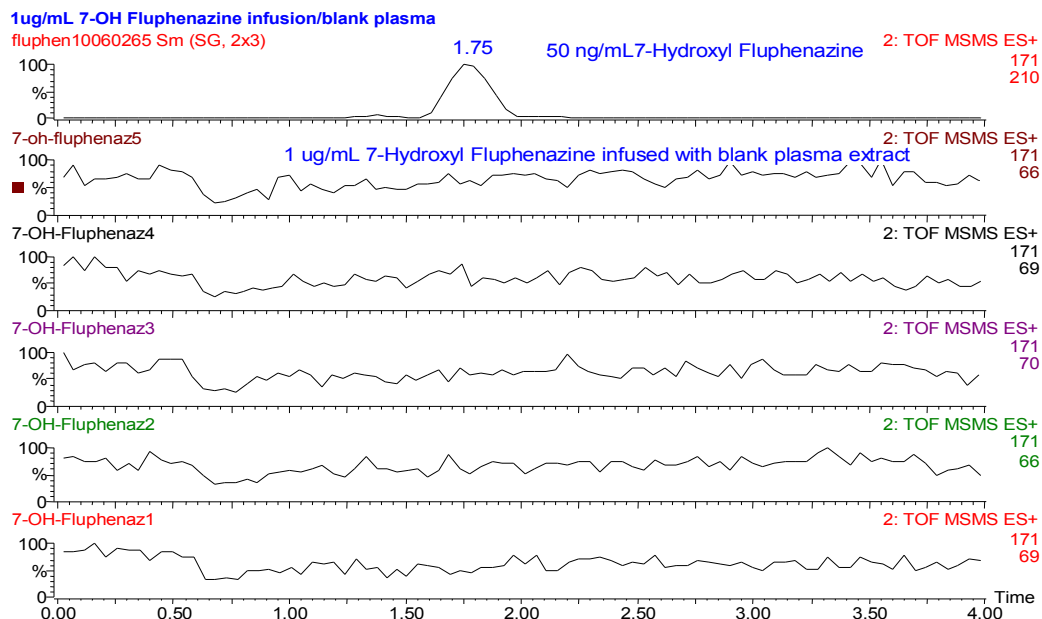


Figure 5. 7-Hydroxyl Fluphenazine infusion experiment demonstrating the absence of method and matrix ionization enhancement or suppression at the retention time of 7-Hydroxyl Fluphenazine (1.75 min.)

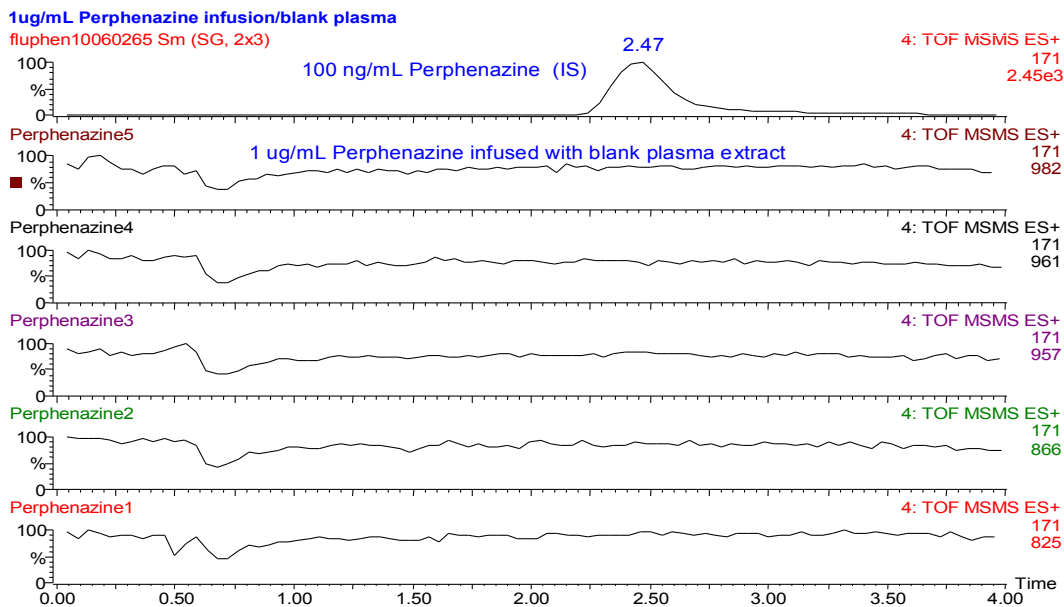


Figure 6. Perphenazine (IS) infusion experiment demonstrating the absence of method and matrix ionization enhancement or suppression at the retention time of Perphenazine (2.47 min.)

Stability of Fluphenazine in Equine Plasma

Room Temperature

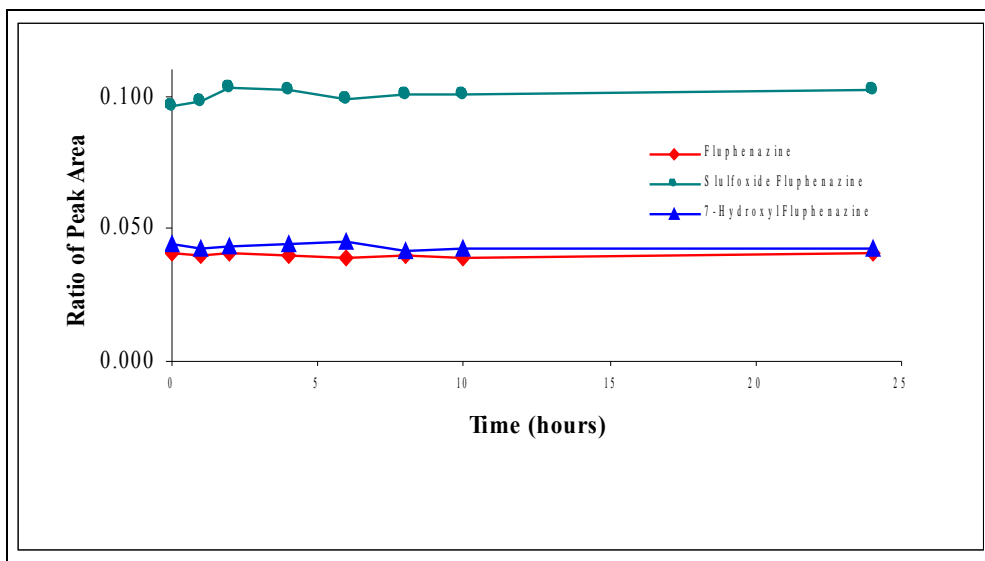


Figure 7. Stability of Fluphenazine and its Metabolites in Equine Plasma at Room Temperature

Freezer Temperature

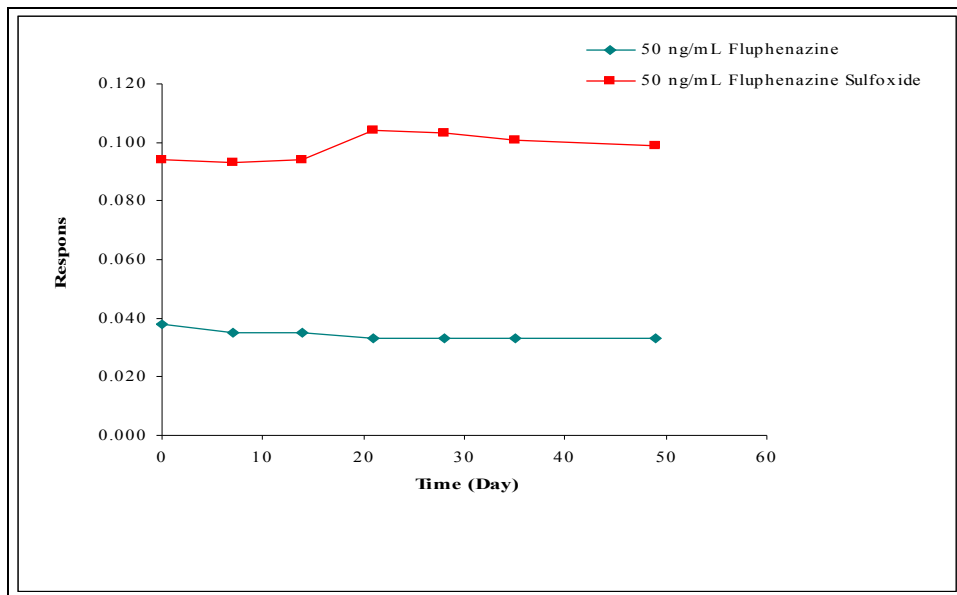


Figure 8. Stability of Fluphenazine and its Metabolites in Equine Plasma at -70°C .

Since the 7-Hydroxy fluphenazine metabolite was not detected/or produced by the horse in sufficient quantity to allow quantification and confirmation, little attention was given to this analyte in the stability study at -70°C .

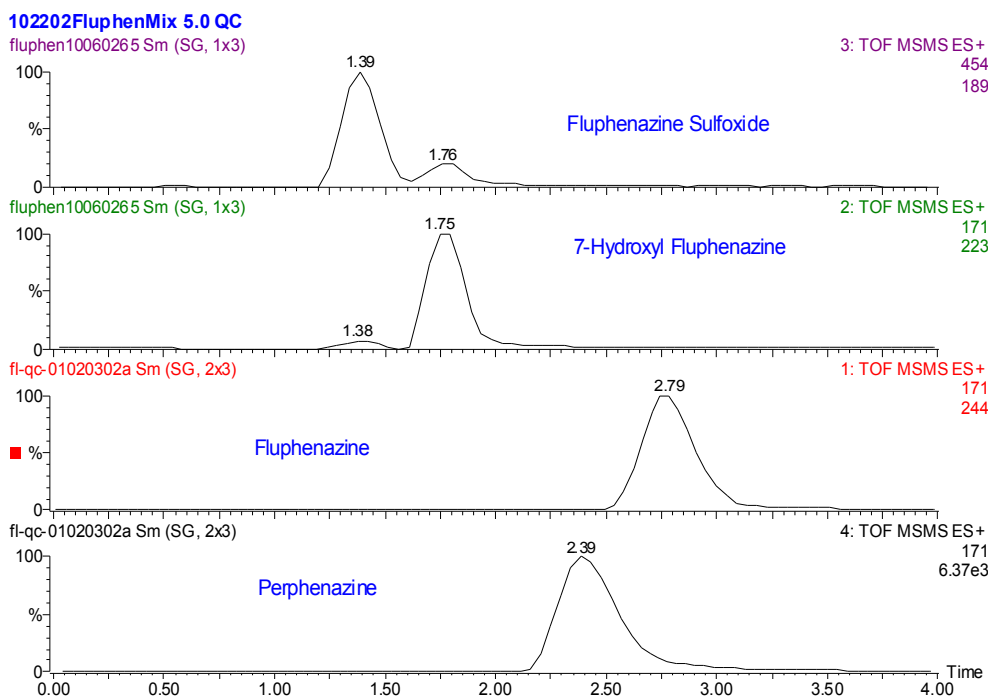


Figure 9. LC-MS Chromatograms of Fluphenazine (second panel from bottom), 7-Hydroxyl Fluphenazine (third-panel from bottom), Fluphenazine Sulfoxide (top panel) and Perphenazine (bottom panel).

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Acknowledged By: _____ Lab Supervisor

102202FluphenMix 5.0 QC

fluphen10060265 30 (1.387) Cm (27:34-43:62)

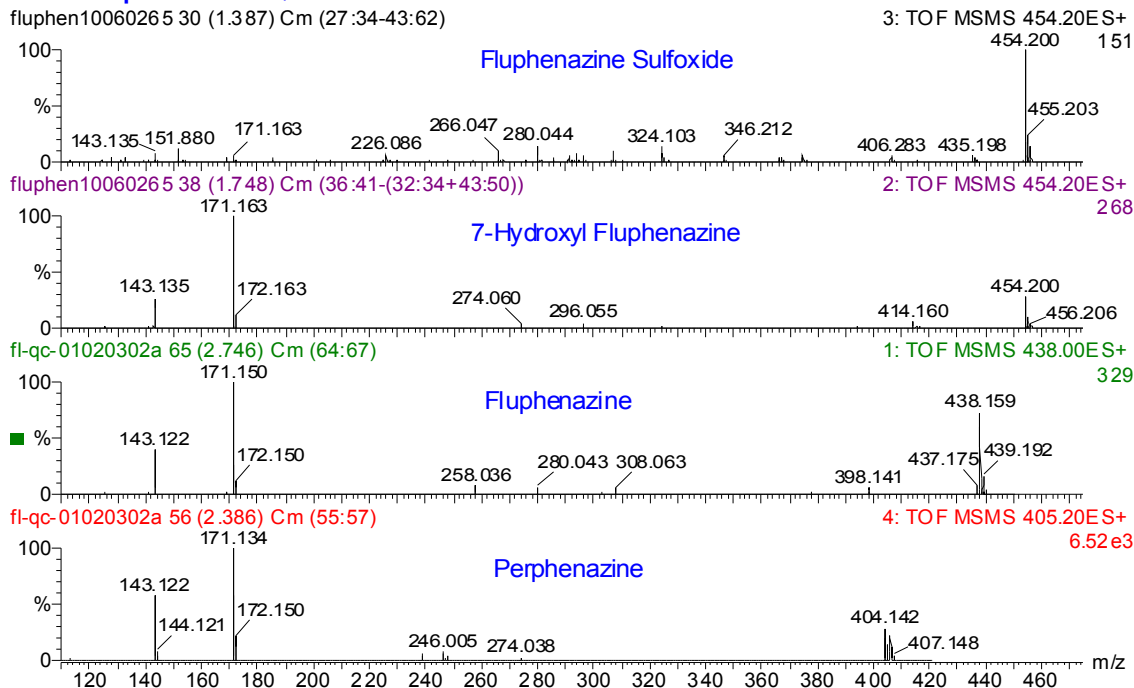


Figure 10: Mass Spectrum of Fluphenazine (second panel from bottom), 7-Hydroxyl Fluphenazine (third-panel from bottom), Fluphenazine Sulfoxide (top panel) and Perphenazine (bottom panel).

Compound 1 name: Fluphenazine Method File: Fluphenazine
 Coefficient of Determination: 0.999713
 Calibration curve: $0.00454951 * x + 0.000153676$
 Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
 Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

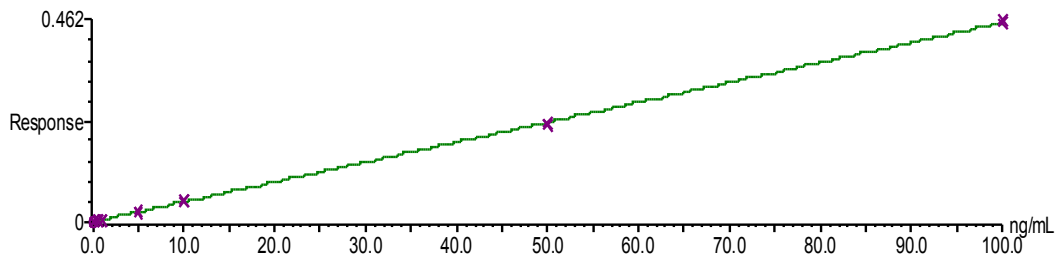


Figure 11. Calibration Curve of Fluphenazine in Equine Plasma

Compound 2 name: Sulfoxide-Fluphenazine Method File: Fluphenazine
 Coefficient of Determination: 0.999900
 Calibration curve: $-4.26943e-5 * x^2 + 0.0112203 * x + -0.000359784$
 Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
 Curve type: 2nd Order, Origin: include, Weighting: 1/x, Axis trans: None

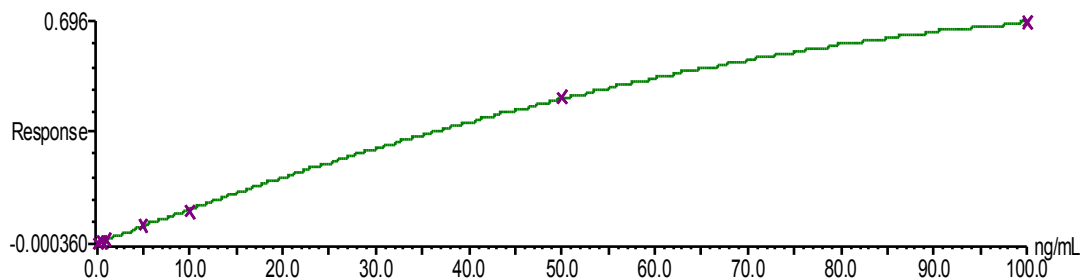


Figure 12. Calibration Curve of Fluphenazine Sulfoxide in Equine Plasma

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Acknowledged By: _____ Lab Supervisor

Compound 4 name: 7-OH-Fluphenazine Method File: Fluphenazine
Coefficient of Determination: 0.999216
Calibration curve: $-1.20252e-6 * x^2 + 0.000294509 * x + -5.37220e-5$
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Include, Weighting: 1/x, Axis: trans: None

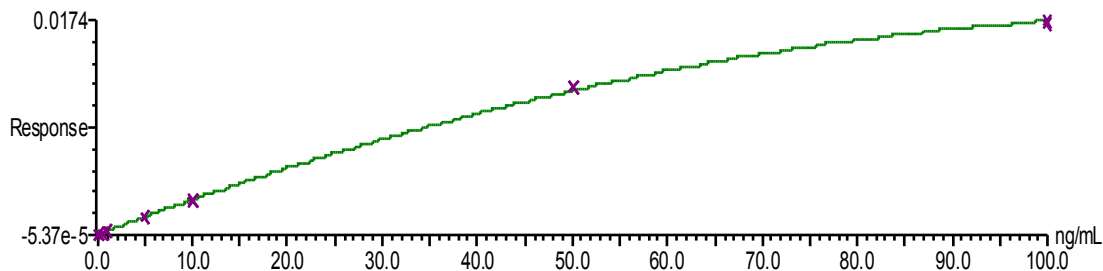


Figure13. Calibration Curve of 7-Hydroxyl Fluphenazine in Equine Plasma

Selectivity of the Method:

To test for selectivity of this method, samples (urine and plasma) were collected from research horses following intravenous or intramuscular administration of fluphenazine hydrochloride or fluphenazine decaonate, respectively. The samples were analyzed as per this SOP. The results are presented below in Figures 14, 15 and 16, and Table 9.

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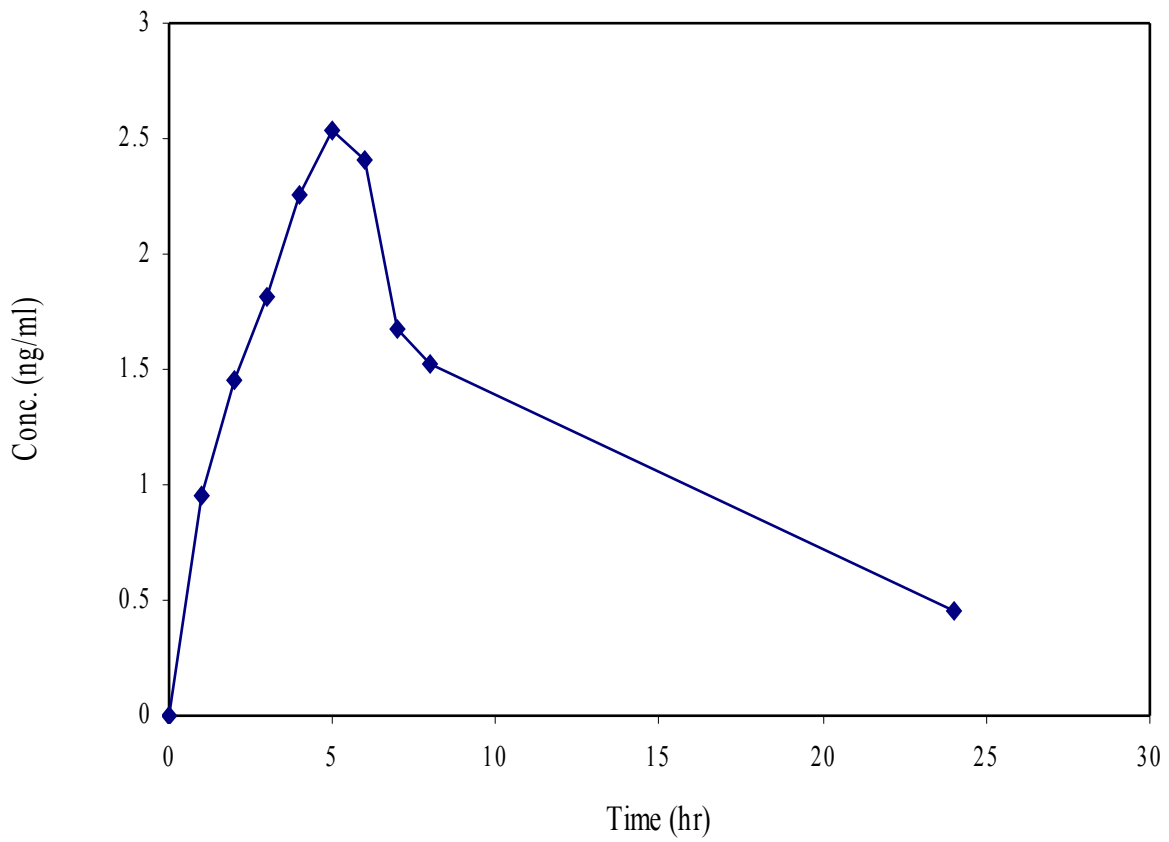


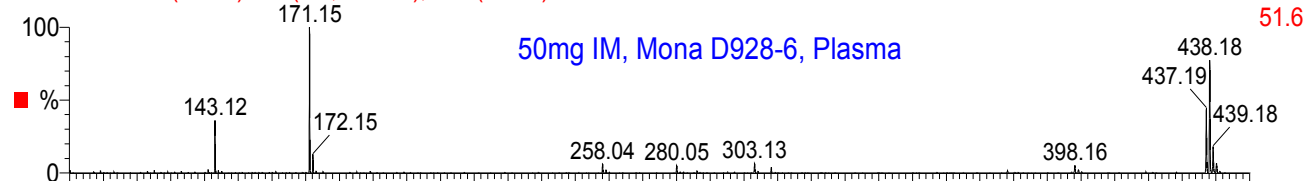
Figure 14. Concentration-time curve of fluphenazine in equine plasma post administration (50 mg, IM)

Fluphenazine mass spectra

50mg IM, Mona D928-6, Plasma

S010132-7 57 (2.612) Sm (Mn, 2x3.00); Cm (55:59)

1: TOF MSMS 438.00ES+
51.6



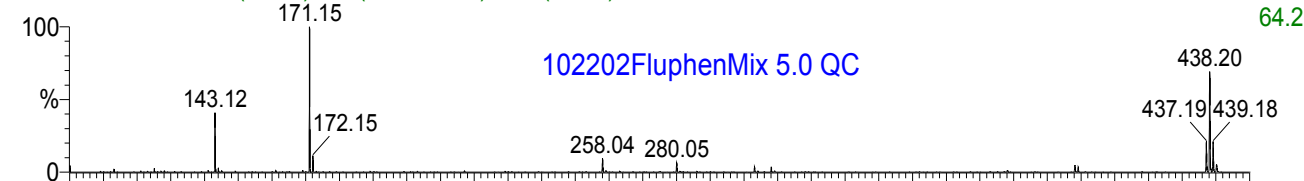
S010132-15 62 (2.682) Sm (Mn, 2x3.00); Cm (61:63)

1: TOF MSMS 438.00ES+
39.4



FL-QC-11210202 57 (2.594) Sm (Mn, 2x3.00); Cm (56:58)

1: TOF MSMS 438.00ES+
64.2



FL-Cal11210204 58 (2.682) Sm (Mn, 2x3.00); Cm (58:60)

1: TOF MSMS 438.00ES+
67.4

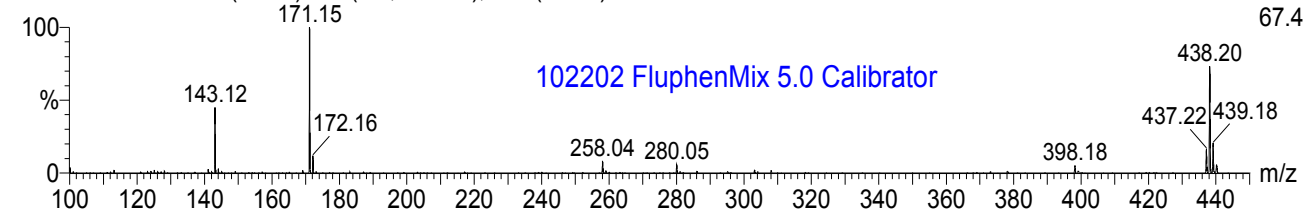


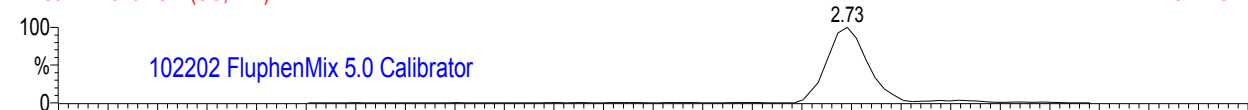
Figure 15. Mass spectra of fluphenazine in plasma of horse (Mona, panel 1 from top to bottom) following IM administration of fluphenazine decaonate (50 mg), urine sample (panel 2), fluphenazine QC at 5 mg/mL (panel 3) and fluphenazine 5 ng/mL calibrator (4 panel).

Fluphenazine chromatograms

50mg IM, Mona D928-6, Plasma

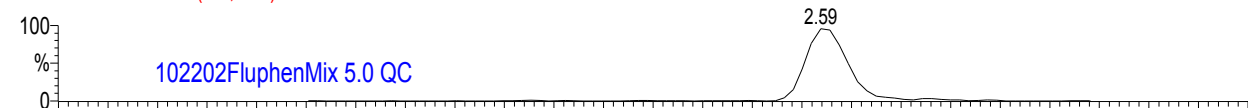
FL-Cal11210204 Sm (SG, 1x2)

1: TOF MSMS ES+
171
205



FL-QC-11210202 Sm (SG, 1x2)

1: TOF MSMS ES+
171
205



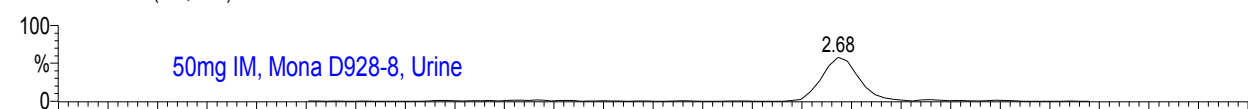
MB11210209

1: TOF MSMS ES+
171
205



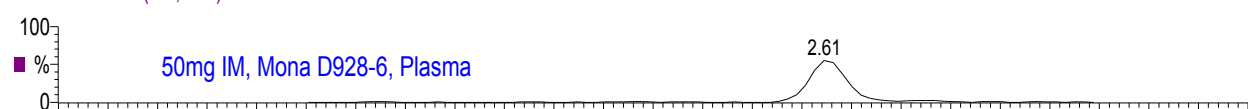
S010132-15 Sm (SG, 1x2)

1: TOF MSMS ES+
171
205



S010132-7 Sm (SG, 1x2)

1: TOF MSMS ES+
171
205



MB11210206

1: TOF MSMS ES+
171
205



BP11210201

1: TOF MSMS ES+
171
205



-1.00 -0.50 0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 Time

Figure 16. Chromatograms (from top to bottom) of fluphenazine showing Calibrator 5 ng/mL (panel, 1), 5 ng/mL QC (panel 2), mobil phase (panel 3), horse urine 8 hr post 50 mg, im injection (panel 4), horse plasma 6 hr post 50 mg im injection (panel 5), mobile phase (panel 6) and that of blank plasma (QNP-23).

Approved By: _____ Laboratory Director

Acknowledged By: _____ Quality Assurance Officer

Acknowledged By: _____ Lab Supervisor

Table 9. Summary of fluphenazine analysis

Tube #	Source	Sample Text	Date	Sample Type	Detected Fluphenazine (ng/mL)
1	S010132-1	50mg IM, Mona D928-0	9/20/2001	Plasma	ND
2	S010132-2	50mg IM, Mona D928-1	9/20/2001	Plasma	0.95
3	S010132-3	50mg IM, Mona D928-2	9/20/2001	Plasma	1.45
4	S010132-4	50mg IM, Mona D928-3	9/20/2001	Plasma	1.81
5	S010132-5	50mg IM, Mona D928-4	9/20/2001	Plasma	2.26
6	S010132-6	50mg IM, Mona D928-5	9/20/2001	Plasma	2.53
7	S010132-7	50mg IM, Mona D928-6	9/20/2001	Plasma	2.41
8	S010132-8	50mg IM, Mona D928-7	9/20/2001	Plasma	1.67
9	S010132-9	50mg IM, Mona D928-8	9/20/2001	Plasma	1.52
10	S010132-10	50mg IM, Mona D928-24	9/20/2001	Plasma	0.45
11	S010132-11	50mg IM, Mona D928-0	9/20/2001	Urine	ND
12	S010132-12	50mg IM, Mona D928-2	9/20/2001	Urine	ND
13	S010132-13	50mg IM, Mona D928-4	9/20/2001	Urine	ND
14	S010132-14	50mg IM, Mona D928-6	9/20/2001	Urine	ND
15	S010132-15	50mg IM, Mona D928-8	9/20/2001	Urine	3.64
16	S010132-16	50mg IM, Mona D928-24	9/20/2001	Urine	0.1
17	P-Dis-25-0	25mg IM, Discretion, 0hr	4/1/1998	Plasma	UD
18	P-Dis-25-1	25mg IM, Discretion, 1hr	4/1/1998	Plasma	0.47
19	P-Dis-35-2	35mg IM, Discretion, 2hr	4/1/1998	Plasma	0.73
20	P-Dis-35-4	35mg IM, Discretion, 4hr	4/1/1998	Plasma	0.65
21	P-Dis-35-6	35mg IM, Discretion, 6hr	4/1/1998	Plasma	0.66
22	P-Dis-35-8	35mg IM, Discretion, 8hr	4/1/1998	Plasma	0.51
23	P-Dis-35-24	35mg IM, Discretion, 24hr	4/1/1998	Plasma	0.42
24	P-Dis-35-54	35mg IM, Discretion, 54hr	4/1/1998	Plasma	0.42
25	P-Dis-50-0	50mg IM, Discretion, 0hr	5/19/1998	Plasma	0.1
26	P-Dis-50-2	50mg IM, Discretion, 2hr	5/19/1998	Plasma	0.45
27	P-Dis-50-4	50mg IM, Discretion, 4hr	5/19/1998	Plasma	0.78
28	P-Dis-50-6	50mg IM, Discretion, 6hr	5/19/1998	Plasma	0.72
29	P-Dis-50-8	50mg IM, Discretion, 8hr	5/19/1998	Plasma	0.56
30	P-Dis-50-24	50mg IM, Discretion, 24hr	5/19/1998	Plasma	0.36
31	D3544-5-0	25mg IM, Fluphenazine Decanoate, 0hr	12/15/1992	Urine	ND
32	D3544-5-2	25mg IM, Fluphenazine Decanoate, 2hr	12/15/1992	Urine	0.08
33	D3544-5-4	25mg IM, Fluphenazine Decanoate, 4hr	12/15/1992	Urine	ND
34	D3544-5-7	25mg IM, Fluphenazine Decanoate, 7hr	12/15/1992	Urine	0.23
35	D3544-5-24	25mg IM, Fluphenazine Decanoate, 24hr	12/15/1992	Urine	ND
36	D3544-5-48	25mg IM, Fluphenazine Decanoate, 48hr	12/15/1992	Urine	ND
37	D3544-5-72	25mg IM, Fluphenazine Decanoate, 72hr	12/15/1992	Urine	ND
38	U-Waltz-0	50mg IM, Waltz, 0hr	2/8/2000	Urine	ND
39	U-Waltz-2	50mg IM, Waltz, 2hr	2/8/2000	Urine	ND
40	U-Waltz-4	50mg IM, Waltz, 4hr	2/8/2000	Urine	ND
41	U-Waltz-6	50mg IM, Waltz, 6hr	2/8/2000	Urine	ND
42	U-Waltz-8	50mg IM, Waltz, 8hr	2/8/2000	Urine	ND

Approved By: _____ Laboratory Director

Acknowledged By: _____ Quality Assurance Officer

Acknowledged By: _____ Lab Supervisor

SEQUENCE OF POSITIVE SAMPLE DATA PACKET for Micromass Q-TOF

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

XV. 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:\FLUPHENAZINE

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

DRUGmddyy.pro for instance:

\\F:\FLUPHENAZINE\FluphenMix121002.pro

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

Once all folders and files are successfully copied and verified, the files in C:\Masslynx\FLUPHENAZINE\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 method 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 backs up this hard drive on a weekly basis.

APPENDIX I. MATERIALS, REAGENTS, AND FORMULAE

I. REAGENTS

Methanol, HPLC grade (Cat. No. A 452-4, Fisher Scientific.)
Methyl-tert Butyl Ether (MTBE), HPLC grade (Cat. No. E127-4, Fisher Scientific.)
Acetonitrile, HPLC grade (Cat. No. A998-4, 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.)
Sodium Hydroxide, 50/50, w/w (Cat. No.SO-S-410, Fisher Scientific.)
Phosphoric Acid, meets A.C.S. Specification (Cat. No. 0260-3, J.T. Baker Chemicals)
Methylene Chloride, HPLC grade (Cat. No. 9715-03, T.J.Baker)
Monobasic Potassium Phosphate, A.C.S reagent (Cat. No. P-0662, Sigma)

II. SOLUTIONS

0.1 M Phosphate Buffer (pH 9.0)

Reagents

Monobasic potassium phosphate (KH_2PO_4)
Water, HPLC grade
10.0 M Sodium Hydroxide

Procedure

Weigh 13.61 g of Monobasic potassium phosphate (KH_2PO_4)
Put 800 mL water in a 1liter glass container. Mix
Adjust pH to 9.0 using 10 M Sodium Hydroxide while stirring.
Add water to bring final volume to1 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.

Methanol:Water:Formic Acid (50:50:1)

Reagents

Methanol, HPLC grade
Formic acid
Water, HPLC grade

Procedure

Add 25 mL of methanol to a liter glass container.
Add 25 mL of water. Mix.
Add 0.5mL of formic acid. Mix.

Storage Requirements

Store at room temperature in a glass container.
Prepare fresh on day of use.

Methanol:Water (50:50, v/v)

Reagents

Approved By: _____ Laboratory Director

Acknowledged By: _____ Quality Assurance Officer

Acknowledged By: _____ Lab Supervisor

Methanol, HPLC grade
Water, HPLC grade

Procedure

Add 25 mL of methanol to a liter glass container.
Add 25 mL of water. Mix.

Storage Requirements

Store at room temperature in a glass container.
Prepare fresh on day of use.

HPLC Solvent A (2 mM Ammonium Acetate:Acetonitrile:Ammonium Hydroxide (95:5:0.01, v/v/v, pH 4.70)

Procedure

Add 1 mL of 2 M ammonium acetate solution to 999 mL water. Mix.
Add 950 mL to a 1000 mL glass bottle. Mix with 50 mL of Acetonitrile and 0.1 mL of ammonium hydroxide. Mix thoroughly before placing on HPLC. The final pH value should be 4.70, if not, adjust pH to 4.70 by using acetic acid or ammonium hydroxide while stirring.

Storage Requirements

Store at room temperature in a glass bottle.

HPLC Solvent B (2 mM Ammonium Acetate:Acetonitrile, 5:95, v/v, pH ≈ 7.0)

Procedure

Add 950 mL of Acetonitrile into a glass bottle (a liter). Mix with 50 mL of 2 mM of ammonium acetate.
Mix the solvent 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, Germany?)

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.)

REFERENCES:

Approved By: _____ Laboratory Director

Acknowledged By: _____ Quality Assurance Officer

Acknowledged By: _____ Lab Supervisor

1. New High-Performance Liquid chromatographic Method for Fluphenazine and Metabolites in Human Plasma, D.W. Hoffman, R.D. Edkins, S.D. Shillcutt and A. Salama. J. Chromatogr. 414(1987), page 504-509
2. The Effects of Sample Preparation Methods on the Variability of the Electrospray Ionization Response for Model Drug Compounds, R. King, R. Bonfiglio, et al, Rapid Communications in Mass Spectrometry, 13(1999), pgs 1175-1185.