

Quantitation of Fluphenazine in Equine Serum Following Fluphenazine Decanoate Administration

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Fluphenazine, a potent antipsychotic used to treat schizophrenia in humans, is used in racehorses as a performance-enhancing drug, and for that reason it has been banned by the Association of Racing Commissioners International. A liquid chromatography–tandem mass spectrometry method for detecting and quantitating fluphenazine in equine serum was developed and validated. The method was then employed to quantitate fluphenazine in serum samples collected from three study horses after intramuscular injection of fluphenazine decanoate. Stability testing showed that fluphenazine is stable in unextracted and processed samples as well as samples that have been subjected to up to three freeze–thaw cycles. The limit of detection and lower limit of quantitation of fluphenazine were determined to be 0.05 and 0.1 ng/mL, respectively. Precision was evaluated based on one-way analysis of variance of replicate quality control samples and was determined to be 27.2% at the 0.2 ng/mL level and 18.1% at the 2 ng/mL level. Bias was determined to be 0.55% at the 0.2 ng/mL level and 3.66% at the 2 ng/mL level. In two of three horses, fluphenazine was detected in serum up to 14 days post-administration. The highest detected concentration of fluphenazine in serum was 1.4 ng/mL.

Introduction

Fluphenazine is a potent antipsychotic used in humans to minimize the episodes of hallucinations and violence that are typically associated with schizophrenia. Its action is primarily due to its high affinity for the dopamine D2 receptor. By binding to the D2 receptor, fluphenazine and other typical antipsychotics block dopamine from interacting with it. This decrease in dopamine activity results in the minimization of psychotic symptoms in humans. The potency of fluphenazine is directly related to its affinity for the D2 receptor. Fluphenazine and other typical antipsychotics have an even higher affinity for the D2 receptor than dopamine, its endogenous ligand. Strongly binding drugs like fluphenazine dissociate from the D2 receptor slowly. This is linked to the development of Parkinson’s-like symptoms, which have been observed in both humans and racehorses given fluphenazine. These adverse reactions may be life threatening and can lead to euthanasia in racehorses.

Fluphenazine has been administered to horses through intramuscular injection to control high-strung behavior by providing long-term sedation. However, it is not approved for veterinary use and is not believed to have any therapeutic value for horses. Furthermore, it has been banned for use in racehorses by the US Equestrian Federation and by the Illinois Racing Board. The Association of Racing Commissioners International (ARCI) categorizes fluphenazine as a Class 2 foreign substance due to its high potential to affect the performance of the racehorse. Most Class 2 foreign substances are drugs ‘intended to alter consciousness or the psychic state of humans and have no approved or indicated use in the horse’ (5). Penalty classification is separate from foreign substance classification and depends on the gravity of the violation. The presence of fluphenazine was classified into Penalty Class A by the ARCI, which recommends a minimum 1-year suspension for the trainer and disqualification and loss of purse for the owner for the first offense. Substances belonging to Penalty Class A carry the harshest punishment for violations.

There is no threshold value for reporting a positive finding of fluphenazine; that is, if the presence of fluphenazine is confirmed in a sample from a racehorse, the sample should be reported as positive and penalties should be applied. Concentrations of fluphenazine in equine serum are typically <1 ng/mL, which is at or below the limit of detection (LOD) for many screening techniques. A useful screening method must be sensitive enough to detect low concentrations of fluphenazine. Immunoassay techniques, including the enzyme-linked immunosorbent assay, are far more sensitive than previously used screening techniques such as thin-layer chromatography. Mass spectrometry provides the advantage of even more sensitivity with limits of detection in the picogram range. Liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) is a rapid and sensitive method that can be used to screen samples, to confirm the presence of fluphenazine and to determine the amount present.

Intrasample differences in extraction efficiency, ionization efficiency and other variables have the potential to impact quantitation data. The use of an internal standard minimizes the effects of these factors and yields more accurate results. Both fluphenazine and perphenazine belong to the piperazynl phenothiazone class of antipsychotics. In this study, perphenazine was chosen due to its structural similarities to fluphenazine. Perphenazine has no approved medical use in race horses, and to the best of our knowledge has never been reported by the racing community.

The aim of this study was to develop and validate an LC–MS–MS method for detecting and quantitating fluphenazine in equine serum and to employ the developed methodology to analyze samples from a controlled fluphenazine decanoate administration to study horses.

Experimental

Fluphenazine administration and sample collection

Three horses were used in this study. All were mares (two thoroughbred and one quarter horse) with ages ranging from 9 to...
15 years. The dosage was based on a body weight of an average horse, which is 450 kg. Fluphenazine decanoate was administered in a dose of 62.5 mg (46.2 mg free base) as an intramuscular injection. The injection solution had a concentration of 25 mg fluphenazine decanoate per milliliter in sesame oil. A serum sample was collected from each horse immediately before the injection. Following the injection, samples were collected at 15 and 30 min, 1, 2, 4, 6, 24, 48, 72 h and 4, 5, 6, 7, and 8 days post-injection. Sample collection stopped at 8 days for one of the horses. Daily sample collection continued in the other two horses until 14 days post-injection.

Materials and reagents
Methanol (Optima LC–MS grade), water (Optima LC–MS grade), methyl tert-butyl ether (MTBE) (HPLC grade), acetonitrile (Optima LC–MS grade), isopropyl alcohol (Optima LC–MS grade), glacial acetic acid (HPLC grade) and formic acid (Optima LC–MS grade) were obtained from Fisher Scientific (Hanover Park, IL, USA). Ammonium acetate (≥99.0%, HPLC grade) was obtained from Sigma-Aldrich (St Louis, MO, USA). Potassium carbonate (Certified ACS grade) was obtained from Fisher Scientific. Fluphenazine dihydrochloride and perphenazine were obtained from Sigma-Aldrich. A control stock solution of fluphenazine in methanol (1 mg/mL) was prepared. This solution was diluted with methanol to yield high (100 ng/mL) and low (10 ng/mL) control working solutions. A standard stock solution of fluphenazine in methanol (1 mg/mL) was also prepared and diluted to yield a standard working solution (50 ng/mL). A stock solution of perphenazine in methanol was prepared and diluted to yield a working solution (50 ng/mL). All control and standard solutions were stored at 4 ± 2 °C. Blank equine serum was purchased from Bioreclamation LLC (Westbury, NY, USA).

Apparatus
The LC–MS system consisted of an Agilent Technologies 6460 Series Triple Quadrupole LC–MS-MS, the Agilent Technologies 1260 Series binary pump, degasser, column compartment and autosampler. The system was equipped with an Agilent Zorbax RX-SIL column (2.1 mm i.d. × 150 mm, 5 μm particle size). The column temperature was held at 40 °C. Agilent’s MassHunter Optimizer software was used to perform product ion scans, to select abundant product ions and to determine the optimum conditions for their detection. Agilent’s MassHunter Quantitative Analysis software was used to quantitate the amount of fluphenazine in a sample using data from a calibration curve.

Ultra-high purity nitrogen was used as the drying, sheath and collision gases. The drying gas temperature and flow were 200 °C and 8 L/min, respectively, and the sheath gas temperature and flow were 250 °C and 11 L/min, respectively. The capillary voltage was set to 3.5 kV. The multiple reaction monitoring operation mode was used for the detection of fluphenazine and perphenazine. The dwell time for each ion transition was 80 ms. Gradient elution was employed in this method and is detailed in Table I. The flow rate was held at 500 μL/min for 5 min and was followed by a post run time of 1 min to allow equilibration. The maximum pressure allowed was 600 bar.

Standard curve and control serum preparations
An eight-point standard curve for fluphenazine was prepared in blank horse serum at the following concentrations: 0.1, 0.2, 0.5, 1, 2, 3, 4 and 5 ng/mL. Serum controls were prepared by spiking blank equine serum with fluphenazine control solution. Six sets of high and low control samples containing fluphenazine (2 and 0.2 ng/mL) were included in each run.

Extraction method
Samples, calibrators and controls (1 mL serum) were extracted using 5 mL of MTBE after addition of 1 mL of 0.5 M K2CO3 (containing 1.0 ng/mL of perphenazine). The samples were mixed on a mechanical shaker for 15 min and then centrifuged for 10 min at 3,500 rpm. The organic layers were transferred to clean drying tubes and evaporated to dryness under nitrogen. The residue from each tube was reconstituted in 100 μL of water containing 0.2% formic acid : isopropyl alcohol (95 : 5) and transferred to an autosampler vial for analysis.
Specificity and carryover

Specificity was evaluated by analyzing three samples of negative serum extracted according to the previously described procedure and three serum samples spiked with fluphenazine (5 ng/mL) and perphenazine (5 ng/mL). The extract was considered negative if no peaks for the target ions had a signal-to-noise ratio greater than 5 : 1 within 0.2 min of the expected retention time based on the injected standards.

Carryover and contamination were evaluated by analyzing three samples of naive serum spiked with fluphenazine (5 ng/mL) followed by three samples of drug-free serum.

Stability testing

Benchtop samples

Stability testing was performed on three samples of blank serum spiked with fluphenazine (2 ng/mL) and left at room temperature for 24 h. These samples were extracted and analyzed with three samples of blank serum spiked with fluphenazine (2 ng/mL) on the day of analysis.

Processed samples

Stability testing was performed on three samples of blank serum spiked with fluphenazine (2 ng/mL), extracted and prepared for analysis according to the previously described procedure, and then left at room temperature for 48 h before being analyzed. These samples were analyzed with three samples of blank serum spiked with fluphenazine (2 ng/mL) and extracted on the day of analysis.

Freeze–thaw samples

Stability testing was performed on three samples of blank serum spiked with fluphenazine (2 ng/mL) and then frozen. These samples were stored at −20 °C for 48 h and then thawed at room temperature. The freeze–thaw cycle was repeated twice. The samples were thawed on the day of analysis, then were extracted and analyzed with three samples of blank serum spiked with fluphenazine (2 ng/mL) on the day of analysis.

Matrix effects and extraction recovery

Matrix effects and extraction recovery were evaluated by preparing three sets of three samples at one concentration of fluphenazine (2 ng/mL): neat standards, blank serum spiked after extraction and blank serum spiked before extraction. The areas of the 438.2 → 171.2 peaks for each set of samples were compared to evaluate the percentage matrix effect, extraction recovery and process efficiency.

Results and discussion

Parent fluphenazine is the primary analyte in equine serum with a mass-to-charge ratio of 438 for the [M+H]^+ peak. The parent ion of the internal standard perphenazine has a mass-to-charge ratio of 404 for the [M+H]^+ peak. Product ion scans were performed while infusing 1 ng/μL of fluphenazine and 1 ng/μL of perphenazine (both in methanol) with a syringe pump. Four abundant product ions were selected for fluphenazine (Supplementary Table SI). The optimum fragmentation voltage and collision energy for detecting each ion transition were determined (Supplementary Figure S1). The transitions monitored were 438.2 → 70.1, 438.2 → 100.1, 438.2 → 171.2 and 438.2 → 143.1. The transition used for quantitation was 438.2 → 143.1. Four abundant product ions were selected for perphenazine (Supplementary Table SII). The optimum fragmentation voltage and collision energy for detecting each ion transition were also determined (Supplementary Figure S2). The transitions monitored were 404.2 → 70.1, 404.2 → 171.2 and 404.2 → 143.1. The transition used for quantitation was 404.2 → 143.1.

Neither fluphenazine nor perphenazine could be detected in the samples of negative control serum, so the method was determined to be selective for these drugs. No traces of fluphenazine or perphenazine were detected in the samples of negative control serum analyzed immediately following spiked samples, so no carryover or contamination was observed for this method.

The spiked samples that were left at room temperature for 24 h were extracted and analyzed in the same run as samples that were spiked and extracted immediately prior to analysis. The areas of the 438.2 → 171.2 peaks for each set of samples were then compared (Supplementary Table SIII). The peak areas of the benchtop samples left at room temperature for 24 h before being extracted and analyzed were within 3% of those of the control samples.

The processed samples left at room temperature for 48 h before analysis were analyzed in the same run as samples that were spiked and extracted immediately prior to analysis. The areas of the 438.2 → 171.2 peaks for each set of samples were then compared (Supplementary Table SIV). The peak areas of the processed samples left at room temperature for 48 h before analysis were 90% of those of samples that were analyzed immediately after being spiked and extracted.

The samples subjected to three freeze–thaw cycles were extracted and analyzed in the same run as samples that were spiked and extracted immediately prior to analysis. The areas of the 438.2 → 171.2 peaks for each set of samples were then compared (Supplementary Table SV). The peak areas of the samples subjected to three freeze–thaw cycles were on average 72% of those of samples that were extracted and analyzed immediately after being spiked.

The matrix effect was evaluated by comparing the areas of the 438.2 → 171.2 peaks of post-extraction spikes with those of neat standards (Supplementary Table SVI). The matrix effect was calculated as a percentage by dividing the average area of the post-extraction spikes by that of the standards, multiplying that value by 100 and subtracting 100 from that value to obtain the final percentage. The matrix effect was determined to be −98%.

Extraction recovery was evaluated by comparing the areas of the 438.2 → 171.2 peaks of preextraction spikes with those of post-extraction spikes (Supplementary Table SVII). Extraction recovery was calculated as a percentage by dividing the average area of the preextraction spikes by that of the standards and multiplying that value by 100. Extraction recovery was determined to be 73%. Process efficiency was evaluated by comparing the peak areas of the same ion transition of preextraction spikes with those of standards (Supplementary Table SVIII). Process efficiency was calculated as a percentage by dividing the average area of the preextraction spikes by that of the standards and multiplying that value by 100. Process efficiency was found to be 0.74%. However, it is believed that low
efficiency and strong matrix effects are acceptable as long as other method-validation data such as the LOD, lower limit of quantitation (LLOQ), precision, stability and bias are satisfactory (9).

Quantification of fluphenazine in blood has some advantages over testing in urine even though urine samples from racehorses are nearly always of significantly greater volume than blood samples. The first advantage is that the concentration of fluphenazine excreted in urine is lower than that of fluphenazine in blood, which is already very low (6). More importantly, quantitation of a drug in blood allows for meaningful conclusions to be drawn about the physiological effect of the drug at that particular concentration. By measuring the drug concentration (antemortem or postmortem) in blood, it can be determined whether the concentration of drug present was subtherapeutic, therapeutic or lethal. The concentration of a drug in urine cannot be reliably correlated with a concentration in blood or physiological effects of the drug.

Agilent’s Masshunter Quantitative Analysis software was used to analyze the quantitation data and to calculate the concentration of fluphenazine in each sample. The ratio of fluphenazine peak area to perphenazine peak area was determined in all fluphenazine standard serum preparations. The peak area ratios were then plotted against the concentration of fluphenazine to obtain the calibration curve (Supplementary Figure S3). The concentration of fluphenazine in each control and administration sample were calculated using the calibration curve.

An LOD for fluphenazine was defined as the concentration at which the signal-to-noise ratio for any of the ion transitions used to identify fluphenazine was less than 5 : 1, and was determined to be 0.05 ng/mL. The LLOQ for fluphenazine was defined as the concentration at which the signal-to-noise ratio for any of the ion transitions used to identify fluphenazine was less than 10 : 1, and it was 0.1 ng/mL. The retention time and the qualifier ion transitions used to identify fluphenazine was less than 5 : 1, and was determined to be 0.05 ng/mL. The LLOQ for fluphenazine was defined as the concentration at which the signal-to-noise ratio for any of the ion transitions used to identify fluphenazine was less than 10 : 1, and it was 0.1 ng/mL. The retention time and the qualifier ion transitions used to identify fluphenazine were determined by multiple injections of the standard. These ratios were calculated using the Agilent MassHunter software for all target ions.

The quality control samples were evaluated by calculating intra- and interday precision, total precision and bias at both levels (0.2 and 2 ng/mL). Six high and six low quality control samples were extracted and analyzed for five different days (Table II). The grand mean for the 30 high quality control samples was 2.07 ng/mL. The grand mean for the 30 low quality control samples was 0.20 ng/mL.

One-way analysis of variance (ANOVA) was performed on the five sets of quality control samples at each level (Supplementary Table SIX). These calculations were used to assess intra-, inter-, and total precision according to Equations 1–3 (8):

$$\text{RSD}_{I}(\%) = \sqrt{\frac{MS_{bg}}{X}} \times 100$$  \hfill (1)

where $X$ is the grand mean and $X$, the mean of observations in each group.

$$\text{RSD}_{bg}(\%) = \sqrt{\frac{MS_{bg} - MS_{bgg}}{nX}} \times 100$$  \hfill (2)

where $MS_{bg}$ is the mean square between groups (determined by one-way ANOVA), $MS_{bgg}$, the mean square within groups (determined by one-way ANOVA), $n$, the number of observations in each group and $X$, the grand mean.

$$\text{RSD}_{I(F)}(\%) = \sqrt{(MS_{bg} + (n + 1)MS_{bgg})/nX} \times 100$$  \hfill (3)

where $RSD_{I(F)}$ (factor-different intermediate precision) is total precision, $MS_{bg}$, the mean square between groups (determined by one-way ANOVA), $MS_{bgg}$, the mean square within groups (determined by one-way ANOVA), $n$, the number of observations in each group and $X$, the grand mean.

Bias was evaluated at each level according to Equation 4. A summary of the calculated intra-, inter-, total precision and bias at each quality control level is given in Table III.

$$\text{Bias} = \frac{X - \bar{X}}{X}$$  \hfill (4)

where $\bar{X}$ is the grand mean and $X$, the theoretical value.

Uncertainty was calculated using an eight-step Simplified Guide to the Expression of Uncertainty in Measurement (GUM) approach (8). An uncertainty budget form was prepared to record these steps (Supplementary Table SX). Sources of uncertainty with standard uncertainty values less than one-third of the largest contributor were considered insignificant and not included when combining standard uncertainties (8). The only significant contributor to the combined uncertainty was the intraday precision as determined from quality control data. The expanded uncertainty (95% confidence level) was determined to be 36%.

| Table II
| Summary of quality control concentrations (ng/mL) |
|------------------|------------------|------------------|------------------|------------------|
|                  | Set 1           | Set 2           | Set 3           | Set 4           | Set 5           |
| High Replicate 1 | 1.32            | 1.48            | 1.45            | 2.07            | 1.45            |
| High Replicate 2 | 1.54            | 1.95            | 1.45            | 2.11            | 1.98            |
| High Replicate 3 | 2.11            | 2.03            | 2.04            | 2.16            | 2.04            |
| High Replicate 4 | 2.21            | 2.28            | 2.06            | 2.41            | 2.04            |
| High Replicate 5 | 2.25            | 2.61            | 2.16            | 2.53            | 2.06            |
| High Replicate 6 | 1.98            | 2.20            | 1.91            | 2.31            | 1.96            |
| Mean             | 1.98            | 2.20            | 1.91            | 2.31            | 1.96            |
| Grand mean       | 2.07            |                 |                 |                 |                 |
| Low Replicate 1  | 0.10            | 0.13            | 0.19            | 0.16            | 0.17            |
| Low Replicate 2  | 0.12            | 0.14            | 0.25            | 0.16            | 0.17            |
| Low Replicate 3  | 0.14            | 0.16            | 0.27            | 0.18            | 0.18            |
| Low Replicate 4  | 0.16            | 0.18            | 0.28            | 0.18            | 0.22            |
| Low Replicate 5  | 0.19            | 0.22            | 0.33            | 0.19            | 0.24            |
| Low Replicate 6  | 0.21            | 0.34            | 0.37            | 0.19            | 0.24            |
| Mean             | 0.15            | 0.19            | 0.28            | 0.18            | 0.20            |
| Grand mean       | 0.20            |                 |                 |                 |                 |

| Table III
| Precision and bias of quality control samples |
|------------------|------------------|------------------|
|                  | QC low (0.2 ng/mL) | QC high (2.0 ng/mL) |
| Intraday precision (%) | 25.4            | 18.0            |
| Interday precision (%)  | 9.66            | 1.76            |
| Total precision (%)     | 27.2            | 18.1            |
| Bias (%)                | 0.55            | 3.66            |

where $RSD_{bg}$ is interday precision (expressed as a percentage), $MS_{bg}$, the mean square between groups (determined by one-way ANOVA), $MS_{bgg}$, the mean square within groups (determined by one-way ANOVA), $n$, the number of observations in each group and $X$, the grand mean.
The concentrations of fluphenazine in each sample collected from three study horses were determined using the calibration curve and are presented in Supplementary Table SXI. This set of data was used to prepare concentration vs. time curves (Figure 2).

As expected, concentrations of fluphenazine in serum were low (in the subnanogram-per-milliliter range). Effects of the drug can be seen even at these low serum concentrations. Baird et al. (1) discussed the behavior of four racehorses that received fluphenazine decanoate through intramuscular injection at doses ranging from 40 to 125 mg. Horses presented with strange posture and movements (oscillating and swinging of the head), tremors, inability to walk normally and reduced respiratory rate. The horse that received the highest dose (125 mg, i.m.) suffered swelling of the face and nostrils, excessive sweating, agitation, and flank biting and experienced difficulty eating. Serum samples were not taken as frequently as in this study. When samples were collected and tested, the concentrations of fluphenazine were in a similar range to what was found in this study with the exception of the horse that received a much higher dose.

The increases and decreases in the serum concentration of fluphenazine over the administration may be due to the formulation used. Fluphenazine decanoate is a depot formulation of the drug, which is intended to release the active component (i.e., fluphenazine) over an extended period of time (1, 2). A study investigating the pharmacokinetics of depot formulations of fluphenazine and haloperidol involved dosing human participants once every 4 weeks for 60 weeks (10). Analytical data from early in the study were not available due to contaminants interfering with the assay, but a concentration vs. time curve was prepared after the eighth dose of fluphenazine decanoate. Although participants were sampled less frequently, the early portion of this concentration vs. time curve appeared similar to that seen in this equine administration, with a peak immediately after dosage and a second peak several days later (10). This was after 8 months of treatment, so fluphenazine already reached the steady-state concentration. Earlier in treatment, the concentration of fluphenazine in plasma may have fluctuated to a greater extent. Unfortunately, the data were not available for comparison with the results from this study.

It is interesting and important to note that fluphenazine was still detected in the serum of two study horses 14 days (336 h) after the administration. This result was also consistent with the previously mentioned case study, in which nanogram-per-milliliter levels of fluphenazine were detected 12 days after the original dose of decanoate formulation (1). This indicates that any racehorse receiving fluphenazine would test positive for the drug for at least 2 weeks after receiving a similar dose. The long period of time required for fluphenazine to be metabolized and excreted provides authorities with a chance to detect any illicit use in racehorses.

Conclusions
A method for detecting and quantitating fluphenazine using LC–MS-MS after a liquid–liquid extraction was developed and validated. The method-validation study demonstrated acceptable lower limits of detection and quantitation, linearity, stability, precision and bias. The analytical methodology is simple and can be used for routine screening and confirmation of fluphenazine in equine serum. This method was then used to quantitate fluphenazine in equine serum samples from a fluphenazine decanoate administration to study horses. The highest concentration of fluphenazine in serum was 1.4 ng/mL. The drug can be detected in equine blood for at least 2 weeks after intramuscular administration. Future study could look at the potential of adding other phenothiazines to the method.

Supplementary data
Supplementary data is available at Journal of Analytical Toxicology online.

References