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Regulatory control of glycopyrrolate in performance horses using validated UHPLC/MS–MS methods

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ABSTRACT

We describe a validated, rapid, sensitive, and specific UHPLC–MS/MS method to detect and quantify glycopyrrolate in 0.5 mL of horse urine. Further, we investigated the elimination of glycopyrrolate in urine after both intravenous and oral administration of clinically relevant doses to Thoroughbred horses. Quantification was performed by weighted, linear regression analysis using a deuterated analogue of glycopyrrolate as internal standard (IS). The method was characterized by a linear range of 5–2500 pg/mL, a lower limit of quantification of 5 pg/mL and a limit of detection of 1 pg/mL. The intra and inter-batch imprecisions were <10% RSD and accuracy of the method ranged between 94 and 104%. Glycopyrrolate remained detectable in urine samples collected through 168 h after intravenous administration and through 24 h after oral administration. Analytical method validation requirements for linearity, specificity, precision, accuracy, stability, dilution integrity, matrix effect, and ruggedness have been fulfilled. The urine method described in this report is simple and efficient and is the first reported method with sufficient sensitivity, accuracy, and precision to regulate the use of glycopyrrolate in urine samples collected more than one day after dosing of horses. Urine to plasma glycopyrrolate concentration ratios were calculated and were approximately 100:1 in samples collected from 24 h through the end of sample collection.

1. Introduction

Glycopyrrolate (GLY) is classified as a class 3 substance by the Association of Racing Commissioners International, Inc., because it has legitimate therapeutic uses in horses but it also may alter racing performance if administered too close to the time of racing. Pharmacologically it is a peripheral anti-muscarinic compound that has been used in a variety of clinical applications, such as treatment of chronic obstructive pulmonary disease (COPD) and as a pre-anesthetic agent to reduce secretions. In horse racing, GLY is potentially exploited for its bronchodilatory effects and favored for its lack of effects on the central nervous system (CNS) compared to other muscarinic antagonists such as atropine. Clinically, GLY is similar to atropine, an exception however, being that it is completely ionized at physiological pH and thus permeates the CNS less extensively compared to its less polar and more lipophilic congeners [1]. The difference is attributed to its quaternary amine structure which increases polarity and reduces membrane permeability.

Analysis of GLY in urine samples collected after oral administration results in concentrations that would have been undetectable with methods described in the literature, due to its low oral bioavailability as predicted from its permanent ionization. Therefore, a method with greater sensitivity is necessary in order to detect GLY administration by this route and to detect parenteral administration for more than a few hours after dosing. Previous reports that focus on the quantitative determination of quaternary ammonium compounds, including GLY, employ volatile ion pairing reagents to extract GLY [2] and other methods fail to achieve detection limits that are necessary for regulatory control of this substance [3,4].

In order to provide the appropriate regulatory control for therapeutic substances that may also have the ability to affect performance, threshold limits must be determined in blood (plasma), urine or both [5–7]. Furthermore, it is important to determine the relationship between urine and plasma concentrations after a single intravenous and clinically relevant dose of GLY.

The following report presents a rapid, sensitive, and selective method for the quantification of GLY in horse urine. We investigated urine GLY concentrations after single intravenous and oral

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doses. Further, we report the plasma concentrations in samples collected from these horses [8] and calculated the urine to plasma concentration ratio at the end of 24 h period after dose administration.

2. Experimental

2.1. Animals

Twenty, adult, Thoroughbreds (6 mares and 14 geldings) ranging in age from 4 to 10 years and weighing from 485 to 602 kg were used in these studies. All horses were dosed intravenously and six of these horses (1 mare and 5 geldings) ranging in age from 8 to 10 years and weighing from 518 to 580 kg were dosed orally following a sufficient washout period. All horses were housed in grass paddocks at the University of Florida (UF) Veterinary Medical Center, kept on a diet of commercially available grain mixture, and had free access to water and hay at all times. Horses were regularly exercised (3 days/week) before and throughout the duration of the study. Details of the conditioning regimen are described elsewhere [8].

For the intravenous study, horses were administered 1 mg (1.66–2.06 μg/kg) of GLY (glycopyrronium bromide, American Regent, Inc., Shirley, NY, USA) into the right jugular vein via needle venipuncture. Oral administration was carried out using 50 mL of 0.2 mg/mL GLY solution for a total of 10 mg orally. All horses were trained to urinate on command and the urine from each horse was collected via the free-catch method into separate, clean, 1 L containers. Urine specimens were stored in 15-mL sterile, disposable, polypropylene centrifuge tubes at −20 °C immediately and at −80 °C within 48 h. Collection times were before drug administration and at 4, 8, 24, 48, 72, 96, and 168 h after intravenous administration and at 2, 4, 6, 8, and 24 h after oral administration. Blood collections and plasma processing are described in a previous report [8]. The experimental protocols, including animal conditioning and drug administration and sample collection, were approved and facilities were inspected periodically by the UF Institutional Animal Care and Use Committee.

2.2. Chemicals and reagents

Glycopyrrolate ((United States Adopted Name (USAN)) is also known as glycopyrronium bromide (Recommended International Nonproprietary Name) [9]. Glycopyrrolate has the elemental composition $C_{19}H_{28}BrNO_3$ and, as such, includes the bromide counter ion. Therefore, concentrations of GLY are reported herein without adjustment for the mass of the bromide ion, consistent with the USAN definition for GLY.

Analytical grade drug standards including glycopyrronium bromide and d_3-glycopyrrolate iodide were obtained from the United States Pharmacopeial Convention (Rockville, MD) and Toronto Research Chemicals (North York, Ontario, Canada), respectively. Reagent grade formic acid was obtained from ACROS Organics (Morris Plains, NJ, USA). All solvents including acetonitrile, methanol, and methylene chloride were HPLC grade and obtained from Thermo Fisher (Pittsburg, PA, USA). All water used was deionized with a resistivity greater than or equal to 18 MΩ and organic content less than 10 ppb.

All stock standard solutions were prepared from solid form and dissolved in acetonitrile. All working standard solutions were diluted to the appropriate concentrations in acetonitrile to prepare calibrators in urine from 1 to 2500 pg/mL. Calibrators and positive control samples were prepared from independently prepared stock solutions. All calibrators and positive control samples were prepared from 1 mL of phosphate buffer (50 mM, pH 7.0) and 0.5 mL of drug-free control horse urine, and fortified with the appropriate volume of GLY working standard solution and 25 μL of d_3-GLY working standard solution. The deuterated GLY analogue was prepared in a working standard solution at a concentration of 0.004 ng/μL. The final IS concentration was 200 pg/mL of urine.

2.3. Sample preparation

In duplicate, a 0.5 mL aliquot of each urine sample was pipetted into 1 mL of phosphate buffer (50 mM, pH 7.0) and 25 μL of 0.004 ng/μL IS working solution in 5-mL disposable, centrifuge tubes. The tubes were centrifuged at 1508 × *g* (2800 rpm) for 12 min and the buffered urine samples were subjected to solid phase extraction. Isolute CBA 3-mL columns (Biotage, Charlottesville, VA, USA) were sequentially conditioned with 2 mL each of methanol, water, and phosphate buffer (50 mM, pH 7.0). Buffered urine specimens were loaded onto the columns and a positive pressure sufficient to achieve a flow rate of no more than 2 mL/min was applied. The columns were sequentially washed with 2 mL each of water, methanol, and methylene chloride. The analyte was eluted with two 1-mL aliquots of 1% formic acid in acetonitrile. The eluate was evaporated under nitrogen on a TurboVap® LV evaporator (Zymark, Hopkinton, MA, USA). Dried sample extracts were then dissolved in 100 μL of acetonitrile:water (10:90) containing 0.1% formic acid and transferred to glass autosampler vials.

2.4. Instrumentation

Ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC/MS–MS) was performed on a TSQ Quantum Ultra mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with a heated electrospray ionization (HESI) source and interfaced with an HTC-PAL autosampler (Leap Technologies, Carrboro, NC, USA). Complete details of the LC/MS–MS instrumental conditions can be found in a previous report for the determination of GLY in horse plasma [8].

2.5. Data analysis

The most abundant ion transition (*i.e.*, m/z 318 → 116) for GLY was used for quantification. The second (*i.e.*, m/z 318 → 58) and third (*i.e.*, m/z 318 → 88) most abundant transitions were used as qualifier transitions (Fig. 1). All standards, controls, calibrators, and samples were prepared in duplicate and peak ion area ratios of the analyte and IS (*i.e.*, m/z 321 → 119) were calculated for each. Individual values of the duplicate samples were averaged. Calibration was performed using a simple least squares linear regression analysis with a $1/C_u$ weighting factor, where C_u was the nominal urine concentration. Quality control and sample acceptance criteria have been specified according to the following guidelines and standard operating procedures of the UF Racing Laboratory, Research Section. The requirement is that the %CV for all calibrators, positive controls, and samples must not exceed 10% (15% at the LLOQ). In addition, for calibrators the difference between the back-calculated concentration and the nominal concentration must not exceed 10% (15% at the LLOQ). All samples that did not meet such criteria were re-analyzed.

2.6. Urine method validation

The method was validated in accordance with the U.S. Food and Drug Administration recommended guidelines [10] for specificity, sensitivity, linearity, accuracy, precision, extraction efficiency and stability. Other parameters such as carryover, dilution integrity and matrix effect were assessed in accordance with the European

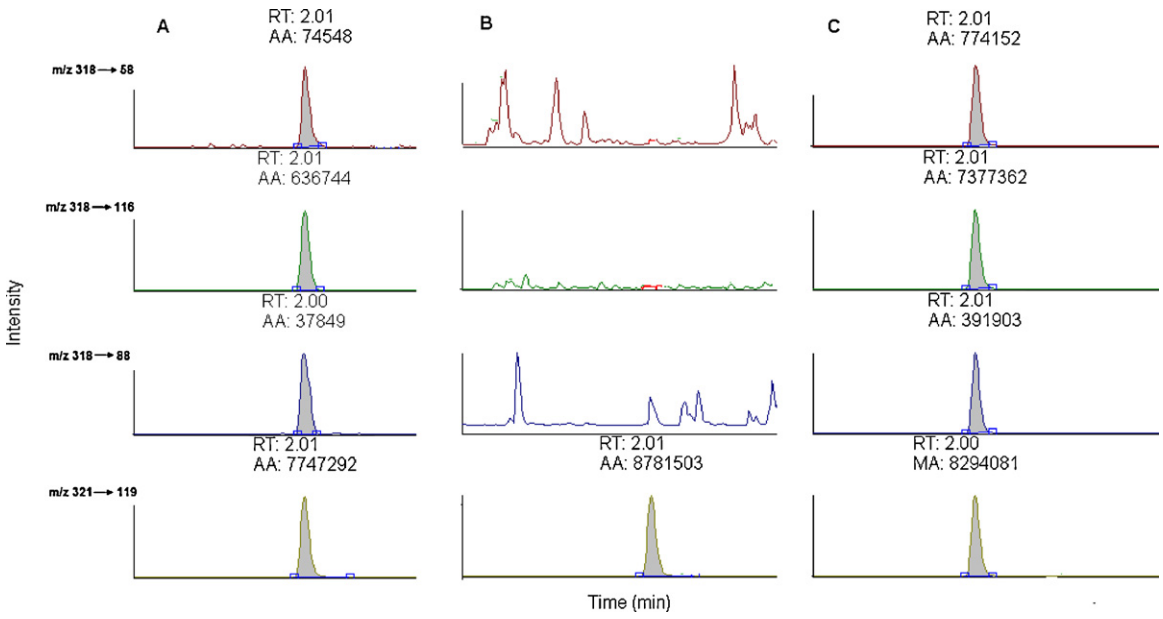

Fig. 1. Extracted ion chromatograms for GLY at the 5 pg/mL calibrator (A), a pre-administration sample (B) and a post administration sample of approximately 50 pg/mL (C).

Medicines Agency recommended guidelines [11]. Each validation and study sample run contained 10 calibrators prepared in drug-free horse urine, three non-fortified (analyte) control samples, and five positive control samples, all prepared in duplicate. Run acceptability was determined by the accuracy and precision of the calibration standards and positive control samples, the coefficient of determination of the standard curve, and evidence for the presence of GLY in the negative control samples.

Specificity of the method was assessed by supplementing positive control horse urine with various licit and potentially interfering substances. The purpose of this study was to determine whether such compounds altered the response of the analyte or IS or both. Three replicates each of five GLY concentrations (25, 125, 500, 750 and 1250 pg/mL) of positive controls samples were evaluated in the presence of 500 ng/mL of phenylbutazone and furosemide, substances that are frequently present in race horse urine specimens.

Sensitivity was assessed by determining the limit of detection (LOD) and lower limit of quantification (LLOQ) for the analyte. LOD was defined as the lowest concentration of analyte that could be detected with acceptable chromatography, the presence of quantifier and qualifier ions each with a signal-to-noise ratio of at least 3, and a retention time within ±0.2 min of the average retention time. LLOQ was the lowest concentration that met the LOD criteria and a signal-to-noise ratio of 10 and an acceptable accuracy and precision as described below. The upper limit of quantification (ULOQ) corresponded to the highest calibrator.

Linearity was assessed using a simple least squares regression with a $1/C_u$ weighting factor to account for homoscedasticity, where C_u was the urine GLY concentration. Evidence of linearity was provided when calibrator quantification was within 15% and 10% of the nominal concentration at the LLOQ, and all other concentrations, respectively. Linearity was also evaluated by plotting the response factor against the nominal concentration, visually inspecting residuals plots, and calculating the coefficient of determination (R^2).

Carryover was evaluated by observing the ion intensities of the characteristic ions of GLY in a negative control urine sample extract analyzed immediately after each of the four highest calibrators. Carryover was determined to have occurred if the apparent GLY concentrations in the negative control samples exceeded the limit of detection.

Accuracy and precision were investigated at five positive control concentrations (5, 125, 500, 750 and 1250 pg/mL). Intra- and inter-batch accuracy and precision were assessed with five replicates per concentration over 1 ($n = 5$) and 4 days ($n = 20$), respectively. An estimate of precision, expressed as percentage relative standard deviation (%RSD), was obtained using a one-way analysis of variance (ANOVA), using Microsoft Excel [12]. Accuracy was determined by comparing the mean ($n = 20$) measured concentration of the analyte to the target or nominal value. Accuracy was expressed as a percent of the target concentration with an acceptance criterion being 100 ± 15% of the nominal concentration.

Matrix effect, extraction efficiency (recovery), and process efficiency were evaluated using the three set method outlined by Matuszewski et al. [13]. The first set (A) consisted of analyte and IS solutions prepared "neat" into a starting mobile phase solution. Set 2 (B) comprised negative control urine extracts that were fortified with analyte and IS solutions after solid phase extraction. The third set (C) was negative control urine fortified with analyte and IS solutions before solid phase extraction. Absolute matrix effect, extraction efficiency, and process efficiency, all expressed as a percentage, were calculated using the following equations:

$$\text{Matrix Effect} \quad (\%) = \left(\frac{B}{A}\right) \times 100 \tag{1}$$

$$\text{Extraction Efficiency} \quad (\%) = \left(\frac{C}{B}\right) \times 100 \tag{2}$$

$$\text{Process Efficiency} \quad (\%) = \left(\frac{C}{A}\right) \times 100 \tag{3}$$

where A, B and C are the mean absolute peak areas obtained with a neat preparation, with urine extracts fortified with analyte and IS solutions after extraction, and with urine fortified with analyte and IS solutions before solid phase extraction, respectively. The process efficiency incorporates matrix effect and provides a more accurate estimation of the analyte recovery. In addition, to evaluate the influence of different sources of matrices on analyte quantification, five different lots of negative control urine were compared.

Concentrations of GLY in urine samples collected immediately after drug administration exceeded the ULOQ. Thus, sample dilutions were required. Therefore, dilution integrity was assessed by supplementing negative control urine with GLY at three

Table 1
Summary of accuracy and precision.

		Nominal concentration (pg/mL)				
		PC1	PC2	PC3	PC4	PC5
Characteristic	Statistic	5	125	500	750	1250
#Results	*N*	20	20	20	20	20
Accuracy	Mean bias (%RE)	0.690	0.556	1.180	0.366	0.253
	[a]LCL	0.283	−0.131	−0.410	−0.484	−0.155
	[b]UCL	1.10	1.242	1.951	1.216	0.661
Precision	Intra-batch (%CV)	1.20	1.146	2.100	1.208	1.498
	Inter-batch (%CV)	1.20	1.146	2.100	1.208	1.498
Accuracy + precision	|Mean| + inter-batch	1.89	1.702	3.281	1.574	1.750
90% expectation	Lower limit (%RE)	−1.40	−1.5	−2.5	−1.8	−2.4
Tolerance interval	Upper limit (%RE)	2.78	2.6	4.8	2.5	2.9

[a] Lower confidence limit for the mean bias.
[b] Upper confidence limit for the mean bias.

concentrations (0.5, 50, and 250.0 ng/mL) and diluting the samples over the range of dilution factors used for the study samples. Dilution factors used and evaluated were 1:2, 1:200, and 1:1000. Dilutional integrity was considered acceptable if replicate ($n = 5$) values were within 100 ± 20% of the nominal concentrations.

Stability of the analyte was evaluated over short-term intervals at 0 °C, −20 °C and −80 °C storage. Long-term stability was evaluated over nearly six months at −80 °C. Freeze–thaw stability was evaluated following three freeze/thaw cycles. Extracted analyte stability was evaluated at 24, 48 and 72 h in 20 °C autosampler conditions. All GLY stability samples were assessed with three replicates at each of three concentrations (5, 100 and 2500 pg/mL).

2.7. Statistical analysis

All *p*-values were determined using a two sample Student's *t*-test and were computed using Microsoft Excel 2010. A *p*-value of less than 0.05 was considered statistically significant. Goodness of fit evaluations were performed using GraphPad Prism™ version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Tolerance intervals for urine and plasma GLY concentrations at different collection times were computed to contain at least 99% of the population with 95% confidence and were calculated using the software program JMP 8.0 (SAS Institute Inc., Cary, NC, USA).

2.8. Plasma GLY determination

Plasma GLY concentrations were determined as previously reported [8].

3. Results

3.1. Urine method validation

No interferences with the determination of the GLY or the IS were detected in the analysis of positive control urine samples fortified with phenylbutazone or furosemide. This high specificity was determined by the retention time of the product ions, which varied ≤0.02 min for both GLY and the IS as well as sufficient accuracy (97–103%) compared to positive control samples that did not contain phenylbutazone or furosemide.

Method linearity was demonstrated with five calibration curves each spanning the range of 5–2500 pg/mL. In all instances ($n = 5$) the coefficient of determination (R^2) was >0.999 and the back-calculated concentrations of GLY in all calibrators were within 15% and 10% of the target concentration for the LLOQ and all other concentrations, respectively (data not shown). The corresponding LOD, LOQ, and ULOQ were 1 (CAL 1), 5 (CAL 2) and 2500 pg/mL (CAL 10) of urine, respectively. Linear regression analysis of the response factors (based on the areas of the quantifier ion) vs. the nominal GLY calibrator concentrations demonstrated a decreasing response with increasing GLY concentrations and a slope of −11.02 (data not shown). However, linear regression analysis of the response factor (based on the ion area ratios) vs. the nominal GLY calibrator concentrations demonstrated a slope that was not different from zero across the range of concentrations (Fig. 2). The intra-batch ($n = 5$) and inter-batch ($n = 20$) imprecisions were < 10% (expressed as %RSD). Accuracy was calculated as the recovery, which was determined to have a range of 94–104% (Table 1).

Glycopyrrolate carryover was observed in one blank injection each following the 1000 and 2500 pg/mL calibrators. However, the carryover was <1% of the total area response and was eliminated completely with the addition of a second consecutive mobile phase only injection. Two mobile phase blank injections were made between sets of calibrators, positive control samples, and research samples throughout the sequence.

Extraction efficiency, taking into account the matrix effect, was determined at 5, 20, 50, 250, and 1000 pg/mL ($n = 5$) for each concentration. It ranged from 91 to 108% for all concentrations, except for the low concentration (5.0 pg/mL), which was 120%. Overall, process efficiency, calculated from the ratio of the pre-extraction over the neat preparations, ranged from 82 to 105%. Absolute matrix effect was observed at all five positive control concentrations with a range of 82–90% (Table 2). Relative matrix effect was evaluated using five different matrix lots. Glycopyrrolate concentrations in positive control samples prepared in each of the five different lots of matrix differed from those of positive control samples prepared in a single lot used for the calibrators by <10%.

Table 3 illustrates GLY storage stability. Stability of GLY in extracted quality control samples over the range of the calibration curve was evaluated under 20 °C autosampler conditions for up to

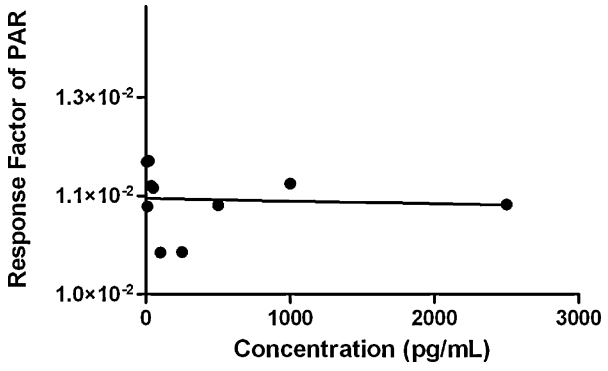

Fig. 2. Plot of the response factor of the peak area ratio (PAR) vs. the nominal calibrator concentrations. Slope = -3.51×10^{-8}, *y*-intercept = 0.0112.

Table 2
Matrix effect, extraction efficiency, and process efficiency data for GLY in horse urine.

Positive control concentration (pg/mL)	Absolute matrix effect (%)	Extraction efficiency (%)	Process efficiency (%)
5	87.4	119.8	104.6
20	87.6	101.0	88.5
50	82.4	107.6	88.6
250	89.8	91.0	81.7
1000	90.4	90.8	82.0

72 h and no appreciable degradation was observed as evidenced by less than a 10% decrease in absolute peak area over the 72 h period for all QC samples. Additionally, stability following three freeze–thaw cycles at −80 °C was demonstrated for all samples as the mean concentrations of GLY in the freeze-thawed replicates did not exceed 10% difference from the mean concentrations of the freshly prepared samples. Stability was evaluated after storage for 147 days at −80 °C at three concentrations and GLY concentrations did not differ more than 5% from those of freshly prepared samples.

Dilutional integrity was evaluated at three dilution factors: 2 (low), 200 (medium) and 1000 (high), at five determinations for each factor, to encompass the range of dilutions that were required for sample analysis. Each dilution was intended to yield a target concentration of 250 pg/mL. Comparing the average of five replicates for each dilution factor with the nominal value produced p-values > 0.05, indicating acceptable accuracy over the range of dilutions tested.

Table 3
Summary of storage stability for GLY in horse urine.

Storage conditions	Positive control concentration (pg/mL)		
	5	100	2500
Fresh samples			
Mean conc. (pg/mL)	4.98	97.8	2559.2
Difference (%)	0.00	0.00	0.00
CV (%)	3.09	4.17	0.28
0 °C (30 days)			
Mean conc. (pg/mL)	5.46	95.2	2553.9
Difference (%)	9.66	−2.66	−0.21
CV (%)	5.10	1.35	2.57
p-Value	0.125	0.326	0.911
−20 °C (60 days)			
Mean conc. (pg/mL)	4.81	91.5	2333.0
Difference (%)	−3.44	−6.49	−8.84
CV (%)	9.47	1.88	4.55
p-Value	0.489	0.171	0.070
−80 °C (147 days)			
Mean conc. (pg/mL)	4.78	93.5	2514.7
Difference (%)	−4.04	−4.47	−1.74
CV (%)	2.66	2.20	2.82
p-Value	0.219	0.068	0.360
Extracts (24 h)			
Mean conc. (pg/mL)	4.92	98.4	2525.4
Difference (%)	−1.12	0.60	−1.32
CV (%)	5.21	2.84	0.71
p-Value	0.824	0.869	0.143
Extracts (48 h)			
Mean conc. (pg/mL)	4.88	98.5	2474.8
Difference (%)	−2.06	0.68	−3.30
CV (%)	1.31	2.99	0.36
p-Value	0.330	0.884	**0.002**
Extracts (72 h)			
Mean conc. (pg/mL)	4.82	96.3	2506.4
Difference (%)	−3.11	−1.56	−2.06
CV (%)	2.83	3.43	1.10
p-Value	**0.006**	0.686	0.051
3 freeze/thaw cycles (−80 °C)			
Mean conc. (pg/mL)	4.82	98.8	2501.0
Difference (%)	−3.27	1.00	−2.27
CV (%)	7.77	0.84	2.54
p-Value	0.646	0.716	0.222

The % difference compares the mean concentration of replicates (n = 3) under the test condition to the mean concentration of replicates prepared fresh. The p-value was determined by a two sample Student's t-test.
Values in bold font are out of specification.

3.2. Administration

Urine GLY concentrations, determined using the method described above, were above the lower limit of quantification in urine samples collected through 96 h in all horses and in all but three samples through 168 h after IV administration. All urine concentrations are reported in Table 4. Peak urine concentrations of GLY were observed at the 4 h collection time for all horses. Urine concentrations of GLY through 168 h are graphed in Fig. 3. The upper limit of the tolerance interval was calculated (n = 20) for each collection time. Notably, the upper limit of the 99/95% tolerance interval at 24 h in urine was 325 pg/mL.

Descriptive statistics and graphical depictions of the plasma GLY concentrations have been reported previously [8]. The current report provides a table of plasma GLY concentrations in order to report the tolerance interval at each time (Table 5). The upper limit of the 99/95% tolerance interval (n = 20) at 24 h in plasma was 3.02 pg/mL.

Table 4
Glycopyrrolate urine concentrations (pg/mL) after intravenous administration of 1 mg.

Horse	Time (h)						
	4	8	24	48	72	96	168
1	45,820	1596	157	71.0	49.8	24.9	8.02
2	5082	794	123	58.1	40.1	23.0	7.03
3	10,160	1366	188	101	58.9	34.9	11.3
4	8396	665	104	57.7	27.5	28.5	8.24
5	11,790	413	138	71.7	36.4	24.1	6.20
6	30,316	482	83.2	45.1	27.9	14.1	4.74
7	25,040	1072	154	124	60.6	13.6	12.0
8	23,939	1092	117	60.6	32.5	26.8	5.28
9	12,932	1631	109	31.2	15.8	10.4	5.70
10	9415	188	79.7	34.1	14.8	13.0	6.25
11	209,198	3675	262	93.8	41.7	35.7	12.8
12	68,240	1135	80.6	34.0	14.5	10.4	4.86
13	64,186	510	103	35.2	16.6	11.4	6.22
14	11,939	423	61.5	17.3	10.4	7.06	3.22
15	111,211	519	145	69.7	34.1	18.5	7.47
16	34,568	2387	196	104	63.3	45.0	14.7
17	173,489	9708	132	75.9	34.7	24.3	11.3
18	119,981	2049	132	117	45.8	18.7	14.5
19	77,124	944	144	80.2	39.6	23.7	10.8
20	43,033	2378	113	52.1	25.6	18.8	5.81
Geomean	32,276	1073	124	59.6	30.7	19.2	7.66
Median	32,442	1082	127	65.1	34.4	20.9	7.25
Min	5082	188	61.5	17.3	10.4	7.06	3.22
Max	209,198	9708	262	124	63.3	45.0	14.7
TI (urine)	692,790	13,364	325	250	134	74.0	24.9

Concentrations in bold font indicate values that are below the current method's LLOQ. These values have been included in calculations to obtain measures of central tendency and dispersion and the tolerance interval.
Geomean—geometric mean; TI—tolerance interval (99%/95%).
*Values have been calculated from previously reported data.

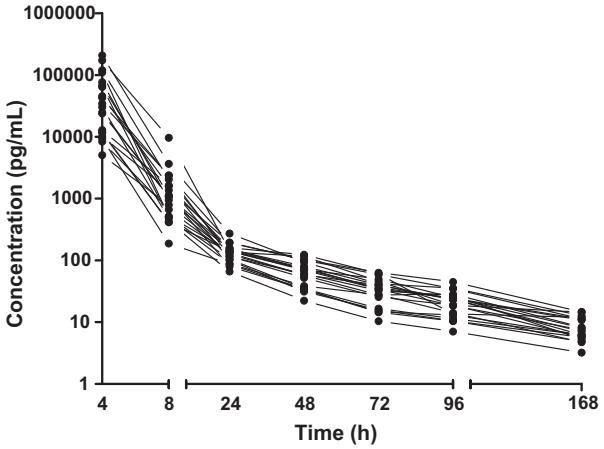

Fig. 3. Plot of urine concentration (pg/mL) vs. time (h) after intravenously administration of GLY (1 mg) to 20 horses.

The urine to plasma concentration ratio of any substance is determined by the renal clearance of that substance and the rate of urine formation:

$$\frac{CL_R}{\dot{V}} = \frac{C_U}{C_P}$$

where CL_R is the renal clearance, $\dot{V}$ is the rate of urine formation, C_U is the urine concentration, and C_P is the corresponding plasma concentration. The urine to plasma GLY concentration ratios for samples collected daily after intravenous administration are shown in Fig. 4. The urine to plasma concentration ratio at 4 and 8 h after administration had a median (range) of 3453.3 (505.8–15969.3) and 232.6 (505.8–15969.3), respectively. From 24 to 168 h after administration the urine to plasma concentration ratio ranged from 90 to 150 with a mean of 131. The mean (n = 20) at 24 h after administration was 103.

Table 5
Glycopyrrolate plasma concentrations (pg/mL) after intravenous administration of 1 mg to each of 20 horses.

Horse	Time (h)						
	4	8	24	48	72	96	168
1	8.27	3.88	1.54	0.510	0.102	0.029	0.093
2	9.36	4.45	1.17	0.850	0.209	0.110	0.069
3	11.9	3.91	1.92	0.970	0.479	0.163	0.119
4	16.6	6.49	2.25	1.21	0.313	0.209	0.097
5	9.40	3.82	0.95	0.376	0.283	0.146	0.080
6	9.69	4.86	0.860	0.294	0.138	0.025	<LOD
7	14.3	5.93	1.98	0.833	0.462	0.270	0.065
8	8.48	3.84	1.42	0.959	0.299	0.116	<LOD
9	9.85	4.39	1.29	0.267	0.346	0.135	0.077
10	10.4	5.88	1.85	0.681	0.519	0.269	0.058
11	13.1	3.94	1.17	0.584	0.219	<LOD	<LOD
12	12.1	3.44	1.27	0.933	<LOD	<LOD	<LOD
13	14.0	5.96	1.55	0.765	0.282	0.115	0.016
14	5.17	4.06	0.744	0.462	0.251	0.108	0.019
15	11.3	5.01	1.15	0.446	0.199	0.111	0.114
16	9.15	7.26	1.07	0.250	0.233	0.077	<LOD
17	10.9	5.81	0.95	0.350	0.073	<LOD	<LOD
18	9.85	4.63	1.22	0.545	0.482	0.174	0.107
19	13.6	7.54	1.76	0.574	0.238	0.149	0.123
20	6.97	4.07	1.06	0.519	0.271	0.115	0.117
Geomean	10.4	4.83	1.30	0.562	0.254	0.117	0.079
Median	10.1	4.54	1.24	0.559	0.271	0.116	0.093
Min	5.17	3.44	0.744	0.250	0.073	0.025	0.016
Max	16.6	7.54	2.251	1.207	0.519	0.270	0.123
TI (plasma)	22.0	9.33	3.02	2.04	1.11	0.787	0.551

Concentrations in bold font indicate values that are below the current method's LLOQ. These values have been included in calculations to obtain measures of central tendency and dispersion and the tolerance interval. For instances where no values were obtained (<LOD), the LOD (0.025 pg/mL) was substituted in order to calculate the tolerance interval [14].
Geomean—geometric mean; TI—tolerance interval (99%/95%).

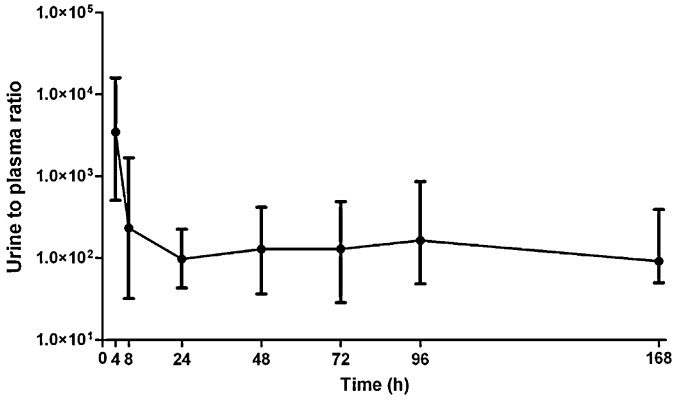

Fig. 4. Plot of median (range) urine to plasma concentration ratios for 20 horses administered a single 1 mg intravenous dose of GLY.

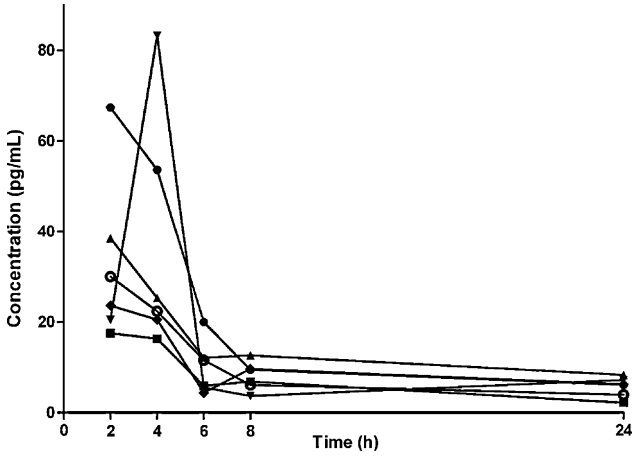

Fig. 5. Plot of concentration (pg/mL) vs. time (h) after oral administration of GLY (10 mg) in horse urine.

3.3. Oral administration

Glycopyrrolate urine concentrations were above the limit of detection in samples collected up to 24 h after oral administration in all horses. Peak urinary concentrations of GLY were observed within 2–4 h after oral administration. Median (range) concentrations at 2 h were 26.8 pg/mL (17.5–67.4). Fig. 5 displays urine concentrations versus time after oral administration of 10 mg to each horse. Median (range) concentrations at 4 h after oral administration were 23.8 pg/mL (16.3–83.2), not very different from the median value (22.4 pg/mL) when the highest value of 83.2 pg/mL was excluded.

4. Discussion

Glycopyrrolate (Robinul-V®) is a peripherally acting anticholinergic drug and effective bronchodilator in horses. Although it may have legitimate therapeutic applications in race horses, its use close to the day of racing is not permitted because of its potential to affect performance during racing. Consequently, glycopyrrolate has been categorized as a class 3 substance by the Association of Racing Commissioners International, Inc. and penalties associated with its use may include disqualification of the horse as well as a fine or

license suspension for the trainer. The currently described analytical method has been demonstrated to be adequate for determining urine GLY concentrations for several days after administration of GLY at clinically relevant doses to horses. In a previous report [3], capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) was compared to LC–MS/MS for the detection of GLY. The authors reported numerous benefits using CE-MS/MS over the conventional LC–MS/MS methods including, but not limited to, improved sensitivity and resolution. Although the dose administered in that study was approximately twice (4 μg/kg) the dose administered in the current study, a limit of detection of 1 ng/mL of urine prevented investigators from detecting GLY beyond 21.5 h whereas the method described in the current study was characterized by a LOD of 1 pg/mL and GLY was detected for 168 h.

We observed that carryover and possible contamination of GLY throughout the entire LC–MS system was possible if comparatively high concentrations were analyzed. Issues relating to GLY sequestration within the system were found to occur largely in the autosampler apparatus consisting of the syringe, injection valve, and wash stations. We therefore incorporated the comprehensive syringe and injection valve rinsing technique outlined above. Glycopyrrolate urine concentrations were not detectable or were below the LOD in pre-administration urine samples from 19 out of 20 horses. An exception was one horse in which the urine sample concentration was 12.2 pg/mL. The presence of GLY in this sample was confirmed through re-analysis and a review of the full scan product spectra. We attribute this finding to an error in the sample collection process or post-collection processing. We suspect that the pre-administration sample was inadvertently contaminated with a post-administration sample from a different collection time. We have reviewed the sample collection and aliquoting processes but have not identified a definitive explanation for this finding.

Urine GLY concentrations in 3 of 20 horses were above the LOD but below the method's LLOQ at 168 h. These values were included in calculations of the 168 h upper limit of the 99%/95% tolerance interval in order to reduce the bias associated with replacing these values with zero, replacing them with the LLOQ or a fraction of the LLOQ, or omitting the values from the calculations [14–16]. A similar analytical method for quantification of GLY in horse plasma has also been developed and validated [8] and differs from the current method only with regard to the sample volume used. Plasma GLY concentrations from the current study are reported here (Table 5) in order to assess the relationship between urine and plasma GLY concentrations. Also, the upper limits of the 99%/95% tolerance intervals, which have not previously been reported for GLY in horse plasma, were calculated in the same manner as those for urine and are reported here. Several plasma measurements for samples collected at 72, 96, and 168 h were below the LOD. Therefore, the plasma method's LOD was substituted for all missing values in order to calculate the tolerance interval [17].

The urine to plasma concentration ratio for any substance is a dimensionless value that is equal to the renal clearance of the substance divided by the volumetric urine flow rate. It is therefore possible to estimate the renal clearance of GLY by multiplying the ratio obtained from the GLY concentrations in paired urine and plasma samples by the measured or estimated volumetric flow rate of urine. The urine flow rate was not measured in the present study because volumetric urine collections were not made. However, normal urine flow rates of 0.52, 0.92, 1.12, and 1.24 mL/h/kg have been reported in healthy horses with no restrictions to feed and water [18–21]. Using these estimates of the urinary flow rate in horses, we estimated renal clearance of GLY by multiplying the urine to plasma concentration ratios at various times by these urine flow rates and present them in Table 6. The renal clearance estimates from 24 to 168 h ranged from 0.84 to 3.43 mL/min/kg and are similar to estimates of the glomerular filtration rate in horses suggesting that GLY is cleared renally primarily by filtration in the horse. Since we do not expect GLY to be reabsorbed in the distal tubules due to its polarity and because GLY is not appreciably bound to plasma proteins, tubular secretion must not account for much of the renal clearance due to the similarity between reported values of glomerular filtration and the estimates of renal clearance of GLY.

The median (range) of the total GLY plasma clearance reported from the pharmacokinetic study was 22.4 mL/min/kg (14.2–31.2) [22]. The total plasma clearance closely approaches estimates of hepatic blood flow in the horse [23] suggesting that GLY is appreciably cleared by metabolic transformation. The observed plasma clearance cannot be attributed exclusively to renal clearance because it exceeds the effective renal plasma flow and therefore the maximum value for renal clearance in the horse [24,25]. Since the renal clearance estimates from these horses are approximately equal to the glomular filtation rate and are substantially lower than estimates of total plasma clearance, it is evident that GLY is substantially cleared by non-renal mechanisms in the horse. The metabolism of glycopyrrolate in the horse and other species has not been extensively investigated. Some investigators have reported that most of the human dose is excreted unchanged in the urine [26] indicating that renal clearance is responsible for much of the plasma clearance in contrast to our finding in the horse.

The estimated value of renal clearance from these studies is affected by the timing of sample collections particularly in the early period after drug administration when concentrations were changing rapidly. For this reason, renal clearance studies often use the plasma concentration at the midpoint of the urine collection interval rather than one at either end for renal clearance calculations. In the study reported here, urine samples were collected at the same times as blood samples so it was anticipated that the ratios in the early period after GLY administration would be affected by the timing of sample collections and it was predicted that the urine to plasma ratios would be higher than those obtained later because plasma concentrations declined very rapidly for the first 8 h after administration. In fact, urine to plasma GLY ratios were higher at 4 and 8 h after administration than they were at any later time. From 24 h after administration, the ratio was relatively constant and was approximately 100 to 1 with a median (range) of 97.3 (83.1–123.5).

The urine to plasma concentration ratio is useful forensically since it permits analysts to predict the concentration in one matrix (*e.g.*, urine) from a result in the other matrix. This is useful in those

Table 6
Median (range) of estimated renal clearance (mL/min/kg) using the range of urinary flow rate estimates from various reports [18–21].

Time (h)	Urinary flow rate (mL/h/kg)			
	0.52	0.92	1.12	1.24
4	29.9 (4.38–138.4)	53.0 (7.76–244.9)	64.5 (9.44–298.1)	71.4 (10.5–330.0)
8	2.02 (0.28–14.48)	3.57 (0.49–25.6)	4.34 (0.60–31.2)	4.81 (0.66–34.5)
24	0.84 (0.37–1.94)	1.49 (0.66–3.43)	1.82 (0.81–4.17)	2.01 (0.89–4.62)
48	1.11 (0.32–3.59)	1.96 (0.56–6.35)	2.39 (0.68–7.74)	2.65 (0.75–8.57)
72	1.13 (0.25–5.03)	3.33 (0.44–8.89)	2.43 (0.53–10.8)	2.69 (0.59–12.0)
96	1.44 (0.42–12.4)	2.54 (0.74–21.9)	3.10 (0.90–26.7)	3.43 (1.00–29.5)
168	1.06 (0.43–5.09)	1.87 (0.76–9.00)	2.27 (0.93–11.0)	2.52 (1.03–12.13)

cases in which the drug is regulated with a plasma threshold but for which screening methods are based on analysis of urine samples. For example, a finding in urine can be dismissed or pursued based on a simple calculation of dividing the estimated urine concentration by the concentration ratio and comparing the predicted plasma concentration to the plasma threshold. Additionally, a laboratory finding in an official post-race sample of comparatively higher urine to plasma ratio may indicate that the drug was administered close to race time.

Samples used to regulate drugs in horse racing are typically collected in the period from 30 to 120 min after the end of the race during which a brief period of increased urine flow rate has been observed [27]. The increase in urine flow rate is likely due to increased renal blood flow in which case the renal clearance and urine flow rate would be expected on increase proportionately. If this is the case, the urine to plasma concentration ratio would be expected to be unchanged.

5. Conclusion

A validated method for the detection and quantification of GLY at low pg/mL concentrations in horse urine is reported. The method was demonstrated to provide reliable quantification and adequate sensitivity for post-race sample analysis and we believe this to be the first reported method for regulatory control of GLY in horse urine. Further, we have demonstrated that GLY was detectable in horse urine for at least 168 h after intravenous administration of a clinically relevant dose and 24 h after oral dosing. Post-race plasma analysis could be complementary to urine analysis in order to provide adequate regulatory control of the use of GLY. The relationship between plasma and urinary glycopyrrolate concentrations following a single intravenous dose is a noteworthy observation that should be considered when evaluating the pharmacologic significance of the presence of glycopyrrolate and regulatory control in official post-race urine samples. The results of this research can be used to develop thresholds and withdrawal guidelines for regulating the use of GLY in the horseracing industry.

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