Validation of a Liquid Chromatography–Tandem Mass Spectrometry Method for Quantification of Glycopyrrolate in Horse Plasma

M.J. Rumpler[1,*], R.A. Sams[1,†], and **P. Colahan[2]**

[1]Department of Physiological Sciences, Florida Racing Laboratory, University of Florida, College of Veterinary Medicine, Gainesville, Florida 32610-0117 and [2]Department of Large Animal Clinical Sciences, University of Florida, College of Veterinary Medicine, Gainesville, Florida 32608-0136

Abstract

A rapid, sensitive, and specific ultra-high-performance liquid chromatography with heated electrospray ionization-tandem mass spectrometry (UHPLC–HESI-MS–MS) method to detect and quantify glycopyrrolate in horse plasma has been developed and validated. We also determined glycopyrrolate in plasma after oral and intravenous administration of clinically relevant doses to Thoroughbred horses. Calibration was accomplished by weighted, linear regression analysis using a deuterated analogue of glycopyrrolate as internal standard (IS). Glycopyrrolate (GLY) and the IS (GLY-d_3) were isolated from plasma matrices via weak cation exchange using a simple solid-phase extraction technique. Chromatographic analysis was achieved by reversed-phase UHPLC on a C_{18} Acquity™ column. Extracts were analyzed in positive electrospray ionization mode and precursor and product ions were detected and quantified by MS–MS using a triple-stage quadrupole (TSQ) instrument. The method was characterized by a linear range of 0.125–25 pg/mL (R^2 > 0.998), a lower limit of quantification of 0.125 pg/mL and a lower limit of detection of 0.025 pg/mL. Recovery of GLY ranged from 78% to 96%, and intra- and interbatch precision were 3.3–14.4%CV and 3.4–14.4%CV, respectively. Glycopyrrolate was stable in plasma for up to 170 days at –80°C, through three freeze/thaw cycles, and for up to 48 h after extraction under 20°C autosampler conditions.

Introduction

Glycopyrrolate (Robinul-V®) is a synthetic anti-cholinergic drug and effective bronchodilator in horses. It has legitimate veterinary clinical applications but also remains a suspected doping agent for performance horses. This quaternary amine is a peripheral anti-muscarinic compound and, as such, can be used therapeutically, primarily to inhibit parasympathetic activity. Currently classified as a class 3 substance by the Association of Racing Commissioners International, Inc., GLY has long been indicated as a performance modifier in horseracing. In racing, GLY is potentially exploited for its dilatory effects on the respiratory tract and likely preferred for its absence of appreciable effects on the central nervous system (CNS) compared to other muscarinic antagonists such as atropine and scopolamine. Although clinically similar to GLY, these compounds penetrate the CNS much more effectively as their tertiary amine structure increases membrane permeability (1). Additionally, as predicted from its structure, GLY has a limited oral bioavailability that is due to the compound's permanent ionization (2).

A method with greater sensitivity than those previously reported was necessary for meaningful pharmacokinetic analysis of GLY. Previous reports that focus on the quantitative determination of GLY and other quaternary ammonium compounds employ volatile ion pairing reagents to extract GLY (3) and fail to achieve detection limits that may be necessary for regulatory control of GLY (4). The following report presents a sensitive and selective method for the direct quantification of GLY in horse plasma.

Experimental

Animals

Twenty adult Thoroughbred horses (6 mares and 14 geldings) in athletic condition 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 6 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 upon completion of the IV study. All horses were housed in grass paddocks at the UF

* Author to whom correspondence should be addressed: Marc J Rumpler, MS, University of Florida College of Veterinary Medicine. Email: mrumpler@ufl.edu.
† Current address: HFL Sport Science, Inc., Lexington, KY 40509.

Veterinary Medical Center (Gainesville, FL), maintained on a diet of commercially available grain mixture, and had open access to water and hay at all times. Horses were regularly exercised (3 days/week) before and throughout the duration of the studies.

Conditioning was achieved at the UF Equine Performance Laboratory using a high-speed Säto treadmill (Equine Dynamics, Lexington, KY). For two months prior to the study, eligible horses were required to meet a fitness goal using a standard training regimen and exhaustion test. This preconditioning training regimen was designed to prepare the horse to complete a mile in 2 min at a steady gallop without undue stress. Horses were evaluated for this goal through a condition test before the start of the study. The standard training regimen continued throughout the course of the study and consisted of trotting for 0.6 km at 4.0 m/s, galloping for 2 km at 8 m/s, and trotting for 0.6 km at 4.0 m/s. The treadmill belt was horizontally orientated one day per week (Monday) and at a 6° inclination two days per week (Wednesday and Friday).

For the intravenous study, all horses were administered 1 mg (1.66–2.06 µg/kg) of GLY (glycopyrronium bromide, American Regent, Shirley, NY) 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 dose of 10 mg orally. Whole blood samples were collected from the left (contralateral) jugular vein via needle venipuncture into partially evacuated tubes containing lithium heparin. Blood samples were stored on ice until the plasma was concentrated by centrifugation (2500–3000 rpm or 776–1318 × *g*) at 4°C for 15 min. Harvesting of plasma took place within 1 h of sample collection and 2–4-mL aliquots of plasma were immediately frozen at –20°C and stored within 24 h at –80°C until analyzed. Collection times were before drug administration and at 4, 8, 24, 48, 72, 96, and 168 h after intravenous administration and at 15, 30, and 45 min and 1, 1.5, 2, 3, 4, 6, 8, and 24 h after oral administration. The experimental protocol, including animal conditioning and drug administration and collection, was approved and facilities were inspected by the University of Florida Institutional Animal Care and Use Committee (IACUC)

Chemicals and reagents

Certified analytical grade drug standards including glycopyrronium bromide and GLY iodide-d_3 were obtained from USP Pharmacopeia (Rockville, MD) and Toronto Research Chemicals (North York, ON, Canada), respectively. Reagent grade formic acid was obtained from ACROS Organics (Morris Plains, NJ). All solvents including acetonitrile, methanol, and methylene chloride were HPLC grade and obtained from Thermo Fisher (Pittsburgh, PA). All water used was deionized with a resistivity greater than or equal to 18 megaohms and organic content less than 10 ppb.

Glycopyrrolate [(United States Adopted Name (USAN)] is also known as glycopyrronium bromide (Recommended International Nonproprietary Name) (5). 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 of the mass for the bromide ion component, consistent with the USAN definition for GLY.

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 yield a calibration curve ranging from 0.025 to 25 pg/mL. Calibrators and positive control samples were prepared from separately prepared stock solutions. Each calibrator and positive control sample was prepared using 1 mL of phosphate buffer and 1 mL of drug-free control horse plasma, and fortified with the appropriate volume of GLY working standard solution and 25 µL of the IS. The IS was prepared in a working standard solution at a concentration of 0.004 ng/µL. The final IS concentration was 100 pg/mL of plasma.

Sample preparation

Duplicate 1-mL aliquots of sample plasma were added to 1 mL of phosphate buffer (50 mM, pH 7.0) and 25 µL of 0.004 ng/µL IS working standard solution in 5-mL disposable centrifuge tubes. If sample dilution was required, an aliquot of the sample was diluted with 0.9% (w/v) saline. The tubes were centrifuged at 1508 × *g* (2800 rpm) for 12 min, and the buffered plasma samples were subjected to solid-phase extraction. Isolute CBA 3-mL columns (Biotage, Charlottesville, VA) were sequentially conditioned with 2 mL each of methanol, water, and phosphate buffer. Buffered plasma specimens were pipetted onto the columns, and a positive pressure sufficient to achieve

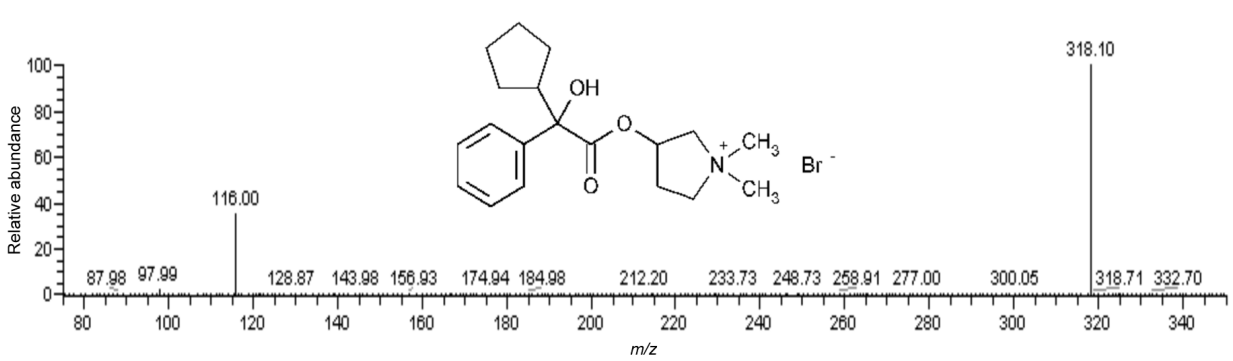

Figure 1. Ion spectrum for glycopyrrolate (*m/z* 318.1) and glycopyrrolate bromide structure.

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 dichloromethane. The analyte was eluted with two 0.5-mL aliquots of 1% formic acid in acetonitrile and the eluate was evaporated under nitrogen on a TurboVap® LV evaporator (Zymark, Hopkington, MA). Sample extracts were then dissolved in 100 µL of 0.1% formic acid in acetonitrile/water (20:80) and transferred to glass autosampler vials.

Instrumentation

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) analysis was performed on a TSQ Quantum Ultra MS (ThermoFisher, San Jose, CA) equipped with an HESI source interfaced with an HTC PAL autosampler (Leap Technologies, Carrboro, NC) and Accela LC pump (ThermoFisher). Xcaliber™ (ThermoFisher) software version 2.0.7 and LCquan (ThermoFisher) version 2.5.6 were used for data acquisition and analysis.

The autosampler syringe was washed before and after injection five times each with 2% formic acid in acetonitrile (wash 1) followed by 10% methanol in water (wash 2). The post injection rinse was followed by an injection valve rinse using five repetitions each of wash 1 followed by wash 2. All rinse solvents were diverted directly into the waste stream after use.

Chromatographic separations were achieved with an Acquity™ UPLC HSS T3 (2.1 mm × 50 mm, 1.8-µm particle size) column and an identically packed 2.1-mm × 5-mm guard column (Waters, Taunton, MA). Gradient elution was begun with a mobile phase of 0.1% (v/v) formic acid in water (80%) (Solvent A) and 0.1% (v/v) formic acid in acetonitrile (20%) (Solvent B). The initial mixture, kept constant at a 500 µL/min flow rate, was held isocratically for 0.5 min, then Solvent A was decreased linearly to 5% and Solvent B increased to 95% over 2.25 min and held for 0.25 min. The mobile phase was then returned to the initial conditions for the remaining 0.5 min for a total run time of 3.5 min. The flow into the MS was diverted from 0 to 0.75 min and 2.5 to 3.5 min. The column temperature was 35°C and 20 µL of the sample extract was injected. Mass spectral data were acquired in positive ion mode using the HESI and the following MS parameters: ESI spray voltage, 4100; vaporizer temperature, 240°C; sheath gas pressure, 40; ion sweep gas, 0; auxiliary gas pressure, 6; capillary temperature, 300°C; tube lens offset, 89; and skimmer offset, 10.

Identification and quantification of the analyte was based on selected reaction monitoring (SRM). Compound specific optimization (tuning) of MS–MS parameters was performed before analyses via direct infusion of the analyte and internal standard dissolved in mobile phase (10 ng/µL each). Tuning for GLY yielded collision energies of 39, 50, and 33 V for transitions 318 → 58, 318 → 88, and 318 → 116, respectively. Tuning for GLY-d_3 yielded a collision energy of 33 and tube lens offset of 118 for transition 321 → 119. The most abundant ion transmission for the analyte was 318 → 116 (Figure 1) and was used for quantification. The second and third most abundant transitions were used as qualifier transitions.

All standards, controls, calibrators, and samples were prepared in duplicate, and ion peak-area ratios of the analyte and IS were calculated for each. Individual values of the duplicate concentrations were averaged. 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 LOQ). In addition, for calibrators the difference between the back-calculated concentration and the nominal concentration must not exceed 10% (15% at the LOQ). All samples that did not meet such criteria were re-analyzed.

Method validation

The method was validated in accordance with the U.S Food and Drug Administration recommended guidelines (6) 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 Medicines Agency recommended guidelines (7). Each validation and study sample run contained ten calibrators prepared in drug-free horse plasma, 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 determined by supplementing negative control plasma with various licit and potentially interfering substances. The purpose of this experiment was to determine whether such compounds altered the response of the analyte or IS. Three replicates each of five concentrations (0.125, 1.25, 5, 12.5, and 22.5 pg/mL) of positive controls samples were evaluated in the presence of phenylbutazone and furosemide, substances that are routinely present in official post race horse samples.

Sensitivity was evaluated by establishing a limit of detection (LOD) and lower limit of quantification (LLOQ) for the analyte. The LOD was defined as the lowest concentration of analyte that could be detected with acceptable chromatography, the presence of quantifier and qualifier ions with a signal-to-noise ratio of at least 3, and a retention time within ±0.2 min of the

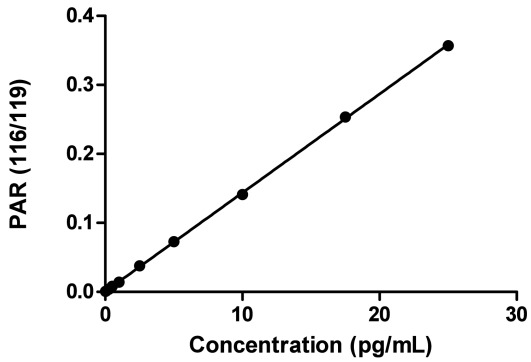

Figure 2. Glycopyrrolate calibration curve weighted 1/*x* ($R^2 > 0.999$).

average retention time. The LLOQ was the lowest concentration that met the LOD criteria but with a signal–to-noise ratio of 10 and acceptable accuracy and precision as defined. The upper limit of quantitation (ULOQ) corresponds to the highest calibration point.

Linearity was assessed using a simple least-squares regression with a $1/C_p$ weighting factor, where C_p was the plasma concentration. Evidence of sufficient linearity was achieved when the coefficient of determination (R^2) was at least 0.998 and calibrator quantification was within 15% and 10% of the nominal concentration at the LLOQ and all other concentrations, respectively. Linearity was further assessed by plotting the response factor against the nominal concentration and visually inspecting the residuals plots.

Carryover was evaluated by observing the peak intensities for the characteristic ions of GLY in a negative plasma sample analyzed immediately after each of the four highest calibrators. Concentrations in the negative plasma samples were calculated, and carryover was determined to occur if the analyte concentrations exceeded the LOD.

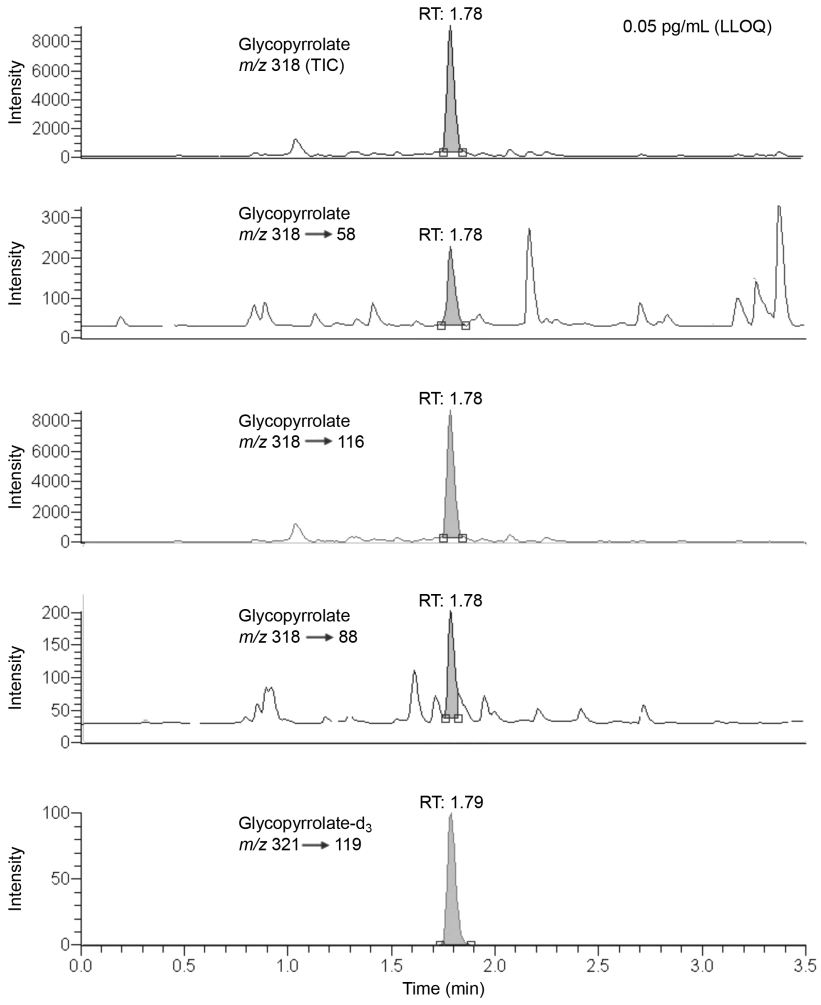

Figure 3. SRM chromatograms for GLY in horse plasma at the LLOQ (0.125 pg/mL) and the IS. TIC = total ion chromatogram.

Accuracy and precision were investigated over the linear dynamic range at five positive control concentrations (0.125, 1.25, 5, 12.5, and 22.5 pg/mL). Intra- and interbatch accuracy and precision were assessed with five replicates per concentration over 1 (n = 5) and 4 (n = 20) days, respectively. An estimate of precision, expressed as percentage relative standard deviation (%RSD), was obtained using a one-way analysis of variance (ANOVA), via Microsoft Excel. Values were required to be within ±15% and ±20% for the lowest positive control concentration. Accuracy was determined by comparing the mean (n = 20) measured concentration of the analyte to the nominal value. Accuracy was expressed as a percent of the nominal concentration with an acceptance criterion being ±20% of the nominal concentration.

Matrix effect, extraction efficiency (recovery), and process efficiency were evaluated using the three set method outlined by Matuszewski et al. (8). The first set (A) consisted of analyte and internal standard solutions prepared "neat" into a starting mobile phase solution. Set 2 (B) was plasma extracts that were fortified with analyte and internal standard solutions following solid-phase extraction. The third set (C) was plasma fortified with analyte and internal standard solutions before solid-phase extraction. Absolute matrix effect, analytical recovery and process efficiency, all expressed as a percentage, were calculated using the following equations:

$$\text{Matrix Effect (\%)} = (B/A) \times 100 \qquad \text{Eq. 1}$$

$$\text{Recovery (\%)} = (C/B) \times 100 \qquad \text{Eq. 2}$$

$$\text{Process Efficiency (\%)} = (C/A) \times 100 \qquad \text{Eq. 3}$$

where A, B, and C are the mean absolute peak areas obtained with a neat preparation, with plasma extracts fortified with analyte and internal standard solutions following extraction and with plasma fortified with analyte and internal standard solutions before solid-phase extraction, respectively.

The process efficiency incorporates matrix effect and provides a more accurate calculation of the analyte recovery. In addition, to evaluate the influence of different sources of matrices on analyte quantification, five different lots of horse plasma were used. Therefore, for each set, a range of positive control samples (standard curve) were prepared in each of five different lots of plasma to generate five slopes (calculated using $y = mx + b$). Relative matrix effect (expressed as percent coefficient of variation) was determined by the variability of slopes within a set.

Concentrations of GLY in plasma samples collected immediately after drug

administration exceeded the upper limit of the calibration range used for validation. Thus, sample dilutions were required. Therefore, dilution integrity was assessed by supplementing negative control horse plasma with GLY at four concentrations (0.02, 1, 5, and 10 ng/mL) and diluting the samples over the range of dilution factors used for the study samples. All dilutions were prepared with 0.9% (w/v) saline using ultrapure (resistivity greater than or equal to 18 megohms and organic content less than 10 ppb) deionized water. Dilution factors evaluated were 1:2, 1:100, 1:500, and 1:1000. Dilutional integrity was considered acceptable if replicate (n = 5) values were within ±20% of 10 pg/mL.

Method ruggedness was investigated to determine whether small variations in sample preparation affected analyte quantitation. Positive control samples at five concentrations (0.125, 1.25, 5, 12.5, and 22.5 pg/mL) were evaluated under test conditions and compared to positive control samples prepared under normal conditions.

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 (1, 5, and 25 pg/mL).

Statistical analysis

Mean plasma concentrations of GLY are reported as mean ± s.d. 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.

Results

Method validation

Method specificity was demonstrated by adding therapeutically relevant but potentially interfering concentrations (1–4 µg/mL) of the permitted substances phenylbutazone and furosemide to quality control samples. No interferences with the determination of the target analyte GLY or the IS were detected in the analysis of plasma samples containing phenylbutazone or furosemide. High selectivity was assessed by the retention time of the product ions, which varied ≤ 0.02 min for both GLY and its internal standard, and the accuracy to the target value for all three concentrations (96–101%) of the control samples.

Calibration linearity (Figure 2) through 10 points was observed over a range of 0.05–25 pg/mL with a coefficient of determination (R^2) of > 0.998 (n = 4). The corresponding LOD, LLOQ (Figure 3), and ULOQ (Figure 4) were 0.025, 0.125, and 25 pg/mL, respectively.

Carryover and possible contamination of GLY throughout the entire LC–MS system occurred and was potentially detrimental to the determination of low concentrations when comparatively high concentrations were analyzed during method validation studies. Glycopyrrolate sequestration within the system had been determined to occur largely in the syringe, injection valve, and wash stations of the autosampler. We therefore incorporated the extensive syringe and injection valve washing steps outlined here. Under these conditions the extent of GLY carryover was below the LOD.

Precision and accuracy of the method were evaluated at five concentrations over the linear dynamic range (0.125, 1.25,

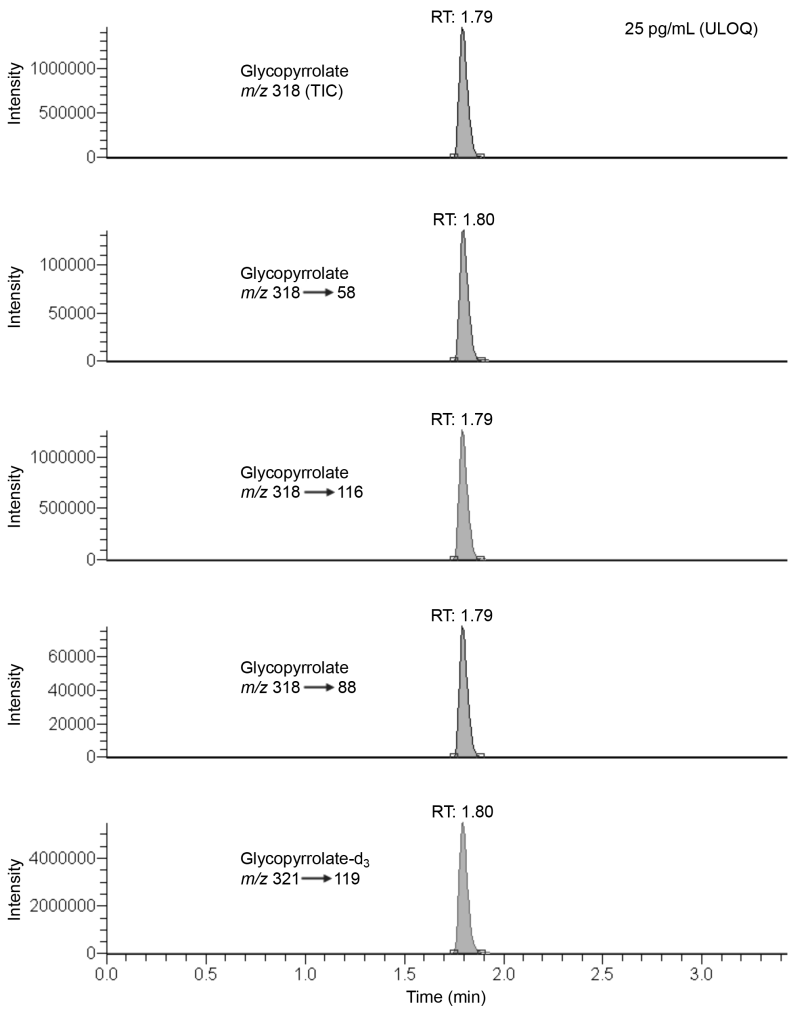

Figure 4. SRM chromatograms for GLY in horse plasma at ULOQ (25 pg/mL) and the IS. TIC = total ion chromatogram.

5.0, 12.5, and 22.5 pg/mL). The interbatch (n = 5) and intrabatch (n = 20) imprecision values were <10% (expressed as %CV). Inaccuracy ranged from 0.5% to 4.7%. The complete summarized results of accuracy and precision are presented in Tables I and II. Calculations required for Table II are those described by Desilva et al. (9).

The matrix effect was evaluated using five different lots of matrix at five concentrations of GLY for five replicates each (n = 5) using the three set experimental design described by Matuszewski et al. (8). Absolute matrix effect was observed in all five lots of plasma with a range of 85–99%. Extraction efficiency, determined at 0.125, 1.25, 5, 12.5, and 22.5 pg/mL (n = 5) for each concentration, ranged from 79% to 96% for all concentrations. Overall process efficiency, taking into account the matrix effect, ranged from 67% to 95% (Table III). Relative matrix effect between five lots of plasma was expressed as coefficient of variance of five slopes generated from five prepared standard curves within a set. These values were less than 6% indicating minimal matrix interferences and increased reliability across different sources of plasma (Table IV).

Dilutional Integrity was evaluated at four dilution factors (1:2, 1:100, 1:500, and 1:1000), with five determinations at each factor, encompassing the range of dilutions that were required for sample analysis. The average back-calculated concentration did not differ from the target concentration more than 5% (Table V).

Method ruggedness was tested to investigate whether small variations in the proposed method affected GLY quantification (Table VI). Small changes in the proportion of the solution used to dissolve the extraction residue and the rinse phase of the extraction had minimal effects on GLY response. We investigated the solid-phase extraction elution step for ruggedness by removing the 1% formic acid from the elution solvent (acetonitrile). The results demonstrated no detectable response for GLY at the concentrations examined (results not shown). In addition, when the elution volume (1 mL) was reduced to 0.5 mL, mean accuracy and precision ranged from 86% to 263% and 5% to 171%, respectively. All concentrations were out of specification for accuracy, precision, or both. Results indicate that the volume of the elution solvent and the presence of formic acid in the elution solvent are crucial variables in the solid-phase extraction process.

The stability of GLY from extracted quality control samples over the range of the calibration curve was evaluated under 20°C autosampler conditions for up to 72 h. The mean GLY concentration after storage for 48 h on the autosampler apparatus differed less than a 10% compared to freshly prepared samples over a 48 h period whereas those determined after storage for 72 h were greater than 10%. Additionally, the stability of GLY through three freeze/thaw cycles at –80°C was demonstrated as no appreciable degradation was found compared to freshly prepared positive control samples. Short-term stability of GLY at three concentrations in plasma after storage at 0°C, –20°C, and –80°C for 14, 60, and 60 days was evaluated.

Table I. Accuracy and Precision

		Intrabatch Statistics					
Sample*	Batch	n	Mean	SD	% CV	%RE	Ancillary Statistics
PC1	1	5	0.131	0.012	9.88	4.45	MS_w† = 0.000
0.125	2	5	0.119	0.014	10.9	-4.70	MS_b = 0.000
	3	5	0.126	0.031	25.0	0.41	MS_t = 0.000
	4	5	0.127	0.012	9.40	1.83	s_t = 0.018
							s_b = 0.000
Intrabatch statistics (Pooled):		5	0.126	0.018	14.4	0.50	p = 4
Interbatch statistics (ANOVA):		20	0.126	0.018	14.4	0.50	
PC2	1	5	1.27	0.069	5.55	1.55	MS_w = 0.006
1.25	2	5	1.26	0.045	3.57	0.73	MS_b = 0.014
	3	5	1.21	0.126	10.1	-3.09	MS_t = 0.007
	4	5	1.34	0.025	1.97	7.33	s_t = 0.085
							s_b = 0.042
Intrabatch statistics (Pooled):		5	1.27	0.076	6.10	1.63	p = 4
Interbatch statistics (ANOVA):		20	1.27	0.087	6.95	1.63	
PC3	1	5	5.15	0.074	1.48	2.98	MS_w = 0.045
5	2	5	4.98	0.187	3.74	–0.47	MS_b = 0.211
	3	5	5.04	0.312	6.23	0.76	MS_t = 0.071
	4	5	5.44	0.203	4.06	8.80	s_t = 0.266
							s_b = 0.182
Intrabatch statistics (Pooled):		5	5.20	0.211	4.23	3.02	p = 4
Interbatch statistics (ANOVA):		20	5.20	0.279	5.58	3.02	
PC4	1	5	12.9	0.345	2.76	2.91	MS_w = 0.222
12.5	2	5	12.8	0.419	3.35	2.07	MS_b = 0.747
	3	5	13.1	0.538	4.30	4.98	MS_t = 0.305
	4	5	13.6	0.554	4.43	9.00	s_t = 0.553
							s_b = 0.324
Intrabatch statistics (Pooled):		5	13.1	0.472	3.77	4.74	p = 4
Interbatch statistics (ANOVA):		20	13.1	0.572	4.58	4.74	
PC5	1	5	22.6	0.526	2.34	0.24	MS_w = 0.542
22.5	2	5	22.7	0.694	3.09	0.87	MS_b = 0.781
	3	5	22.7	0.712	3.16	0.70	MS_t = 0.580
	4	5	23.4	0.951	4.23	4.08	s_t = 0.762
							s_b = 0.219
Intrabatch statistics (Pooled):		5	22.8	0.736	3.27	1.47	p = 4
Interbatch statistics (ANOVA):		20	22.8	0.768	3.41	1.47	

* Sample concentrations are in pg/mL.

† Abbreviations: MS_w, ANOVA mean square for intrabatch samples; MS_b, ANOVA mean square for interbatch samples; MS_t, ANOVA mean square for all samples; s_t, ANOVA variance component for all samples; and s_b, ANOVA variance component for interbatch samples.

Table II. Summary of Accuracy and Precision

Characteristic	Statistic	Nominal Concentration PC1 0.125 (pg/mL)	PC2 1.25 (pg/mL)	PC3 5.0 (pg/mL)	PC4 12.5 (pg/mL)	PC5 22.5 (pg/mL)
# Results	*N*	20	20	20	20	20
Accuracy	Mean bias (%RE)	0.5	1.6	3.0	4.7	1.5
	*LCL	–5.622	–5.222	–3.521	–0.182	–1.324
	†UCL	6.622	8.479	9.558	9.660	4.267
Precision	Intrabatch (%CV)	14.4	6.1	4.2	3.8	3.3
	Interbatch (%CV)	14.4	7.0	5.6	4.6	3.4
Accuracy + Precision	|Mean| + Interbatch	14.899	8.581	8.603	9.317	4.886
90% Expectation	Lower limit (%RE)	–24.6	–11.2	–7.9	–3.9	–4.7
Tolerance interval	Upper limit (%RE)	25.6	14.4	13.9	13.4	7.6

* Lower confidence limit for the mean bias.
† Upper confidence limit for the mean bias.

Table III. Matrix Effect, Extraction Efficiency, and Process Efficiency Data for Glycopyrrolate in Horse Plasma

Positive Control Concentration (pg/mL)	Absolute Matrix Effect (%)	Extraction Efficiency (%)	Process Efficiency (%)
0.125	85.3	78.5	66.9
1.25	97.6	90.5	88.4
5	98.7	95.7	94.5
12.5	99.0	91.0	90.1
22.5	97.3	95.2	92.6

Table IV. Relative Matrix Effect*

	Neat Standards (set 1)	Plasma Extracts Fortified After Extraction (set 2)	Plasma Extracts Fortified Before Extraction (set 3)
Slope†	0.0100	0.0098	0.0097
SD	0.00017	0.00055	0.00045
%CV	1.73	5.57	4.64

* For each set, five different standard curves using five different matrix lots were prepared. Each standard curve was constructed using five concentrations (positive controls).
† Mean values of five slopes ($n = 5$), each obtained in a different plasma matrix lot. The slope of a standard curve was calculated using $y = mx + b$.

Table V. Dilution Integrity

Dilution Factor*	% CV ($n = 5$)	% Difference From Nominal Concentration
2	4.12	1.75
100	3.49	2.91
500	3.32	–2.04
1000	2.41	–4.24

* Diluted concentrations were multiplied by the appropriate dilution factor to obtain a mean ($n = 3$) sample concentration. This value was compared to the nominal concentration of the positive control prior to dilution.

Long-term storage stability was evaluated at –80°C for 170 days at these concentrations. All validation study results for stability are reported in Table VII.

Intravenous administration

Glycopyrrolate in plasma extracts was detectable for up to 168 h after intravenous administration (Figure 5). The mean plasma ± s.d. concentration at 24 h after dosing was 1.36 ± 0.41 pg/mL.

Oral administration

Glycopyrrolate was detected in plasma samples collected after oral administration of 10 mg of GLY in aqueous solution. We have discovered from previous investigations that the GLY urine to plasma concentration ratio averages about 100 following intravenous administration. We have previously reported that the mean ± s.d. peak urinary concentration observed 2–4 h after a 10-mg oral dose was 43 ± 26 pg/mL (10). Therefore, we hypothesized that plasma concentrations of GLY following an oral dose of 10 mg would be below the limit of quantitation 2–4 h after dosing. The measured mean peak concentrations were 4.7 ± 2.6 pg/mL and occurred at 15 min after dosing. At 1 h after dosing, the plasma GLY concentrations in all horses were below 0.5 pg/mL and those at all other collection times up to 24 h were determined to be less than the LOD.

Discussion

We have demonstrated that identification of GLY in post race plasma samples can be accomplished at concentrations less than a picogram per milliliter using common analytical laboratory procedures and modern analytical instrumentation. Further, the method has undergone the necessary validation procedures for reliable analysis, and we believe this to be the first reported method for determination of GLY with adequate sensitivity for detailed pharmacokinetic analysis and the appropriate regulatory control of this drug.

Analysis of GLY on the Acquity UPLC HSS T3 was performed using higher flow rates (0.5 mL/min) and shorter run times (3.5 min) with increased efficiency and no apparent loss in sensitivity or reproducibility. Tandem MS provided excellent selectivity for this compound.

Fourteen of 20 horses exhibited detectable concentrations of GLY in plasma at 168 h. However, 12 horses exhibited concen-

Table VI. Ruggedness Evaluation

Sample Preparation Condition Tested	Positive Control Concentration (pg/mL) 0.125 Mean accuracy* (%) w/precision, CV (%)	1.25 Mean accuracy (%) w/precision, CV (%)	5 Mean accuracy (%) w/precision, CV (%)	12.5 Mean accuracy (%) w/precision, CV (%)	22.5 Mean accuracy (%) w/precision, CV (%)
Rinse with 1 mL of water, MeOH, DCM instead of 2 mL water, MeOH, DCM	104.0 (12.90)	109.1 (2.33)	93.0 (2.86)	95.9 (2.57)	89.5 (1.66)
SPE, rinse with water and MeOH (no DCM)	101.3 (10.40)	106.0 (8.72)	94.6 (8.20)	98.4 (2.42)	93.1 (1.33)
Dissolved in 70:30 instead of 80:20 water/acetonitrile (0.1% formic acid)	107.2 (8.60)	98.2 (8.32)	92.4 (3.23)	93.3 (3.47)	92.2 (1.46)
Dissolved in 90:10 instead of 80:20 water/acetonitrile (0.1% formic acid)	102.4 (3.92)	108.1 (5.59)	94.6 (0.59)	98.5 (2.26)	93.9 (1.81)
Eluted with 0.5 mL instead of 1.0 mL	240.9 (120.4)	263.2 (171.2)	85.5 (69.1)	101.9 (5.40)	16.9 (87.1)

* Mean accuracy (%) is obtained by comparing the mean (n = 3) measured concentration under test conditions to the mean measured concentration of positive control samples prepared under normal conditions.

Table VII. Storage Stability*

Storage Conditions	Positive Control Concentration (pg/mL) 1	5	25
Fresh samples			
Mean conc. (pg/mL)	1.00	5.05	24.47
Difference (%)	0.00	0.00	0.00
CV (%)	3.76	1.21	1.76
0°C (14 days)			
Mean conc. (pg/mL)	0.97	4.91	24.8
Difference (%)	–2.67	–2.86	1.36
CV (%)	4.89	1.84	3.92
p value	0.426	0.044	0.720
–20°C (60 days)			
Mean conc. (pg/mL)	0.98	4.93	25.5
Difference (%)	–2.03	–2.34	4.03
CV (%)	7.27	1.71	5.04
p value	0.431	0.281	0.361
–80°C (60 days)			
Mean conc. (pg/mL)	1.04	4.87	24.7
Difference (%)	4.68	–3.61	0.85
CV (%)	3.43	0.89	1.94
p value	0.369	0.004	0.692
–80°C (170) days)			
Mean conc. (pg/mL)	1.08	4.88	23.7
Difference (%)	8.64	–3.35	–3.22
CV (%)	1.49	3.92	5.0
p value	0.108	0.364	0.472

Storage Conditions	Positive Control Concentration (pg/mL) 1	5	25
Extracts (24 h)			
Mean conc. (pg/mL)	0.98	4.89	23.3
Difference (%)	–1.60	–3.30	–4.64
CV (%)	5.76	8.38	4.46
p value	0.632	0.592	0.143
Extracts (48 h)			
Mean conc. (pg/mL)	0.93	4.65	23.1
Difference (%)	–6.21	–8.01	–5.66
CV (%)	5.49	3.24	9.57
p value	0.025	0.077	0.314
Extracts (72 h)			
Mean conc. (pg/mL)	0.90	4.04†	22.1
Difference (%)	**–10.0**	**–20.1**	–9.75
CV (%)	5.74	**15.8**	**11.6**
p value	0.169	n/a‡	0.257
Three freeze/thaw cycles (–80°C)			
Mean conc. (pg/mL)	0.98	4.98	24.08
Difference (%)	–1.46	–1.48	–1.58
CV (%)	7.81	2.29	2.50
p value	0.747	0.135	0.552

* 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 are out of specification.
† Value was determined with two replicates instead of three because of a failed injection.
‡ p value could not be generated with unequal number of replicates.

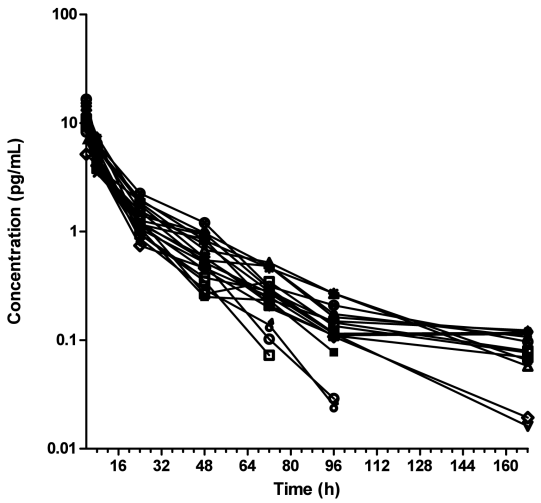

Figure 5. Plasma GLY concentration from 4 to 168 h in 20 horses.

trations above the limit of quantitation but not exceeding 0.15 pg/mL. Using the method described in the current study, the likelihood of quantitating GLY beyond 168 h is small. Therefore, additional timepoints are not likely to benefit the researcher. Moreover, the authors could find few circumstances where it is necessary to detect GLY beyond 7 days.

Glycopyrrolate plasma concentrations decreased precipitously within 24 h following intravenous administration in the horse. A more thorough investigation into GLY pharmacokinetics has been reported (11). We found that GLY is minimally detectable in plasma with the current method after a 10-mg oral dose. We are aware that GLY has low oral bioavailability due to its permanent ionization and are therefore not surprised by this finding.

Conclusions

In summary, a fully validated method suitable for the identification and quantification of GLY in horse plasma after intravenous administration of clinically relevant doses is reported. The method provides reliable quantification and adequate sensitivity for post-race sample and pharmacokinetic analysis. Further, we demonstrated that GLY is detectable in horse plasma for at least 168 h after intravenous administration of a clinically relevant dose. The results of this research could support the development of thresholds and withdrawal guidelines for regulating the use of GLY in the horseracing industry.

Acknowledgments

The authors wish to thank the Racing Medication and Testing Consortium, Inc. (Lexington, KY) and the Florida Division of Pari-Mutuel Wagering (Tallahassee, FL) for financial support for this work. We also thank the veterinary and research personnel at the University of Florida Equine Performance Laboratory for performing the treadmill exercise and sample collections.

References

1. A.G. Proakis and G.B. Harris. Comparative penetration of glycopyrrolate and atropine across the blood-brain and placental barriers in anesthetized dogs. *Anaesthesiol*. **48:** 339–344 (1978).
2. P. Rautakorpi, T. Manner, T. Ali-Melkkila, T. Kaila, K. Olkkola, and J. Kanto. Pharmacokinetics and oral bioavailability of glycopyrrolate in children. *Pharmacol. Toxicol.* **83:** 132–134 (1998).
3. M.L. Storme, R.S. t'Kindt, W. Goeteyn, K. Reyntjens, and J.F. Van Bocxlaer. Quantitative determination of glycopyrrolate in human plasma by liquid chromatography–electrospray ionization mass spectrometry: the use of a volatile ion-pairing agent during both liquid–liquid extraction and liquid chromatography. *J. Chromatogr. B* **876:** 24–30 (2008).
4. L.C. Matassa, D. Woodard, R.K. Leavitt, P. Firby, and P. Beaumier. Solid-phase extraction techniques for the determination of glycopyrrolate from equine urine by liquid chromatography–tandem mass spectrometry and gas chromatography–mass spectrometry. *J. Chromatogr.* **573:** 43–48 (1992).
5. World Health Organization. Guidelines on the use of international nonproprietary names for pharmaceutical substances. WHO/PHARMS/NOM 1570, World Health Organization, Geneva, Switzerland, 1997.
6. U.S. Department of Health and Human Services, Food and Drug Administration. Guidance for Industry. Bioanalytical Method Validation. May 2001.
7. European Medicine Agency (EMEA), Committee for Medicinal Products for Human Use (CHMP). Guideline on Validation of Bioanalytical Methods, May 2010 (draft).
8. B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Anal. Chem.* **75:** 3019–3030 (2003).
9. B. Desilva, W. Smith, R. Weiner, M. Kelly, J. Smolec, B. Lee, M. Khan, R. Tacey, H. Hill, and A. Celniker. Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* **20:** 1885–1900 (2003).
10. M.J. Rumpler, R.A. Sams, and P. Colahan. Regulatory control of glycopyrrolate in performance horses. *J. Chromatogr. B*, in press.
11. M.J. Rumpler, R.A. Sams, and P. Colahan. Pharmacokinetics of glycopyrrolate following intravenous administration in the horse. *J. Vet. Pharmacol. Ther.* Article first published online 2 MAR 2011. DOI: 10.1111/j.1365-2885.2011.01272.x (2011).