PHARMACOKINETICS AND PHARMACODYNAMICS OF GLYCOPYRROLATE IN THE HORSE

By

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To Tyler and Evelyn:
Whatever it is you wish to accomplish in life, may you pursue it with passion, attack it with perseverance, triumph with success and reminisce with pride.

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λ_z	elimination rate constant
A	intercept at time=0 for the first phase
ACh	acetylcholine
ACN	acetonitrile
AIC	Akaike Information Criteria
Alpha	slope for the first phase of the model equation
ANOVA	analysis of variance
AORC	Association of Official Racing Chemists
ARCI	Association of Racing Commissioners International
ARE	amount remaining to be excreted
AUC	area under the curve
AUMC	area under the moment curve
B	intercept at time=0 for the second phase
Beta	slope for the second phase of the model equation
BLQ	below the limit of quantitation
bpm	beats per minute
C	concentration
C_b	bound concentration
C_e	effect site drug concentration
C_f	free concentration
C_{last}	observed plasma drug concentration at the last timepoint
C_{max}	maximum plasma drug concentration
C_p	plasma drug concentration
$C_{p(last)}$	last measured plasma drug concentration

C_{pss}	plasma drug concentration at steady-state
C_t	total concentration
C_u	urine drug concentration
CBA	carboxy propyl phase
CBC	complete blood count
CID	collision induced dissociation
Cl	total clearance
Cl_P	plasma clearance
Cl_R	renal clearance
Cl_H	hepatic clearance
CRI	continuous rate infusion
CV	coefficient of variation
D	dose
DCM	dichloromethane
E	Effect
E_H	extraction ratio
E_0	baseline effect
E_{max}	maximum effect produced (efficacy)
ECG	electrocardiography
EC_{50}	concentration that produces half of the maximal effect (potency)
EE	extraction efficiency
ESI	electrospray ionization
FA	formic acid
FDA	Food and Drug Administration
fu	fraction unbound or free drug fraction

Gamma	slope for the third phase of the model equation
GC	gas chromatography
GLY	glycopyrrolate
h	hour
HESI	heated electrospray ionization
HR	heart rate
IS	internal standard
IV	intravenous
k_{12}	transfer-rate constant from the first to the second compartment
k_{21}	transfer-rate constant from the second to the first compartment
k_{10}	elimination-rate constant from the first compartment
k_{1e}	transfer-rate constant from the first to the effect compartment
k_a	absorption-rate constant
k_{e0}	elimination-rate constant from the effect compartment
LC	liquid chromatography
LLE	liquid-liquid extraction
LLOD	lower limit of detection
LLOQ	lower limit of quantitation
ME	matrix effect
MeOH	methanol
mg	milligrams
min	minutes
mL	milliliters
MRM	multiple reaction monitoring
MRT	mean residence time

MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
m/z	mass to charge ratio
n	sigmoidicity factor (hill coefficient)
NA	nonspecific adsorption
ng	nanograms
PAR	peak area ratio
PB	protein binding
PD	pharmacodynamics
PE	process efficiency
pg	picograms
PK	pharmacokinetics
R^2	coefficient of determination
R_o	rate of intravenous infusion
RP	reversed phase
RMTC	Racing Medication Testing Consortium
RR	respiratory rate
RSD	relative standard deviation
SBC	Schwarz Bayesian Criteria
SD	standard deviation
S/N	signal to noise ratio
SOP	standard operating procedure
SPE	solid phase extraction
t	time

$t_{1/2}$	half-life
$t_{1/2}ke0$	half-life for equilibration
t_{max}	time to reach peak plasma concentration
TI	tolerance interval
TIC	total ion chromatogram
TSQ	triple stage quadrupole
UF	ultrafiltration
µg	micrograms
ULOQ	upper limit of quantitation
$\dot{V}$	rate of urine formation
V_2	volume of distribution of the second compartment
V_3	volume of distribution of the third compartment
Vd	volume of distribution
Vd_{ss}	volume of distribution at steady state
Vc	volume of central compartment
Vz	apparent volume of distribution during the terminal phase

Abstract of Dissertation Presented to the Graduate School
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THE PHARMACOKINETICS AND PHARMCODYNAMICS OF GLYCOPYRROLATE IN THE HORSE

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Chair: Tom Vickroy
Major: Veterinary Medical Sciences

Glycopyrrolate (GLY) is a muscarinic receptor antagonist typically used in veterinary medicine to inhibit gastrointestinal motility and block the effects of vagal stimulation during anesthesia. It is reportedly used in horseracing to reduce bronchial secretions despite the fact that it is a prohibited substance. Regulating legitimate medications in horseracing relies on the development of industry threshold limits and recommended withholding times. Therefore pharmacokinetics studies are important for determining drug plasma and urine concentrations following a typical often clinically relevant dosage.

The following research describes a rapid, sensitive, selective and fully validated liquid-chromatography mass spectrometry analytical method for the detection and quantification of GLY in horse plasma and urine. The Pharmacokinetics of GLY have been evaluated after a 1 mg intravenous dose in Thoroughbreds and Standardbreds. Additionally, the pharmacodynamics have been evaluated following a clinically relevant intravenous infusion (8 µg/kg) in Thoroughbreds. Further, we investigated the elimination of GLY in urine after both intravenous and oral administration of clinically relevant doses to Thoroughbred horses. Pharmacokinetic parameters were best estimated using a three-compartment model for plasma concentration versus time data from 0-24 h, although GLY was detectable for 168 h after administration. GLY

disposition in the horse was characterized by a steep decline in plasma concentration immediately after dosing, extensive distribution from the central compartment, rapid clearance, and a prolonged terminal elimination phase. GLY remained detectable in urine samples collected through 168 h after intravenous administration and through 24 h after oral administration.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Overview and History

Glycopyrrolate (GLY) is a synthetic anticholinergic compound with a chemical name 3[(cyclopentylhydroxyphenylacetyl)oxy]-1, 1-dimethyl pyrrolidinium bromide that was derived from several naturally occurring alkaloids of the "belladonna" (*Solanaceae*) plants. It was synthesized in the 1950s in an effort to develop safer anticholinergic compounds that could not easily penetrate the central nervous system (CNS) where they may impart adverse effects (Franko & Lunsford, 1960). The compound's reduced permeability was accomplished with the addition of a quaternary ammonium substituent group that is permanently ionized at physiological pH values.

Anticholinergic agents competitively inhibit the actions of acetylcholine (ACh) in tissues innervated by postganglionic cholinergic nerve terminals and on smooth muscles that respond to ACh but lack cholinergic innervation (Grum & Osborne, 1991). The prototypical drug in this class is atropine, which, along with its analogues, competes predominately at the muscarinic receptor with little action at the nicotinic receptor (Doods *et al*., 1987). Thus, these compounds are also known as muscarinic antagonists or antimuscarinic drugs. Cholinergic antagonists all contain similar structural elements such as a cationic head group with a tertiary or quaternary ammonium group; alicyclic or aromatic rings, for hydrophobic interactions with the receptor; an interconnecting structural moiety such as an amide or ester; and one or more hydroxyl groups (Wess *et al*., 1990).

The two most frequently used anticholinergics drugs are atropine and scopolamine, both isolated from *Atropa belladonna* and having long medicinal histories. They are organic esters formed by the combination of an aromatic acid, tropic acid, and a complex organic base, either

tropine as in the case of atropine (Figure 1-1) or scopine in the case of scopolamine (Figure 1-2). Scopine differs from tropine only in having an epoxyl group in the tropine structure. The intact ester of tropine and tropic acid is essential for the antimuscarinic action of atropine, since neither the free acid nor the base exhibit significant anticholinergic activity. The presence of a free OH group in the acid portion of the ester also is important for activity. Substitution of other aromatic acids for tropic acid modifies but does not necessarily abolish the anticholinergic activity.

Both atropine and scopolamine contain a tertiary ammonium group (pKa 7.5-10.2) which allows the drug to permeate biological membranes causing unwanted side effects as a result of interaction with central nervous system receptors when the drugs were used to treat peripheral conditions. The introduction of GLY into human medicine provided an antimuscarinic agent that was specifically designed to penetrate barriers less effectively and to act in the periphery without causing side effects that could outweigh the potential benefits. To date, GLY has been used as an alternative to these antimuscarinic agents in the treatment of several conditions of the cardiovascular, respiratory, glandular and gastrointestinal systems.

Basic Pharmacology of Glycopyrrolate

Glycopyrronium occurs as a white, odorless crystalline powder and is known as GLY when it is accompanied by a bromide counter ion in order to maintain electric neutrality. GLY consists of two components (mandelic acid and an organic base) bound together by an ester linkage. The quaternary ammonium substituent was formed with the addition of a second methyl group on the tertiary nitrogen atom (Franko & Lunsford, 1960). The compound is water soluble due to the quaternary amine structure (Figure 1-3). The partition coefficient of GLY in an n-octanol/water system is 0.304 (log10 P=-1.52) at room temperature (24°C).

Muscarinic receptor antagonists block the effects of ACh on muscarinic cholinergic receptors in the peripheral ganglia as well as in the CNS. GLY differs from other muscarinic antagonists because it has difficulty permeating biological membranes due its highly polar quaternary ammonium group and its permanent ionization at physiological pH (Franko, 1962). In contrast to its naturally occurring analogues, it is poorly absorbed orally (Rautakorpi *et al*., 1998), does not cross the placental barrier (Proakis & Harris, 1979) and does not interfere with central nervous system (CNS) cholinergic function (Ali-Melkkila *et al*., 1990). Consequently, without such membrane permeating potential, GLY produces markedly reduced CNS effects compared to its more lipophilic congeners, atropine and scopolamine (Shereff, 1985).

Peripheral cholinergic receptors are predominately present in the autonomic effector cells of smooth muscle, cardiac muscle, the sinoatrial node, the atrioventricular node, exocrine glands, and, to a lesser extent, in the autonomic ganglia (Brown & Taylor, 2006). However, due to the nonselective blockade of muscarinic receptors in other organ systems, GLY also inhibits a number of parasympathetically mediated functions, causing decreased airway and gastrointestinal secretions, bronchodilation, and inhibition of gastrointestinal motility (Barocelli *et al*., 1993). GLY competitively antagonizes the effect of ACh on the muscarinic receptors by occupying post-synaptic receptor sites (Lau & Szilagyi, 1992). It has a high affinity for the muscarinic receptor family (with little effect at nicotinic sites) with little selectivity for any of the receptor subtypes (Haddad, *et al*., 1999). At present there are five well defined subtypes of muscarinic cholinergic receptors in humans, denoted M1 to M5, each encoded by a unique gene and differing in their amino acid sequence (Bonner *et al*., 1987).

Until the early 1980s, all muscarinic receptors were thought to be alike given the lack of muscarinic agonists or antagonists presenting specific effects (Goyal, 1989). Since then, different

muscarinic receptor subtypes have been shown to be responsible for mediating the effects of ACh on heart rate, smooth muscle contractile activity, central nervous system activity and exocrine gland function. M1 and M4 receptors are predominately located in the brain and are involved with behavioral and cognitive functions (Hammer & Giachetti, 1982). M2 receptors are the only subtype located in the cardiac smooth muscle (Caulfield, 1993) and the M3 subtype exists in the glandular tissue as well as in the smooth muscle of the airways (Doods *et al*., 1987), gastrointestinal tract (Eglen R., 2001) and urinary bladder (Hedge, 2006). Little research has been conducted on M5 receptors but they are also believed to be physiologically relevant in the CNS (Yamada *et al*., 2001).

Functional studies in humans suggest that parasympathetically mediated intestinal smooth muscle contraction occurs via M3 receptor stimulation (Eglen *et al*., 1992), whereas M2 receptor stimulation may be involved in abrogating sympathetically mediated smooth muscle relaxation (Elgen *et al*., 1996). Radioligand binding studies have shown the presence of receptor subtypes M2 and M3 in intestinal smooth muscle of guinea pigs and dogs (Giraldo *et al*., 1987; Zhang *et al*., 1991). In the horse species, the M3 receptors are responsible for mediating ACh induced tracheal (van Nieuwastadt *et al*., 1997) and jejunal (Teixeira-Neto *et al*., 2011) smooth muscle contraction. Even though relaxation of the airway of the horse by M3 receptor antagonists may have potential therapeutic implications in horses presented with heaves, M3 receptor antagonism may also have a major inhibitory effect on bethanechol-induced smooth muscle contraction in the horse (Marti *et al*., 2005).

Although GLY, atropine and scopolamine are non-specific antagonists at these receptors, atropine has a two-fold preference for M1 (CNS) receptors (Gomez *et al*., 1995). M3 receptors (glandular secretion, blockade producing a reduction in salivation) are much more sensitive to

anticholinergics than M2 receptors (cardiac, blockade causing an increase in heart rate). Thus, for all three drugs, a greater dose is needed to prevent bradycardia than to reduce salivation (Jongerius *et al*., 2003).

Therapeutic and Clinical Applications of Glycopyrrolate

GLY and other muscarinic cholinergic antagonists, such as atropine have been used clinically for several decades for a variety of conditions as a result of their receptor non-selectivity. GLY is administered almost exclusively by the parenteral route, due to its poor absorption from the gastrointestinal tract. GLY was introduced in 1961 as a long acting anticholinergic with initial reports confined to gastroenterology to reduce the volume and free acidity of gastric secretions (Sun, 1962; Moeller, 1962), until 1970 when Boatright *et al*., reported on its preoperative uses to decrease complication of gastric juice aspiration during tonsillectomy (Boatright *et al*., 1970).

GLY protects against the peripheral muscarinic effects (e.g. bradycardia and excessive secretions) of cholinergic agents such as neostigmine and pyridostigmine which are given to reverse the neuromuscular blockade due to non-depolarizing muscle relaxants (Gyermek, 1975; Ramamurthy, 1972). Atropine is often used in emergency situations but the lack of CNS side effects and better matched onset and offset time with neostigmine makes GLY the agent of choice in reversal of neuromuscular blockade, especially in neonates and the elderly (Salem & Ahearn, 1986). Typical doses for adults are 0.2 mg intravenously per 1 mg of neostigmine or the equivalent dose of pyridostigmine and a dose of 0.01-0.015 mg intravenously with 0.05 mg/kg neostigmine or equivalent dose of pyridostigmine.

In anesthesia GLY is indicated for use as a preoperative antimuscarinic agent to reduce salivary, tracheobronchial, and pharyngeal secretions, to reduce the volume and free acidity of

gastric secretions, and to block cardiac vagal inhibitory reflexes during induction of anesthesia and intubation. GLY injectable may be used intra-operatively to counteract drug-induced or vagal traction reflexes with the associated arrhythmias. In small animals GLY has been used with success to treat vagally mediated bradycardia during anesthesia without causing major complications in other organ systems (Dyson *et al*., 1999). In horses, however, GLY has been reported to cause prolonged intestinal hypomotility and colic when used to prevent intraoperative bradycardia (Singh *et al*., 1997; Teixeira-Neto *et al*., 2004). Therefore clinicians have taken a conservative approach when using GLY during intra-operative procedures. Typical does for adult humans are 0.2 mg to 0.4 mg intravenously or intramuscularly before the induction of anesthesia. Alternatively, a dose of 4-5 µg/kg of body weight up to a maximum of 0.4 mg may be used. In dogs and horses typical pre-anesthetic doses range from 5-10 µg/mg of body weight.

GLY's antisialagogue properties have been known for several decades (Wyant & Kao, 1974; Mirakhur *et al*., 1978). More recently, GLY has been investigated to treat sialorrhea (excessive drooling) in adolescents with neurological disorders (Tscheng, 2002) and adults with Parkinson disease (Arbouw *et al*., 2010). Despite some reports detailing only a modest improvement (Blasco & Stansbury, 1996) and adverse effects (Madan & Beck, 2006), the medication is well tolerated (Stern, 1997) and a liquid oral formulation (Cuvposa®) has recently been FDA approved for this purpose based on encouraging clinical trial results (Zeller *et al*., 2012; Zeller *et al*., 2012). Similarly, GLY has been used topically for the treatment of hyperhidrosis (excessive sweating) (Seukeran & Highet, 1998; Luh & Blackwell, 2002; Shaw *et al*., 1997; Atkin & Brown, 1996; Bajaj & Langtry, 2007), an embarrassing condition that effects a small population.

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation caused by persistent inflammatory processes in the airways. An increased cholinergic tone mediates different pathophysiological features of COPD, such as bronchoconstriction and mucus hypersecretion, mostly through activation of the human muscarinic (M3) receptor subtype (Brown & Taylor, 2006). Clinical studies have shown that inhaled GLY displays bronchodilator activity in COPD and asthmatic patients, an effect apparently lasting for 8-12 h (Walker *et al*., 1987) (Tzelepis *et al*., 1996) (Schroeckenstein *et al*., 1988). Additionally, nebulized GLY has been found to have a synergistic effect when combined with albuterol in treating acute exacerbations of asthma (Gilman *et al*., 1990) (Cydulka & Emerman, 1994) and COPD (Cydulka & Emerman, 1995) It has also been demonstrated to be a more effective bronchodilator over a longer duration when compared to atropine, for the treatment of exercise induced asthma (Johnson *et al*., 1984).

In normal human subjects, GLY has been shown to cause prolonged bronchodilation after intravenous (10 µg/kg) (Gal & Suratt, 1981) and nebulized (Gal *et al*., 1984) (Alex *et al*., 1999) administration without the systemic anticholinergic cardiac effects of the shorter acting atropine. It was shown that GLY inhibited electrical field stimulation-induced contractions of human and guinea pig isolated airways with a longer duration of action than ipratropium and that both agents exhibited no selectivity toward M1, M2, and M3 receptors (Giraldo *et al*., 1987). Other human studies have demonstrated that intratracheally administered GLY provides superior bronchoprotection and slower receptor dissociation over ipratropium bromide in patients with asthma (Hansel *et al*., 2005) and COPD (Ogoda *et al*., 2011). There is also optimism for products under development following clinical trials for an inhaled therapeutic of GLY alone (Sechaud *et*

al., 2012) and a combination of GLY with a long acting beta-2-agonist (formoterol) to treat COPD.

GLY has not been reported to be used routinely to treat respiratory conditions in horses. Atropine was used to a limited extent before the development of more selective drugs, such as clenbuterol with reduced side effects (Murphy *et al*., 1980). Instead most research in horses is devoted to β2-selective agonists because of their potent and prolonged duration of action with limited adverse effects (Adams, 2001). GLY is reportedly used to reduce bronchial secretions in race horses, an effect that has benefited human patients in surgery (Sengupta *et al*., 1980) and in trauma with life-threatening conditions (Bhalla *et al*., 2011).

GLY is also preferred in obstetrics due to its lack of propensity to reach neonates (Abboud *et al*., 1981). GLY has been investigated for its effect on hypotension during anesthesia conducted for caesarean section, but has been met with conflicting results. Previous studies found that GLY reduced (Ure *et al*., 1999), increased (Quiney & Murphy, 1995) or had no effect (Yentis *et al*., 2000) (Rucklidge *et al*., 2002) on maternal hypotension after spinal anesthesia for caesarean section. Studies evaluating GLY as a prophylactic antiemetic for spinal anesthesia have shown that the drug is of little benefit for this purpose (Quiney & Murphy, 1995) (Thakur *et al*., 2011).

Analytical Methodology

Early investigations into human and horse GLY disposition used a radioreceptor assay (Kaila *et al*., 1990) or an enzyme-linked immunoabsorbent assay (Leavitt *et al*., 1991). Pharmacokinetic studies could not be performed earlier due to the availability of methods that were too complex (Kaltiala *et al*., 1974) or demonstrated inadequate sensitivity (Murray *et al*., 1984). By their nature, radioreceptor and immunoassays are rapid, highly sensitive, reliable,

precise, accurate and simple to perform. Their primary disadvantage relates to specificity, since any substance having an appreciable affinity to the receptor site will displace the specifically bound radioligand. The use of such techniques has been eclipsed by hyphenated separation and mass spectrometric methods due to their ability to identify and quantify a substance.

For many years gas chromatography (GC) has cemented its role as an analytical powerhouse in analytical chemistry/toxicology and is likely the most widely used tool in analytical laboratories. However, analysis of heat labile and polar compounds, long analysis time and tedious sample preparation procedures have left room for much innovation. Gas chromatography has been successfully used for GLY detection in horse urine (Matassa *et al*., 1992). The method employs a selective solid phase extraction and can be sensitive down to 250 pg/mL. However, a large sample volume (50 mL) and an intensive derivatization procedure put a strain on repeat/duplicate analysis and the analyst.

Liquid chromatography (LC) is quickly winning favor among investigators as an alternative to GC methods. This is evident by the number of published method validation studies performed using LC-MS compared to GC-MS. In recent years researchers have found that LC/MS can impart a significant impact on a laboratory's capabilities (Alder *et al*., 2006). Although the field of drug discovery was among the first to recognize the instrument's capabilities (Lee & Kerns, 1999), analytical drug detection laboratories have been rapidly validating methods over the past several years to incorporate this technology into routine screening and confirmation procedures (Maurer, 2005; Maurer H., 2005). Limitations for LC-MS include ion suppression (Cappiello *et al*., 2008) and cost. In the most recent work Storme *et al*. (2008) used liquid chromatography-tandem mass spectrometry to quantify GLY, using a method that employed volatile ion pairing reagents to extract GLY from human plasma (Storme *et al*.,

2008). In this method time-of-flight (TOF) mass spectrometry was used. A structurally similar compound, mepenzolate, was used as the internal standard. While sensitivity was substantially improved (100 pg/mL) compared to previous methods, there appeared to be unwanted matrix effects and recovery remained unacceptable for the objectives proposed in this research.

Mass spectrometry has become the analytical tool of choice for the confirmation of small molecules because it allows the unequivocal identification and quantification of substances at very low concentrations in complex mixtures such as plasma and urine. Tandem mass spectrometry (MS^n), which incorporates consecutive stages of MS analysis can derive structural information (Chen *et al*., 2007) and further improve compound selectivity by mapping fragmentation pathways (Watson & Sparkman, 2007).

Glycopyrrolate Doping in Race Horses

The detection and confirmation of identity of drugs and metabolites in biological specimens of racing animals is a challenging analytical undertaking. Laboratories are tasked with the goal to detect and quantify substances that may affect performance. This includes compounds that may enhance the physical capabilities and or provide legitimate therapeutic value. For substances that provide no therapeutic benefits, their presence alone may result in regulatory sanctions.

GLY is designated a class 3 substance by the Association of Racing Commissioners International (ARCI), Inc. (Association of Racing Commissioners International, 2011), and regulated in racing horses due to its potential to affect performance and degree of therapeutic value. Although it has several veterinary clinical applications by inhibiting parasympathetic activity, its use near race day is prohibited and positive reports from post-horserace samples in the US are relatively common (reference RMTC). Accordingly, the American Association of

Equine Practitioners (AAEP) has identified GLY as a therapeutic substance used by race track practitioners for legitimate therapeutic purposes. Consequently, the Racing Medication and Testing Consortium (RMTC) has provided financial support for studies of the disposition of GLY as part of its efforts to acquire reliable data upon which to establish thresholds and withdrawal time recommendations for therapeutic substances used in racing horses.

A threshold limit is a concentration, either in plasma, serum or urine that, if exceeded, will result in a positive finding. Whereas threshold limits cannot easily be interpreted by non-scientists, a drug withdrawal time is also recommended by regulatory bodies. A withdrawal time is an estimated time period in which veterinarians or horseman should withhold a medication before the start of a race. While threshold limits and withdrawal times may be determined from properly designed and sophisticated pharmacokinetic studies, it is important to determine the duration of pharmacological actions relative to plasma drug concentrations for compounds that may alter performance.

According to the ARCI guidelines a class 3 substance has potential medicinal value with the possibility to affect performance (Short *et al*., 1998). 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 as described above. The pharmacokinetics and pharmacodynamics of GLY and other anticholinergics substances have been studied thoroughly in humans. Several accounts of the effects of GLY have been studied in horses (Dyson *et al*., 1999; Singh *et al*., 1997; Teixeira Neto *et al*., 2004) but there are no reports that have combined pharmacokinetic and pharmacodynamic analysis and have performed predicative modeling. Such studies may benefit the development of regulatory guidelines and clinical research.

Hypothesis

A sensitive and selective quantitative analytical method can be developed to enable accurate determination of GLY in plasma and urine in the horse after clinically relevant doses. Further the method will allow meaningful pharmacokinetic analysis and the development of an integrated pharmacokinetic-pharmacodynamic (PK-PD) model to describe the complete time course of common clinical effects. To test the hypothesis of the study, the following specific aims were purposed:

Specific Aim 1: To develop and fully validate a quantitative liquid chromatography tandem mass spectrometric method for the confirmation of GLY in horse plasma and urine.

Specific Aim 2: To investigate and characterize the pharmacokinetic profile after intravenous and oral administration to horses.

Specific Aim 3: To investigate the pharmacodynamic effects after a continuous rate intravenous infusion in horses.

Specific Aim 4: To apply a mathematical model to correlate GLY plasma concentrations with the pharmacological response.

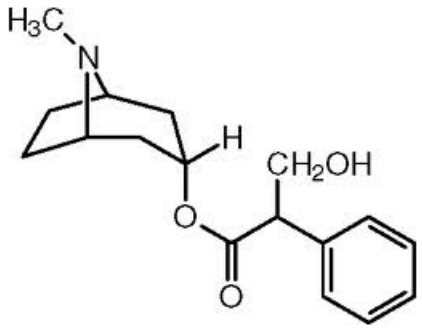

Figure 1-1. Chemical structure of atropine.

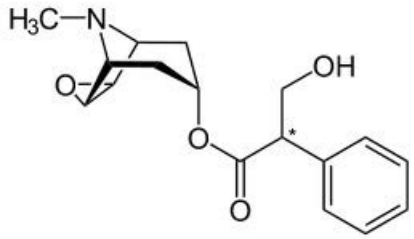

Figure 1-2. Chemical structure of scopolamine.

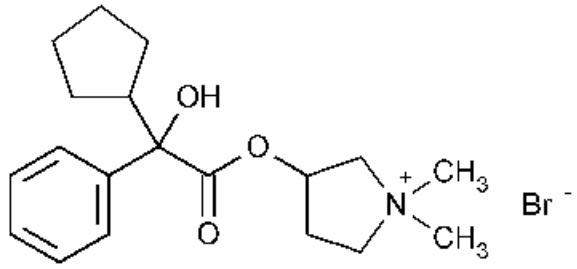

Figure 1-3 Chemical structure of GLY.

CHAPTER 2
ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

The physiochemical properties of glycopyrrolate (GLY) present certain analytical challenges. Its quaternary ammonium structure can increase the difficulty of isolating and extracting it from biological fluids. Recovery of the compound could only be done with a selective sorbent and optimized pH conditions, solvent wash steps and the selective elution of analytes. While the linearity of previous methods was sufficient for routine detection, a larger dynamic range would likely be necessary for the proposed analysis of samples collected after intravenous and oral administration of GLY to the horse. Therefore, we sought a stable isotope labeled analogue for use as an internal standard to appropriately track the analyte and possibly reduce matrix effects. Finally, the intention for this work was to produce reliable and meaningful pharmacokinetic analysis.

Selection of an appropriate internal standard (IS) is important to the development of a quantitative assay as in the case of LC-MS/MS (Avery, 2003). During method development, the selection of the IS should be such that it will adequately track the analyte throughout sample analysis. An IS is added to samples to compensate for unavoidable sample losses (e.g., extraction transfer losses, ionization effects and injection issues) and to account for the presence of competing substances in the extracts and to minimize their influence. Thus, a reliable IS should be structurally similar to the analyte of interest. Typically the best alternative, if available, is a stable-isotopically labeled analogue of the analyte (Jian *et al*., 2010; Stokvis *et al*., 2005).

GLY may be chromatographed on a wide range of analytical LC columns. Ideally, the best match would offer superior polar retention, aqueous and acidified mobile phase compatibility, rapid analysis and the ability to sustain high pressures, although the latter in not a necessity. Column selection is based upon the likelihood of accommodating these features. Use of a guard

column is necessary to prevent unwanted matrix components from entering the analytical column, thus extending its life. Ultra-performance liquid chromatography (UPLC), a recent advancement (Sherma, 2005) is appropriate for this method because the columns are packed with smaller diameter particles requiring a shorter column length and facilitating faster run times with comparable resolution to traditional LC columns (Fan *et al*., 2007) (Wren & Tchelitcheff, 2006).

Analytical method validation is required across several disciplines and is essential in order to acquire reliable data for analyst interpretation (Stöckl *et al*., 2009). Without such guidelines for analytical integrity, assay determinations are capricious and could impose speculative and/or unsupported conclusions. Analysts, laboratories and even organizations may become accountable for misleading, inaccurate or erroneous information. From a medico-legal perspective such misinformation may result in unsubstantiated legal ramifications. Several governing agencies have published requirements and recommendations for validation studies (CITAC/EURACHEM, 2002; Guidance for Industry, Bioanalytical Method Validation, 2001; Guideline on Validation of Analytical Methods, 2009; General Requirements for the Competence of Testing and Calibration Laboratories). Consequently, the validation of bioanalytical methods has become a rate-limiting step for the reliable analysis of research samples.

Methods

Chemicals and Reagents

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 were obtained from Thermo Fisher (Pittsburg, PA, USA).

Standard Preparation

GLY ((United States Adopted Name (USAN)) is also known as glycopyrronium bromide (Recommended International Nonproprietary Name) (Organization, 1997), (Council). GLY 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 solutions used to prepare calibrators and controls were prepared from certified reference standards. Drug standards including GLY and GLY iodide-d_3 were obtained from United States Pharmacopeial Convention (Rockville, MD, USA) and Toronto Research Chemicals (North York, Ontario, Canada), respectively.

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 and in plasma from 1-2500 pg/mL and 0.05–25 pg/mL, respectively. Working standard solutions used to prepare calibrators and positive control samples were prepared from independently prepared stock solutions.

For plasma analysis each calibrator and positive control sample was prepared using 1 mL of phosphate buffer (50 mM, pH 7.0) and 1.0 mL of drug-free control horse plasma, and fortified with the appropriate volume of GLY working standard solution (Table 2-1) and 25 µL of GLY-d_3 standard solution. The deuterated GLY analog was prepared in a working standard solution at a concentration of 0.004 ng/µL. The final internal standard concentration was 100 pg/mL of plasma.

For urine analysis each calibrator and positive control sample was prepared using 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 (Table 2-2) and 25 µL of GLY-d_3 standard solution. The deuterated GLY analog was prepared in a working standard solution at a concentration of 0.004 ng/µL. The final internal standard concentration was 200 pg/mL of urine.

Solid Phase Extraction

The tubes were centrifuged at 1508 x *g* (2800 rpm) for 12 min and the buffered plasma and 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). Samples were loaded onto the columns and a positive pressure sufficient to achieve a flow rate of no more than 2 mL per minute 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, USA). Sample extracts were then dissolved in 100 µL of 0.1% formic acid in acetonitrile:water (10:90) and transferred to glass autosampler vials.

Liquid Chromatography

Chromatographic separations were achieved with an Acquity™ UPLC HSS T3 (5 mm x 2 mm x 1.8 µm) column and a 2.1 mm x 5 mm, identically packed, guard column (Waters, Taunton, MA, USA). 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 (Table 2-3). The flow into the mass spectrometer was diverted

from 0 - 0.75 min and 2.5 - 3.5 min. The column temperature was 35°C and 20 µL of the sample extract dissolved in 100 µL of acetonitrile:water (10:90) containing 0.1% formic acid was injected.

All LC-MS/MS analyses were performed on a Triple Stage Quadrupole (TSQ) Quantum Ultra mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with a heated electrospray ionization (HESI) source and interfaced with a HTC PAL autosampler (Leap Technologies, Carrboro, NC, USA) and Accela LC pump (ThermoFisher). Xcaliber™ software version 2.0.7 and LCquan™ 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.

Mass spectral data were acquired in positive ion mode using a heated electrospray ionization technique with the following MS parameters: ESI spray voltage; 4100, vaporizer temperature; 240 °C, sheath gas pressure; 40 (arbitrary units), ion sweep gas; 0 (arbitrary units), auxiliary gas pressure; 6 (arbitrary units), capillary temperature; 300 °C, tube lens offset; 89 V and skimmer offset; 0 V.

Data Analysis

Identification and quantification of the analyte were based on selected reaction monitoring (SRM). Compound specific optimization (tuning) of MS/MS parameters was performed before analyses via direct infusion of 10 ng/µL each of the analyte and internal standard dissolved in mobile phase. Tuning for GLY yielded collision energies of 39, 50, and 33 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 (i.e., m/z 318→116) for the analyte was used for quantification. The second and third most abundant transitions were used as qualifier transitions. Figure 2-3 represents the ion spectrum for GLY.

All standard solutions, controls, calibrators, and test samples were prepared in duplicate and peak ion area ratios of the analyte and internal standard were calculated for each. Individual values of the duplicate concentrations were averaged. Calibration was performed using a simple least squares linear regression analysis with a 1/C weighting factor, where C was the nominal plasma or urine concentration. Quality control and sample acceptance criteria have been specified according to the following requirements and standard operating procedures of the UF Racing Laboratory, Research Section. The requirement is that the %CV for all calibrators, positive controls, and test 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 these criteria were re-analyzed.

Method Validation

The method was validated in accordance with the U.S Food and Drug Administration recommended guidelines (Guidance for Industry, Bioanalytical Method Validation, 2001) 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 (EURACHEM, 1998). Also, I have consulted additional reference materials such as those referenced above for guidance with method validation procedures and specifications.

System Suitability

System suitability is a valuable component of any analytical procedure and ensures the performance of the analytical system(s) (Briscoe *et al*., 2007). Each validation and test sample batch contained ten calibrators prepared in plasma or urine, three non-fortified (analyte) control samples, and five analyte and internal standard fortified positive control samples spanning the range of the calibration line, all prepared in duplicate. The three non-fortified analyte control samples consisted of a reagent blank (diluent only), matrix blank (matrix + diluent), and negative control (internal standard + matrix + diluent). Run acceptability was determined by the accuracy, precision and minimum acceptance criteria (as described below) of the calibration standards and positive control samples, the coefficient of determination of the standard curve, and the absence or degree of GLY present in the negative control samples. If carryover or contamination existed in the negative control samples, attempts were made to minimize this effect before analyzing the calibration curve.

All calibration points included in the curve calculation must adhere to the following minimum acceptance criteria:

1. Each duplicate measurement from each calibration point to be included in the calibration curve must generate valid data (see conditions below).
2. For the average of the replicate determinations the absolute difference between the back-calculated and nominal (intended) concentration must be <15% for the LOQ standard and <10% for all other standards.
3. The coefficient of variation between duplicate measurements of the same standard must be <10%.
4. For the entire batch to qualify, a minimum of 75% of the calibrators or at least six points must be included and within the aforementioned limits. Additional measurements falling outside of these limits may be excluded from calculation provided this does not adversely affect the model.

If one of the calibrators does not meet any of the criteria outline in points 1-3, the calibrator should be rejected and the calibration curve without the failed calibration standard should be re-evaluated, and regression analysis performed. Removal of additional calibrators can be performed under the condition that the calibration curve adheres to point 4. If the criteria in point 4 are not met, the analytical run should be rejected.

The following conditions outline minimum acceptance criteria for positive control samples:

1. For the average of the replicate determinations the absolute difference between the back-calculated and nominal (intended) concentration must be <10% for all positive control standards.

2. The coefficient of variance between duplicate measurements of the same standard must be <10%.

3. The concentrations of the positive control samples must be spaced across the calibration curve and span the range of the unknown samples as per FDA Guidance (Guidance for Industry, Bioanalytical Method Validation, 2001).

4. A minimum of 3 out of 5 (60%) of positive control samples must meet the requirements in points 1-3 above. Failure to meet the criteria should result in the rejection of the analytical run.

The following criteria describe conditions under which calibrators or positive controls may generate no valid data:

1. Improper sample collection or preparation procedures.

2. A power failure occurs during injection, analysis, or data acquisition for the calibrators or positive controls in question.

3. A mechanical injection failure prevents the necessary volume of sample from being introduced into the system.

4. A lack or loss of sample leads to an incomplete injection of a fraction of the required sample volume.

Sample Acceptance Criteria

During sample analysis all test samples were bracketed by positive control samples to ensure accuracy throughout the run. Test samples were always prepared in duplicate. Reanalysis was necessary if samples did not meet the acceptance criteria outlined below. Samples that did not generate valid data due to a mechanical failure or power outage were re-tested. Data that appeared anomalous in the context of other measurements or variable compared to duplicate measurements may be accepted provided it meets all criteria outlined in this document.

Acceptance criteria guidelines:

1. The samples must be prepared with an established validated protocol. In the case of method development, the sample preparation, including concentrations used, necessary dilutions and extraction technique, must be thoroughly documented in the researcher's laboratory notebook.
2. The sample(s) must be preceded with a successful and appropriate standard curve and a set of negative and positive controls.
3. Each sample duplicate must generate valid data (see below for conditions).
4. The coefficient of variation between duplicate measurements of the same sample must be <10%.

Duplicate measurements must occur consecutively with or without a blank in between.

The following conditions describe when no valid data could be generated:

1. The sample mixture was not fortified with the proper concentration of appropriate internal standard during the sample preparation procedure.
2. The instrument experiences a mechanical failure or power outage during sample injection or data acquisition.
3. A lack or loss of sample leads to an incomplete injection of a fraction of the required sample volume.

Linearity and Range

The linearity defines the ability of the method to obtain test results proportional to the concentration of the analyte (Green, 1996). Linearity was assessed using a simple least squares

regression with a $1/C_p$ or $1/C_u$ weighting factor to compensate for heteroscedasticity (Almeida *et al*., 2002), where C_p and C_u were the nominal concentration of GLY in plasma and urine calibrators, respectively. Evidence of linearity was provided when calibrator quantification was within 15% and 10% of the nominal concentration at the lower limit of quantification (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).

Sensitivity

Sensitivity was assessed by determining the limit of detection (LOD) and lower limit of quantification (LLOQ) for the analyte using the proposed method. The limit of detection 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. The lower limit of quantification 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 below. Both LOD and LLOQ were determined with decreasing analyte concentrations in fortified plasma and urine. The upper limit of quantification (ULOQ) was defined as the concentration of the highest calibration point.

Accuracy and Precision

Accuracy is defined as the degree of closeness of the measured concentration to the true concentration. Precision is the degree of scatter for repeated measured concentrations from one homogenous sample (Bansal *et al*, 2007). Accuracy and precision were investigated at five positive control concentrations for plasma (0.125, 1.25, 5, 12.5, and 22.5 pg/mL) and urine (5, 125, 500, 750 and 1250 pg/mL). Intra- and inter-batch accuracy and precision were assessed in

each matrix 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 (Desilva *et al*., 2003). Precision estimates were required to be within ±20% for the lowest positive control concentration and ±15% for all other positive control concentrations. 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% ±20% of the nominal concentration.

Carryover

Carryover, a common issue encountered with LC-MS/MS, is caused by residual analyte from a sample analyzed earlier in the run sequence (Hughes *et al*., 2007). Eliminating or minimizing carryover confirms that a high-concentration sample will not contribute to the quantification of the next sample (Clouser-Roche *et al*., 2008). Carryover was evaluated by observing the ion intensities of the characteristic ions of GLY in a negative plasma and urine sample analyzed immediately after each of the four highest calibrators. Concentrations in the negative plasma and urine samples were calculated and carryover was determined to occur if the analyte concentrations exceeded the limit of detection.

Matrix Effect, Extraction Efficiency, Process Efficiency

Matrix effect, extraction efficiency (recovery), and process efficiency were evaluated using the three set method outlined by Matuszewski *et al*. (Matuszewski *et al*., 2003). The first set (A) consisted of analyte and internal standard solutions prepared “neat” into a starting mobile phase solution. Set 2 (B) was negative control plasma or urine extracts that were fortified with analyte and internal standard solutions following solid phase extraction. The third set (C) was negative

control plasma or urine fortified with analyte and internal standard solutions before solid phase extraction. Absolute matrix effect, extraction efficiency, and process efficiency, all expressed as a percentage, were calculated using the following equations:

$$\text{Matrix Effect (\%)} = (B/A) \times 100 \quad (2\text{-}1)$$

$$\text{Extraction Efficiency (\%)} = (C/B) \times 100 \quad (2\text{-}2)$$

$$\text{Process Efficiency (\%)} = (C/A) \times 100 \quad (2\text{-}3)$$

where A, B and C are the mean absolute peak areas obtained with a neat preparation, with plasma or urine extracts fortified with analyte and internal standard solutions following extraction, and with plasma or urine fortified with analyte and internal standard solutions before solid phase extraction, respectively. The process efficiency incorporates matrix effect and provides a more accurate estimation of the analyte recovery than does extraction efficiency. In addition, to evaluate the influence of different sources of matrices on analyte quantification, five different lots of negative control plasma or urine were compared (Matuszewski B. , 2006).

Dilution Integrity

It was presumed that concentrations of GLY in plasma and in urine samples collected immediately after drug administration would exceed the upper limit of the calibration range, for the respective methods, used for validation. Proving that dilution does not affect quantification is essential for obtaining accurate results for high-concentration specimens Hence, sample dilutions were likely required for both matrices and an appropriate validation procedure is recommended (Bansal *et al*., 2007). Dilution integrity was assessed by supplementing negative control 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. 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.

Dilution integrity for urine samples was assessed by supplementing negative control urine with GLY at four concentrations (0.05, 2.5, 10.0 ng/mL) and diluting the samples over the range of dilution factors used for the study samples. Dilution factors used and evaluated were 1:5, 1:250, and 1:1000. Dilutional integrity was considered acceptable if replicate (n=5) values were within ±20% of 10 pg/mL. All dilutions, in both plasma and urine, were prepared using ultra-pure (resistivity greater than or equal to 18 megaohms and organic content less than 10 ppb) de-ionized water.

Specificity

Specificity is the ability of the method to accurately measure the analyte response in the presence of all potential sample components (Kushnir *et al*., 2005). Specificity of the method was assessed by supplementing negative control horse plasma or urine with various therapeutic substances that are allowed in horse racing if present at a concentration less than the regulatory threshold. The purpose of this study was to determine whether such compounds altered the response of the analyte or internal standard or both. For plasma 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 1-4 µg/mL of phenylbutazone and furosemide, substances that are frequently present in post-race horse specimens. For urine 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 each of phenylbutazone and furosemide.

Stability

The evaluation of stability under the appropriate conditions is necessary in order to verify chemical integrity and assure reliable quantification results throughout the entire analytical process. The stability test conditions are determined by the method and length of storage of the biological samples. Stability of the analyte in both plasma and urine was evaluated over short-term intervals at 0 °C, -20 °C and -80 °C storage. Long-term stability was evaluated over six, twelve and eighteen 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 for plasma (1, 5 and 25 pg/mL) and urine (5, 100, 2500 pg/mL).

Ruggedness

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

Stock and Working Standard Solution Stability

As noted above all stock and working standard solutions were prepared in methanol. These solutions are independent from the dry certified drug standard. Therefore prepared stock and working standard solutions cannot be assigned a similar expiration date as the dry standard. One stock solution and four working standard solutions that are routinely prepared from the stock solution were evaluated for stability over several intervals for up to 2 yr under 0°C conditions. All solutions were compared to one fresh stock solution prepared directly from the dry reference

material and fresh working standards that had been prepared from the fresh stock solution (Nowatzke & Woolf, 2007).

Analyte Confirmation

In order to demonstrate that the GLY in a specimen obtained from a treated horse is chemically identical to the certified reference standard used for calibration, the ion intensity ratios were compared. The GLY product ions used for analyte confirmation were at *m/z* 58, 88, and 116. An ion intensity ratio was calculated according to peak height and relative abundance of the total ion chromatogram (TIC) as previously described (de Zeeuw, 2004). As such, the ion intensity ratio for the reference standard (calibrators and positive controls) were compared to the ion intensity ratio for unknown substance in a plasma sample using the following equation:

$$\text{Ion intensity ratio similarity (\%)} = (R_{unknown}/R_{standard}) \times 100 \qquad (2\text{-}4)$$

where $R_{unknown}$ represents the ion intensity ratio for the unknown substance present in a plasma specimen obtained from a horse treated with GLY and $R_{standard}$ represents the ratio for the GLY certified reference standard used to prepare the calibrators and positive control samples.

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).

Results

GLY is a quaternary ammonium compound and as such contains a permanent positive charge. Thus, weak cationic exchange is the preferred method of extraction because the quaternary ammonium moiety makes the compound difficult to isolate by other means. The

cation exchange sorbent (CBA-carboxyl propyl phase) has a pKa of 4.8 (Figure 2-1). Therefore the sorbent is negatively charged at pH 6.8 and greater and neutralized at pH 2.8 and lower. GLY is adsorbed onto the stationary phase due to the attraction between the negative charge on the sorbent and the positive charge of GLY (Figure 2-2(A)). Interfering matrix components can be eluted with methanol washes by interrupting hydrophobic interactions without concomitant loss of cationic analytes. Final elution of the drug can be achieved by disrupting the ionic interactions by suppressing ionization of the sorbent while ensuring that hydrophobic interactions between the phase and the target analyte do not occur (Figure 2-2(B)).

Method Validation

Linearity

Plasma: Method linearity for GLY in plasma was demonstrated with five calibration curves each spanning the range of 0.025-25 pg/mL. In all instances (n=5) a coefficient of determination (R2) of >0.998 was obtained (Table 2-4). The back-calculated concentrations of GLY in calibrators were within 85-115% and 90-110% of the target concentration for the LLOQ and all other concentrations, respectively (Table 2-5). Figure 2-4 illustrates a standard curve taken from the linearity experiments. Linear regression analysis of the response factors (based on the areas of the quantifier ion) vs. the nominal GLY calibrator concentrations demonstrated a slight decrease in response with increasing GLY concentrations and a slope of -12.86 (Figure 2-5). Linear regression analysis of the response factor (based on the ion area ratios) vs. the nominal GLY calibrator concentrations demonstrated a response that was not statistically different from zero across the range of concentrations (Figure 2-6).

Urine: Method linearity was demonstrated with five calibration curves each spanning the range of 5-2500 pg/mL. In all instances (n=5) a coefficient of determination (R2) of >0.999 was

obtained (Table 2-6). The back-calculated concentrations of GLY in calibrators were within 85-115% and 90-110% of the target concentration for the LLOQ and all other concentrations, respectively (Table 2-7). Figure 2-7 illustrates a standard curve taken from the linearity experiments. 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 (Figure 2-8). However, linear regression analysis of the response factor (based on the ion area ratios) vs. the nominal GLY calibrator concentrations demonstrated a response that was not statistically different from zero across the range of concentrations (Figure 2-9).

Sensitivity

Plasma: The corresponding LOD, LOQ and ULOQ were 0.025 (CAL 1), 0.125 (CAL 2) and 25 pg/mL (CAL 10) of plasma, respectively. Chromatograms for the LLOQ and ULOQ are shown in Figure 2-10 and Figure 2-11, respectively.

Urine: The corresponding LOD, LOQ and ULOQ were 1 (CAL 1), 5 (CAL 2) and 2500 pg/mL (CAL 10) of urine, respectively. Chromatograms for the LLOQ and the ULOQ are shown in Figure 2-12 and Figure 2-13, respectively.

Accuracy and Precision

Plasma: Precision and accuracy of the method were evaluated at five concentrations over the linear dynamic range (0.125, 1.25, 5, 12.5, 22.5 pg/mL) and results are provided in Table 2-8. The intra-batch (n=5) and inter-batch (n=20) precisions, expressed as %RSD were < 10. Accuracy was calculated as the percentage difference from the mean measured values to the target value and was determined to have a range of 94-104% (Table 2-9).

Urine: Precision and accuracy of the method were evaluated at five concentrations over the linear dynamic range (25, 125, 500, 750, 1250 pg/mL) and results are provided in Table 2-10. The intra-batch (n=5) and inter-batch (n=20) precisions, expressed as %RSD were < 10. Accuracy was calculated as the percentage difference from the mean measured values to the target value and was determined to have a range of 94-104% (Table 2-11).

Carryover

Plasma: Carryover and possible contamination of GLY throughout the entire LC-MS/MS system occurred and was potentially detrimental to the determination of low concentrations when comparatively high concentrations were analyzed during method validation studies. GLY 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 above. Under these conditions the extent of GLY carryover was <20% of the LOD.

Urine: GLY carryover was observed in one blank injection each following the 1000 and 2500 pg/mL calibrators. However, this carryover was <1% of the total area response and was eliminated with the addition of a second consecutive mobile phase only injection. Hence, study sample duplicates were separated by a minimum of two blank mobile phase injections.

Matrix Effect, Extraction Efficiency, Process Efficiency

Plasma: The matrix effect was evaluated using five different lots of matrix at five concentrations (0.125, 1.25, 5, 12.5, and 22.5 pg/mL) of GLY for five replicates each (n=5) using the three set experimental design described by Matuszewski *et al*. Absolute matrix effect was observed in all five lots of plasma with a range of 85-99%. Extraction efficiency ranged from 79 - 96% for all concentrations. Overall process efficiency, taking into account the matrix

effect, ranged from 67 - 95%). Relative matrix effect between five lots of plasma was expressed as coefficient of variation 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 2-13).

Urine: 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 – 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 82-105%. Absolute matrix effect was observed in all five positive control concentrations with a range of 82-90% (Table 2-14). Relative matrix effect between five lots of urine was expressed as coefficient of variation of five slopes generated from five prepared standard curves within a set. These values were less than 4% indicating minimal matrix interferences and increased reliability across different sources of urine (Table 2-15).

Dilution Integrity

Plasma: 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%. Comparing the average of five replicates for each dilution factor with the nominal value produced p-values > 0.05 (Table 2-16).

Urine: Dilutional Integrity was evaluated at three dilution factors 5 (low), 250 (medium) and 1000 (high), at five determinations for each factor, to encompass the range of dilutions that were required for sample analysis. The average back-calculated concentration did not differ from

the target concentration more than 10%. Comparing the average of five replicates for each dilution factor with the nominal value produced p-values > 0.05 (Table 2-17).

Specificity

Plasma: No interferences with the determination of the target analyte GLY or the IS were detected in the analysis of plasma samples fortified with 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.

Urine: 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. The identity of GLY in plasma and urine was confirmed by comparing the product ion intensity ratio. The product ions used for compound identification were m/z 116, 88, and 58. The ion intensity ratio was obtained using the peak area and calculated relative to the total ion chromatograph (see Analyte Confirmation).

Stability

Plasma: 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 hours. The mean GLY concentration after storage for 48 h on the autosampler tray differed less than a 10% compared to freshly prepared samples whereas those determined after storage for 72 hours 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. Long-term storage stability was evaluated at -80 °C for 170 days at these concentrations. Other validation study results for stability are reported in Table 2-18.

Urine: 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 hours. The mean GLY concentration after storage for 72 h on the autosampler tray differed less than 5% compared to freshly prepared samples. 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 was assessed at three concentrations in urine after storage at 0 °C, and -20 °C for 30 and 60 days, respectively. Long-term storage stability was evaluated at -80 °C for 170 days at these concentrations. Other validation study results for stability are reported in Table 2-19.

Ruggedness

Plasma: Changes in the composition of the solution used to dissolve the extraction residue and the volume of the rinse phase of the solid phase extraction procedure 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. However, when the elution volume (1 mL) was reduced to 0.5 mL, mean accuracy and precision ranged from 85.5-263.2% and 5.4-171.2%. 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 critical variables in the solid phase extraction process. For entire results refer to Table 2-20.

Analyte Confirmation

Figure 2-15 illustrates a graphical comparison of the product ion intensity ratios between the GLY standard (A) the unknown test plasma (B) and urine (C) samples. The ion intensity ratio similarity for all three GLY product ions between the standard and plasma samples are presented in Table 2-21 and range from 82-106%. The ion intensity ratio similarity for all three GLY product ions between the standard and urine samples are presented in Table 2-22 and range from 94-101%.

Discussion

GLY has been extracted from biological matrices using solid phase (SPE) (Matassa *et al*., 1992) and liquid-liquid extractions (LLE) (Storme *et al*., 2008; Tang *et al*., 2001). Liquid-liquid extractions are difficult to automate and often require large volumes of hazardous solvent. The liquid-liquid extraction procedure is labor intensive, time consuming and often less reproducible than SPE procedures. Additionally, the cationic properties of GLY are not ideal for partitioning into the liquid phase. Tang *et al*. presented a liquid-liquid extraction (LLE) for the screening and confirmation of eight quaternary ammonium compounds in horse urine, including GLY which was characterized by a variable but modest recovery (74%). Solid phase extraction produces cleaner extracts, greater recoveries and is available for automation (Zief & Kokodkar, 1994). Such advantages and the wide availability of sorbent chemistries have made solid phase extraction ideal for this analysis (Wynne, 2000).

The goal of the method development process was to make improvements where necessary to existing methodologies and to utilize newer technologies to accommodate the specific aims

for this project. Linearity and range of the method have been improved and increased by the addition of an internal standard. The methods use a stable-isotope labeled analogue of the analyte of interest (GLY) to account for the presence of competing substances in the extracts and to minimize their influence. Our experience with GLY before the use of this internal standard was that the methods were not adequate in terms of accuracy and precision. A solid phase extraction for the isolation and purification of GLY has increased recovery and reduced unwanted matrix interferences. The sample extraction technique has also been simplified with the use of a commonly available sorbent type and minimal procedural steps. Method analysis time has been reduced with the incorporation of a short but efficient UPLC analytical column while maintaining excellent peak shape and resolution. Sensitivity and selectivity for the analyte have been improved by the choice of instrumentation, a TSQ mass spectrometer.

In a previous report (Storme *et al*., 2008), a capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) was compared to LC-MS/MS for the detection of GLY in human plasma. The authors reported numerous benefits using CE-MS/MS over the conventional LC-MS/MS methods including, but not limited to, improved sensitivity and separation with a limit of detection of 1 ng/mL. For comparison, the LLOQ for the horse plasma method described above is 0.125 pg/mL. The advantages of CE-MS are well documented (Suntornsuk, 2007), but its use has been stifled because of a history of poor interfacing issues with an MS detector (Hommerson *et al*., 2011). While these problems have been largely eliminated, many laboratories are not equipped and do not have trained operators for CE instrumentation. Additionally, CE-MS method validations are uncommon, thus forensic laboratories are less likely to incorporate them into their standard operating procedures. The current methods also improve on previously published LC-MS/MS methods for the quantification of GLY in urine and plasma by including a

thorough method validation study and clearly describing identification criteria and procedures to quantify analytes of interest. Additionally, carryover, dilution integrity, and specificity with respect to potentially interfering exogenous analytes were included in the current validation but not in previously published reports (Table 2-23).

Table 2-1. Preparation of working standard solutions for plasma analysis.

Calibrators	Concentration	Working Standard Concentration	Volume of working standard
	(pg/mL)	(ng/µL)	(µL)
CAL-1	0.025	0.00000125	20
CAL-2	0.05		40
CAL-3	0.25	0.0000125	20
CAL-4	0.5		40
CAL-5	1.0		80
CAL-6	2.5	0.000125	20
CAL-7	5.0		40
CAL-8	10.0		80
CAL-9	17.5		140
CAL-10	25.0	0.00125	20
Positive Controls			
PC-A	0.125	0.000005	25
PC-B	1.25	0.00005	
PC-C	5.0		100
PC-D	12.5	0.0005	25
PC-E	22.5		45

Table 2-2. Preparation of working standard solutions for urine analysis.

Calibrators	Concentration	Working Standard Concentration	Volume of working standard
	(pg/mL)	(ng/µL)	(µL)
CAL-1	5		20
CAL-2	10	0.000125	40
CAL-3	20		80
CAL-4	35		140
CAL-5	50		20
CAL-6	100	0.00125	40
CAL-7	250		100
CAL-8	500		20
CAL-9	1000	0.0125	40
CAL-10	2500		100
Positive Controls			
PC-A	0.125	0.0005	5
PC-B	1.25		12.5
PC-C	5.0	0.005	50
PC-D	12.5		75
PC-E	22.5	0.05	12.5

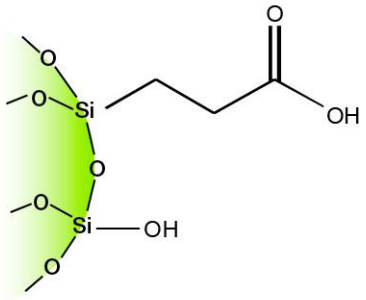

Figure 2-1. A weak cation-exchange sorbent that consists of a carboxylic acid group bonded to the surface of a silica particle (pH ≤ 2.8)

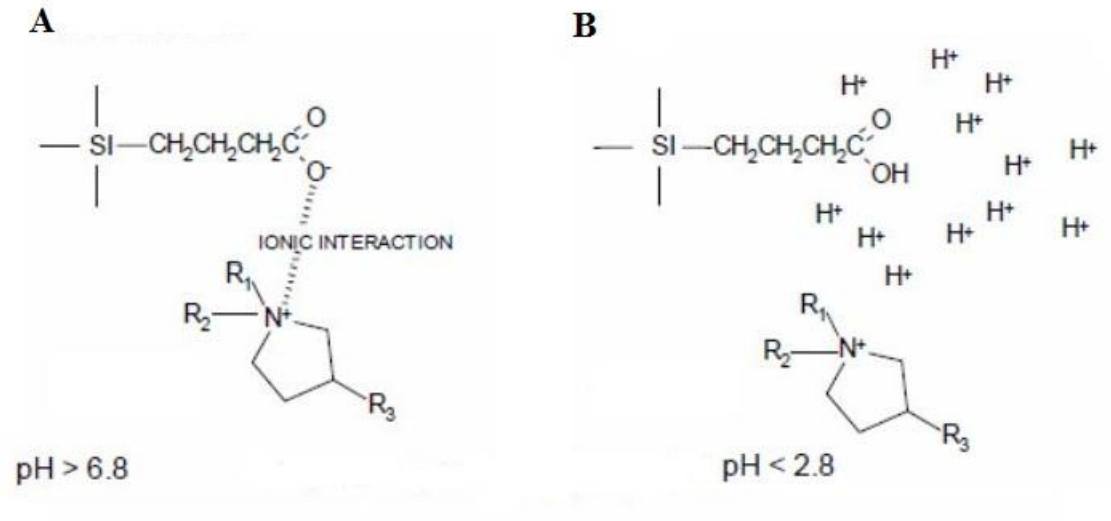

Figure 2-2. Solid phase extraction of GLY. A) At pH ≥ 6.8, the sorbent is essentially 100% ionized. Retention is due to ionic interactions. B) At pH ≤ 2.8 the charge on the sorbent is neutralized, and the elution is facilitated by the addition of a high ionic strength solvent. The analyte elutes from the sorbent because there are no forces to retain it.

Table 2-3. Gradient table for GLY LC method. A – ACN with 0.1% formic acid; B – DI Water with 0.1% formic acid.

Time (min)	A (%)	B (%)	Flow Rate (µL/min)
0.00	80.0	20.0	500
0.50	80.0	20.0	500
2.75	5.0	95.0	500
3.00	5.0	95.0	500
3.01	80.0	20.0	500
3.50	80.0	20.0	500

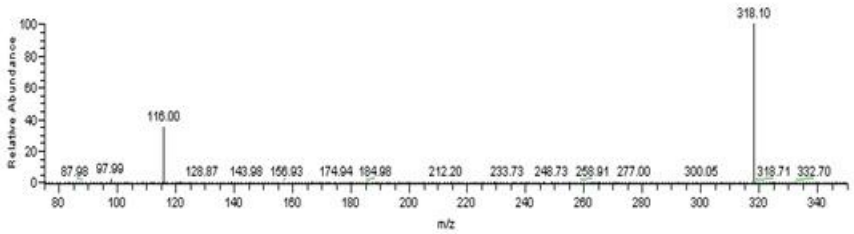

Figure 2-3. Ion spectrum for GLY (m/z 318.1)

Table 2-4. Characteristics of plasma GLY calibration curves (n=5) used for linearity assessment.

Run	Y-intercept	Slope	R^2
Set 1	0.00115	0.00960	0.9997
Set 2	0.00220	0.00904	0.9994
Set 3	0.00003	0.00702	0.9984
Set 4	0.00182	0.00631	0.9997
Set 5	0.00005	0.00934	0.9994

Table 2-5. Plasma GLY calibrator concentration taken from the accuracy and precision studies.

	Nominal Concentration (pg/mL)									
A&P Run	0.025	0.05	0.25	0.5	1.0	2.5	5.0	10.0	17.5	25.0
Set 1	0.025	0.049	0.244	0.506	1.02	2.54	5.01	9.93	17.6	25.0
Set 2	0.024	0.054	0.256	0.484	0.964	2.47	5.04	10.0	17.5	25.0
Set 3	0.026	0.054	0.237	0.492	0.954	2.40	5.01	10.0	17.7	25.0
Set 4	0.025	0.048	0.273	0.518	0.983	2.37	4.91	10.0	17.5	25.2
Set 5	0.028	0.053	0.237	0.453	0.907	2.60	5.00	10.0	17.6	24.9
Mean	0.026	0.052	0.249	0.500	0.965	2.44	4.99	10.0	17.6	25.0
SD	0.00	0.00	0.02	0.02	0.04	0.07	0.05	0.05	0.07	0.10
%RSD	7.10	6.17	6.07	3.03	4.28	3.00	0.94	0.48	0.38	0.41
%RE	2.57	3.40	-0.21	0.04	-3.45	-2.27	-0.15	0.04	0.40	0.01
n	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0

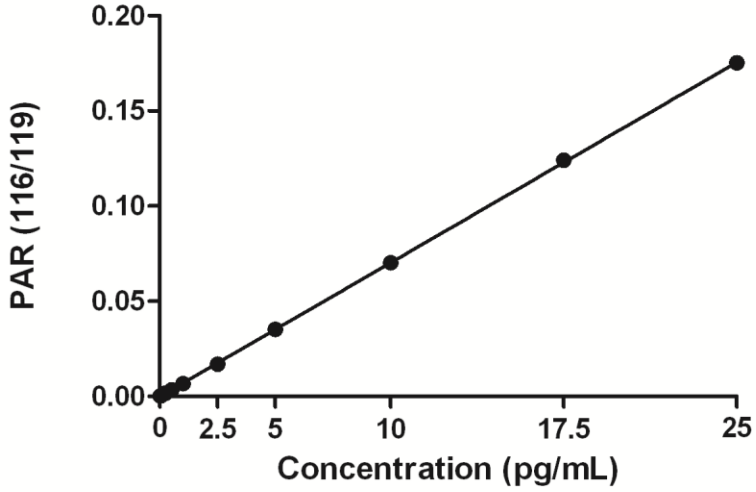

Figure 2-4. GLY calibration curve weighted 1/x (R^2 > 0.999) corresponding to Set 1. PAR – Peak Area Ratio.

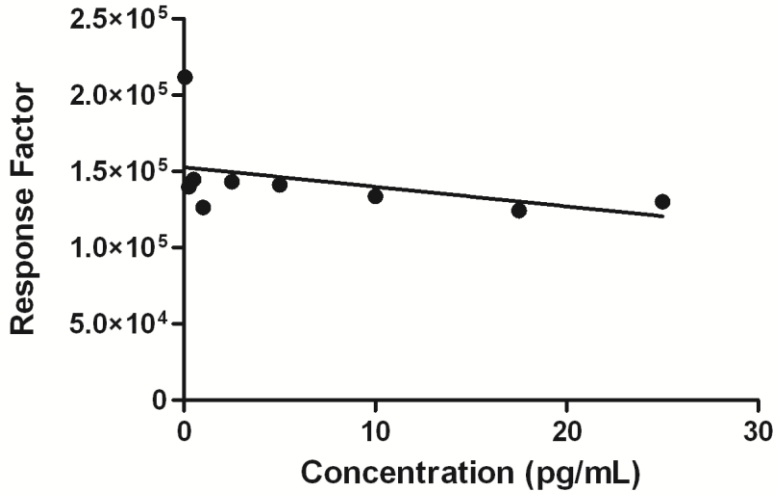

Figure 2-5. Plot of the response factor vs. the nominal calibrator concentrations. Slope = -12.86, y-intercept = 152766.

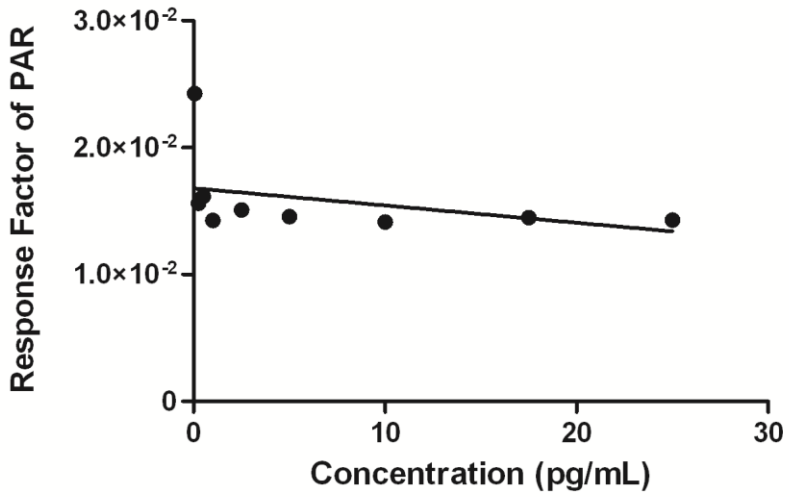

Figure 2-6. Plot of the response factor of the peak area ratio (PAR) vs. the nominal calibrator concentrations. Slope = -1.36×10^{-4}, y-intercept = 0.01680.

Table 2-6. Characteristics of urine GLY calibration curves (n=5) used for linearity assessment.

Run	y-intercept	Slope	R^2
Set 1	-0.01002	0.00429	0.99990
Set 2	-0.00393	0.00407	0.99995
Set 3	-0.00265	0.00394	1.0000
Set 4	-0.00354	0.00406	0.99995
Set 5	-0.00144	0.00514	0.99970

Table 2-7. Urine GLY calibrator concentration taken from the accuracy and precision studies.

	Nominal Concentration (pg/mL)									
A&P Run	5	10	20	35	50	100	250	500	1000	2500
Set 1	5.43	10.5	19.4	33.2	48.9	97.7	246.4	499.9	1004.9	2503.6
Set 2	5.42	10.0	19.4	34.6	50.7	98.5	237.9	500.0	998.8	2514.6
Set 3	5.19	10.1	19.5	33.3	50.1	101.2	249.3	507.0	1003.1	2491.6
Set 4	5.24	10.3	19.7	33.8	49.5	98.2	248.3	505.4	1004.5	2495.3
Set 5	5.04	9.56	19.2	33.3	49.4	98.2	254.2	499.2	1003.5	2498.5
Mean	5.26	10.1	19.4	33.6	49.7	98.8	247.2	502.3	1002.9	2500.7
SD	0.17	0.34	0.17	0.59	0.68	1.39	5.94	3.63	2.43	8.92
%RSD	3.14	3.38	0.85	1.76	1.37	1.41	2.40	0.72	0.24	0.36
%RE	5.27	0.79	-2.76	-3.88	-0.59	-1.22	-1.12	0.46	0.29	0.03
n	5	5	5	5	5	5	5	5	5	5

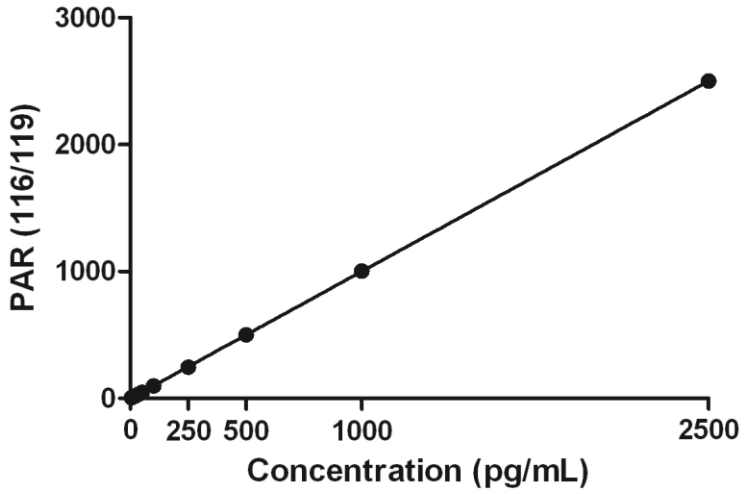

Figure 2-7. GLY calibration curve weighted 1/x (R2 > 0.999) corresponding to Set 1. PAR – Peak Area Ratio.

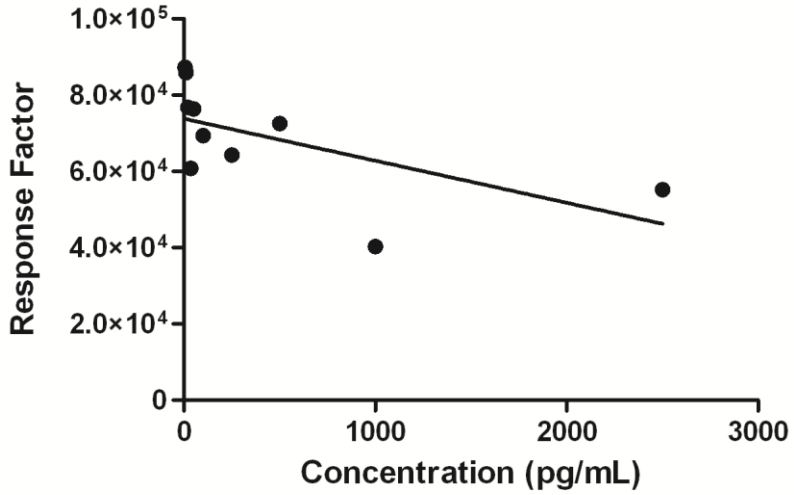

Figure 2-8. Plot of the response factor vs. the nominal calibrator concentrations. Slope = -11.02, y-intercept = 73825.

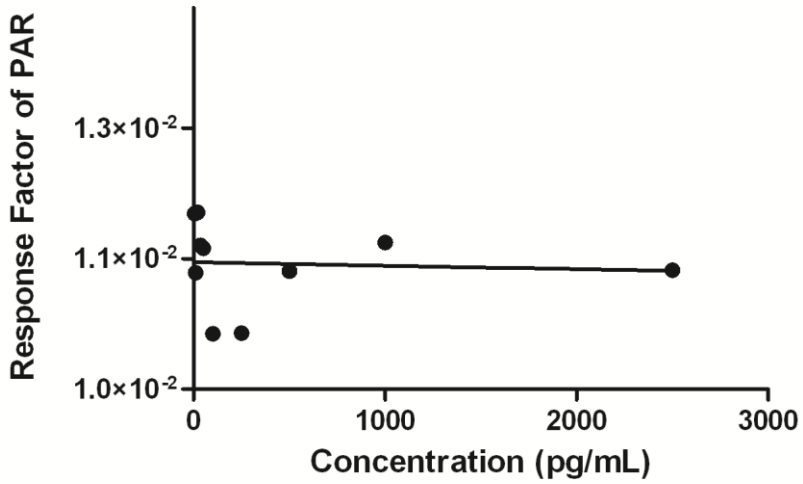

Figure 2-9. Plot of the response factor of the peak area ratio (PAR) vs. the nominal calibrator concentrations. Slope = -3.51 x 10-8, y-intercept = 0.0112.

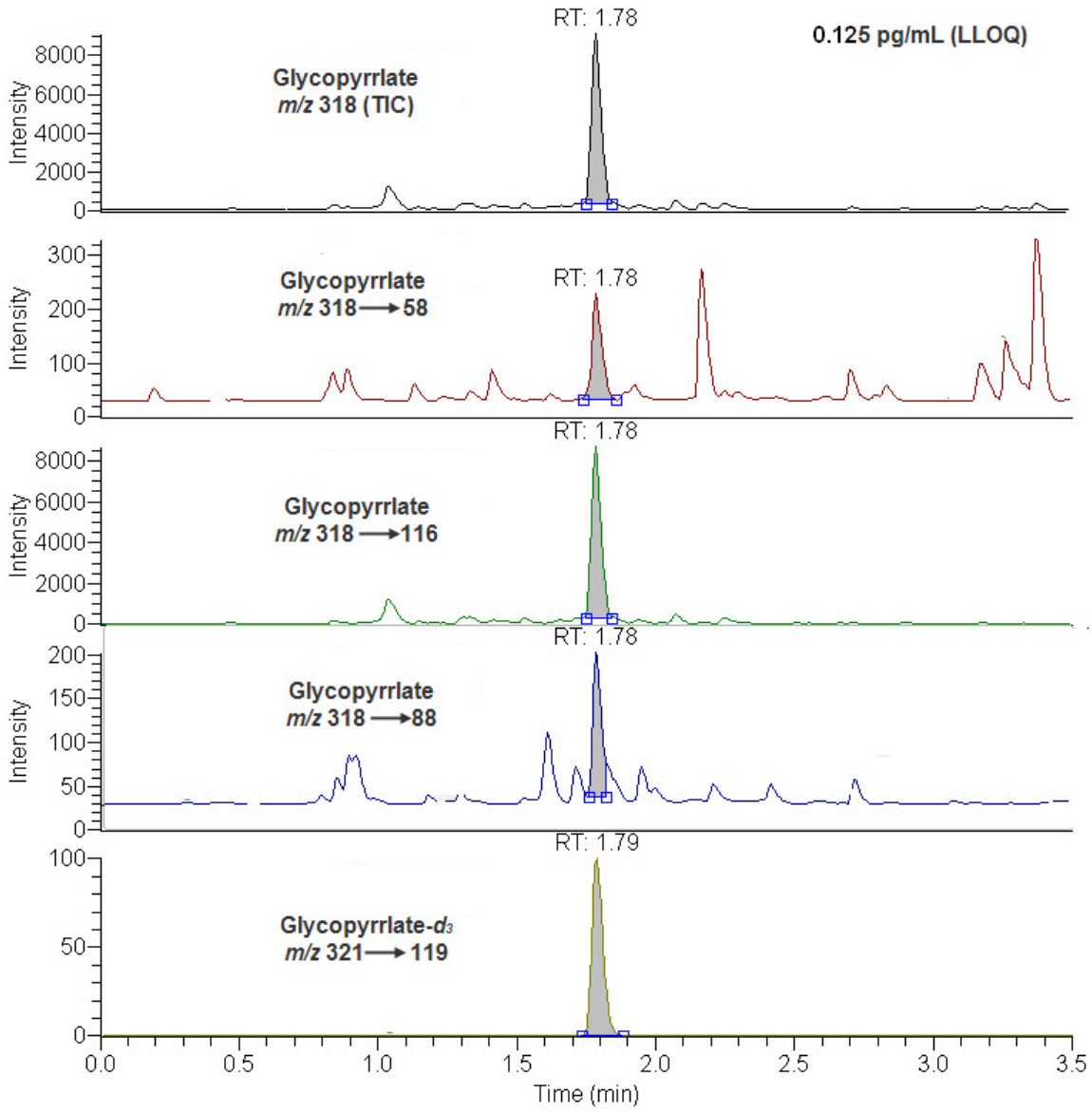

Figure 2-10. SRM chromatograms for GLY in horse plasma at the LLOQ (0.125 pg/mL) and the deuterated internal standard. TIC- Total Ion Chromatogram.

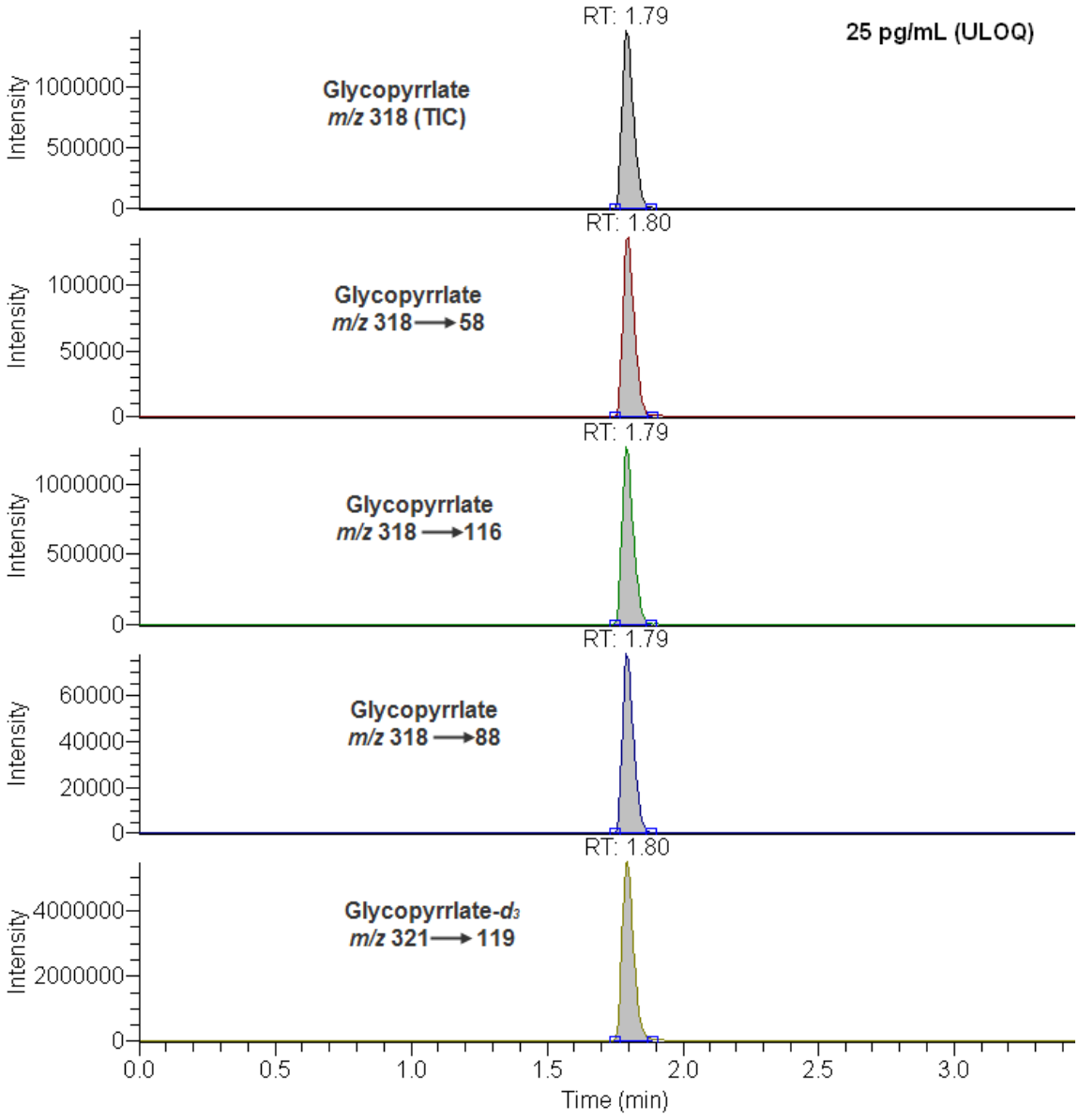

Figure 2-11. SRM chromatograms for GLY in horse plasma at ULOQ (25 pg/mL) and the deuterated internal standard. TIC- Total Ion Chromatogram.

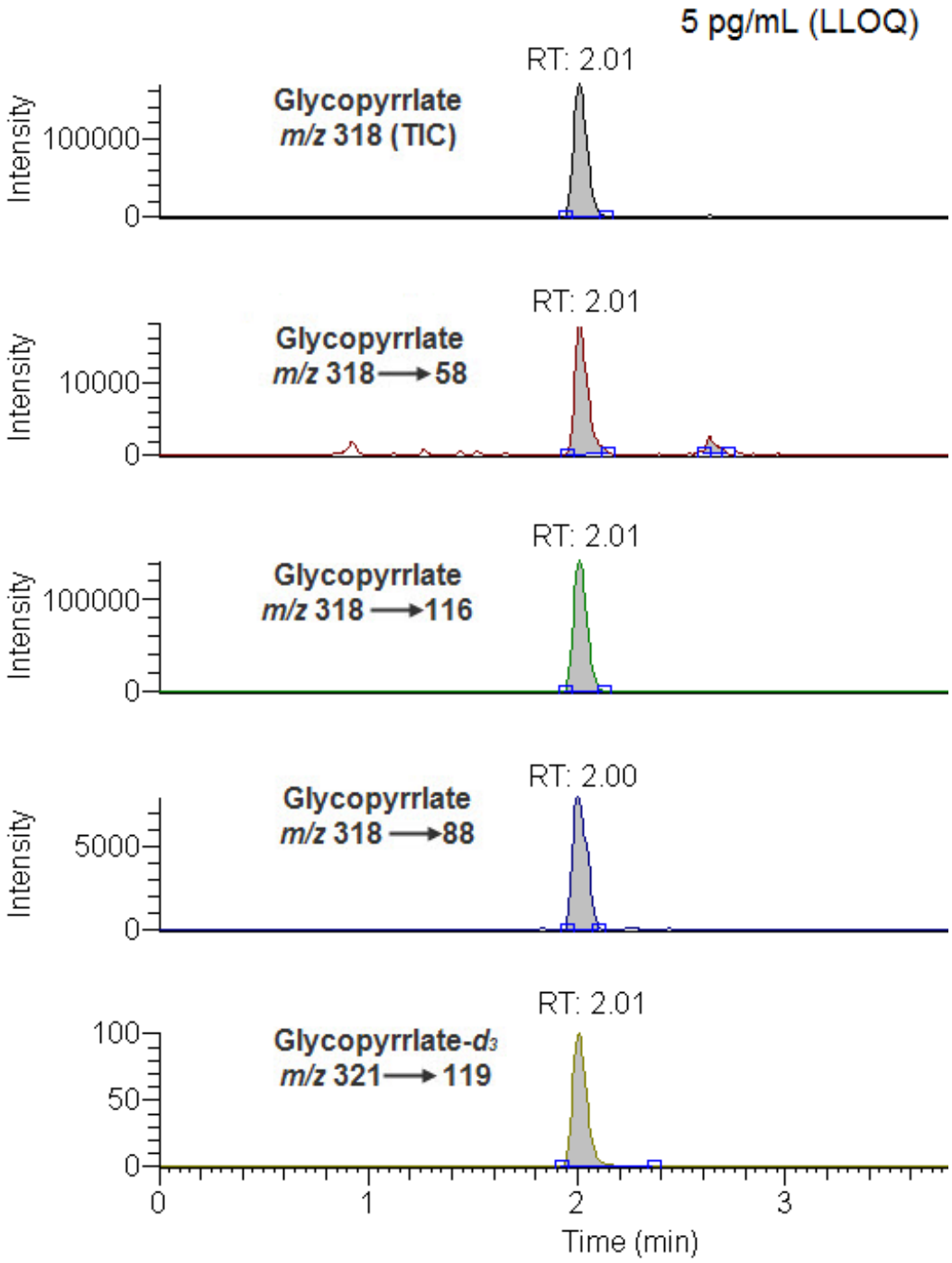

Figure 2-12. SRM chromatograms for GLY in horse urine at LLOQ (5 pg/mL) and the deuterated internal standard. TIC- Total Ion Chromatogram.

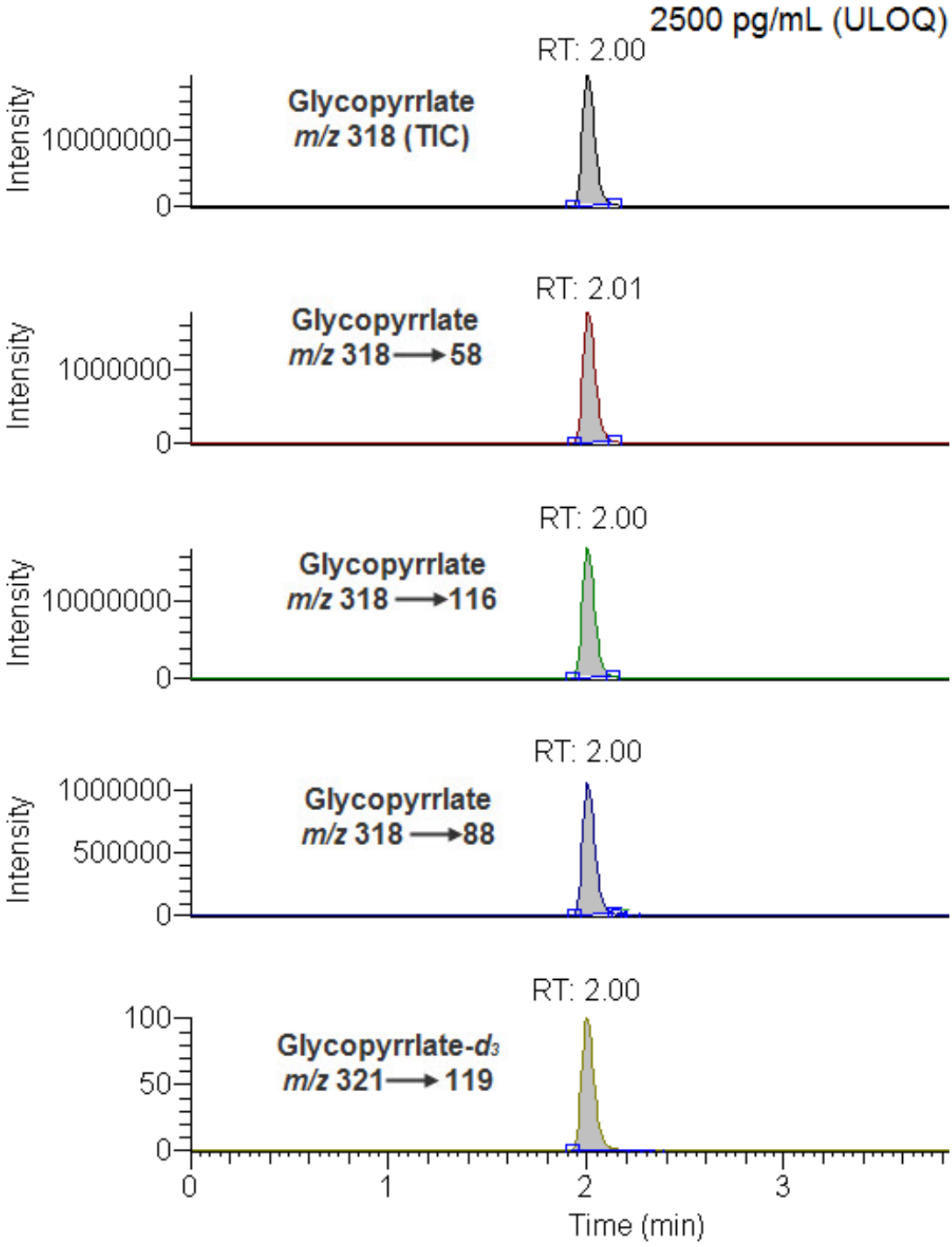

Figure 2-13. SRM chromatograms for GLY in horse urine at ULOQ (2500 pg/mL) and the deuterated internal standard. TIC- Total Ion Chromatogram.

Table 2-8. Accuracy and Precision (plasma)

			Intra-batch Statistics					
Sample	Batch	n	Mean	SD	% CV	%RE	Ancillary Statistics	
PC1	1	5	0.131	0.012	9.88	0.080	MS_w =	0.000
0.125	2	5	0.119	0.014	10.9	0.975	MS_b =	0.000
	3	5	0.126	0.031	25.0	-0.052	MS_t =	0.000
	4	5	0.127	0.012	9.40	1.037	s_t=	0.018
							s_b =	0.000
Intra-batch statistics (Pooled).		5	0.126	0.018	14.4	0.50	p =	4
Inter-batch 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
Intra-batch statistics (Pooled):		5	1.27	0.076	6.10	1.63	p =	4
Inter-batch 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
Intra-batch statistics (Pooled):		5	5.20	0.211	4.23	3.02	p =	4
Inter-batch statistics (ANOVA):		20	5.20	0.279	5.58	3.02		

Table 2-8: Continued

		Intra-batch Statistics						
Sample	Batch	n	Mean	SD	% CV	%RE	Ancillary Statistics	
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
Intra-batch statistics (Pooled):		5	13.1	0.472	3.77	4.74	p =	4
Inter-batch statistics (ANOVA):		20	13.1	0.572	4.58	4.74		
PC5	1	5	22.6	0.526	2.34	0.241	MS_w =	0.542
22.5	2	5	22.7	0.694	3.09	0.872	MS_b =	0.781
	3	5	22.7	0.712	3.16	0.702	MS_t =	0.580
	4	5	23.4	0.951	4.23	4.08	s_t=	0.762
							s_b =	0.219
Intra-batch statistics (Pooled):		5	22.8	0.736	3.27	1.47	p =	4
Inter-batch statistics (ANOVA):		20	22.8	0.768	3.41	1.47		

Sample concentrations are in pg/mL. MS_w - ANOVA mean square for intra-batch samples; MS_b - ANOVA mean square for inter-batch samples; MS_t - ANOVA mean square for all samples; s_t - ANOVA variance component for all samples; s_b - ANOVA variance component for inter-batch samples

Table 2-9. Summary of Accuracy and Precision (plasma)

		Nominal Concentration (pg/mL)				
		PC1	PC2	PC3	PC4	PC5
Characteristic	Statistic	0.125	1.25	5	12.5	22.5
# Results	N	20	20	20	20	20
Accuracy	Mean Bias (%RE)	0.533	1.62	3.05	4.75	1.56
	*LCL	-5.62	-5.22	-3.52	-1.82	-1.32
	**UCL	6.62	8.48	9.56	9.66	4.27
Precision	Intra-batch (%CV)	14.4	6.15	4.20	3.84	3.39
	Inter-batch (%CV)	14.4	7.00	5.60	4.60	3.45
Accuracy + Precision	|Mean| + Inter-batch	14.9	8.58	8.60	9.32	4.89
90% Expectation Tolerance Interval	Lower Limit (%RE)	-24.6	-11.2	-7.91	-3.92	-4.74
	Upper Limit (%RE)	25.6	14.4	13.9	13.4	7.61

*Lower Confidence Limit for the Mean Bias; **Upper Confidence Limit for the Mean Bias

Table 2-10. Accuracy and Precision (urine)

		Intra-batch Statistics						
Sample	Batch	n	Mean	SD	% CV	%RE	Ancillary Statistics	
PC1	1	5	5.04	0.039	0.780	0.840	MSw =	0.004
5	2	5	5.02	0.075	1.50	0.400	MSb =	0.001
	3	5	5.03	0.074	1.48	0.560	MSt =	0.004
	4	5	5.05	0.063	1.25	0.960	st=	0.060
							sb =	0.000
Intra-batch statistics (Pooled).		5	5.04	0.060	1.20	0.690	p =	4
Inter-batch statistics (ANOVA):		20	5.04	0.060	1.20	0.690		
PC2	1	5	125.1	1.53	1.221	0.037	MSw =	2.17
125	2	5	126.4	1.28	1.022	1.09	MSb =	1.45
	3	5	125.7	1.14	0.910	0.538	MSt =	2.05
	4	5	125.7	1.85	1.477	0.556	st=	1.43
							sb =	0.00
Intra-batch statistics (Pooled):		5	125.7	1.43	1.146	0.556	p =	4
Inter-batch statistics (ANOVA):		20	125.7	1.43	1.146	0.556		
PC3	1	5	507.6	18.6	3.728	1.52	MSw =	125.5
500	2	5	503.5	5.19	1.038	0.71	MSb =	29.3
	3	5	508.3	9.59	1.918	1.67	MSt =	110.3
	4	5	504.1	5.96	1.193	0.83	st=	10.5
							sb =	0.182
Intra-batch statistics (Pooled):		5	505.9	10.5	2.10	1.18	p =	4
Inter-batch statistics (ANOVA):		20	505.9	10.5	2.10	1.18		

Table 2-10: Continued

		Intra-batch Statistics					
Sample	Batch	n	Mean	SD	% CV	%RE	Ancillary Statistics
PC4	1	5	757.7	11.42	1.523	1.028	MSw = 82.42
750	2	5	753.1	8.916	1.189	0.416	MSb = 80.27
	3	5	747.9	7.785	1.038	-0.275	MSt = 82.08
	4	5	752.1	7.685	1.025	0.294	st= 9.060
							sb = 0.000
Intra-batch statistics (Pooled):		5	752.7	9.060	1.208	0.366	p = 4
Inter-batch statistics (ANOVA):		20	752.7	9.060	1.208	0.366	
PC5	1	5	1253.6	33.59	2.687	0.291	MSw = 406.5
1250	2	5	1257.5	15.06	1.204	0.600	MSb = 51.36
	3	5	1250.5	10.44	0.835	0.041	MSt = 350.5
	4	5	1251.0	12.75	1.020	0.079	st= 18.72
							sb = 0.000
Intra-batch statistics (Pooled):		5	1253.2	18.72	1.498	0.253	p = 4
Inter-batch statistics (ANOVA):		20	1253.2	18.72	1.498	0.253	

Sample concentrations are in pg/mL. MS_w - ANOVA mean square for intra-batch samples; MS_b - ANOVA mean square for inter-batch samples; MS_t - ANOVA mean square for all samples; s_t - ANOVA variance component for all samples; s_b - ANOVA variance component for inter-batch samples

Table 2-11. Summary of Accuracy and Precision (urine)

		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	1.18	0.366	0.253
	*LCL	2.83	-0.131	-0.410	-0.484	-0.155
	**UCL	1.10	1.24	1.95	1.22	0.661
Precision	Intra-batch (%CV)	1.20	1.15	2.10	1.21	1.49
	Inter-batch (%CV)	1.20	1.15	2.10	1.21	1.49
Accuracy + Precision	|Mean| + Inter-batch	1.89	1.70	3.28	1.57	1.75
90% Expectation Tolerance Interval	Lower Limit (%RE)	-1.40	-1.50	-2.54	-1.78	-2.41
	Upper Limit (%RE)	2.78	2.60	4.82	2.47	2.89

*Lower Confidence Limit for the Mean Bias;
**Upper Confidence Limit for the Mean Bias

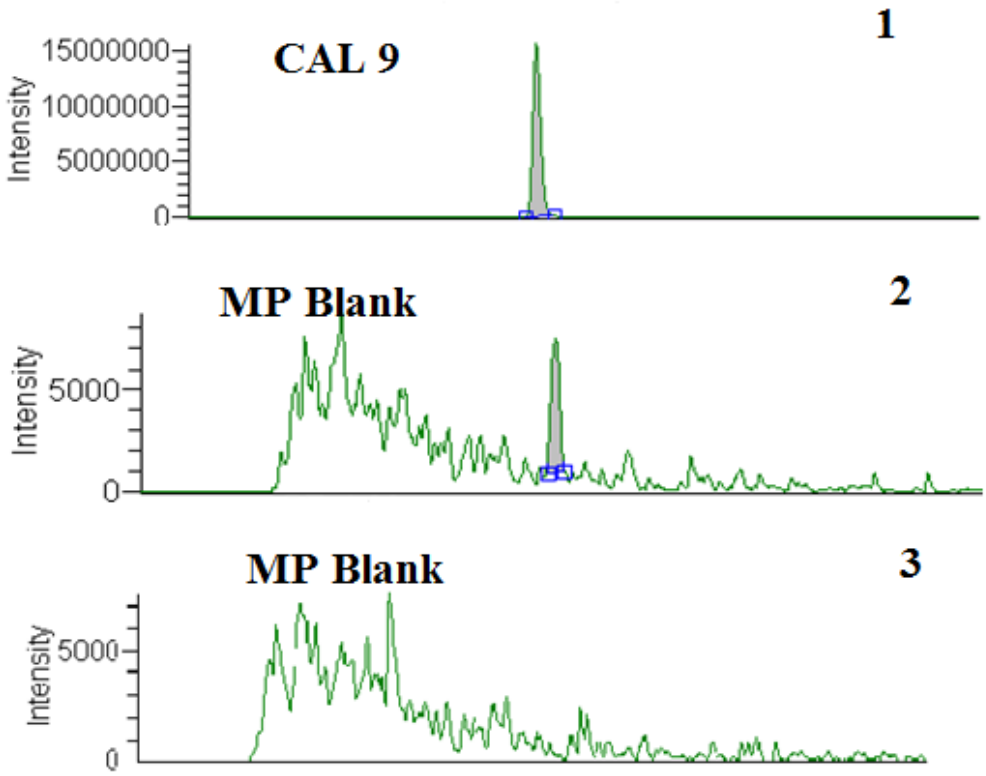

Figure 2-14. GLY present as carryover in a mobile phase blank (2) following CAL 9 (1000 pg/mL) (1) and a second consecutive mobile phase blank (3) exhibiting no further GLY carryover.

Table 2-12. Matrix effect, Extraction Efficiency, and Process Efficiency data for GLY 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 2-13. Relative matrix effect in plasma.

	Neat Standards (set 1)	Plasma extracts fortified after-extraction (set 2)	Plasma extracts fortified before extraction (set 3)
Slope[a]	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 plasma matrix lots were prepared. Each standard curve was constructed using five concentrations (positive controls). [a] 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 2-14. 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

Table 2-15. Relative matrix effect in urine.

	Neat Standards (set 1)	Plasma extracts fortified after-extraction (set 2)	Plasma extracts fortified before extraction (set 3)
Slope[a]	0.0052	0.0045	0.0044
SD	0.0001	0.0001	0.0001
%CV	2.09	1.79	3.06

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

Table 2-16. Plasma sample dilution integrity.

Dilution Factor*	% CV (n=5)	% difference from nominal concentration	*p*-value
2	4.12	1.75	0.521
100	3.49	2.91	0.117
500	3.32	-2.04	0.124
1000	2.41	-4.24	0.181

*Diluted concentrations were multiplied by the appropriate dilution factor to obtain a mean (n=5) sample concentration. This value was compared to the nominal concentration of the positive control prior to dilution.

Table 2-17. Urine sample dilution integrity.

Dilution Factor*	% CV (n=5)	% difference from nominal concentration	*p*-value
5	4.35	8.31	0.09
250	2.89	2.75	0.445
1000	2.67	-3.03	0.295

*Diluted concentrations were multiplied by the appropriate dilution factor to obtain a mean (n=5) sample concentration. This value was compared to the nominal concentration of the positive control prior to dilution.

Table 2-18. Storage stability of GLY in plasma.

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	0.144	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	0.094	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
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
3 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 are out of specification. *Value was determined with two replicates instead of three due to a failed injection. **p-value could not be generated with unequal number of replicates.

Table 2-19. Storage stability of GLY in 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 are out of specification.

Table 2-20. Method ruggedness evaluation.

Sample preparation condition tested	Positive control Concentration (pg/mL) 0.125	1.25	5	12.5	22.5
	Mean accuracy (%) with 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 30:70 instead of 80:20 ACN:Water (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 10:90 instead of 80:20 ACN:Water (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)
Eluted with ACN instead of ACN w/ 1% FA	< LOD	< LOD	< LOD	< LOD	< LOD

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 standard conditions. Precision of the replicates is expressed as coefficient of variation (% CV).

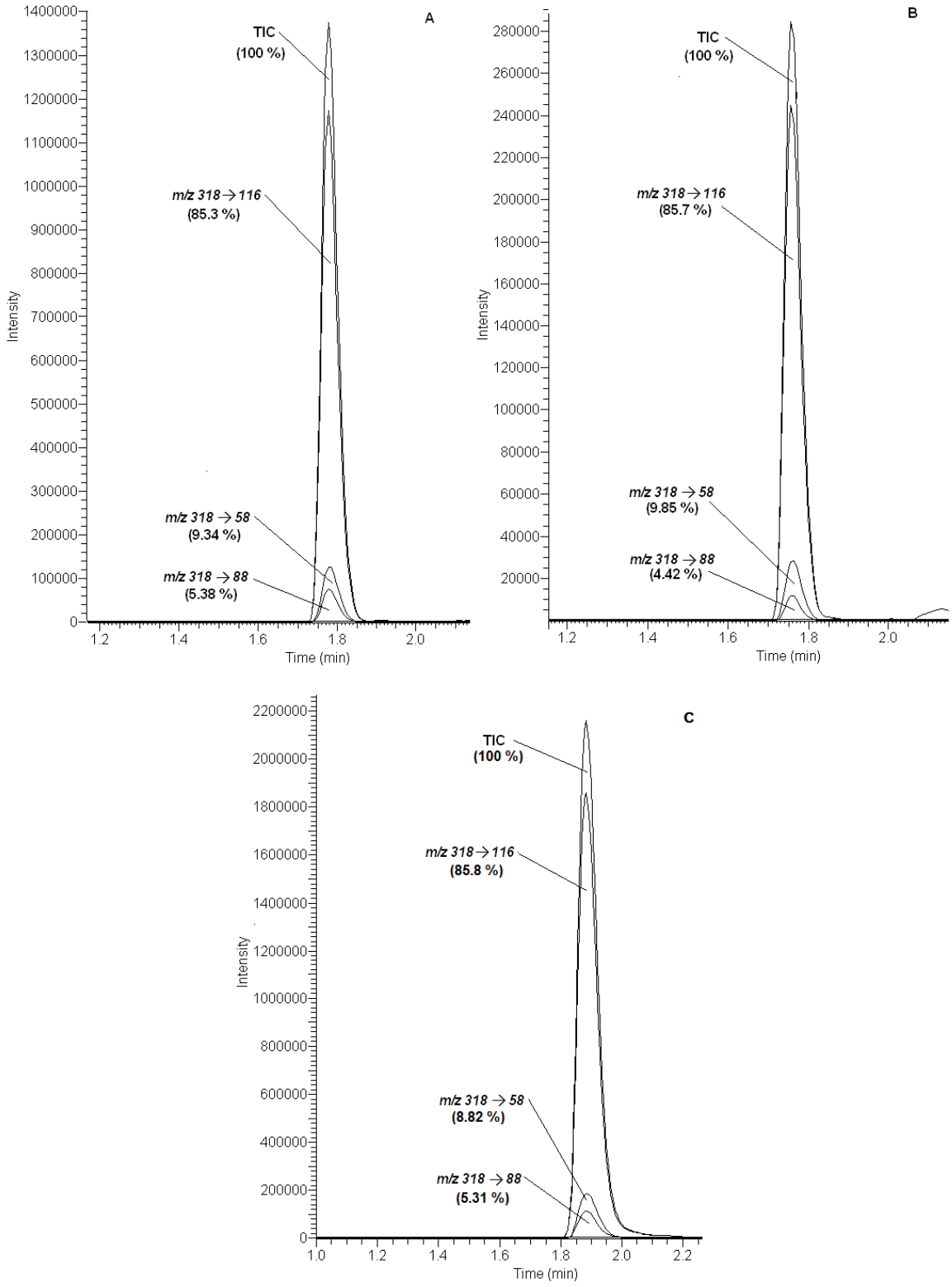

Figure 2-15. Comparison of the ion intensity ratios between a GLY reference standard (25 pg/mL) (A), a plasma (B) and urine (C) sample after GLY administration.

Table 2-21. Determination of the product ion ratio for confirmation of the presence of GLY in horse plasma.

Product ions	Intensity Ratio $_{standard}$ (n=10)	Intensity Ratio $_{unknown}$ (n=10)	Ion Intensity Ratio Similarity (%)
58	9.34 ± 0.67	9.85 ± 0.45	105.5
88	5.38 ± 0.96	4.42 ± 0.63	82.2
116	85.3 ± 0.34	85.7 ± 0.21	100.2

TIC was used as the denominator in calculating the ion intensity ratio

Table 2-22. Determination of the product ion ratio for confirmation of the presence of GLY in horse urine.

Product ions	Intensity Ratio $_{standard}$ (n=10)	Intensity Ratio $_{unknown}$ (n=10)	Ion Intensity Ratio Similarity (%)
58	9.34 ± 0.67	8.82 ±	94.4
88	5.38 ± 0.96	5.31 ±	98.7
116	85.3 ± 0.34	85.8 ±	100.6

Table 2-23. Comparison of evaluated method validation parameters between the current study and three previously published reports.

	[1]Current Study	[2]Storme *et al*., 2008	[3]Tang *et al*., 2001	[3]Matassa *et al*., 1992
Sensitivity	X	X	X	X
Linearity	X			
Carryover	X			
Accuracy	X	X	X	X
Imprecision	X	X	X	X
Recovery	X	X	X	X
Matrix Effect	X	X		
Process Efficiency	X	X		
Matrix Interference	X			
Dilution Integrity	X			
System Suitability	X			
Specificity	X			
Stability	X			
Ruggedness	X			X
Measurement Uncertainty	X			

[1] Method validation study performed in horse plasma and urine,
[2] Method validation study performed in human plasma,
[3] Method validation study performed in horse urine.

CHAPTER 3
PHARMACOKINETICS OF GLYCOPYRROLATE IN THOROUGHBREDS

Until the early 1990s, the pharmacokinetics of glycopyrrolate (GLY) were poorly understood primarily due the lack of sufficiently sensitive analytical methods (Kaltiala *et al*., 1974; Murray *et al*., 1984) when Kaila *et al*. introduced a radioreceptor assay for this purpose (Kaila *et al*., 1990). Since then GLY pharmacokinetics have been reported mainly in adult humans through intramuscular (Ali-Melkkila *et al*., 1990; Ali-Melkkila *et al*., 1993) and intravenous (Ali-Melkkila *et al*., 1989) administration and in children (Rautakorpi, *et al*., 1994; Rautakorpi *et al*., 1998).

To the author's knowledge, meaningful pharmacokinetic studies of this drug in the horse following rumored useage at performance racetracks have not been reported due to limitations in sensitivity of the methods that are commonly used. Yet positive post-race findings for GLY have surfaced at a steady rate in recent years. Therefore this study investigated the disposition of GLY following intravenous administration of a 1 mg dose in the horse, a dose thought to be used during the prohibited administration period before racetime.

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 (Tobin *et al*., 1999; Kollias-Baker, 2001; Report, 1991). Furthermore, it is important to determine the relationship between urine and plasma concentrations after a single intravenous and clinically relevant dose of GLY. Such investigations could contribute to the RMTC effort to establish a plasma threshold and to recommend a withdrawal time for this drug in race horses.

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 such 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.

Methods

Animals

Twenty, adult, athletically conditioned Thoroughbreds (6 mares and 14 geldings) ranging in age from 4-10 years and weighing from 485-602 kg were used in these studies. Eight horses (1 mares and 7 geldings) ranging in age from 4-10 years and weighing from 502-580 kg were used in the pharmacokinetic studies. All horses were dosed intravenously and six of these horses (1 mare and 5 geldings) ranging in age from 8-10 years and weighing from 518-580 kg were dosed orally following a sufficient (30-day) washout period. All horses were housed in grass paddocks at the UF Veterinary Medical Center, kept 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 study.

Conditioning

All horses were conditioned using a high-speed Säto treadmill (Equine Dynamics, Lexington, KY, USA) at the UF Equine Performance Laboratory. For two months before the study, horses were conditioned using a standard training regimen and were subjected to an exhaustion test. The conditioning regimen was designed to prepare the horses 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 dose administration study. The standardized 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).

Dosing

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. Oral administration was carried out using 50 mL of 0.2 mg/mL GLY solution for a total of 10 mg orally. Demographics for the current study are summarized in (Table 3-1).

Specimen Collection

Plasma: Whole blood samples were collected from the left 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 x g) at 4 °C for 15 min. Harvesting of plasma took place within 1 hour 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. For horses included in the pharmacokinetic studies, collection times included a sample collection before drug administration and 5, 10, 15, 20, 30 and 45 min and 1, 2, 3, 4, 6, 8, 24, 48, 72, 96, and 168 h after intravenous administration. Collections times for oral administration were before drug administration and at 15, 30, 45, 60 and 90 min and 2, 3, 4, 6, 8 and 24 h after dosing.

Urine: 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 divided into single use aliquots in 15-mL sterile, disposable, polypropylene centrifuge tubes and stored 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. For oral administration collection times were before drug administration and at 2, 4, 6, 8, and 24 h after dosing.

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).

Pharmacokinetic Analysis

Nonlinear least squares regression analysis was performed on plasma GLY concentration versus time data and pharmacokinetic parameters for all horses were estimated with both noncompartmental (NCA) and compartmental analysis using Phoenix WinNonlin® 6.1 (Pharsight, St. Louis, MO, USA). For compartmental analysis, the Gauss–Newton (Levenberg and Hartley) method was used and goodness of fit and the appropriate weighting factor were selected based on the coefficients of variation, Akaike's Information Criterion (Yamaoka, 1978) and Schwarz's Bayesian Criterion (Schwarz, 1978) as well as visual analysis of the graphical output (including residual plots). Secondary parameters calculated include area under the curve (AUC), terminal half-life ($t_{1/2\gamma}$), apparent volumes of distribution, total plasma clearance (Cl_p), and micro distribution rate constants. For the NCA analysis, the area under the plasma concentration vs. time curve (AUC_{0-24}) from time 0 to 24 h was calculated using the log-linear trapezoidal method with linear interpolation. The pharmacokinetic parameters calculated

included the observed maximum plasma concentration (C_{max}), area under the plasma concentration vs. time curve to the last determined plasma concentration (AUC_t), terminal half-life ($t_{1/2}$), total plasma clearance (Cl_p), mean residence time (MRT), and steady state volume of distribution (V_{ss}). All calculations for pharmacokinetic parameters were based on methods described by Gibaldi and Perrier (Gibaldi & Perrier, 1982). All pharmacokinetic parameter estimates were calculated for each horse and values are reported as median and range (minimum-maximum).

Statistical Analysis

Plots of urine and plasma concentrations were performed using GraphPad Prism™ version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) and plasma and urine concentrations of GLY are expressed as mean and standard deviation. Tolerance intervals for urine and plasma 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).

Results

Intravenous Administration

Plasma: After intravenous administration of 1 mg, plasma GLY was detectable for up to 168 h in fourteen of the twenty horses used for the study. The mean plasma ± s.d. concentration at 24 h after dosing was 1.36 ± 0.41 pg/mL. The upper limit of the tolerance interval was calculated (n=20) for each collection interval. Notably, the upper limit of the 99/95% tolerance interval (n=20) at 24 h in plasma was 3.02 pg/mL. Plasma concentrations and descriptive statistics at collection times for all horses (n=20) are summarized in Table 3-2.

For subjects (n=8) with a more extensive blood collections, pharmacokinetic analysis was performed. After intravenous administration of 1 mg of GLY, the observed plasma concentration versus time profile could be best described by a three-compartment model (Figure 3-1). The derived equation (Jacobs, 1988) based on macro constants for this model is:

$$C_t = Aexp^{-\alpha \cdot t} + Bexp^{-\beta \cdot t} + Cexp^{-\gamma \cdot t} \quad (3\text{-}1)$$

where C_t is the plasma concentration at time (t), A, B and C are the zero time intercepts for the first, second and third phases. Further, α, β and γ are the exponential terms for each phase and exp is the base of the natural logarithm (Gabrielsson & Weiner, 2007). The weighting factor chosen with this model was 1/ (Y^2) where Y was the observed plasma concentration. A three compartment model was chosen over a two-compartment model based on visual observations of the observed and predicted concentrations versus time for a two (Figure 3-2) and three (Figure 3-3) compartment analysis and other diagnostic criteria (Table 3-5). Estimates for a number of pharmacokinetic parameters following noncompartmental and compartmental model analysis are reported in Table 3-3 and Table 3-4, respectively. Plasma GLY concentration vs. time plots for all eight horses are depicted in Figure 3-4 and Figure 3-5. The drug concentrations remained above the method's LLOQ (0.125 pg/mL) for all horses through 168 h after dosing (Figure 3-6).

Urine: Urine GLY concentrations, determined using the method described above, were above the lower limit of quantification (5 pg/mL) 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 3-6. Peak urine concentrations of GLY were observed at the 4 h collection time for all horses. Urine concentrations (n=20) of GLY through 168 h are graphed in Figure 3-7. The upper limit of the tolerance interval was calculated for each collection time. Notably, the upper limit of the 99/95% tolerance interval at 24 h in urine was 325 pg/mL.

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

$$\frac{CL_R}{\dot{V}} = \frac{C_U}{C_P} \quad (3\text{-}2)$$

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 Figure 3-9. 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 24-168 h after administration the urine to plasma concentration ratio ranged from 90-150 with a mean of 131. The mean (n=20) at 24 h after administration was 103.

Oral Administration

Plasma: GLY was detected in plasma samples collected after oral administration of 10 mg of GLY in aqueous solution. 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 LLOQ. Therefore, bioavailability could not be calculated using the conventional methods as described in Gibaldi and Perrier (Gibaldi & Perrier, 1982). Figure 3-10 displays plasma concentrations versus time after oral administration of 10 mg to each horse. Figure 3-11 displays the plasma concentrations versus time following oral and intravenous administration.

Urine: GLY 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 were 26.8 (17.5-67.4) pg/mL. Figure 3-12 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 (16.3-83.2) pg/mL, not very different from the median value (22.4 pg/mL) when the highest value of 83.2 pg/mL was excluded.

Discussion

The data indicate that GLY disposition in the horse exhibited tri-exponential disappearance from plasma after intravenous administration. This is characterized by an early rapid decline in plasma concentrations followed by a slow terminal phase with concentrations above the LLOQ of the method for up to 168 h. All horses exhibited plasma concentrations above 1 ng/mL 5 min after drug administration followed by a precipitous decline through 20 min. Although the three-compartment model estimates for C_{max} are higher than the noncompartmental estimates due to the extrapolation back to time 0 in the compartmental model, it is believed that the inclusion of these values in the model is necessary to describe the disposition of GLY (Beaufort *et al*., 1999). Moreover, data in humans suggest a similar pharmacokinetic profile (Penttila *et al*., 2001). Schenin *et al*., have reported a rapid initial distribution/elimination phase with a two compartment model in humans following a single intravenous 5 µg/kg dose (Scheinin *et al*., 1999). However, an insufficient analytical method may have prevented the discovery of an additional decay phase.

Relevant factors in the rapid decline in the plasma GLY concentration were the large volume of distribution and the swift transfer into the tissue compartment and not due to renal clearance. The long terminal half-life is the result of the distribution into a deeper third compartment. Noncompartmental analysis, a model independent approach, was also performed and provided physiologically reasonable parameter estimates. However, the median (range) volume of distribution based on the terminal phase (V_z) was unrealistically large (14.4 (7.56-

27.9) L/kg), likely accounted for by the rapid elimination during the initial phase and low plasma GLY concentrations during the terminal phase (Toutain & Bousquet-Molou, 2004). Moreover, NCA analysis typically provides limited information regarding the plasma drug concentration vs. time profile due to the numerous assumptions made (Rosenbaum, 2011).

Total plasma clearance is attributed to hydrolysis of GLY and renal clearance. While this study has revealed that some GLY is eliminated unchanged in the urine, we did not perform volumetric urine collections in this study and therefore cannot estimate renal clearance of GLY. The median (range) of the total GLY plasma clearance reported from the pharmacokinetic study was 22.4 (14.2-31.2) mL/min/kg and is approximately equal to previous estimates of hepatic blood flow in the horse (Dyke *et al*., 1998), 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 (Kohn and Strasser, 1986; Woods, 2000). Since the renal clearance estimates from these horses are approximately equal to the glomerular filtration 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 GLY 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 (Kaltiala *et al*., 1974) indicating that renal clearance is responsible for much of the plasma clearance in contrast to our finding in the horse. In contrast, more recent studies in humans, following a single intravenous dose, estimate plasma clearance values to be 16.8 ± 3.83 (mean ± s.d.) and 18.1 (10-23.8) (median and range) mL/min/kg (Penttila *et al*., 2001; Rautakorpi, *et al*., 1994), closely approximating human hepatic blood flow (Davies & Morris, 1993). Further, these high total clearance estimates exceed the

effective renal blood flow rate in man and rule out the possibility that GLY clearance can be mostly attributed to renal mechanisms.

The upper limits of the 99/95 tolerance intervals for GLY in horse plasma were calculated and reported. 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 (Hornung & Reed, 1990).

GLY urine concentrations were not detectable or were below the LOD in pre-administration urine samples from nineteen out of twenty 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. This finding can likely be attributed to an error in the sample collection process or post-collection processing. It is suspected that the pre-administration sample was inadvertently contaminated with a post-administration sample from a different collection time. A review of the sample collection and aliquot handling procedures has been performed but has 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 (Beal, 2001; Duval & Karlsson, 2002; Ahn *et al*., 2008).

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 (Tasker, 1967; Watson *et al*., 2002; Thurmon *et al*., 1984; Rumbaugh *et al*., 1982). 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 report them in Table 3-7. The renal clearance estimates from 24-168 h ranged from 0.84-3.43 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 GLY is not expected 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 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 hours 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).

Samples used to regulate drugs in horse racing are typically collected in the period from 30-120 min after the end of the race during which a brief period of increased urine flow rate has been observed (Schott *et al*., 1991). 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 to increase proportionately. If this is the case, the urine to plasma concentration ratio would be expected to be unchanged during this period.

Analysis of GLY in plasma samples collected after a 10 mg oral administration resulted in concentrations that would have been undetectable with methods described in the literature, due to its extremely low oral bioavailability as predicted from its permanent ionization. In the only other account, Rautakorpi *et al*. report a median (range) bioavailability of 3.3% (1.3-13.3) following a 50 µg/kg dose in children (Rautakorpi *et al*., 1998). Therefore, a method with greater sensitivity is necessary in order to detect GLY administration by this route for more than a few hours after dosing.

Table 3-1. Demographics of study subjects. Each number represents a horse.

Horse	Gender	Age (yr)	Weight (kg)	IV Administration	Oral Administration	PK Analysis
1	G	9	549	X	X	X
2	G	9	570	X	X	X
3	G	9	546	X	X	X
4	G	9	545	X	X	X
5	G	7	580	X	X	X
6	G	9	518	X	X	X
7	G	7	494	X		
8	G	9	571	X		X
9	G	7	502	X		X
10	G	9	534	X		
11	G	6	602	X		
12	G	6	538	X		
13	G	6	536	X		
14	G	4	495	X		
15	M	5	554	X		
16	M	6	485	X		
17	M	7	602	X		
18	M	6	565	X		
19	M	6	518	X		
20	M	4	515	X		

M- Mare, G-Gelding

Table 3-2. Plasma GLY concentrations (pg/mL) following intravenous administration of 1 mg to each of 20 horses.

	Time (h)						
Horse	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.
Geomean – geometric mean
TI – tolerance interval (99%/95%)

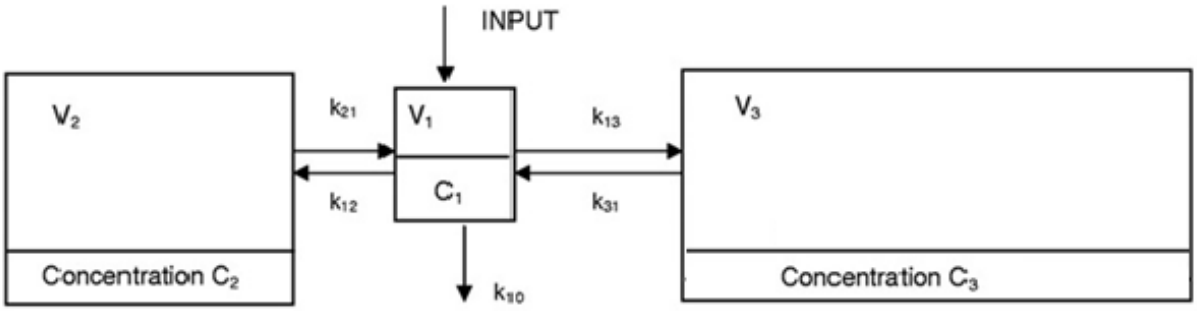

Figure 3-1. Illustration of a three-compartment model.

Table 3-3. Pharmacokinetic parameter estimates of GLY, determined using noncompartmental analysis, following intravenous administration of 1mg to eight (n=8) healthy adult Thoroughbred horses.

	Horse										
Parameter	1	2	3	4	5	6	7	8	Median	Min	Max
λ_z (h^{-1})	0.097	0.066	0.089	0.102	0.084	0.082	0.067	0.054	0.083	0.066	0.102
$t_{1/2}\lambda_z$ (h)	7.14	10.5	7.79	6.78	8.28	8.48	10.4	12.9	8.38	6.78	12.9
C_{max} (ng/mL)	5.48	4.72	4.21	8.27	5.14	4.07	2.43	4.55	4.64	2.43	8.27
C_{last} (ng/mL) $x10^{-3}$	1.11	1.54	1.17	0.860	1.25	0.953	2.25	1.92	1. 21	0.860	2.25
AUC_{0-24} (h*ng/mL)	1.67	1.40	1.38	2.49	1.54	1.43	0.953	1.50	1.46	0.953	2.49
$AUC_{0-\infty}$ (h*ng/mL)	1.68	1.42	1.40	2.50	1.55	1.44	0.987	1.53	1.49	0.987	2.50
V_z (L/kg)	12.5	19.4	14.1	7.56	13.1	14.6	27.9	22.2	14.4	7.56	27.9
Cl_P (mL/min/kg)	20.3	21.3	21.0	12.9	18.3	19.9	31.0	19.9	20.1	12.9	31.0
$AUMC_{0-24}$ (h*h*ng/mL)	1.06	0.963	1.04	0.952	0.990	0.783	1.40	1.20	1.01	0.783	1.40
MRT_{0-24} (h)	0.636	0.688	0.750	0.383	0.644	0.548	1.47	0.798	0.666	0.383	1.47
V_{ss} (L/kg)	1.05	1.68	1.35	0.383	1.08	1.00	5.13	2.10	1.22	0.383	5.12

λ_z, elimination rate constant; $t_{1/2}\lambda_z$, terminal half-life; C_{max}, observed maximum plasma GLY concentration; C_{last}, observed plasma GLY concentration at 24 h; AUC_{0-24}, area under the plasma concentration vs. time curve from time 0 to 24 h; V_z, volume of distribution based on the terminal phase; Cl_P, observed total plasma clearance; $AUMC_{0-24}$, area under the first moment curve from time 0 to 24 h; MRT_{0-24}, mean residence time from time 0 to 24 h; V_{ss}, volume of distribution at steady state;

Table 3-4. Pharmacokinetic parameter estimates of GLY, determined using a three-compartmental model, following intravenous administration of 1 mg to eight (n=8) healthy adult Thoroughbred horses.

	Horse										
Parameter	1	2	3	4	5	6	7	8	Median	Min	Max
A (ng/mL)	9.72	5.96	7.49	27.3	9.24	22.8	4.51	7.32	8.37	4.51	27.3
B (ng/mL)	0.436	0.076	0.371	2.23	0.281	0.954	0.935	0.331	0.404	0.076	2.23
C (ng/mL)	0.012	0.069	0.011	0.014	0.011	0.098	0.015	0.071	0.011	0.069	0.015
Alpha (h^{-1})	9.16	6.77	7.30	17.3	8.73	23.5	10.8	8.86	9.01	6.77	23.5
Beta (h^{-1})	1.73	0.809	1.53	3.86	1.57	2.95	2.45	1.09	1.65	0.809	3.86
Gamma (h^{-1})	0.101	0.063	0.095	0.119	0.092	0.102	0.080	0.056	0.094	0.056	0.119
C_{max} (ng/mL)	10.2	6.04	7.88	29.5	9.54	23.8	5.46	7.66	8.71	5.46	29.5
V_1 (L/kg)	0.201	0.302	0.223	0.065	0.179	0.073	0.336	0.239	0.212	0.065	0.336
K_{21} (h^{-1})	2.05	0.884	1.80	4.88	1.79	3.78	3.89	1.43	1.93	0.884	4.88
K_{31} (h^{-1})	0.110	0.070	0.104	0.125	0.101	0.110	0.098	0.062	0.102	0.062	0.125
K_{10} (h^{-1})	7.08	5.58	5.69	13.0	7.03	17.1	5.58	6.10	6.56	5.58	17.1
K_{12} (h^{-1})	1.12	0.505	0.838	2.60	0.830	4.32	2.58	1.76	1.44	0.505	4.32
K_{13} (h^{-1})	0.628	0.603	0.494	0.668	0.646	1.23	1.19	0.656	0.651	0.494	1.23
K_{10}_HL (h)	0.098	0.124	0.122	0.053	0.099	0.041	0.124	0.114	0.106	0.041	0.124
$t_{1/2\alpha}$ (h)	0.076	0.102	0.095	0.040	0.079	0.030	0.064	0.078	0.077	0.030	0.102
$t_{1/2\beta}$ (h)	0.401	0.857	0.454	0.180	0.441	0.235	0.283	0.635	0.421	0.180	0.857
$t_{1/2\gamma}$ (h)	6.89	11.0	7.28	5.82	7.52	6.77	8.61	12.5	7.40	5.82	12.5
$AUC_{0\text{-}24}$ (h*ng/mL)	1.43	1.08	1.39	2.27	1.36	1.39	0.979	1.26	1.37	0.979	2.27
Cl_P (mL/min/kg)	23.8	28.0	21.1	14.2	20.9	20.7	31.2	24.3	22.4	14.2	31.2
$AUMC_{0\text{-}24}$ (h*h*ng/mL)	1.47	1.98	1.52	1.20	1.52	1.09	2.43	2.65	1.51	1.09	2.65
V_{ss} (L/kg)	1.46	3.07	1.39	0.449	1.41	0.967	4.64	3.08	1.43	0.449	4.64
V_2 (L/kg)	0.110	0.172	0.104	0.035	0.083	0.083	0.222	0.295	0.107	0.035	0.295
V_3 (L/kg)	1.15	2.60	1.06	0.349	1.14	0.812	4.08	2.54	1.15	0.349	4.08

A,B and C, intercepts at t=0 for the model equation; alpha, beta and gamma, slopes for the model equation; C_{max}, extrapolated plasma GLY concentration at time 0; V_1, V_2, V_3, volumes of the central, second and third compartments, respectively; k_{21}, k_{31}, k_{12}, k_{13}, distribution rate constants; k_{10}, elimination rate constant; $t_{1/2\alpha}$, phase 1 half-life; $t_{1/2\beta}$, phase 2 half-life; $t_{1/2\gamma}$, phase 3 half-life; AUC, area under the plasma concentration vs. time curve; Cl_P, total plasma clearance; AUMC, area under the first moment curve; V_{ss}, volume of distribution at steady state.

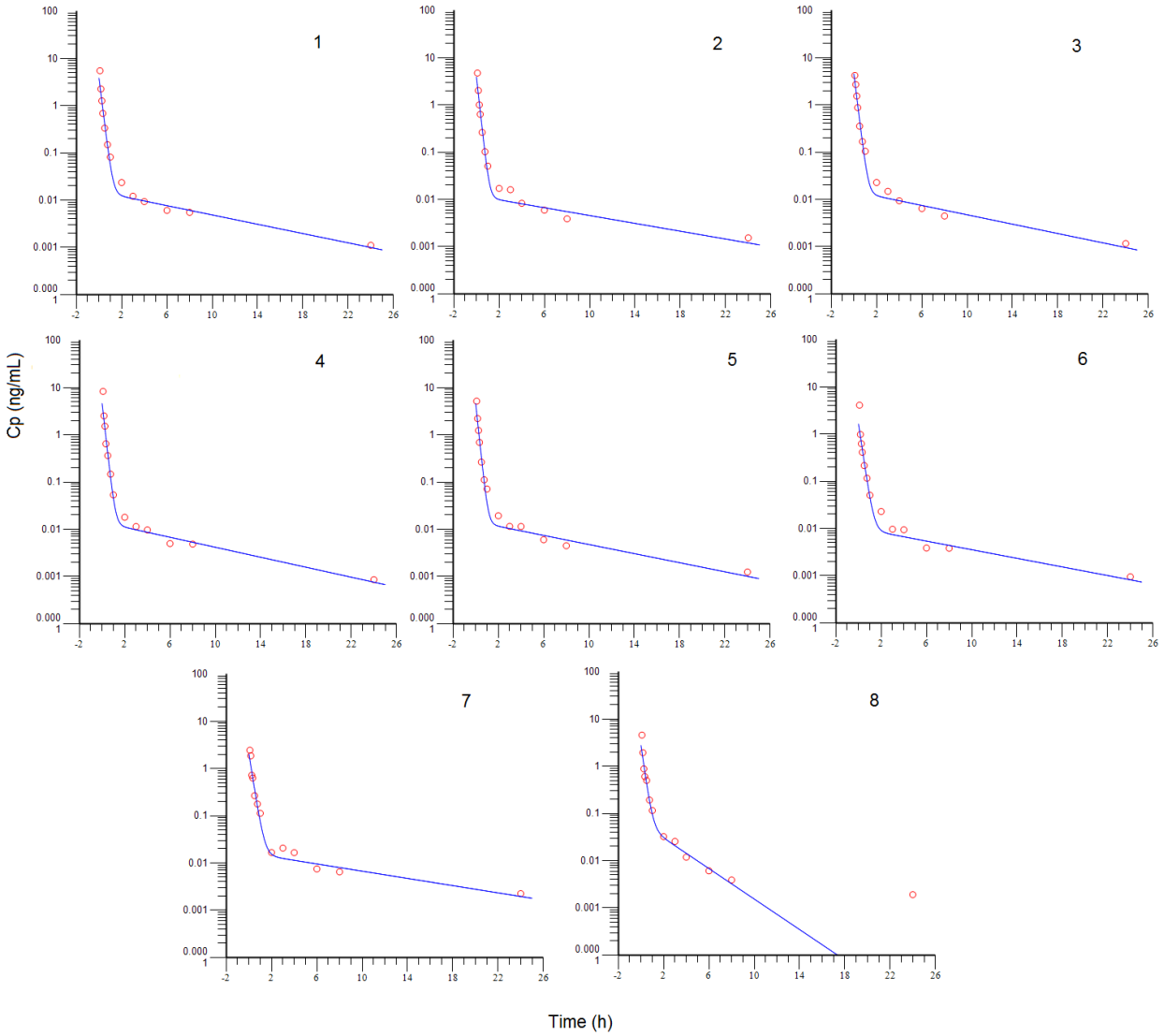

Figure 3-2. Observed (open circles) and the predicted concentrations (line) versus time when a two-compartment model is applied.

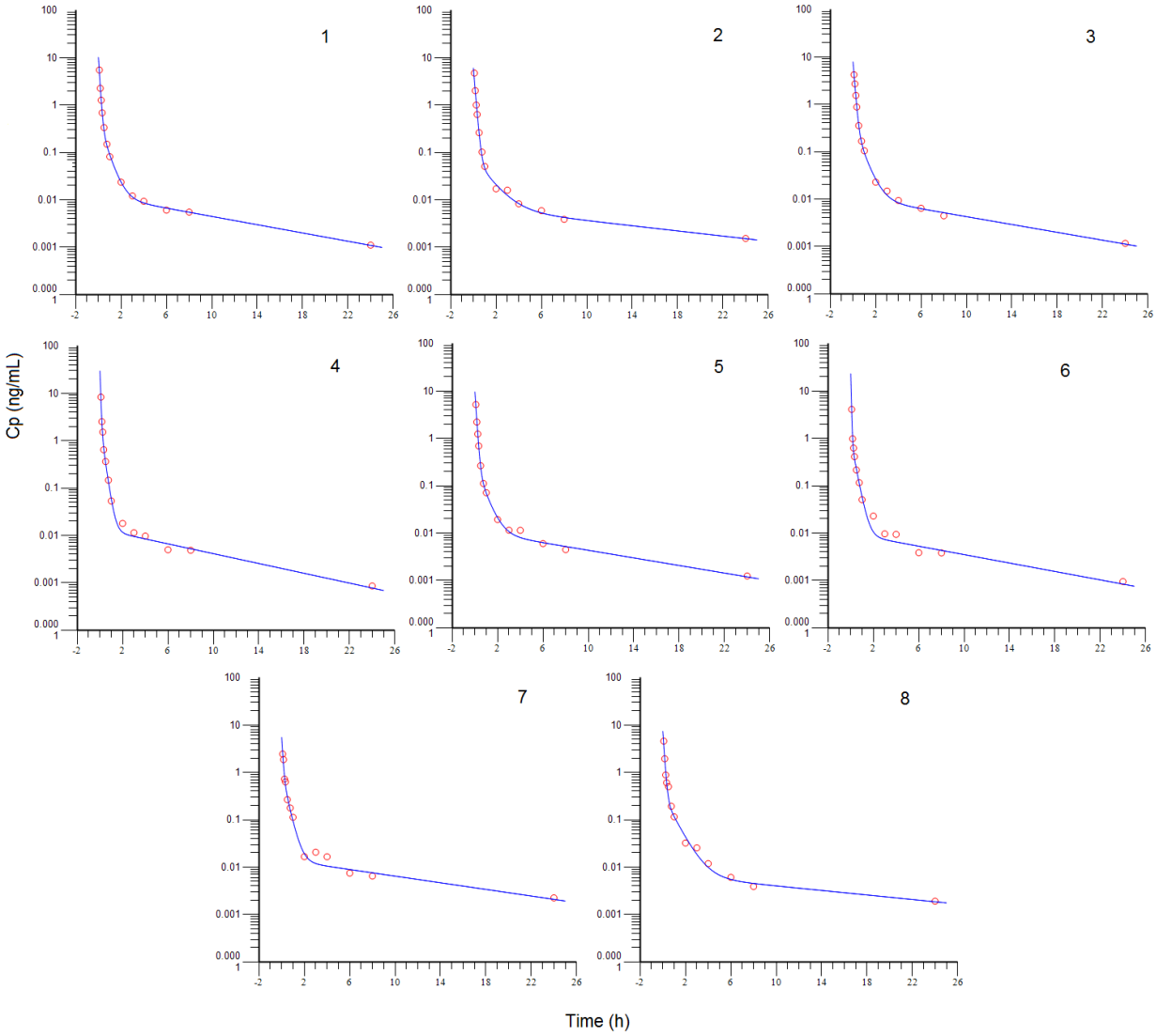

Figure 3-3. Observed (open circles) and the predicted concentrations (line) versus time when a three-compartment model is applied.

Table 3-5. Model comparison using diagnostic criteria.

	2-compartment		3-compartment	
Subject #	AIC	SBC	AIC	SBC
1	-138.8	-136.5	-167.9	-164.5
2	-129.9	-127.6	-143.5	-140.1
3	-137.1	-134.9	-159.1	-155.7
4	-143.4	-141.1	-152.1	-148.8
5	-134.9	-132.6	-155.4	-151.9
6	-138.0	-135.8	-142.4	-139.0
7	-120.3	-118.1	-123.4	-120.1
8	-118.9	-116.6	-129.4	-126.0

AIC – Akaike's Information Criteria
SBC – Schwarz Bayesian Criteria

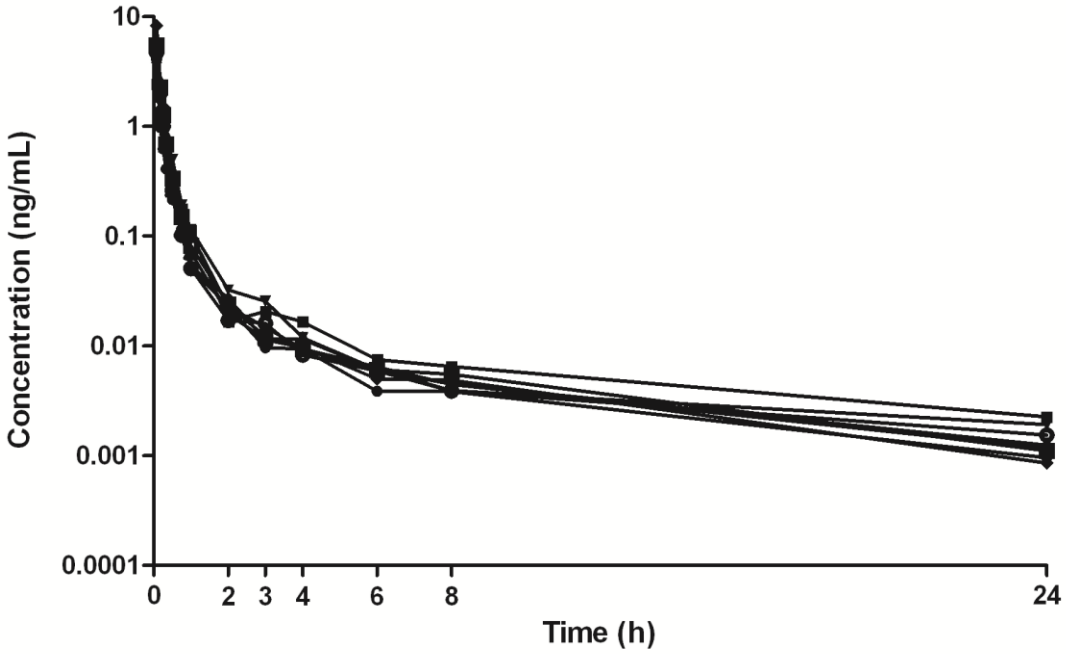

Figure 3-4. Plasma concentration (ng/mL) vs. time (h) data from 0-24 h and for GLY administered intravenously to eight healthy athletic adult Thoroughbreds.

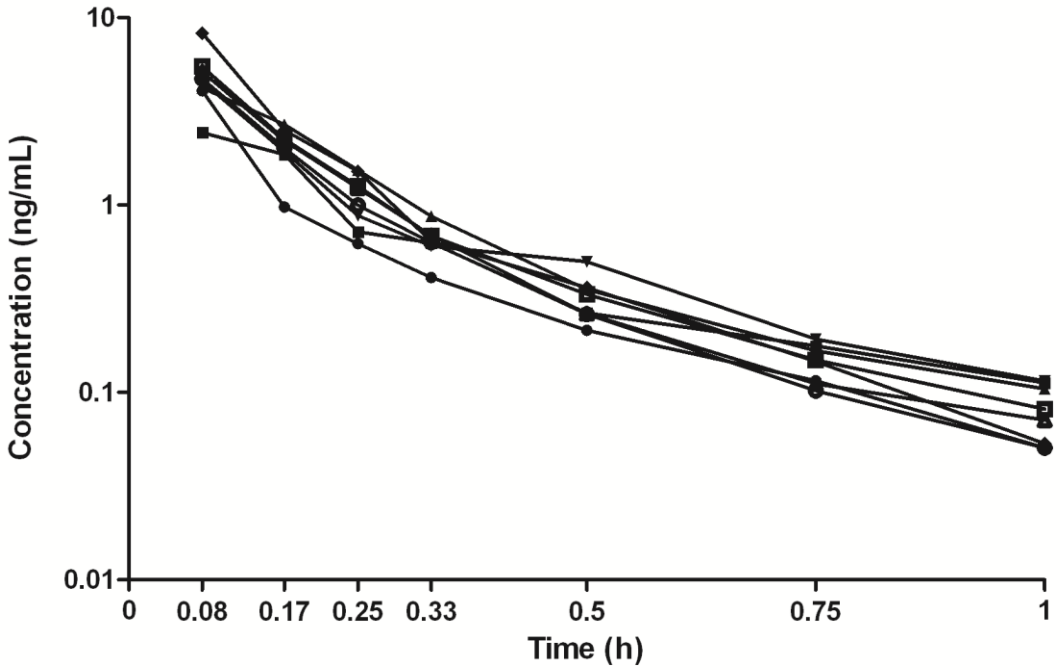

Figure 3-5. Plasma concentration (ng/mL) vs. time (h) data from 0-1 h and for GLY administered intravenously to eight healthy athletic adult Thoroughbreds.

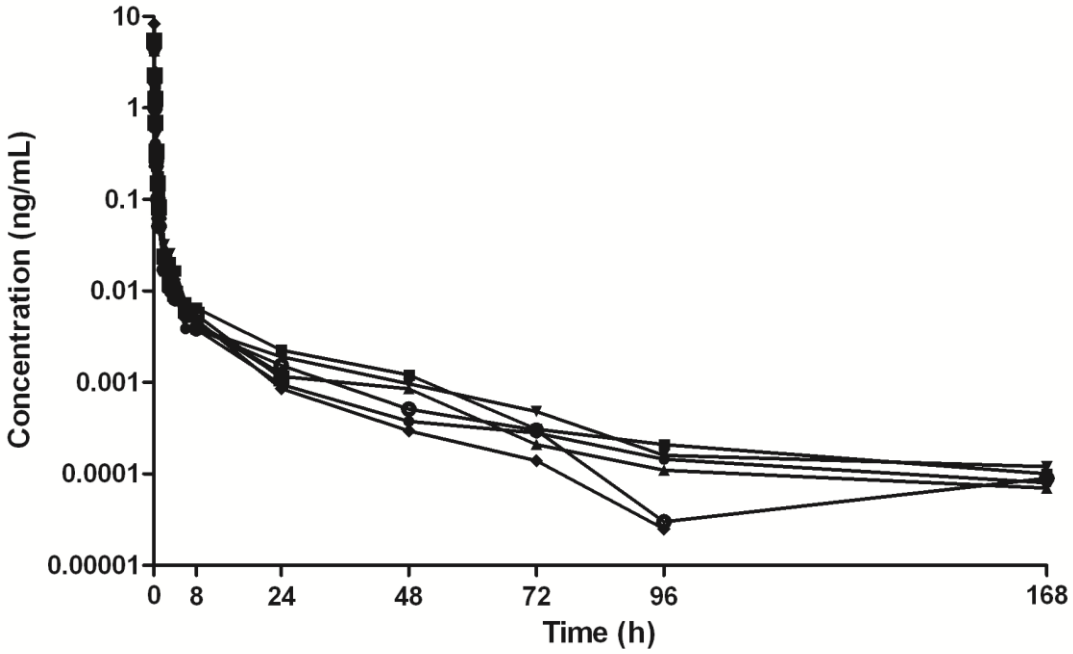

Figure 3-6. Plasma concentration (ng/mL) vs. time (h) data from 0-168 h and for GLY administered intravenously to eight healthy athletic adult Thoroughbreds.

Table 3-6. Urine GLY concentrations (pg/mL) following intravenous administration of 1 mg to each of 20 horses.

	Time (h)						
Horse	4	8	24	48	72	96	168
1	45820	1596	157	71.0	49.8	24.9	8.02
2	5082	794	123	58.1	40.1	23.0	7.03
3	10160	1366	188	101	58.9	34.9	11.3
4	8396	665	104	57.7	27.5	28.5	8.24
5	11790	413	138	71.7	36.4	24.1	6.20
6	30316	482	83.2	45.1	27.9	14.1	4.74
7	25040	1072	154	124	60.6	13.6	12.0
8	23939	1092	117	60.6	32.5	26.8	5.28
9	12932	1631	109	31.2	15.8	10.4	5.70
10	9415	188	79.7	34.1	14.8	13.0	6.25
11	209198	3675	262	93.8	41.7	35.7	12.8
12	68240	1135	80.6	34.0	14.5	10.4	4.86
13	64186	510	103	35.2	16.6	11.4	6.22
14	11939	423	61.5	17.3	10.4	7.06	3.22
15	11121	519	145	69.7	34.1	18.5	7.47
16	34568	2387	196	104	63.3	45.0	14.7
17	173489	9708	132	75.9	34.7	24.3	11.3
18	119981	2049	132	117	45.8	18.7	14.5
19	77124	944	144	80.2	39.6	23.7	10.8
20	43033	2378	113	52.1	25.6	18.8	5.81
Geomean	32276	1073	124	59.6	30.7	19.2	7.66
Median	32442	1082	127	65.1	34.4	20.9	7.25
Min	5082	188	61.5	17.3	10.4	7.06	3.22
Max	209198	9708	262	124	63.3	45.0	14.7
TI (urine)	441837	9232	282	203	108	60.7	20.3

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.

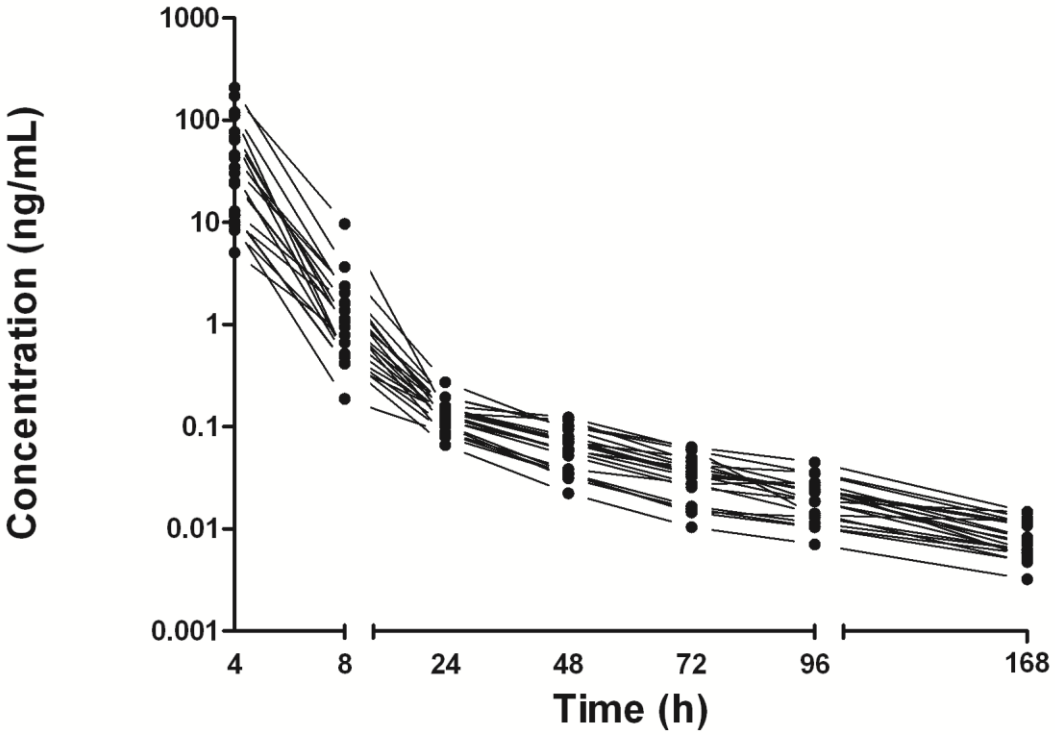

Figure 3-7. Plot of urine concentration (ng/mL) vs. time (h) after intravenous administration of GLY (1 mg) to 20 horses.

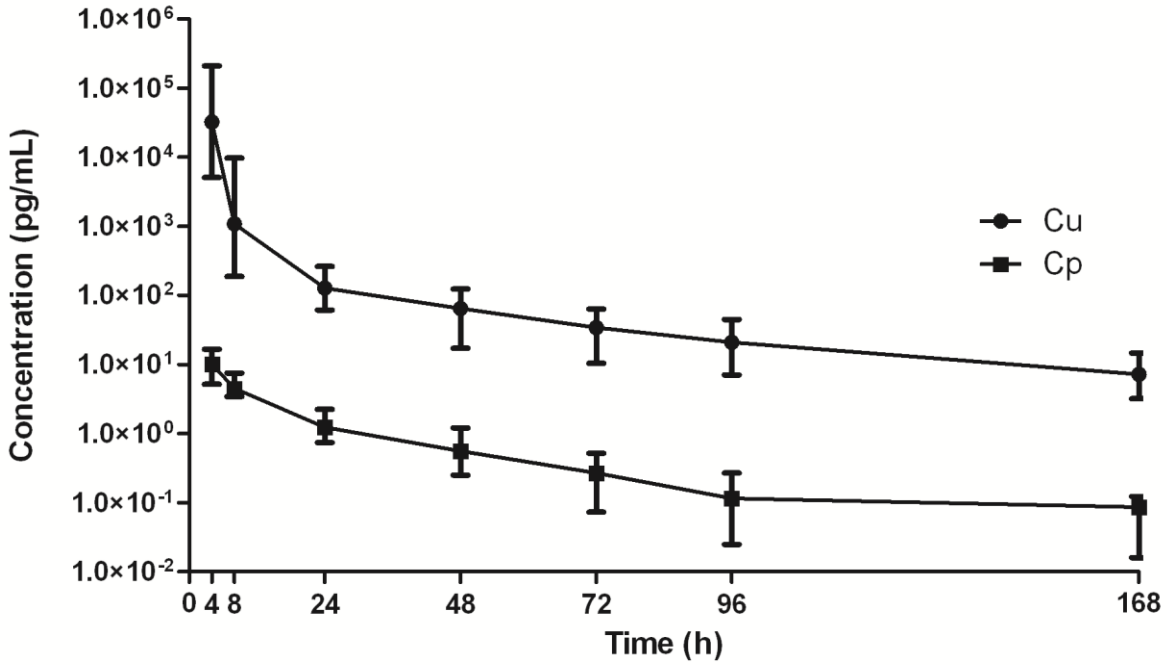

Figure 3-8. Plot of median (range) urine (●) and plasma (■) concentrations for 20 horses administered a single 1 mg intravenous dose of GLY.

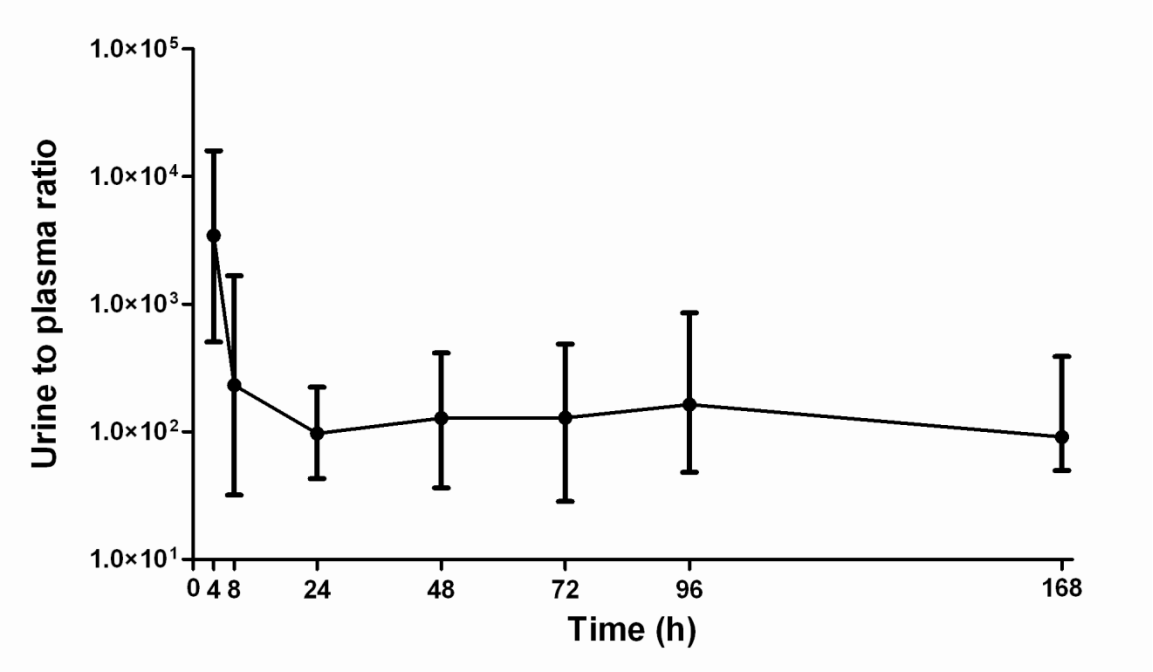

Figure 3-9. Plot of median (range) urine to plasma concentration ratios for 20 horses administered a single 1 mg intravenous dose of GLY.

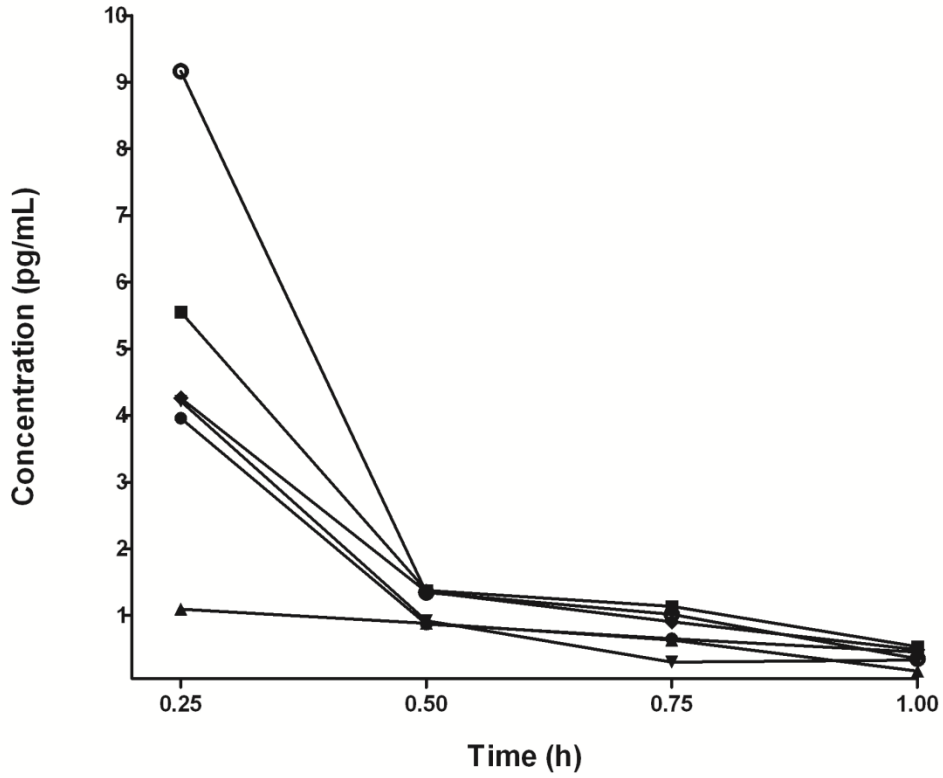

Figure 3-10. Plot of plasma concentration (pg/mL) vs. time (h) after oral administration of GLY (10 mg/mL) to six horses.

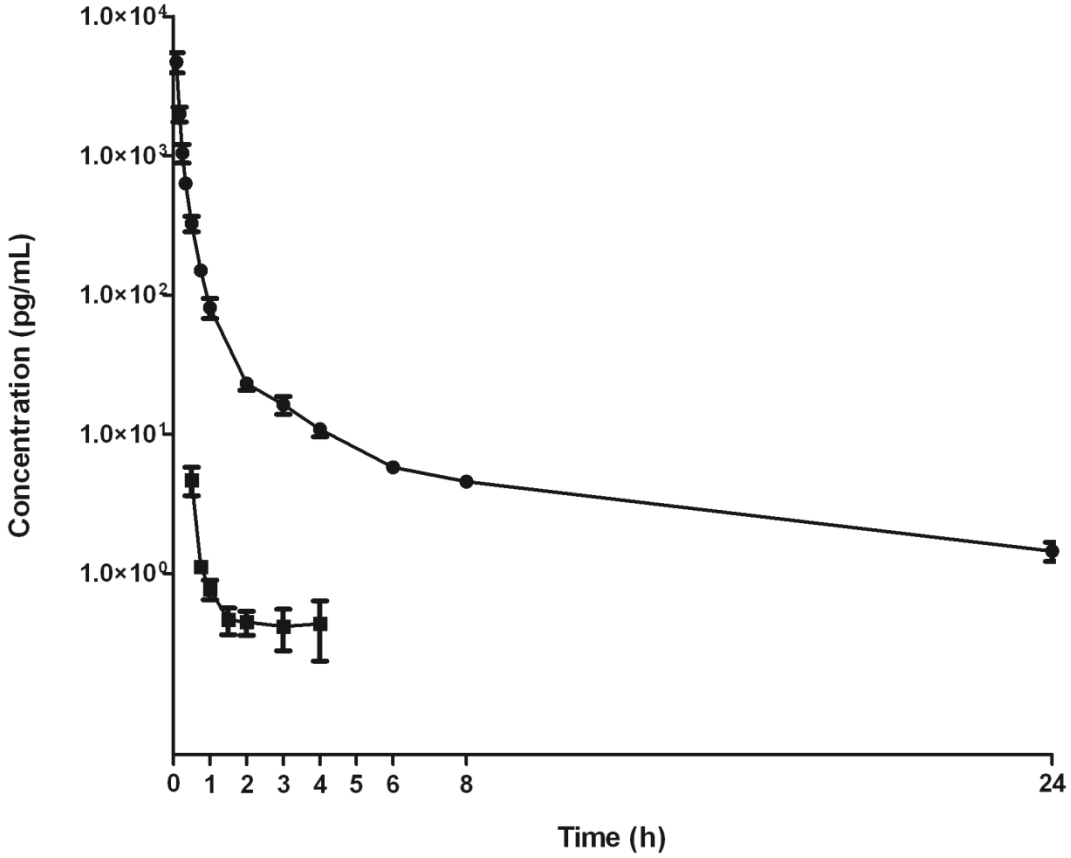

Figure 3-11. Plot of concentration (pg/mL) vs. time (h) of intravenous (●) and orally (■) administered GLY in horse (*n*=6) plasma.

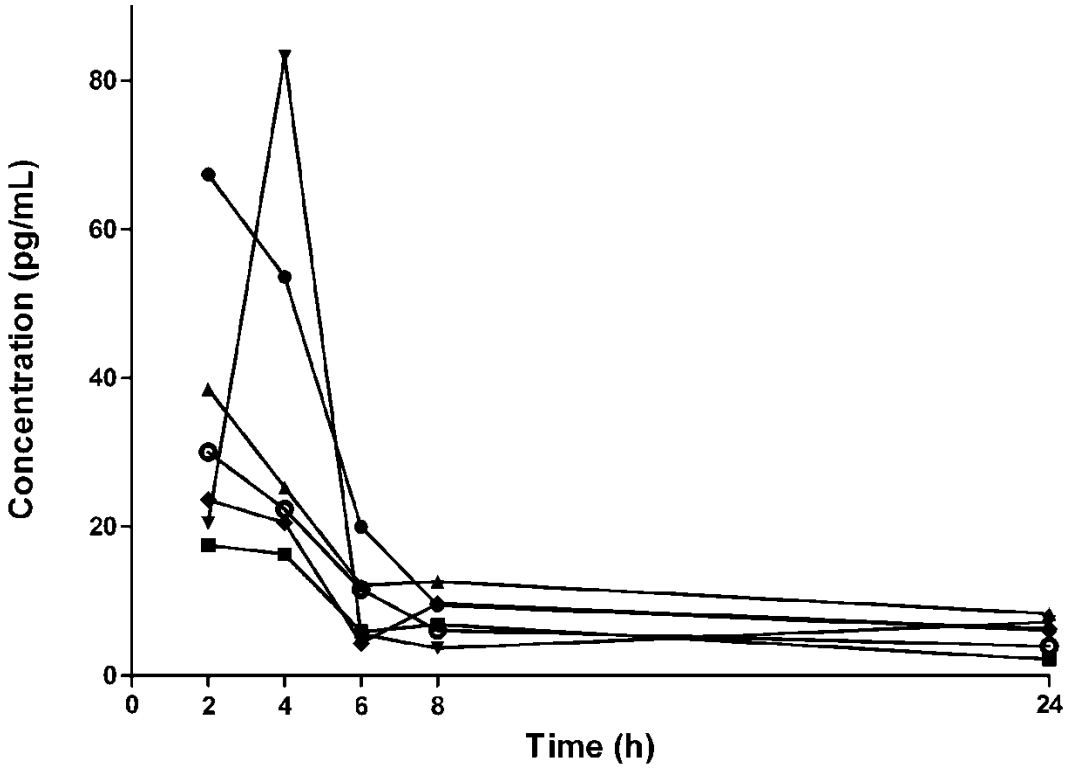

Figure 3-12. Plot of concentration (pg/mL) vs. time (h) of orally administered GLY (10 mg/mL) in horse (n=6) urine.

Table 3-7. Median (range) of estimated renal clearance (mL/min/kg) using the range of urinary flow rate estimates from various reports.

	Urinary Flow Rate (mL/h/kg)			
Time (h)	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)

CHAPTER 4
PHARMACOKINETICS OF GLYCOPYRROLATE IN STANDARDBREDS

We hypothesized that the urinary clearance of unchanged glycopyrrolate (GLY) accounts for a minor fraction of the total clearance because the estimated total plasma clearance is substantially greater than effective renal plasma flow and approaches estimates of hepatic blood flow in the horse. However, our previous studies did not employ an experimental design that would permit estimation of the renal clearance. This requires volumetric urine collections for a specified time period following drug administration. Once this is done, renal clearance may be calculated using several methods, all of which are based upon the rate of unchanged GLY excretion and the plasma GLY concentration.

The presence and degree of plasma esterase activity in the blood may contribute to GLY elimination (Chen & Hsieh, 2005). Compounds containing ester groups, such as GLY, may be particularly susceptible to plasma esterases, a heterogeneous family of enzymes that catalyze the hydrolysis of esters (Satoh *et al*., 2002). Other studies have indicated a minimal inhibitory effect of GLY on plasma cholinesterases in humans, but did not determine the presence or extent of GLY hydrolysis by these or other enzymes (Mirakhur, 1985; Zsigmond *et al*., 1985). The following study will determine whether plasma hydrolysis occurs and the extent to which this pathway contributes to GLY clearance in the horse.

Additionally, this study uses a different horse breed (Standardbred) from the previous (Thoroughbred) study. This will allow a breed comparison of pharmacokinetic disposition and parameter estimates. We hypothesize that there are no major differences in pharmacokinetic disposition between the two horse breeds.

Methods

Animals

Six, healthy, adult, and athletically conditioned Standardbreds (1 mare and 5 geldings) ranging in age from 4-9 years and weighing from 445-510 kg were used in these studies. All horses were fed a diet of commercially available grain mixture and were housed indoors at the UF Veterinary Medical Center in climate-controlled stalls for 1 h before and 24 h after drug administration. From 24 h after administration until the conclusion of the study (168 h) all horses were turned out to grass paddocks 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 study. The conditioning protocol is described in detail in Chapter 2.

Dosing

All horses were administered 1 mg (1.96-2.25 µg/kg) of GLY (glycopyrronium bromide, American Regent, Inc., Shirley, NY, USA) intravenously into the right jugular vein. Demographics for the study animals are presented in Table 3-1.

Specimen Collection

Plasma: Whole blood samples were collected using the procedures described in Chapter 3. Collection times were before drug administration and at 5, 10, 15, 20, 30 and 45 min and 1, 2, 3, 4, 6, 8, 24, 48, 72, 96, and 168 h after intravenous administration.

Urine: 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. Total urine volume was collected through 24 h. Designated collection intervals were every hour. If a subject produced no urine during a collection interval, the next available specimen was taken. Additional urine collections occurred at 48, 72, 96 and 168 h following administration. Urine specimens were

aliquoted into 15-mL sterile, disposable, polypropylene centrifuge tubes and stored at -20°C immediately and at -80°C within 48 h.

Plasma Esterase Stability

One hundred milliliters of venous blood was collected from a 3 yr old gelding into tubes containing lithium heparin (BD Vacutainer, 10 mL, Becton Dickinson, Franklin Lakes, NJ, USA). The horse had been drug-free for the previous 30 days and was considered healthy, based upon physical examination, complete blood count, serum chemistry profile, and plasma fibrinogen concentration.

Fresh harvested plasma was obtained within 1 h of blood collection through centrifugation of the blood samples for 15 min at 2,000 *g*. All plasma was pooled and the pH was adjusted to 7.4, if necessary. Stock and working standard solutions of GLY and GLY-d_3 were prepared according to the procedures outlined above in Chapter 2. Calibrators and positive controls were prepared according to Table 2-1 using the fresh plasma obtained for this experiment.

For each of fourteen timepoints 4 mL of fresh plasma was aliquoted to a CryoTube™ vial (Nunc, Roskilde, Denmark). The tubes were fortified with 32 µL of 0.00125 ng/µL GLY working standard solution for a final concentration of 10 pg/mL. The zero time sample was immediately capped and flash frozen using liquid nitrogen and stored at -20°C until analysis. The remaining samples were capped and incubated in a water bath at 38°C for 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min, flash frozen in liquid nitrogen upon removal and stored in -20°C until analyzed. All samples were analyzed in duplicate according to the LC-MS/MS method outlined in Chapter 2.

Pharmacokinetic Analysis

Nonlinear least squares regression analysis was performed on plasma GLY concentration versus time data and pharmacokinetic parameters for all horses were estimated with compartmental analysis using Phoenix WinNonlin® 6.1 (Pharsight, St. Louis, MO, USA). The Gauss–Newton (Levenberg and Hartley) method was used and goodness of fit and the appropriate weighting factor were selected based on the coefficients of variation, Akaike's Information Criterion (AIC) (Yamaoka *et al*., 1978) and Schwarz's Bayesian Criterion (SBC) (Schwarz, 1978) as well as visual analysis of the graphical output (including residual plots). Secondary parameters calculated include area under the curve (AUC), terminal half-life ($t_{1/2\gamma}$), apparent volumes of distribution, total plasma clearance (Cl_p), and micro distribution rate constants. All calculations for pharmacokinetic parameters were based on methods described by Gibaldi and Perrier (Gibaldi & Perrier, 1982). All pharmacokinetic parameter estimates were calculated for each horse and values are reported as median and range (minimum-maximum).

Statistical Analysis

Plots of urine and plasma concentrations were performed using GraphPad Prism™ version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) and plasma and urine concentrations of GLY are expressed as mean and standard deviation. Mann-Whitney U and Kruskall-Wallis rank-sum tests were used for statistical comparisons (nonparametric) of pharmacokinetic parameters between breeds (Powers, 1990; Hollander & Wolfe, 1973). Analysis of variance (ANOVA) was used for parametric analysis. A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using JMP Version 8.0 (SAS Institute Inc., Cary, NC, USA)

Results

Plasma: After intravenous administration of 1 mg of GLY, the observed plasma concentration versus time profile could be best described by a three-compartment model. The equation based on macro constants for this model is:

$$C_t = A exp^{-\alpha \cdot t} + B exp^{-\beta \cdot t} + C exp^{-\gamma \cdot t} \quad (4\text{-}1)$$

where C_t is the plasma concentration at time (t), A, B and C are the zero time intercepts for the first, second and third phases. Further, α, β and γ are the exponential terms for each phase and *exp* is the base of the natural logarithm (Gabrielsson & Weiner, 2007). The weighting factor chosen with this model was 1/ (Y^2) where Y was the observed plasma concentration. The model was chosen based on visual inspection of the observed and predicted concentration versus time graphs for a two (Figure 4-1) and three (Figure 4-2) compartment analysis and other diagnostic criteria (Table 4-2). Plasma GLY concentration vs. time plots for all six horses are depicted in Figure 4-3 and Figure 4-4. The drug concentrations remained above the method's LLOQ (0.125 pg/mL) for all horses through 48 h and only two horses 168 h after dosing (Figure 4-5). Mean (SD) plasma concentrations are presented in Table 4-3

The median (range) C_{max} in six Standardbreds following a 1 mg intravenous dose of GLY was 12.4 (10.0-19.0) ng/mL. Median (range) estimates for plasma clearance (Cl_p), volume of distribution of the central compartment (V_1) and area under the plasma concentration-time curve (AUC_{0-24}) were 16.7 (13.6-21.7) mL/min/kg, 0.167 (0.103-0.215) L/kg, and 2.13 (1.67-2.57) ng*h/mL, respectively. Estimates for all of the pharmacokinetic parameters following compartmental model analysis are reported in Table 4-4.

The median (range) values for pharmacokinetic parameter estimates for Standardbreds and Thoroughbreds are presented in Table 4-5. The AUC_{0-24}, MRT, and terminal half-life of the Standardbred horses were significantly different when compared to those of six Thoroughbreds

(Table 4-6). Figure 4-6 illustrates the distribution of twelve horses separated by breed for each of six PK parameter estimates. Statistical comparisons were made using the Mann Whitney test (Powers, 1990).

With regard to plasma hydrolysis, the measured concentrations of GLY in plasma incubated at 38 degrees for various times did not differ from the fortified concentrations by more than 10% and the GLY concentrations of all incubated samples differed from the zero-time sample by < 5%. Average concentrations of GLY for duplicate analysis at the end of each incubation period through 360 min ranged from 9.05–10.3 pg/mL (Figure 4-7).

Urine: For five of six horses, urine GLY concentrations peaked at the first collection interval. Complete urine results for each horse are provided in Table 4-7. The total GLY accumulated urinary excretion theough 24 h for six horses had a median (range) of 0.140 (0.113-0.246) mg or 14 (11.3-24.6)% of the total administered dose of 1 mg. Cumulative urinary excretion of GLY for each horse is shown in Figure 4-8. Greater than 95% of GLY that was renally cleared was excreted within 4 h after intravenous administration (Figure 4-9). The urinary GLY excretion rate is plotted for each horse in Figure 4-10. Median (range) renal clearance for all six horses was 2.65 (1.92-3.59) mL/min/kg. Renal clearance represented approximately 11.3-24.7 % of the total plasma clearance.

Discussion

The pharmacokinetic profile of a single intravenous administration of GLY in Standardbred horses followed a tri-exponential decay, similar to its disposition in the Thoroughbred horse (Figure 4-11). A three-compartment model compared to a two-compartment model was a superior model fit based on graphical inspection and lower diagnostic values for both AIC and SBC. GLY disposition was characterized by a rapid drop in plasma concentrations

beginning immediately after administration, a small volume of distribution and a slow terminal elimination attributed to the redistribution of GLY from the third compartment to the central compartment.

Although the Thoroughbred and Standardbred horses showed a similar GLY disposition following intravenous administration, differences in some pharmacokinetic parameter estimates were evident when compared using a nonparametric rank sum test. The difference in AUC_{0-24} was attributed to the larger CL_T exhibited in Standardbreds (16.7 (13.6-21.7) mL/min/kg) compared to Thoroughbreds (22.4 (14.2-31.2) mL/min/kg). While an identical dose was used for both studies, a reason for the difference in peak plasma concentrations is unknown other than the wide degree of subject variability for parameter estimates in both studies. Additionally, while differences in drug distribution between donkeys and horses have been reported for phenylbutazone (Mealey *et al*., 1997), guaifenesin (Matthews *et al*., 1997) and sulphamethoxasole (Peck *et al*., 2002) there is little data detailing pharmacological differences, if any, between Standardbred and Thoroughbred horse breeds.

The cumulative excretion of unchanged GLY in horse urine indicates that less than 25% of the total dose is cleared through renal mechanisms. This contrasts with pharmacokinetic studies in humans that have reported over 80% (Kaltiala *et al*, 1974) and nearly 50 % (Ali-Melkkila *et al*., 1990) of the intramuscularly administered dose was excreted unchanged in the urine. Consistent with the present research, however, the latter study in humans reported that unchanged GLY was eliminated within three hours of administration. The amount remaining to be excreted (ARE) plots (Figure 4-9) demonstrate that, for this study, over 95% of the unchanged GLY was excreted within 4 h after drug administration. However, it is recognized that ARE calculations are subject to error if complete urine collections are not made. Since this study relied

on the "free-catch" method of collection and did not use catheterization, collection volumes may not be completely accurate due to incomplete bladder emptying. Yet the cumulative excretion remains unaffected by this limitation.

Another method for analyzing urine data, for half-life ($t_{1/2}$) and elimination rate constant (k_{el}), is to plot the average rate of excretion against the time, which in most cases is best represented by the midpoint of the collection interval. The quality of the excretion rate plot has limitations, namely an uncertainty of complete bladder emptying and the need to collect accurately timed urine samples over short intervals, relative to the elimination half-life of the drug (Gibaldi, 1986). However, a significant advantage of the rate of excretion plot is that each data point is essentially independent, especially if the bladder is fully voided for each sample. A missed sample or data points is not critical to this analysis. The GLY excretion rate plots (Figure 4-10) showed a large degree of scatter when displayed on a semi-log plot.

Renal clearance of a particular drug can be determined by several methods, all of which are based on the relationship between the excretion rate and the plasma concentration as demonstrated in Equation 4-2.

$$\frac{dAu}{dt} = Cl_R \cdot C_p \tag{4-2}$$

where Au is the amount of drug in the urine, t is the time interval, Cl_R is the renal clearance and C_p is the plasma concentration. As shown in the equation above, renal clearance is the constant of proportionality between the rate of excretion and the plasma concentration. Thus, a plot of the rate of excretion against the corresponding plasma concentration yields a straight line with a slope equal to renal clearance. Renal clearance is calculated, using the rate of excretion method for each time interval in Table 4-8.

The maximum rate at which GLY can be filtered by the glomerulus is the glomerular filtration rate (GFR). Estimates of the GFR can be obtained by measuring creatinine because the substance is filtered but neither secreted nor reabsorbed. Creatinine clearance is calculated similar to renal clearance in the equation above (4-2) using plasma and urine creatinine measurements and the urine volume taken in the time interval (Table 4-8). We have determined the creatinine clearance to range between 1.8-2.0 mL/min/kg. This indicates that secretion of GLY from the blood into the tubular lumen is likely because estimates for GLY renal clearance are higher than the GFR (creatinine clearance). Reabsorption of unchanged GLY from the tubular lumen back into the blood via passive diffusion is unlikely due to the compound's polarity. Therefore, this data suggests that GLY undergoes renal excretion through glomerular filtration and tubular secretion.

Plasma esterase activity appeared to have little or no effect on the concentration of GLY, ruling out the possibility that a substantial proportion of the drug was being hydrolyzed in the plasma (La Du, 1972), such as the case with other ester containing compounds (Lehner *et al*., 2000; Jordan *et al*., 1996; Egan, 1995). Knowing this and in light of the urinary GLY excretion data, it can be hypothesized that the majority of non-renal clearance is hepatic clearance, and further, the majority of total clearance is attributed to hepatic clearance as demonstrated using the following equation (Wilkinson *et al*., 1987).

$$\mathrm{Cl} = \mathrm{Cl_R} + \mathrm{Cl_H} + \mathrm{Cl_{other}} \qquad (4\text{-}3)$$

where Cl is the total body clearance, $\mathrm{Cl_R}$ is the renal clearance, $\mathrm{Cl_H}$ is the hepatic clearance, and $\mathrm{Cl_{other}}$ represents the sum of all other clearance processes. Thus hepatic clearance may be estimated from this study by subtracting $\mathrm{Cl_R}$ from Cl, assuming that $\mathrm{CL_{other}}$ is relatively small. Total plasma clearance in Standardbreds was characterized by a median (range) of 16.7

(13.6-21.7) mL/min/kg. Therefore, using the renal clearance estimated from this study of 2.65 (1.92-3.59) mL/min/kg, non-renal clearance was approximately 14.1 (11.7-18.1) mL/min/kg.

The median (range) estimates of total plasma clearance in both Thoroughbreds (22.7 (14.2-31.2) mL/min/kg) and Standardbreds (16.7 (13.6-21.7) mL/min/kg) closely approximates previous estimates of liver blood flow in the horse (15-20 mL/min/kg) (Dyke *et al*., 1998). Under such conditions whereby the plasma clearance is equivalent to the hepatic blood flow, the drug is said to be highly extracted by the liver (Toutain & Bousquest-Molou, 2004).

$$E_H = \frac{Cl_H}{Q} \tag{4-4}$$

where Cl_H is the hepatic blood clearance, Q is the liver blood flow, and E_H is the hepatic extraction ratio, a term representing the fraction of dose undergoing metabolism and thus irreversibly removed. Additionally, for drugs with a high extraction ratio, changes in liver blood flow are a major determinant of hepatic clearance. Also, the degree of, or changes in the protein binding of GLY have little influence on hepatic clearance of drugs with a high extraction ratio. In the horse, GLY is extensively metabolized in the liver by hydrolysis of the ester moiety to 2-cyclopentyl-2-hydroxy-2-phenylacetic acid (cyclopentylmandelic acid) and 1,1-dimethyl-3-hydroxypyrrolidine-1-ium. Although not statistically different it's possible that the decrease in total plasma clearance observed for Standardbreds compared to Thoroughbreds was due to decreased liver blood flow as a result of confinement (anxiety) in an indoor stall for 24 h during the study.

Table 4-1. Demographics of Standardbred study subjects. Each number represents a horse.

Horse	Gender	Age (yr)	Weight (kg)	IV Administration	Total Urine Collection	PK Analysis
1	M	4	445	X	X	X
2	G	7	508	X	X	X
3	G	8	510	X	X	X
4	G	9	457	X	X	X
5	G	4	450	X	X	X
6	G	4	478	X	X	X

M- Mare, G-Gelding

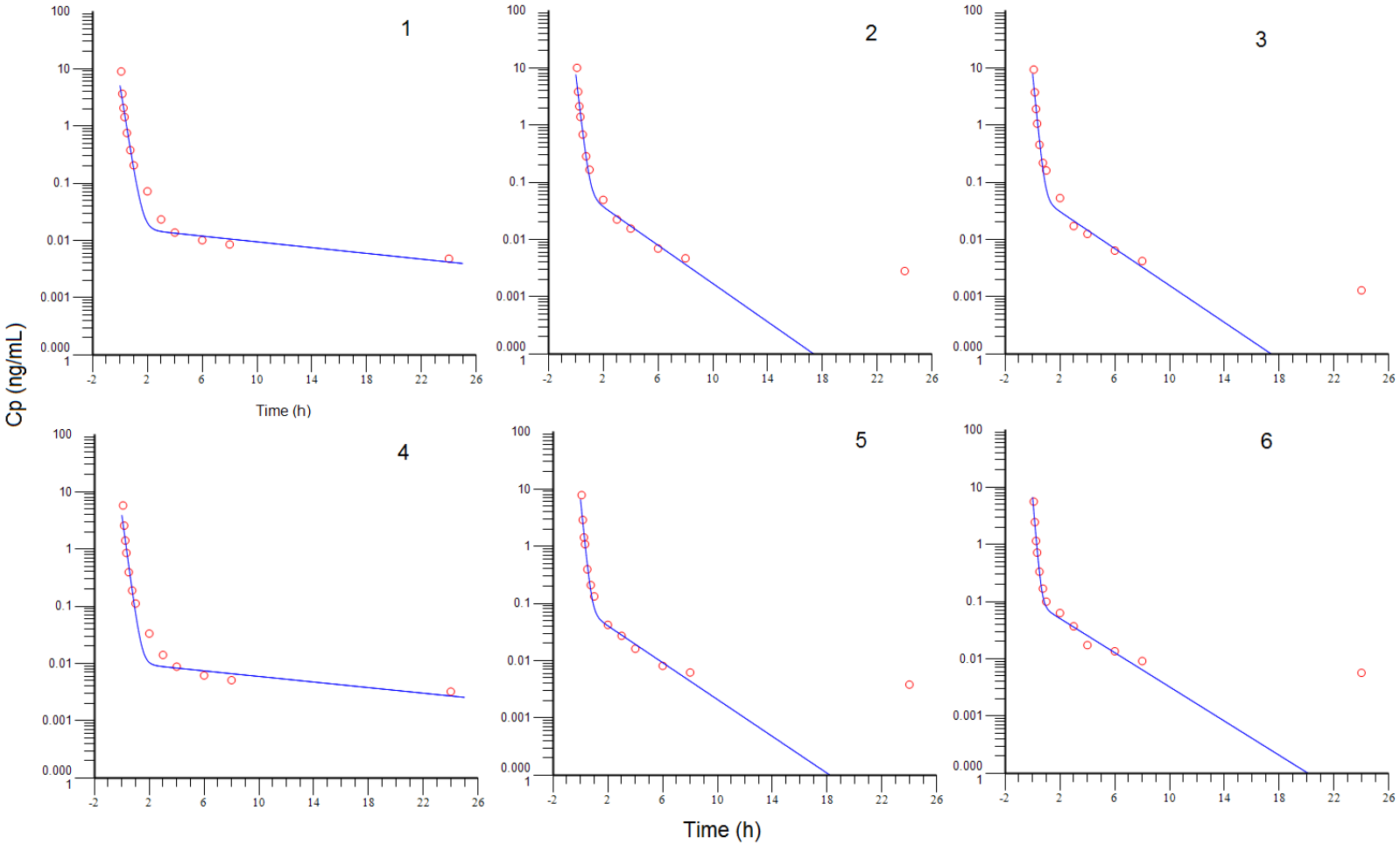

Figure 4-1. Observed concentrations (open circles) and the predicted concentrations (line) versus time when a two-compartment model is applied to six Standardbred horses.

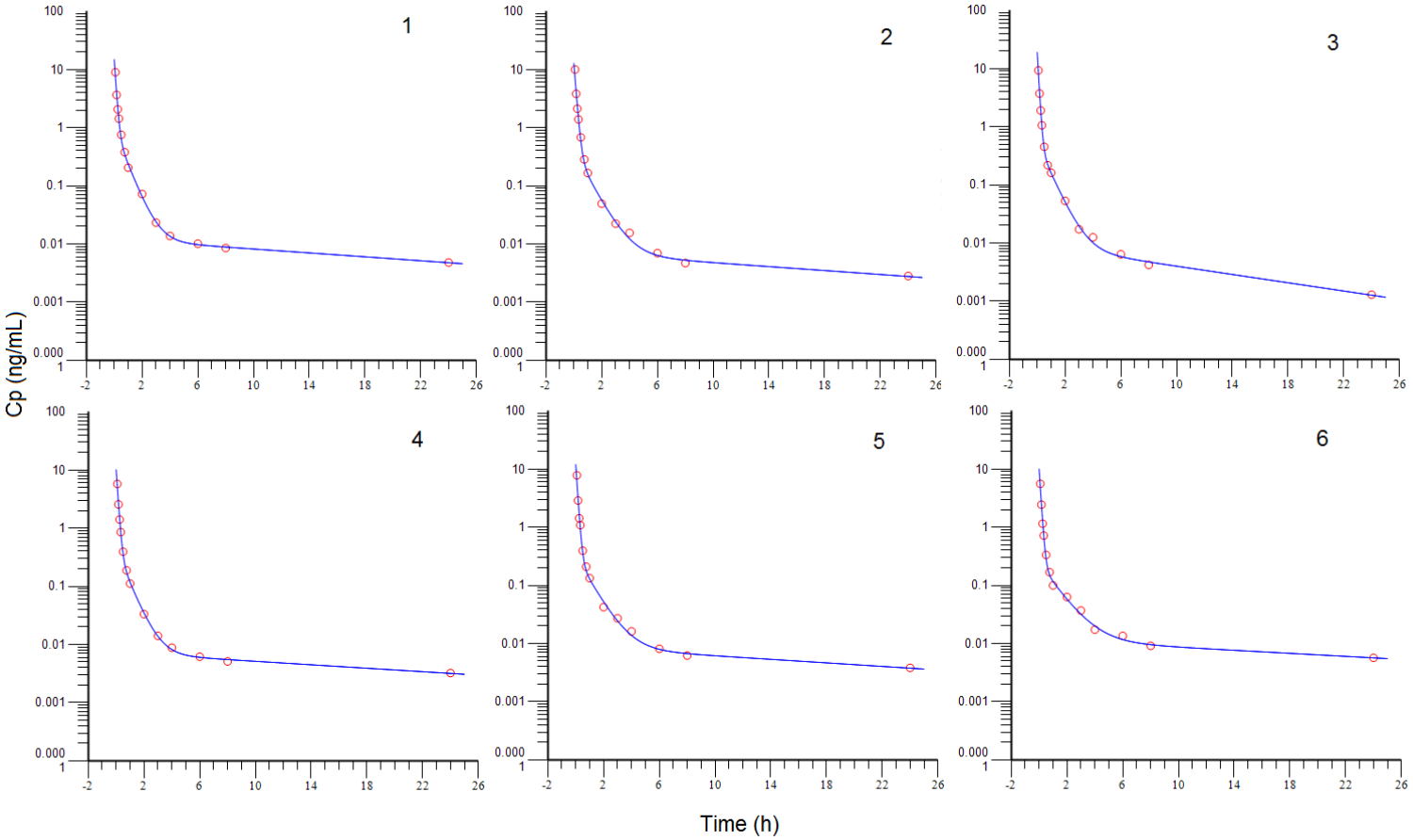

Figure 4-2. Observed (open circles) and the predicted concentrations (line) versus time when a three-compartment model is applied to six Standardbred horses.

Table 4-2. Diagnostic values for a three-compartment model fit to the concentration vs. time data for each horse.

	2-compartment		3-compartment	
Subject #	AIC	SBC	AIC	SBC
1	-101.7	-99.4	-129.6	-126.2
2	-111.8	-109.5	-132.6	-129.2
3	-124.7	-122.4	-159.5	-156.1
4	-112.3	-109.9	-152.7	-149.2
5	-103.8	-101.6	-125.5	-122.1
6	-93.9	-91.6	-120.1	-116.7

AIC – Akaike's Information Criteria
SBC – Schwarz Bayesian Criteria

Table 4-3. Plasma concentration of GLY after a single intravenous dose of 1 mg to each of 6 Standardbred horses.

Time (h)	Mean ± SD (ng/mL)	Range (ng/mL)
0	< LOD	<LOD
0.08	8.13 ± 0.549	7.44-8.93
0.17	3.44 ± 0.491	2.94-4.38
0.25	1.70 ± 0.259	0.899-1.38
0.33	1.17 ± 0.172	0.485-0.622
0.50	0.565 ± 0.048	0.292-0.433
0.75	0.361 ± 0.060	0.142-0.284
1	0.199 ± 0.054	0.047-0.179
2	0.117 ± 0.057	0.020-0.033
3	0.028 ± 0.006	0.015-0.018
4	0.016 ± 0.001	0.006-0.017
6	0.010 ± 0.004	0.006-0.012
8	0.008 ± 0.002	0.003-0.006
24	0.004 ± 0.001	0.001-0.005
48	0.003 ± 0.001	0.001-0.004
72	0.003 ± 0.001	0.001-0.004
96	0.002 ± 0.001	0.001-0.003
168	0.002 ± 0.001	0.001-0.004

Time 0 = immediately before drug administration.

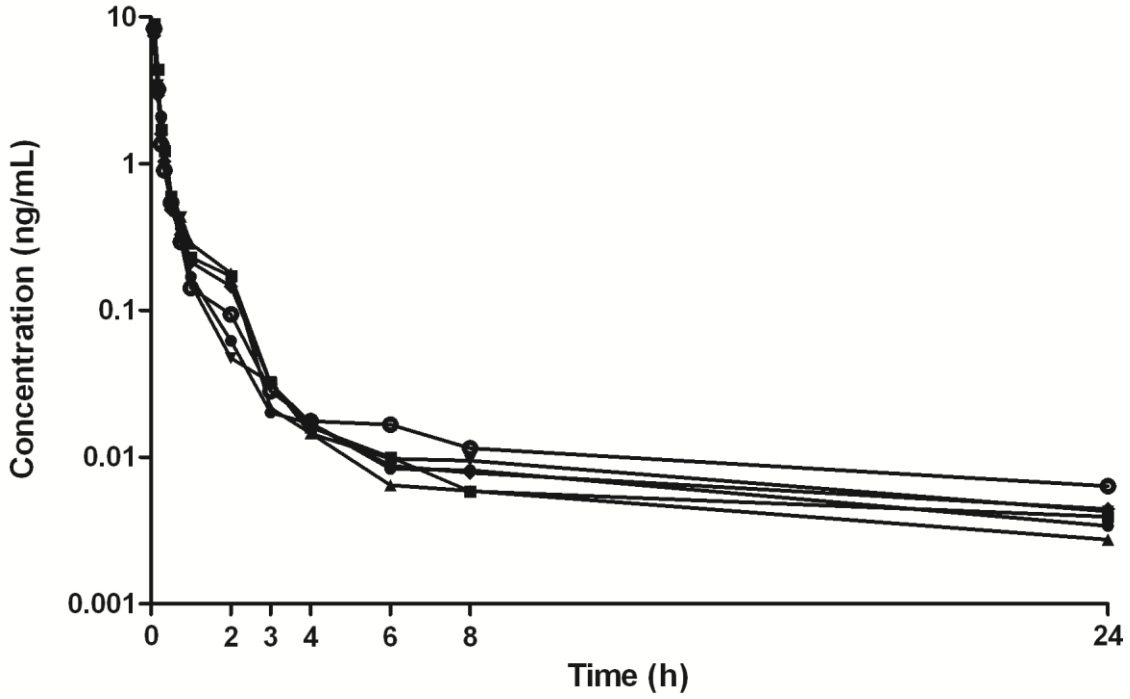

Figure 4-3. Plasma concentration (ng/mL) vs. time (h) data from 0-24 h and for GLY administered intravenously to six healthy athletic adult Standardbreds.

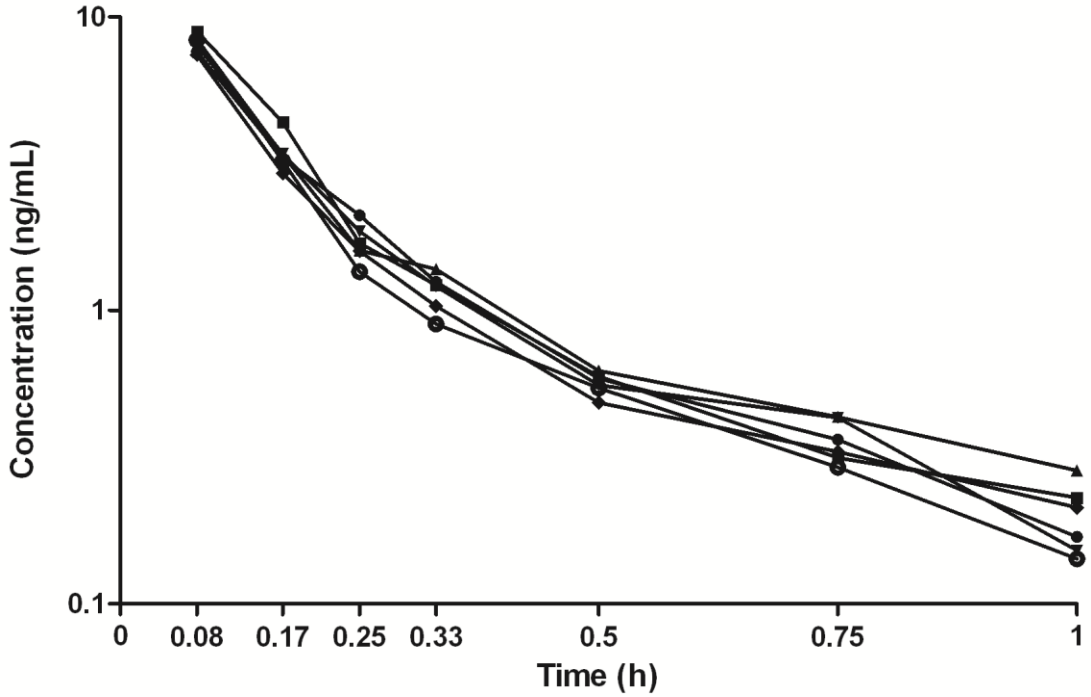

Figure 4-4. Plasma concentration (ng/mL) vs. time (h) data from 0-1 h and for GLY administered intravenously to six healthy athletic adult Standardbreds.

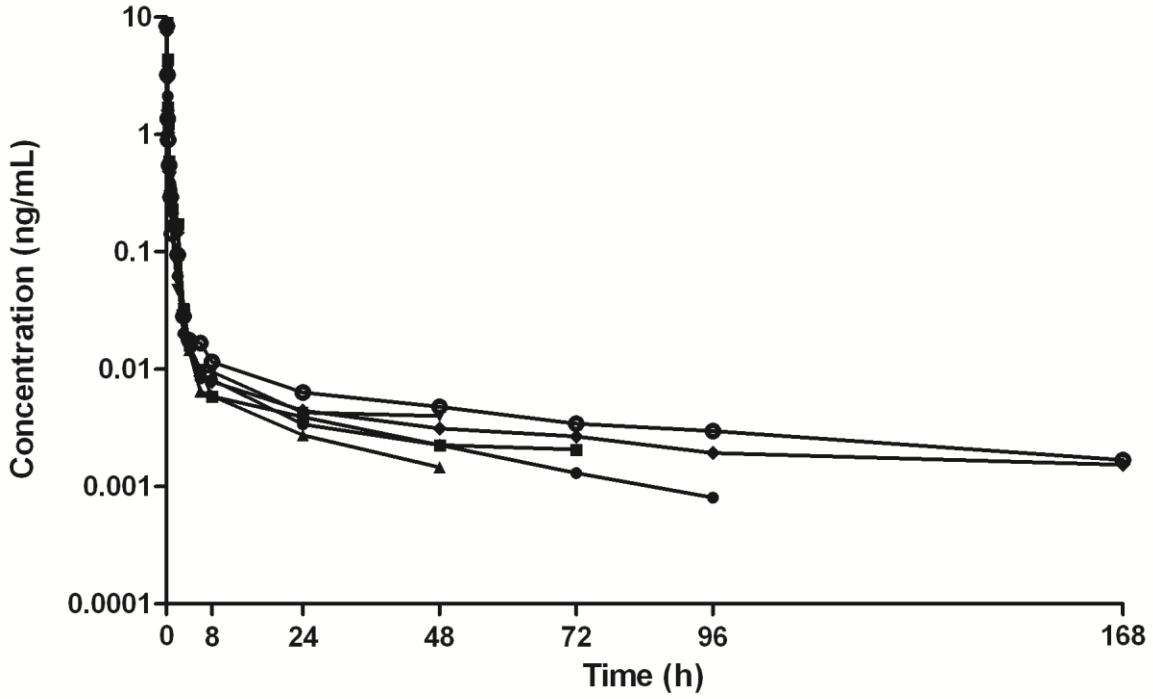

Figure 4-5. Plasma concentration (ng/mL) vs. time (h) data from 0-168 h and for GLY administered intravenously to six healthy athletic adult Standardbreds.

Table 4-4. Pharmacokinetic parameter estimates of GLY, determined using a three-compartmental model, following intravenous administration of 1 mg to six (n=6) healthy adult Standardbred horses.

	Horse						Median	Min	Max.
Parameter	1	2	3	4	5	6			
A (ng/mL)	13.9	12.3	18.5	9.70	11.7	9.78	12.0	9.70	18.5
B (ng/mL)	0.912	0.401	0.521	0.419	0.313	0.229	0.410	0.229	0.912
C (ng/mL)	0.012	0.007	0.009	0.007	0.009	0.012	0.009	0.007	0.012
Alpha (h^{-1})	8.61	7.06	9.88	8.431	8.39	8.75	8.52	7.06	9.88
Beta (h^{-1})	1.41	1.04	1.24	1.35	0.978	0.791	1.14	0.791	1.41
Gamma (h^{-1})	0.039	0.039	0.082	0.034	0.035	0.030	0.037	0.030	0.082
C_{max} (ng/mL)	14.8	12.7	19.0	10.1	12.0	10.0	12.4	10.0	19.0
V_1 (L/kg)	0.152	0.154	0.103	0.216	0.185	0.209	0.167	0.103	0.215
K_{21} (h^{-1})	1.85	1.23	1.48	1.64	1.17	0.975	1.36	0.975	1.85
K_{31} (h^{-1})	0.044	0.042	0.086	0.039	0.040	0.038	0.041	0.038	0.086
K_{10} (h^{-1})	5.76	5.509	7.92	6.05	6.11	5.58	5.91	5.51	7.92
K_{12} (h^{-1})	1.63	0.90	1.35	1.22	1.21	1.48	1.29	0.904	1.63
K_{13} (h^{-1})	0.776	0.456	0.368	0.859	0.873	1.50	0.817	0.368	1.50
K_{10}_HL (h)	0.120	0.126	0.088	0.114	0.113	0.124	0.117	0.088	0.126
$t_{1/2\alpha}$ (h)	0.080	0.098	0.070	0.082	0.083	0.079	0.081	0.070	0.098
$t_{1/2\beta}$ (h)	0.492	0.666	0.557	0.514	0.709	0.876	0.612	0.492	0.876
$t_{1/2\gamma}$ (h)	17.9	17.7	8.46	20.2	19.8	23.1	18.9	8.46	23.1
AUC_{0-24} (h*ng/mL)	2.57	2.31	2.40	1.67	1.96	1.80	2.13	1.67	2.57
Cl_T (mL/min/kg)	14.5	14.1	13.6	21.7	18.9	19.4	16.7	13.6	21.7
$AUMC_{0-24}$ (h*h*ng/mL)	8.73	5.23	1.88	6.54	7.62	13.5	7.08	1.88	13.5
V_{ss} (L/kg)	2.96	1.92	0.640	5.10	4.41	8.73	3.69	0.640	8.73
V_2 (L/kg)	0.133	0.113	0.094	0.160	0.191	0.317	0.146	0.094	0.317
V_3 (L/kg)	2.68	1.66	0.443	4.72	4.03	8.20	3.36	0.443	8.20

A,B and C, intercepts at t=0 for the model equation; alpha, beta and gamma, slopes for the model equation; C_{max}, extrapolated plasma GLY concentration at time 0; V_1, V_2, V_3, volumes of the central, second and third compartments, respectively; k_{21}, k_{31}, k_{12}, k_{13}, distribution rate constants; k_{10}, elimination rate constant; $t_{1/2\alpha}$, phase 1 half-life; $t_{1/2\beta}$, phase 2 half-life; $t_{1/2\gamma}$, phase 3 half-life; AUC, area under the plasma concentration vs. time curve; Cl_P, total plasma clearance; AUMC, area under the first moment curve; V_{ss}, volume of distribution at steady state.

Table 4-5. Comparison of pharmacokinetics parameters of Standardbreds and Thoroughbreds.

	Standardbreds			Thoroughbreds		
Parameter	Median	Min	Max	Median	Min	Max
A (ng/mL)	12.0	9.70	18.5	8.37	4.51	27.3
B (ng/mL)	0.410	0.229	0.912	0.404	0.076	2.23
C (ng/mL)	0.009	0.007	0.012	0.011	0.069	0.015
Alpha (h^{-1})	8.52	7.06	9.88	9.01	6.77	23.5
Beta (h^{-1})	1.14	0.791	1.41	1.65	0.809	3.86
Gamma (h^{-1})	0.037	0.030	0.082	0.094	0.056	0.119
C_{max} (ng/mL)	12.4	10.0	19.0	8.71	5.46	29.5
V_1 (L/kg)	0.170	0.103	0.215	0.212	0.065	0.336
K_{21} (h^{-1})	1.36	0.975	1.85	1.93	0.884	4.88
K_{31} (h^{-1})	0.041	0.038	0.086	0.102	0.062	0.125
K_{10} (h^{-1})	5.90	5.51	7.92	6.56	5.58	17.1
K_{12} (h^{-1})	1.28	0.904	1.63	1.44	0.505	4.32
K_{13} (h^{-1})	0.817	0.368	1.50	0.651	0.494	1.23
K_{10}_HL (h)	0.117	0.088	0.126	0.106	0.041	0.124
$t_{1/2\alpha}$ (h)	0.081	0.070	0.098	0.077	0.030	0.102
$t_{1/2\beta}$ (h)	0.612	0.492	0.876	0.421	0.180	0.857
$t_{1/2\gamma}$ (h)	18.9	8.46	23.1	7.40	5.82	12.5
AUC_{0-24} (h*ng/mL)	2.13	1.67	2.57	1.37	0.979	2.27
Cl_p (mL/min/kg)	16.7	13.6	21.7	22.4	14.2	31.2
$AUMC_{0-24}$ (h*h*ng/mL)	7.08	1.88	13.5	1.51	1.09	2.65
MRT (h)	3.64	0.784	7.50	1.43	0.449	4.64
V_{ss} (L/kg)	3.69	0.640	8.73	0.107	0.035	0.295
V_2 (L/kg)	0.146	0.094	0.317	1.15	0.349	4.08
CLD2	3.92	2.32	5.15	8.37	4.51	27.3
V_3 (L/kg)	3.36	0.443	8.20	0.404	0.076	2.23
CLD3	2.33	0.634	5.21	0.011	0.069	0.015

Min-minimum (*n*=6)
Max-maximum (*n*=6)

Table 4-6. Calculated p-values using the Mann-Whitney U test for comparisons of pharmacokinetic parameter estimates between horse breeds.

PK Parameter Estimate	*p*-value	Significance
Cl_p	0.065	No
$AUC_{0\text{-}24}$	0.015	Yes
V_1	0.394	No
C_{max}	0.392	No
MRT	0.041	Yes
$t_{1/2\gamma}$	0.015	Yes

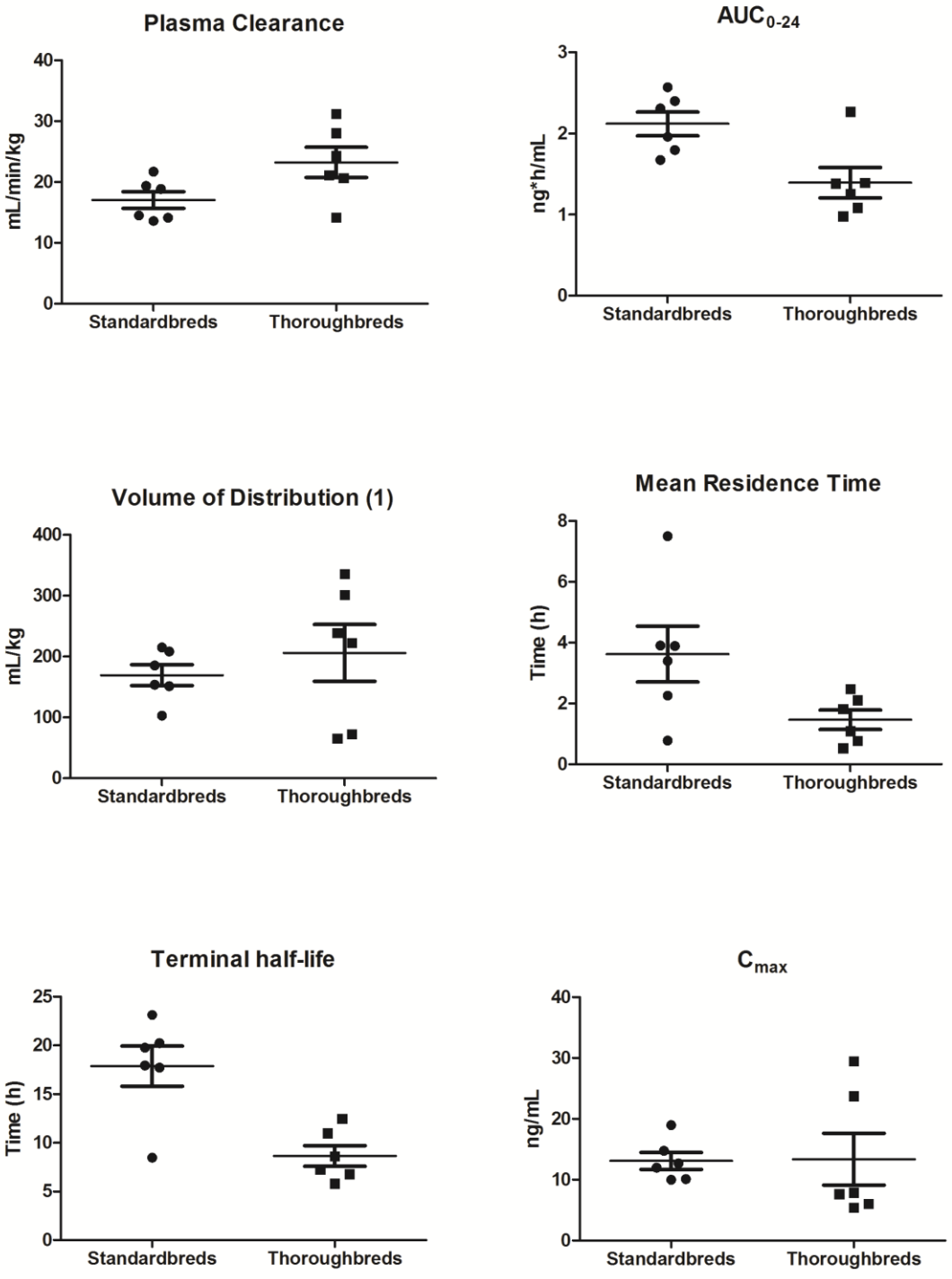

Figure 4-6. Distribution of PK parameter estimates in Standardbreds (*n*=6) and Thoroughbreds (*n*=6).

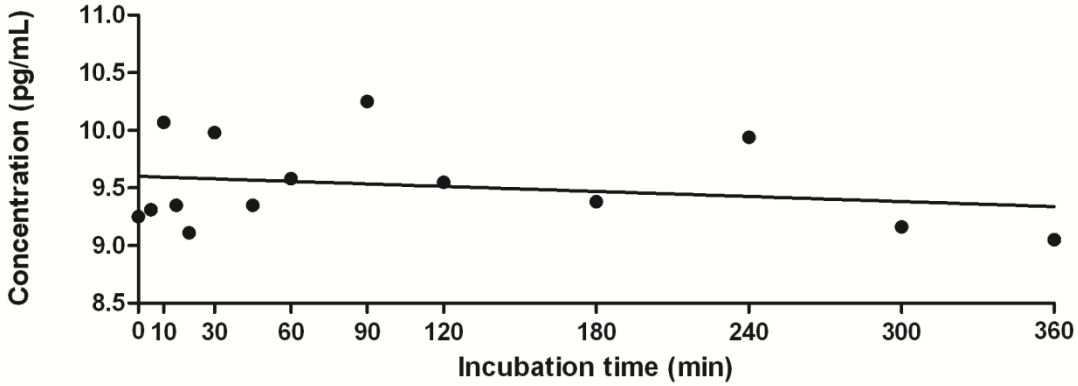

Figure 4-7. Linear regression of concentration vs. incubation time demonstrating the degree of plasma esterase activity on GLY in horse plasma.

Table 4-7. Urine GLY excretion data following intravenous administration of 1 mg to eight (n=6) Standardbred horses.

Time Interval (h)	Δt (h)	Urine Volume (mL)	Urine Concentration (ng/mL)	Amount Excreted ΔU (µg)	Cumulative Amount Excreted (µg)	Midpoint time (h)	Rate of excretion (ΔU/Δt) (µg/h)	A.R.E. (µg)
Subject 1								
0.5-1	0.5	950	176.6	167.7	167.7	0.75	335.5	78.3
1-2	1.0	40	103.0	4.12	171.9	1.5	4.12	74.1
2-3	1.0	720	94.2	67.8	239.7	2.5	67.8	6.33
3-4	1.0	90	26.1	2.35	242.0	3.5	2.35	3.98
4-6	2.0	80	8.64	0.692	242.7	5	0.346	3.29
8-12	4.0	750	2.46	1.84	244.6	10	0.461	1.44
12-16	4.0	1270	0.797	1.01	245.6	14	0.253	0.430
16-20	4.0	480	0.687	0.330	245.9	18	0.082	0.100
20-24	4.0	180	0.557	0.100	246.0	22	0.025	0.000
Subject 2								
0-0.5	0.5	440	189.5	83.4	83.4	0.25	166.8	113.5
0.5-1	0.5	460	86.1	39.6	123.0	0.75	79.3	73.9
1-2	1.0	1780	32.1	57.1	180.1	1.5	57.1	16.7
2-3	1.0	600	15.9	9.53	189.7	2.5	9.53	7.21
4-6	2.0	570	6.37	3.63	193.3	5	1.81	3.59
8-12	4.0	740	2.38	1.76	195.1	10	0.440	1.83
12-16	4.0	1270	0.916	1.16	196.2	14	0.291	0.662
16-20	4.0	800	0.565	0.452	196.7	18	0.113	0.210
20-24	4.0	470	0.447	0.210	196.9	22	0.053	0.000
Subject 3								
0.5-1	0.5	1040	18.7	19.5	19.5	0.75	39.0	121.8
1-2	1.0	740	27.2	20.1	39.6	1.5	20.1	101.6
2-3	1.0	1370	29.5	40.4	80.0	2.5	40.4	61.3
3-4	1.0	2210	26.9	59.4	139.4	3.5	59.4	1.83
8-12	4.0	720	0.922	0.664	140.1	10	0.166	1.17
16-20	4.0	2750	0.339	0.931	141.0	18	0.233	0.237
20-24	4.0	1720	0.138	0.237	141.3	22	0.059	0.000

Table 4-7. Continued

Time Interval (h)	Δt (h)	Urine Volume (mL)	Urine Concentration (ng/mL)	Amount Excreted ΔU (µg)	Cumulative Amount Excreted (µg)	Midpoint time (h)	Rate of excretion (ΔU/Δt) (µg/h)	A.R.E. (µg)
Subject 4								
0.5-1	0.5	860	125.6	108.0	108.0	0.75	216.1	14.6
1--2	1.0	240	36.2	8.70	116.7	1.5	8.70	5.88
4--6	2.0	570	7.45	4.249	121.0	5	2.12	1.63
8--12	4.0	950	0.781	0.742	121.7	10	0.185	0.892
12--16	4.0	940	0.642	0.604	122.3	14	0.151	0.289
16-20	4.0	680	0.239	0.162	122.5	18	0.041	0.126
20-24	4.0	780	0.162	0.126	122.6	22	0.032	0.000
Subject 5								
0-0.5	0.5	140	345.0	48.3	48.3	0.25	96.6	90.4
0.5-1	0.5	250	286.6	71.7	120.0	0.75	143.3	18.8
1-2	1.0	150	65.5	9.83	129.8	1.5	9.83	8.95
2-3	1.0	170	26.3	4.48	134.3	2.5	4.48	4.48
3-4	1.0	90	13.5	1.21	135.5	3.5	1.21	3.27
4-6	2.0	350	4.72	1.65	137.1	5	0.827	1.61
8-12	4.0	830	0.698	0.579	137.7	10	0.145	1.03
12-16	4.0	860	0.503	0.432	138.2	14	0.108	0.600
16-20	4.0	820	0.317	0.260	138.4	18	0.065	0.341
20-24	4.0	1240	0.275	0.341	138.8	22	0.085	0.000
Subject 6								
0-0.5	0.5	1140	69.9	79.7	79.7	0.25	159.3	33.7
0.5-1	0.5	490	37.4	18.3	98.0	0.75	36.7	15.3
8-12	4.0	1870	7.89	14.8	112.8	10	3.69	0.564
12-16	4.0	650	0.412	0.268	113.0	14	0.067	0.297
16-20	4.0	620	0.242	0.150	113.2	18	0.038	0.146
20-24	4.0	760	0.193	0.146	113.3	22	0.037	0.000

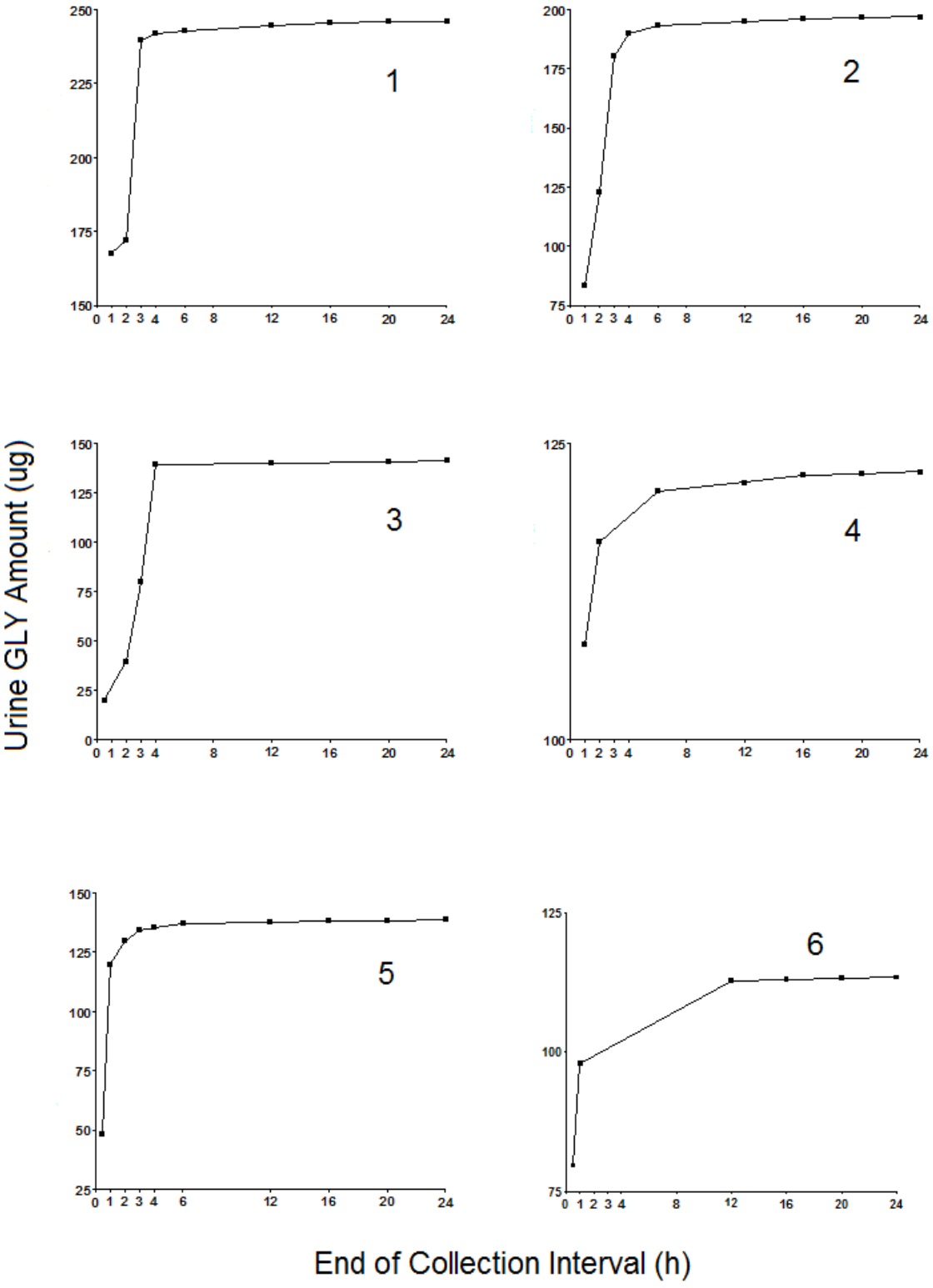

Figure 4-8. Cumulative GLY excretion in six horses administered 1 mg of GLY intravenously.

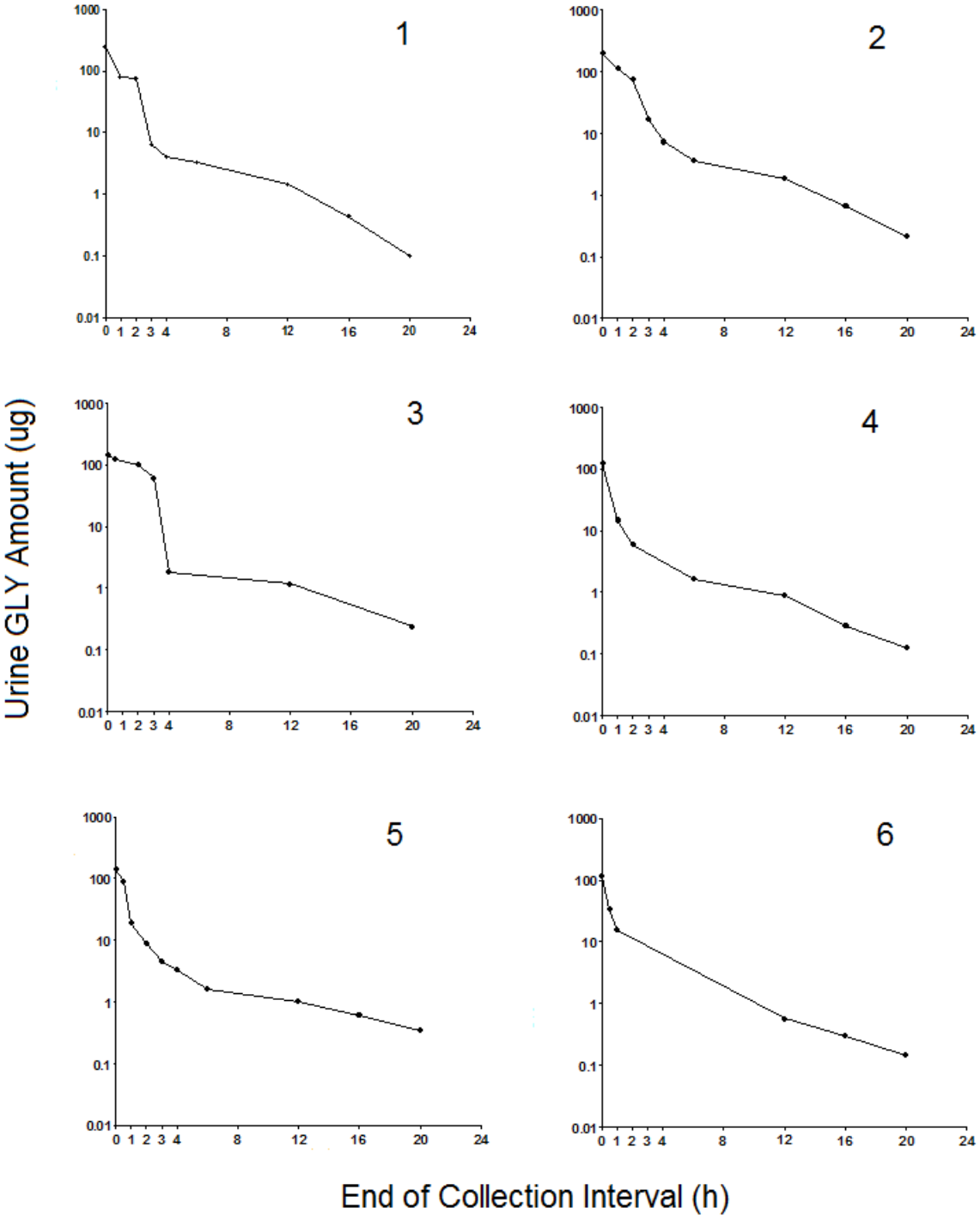

Figure 4-9. Amount remaining to be excreted (ARE).

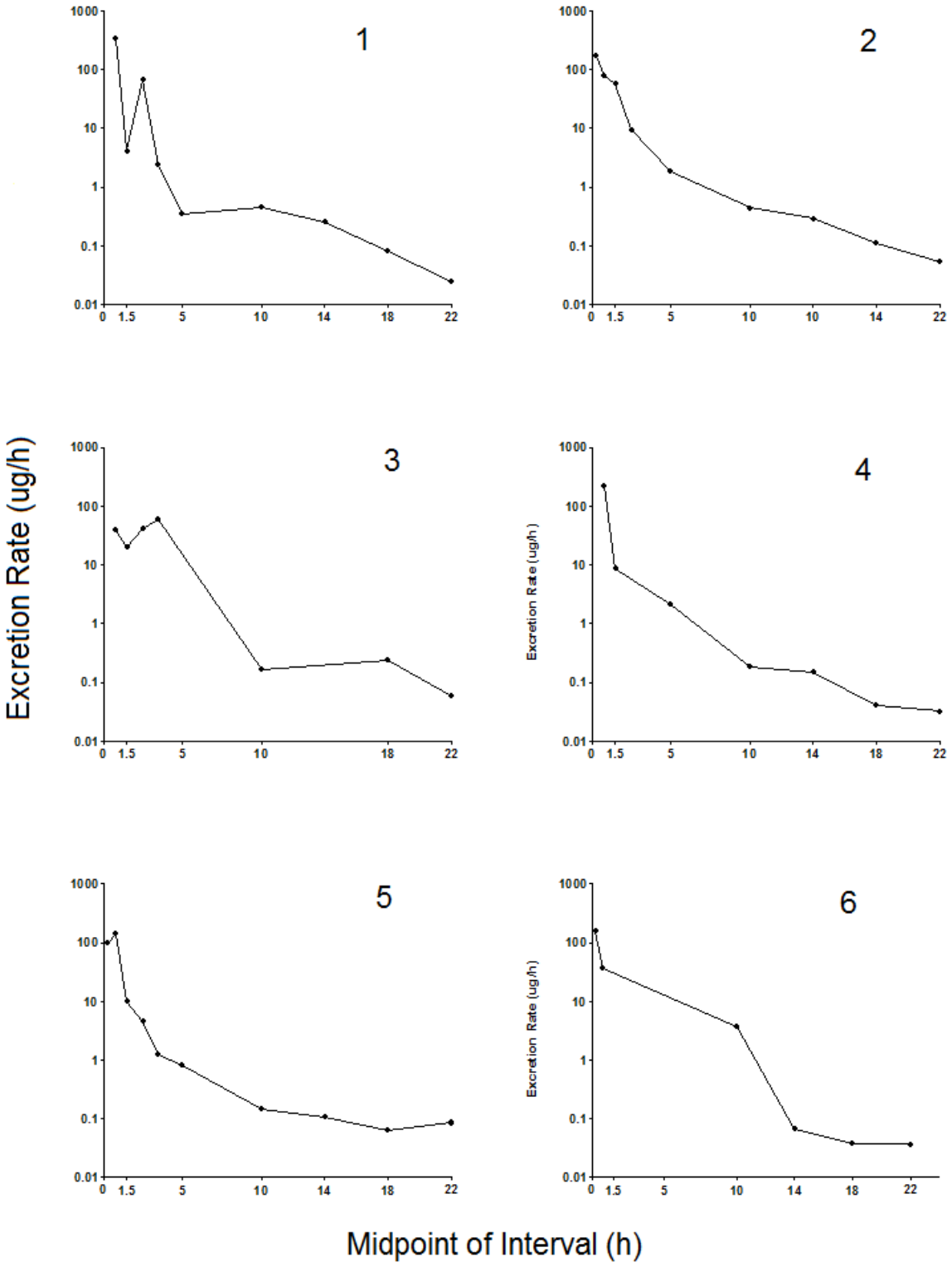

Figure 4-10. Urinary excretion rate of GLY following a single 1 mg intravenous dose to six Standardbred horses.

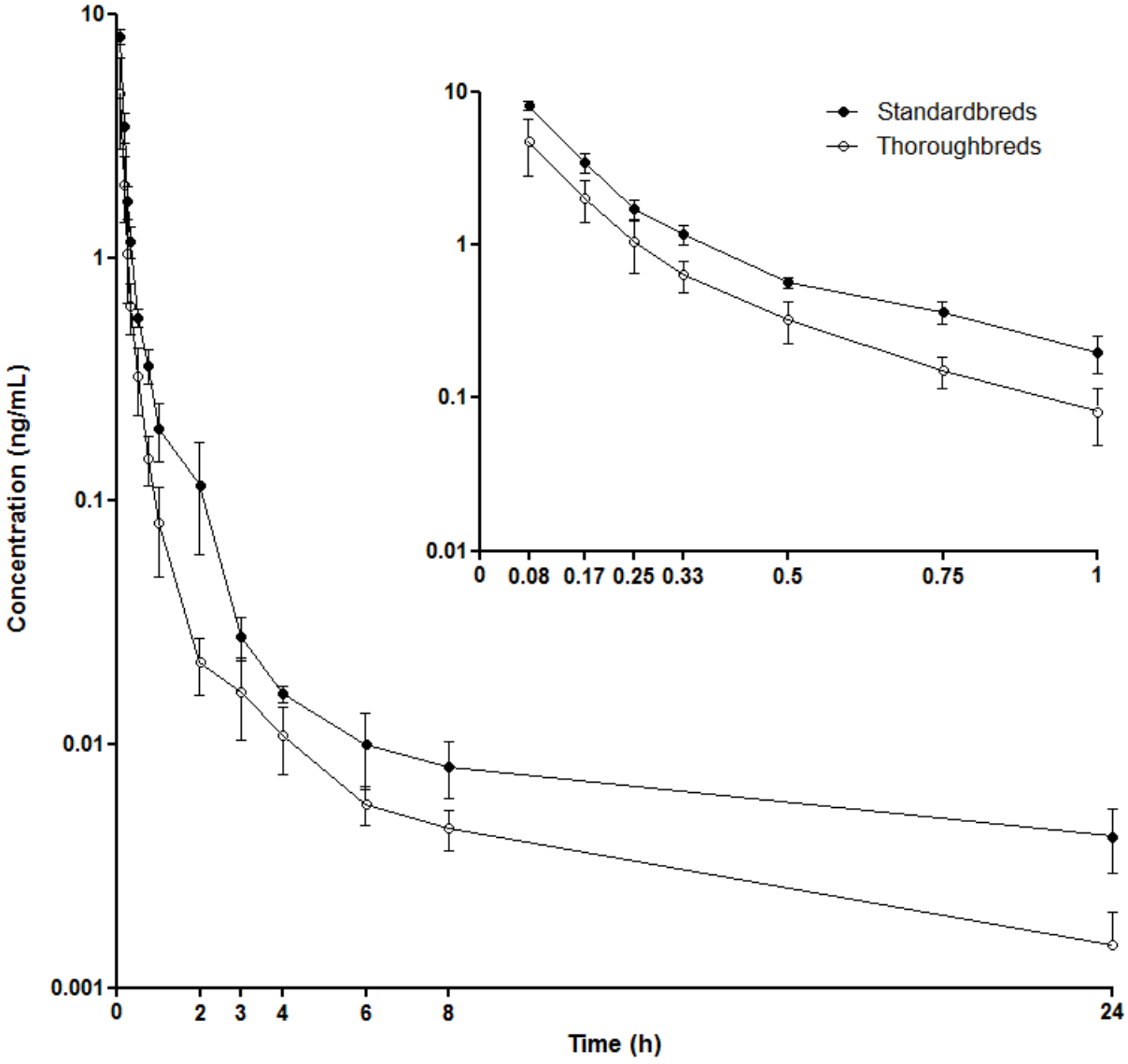

Figure 4-11. Mean (SD) plasma concentration (ng/mL) vs. time (h) data from 0-24 h and for GLY administered intravenously to six Standardbred (●) and six Thoroughbred (○) horses.

Table 4-8. Urine GLY renal clearance following intravenous administration of 1 mg to six (n=6) Standardbred horses.

Time Interval (h)	Δt (h)	Cp at midpoint (ng/mL)	Renal Clearance (mL/min/kg)	Urine Creatinine (mg/dL)	Plasma Creatinine (mg/dL)	Creatinine Clearance (mg/mL/min)
Subject 1						
0.5-1	0.5	0.350	35.89			
1-2	1.0	0.121	1.270			
2-3	1.0	0.038	67.139			
3-4	1.0	0.017	5.1			
4-6	2.0	0.011	1.207			
8-12	4.0	0.008	2.1	239.9	1.2	6.2
12-16	4.0	0.007	1.4			
16-20	4.0	0.006	0.5			
20-24	4.0	0.005	0.2	318.1	1.3	1.8
Subject 2						
0-0.5	0.5	2.420	2.261			
0.5-1	0.5	0.252	10.3			
1-2	1.0	0.091	20.6			
2-3	1.0	0.036	8.7			
4-6	2.0	0.008	7.4			
8-12	4.0	0.005	3.01	197.2	1	6.08
12-16	4.0	0.004	2.33			
16-20	4.0	0.003	1.06			
20-24	4.0	0.003	0.58	265.3	1.1	4.723
Subject 3						
0.5-1	0.5	0.225	5.670			
1-2	1.0	0.089	7.4			
2-3	1.0	0.031	43.150			
3-4	1.0	0.013	144.0			
8-12	4.0	0.004	1.36	144.5	1.2	3.61
16-20	4.0	0.002	3.657			
20-24	4.0	0.001	1.294	115.9	1.2	6.922

Table 4-8. Continued

Time Interval (h)	Δt (h)	Cp at midpoint (ng/mL)	Renal Clearance (mL/min/kg)	Urine Creatinine (mg/dL)	Plasma Creatinine (mg/dL)	Creatinine Clearance (mg/mL/min)
Subject 4						
0.5-1	0.5	0.176	44.6			
1-2	1.0	0.062	5.10			
4-6	2.0	0.007	11.7			
8-12	4.0	0.005	1.31	294.1	1.3	8.95
12-16	4.0	0.004	1.226			
16-20	4.0	0.004	0.38			
20-24	4.0	0.003	0.34	227.3	1.3	5.683
Subject 5						
0-0.5	0.5	1.680	2.130			
0.5-1	0.5	0.180	29.448			
1-2	1.0	0.081	4.5			
2-3	1.0	0.035	4.717			
3-4	1.0	0.018	2.5			
4-6	2.0	0.010	3.2			
8-12	4.0	0.006	0.9	157.1	1.1	4.94
12-16	4.0	0.005	0.746			
16-20	4.0	0.005	0.516			
20-24	4.0	0.004	0.779	215.9	1	11.155
Subject 6						
0-0.5	0.5	1.295	4.3			
0.5-1	0.5	0.152	8.429			
8-12	4.0	0.009	14.7	192.6	1	15.0
12-16	4.0	0.008	0.3			
16-20	4.0	0.007	0.2			
20-24	4.0	0.006	0.21	197.5	1.2	5.21

CHAPTER 5
PHARMACODYNAMICS OF GLYCOPYRROLATE IN THOROUGHBREDS

Muscarinic antagonists, such as glycopyrrolate (GLY), cause positive chronotropism and dromotropism (improved atrioventricular conduction) by competitively blocking the effects of acetylcholine at muscarinic receptors in the heart (Ali-Melkklia *et al*., 1991). However, because of the nonselective blockade of muscarinic receptors in other organ systems, GLY also inhibits a number of parasympathetically mediated functions, causing decreased airway and gastrointestinal secretions, bronchodilation, and inhibition of gastrointestinal motility (Fuder & Meincke, 1993).

During the pharmacokinetic studies outlined in Chapters 3 and 4, the administration of a single intravenous dose of 1 mg produced no apparent clinical or behavioral effects in horses. Although these studies did not specifically monitor clinical effects associated with the drug, it was assumed that the low dosage and rapid metabolism had induced only transient effects and none that were observable without dedicated instrumentation for a measureable amount of time.

Pharmacokinetic parameters representing drug disposition may be substantially affected by the extent of plasma protein binding (Schmidt *et al*., 2010). It has been mathematically demonstrated that the volume of distribution, clearance, half-life and hepatic bioavailability have the potential to be influenced by the fraction of the drug that is unbound (Rowland & Tozer, 1995). Unbound drugs are free and available for extensive distribution into the tissue and thus pharmacologically active. From a pharmacodynamic perspective, clinical effects of most drugs are often minimally affected due to changes in plasma protein binding. Benet and Hoener (2002) make evident that only drugs which are given intravenously and primarily eliminated through hepatic mechanisms or orally and eliminated through non-hepatic mechanism are subject to changes in clinical effects due to an increased unbound percentage (Benet & Hoener, 2002). In

order to further characterize the pharmacology and disposition of GLY in the horse, an *in vitro* experiment was conducted to determine the extent of plasma protein binding at various concentrations.

No reports of the pharmacokinetics of GLY following a clinically relevant dosage in horses are found in the literature. Thus, data correlating plasma concentrations of GLY with the pharmacodynamics are not available, leaving a void of information for clinicians as well as agencies that regulate the drug in performance horses. The purpose of this study was to investigate the pharmacokinetics and pharmacodynamics (PK-PD) following a continuous rate infusion of GLY to the horse. Additionally, we developed a model that offers a plasma concentration-effect predictive relationship for regulators who encounter plasma or urine concentrations in post- performance specimens.

Methods

Animals

Six, adult, Thoroughbred geldings ranging in age from 9-11 years and weighing from 540-595 kg were used in these studies (Table 5-1). All horses were determined to be healthy prior to the study based on physical examination, complete blood count (CBC), horse blood chemistry panel, urine analysis, indirect blood pressure, and electrocardiography (ECG). One week before the start of the study two horses per day for three days underwent a full 5 hour mock-up of the infusion study in order to acquaint them with the equipment, procedures and handling, thereby reducing the potential behavioral effects or stress of the experiment. On the morning of the study, horses were weighed and allowed to feed on a commercially available grain mixture 2 h before drug administration. Each horse was housed indoors at the University of Florida (UF) Veterinary Medical Center, in an individual climate controlled (26°C) stable throughout the

dosing and direct observation period and had open access to water at all times. Following the direct observation period horses were released outdoors to grass paddocks. The experimental protocol, including drug administration and sample collection, was approved and facilities were inspected periodically by the UF Institutional Animal Care and Use Committee.

Dosing

The study consisted of a 2-way crossover design wherein the six participating horses each received a continuous rate infusion (CRI) of GLY and a saline (0.9% NaCl) control with a 10 day washout period between treatments. Thus, each horse served as its own control. On the days during which drug administration took place, the study always began at 0800 in order to reduce variability associated with circadian rhythm changes. Baseline observations took place for1 h (0800-0900) before drug administration. Following the baseline observation period, horses were administered GLY (glycopyrronium bromide, American Regent, Inc., Shirley, NY) using local lidocaine anesthesia and a 14-guage catheter aseptically placed into the right jugular vein, at an intravenous CRI (Medex 3010, Duluth, GA) of 4 µg/kg per hour for 2 h for a total drug dose of 8 µg/kg. The dose was based on previous pharmacokinetic analysis (Chapter 3) in a similar group of horses and was intended to achieve steady state conditions. After the end of the infusion period all horses were directly observed in the stalls until the first bowl movement was passed. After a satisfactory general health assessment for abdominal discomfort and colic, the horses were released to outside paddocks.

Specimen Collection

Plasma: Whole blood samples were collected from the contralateral left 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 x g) at 4 °C for 15 min. Harvesting of plasma took place within 1 hour 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 relative to the start of the infusion and included a sample collection before drug administration and at 5, 10, 15, 20, 30, 45, 60, 90, 120 (end of infusion), 122.5, 125, 130, 135, 140, 150, 165 min and 3, 3.5, 4, 5, 6, 8, 10, 14, and 26 h after the start of the infusion. Samples were stored for no longer than 6 weeks under -80 °C (4 weeks) and -20 °C (2 weeks) conditions which were well within the validated stability limitations previously reported in Chapter 2.

Urine: 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 aliquoted into 15-mL sterile, disposable, polypropylene centrifuge tubes and stored at -20°C immediately and at -80°C within 48 h. Collection times are relative to the start of the infusion and included a sample collection before drug administration and at 1, 2, 3, 4, 6, 8, 10, 14, and 26 h after the start of the infusion.

Determination of Plasma Protein Binding

One hundred milliliters of venous blood was collected from each of six horses, ages 3-8 yrs old, into tubes containing lithium heparin Vacutainer, 10 mL, Becton Dickinson, Franklin Lakes, NJ, USA). Each horse had been drug-free for the previous 30 days and was considered healthy, based upon physical examination, complete blood count, serum chemistry analysis, and plasma fibrinogen concentration.

The storage of plasma has been known to affect the plasma protein binding of certain drugs (Paxton, 1981). For this reason fresh harvested plasma was obtained within 1 h of blood collection through centrifugation of the blood samples for 15 min at 2,000 *g*. All plasma was

pooled and the pH was adjusted to 7.4. The extent of GLY plasma protein binding was determined by UF methodology as described previously (Wright *et al*., 1996; Sebille *et al*., 1990).

Stock and working standard solutions of GLY and GLY-d_3 were prepared according to the procedures outlined above in Chapter 2. Calibrators were prepared using the fresh plasma obtained for this experiment. A set of positive control samples was prepared in triplicate for each horse (n=6) in fresh plasma and a set of protein-free negative control samples was prepared in phosphate buffered saline (pH 7.4), to achieve end-concentrations of 25, 10, 5, 1, 0.5 and 0.1 ng/mL. A 1-mL aliquot of each plasma dilution and of the negative control solution was then transferred into a CryoTube™ vial (Nunc, Roskilde, Denmark), incubated at 37°C for 20 min, flash-frozen in liquid nitrogen, and placed in a freezer at −20°C until analyzed. A second 1-mL aliquot of each sample was transferred into the sample reservoir of the Centrifree® ultrafiltration device (Millipore Corp, Bedford, MA, USA) and incubated at 37°C for 10 min to allow drug-plasma protein binding equilibrium. The samples were then centrifuged at 2,000 ***g*** and 37°C for 15 min. After centrifugation, the filtrate cup was disconnected from the filtration device, sealed with a cap, flash frozen, and stored in a freezer at −20°C until analyzed.

Concentrations of GLY in the unfiltered samples and the filtered samples were determined by use of high-performance liquid chromatography-tandem mass spectrometry as described above in chapter 2. The concentration of protein-bound drug (C_b) was calculated as:

$$C_b = C_t - C_f, \quad (5\text{-}1)$$

where C_t is the total GLY concentration and C_f is the concentration of the free fraction of GLY. The percent plasma protein binding (PB) was calculated as:

$$PB\ (\%) = C_b/C_t \times 100, \quad (5\text{-}2)$$

Similarly, the degree of nonspecific adsorption (NA) of GLY to the filtration device was determined based on the phosphate buffered saline diluted, protein-free negative control samples as:

$$NA = C_b/C_t \times 100. \quad (5\text{-}3)$$

Pharmacokinetic Analysis

Nonlinear least squares analysis was performed on plasma GLY concentration versus time data and pharmacokinetic parameters for all horses were estimated with compartmental analysis using Phoenix WinNonlin® 6.1 (Pharsight, St. Louis, MO, USA). The Gauss–Newton (Levenberg and Hartley) method was used and goodness of fit and the appropriate weighting factor were selected based on the coefficients of variation, Akaike's (AIC) (Yamaoka *et al*., 1978) and Schwarz's Bayesian (Schwarz, 1978) Criteria (SBC) as well as visual analysis of the graphical output (including residual plots). Secondary parameters calculated include area under the curve (AUC), terminal half-life ($t_{1/2\gamma}$), mean residence time (MRT), apparent volumes of distribution, total plasma clearance (Cl_p), and microdistribution rate constants. All calculations for pharmacokinetic parameters were based on methods described by Gibaldi and Perrier (1982). The plasma drug concentration at steady-state (C_{pss}) was calculated as:

$$C_{pss} = R_o/Cl_p \quad (5\text{-}4)$$

where R_o is the drug infusion rate and Cl_p is the systemic (total) drug clearance. All pharmacokinetic parameters were calculated separately for each horse and values are reported as median and range (minimum-maximum).

Physiological Endpoints

Horses were kept under constant direct observation from 0800 (1 h before administration) until 1300 (2 h following the end of the infusion). Any clinical signs of drug response were

carefully monitored and recorded. Heart rate and respiratory rate were recorded for baseline every 10 min for 1 h before drug administration and again every 10 min for 4 h after the start of the infusion. Heart rate was measured using a telemetric device fastened with a girth for continuous monitoring. Respiratory rate was measured by direct counting.

Defecation, urinary incidence, and stool consistency were recorded throughout the observation period. Horses were retained in indoor stalls until the first bowel movement after the end of drug administration was recorded. Following a routine health evaluation, horses were turned out to pasture. In addition to direct observation, all subjects were video recorded from 0800 (1 h before administration) until 1300 (2 h after administration) for a total of 5 h. Abnormal behavior was evaluated based on a detailed rubric (Price *et al*., 2003).

PK-PD Modeling

The relationship of the plasma GLY concentrations vs. pharmacodynamic effect was assessed for heart and respiratory rate each. Pharmacokinetic-pharmacodynamic linked analysis was performed using Phoenix WinNonlin® 6.1 (Pharsight, St. Louis, MO, USA). The appropriate model was selected based on AIC and a visual inspection of the fitted graph. Minimum Akaike Information Criteria estimates were applied to discriminate the best fitting model and improved fit of data was achieved by re-weighting.

Statistical Analysis

Plasma GLY concentrations and pharmacokinetic parameter estimates are reported as mean ± standard deviation and median and range (minimum-maximum), respectively Differences in plasma PB between different nominal GLY concentrations were assessed using one-way repeated measures ANOVA with Tukey's *post hoc* test. Differences between treatment and control groups for physiological endpoints were assessed using two-way ANOVA with

Bonferroni's *post hoc* test. All analyses were performed using commercial statistical software (Microsoft® Office Excel 2003, Microsoft Corporation, Redmond, WA, USA; and GraphPad Prism™ version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Protein Binding

Percent of GLY bound to plasma proteins under a range of plasma concentrations (0.1-25 ng/mL) were between 37 – 44% and are summarized in Table 5-2. The extent of nonspecific adsorption was -0.9-2.9% suggesting that adsorption of GLY to the filtration device was negligible.

Pharmacokinetics

GLY plasma concentrations for all six horses are presented in Table 5-3 and plotted in Figure 5-1. A composite plasma GLY concentration vs. time plot for all six horses from 0 until 24 h after the discontinuation of the infusion (Figure 5-2) and from 0 until 4 h after the discontinuation of the infusion (Figure 5-3) is depicted and represents mean ± SEM.

After the discontinuation of a two hour CRI administration of 4 µg/kg/hr of GLY, the observed plasma concentration versus time profile could be best described by a three-compartment model. The equation based on macro constants for this model is:

$$C_t = Ae^{-\alpha \cdot t} + Be^{-\beta \cdot t} + Ce^{-\gamma \cdot t} \quad (5\text{-}5)$$

where C_t is the plasma concentration at time (t), A, B and C are the zero time intercepts for the first, second and third phases. Further, α, β and γ are the exponential terms for each phase and exp is the base of the natural logarithm (Gabrielsson & Weiner, 2007). The weighting factor chosen with this model was $1/(Y^2)$ where Y was the observed plasma concentration. Plasma

GLY concentration versus time curves demonstrating observed and model predicted concentrations are illustrated in Figure 5-4.

Pharmacokinetic parameter estimates determined from compartmental model analysis are presented in Table 5-4. After the infusion was discontinued the plasma GLY concentration declined rapidly exhibiting a tri-exponential decay, similar to the disposition following a bolus intravenous dose of 1 mg (Chapter 3 & 4). The initial distribution phase was characterized by an estimated median half-life ($t_{1/2\alpha}$) of 0.12 h (7.2 min) and the rapid ($t_{1/2\beta}$) and slower ($t_{1/2\gamma}$) elimination phases were 0.78 h and 13.2 h, respectively. The median (minimum-maximum) plasma GLY steady state concentration was 5.10 (3.90-6.18) ng/mL.

Physiological Endpoints

The heart rates during the direct observation period for GLY and saline administrations for each subject are plotted over time in Figure 5-5. The mean heart rate of the GLY treated group differed significantly from that of the control group beginning approximately 50 min after the start of the drug infusion and ending 40 min after the discontinuation of the infusion. A plot of the mean heart rate (beats per minute) over time for the treated and control groups is displayed in Figure 5-6.

Individual plots demonstrating the respiratory rate response from the treated and control experiments are presented in Figure 5-7. The mean respiratory rate of the GLY treated group differed significantly from that of the control group beginning approximately 20 min after the start of the drug infusion and ending 20 min before the discontinuation of the infusion. A plot of the mean (SD) respiratory rate (breathes per minute) over time for the treated and control groups are displayed in Figure 5-8.

All horses that received GLY exhibited slowed gastrointestinal motility and reduced defecation incidence compared to the control group. Figure 5-9 illustrates the frequency of bowel movements during the entire 5 h direct observation period and Figure 5-10 displays the same data in separate time bins. It is evident that bowel movements in the treatment group are occurring during the pre-administration period and the first hour of the drug infusion, presumably before the drug exerts its effects on the gastrointestinal tract. The treated horses experienced extended periods until the first bowel movement since the first hour of the drug infusion. The mean ± SD time elapsed until the first bowel movement for the treated horse was 6 ± 2.03 h (Figure 5-11) with one horse experiencing a 9 h period without defecation.

During the study it was observed that all horses had exhibited a loss of appetite and refused treats within sixty minutes after the start of the infusion while control horses readily accepted them throughout. All horses showed mild behavioral changes in the form of shifting weight from one leg to the other and occasional muscle fasciculations. Two horses showed signs of colic and received flunixin. These horses were evaluated on the following morning and were found to be in normal condition and medically sound.

PK-PD Modeling

Examination of plots of GLY plasma concentrations against the corresponding heart rate effects revealed that these pharmacodynamic responses were lagging behind the plasma concentrations (Figure 5-12). In order to accommodate this temporal disconnect, the model chosen included an effect compartment, and the relationship between the effect compartment concentration and response was assumed to be linear (Meibohm & Derendorf, 1997).

Effect compartment modeling with heart rate effects following GLY administration was performed using a sigmoidal E_{max} model with baseline effect (Figure 5-13), in which the

individual pharmacokinetic parameter estimates obtained were used as constants. The equation for the effect site concentration (C_e) during and after the constant GLY infusion is,

$$C_e = \frac{k_e \cdot k_{e0}}{V_c} \cdot \left[\begin{array}{l} \frac{(k_{21}-\lambda_1)(k_{31}-\lambda_1)\left(1-e^{-\lambda_1 \cdot T}\right)\cdot e^{-\lambda_1 \cdot t}}{\lambda_1(\lambda_2-\lambda_1)(\lambda_3-\lambda_1)(k_{e0}-\lambda_1)} + \frac{(k_{21}-\lambda_2)(k_{31}-\lambda_2)\left(1-e^{-\lambda_2 \cdot T}\right)\cdot e^{-\lambda_2 \cdot t}}{\lambda_2(\lambda_1-\lambda_2)(\lambda_3-\lambda_2)(k_{e0}-\lambda_2)} \\ + \frac{(k_{21}-\lambda_3)(k_{31}-\lambda_3)\left(1-e^{-\lambda_3 \cdot T}\right)\cdot e^{-\lambda_3 \cdot t}}{\lambda_3(\lambda_1-\lambda_3)(\lambda_2-\lambda_3)(k_{e0}-\lambda_3)} + \frac{(k_{21}-k_{e0})(k_{31}-k_{e0})\left(1-e^{-k_{e0} \cdot T}\right)\cdot e^{-k_{e0} \cdot t}}{k_{e0}(\lambda_2-k_{e0})(\lambda_3-k_{e0})(\lambda_3-k_{e0})} \end{array} \right] \quad (5\text{-}6)$$

where k_0 is the zero-order infusion rate, k_{e0} is the elimination rate constant from the hypothetical effect compartment and V_c, k_{21}, k_{31}, λ_1, λ_2 and λ_3 are the modeled PK parameters, T is the elapsed time during the infusion (after the infusion T=2 h) and t is time after the infusion (Coburn, 1981). Equation 5-6 and Equation 5-7

$$E = E_0 + \frac{E_{max} \times C_e^n}{EC_{50} + C_e^n} \quad (5\text{-}7)$$

were modeled simultaneously, which provided estimates of the baseline effect (E_0), maximal drug effect (E_{max}), concentration at 50% of Emax (EC_{50}), sigmoidicity factor (n, hill coefficient) and k_{e0} for each horse (Holford & Sheiner, 1981).

Hysteresis for the effect of heart rate can also be illustrated when concentration and effect are plotted versus time for each subject (Figure 5-14) and for the entire group (Figure 5-15). The observed and predicted pharmacological response during the direct observation period from the proposed PK-PD linked model for each subject is plotted in Figure 5-16. Linked model PD estimates are presented in Table 5-5.

Discussion

Ultrafiltration is a commonly used technique to determine the binding of a drug to plasma proteins because of its rapidity and ease of use. It utilizes a two chambered reservoir separated by a filter membrane that allows passage of the drug but not that of drug bound to plasma proteins. Drug fortified plasma is pipetted into the upper reservoir and the sample is centrifuged.

Free drug is filtered into the lower reservoir and its concentration subtracted from the total fortified concentration in order to calculate a percentage of the drug which is protein bound. The primary disadvantage includes the non-specific binding of the free drug to the filter membrane (Howard *et al*., 2010). Protein binding of GLY appeared to decrease with increasing concentrations indicating possible drug saturation at protein binding sites in horses. The degree of plasma protein binding of GLY determined in this study suggests that the potential for displacement interactions with other drugs is unlikely to be clinically significant. However, we did not specifically investigate the influence of GLY metabolites or other drugs on the protein binding of GLY.

Since GLY is approximately 40 % protein bound in the horse, previous estimates of glomerular filtration may be overstated. Only the free form of GLY may be filtered by the glomerulus. Therefore in order to calculate the amount of GLY that undergoes filtration, the fraction unbound (fu) must be multiplied by the glomerular filtration rate (GFR). Therefore approximately 1.2 mL/min/kg is estimated to undergo filtration while the remainder is likely to be actively secreted in the tubular lumen.

GLY disposition in the horse following a CRI resulted in a rapid decrease in plasma concentrations early and a prolonged terminal elimination, although this study did not look at GLY plasma or urine concentrations beyond 24 h following the end of the infusion. This profile was similar to that reported following a single rapid bolus administration in horses in Chapters 2 and 3. GLY distributed very rapidly from the central to the peripheral compartments as demonstrated by the initial distribution phase median half-life ($t_{1/2\alpha}$) of 0.12 h (7.2 min). Evidence of wide distribution and prolonged elimination were noted with the rapid ($t_{1/2\beta}$) and slower ($t_{1/2\gamma}$) elimination phases of 0.78 h and 13.2 h, respectively. Compared to single

intravenous injections, GLY plasma concentration versus time curves demonstrated little variability between subjects after a continuous rate intravenous infusion.

GLY's effect on heart rate has been previously studied in horses following a 2.5, 5 and 10 µg/kg intravenous dose. Mean heart rate (n=5) after the 2.5 µg/kg dose did not demonstrate a significant difference from the control group for measurements taken up to 120 min. However, the 5 and 10 µg/kg doses both produced significantly elevated mean heart rates from the control group beginning 5 min after and ending 60 min after treatment in awake horses (Singh *et al*., 1997), in agreement with the current study. In other studies with horses GLY is used to attenuate the cardiovascular depressive effects of anesthetic agents, such as xylazine. Singh *et al.* (1995) report a 2.5 µg/kg dose is effective at reducing atrioventricular bock, but doses such as 5 and 10 µg/kg were associated with a profound loss of gastrointestinal motility and therefore determined unsafe. Teixeira et al (2003) noted a 53 % increase in cardiac output over the control group when horses (n=6) were intravenously administered 5 µg/kg of GLY and anesthetized with xylazine, while low intestinal auscultation scores were evident. A third study in anesthetized horses recognized a significant increase in mean heart rate after 5 µg/kg intravenously but also cautioned unwanted gastrointestinal effects (Dyson *et al.*, 1999).

To the author's knowledge respiratory effects in horses following a clinically relevant dose of GLY have not been reported in horses. However, in humans intravenous GLY has been shown to cause bronchodilatation (Gal and Suratt, 1981), while nebulized administration caused a longer duration of bonchodilatation without the systemic anticholinergic effects of inhaled atropine (Gal *et al*., 1984; Walker *et al*., 1987). In the horse GLY would be expected to antagonize muscarinic receptors on the airway smooth muscle and submucosal glands, to cause bronchodilation and reduced mucus secretions (Coulson and Fryer, 2003). Pharmacokinetic-

pharmacodynamic modeling was not performed using respiratory rate as a physiological parameter in these studies. However, respiratory rate and GLY plasma concentrations were plotted against time for each (Figure 5-17) and all (Figure 5-18) subjects In contrast to heart rate, a clockwise hysteresis (proteresis) was observed between GLY concentrations and the physiological endpoint of respiratory rate (Figure 5-19). Proteresis generally occurs when a subject develops tolerance for a given compound. Despite the limited value of respiratory rate as an indicator of airway function, changes were evident between the treatment and control groups.

The depressant effects of muscarinic receptor antagonists on intestinal motility have been documented and the horse is particularly sensitive to the gastrointestinal effects of these compounds. GLY has been used successfully to treat vagally mediated bradycardia during anesthesia in small animals (Dyson & James-Davies, 1999) and in goats (Pablo *et al*., 1995) without causing major complications in other organ systems. Yet in horses, doses as low as 5 µg/kg of GLY have demonstrated lower auscultation scores and in some case intestinal impaction and colic (Dyson et al., 1999; Singh *et al*., 1997; Teixeira Neto *et al*., 2004; Singh *et al*., 1995).

This study demonstrates that GLY has the potential to slow defecation incidence and frequency in the period following drug administration compared to horses administered saline. Although in this study, motility was not directly quantified, it is assumed that a reduced fecal frequency and extended periods of withheld defecation are the result of gastrointestinal hypomotility. As such, it has been demonstrated through this and other studies that GLY should be used conservatively at CRI doses below 10 µg/kg, especially in horses with pre-existing gastrointestinal conditions or those undergoing surgery.

This study attempted to evaluate behavioral effects associated with the administration of GLY to the horse and the subjects were video monitored for five hours beginning from the time they were led into the stalls. There was no clear evidence of behavioral changes with any of the subjects during this period. Although the subject's behavior varied while housed, all subjects appeared more relaxed when a companion animal was housed in an adjacent stall for the duration of the observation period. Behavioral variation ranged from circling the stall continuously to standing still.

There was obvious hysteresis observed when heart rate and plasma GLY concentrations were plotted against time and when the effect was plotted against plasma GLY concentrations that was minimized by the incorporation of an effect compartment model (Figure 5-20). This finding is unusual mainly because cardiac receptors are located in the periphery and GLY concentrations at the effect site are expected to be similar to measured concentrations. While hysteresis appears to be a characteristic of PK-PD modeling of several drugs active in the central nervous system (Mandema *et al*., 1991; Danhof *et al*., 1992), the current study does not permit us to establish whether distribution is the major determinant of the observed hysteresis. However, the rationale for this delay is easily understood if one assumes that the resulting pharmacological effect or response is preceded by drug distribution to the site of action. Nonetheless, since GLY is unlikely to cross the central nervous system barrier due to its polarity, a temporal delay due to drug distribution to these sites is unlikely. Also, there is no consistent report demonstrating the role of other factors in the biophase equilibration of GLY which are known to cause hysteresis, such as coupling mechanisms and effectuation process that follow the drug-receptor interaction.

The hysteresis has been successfully modeled by the effect-compartment approach, which postulates the existence of a hypothetical effect compartment linked to the plasma site by a first-

order process (k_{e0}). This approach is based on the assumption that distribution kinetics between plasma and effect site are linear and that the same effect-site concentration always evokes the same response, independent of time. This assumption may not hold when active metabolites are formed or when there is development of acute tolerance. However, there are no reports indicating that active metabolites of GLY are formed in humans (Kaila *et al*., 1990) or horses (Matassa *et al*., 1992). Moreover, there is no evidence of the development of tolerance toward the anticholinergic effects of GLY in these models.

In the case of purely peripheral effects, a more direct link of the plasma pharmacokinetics to the corresponding pharmacodynamic effect would have been expected. However, the physiological situation may be more complex. A long equilibration half-life can also ensue from a high affinity of the drug for the cardiac tissue leading to prolongation of the (peripheral) effects of GLY on the heart. Moreover, heart rate is determined by complex peripheral cardiovascular regulatory systems, and changes in one part of the system, such as muscarinic blockade, will likely cause compensatory changes in the components.

Table 5-1. Demographics of PK-PD study subjects.

Subject #	Gender	Age (yr)	Weight (kg)	CRI Administration	PK Analysis	PD Analysis
1	G	12	530	X	X	X
2	G	11	590	X	X	X
3	G	12	560	X	X	X
4	G	12	548	X	X	X
5	G	12	540	X	X	X
6	G	12	550	X	X	X

Table 5-2. Percent protein binding (mean ± SD) of GLY in the plasma of six (*n*=6) healthy horses.

	Nominal plasma GLY concentration (ng/mL)					
Analyte	0.1	0.5	1	5	10	25
Protein Binding (%)	42.9 ± 8.9	43.5 ± 3.4	44.1 ± 2.1	37.3 ± 3.2	40.1 ± 5.8	37.5 ± 7.5
Nonspecific Adsorption (%)	1.5 ± 2.2	2.3 ± 1.9	0.9 ± 1.1	3.3 ± 3.6	2.9 ± 1.1	1.8 ± 1.4

Table 5-3. Plasma GLY concentrations (ng/mL) for six Thoroughbred horses following a two-hour CRI of 8 µg/kg.

	Plasma GLY Concentration (ng/mL)										
	Subject #										
Time (h)	1	2	3	4	5	6	Mean	SD	Median	MIN	MAX
0.08	1.29	1.45	1.30	1.06	1.56	1.63	1.38	0.209	1.38	1.06	1.63
0.17	2.77	3.02	3.08	2.96	2.78	3.02	2.94	0.131	2.99	2.77	3.08
0.33	3.47	3.59	3.92	3.90	4.05	4.20	3.85	0.278	3.91	3.47	4.20
0.50	3.67	3.60	4.22	4.38	4.54	4.73	4.19	0.461	4.30	3.60	4.73
0.67	3.92	3.98	4.49	4.78	4.87	5.14	4.53	0.496	4.64	3.92	5.14
1.00	4.39	4.28	5.09	5.31	5.15	5.49	4.95	0.498	5.12	4.28	5.49
1.50	4.77	4.66	5.31	6.06	5.90	5.62	5.39	0.581	5.47	4.66	6.06
2.00	4.86	4.72	5.35	5.99	6.04	5.88	5.47	0.584	5.62	4.72	6.04
2.04	2.72	2.91	3.83	4.75	4.93	5.45	4.10	1.12	4.29	2.72	5.45
2.08	2.17	3.20	2.95	4.91	3.31	4.15	3.45	0.957	3.26	2.17	4.91
2.17	1.26	1.47	1.77	2.74	2.17	2.98	2.06	0.693	1.97	1.26	2.98
2.25	0.793	0.755	1.29	1.78	1.49	2.11	1.37	0.538	1.39	0.755	2.11
2.33	0.531	0.533	0.933	1.13	1.05	1.29	0.911	0.316	0.991	0.531	1.29
2.50	0.340	0.276	0.541	0.713	0.649	0.896	0.569	0.234	0.595	0.276	0.896
2.75	0.220	0.165	0.414	0.393	0.351	0.616	0.360	0.160	0.372	0.165	0.616
3	0.133	0.112	0.265	0.253	0.254	0.463	0.247	0.125	0.253	0.112	0.463
3.5	0.080	0.053	0.174	0.148	0.134	0.214	0.134	0.059	0.141	0.053	0.214
4	0.055	0.041	0.124	0.096	0.086	0.147	0.092	0.040	0.091	0.041	0.147
5	0.027	0.021	0.049	0.067	0.043	0.082	0.048	0.023	0.046	0.021	0.082
6	0.020	0.017	0.034	0.040	0.030	0.051	0.032	0.013	0.032	0.017	0.051
8	0.014	0.012	0.013	0.023	0.021	0.030	0.019	0.007	0.018	0.012	0.030
10	0.010	0.008	0.009	0.015	0.015	0.021	0.013	0.005	0.012	0.008	0.021
14	0.006	0.004	0.006	0.008	0.011	0.010	0.007	0.003	0.007	0.004	0.011
26	0.003	0.002	0.003	0.006	0.007	0.008	0.005	0.002	0.005	0.002	0.008

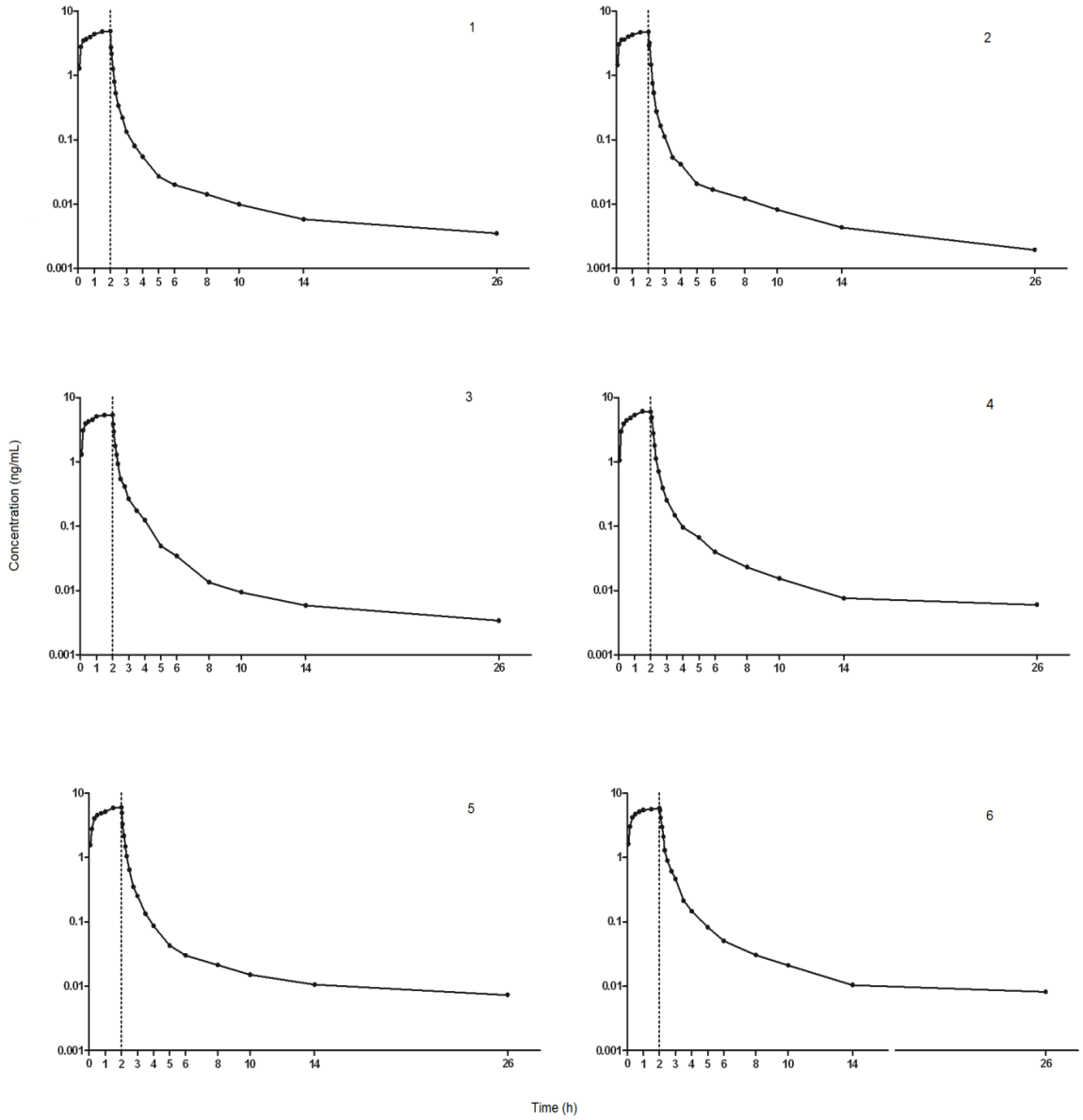

Figure 5-1. Plasma concentration (ng/mL) vs. time (h) data from 0-26 h and for GLY administered by an intravenous infusion in each of six healthy adult Thoroughbreds.

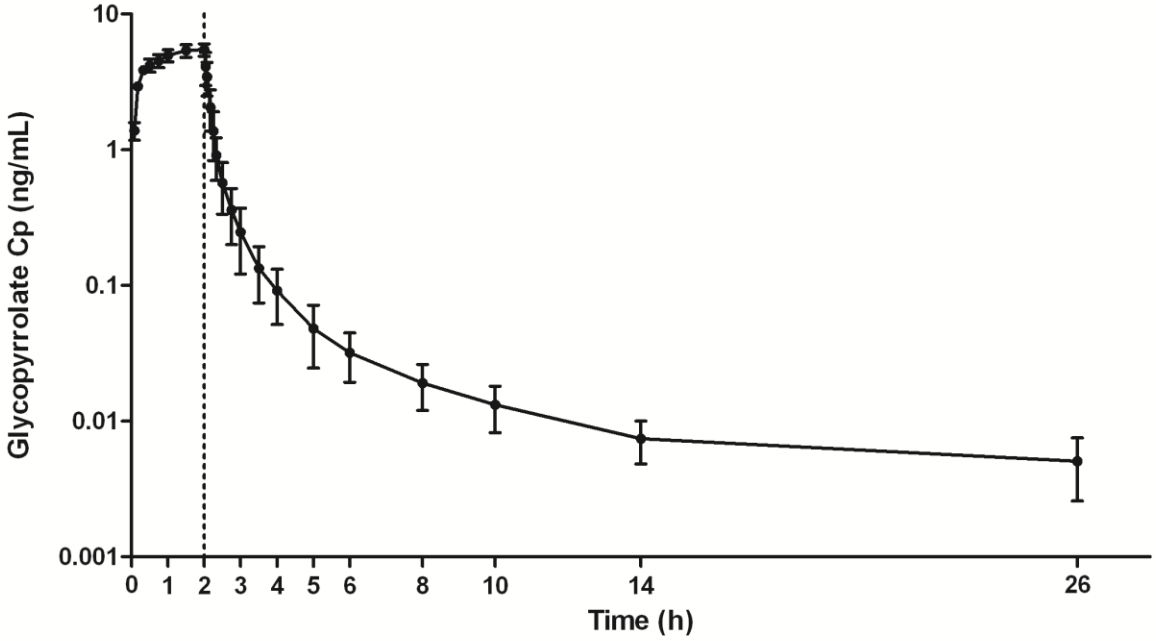

Figure 5-2. Plasma concentration (ng/mL) vs. time (h) data from 0-26 h and for GLY administered by an intravenous infusion in six healthy adult Thoroughbreds.

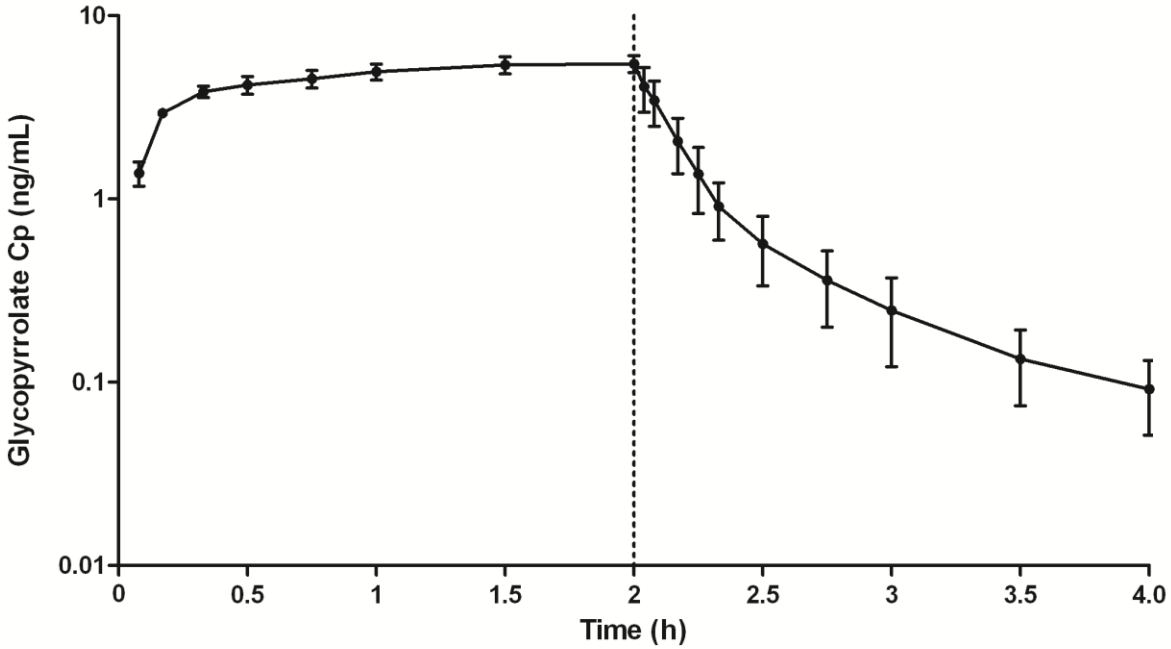

Figure 5-3. Plasma concentration (ng/mL) vs. time (h) data from 0-4 h and for GLY administered by an intravenous infusion in six healthy adult Thoroughbreds.

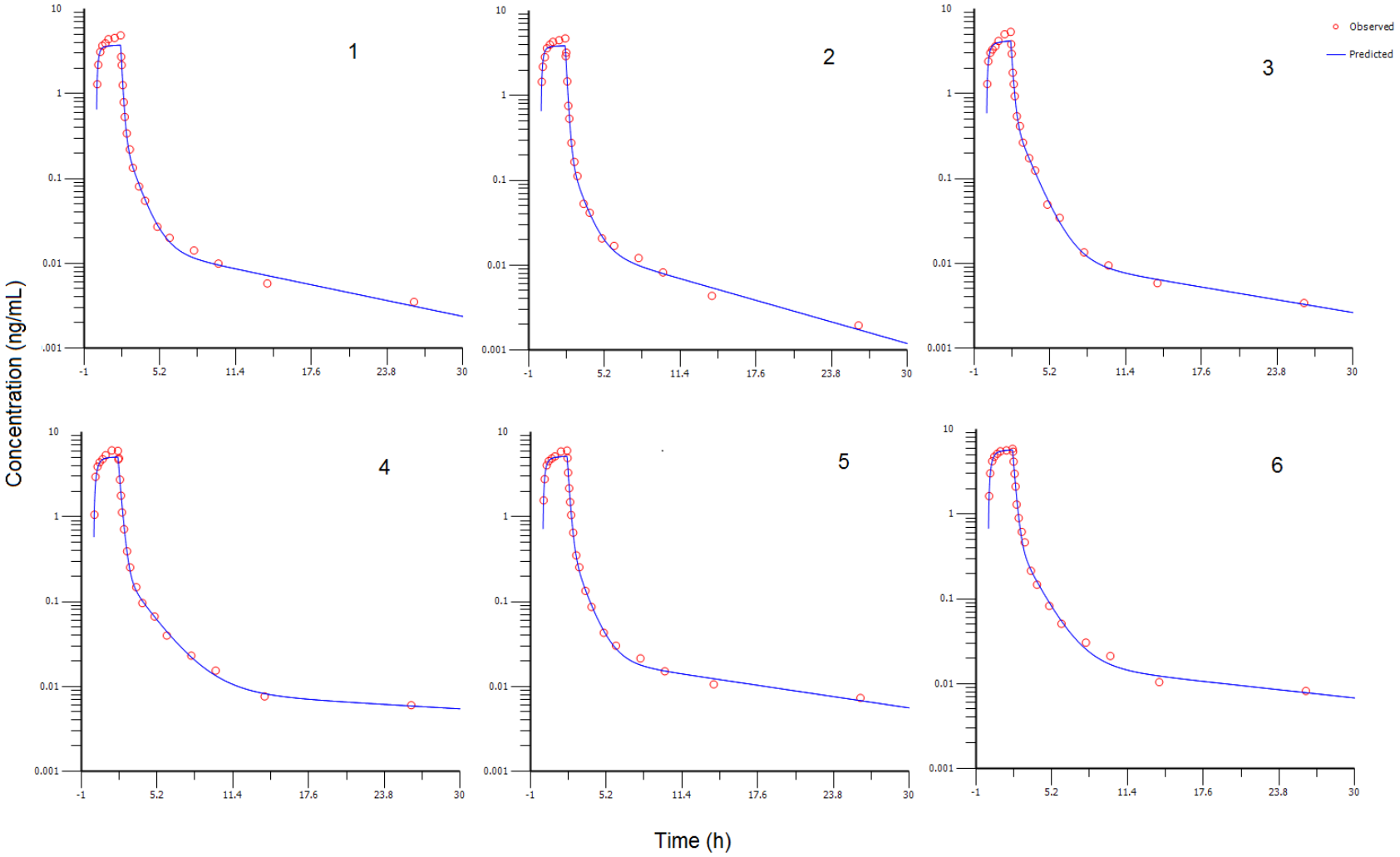

Figure 5-4. Observed (circles) and predicted (line) plasma GLY concentrations after a two-hour intravenous infusion of 8 µg/kg of body weight to six (n=6) healthy adult Thoroughbred horses and pharmacokinetic analysis using a three-compartment model.

Table 5-4. Pharmacokinetic parameter estimates of GLY, determined using a three-compartmental model, following a two-hour intravenous infusion of 4 µg/kg/h of body weight to six (n=6) healthy adult Thoroughbred horses.

	Subject #								
Parameter	1	2	3	4	5	6	Median	Min	Max.
A (ng/mL)	47.7	47.7	41.6	40.8	51.0	47.3	47.5	40.8	51.0
B (ng/mL)	0.806	0.556	1.13	0.367	1.19	0.928	0.867	0.367	1.19
C (ng/mL)	0.018	0.018	0.013	0.010	0.023	0.019	0.018	0.010	0.023
Alpha (h^{-1})	7.03	6.59	5.73	4.22	5.50	4.56	5.61	4.22	7.03
Beta (h^{-1})	1.05	1.071	0.822	0.473	0.995	0.650	0.908	0.473	1.07
Gamma (h^{-1})	0.072	0.095	0.056	0.019	0.050	0.036	0.053	0.019	0.095
C_{max} (ng/mL)	3.74	3.86	4.20	5.08	5.17	5.73	4.64	3.74	5.73
V_1 (L/kg)	0.165	0.166	0.187	0.194	0.153	0.166	0.166	0.153	0.194
K_{21} (h^{-1})	1.150	1.135	0.952	0.507	1.10	0.725	1.02	0.507	1.15
K_{31} (h^{-1})	0.075	0.097	0.057	0.020	0.052	0.038	0.055	0.020	0.097
K_{10} (h^{-1})	6.22	6.07	4.82	3.76	4.77	3.91	4.80	3.76	6.22
K_{12} (h^{-1})	0.511	0.308	0.650	0.246	0.413	0.399	0.406	0.246	0.650
K_{13} (h^{-1})	0.203	0.147	0.127	0.175	0.210	0.172	0.173	0.127	0.210
K_{10}_HL (h)	0.112	0.114	0.144	0.184	0.145	0.177	0.145	0.112	0.184
$t_{1/2\alpha}$ (h)	0.099	0.105	0.121	0.164	0.126	0.152	0.124	0.099	0.164
$t_{1/2\beta}$ (h)	0.660	0.647	0.843	1.47	0.697	1.07	0.770	0.647	1.47
$t_{1/2\gamma}$ (h)	9.59	7.32	12.4	35.8	14.0	19.1	13.2	7.32	35.8
AUC_{0-24} (h*ng/mL)	7.80	7.94	8.88	10.9	10.9	12.3	9.91	7.80	12.3
Cl_p (mL/min/kg)	17.1	14.1	15.0	12.2	12.2	10.8	13.6	10.8	17.1
$AUMC_{0-24}$ (h*h*ng/mL)	13.0	5.23	16.1	40.3	23.4	31.5	19.7	11.6	40.3
V_{ss} (L/kg)	0.686	0.462	0.729	1.96	0.831	1.01	0.780	0.462	1.963
V_2 (L/kg)	0.073	0.045	0.128	0.094	0.058	0.091	0.082	0.045	0.128
V_3 (L/kg)	0.448	0.251	0.414	1.67	0.620	0.751	0.534	0.251	1.675
C_{pss} (ng/mL)	3.90	4.73	4.45	5.48	5.48	6.18	5.10	3.90	6.18

A,B and C, intercepts at t=0 for the model equation; alpha, beta and gamma, slopes for the model equation; C_{max}, extrapolated plasma GLY concentration at time 0; V_1, V_2, V_3, volumes of the central, second and third compartments, respectively; k_{21}, k_{31}, k_{12}, k_{13}, distribution rate constants; k_{10}, elimination rate constant; $t_{1/2\alpha}$, phase 1 half-life; $t_{1/2\beta}$, phase 2 half-life; $t_{1/2\gamma}$, phase 3 half-life; AUC, area under the plasma concentration vs. time curve; Cl_P, total plasma clearance; AUMC, area under the first moment curve; V_{ss}, volume of distribution at steady state; C_{pss}, plasma GLY concentration at steady state.

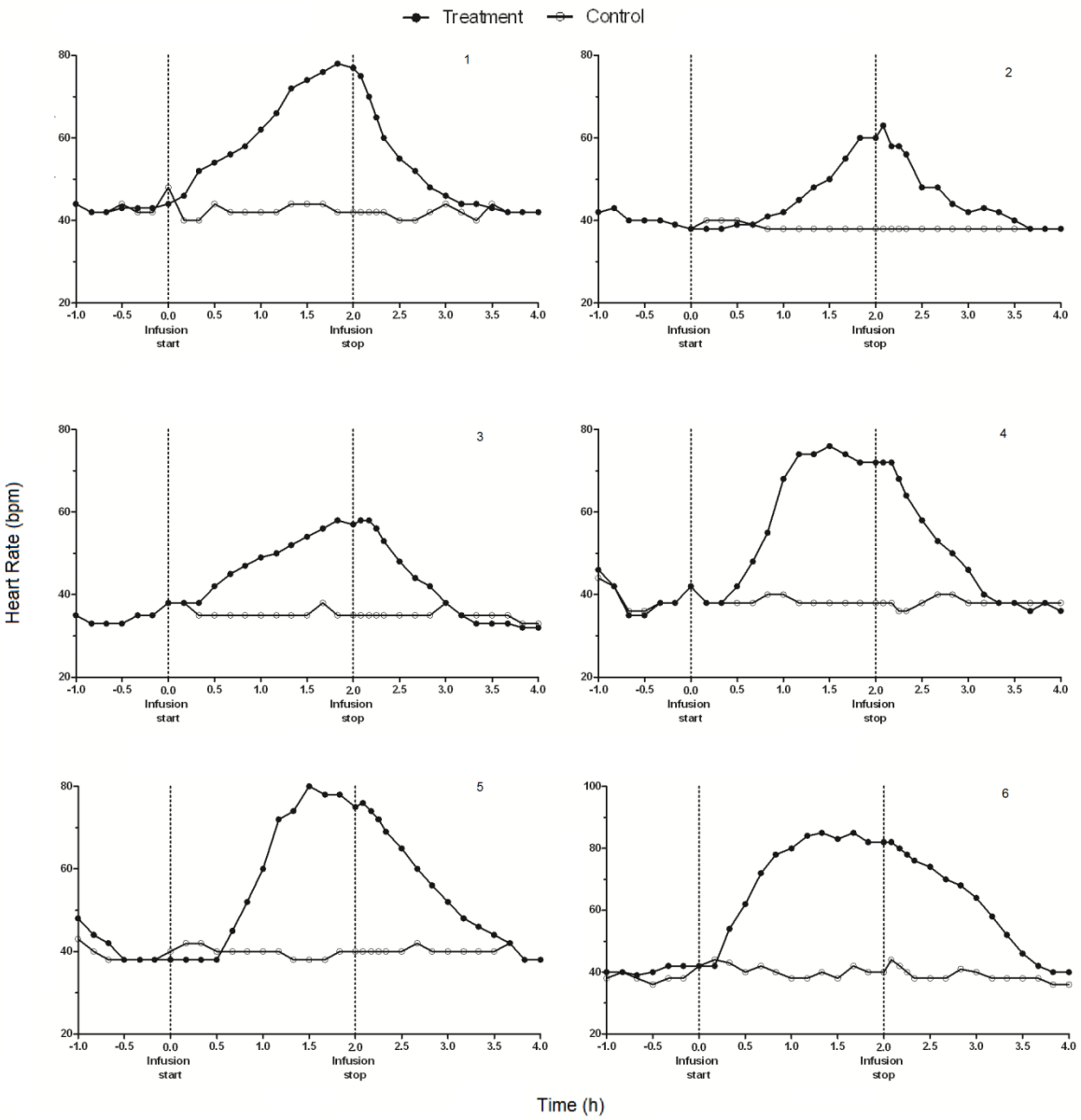

Figure 5-5. Heart rate for each subject following a 2 h CRI of GLY.

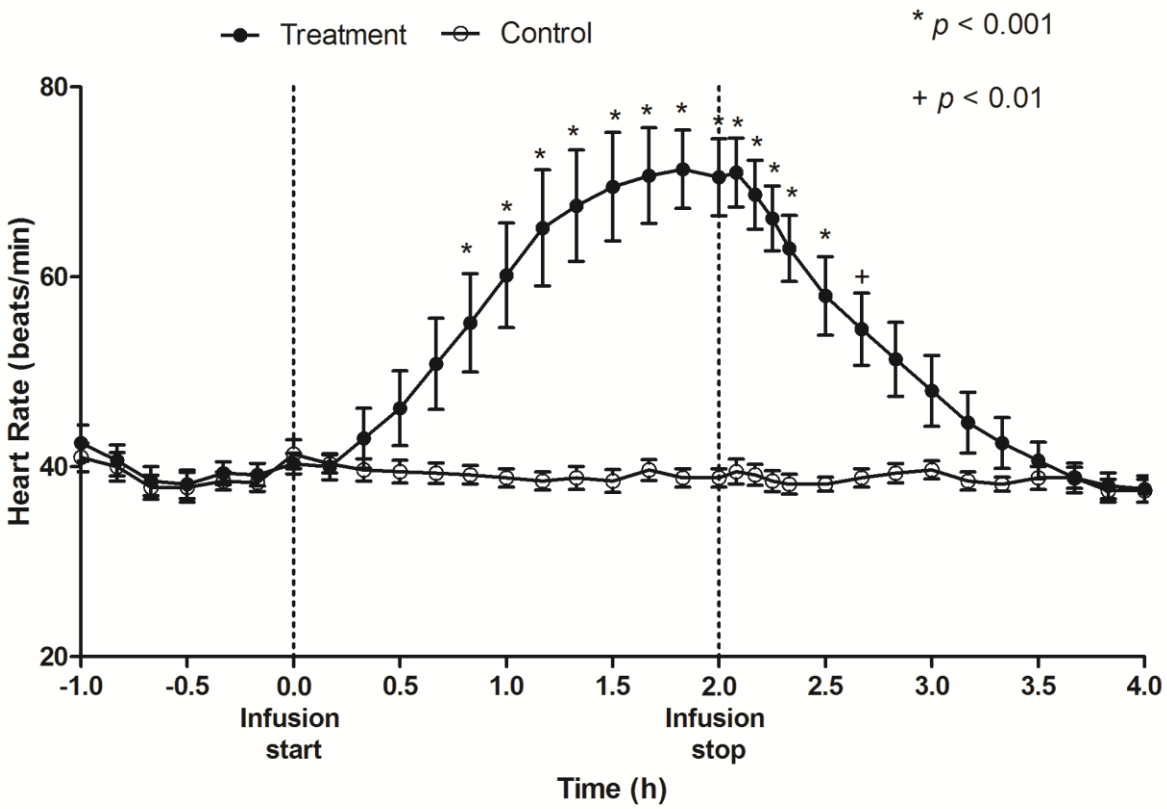

Figure 5-6. Mean (SD) heart rate (bpm) for six horses during the direct observation period.

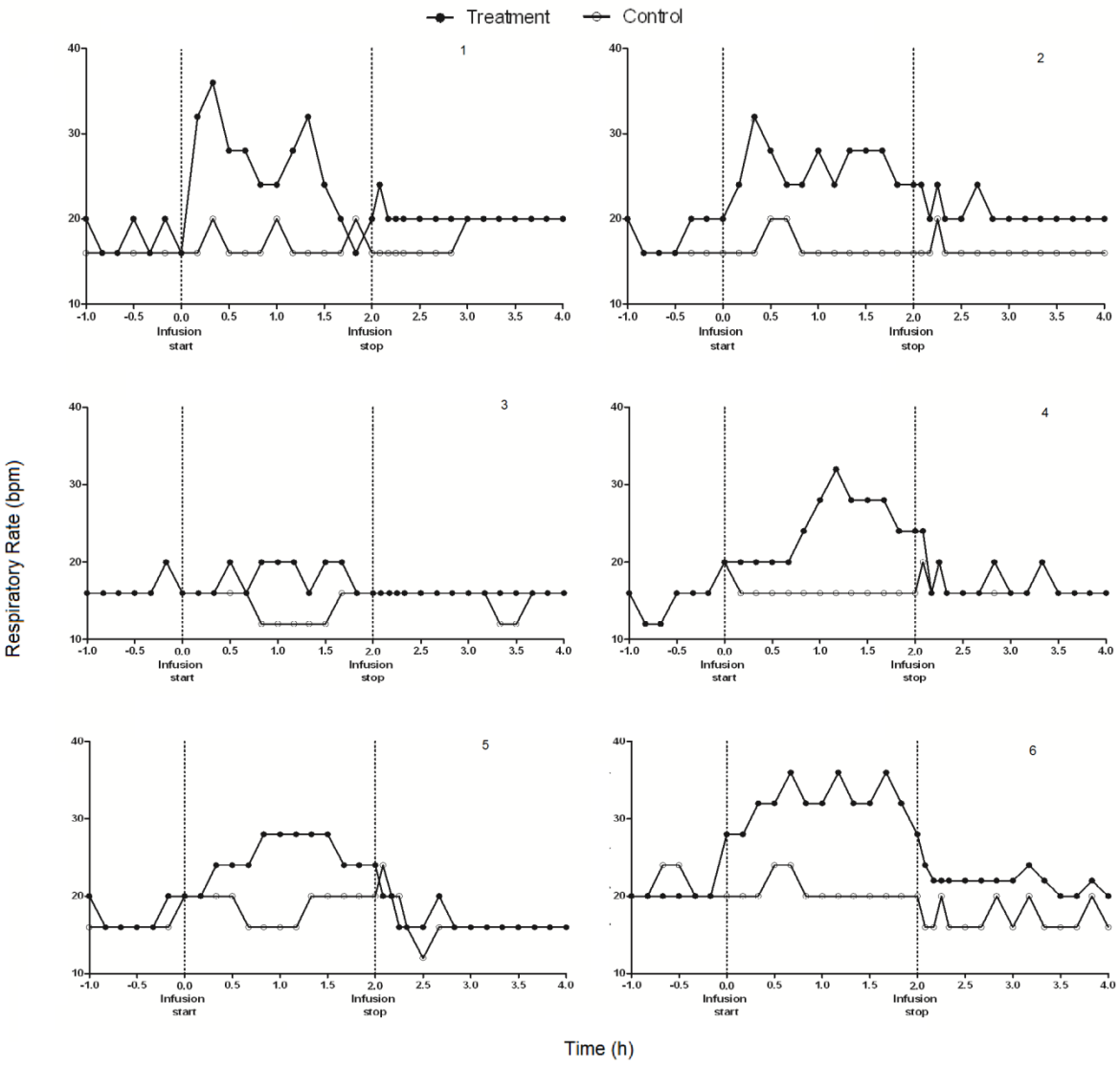

Figure 5-7. Respiratory rate for each subject following a 2 h CRI of GLY.

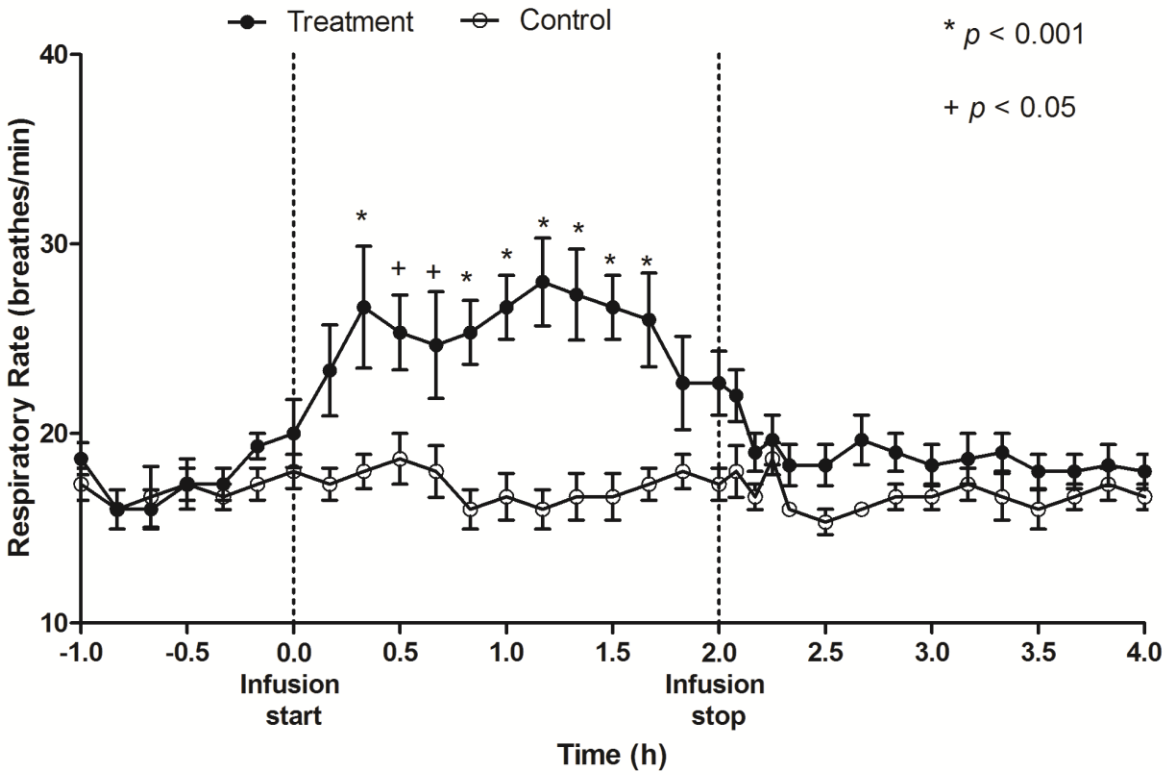

Figure 5-8. Mean (SD) respiratory rate (breathes/min) for six horses during the direct observation period.

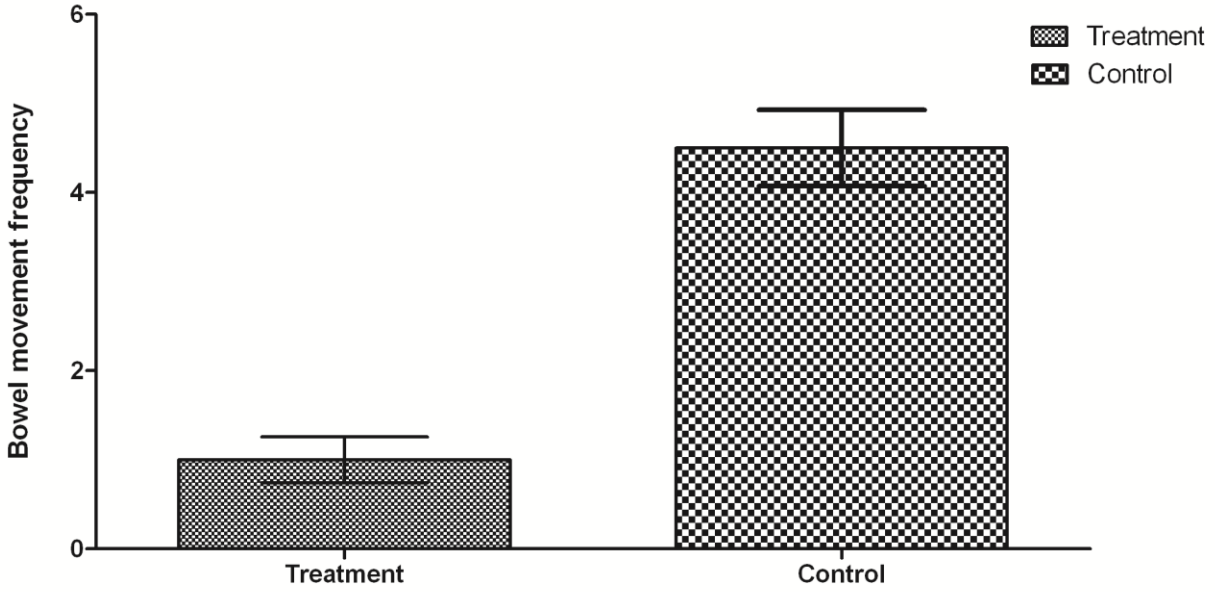

Figure 5-9. Mean (SD) frequency of bowel movements for the entire direct observation period (t_0 = start of the infusion) for six healthy horses administered GLY (8 µg/kg of body weight) over a two-hour intravenous infusion.

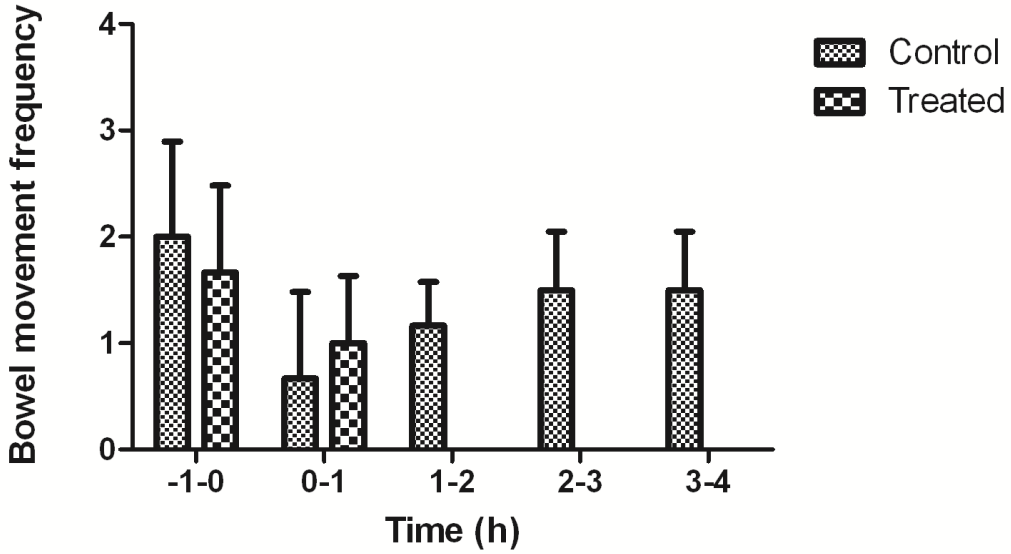

Figure 5-10. Mean (SD) frequency of bowel movements for the entire direct observation period (t_0 = start of the infusion) for six healthy horses administered GLY (8 µg/kg of body weight) over a two-hour intravenous infusion.

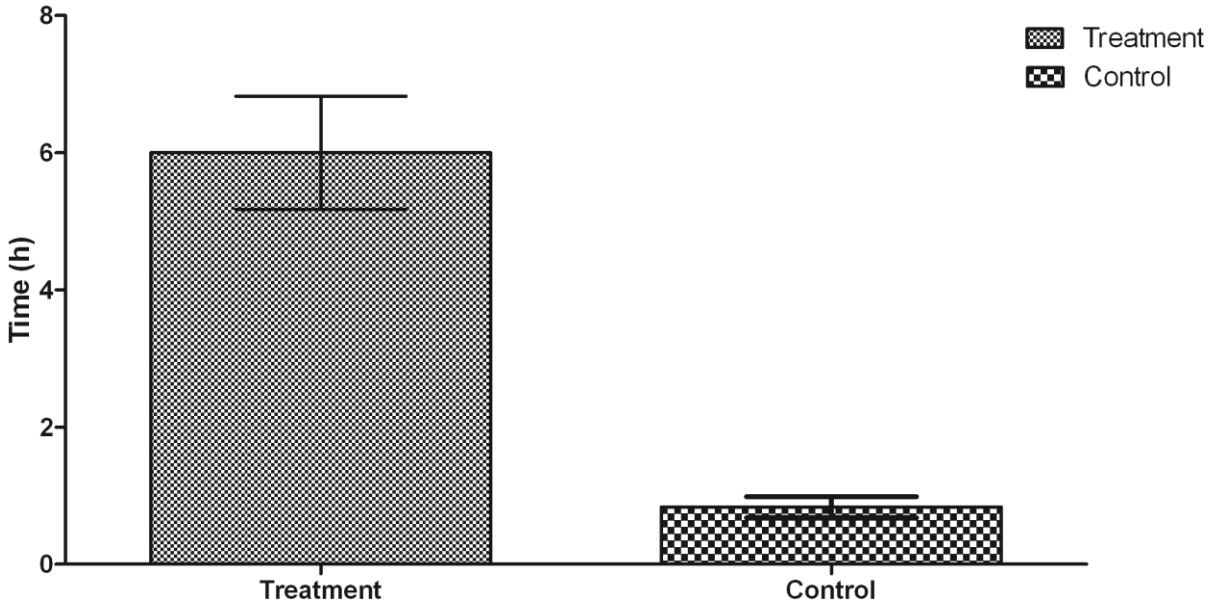

Figure 5-11. Mean (SD) time elapsed from the discontinuation of the infusion (t=0) until the first bowel movement for six healthy horses administered GLY (8 µg/kg of body weight) over a two-hour intravenous infusion.

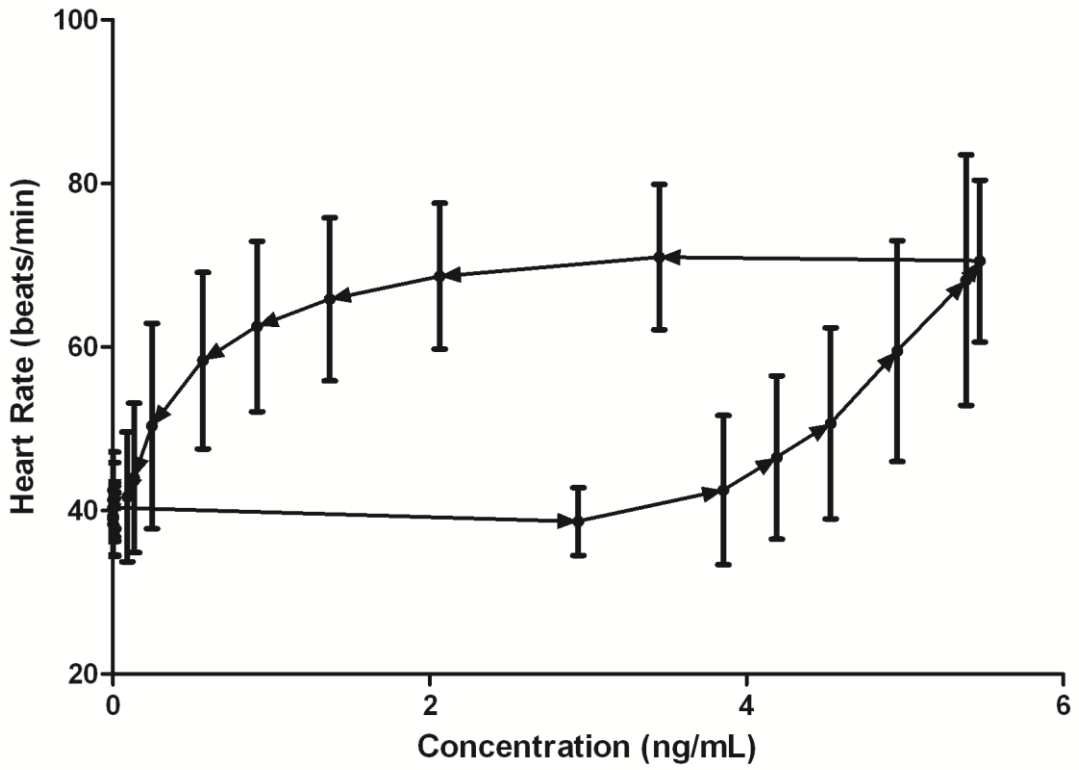

Figure 5-12. Hysteresis (counterclockwise) plot demonstrating that the there is a temporal lag between the physiologic endpoint (heart rate) and plasma GLY concentration. Heart rate represents mean (SD) of all subjects.

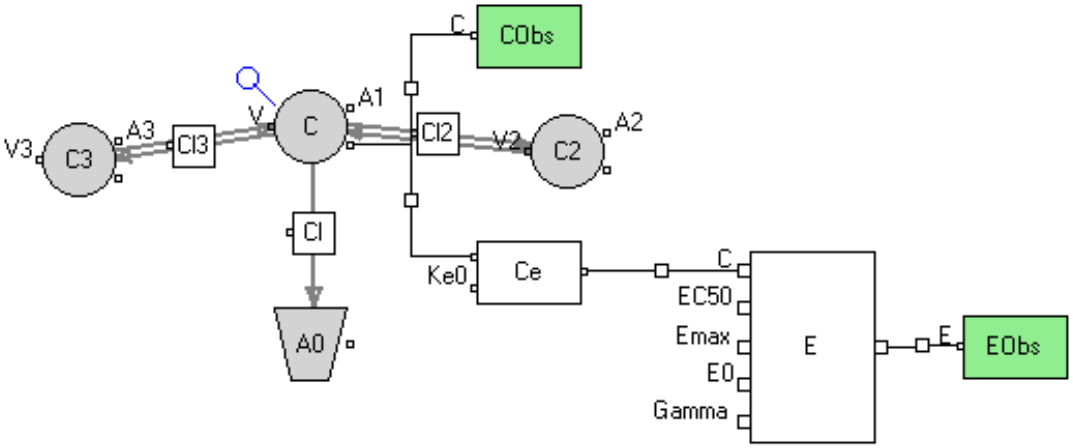

Figure 5-13. The effect compartment model.

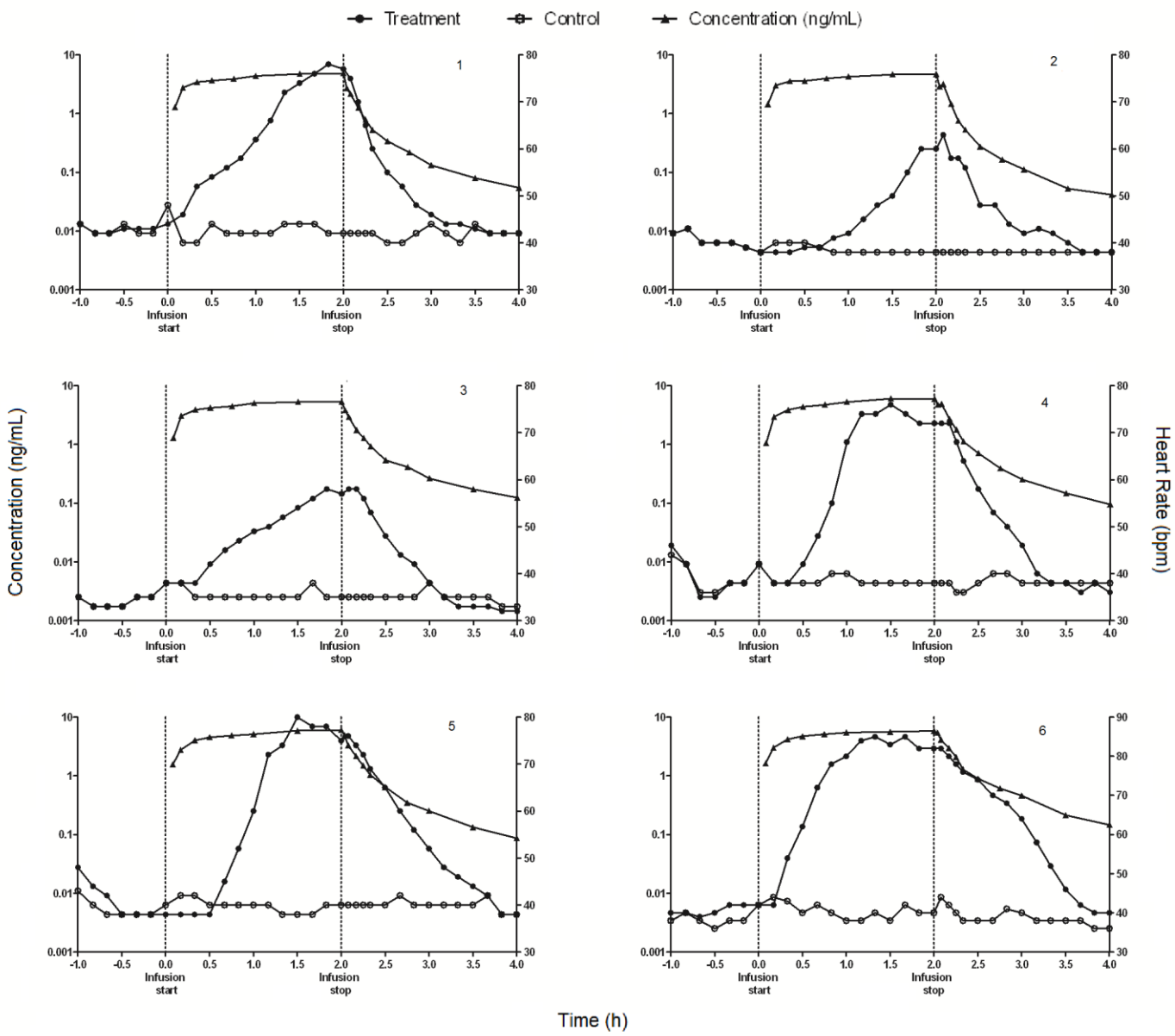

Figure 5-14. Mean plasma GLY concentration and mean heart rate effects over time for each subject. Error bars are removed for clarity.

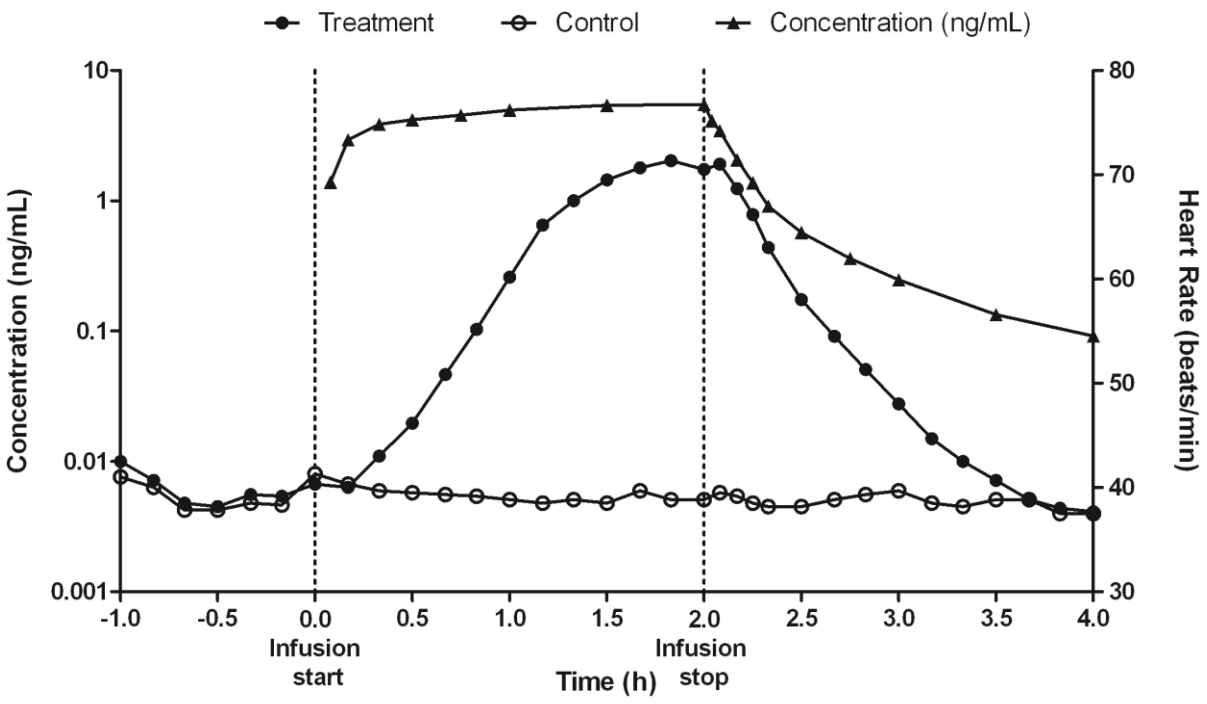

Figure 5-15. Plot demonstrating that the there is a temporal lag between the mean physiologic endpoint (heart rate) and mean plasma GLY concentration. Error bars are removed for clarity.

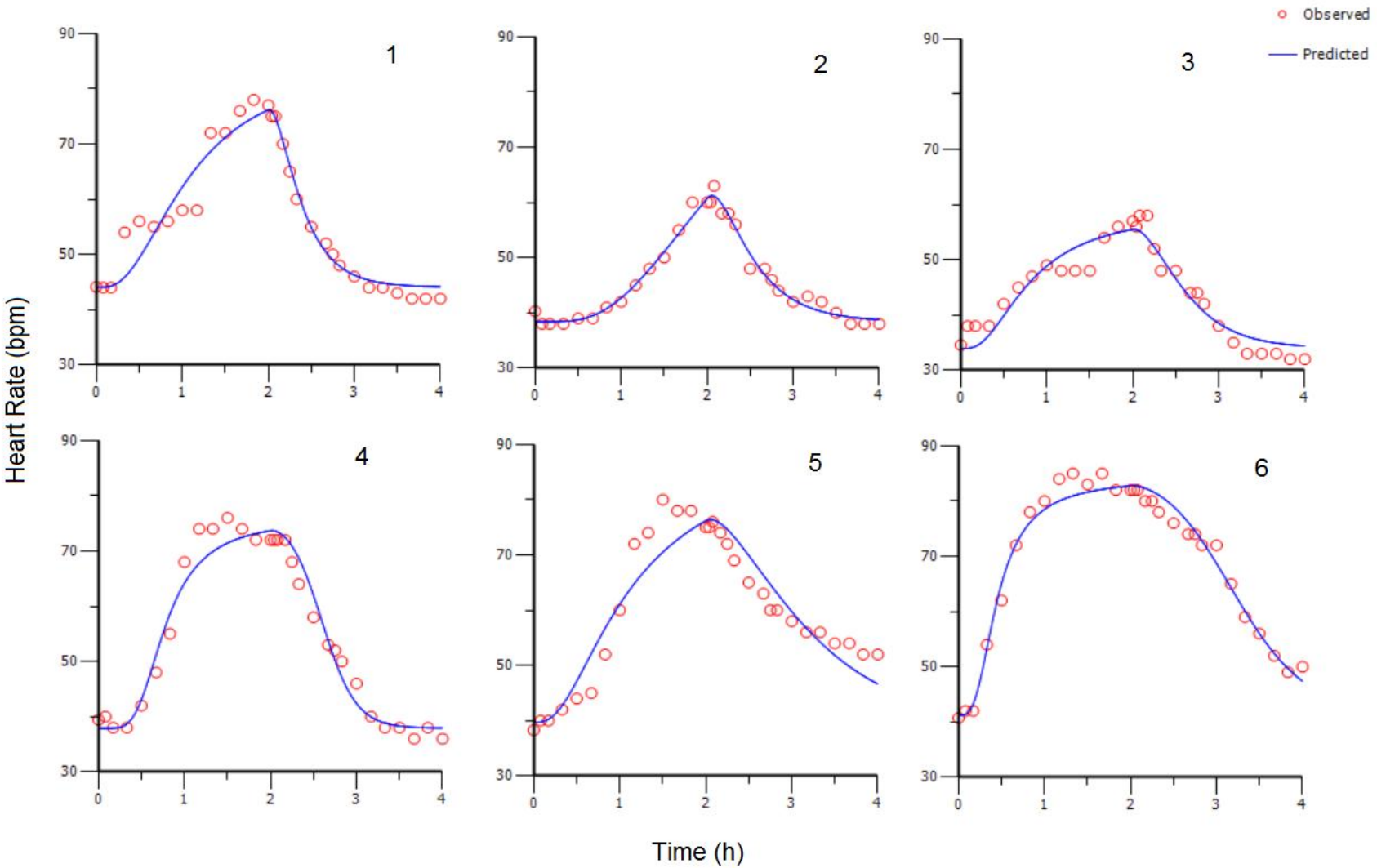

Figure 5-16. PK-PD linked model fit for heart rate for each of six horses.

Table 5-5. Pharmacodynamic model parameters.

	Subject #								
Parameter (units)	1	2	3	4	5	6	Median	Minimum	Maximum
E_{max} (bpm)	87.2	73.5	49.9	41.9	61.9	46.0	55.9	41.9	87.2
EC_{50} (ng/mL)	4.66	3.60	4.72	3.22	3.47	1.76	3.54	1.76	4.72
E0 (bpm)	44.5	38.6	33.9	37.9	40.3	41.3	39.5	33.9	44.5
n	2.81	3.99	1.87	3.70	1.96	2.01	2.41	1.87	3.99
ke0 (h^{-1})	1.61	0.697	1.63	1.58	0.811	1.41	1.49	0.697	1.63
$t_{1/2}$ke0 (h)	0.430	0.994	0.425	0.439	0.855	0.491	0.465	0.425	0.994

E_{max}, maximal effect; EC_{50}, plasma drug concentration producing 50% of E_{max}; n, Hill coefficient; k_{e0}, rate constant of equilibrium of drug compartment; $t_{1/2}k_{e0}$, half-life of equilibrium of drug in effect compartment.

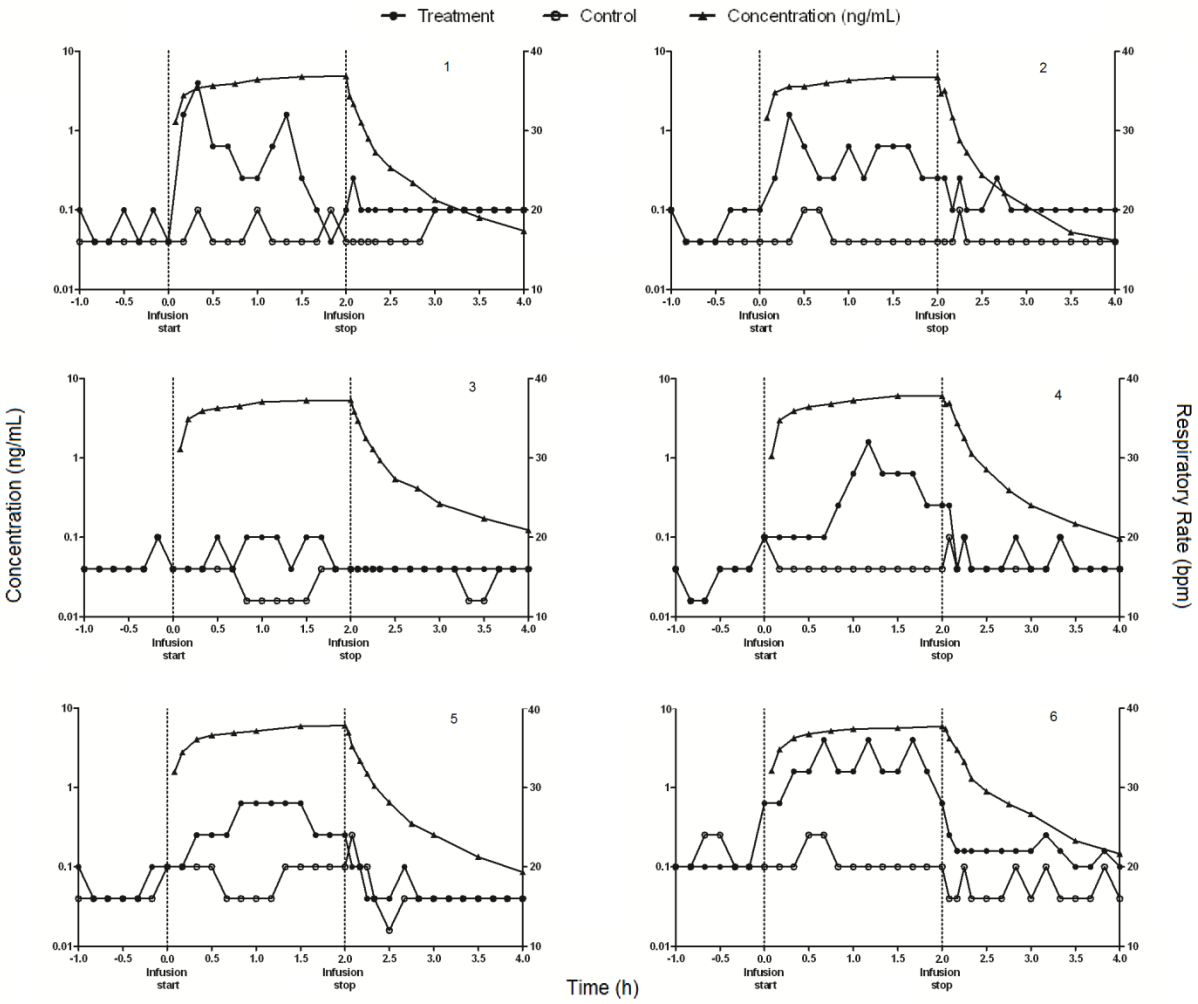

Figure 5-17. Mean plasma GLY concentration and mean respiratory rate effects over time for each subject. Error bars are removed for clarity.

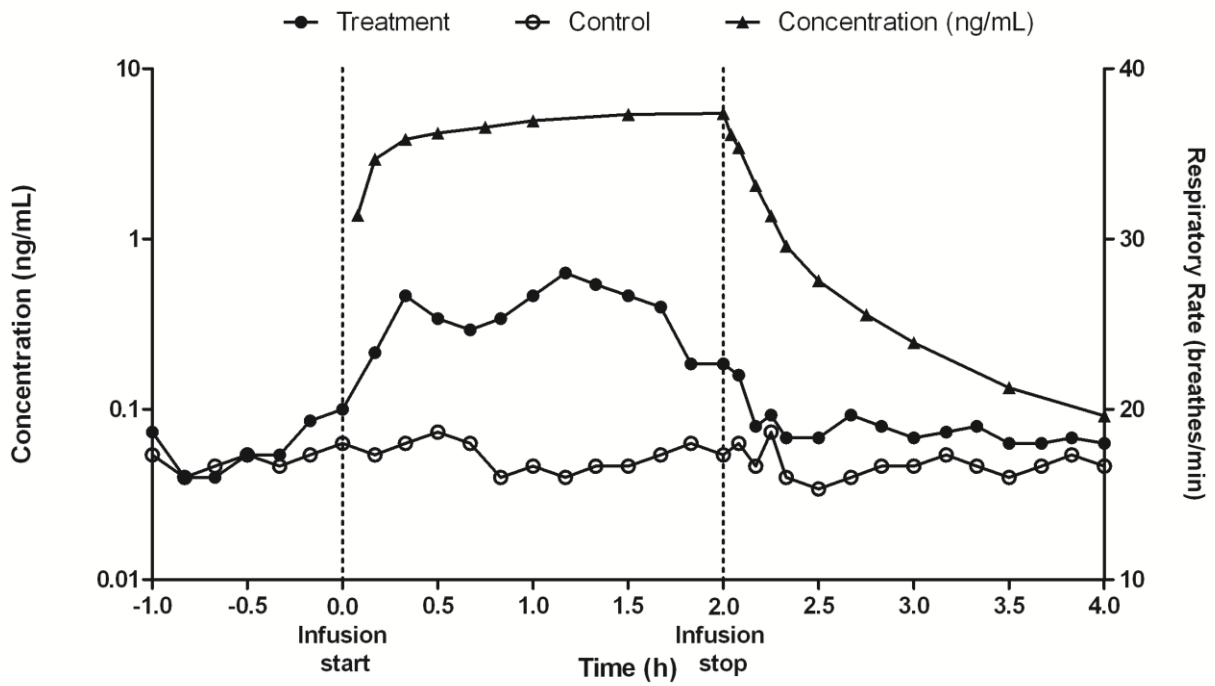

Figure 5-18. Plot demonstrating that the mean maximum effect for the physiologic endpoint (respiratory rate) occurs before mean maximum plasma GLY concentration. Error bars are removed for clarity.

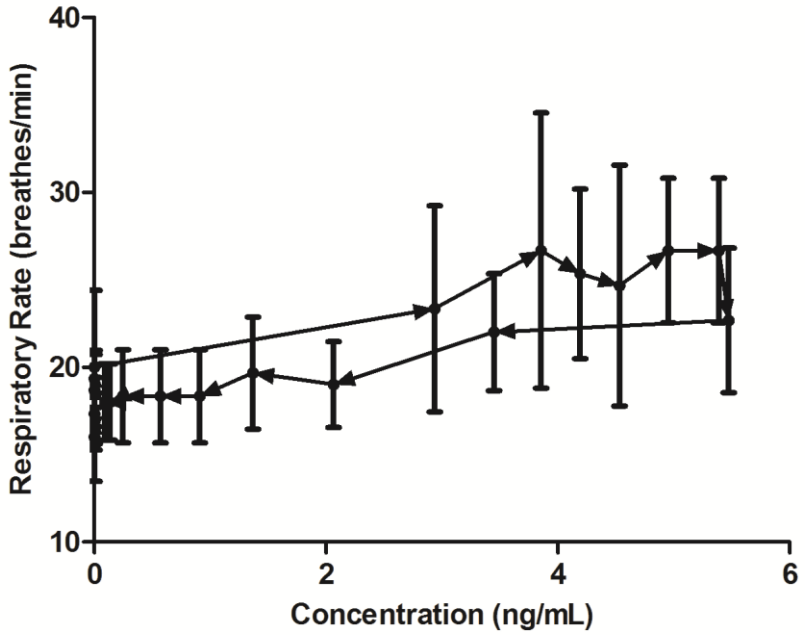

Figure 5-19. Mean (SD) respiratory rate as a function of concentration for six horses demonstrating proteresis (clockwise) plot.

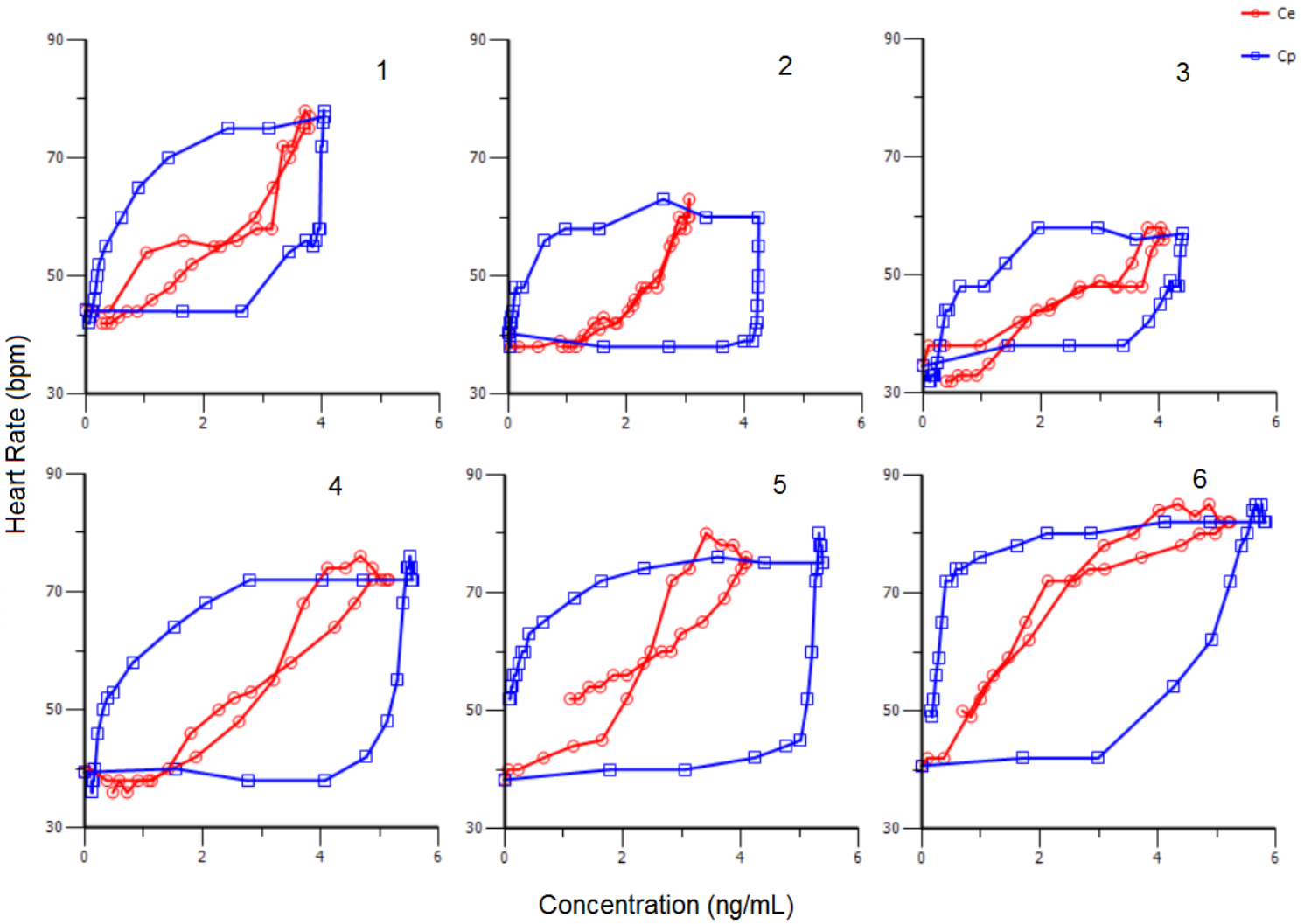

Figure 5-20. Individual hysteresis showing the plasma concentration and the predicted effect compartment concentration after the PK-PD link model was applied.

CHAPTER 6
CONCLUSIONS AND FINAL REMARKS

Analytical Methodology

The results of method development studies described herein have demonstrated that the identification of glycopyrrolate (GLY) in horse plasma and urine samples can be accomplished at sub picogram per milliliter concentrations using common analytical laboratory procedures and modern analytical instrumentation. Moreover, the methods described, have undergone the necessary validation procedures to demonstrate that they are acceptable for their intended use, for quantification of GLY in horse plasma and urine, and meet the minimum requirements set forth by United States Food and Drug Administration (USFDA) (Guidance for Industry, Bioanalytical Method Validation, 2001). The methods, as described, have been successfully applied to the analysis of plasma and urine specimens collected from research horses after administrations of GLY for elimination and pharmacokinetic-pharmacodynamic studies. Currently, the reported methods for the determination of GLY in horse plasma and urine are believed to be the first reported with adequate sensitivity and selectivity for regulatory control of this drug in performance horses. Such investigations could contribute to the RMTC effort to establish a plasma threshold and to recommend a withdrawal time for this drug in race horses.

Further improvements to the described methods can be accomplished with advanced sample preparation techniques and more sophisticated instrumental technologies. Yet, for the purposes of clinical evaluations and for the regulatory control of this drug as a performance enhancer in both humans and horses the method is adequate and robust.

Pharmacokinetics and Pharmacodynamics

To the author's knowledge, the pharmacokinetics of GLY in the Thoroughbred or Standardbred horse have not previously been investigated. These studies have suggested that the

plasma pharmacokinetics of GLY in the horse following a single intravenous and clinically relevant dose can be characterized by a three-compartment mammillary model. Extensive distribution from the central compartment, rapid clearance and prolonged terminal half-life were observed. 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 GLY concentrations following a single intravenous dose is a noteworthy observation that should be considered when evaluating the pharmacologic significance of the presence of GLY 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.

The plasma pharmacokinetics of GLY have also been demonstrated in Standardbred horses and PK parameter estimates have been compared between two different breeds of horses. Obvious differences are apparent between breeds. However, differences in study environments may contribute to such differences in PK parameter estimates. Further studies are needed to confirm differences in drug distribution among breeds.

Clearance of GLY in the horse occurs predominately in the liver and contrasts with previous data obtained from human studies. Renal clearance of unchanged GLY accounts for approximately 10-25% of the total clearance of GLY and plasma hydrolysis of the drug does not occur appreciably in horses. Active secretion of GLY into the renal tubular lumen is unlikely because the rate of excretion does not exceed rate of glomerular filtration and unchanged GLY is not expected to be reabsorbed in the nephron due to the compound's polarity. Further, the effect

of protein binding on the clearance of GLY is thought to be minimal because of the limited degree to which the drug is renally cleared.

The results show that the use of concomitant PK-PD modeling provided independent, accurate information about the transport of GLY to the site of action, understanding about the nature of the observed effects and the underlying concentration-effect relationship. Heart rate is significantly increased for at least 60 min following IV administration. Respiratory rate is also affected but the effect has a shorter duration of action. Defecation incidence and frequency are slowed following GLY administration and the drug must be used conservatively due to intestinal complications.

Regulatory Control

GLY is a class 3 drug as categorized by the ARCI and therefore its identification in official post-race track samples may lead to violations and sanctions for the trainer or owner or both. However, GLY is also well documented in the medical and scientific literature to be an effective human and veterinary therapeutic drug. In order to allow for the legitimate treatment of horses before racing or performance events, withdrawal periods or threshold limits (allowable drug concentrations) for each regulated drug may be set by the various associations.

Concentrations of GLY have been documented to be present in samples taken from post-competition horses. Therefore, it is imperative to determine drug concentrations which no longer provide a performance-altering effect. The capability to detect and quantify small amounts or concentrations of drug substances is critically important for research and regulatory purposes. However, for routine drug analysis in performance horses such sensitivity may reveal pharmacologically insignificant concentrations of drugs that were legitimately used for therapeutic purposes.

One way in which analytical testing laboratories respond to industry concerns over drug use in performance horses is to develop and validate methods and utilize instruments that offer superior sensitivity. Although such increases in sensitivity are necessary for substances that have no practical medicinal value, therapeutic medications should undergo a different level of scrutiny. In fact, it is not uncommon to detect small concentrations of therapeutic medications and it can be argued that the presence of such substances at small concentrations will have no observed effect on a horse's performance.

As part of this drug monitoring process, bioanalytical methods must be available to determine drug concentrations in plasma and urine. Since bioanalytical methods have become highly sensitive, physiologically relevant concentrations of drugs must be determined in order to regulate threshold drug concentrations in plasma and urine that are to be referenced for drug testing, instead of relying on the detection limits of the analytical methods. The investigations detailed above, describe GLY plasma and urine concentrations and disposition following different dosing regimens and can provide regulatory agencies with sufficient data to support certain threshold limits and withdrawal guidelines for the horserace industry.

APPENDIX A
DRUGS AND INSTRUMENT

Figure A-1. GLY (Robinul-V®) injectable.

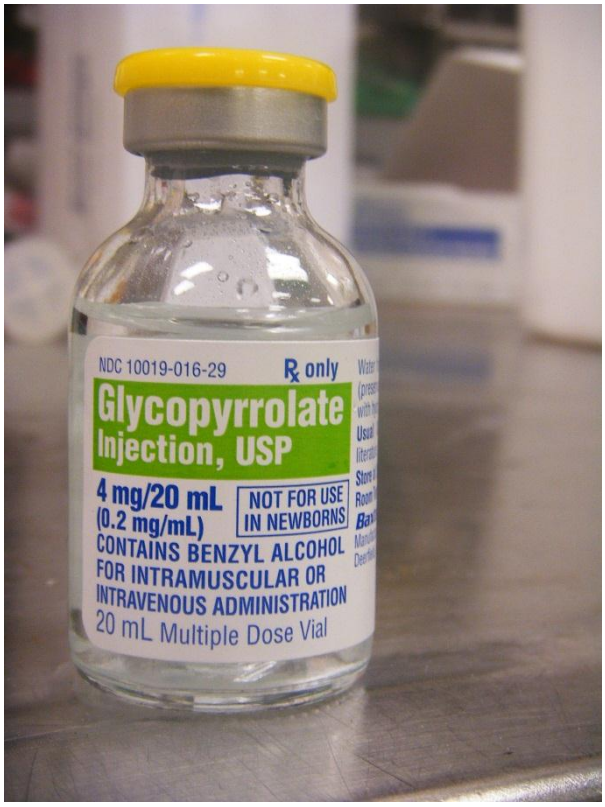

Figure A-2. Generic GLY injectable used for intravenous injection.

Figure A-3. Robinul-V tablets (1 mg each).

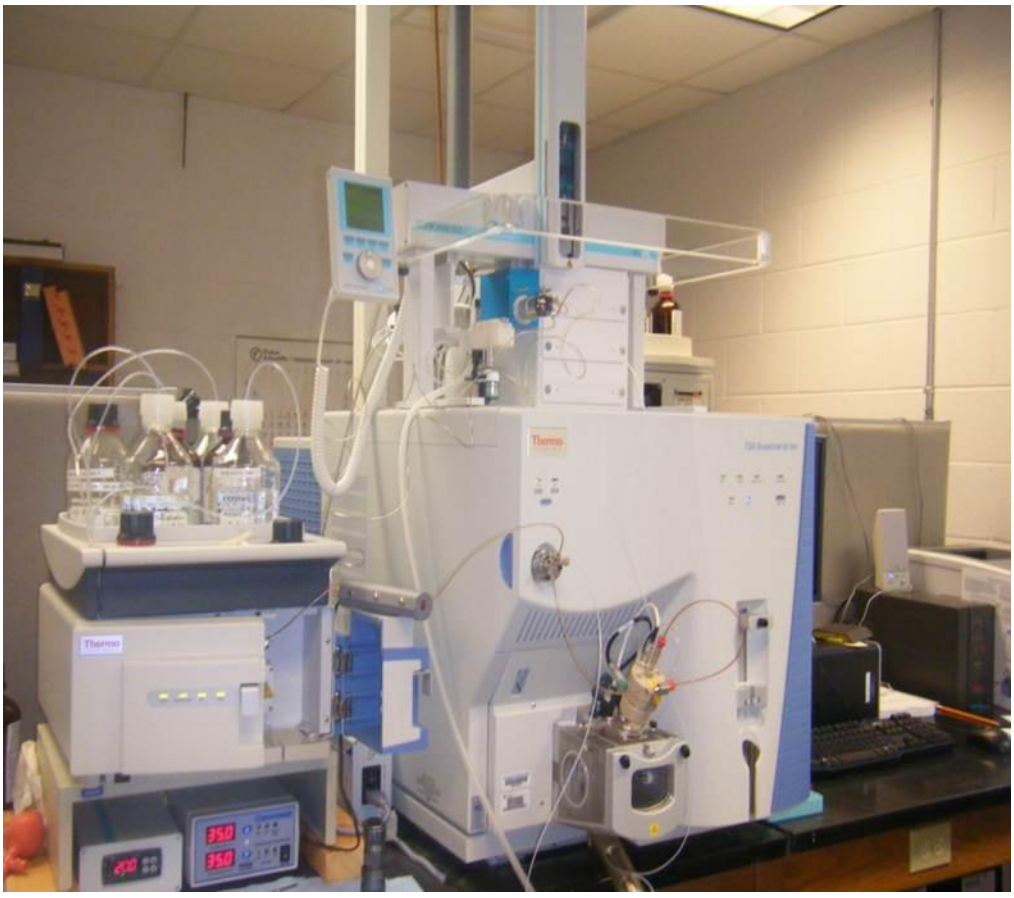
Figure A-4. Triple Stage Quadrupole (TSQ) Quantum Ultra mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with a heated electrospray ionization (HESI) source and interfaced with a HTC PAL autosampler (Leap Technologies, Carrboro, NC, USA) and Accela LC pump (ThermoFisher)

APPENDIX B
HORSE PICTURES

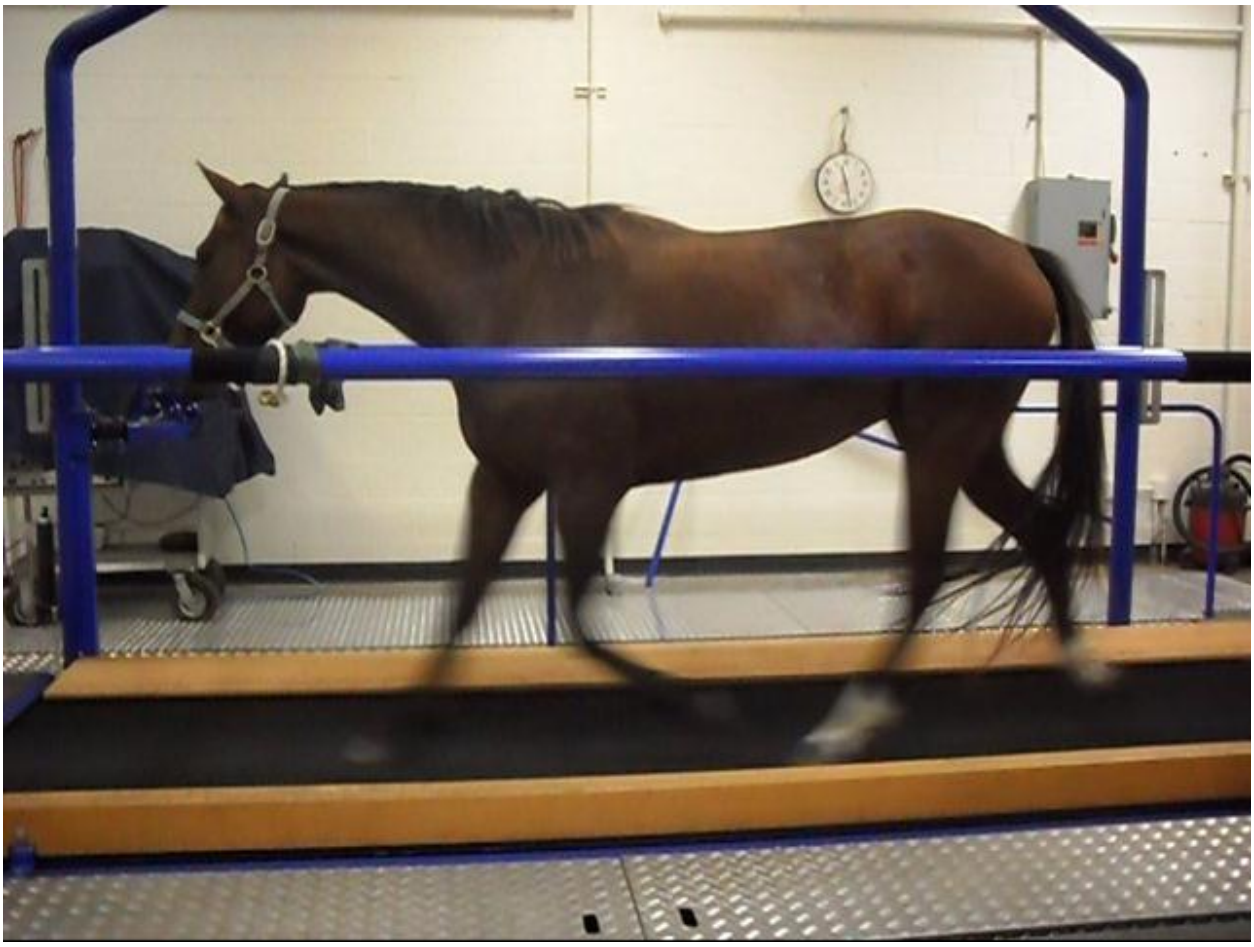

Figure B-1. Study horse being conditioned on a treadmill.

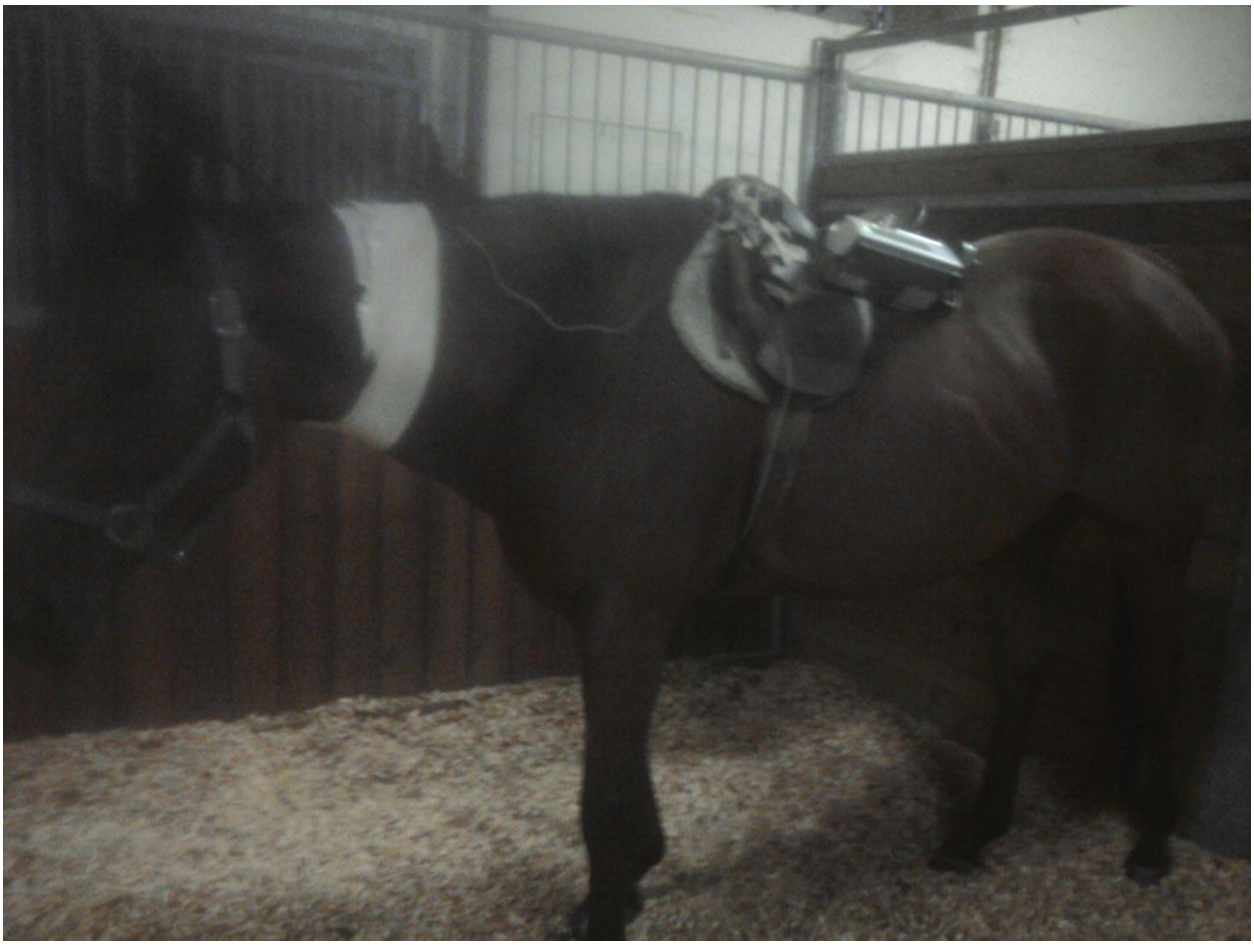

Figure B-2. Study horse receiving an intravenous infusion of GLY.

Figure B-3. During the PK-PD study, horses were housed in pairs, reducing anxiety and normalizing behavior.

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BIOGRAPHICAL SKETCH

Marc Jason Rumpler was born in August 1978, in Troy, New York. The younger of two sons, he graduated with his high school diploma from LaSalle Institute (grades 7-12), an all-male catholic military school, receiving exclusively average grades. Rumpler was admitted to the Bouvé College of Health Sciences at Northeastern University (Boston, Massachusetts) in August 1996, with an undetermined major and later went on to join the toxicology program under the guidance of Robert Schatz, PhD. From January of 1998 until May of 2001, Rumpler interned with several organizations including Biogen Idec, Inc. and the University of Massachusetts (UMass) Medical School (Worcester, MA). Rumpler graduated in 2001 with a Bachelor of Science degree in toxicology and was chosen by his peers to represent his class with a speech at commencement. After several years in private industry performing wet chemistry and routine analytical methods and procedures, Rumpler entered the University of Florida online program for forensic toxicology. In 2007, Rumpler earned his Certificate in Forensic Toxicology and one year later, a Master of Science degree in Forensic Toxicology. Later in 2008, Marc was accepted into the College of Veterinary Medicine at the University of Florida as a Physiological Sciences doctoral student. Here he was introduced to the Florida Racing Laboratory under the mentoring of Drs. Richard Sams and Nancy Szabo. Over four years, Marc was fortunate to participate on several different projects between the Racing and Equine Performance Laboratories. In March 2012 he was awarded “Excellence in Doctoral Studies” honor by the University Of Florida College Of Veterinary Medicine. In May 2012, Marc received his Ph.D. and later assumed a post-doctoral position with the National Institute on Drug Abuse (Baltimore, Maryland) under the guidance of Dr. Marilyn Huestis. Marc was the first person in his family to obtain a four year degree, and the first to obtain a graduate degree. Marc has a wife of 10 years and two children ages 3 and 14 at the time of this writing.