PHARMACOKINETICS OF ALBUTEROL SULFATE IN THOROUGHBRED VERSES STANDARDBRED HORSES

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
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To Mom and Dad: Thank you for all your love and support you have given me throughout my educational journey. To Jeremy, my brother and role model: Thank you for helping me push beyond academic challenges. To Kimberly, my sister and best friend: Thank you for constantly showing me how far a little love, hope, and faith can take you.

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AUC	area under the curve, the integral of the concentration-time curve (after a single dose or in steady state)
cAMP	cyclic adenosine monophosphate
CL	the volume of plasma cleared of the drug per unit time
INH	inhalation administration
IV	intravenous administration
LLOQ	lower limit of quantification
LOD	limit of detection
PO	oral administration

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Albuterol sulfate is a short-acting beta2 adrenergic agonist that acts as a bronchodilator used to treat a respiratory complication in horses known as recurrent airway obstruction (RAO). RAO is an allergic response from environmental allergens causing chronic coughing and dyspnea. Since RAO can limit a horse's performance, albuterol is given however, improper use or doping is becoming frequent in hopes to enhance a horse's performance. Therefore, agencies that regulate equine competitions need reliable pharmacokinetic information on albuterol. In order to understand the disposition of albuterol, we established 2 hypotheses to be tested: the route of administered albuterol sulfate would affect blood concentration over time; and the breed of the horse would influence the pharmacokinetics. Twelve Thoroughbred (n= 6) and Standardbred (n= 6) horses were randomly assigned to a 3-way cross over study. Albuterol was administered by three different routes at dose rates appropriate for each route (PO: 0.08 mg/kg, INH: 1 puff/50 kg, IV: 2 ug/kg). Whole blood samples were collected from the left jugular vein via needle venipuncture into lithium heparin evacuated tubes. Samples were collected before administration then at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 360, and 480, min; and 12, 16, 24 hours until day 7 after

administration. Plasma was aliquoted into 5mL a cryogenic vial, frozen then stored at –80 Celsius. All samples were analyzed by MS/MS Triple Stage Quadrupole mass spectrometer. Results showed that interaction between time and route were highly significant for the free concentration ($p= 0.0061$) and total concentration ($p= 0.0028$). Intravenous administration had a rapid response, while inhalation administration rate was steadier over time. Oral administration free concentration could not be detected because it followed the first pass mechanism. Data proved breed is not a significant factor (free, $p= 0.4718$) (total $p= 0.2082$). Adverse effects observed were muscle twitching, sweating, and coughing were observed in both breeds. Overall, the pharmacokinetics of albuterol sulfate in Thoroughbred and Standardbred horses can help prevent improper use and ensure it will be used correctly for the health corrections in the horse.

CHAPTER 1
INTRODUCTION

Albuterol sulfate is a short-acting beta2 adrenergic agonist that acts as a bronchodilator. When albuterol sulfate binds to beta2 adrenergic receptors in the lungs, these G proteins receptors activate adenyl cyclase resulting in an increase in cyclic AMP (cAMP). By increasing levels of cAMP, protein kinase A is activated which subsequently phosphorylates myosin light chain kinase making it become inactivated. An increase in inactivated phosphorylated myosin light chain kinase inhibits the phosphorylation of the myosin light chains and promotes relaxation of bronchial smooth muscles. As a result of smooth muscle relaxation, the airway lumen expands in diameter leading to a decrease in airway resistance and thus an increase in airflow. Therefore, ultimately resulting in a larger intake of oxygen. (Equine Exercise Physiology 5, equine vet. J.,supple.30 (1999) 575-580).

Albuterol sulfate has been used to treat a respiratory complication in horses known as recurrent airway obstruction (RAO). RAO is an allergic response from environmental allergens such as hay dust and moldy straw bedding and is characterized by a chronic cough, dyspnea, and inflammation of the airways (International Veterinary Information Services, Recurrent Airway Obstruction: Heaves, P. Lekeus (Ed.), 30 Nov 2001). Horses tend to exhibit a poor body condition due to an increased resting energy expenditure imposed by the increased respiratory effort required to compensate for the restricted airways (Am J Vet Res. 1999 Jun;60 (6):689-93).

Albuterol is a proven effective therapy for the relief of RAO. Since RAO limits a horse’s performance, the use of albuterol sulfate is becoming more frequently used in athletic horses. Due to the potential benefits, albuterol is being used as a doping agent

in hopes to enhance athletic performance. However, there is little scientific data confirming a performance enhancing effect. Therefore, agencies that regulate equine competitions need reliable pharmacokinetics for albuterol sulfate to establish threshold and withdraw times. Therefore, more information on this drug will provide a therapeutic dosing regiment that will not violate racing regulations.

In order to understand the disposition of albuterol sulfate we established 2 hypotheses to be tested: the route of administered albuterol sulfate would affect blood concentration over time; and the breed of the horse would influence the pharmacokinetics. To test these hypotheses, athletically conditioned Thoroughbred and Standardbred horses were administered albuterol sulfate by three different routes at doses appropriate for each assigned route (PO: 0.08mg/kg, INH: 1 puff/50 kg, IV: 2ug/kg). A total of twelve Thoroughbred (n=6) and Standardbred (n=6) horses (mares and geldings) were administered albuterol in a randomly assigned 3-way cross over study. All horses received albuterol by all routes of administration and served as their own control.

CHAPTER 2
MATERIALS AND METHODS

Twelve healthy Thoroughbred and Standardbred horses, mean age 8 years, average weight 515 kg, were all housed in a small paddock at the University of Florida Veterinary Hospital (Gainesville, FL) and were fed a commercial concentrate feed twice daily with constant access to hay and water. Horses received routine health care and physically conditioned by exercising on a treadmill adhering to an exercise routine that consisted of a walk at 4 m/s for 2.5 min (0.6 km) followed by a trot at 8 m/s for 2.25 min (3.0 km) then decreased back to 4 m/s for another 2.5 min (0.6 km) for a cool down. All horses performed this exercise regiment three days a week before and after drug administration. All horses were also physically fit before drug administration which was done by stress testing each horse under a strenuous exercise routine.

Albuterol dose delivery mechanisms varied with route. Oral administration consisted of 2 mg/5mL of a clear, thick syrup given in mouth with a syringe. Inhalation dosing was administered by a metered inhaler-mask delivering 90 mcg per activation. IV administration was given in the right jugular vein delivering 2 ug/kg approximately 5mL. The three-way cross over study was designed to limit external variables and keep a controlled environment. For example three horses of the same breed received the same route of administration when another three horses again of another breed received a different route of administration. Whole blood samples were collected from the left jugular vein via needle venipuncture into partially evacuated collection tubes containing lithium heparin. Sample were collected before administration and at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 360, and 480, min; and 12, 16, 24, 36, 48, 72, 96, 120, 144, and 168 hour (day 7) after administration. Blood samples were kept on ice until

centrifuged at 1400 RPM, in a 4 degree Celsius centrifuge for 15 minutes. Plasma was then aliquoted into 5 mL cryogenic vials and immediately frozen at –20 degrees Celsius and then stored at –80 Celsius within 48 hours. All samples were analyzed by MS/MS Triple Stage Quadrupole mass spectrometer to measure the pharmacokinetics of albuterol. The University of Florida Institutional Animal Care and Use Committee approved this experimental protocol.

Chemicals and Reagents

Certified analytical drug standards such as albuterol and albuterol-d_3 (IS) were obtained from Cerrilliant (Round Rock, TX) and CDN Isotopes (Quebec, Canada), Reagent grade formic acid was purchased from ACROS Organics (Morris Plains, NJ). All solvents: acetonitrile, methanol, and methylene chloride were HPLC grade and obtained from Thermo Fisher (Pittsburg, PA). All water used was de-ionized with a resistivity greater than or equal to 18 megaohms and organic content less than 10 ppb.

Stock standard solutions were prepared from solid form then dissolved in methanol. The working standard solutions were diluted to appropriate concentrations in methanol to yield a calibration curve ranging from 0.025-50 ng/mL. Calibrator and positive control working standard solutions were prepared from independent stock solutions. Each calibrator and positive control sample was prepared using 1.0 mL of ammonium acetate (0.1 M, pH 8.4) and 1-mL of drug-free control horse plasma, and fortified with the appropriate volume of albuterol working standard solution and 20 µL of IS working standard solution. The IS was prepared in a working standard solution at a concentration of 0.5 ng/µL. The final IS concentration was 10 ng/mL of plasma (Table 2-1).

Sample Preparation

For the analysis of unattached albuterol, duplicates of 1-mL aliquots of sample plasma were added to 1 mL of ammonium acetate buffer (0.1 M, pH 8.4) and 20 µL of 0.5 ng/µL internal standard working solution in 5-mL disposable, centrifuge tubes. The tubes were centrifuged at 1508 x *g* (2800 rpm) for 12 min and the buffered plasma samples were subjected to solid phase extraction. For the analysis of conjugated albuterol, 1-mL aliquots of sample plasma were added to 0.5 mL of 0.9 % saline solution, 1 mL of acetate buffer (1 M, pH 5.0) and 20 µL of 0.5 ng/µL internal standard working solution in 5-mL disposable, screw-top centrifuge tubes. A 0.5 mL aliquot (5000 U) of β-glucuronidase (*Helix Pomatia*) reagent solution (Sigma, St. Louis, MO) was added and adjusted to pH 4.5-5.0 by diluting the ammonium hydroxide. To trigger enzyme hydrolysis all tubes were placed in a 52 °C water bath for 2-3 hours. Samples were cooled for 10 minutes until at room temperature. All plasma samples were adjusted to pH 8.4 using sodium hydroxide, centrifuged at 1508x *g* (2800 rpm) for 12 minutes, and then used for solid phase extraction.

Strata-X-C 3-mL columns (Phenomenex, Torrence, CA) were conditioned with 2 mL of methanol and ammonium acetate buffer (0.1 M, pH 8.4). The buffered plasma specimens were placed onto the columns and a positive pressure was applied to achieve a flow rate (2 mL per minute). Columns were washed with 2 mL of ammonium acetate buffer (0.1 M, pH 8.4) followed by methanol. The analyte was eluted with two 0.5 mL aliquots of 5:95 ammonium hydroxide:methanol ratio. The elute was then evaporated with nitrogen on a TurboVap® LV evaporator (Zymark, Hopkington, MA). Sample extracts were dissolved in 100 µL of 0.1% (v/v) formic acid in methanol:water (10:90) then transferred to glass autosampler vials.

Instrumentation

LC/MS/MS analysis was performed on a Triple Stage Quadrupole (TSQ) Quantum Ultra mass spectrometer (ThermoFisher, San Jose, CA) equipped with a heated electrospray ionization (HESI) source and interfaced with a HTC PAL autosampler (Leap Technologies, Carrboro, NC) and Accela LC pump (ThermoFisher, San Jose, CA). Xcaliber™ software version 2.0.7 and LCquan version 2.5.6 were used for data analysis. Chromatographic separations were achieved with a Zorbax™ RX-C8 (2.1 x 150 mm x 5 µm) column (Agilent, Santa Clara, CA). Gradient elution (Table 2-2) began with a mobile phase of 0.1% (v/v) formic acid in water (80%) (Solvent A) and 0.1% (v/v) formic acid in methanol (20%) (Solvent B). The initial mixture was kept constant at a 250 µL/min flow rate and held isocratically for 0.5 minutes. Solvent A was decreased linearly to 0% and Solvent B increased to 100 % over 2.5 minutes and remained for 0.5 minutes. The mobile phase was then returned to the initial conditions for the remaining 1.0 minute (total run time of 4.0 minutes). The flow into the mass spectrometer was diverted from 0- 1.0 minute and 3.0 – 4.0 minutes. The column temperature was 40°C and 10 µL of the sample extract dissolved in 100 µL of methanol:water (10:90) containing 0.1% formic acid was injected. Mass spectral data were acquired in positive ion mode using the HESI and MS parameters: ESI spray voltage- 4100, vaporizer temperature-240°C, sheath gas pressure- 50, ion sweep gas- 2, auxiliary gas pressure- 25, capillary temperature- 300°C, tube lens offset- 89, and skimmer offset- 10.

Identification and quantification of the analyte were based on selected reaction monitoring (SRM). Compound specific optimization 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 (Table 2-3). Tuning for albuterol yielded

collision energies of 19, 13, and 10 for transitions 240→148, 240→166, and 240→222, respectively.

The most abundant ion transmission (*i.e*., m/z 240→148) for the analyte was used for quantification. Other transitions were used as qualifier transitions (Table 2-4). All standards, controls, calibrators, and samples were prepared in duplicate. Peak ion area ratios of the analyte and internal standard were calculated. Calibration was performed using a simple least squares linear regression analysis with a $1/C_p$ weighting factor, where C_p was the nominal plasma concentration. Quality control and sample acceptance criteria have been specified according to the following guidelines and standard operating procedures of the UF Racing Laboratory, Research Division. The limit of detection (LOD) was set at 0.025 ng/mL. and the lower limit of quantification (LLOQ) was 0.05 ng/mL. The requirement is that the %CV for all calibrators, positive controls, and samples must not exceed 10% (15% at the LLOQ). In addition, for calibrators the difference between the back-calculated concentration and the nominal concentration do not exceed 10% (15% at the LLOQ). All samples that did not meet such criteria were re-analyzed.

The University of Florida Bio-statistical Consulting Lab (Gainesville, Florida) provided statistical analysis of data. General estimating equations (SAS PROC GENMOD) were used to determine whether of drug administration and/or horse breed were a significant factor in the drug concentrations at the specified collection time points. A linear model was used to take the natural log of concentration for each outcome variable. Route, horse breed, time, and a two-way interaction were evaluated. Horse breed was considered to be a random factor. The variance was analyzed with an

exchangeable correlation matrix. Pheonix WinNonlin was also used to determine if albuterol sulfate functions in a non-compartment or compartment model.

Table 2-1. Preparation of calibrator

Calibrators	Concentration (ng/mL)
CAL-1	0.025
CAL-2	0.050
CAL-3	0.1
CAL-4	0.5
CAL-5	1
CAL-6	5
CAL-7	10
CAL-8	50
PC-A	0.05
PC-B	0.25
PC-C	2.5
PC-D	25
PC-E	50

Table 2-2. Gradient table for ALB LC method. A – Methanol 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	250
0.50	80.0	20.0	250
2.50	0	100	250
3.00	0	100	250
3.01	80.0	20.0	250
4.00	80.0	20.0	250

Table 2-3. Parameters for SRM acquisition obtained from ALB component tune file

Precursor Mass	Product Ion Mass	Scan Time (s)	Collision Energy (v)	Tube Lens Offset (v)
240.110	147.999	0.1	19	125
240.110	165.989	0.1	14	125
240.110	222.054	0.1	10	125
243.120	151.010	0.1	19	148
243.120	169.023	0.1	14	148

Table 2-4. Quantifying and qualifying ions for analysis of albuterol in extracts of horse plasma

Analyte	Quantifying ions (amu)	Qualifying ions (amu)
albuterol	148	166, 222
albuterol-d_3	151	169, 225

CHAPTER 3
RESULTS

The interaction between time and route of albuterol sulfate are highly significant (p=0.0061). This meant that the free drug concentration over time varied differently for each route of administration. For intravenous administration, the exponential decay was rapid, revealing that within one hour most of the drug had circulated through the body. When administered by inhalation, the exponential decay had a slope value near zero indicating that the rate of elimination was slower and steadier over time (in Figure 3-1).

Administration route was a predictor of the total concentration at each time point (p= 0.0008). All horses and time points had observed mean free concentrations for INH= 0.072±0.054 and observed mean concentration for IV= 0.51±0.64. Even though samples were taken until day 7 after administration, drug concentration could only be detected up to 36 hours for IV and INH. Data showed that IV administration had a rapid concentration, but INH had a more direct effect. Oral administration could not be detected because it was absorbed in the body almost as quickly as it was eliminated through the body, meaning it had concentrations below LOQ (in Figure 3-1). Mean total concentration over time was significantly different for different administration methods (0.0028). Also, administration method was a highly significant predictor of total concentration (p= 0.0033). All horses and time points had observed mean concentration for INH= 0.085±0.049, for IV= 0.67±0.49.

Breed of horse is not a significant predictor of free concentration (p= 0.4718) and total concentration (p= 0.2082). The rate of absorption and the rate of elimination for both IV and INH routes were very similar in both Thoroughbred and Standardbred horses.

The total drug concentration over time (elimination curve) was also significantly different for each route of administration (p= 0.0028) (in Figure 3-3). Therefore, route was a predictor of total drug concentration (p= 0.0033). Results indicated that at any given time point, total concentration of IV administration was 0.65 higher than INH (95% Cl= [0.500, 0.805]). INH was 1.52 lower then for the proposed method (95% Cl= [1.79, -1.26]).

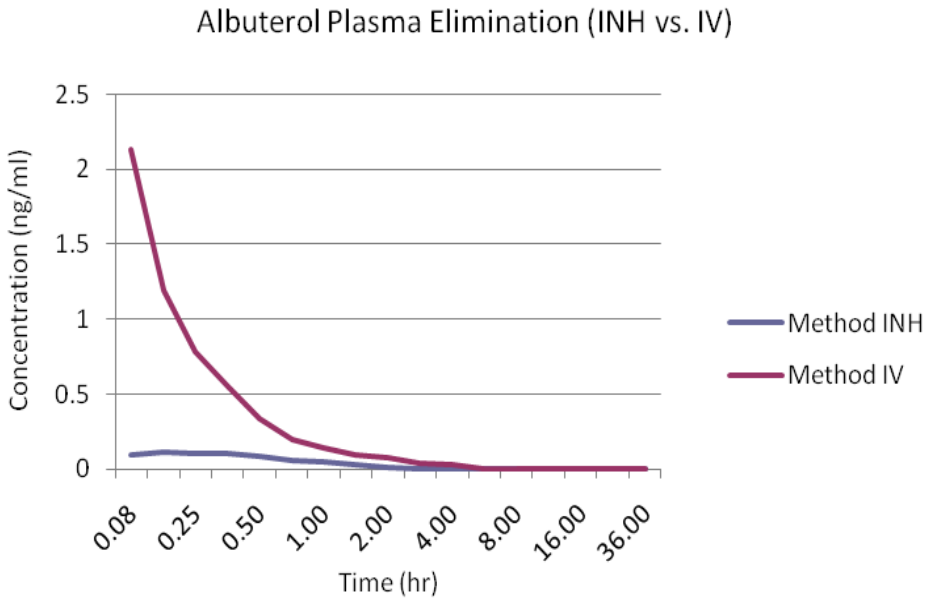

Figure 3-1 Elimination of mean free concentration of Albuterol following IV and INH administrations

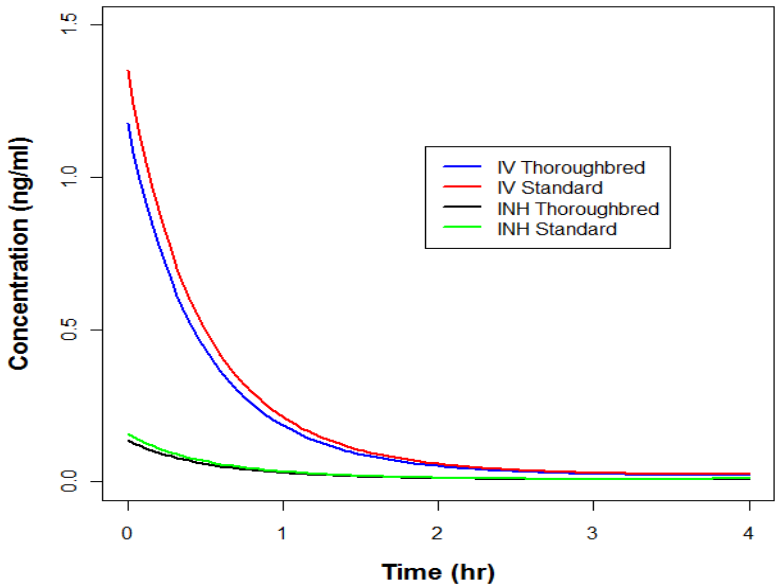

Figure 3-2 Estimated mean free concentration of albuterol over time for each horse

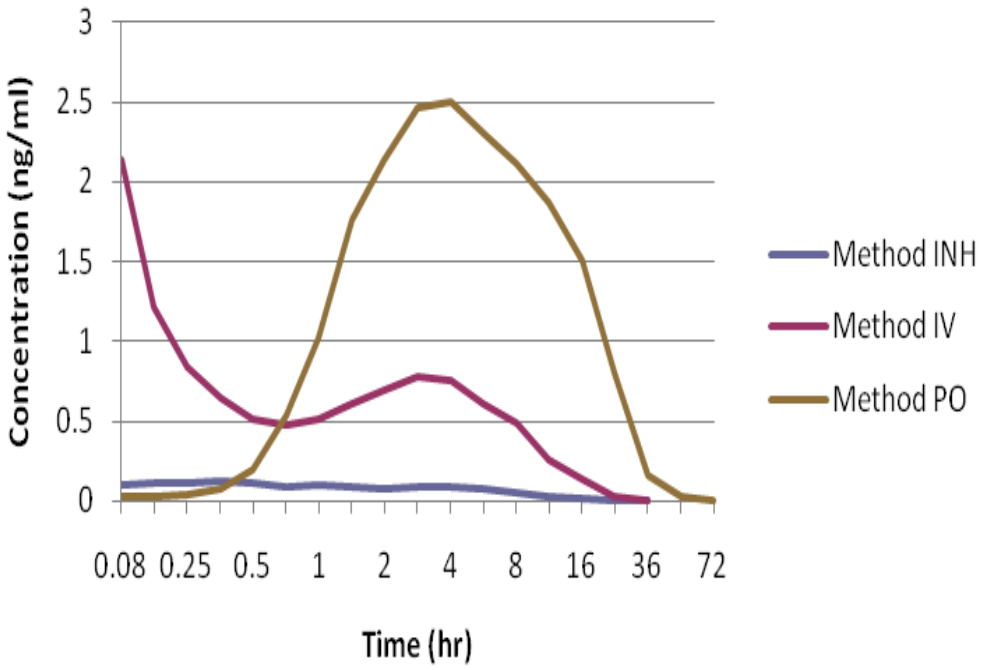

Figure 3-3 The mean total concentration varied with time in each horse

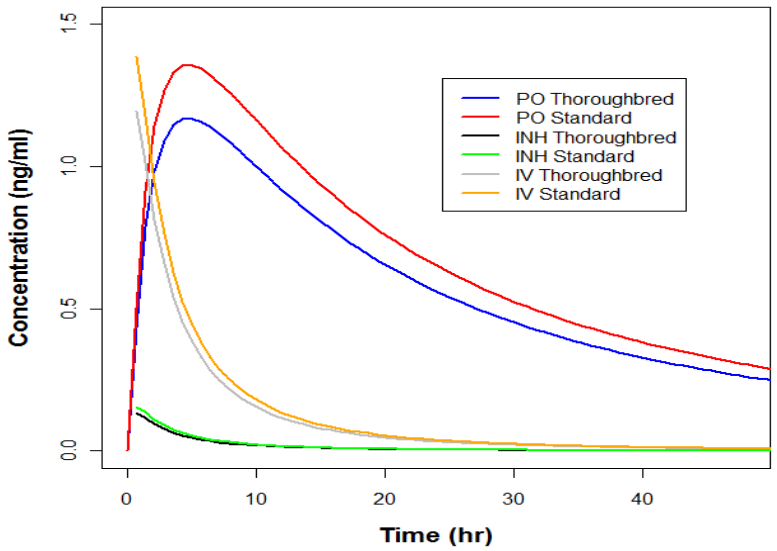

Figure 3-4 Liner model predication of how the free mean concentration varied with horses

CHAPTER 4
DISCUSSION

Albuterol sulfate varied with route. Intravenous administration had a quick response (immediately up to 2 hours), with inhalation following and oral administration unable to be detected. Furthermore, the elimination of albuterol also differed with the various routes administrations. Drug concentrations were observed up to 36 hours after administration. Clearance levels or the volume of blood cleared of drug per unit time averaged at 30.0 mL/min/kg for all horses. For oral administration, the clearance rate is in close range to the max flow rate meaning the drug is being cleared almost as fast as it is traveling through the body. The area under the curve (AUC) were similar for each route, averaging at 0.92 h*ng/mL for all horses. This means that the relative efficiency of the drug is apparent to the volume of distribution. The distribution of a drug is determined by rate of perfusion, plasma proteins, and tissue membranes. Intravenous and inhalation administrations acted in similar mechanisms on the body, while oral administration did not. Phoenix, a mathematical model was used to describe how this drug moved throughout the body and/or a specific compartment of a system. Analyses demonstrated the diffusion of albuterol sulfate within bodily tissues. Albuterol traveled quickly to the lungs (compartment 1) where it acted on the bronchial smooth muscle. Dilation and relaxation of the epithelial cells occurred thus allowed more intake of oxygen. Albuterol occurred as a conjugation reaction, meaning it produced an active metabolite. The metabolite bound to an inactive sulfate ester. When albuterol is bound in the body its total concentration can be measured therefore, an effect of the drug can be observed. After absorption, albuterol continued to travel throughout the body targeting the airways and circulated for excretion. Elimination of albuterol occurred by

metabolic conversion and excretion through the renal system in particular the kidneys, which was defined as compartment 2. The goal of metabolism is to enhance solubility. The kidneys filtered the unwanted waste or albuterol particles and excrete the drug thorough urine.

When administered orally, albuterol did not follow this two-compartment model because it was not absorbed in the blood stream. Therefore, it circulated the body as a free unattached particle. This confirmed the first pass mechanism, proving that the drug concentrations are greatly reduced before it reaches the circulatory system. This mechanism is an estimate of the area under the curve of a drug concentration over time and is dependent on the total drug exposure time. Unattached albuterol sulfate is less effective, thus it produced a reduced concentration in plasma when compared to inhalation and intravenous administration. Both inhalation and intravenous routes avoided this first pass mechanism because they entered directly or rapidly through the blood stream. Data showed the primary action of albuterol sulfate was to stimulate adenyl cyclase which catalyzed cAMP. As a result respiratory and cardiovascular effects occurred. Documented clinical effects were muscle twitching and excessive sweating. This was due to the stimulation of the beta2 adrenergic receptors (J.Bailey, P.Colahan, P.Kublist and L.Pablo. Effect of inhaled B2 adrenoceptor agonist, albuterol sulfate, on performance of horses. Equine Veterinary Journal, Volume 31, Issue S30, pages 575-580, July 1999).

CHAPTER 5
CONCLUSION

Overall, albuterol concentration did vary over time. Albuterol sulfate was most effective when administered intravenously since it immediately enters the blood stream. Administration via inhalation was effective, but had a more steady distribution. Both intravenous and inhalation administration functioned in a two compartment system which consisted of the respiratory and renal system. Oral administration did not operate in this model due to the first pass mechanism and did not have an adequate means of treatment. The results were similar between both Thoroughbred and Standardbred confirming the breeds had similar physiology of the species. Also data confirmed that breed does not affect the “fate” of albuterol, but route was a significant factor when determining the effectiveness of this drug. Studying the pharmacokinetics of albuterol sulfate in Thoroughbred and Standardbred horses gave knowledge on the threshold and withdrawal times of the drug. The clinically observed effects proved that albuterol had adverse effects, however none were performance inhibiting nor performance enhancing. The extent of effect and duration can allow albuterol to be used at a therapeutic drug. By knowing albuterol’s peak concentrations times we know its elimination rate in which will provide proper dosage at the race track. Understanding how this drug behaves in the body can help prevent improper use of albuterol sulfate and ensure it will be used correctly for the health corrections in Thoroughbred and Standardbred horses.

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BIOGRAPHICAL SKETCH

Allison Hreha was born and raised in Wayne, New Jersey as the second child to Albert and Patricia Hreha. She has two wonderful siblings Kimberly and Jeremy. Her family is very close to each other and she would not be the person she is today without them. In wanting to make a career for herself, she made the tough decision of moving to Florida to attend the University of Florida. Making the adjustment was difficult for her, but also very rewarding as she found her passion for veterinary medicine and love for horses. She graduated in December 2009 with bachelors in animal biology; concentration in equine science. She obtained a job at the University of Florida Veterinary Hospital in the Equine Performance Lab, which opened new doors for her. This job experience gave her the opportunity and access to be able to further her education by pursuing a master degree in veterinary pharmacology. She hopes to use this degree in helping her find an exceptional job in the related field or even some day attend veterinary medicine school.