

## Pharmacokinetics and pharmacodynamics of butorphanol following intravenous administration to the horse

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Butorphanol is a narcotic analgesic commonly used in horses. Currently, any detectable concentration of butorphanol in biological samples collected from performance horses is considered a violation. The primary goal of the study reported here was to update the pharmacokinetics of butorphanol following intravenous administration, utilizing a highly sensitive liquid chromatography-mass spectrometry (LC-MS) assay that is currently employed in many drug-testing laboratories. An additional objective was to characterize behavioral and cardiac effects following administration of butorphanol. Ten exercised adult horses received a single intravenous dose of 0.1 mg/kg butorphanol. Blood and urine samples were collected at time 0 and at various times for up to 120 h and analyzed using LC-MS. Mean  $\pm$  SD systemic clearance, steady-state volume of distribution, and terminal elimination half-life were  $11.5 \pm 2.5$  mL/min/kg,  $1.4 \pm 0.3$  L/kg, and  $5.9 \pm 1.5$  h, respectively. Butorphanol plasma concentrations were below the limit of detection (LOD) (0.01 ng/mL) by 48 h post administration. Urine butorphanol concentrations were below the LOD (0.05 ng/mL) of the assay in seven of 10 horses by 120 h post drug administration. Following administration, horses appeared excited as noted by an increase in heart rate and locomotion. Gastrointestinal sounds were markedly decreased for up to 24 h.

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### INTRODUCTION

Butorphanol is a narcotic analgesic agent used for treating both superficial and visceral pain in horses (Kalpravidh *et al.*, 1984a; Muir & Robertson, 1985). It is a synthetic, centrally acting agonist-antagonist with activity at both mu and kappa opioid receptors. Sellon and colleagues have previously studied and reported the pharmacokinetics of butorphanol following both intravenous (Sellon *et al.*, 2001) and intramuscular administration (Sellon *et al.*, 2009) to adult horses. Following intravenous administration, the authors reported an elimination half-life ranging from 18 to 90.4 min following a 0.1 mg/kg dose and  $7.8 \pm 5.1$  h following administration of 0.08 mg/kg. However, in the first study, butorphanol plasma concentrations fell below the limit of quantitation (LOQ) of the assay within 2 h of administration (Sellon *et al.*, 2001), and in the second study, butorphanol concentrations were still quantifiable by the termination of sample collection, making both studies less than ideal for establishing a withdrawal time prior to performance.

Butorphanol is a prohibited drug in horseracing and horse showing. Currently, butorphanol is classified as a Class 3 (Penalty Class B) foreign substance by the Association of Racing Commissioners International (ARCI). Its presence in a regulatory sample is prohibited and its use prior to racing or showing is restricted; therefore, it is important to establish an appropriate withdrawal time prior to competition to prevent inadvertent positives. Additionally, with the temporal increase in sensitivity of analytical instruments utilized by drug-testing laboratories more currently, it is necessary to reassess the pharmacokinetics of drugs regulated in performance horses to establish relevant withdrawal times, especially as they relate to drugs such as butorphanol, which can be violations at any detectable level. Previous studies have also only assessed butorphanol concentrations in blood (Sellon *et al.*, 2001); however, in many racing jurisdictions, urine is the matrix of choice for testing for illegal substances, necessitating study of butorphanol concentrations in urine. Accordingly, the primary objective in the present study was to quantitate plasma and urine concentrations of

butorphanol following intravenous administration of 0.1 mg/kg using a highly sensitive LC-MS method and to update published butorphanol pharmacokinetic values. LC-MS technology is becoming more commonly used by drug-testing laboratories to analyze the biological samples collected from racehorses and show horses.

Of additional concern to regulatory officials is the rumored use of a 'hub' dose prior to racing. Presumably, in the case of butorphanol, administration of a small dose might 'take the edge off' prior to competition. As the dose given is small and subsequent plasma concentrations are low, administration may avoid detection by drug-testing laboratories. Therefore, an additional objective of the current study was to assess the detection capabilities of the analytical assay used by many regulatory laboratories, following administration of low doses of butorphanol.

The third objective of the current study was to describe behavioral and cardiovascular effects of butorphanol following intravenous administration and to correlate plasma butorphanol concentrations with these effects. Butorphanol has been reported to cause excitation in horses in a dose-dependent manner (Robertson *et al.*, 1981). Robertson *et al.* (1981) noted an inconsistent increase in ambulatory activity between horses following intravenous administration of 0.1 mg/kg while a consistent increase in all horses was noted at 0.2 mg/kg butorphanol. Similarly, Nolan *et al.* (1994) also noted an increase in ambulatory activity following butorphanol administration to ponies, albeit at a much lower intravenous dose (0.05 mg/kg). Cardiovascular effects of butorphanol have been studied previously (Robertson *et al.*, 1981; Kalpravidh *et al.*, 1984b; Sellon *et al.*, 2001); however, the results are highly variable between these studies.

## MATERIALS AND METHODS

### *Animals*

Ten healthy, exercised adult Thoroughbred horses (five mares and five geldings with a mean  $\pm$  SD weight of 500  $\pm$  25 kg and an age of 3.8  $\pm$  0.8 years) were studied. All horses are part of the research herd at the University of California, Davis, and presently studied horses were exercised 5 days a week, following standard protocols established by our laboratory. Briefly, the protocol includes exercising horses utilizing a high-speed treadmill (Mustang 2200; Graber AG, Switzerland). The horses are worked in the following manner: walk 3 min at 1.9 m/sec; trot 5 min at 3.5 m/sec; canter 2 min at 7 m/sec; canter 2 min at 9 m/sec; walk 3 min at 1.9 m/sec.

Food was withheld for 12 h prior to and for 12 h following drug administration. Water was available *ad libitum* throughout the study. Before beginning the study, horses were determined healthy and free of cardiovascular diseases by physical examination, complete blood count and a serum biochemistry panel that included aspartate aminotransferase, creatinine phosphokinase, alkaline phosphatase, total bilirubin, sorbitol dehydrogenase, blood urea nitrogen, and creatinine. Blood analyses were

performed by the Clinical Pathology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital of the University of California, Davis, using the laboratory's standard protocols. Horses did not receive any other medications for at least 2 weeks prior to conducting this study. This study was approved by the Institutional Animal Care and Use Committee of the University of California at Davis.

### *Instrumentation and drug administration*

A 14-gauge catheter was aseptically placed in each external jugular vein. The right jugular vein catheter was used for drug administration while the contralateral catheter was used for sample collection. Each horse was weighed immediately prior to drug administration and received a single intravenous dose of 0.1 mg/kg butorphanol (Torbugesic, Fort Dodge, IA, USA), followed by 5–10 mL of a heparinized saline flush. The right jugular vein catheter was removed following dosing.

The following week, two of the horses studied previously were randomly selected for a micro-dosing study. In this study, 1 mg (total dose) of butorphanol ('hub' dose, based on the volume of the hub of a 20-G needle) was administered to one horse and 5 mg (total dose) to a second horse. Butorphanol was administered directly into the vein. Samples for analysis of butorphanol concentrations were collected from a catheter, placed as described above.

### *Sample collection*

Blood samples were collected at time 0 (prior to drug administration) and at 5, 10, 15, 30, and 45 min and 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 18, 24, 36 and 48 h post butorphanol administration. For the micro-dosing portion of the study, samples were collected at 0, 5, 10, 15, 30, and 45 min and 1, 1.5, 2, 2.5, 3, and 4 h. Catheters used for sample collection were removed following collection of the 24 h sample (left side), and the remaining samples were collected by direct venipuncture. Blood samples were collected into EDTA blood tubes (Kendall/Tyco Healthcare, Mansfield, MA, USA) and were centrifuged at 3000 *g* for 10 min. Plasma was immediately transferred into storage cryovials (Phenix Research Products, Chandler, NC, USA) and stored at  $-20$  °C until analysis (2 weeks).

Urine was collected either by free catch (geldings or via urinary catheters (mares) at 24, 48, 72, 96, and 120 h post drug administration. For the micro-dosing portion of the study, samples were collected at 1, 2, 3, and 4 h post butorphanol administration. When free catch collection was utilized, samples were collected as close to the timepoints as possible and the actual time of collection was recorded. Urine samples were stored at  $-20$  °C (approximately 1 week) until analysis.

### *Physiologic responses and behavioral monitoring*

Each horse was equipped with two Step Monitors (SAM3, Seattle, WA, USA) programmed to count the number of steps taken each minute. Monitors were fastened by a velcro strap to the lateral

side of the left lower front and hind leg of each horse. The distal portions of all four legs were wrapped with polo wraps, to minimize favoring of one leg over another. The number of steps were recorded each minute for a minimum of 30 min prior to and 4 h post butorphanol administration. Chin to ground distance was also monitored for all horses prior to and for 4 h post drug administration. The distance from the animal's muzzle to the ground was measured using a measuring tape. Gastro-intestinal borborygmi were recorded as normal, increased, decreased, or absent after auscultation in all four quadrants prior to and at 10, 15, 30, and 45 min and 1, 2, 4, 6, 8, and 24 h post butorphanol administration. Defecation incidence as well as stool consistency was recorded throughout the sampling period. Additional notable physiologic or behavioral observations were recorded throughout the course of the study.

Horses were also equipped with a Holter monitor (Forrest Medical, East Syracuse, NY, USA) to assess any potential effect on the cardiovascular system. Heart rate and rhythm were recorded continuously for a minimum of 30 min before and 4 h post drug administration. Heart rate was determined at predetermined timepoints via manual counting of P-QRS-T complexes over a 1-min time period. The percentage of atrial signals blocked by the atrio-ventricular node before and after butorphanol administration was calculated using the formula (atrial rate – ventricular rate)/atrial rate. The atrial and ventricular rates were determined by manually counting P waves and P-QRS-T complexes, respectively, over a 1-min period at predetermined timepoints.

#### *Statistical analysis*

Data were summarized as mean  $\pm$  SD. Statistical analyses using commercially available software (SAS, Cary, NC, USA) were performed to assess significant differences in chin to ground distance, step count, heart rate, and %AV block both pre- and postbutorphanol administration for individual horses. Raw data for all variables were checked for normality using the Wilk–Shapiro test and then log transformed as necessary to bring the residual distribution in close agreement with a normal distribution. Data for all variables were subsequently analyzed using a mixed model ANOVA with repeated measures. Significance was set a  $P < 0.05$ .

#### *Butorphanol plasma sample analysis*

The analytical reference standard for butorphanol was bought from Sigma-Aldrich (St Louis, MO, USA). The internal standard levorphanol was bought from VWR (Radnor, PA, USA). Stock solutions of butorphanol and levorphanol were prepared at 1 mg/mL in methanol and acetonitrile, respectively.

The concentration of butorphanol was measured in plasma by LC-MS. Quantitative analysis of plasma was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled with a turbulent flow chromatography system (TFC TLX4 Thermo Scientific) having 1100 series liquid chromatography system (Agilent Technologies, Palo Alto,

CA, USA). Chromatography employed an ACE 3 C18 10 cm  $\times$  2.1 mm, 3  $\mu$ m, column (Mac-Mod Analytical, Chadds Ford, PA, USA) and a gradient of acetonitrile in water with a constant 0.2% formic acid at a flow rate of 0.35 mL/min. The initial acetonitrile (ACN) concentration was held at 3% for 1.9 min and ramped up to 95% over 4.4 min and held for 1.7 min before re-equilibrating for 2.9 min at initial conditions. Butorphanol working solutions were prepared by dilution of the stock solution with methanol to concentrations of 0.001, 0.01, 0.1, 1, and 10 ng/mL. Plasma calibrators were prepared by adding the working standard solution into drug-free plasma to final concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 300, and 400 ng/mL. All plasma calibration curves used a quadratic equation for nonlinear regression as best fit with a 1/X weighting. All curves gave correlation coefficients of ( $R^2$ ) of 0.99 or better. The technique was optimized to provide limits of detection of 0.01 ng/mL and limits of quantitation of 0.05 ng/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (plasma fortified with analyte at three concentrations within the standard curve) were included with each sample set as an additional check of accuracy. The intraday accuracy (% of nominal concentration) for the QC samples was 125, 113, and 108% for 0.2, 4, and 20 ng/mL, respectively. The interday accuracy was 114, 113, and 106% for 0.2, 4, and 20 ng/mL, respectively. The intraday precision (% relative standard deviation) was 9, 7, and 4% for 0.2, 4, and 20 ng/mL, respectively, and 12, 6, and 5% for interday precision, respectively.

Plasma samples were measured both with and without enzyme hydrolysis to assess both conjugated and free plasma butorphanol concentrations, respectively. Plasma concentrations without hydrolysis represent free butorphanol concentrations while conjugated butorphanol concentrations were determined by calculating the difference between samples analyzed with and without enzyme hydrolysis at each timepoint sampled. Prior to analysis, 1 mL of plasma was diluted with 200  $\mu$ L of water containing 50 ng/mL of levorphanol as an internal standard. The samples were vortexed briefly to mix, and 50  $\mu$ L was injected into the TFC system equipped with Cyclone-P 0.5  $\times$  50 mm turbulent flow columns (Thermo Scientific). Analytes were eluted from the turboflow column onto the analytical column with a 100  $\mu$ L loop volume of 90% acetonitrile in water both with 0.2% formic acid. The plasma samples were analyzed with and without enzyme hydrolysis for comparison. For plasma samples that were enzyme hydrolyzed, 1 mL of plasma was diluted with 400  $\mu$ L of  $\beta$ -glucuronidase enzyme (10 000 units/mL; Sigma-Aldrich) in 1.6 M acetate buffer at pH 5.0, containing the internal standard at 25 ng/mL. The samples were heated in an oven for 3 h at 50  $^{\circ}$ C, allowed to cool to room temperature, and 50  $\mu$ L was injected into the TFC system as described above for nonhydrolyzed plasma treatment.

Detection and quantitation was conducted using selective reaction monitoring (SRM) of initial precursor ion for butorphanol [mass to charge ratio 328.2 ( $m/z$ )] and the internal standard levorphanol (258.2  $m/z$ ). The response for the major

product ions for butorphanol ( $m/z$  310.3) and the internal standard ( $m/z$  133, 157, 201) were plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate these analytes in all urine and plasma samples.

#### Butorphanol urine sample analysis

The urine samples were analyzed with and without enzyme hydrolysis for comparison. Urine concentrations without hydrolysis represent free butorphanol concentrations while conjugated butorphanol concentrations were determined by calculating the difference between samples analyzed with and without enzyme hydrolysis at each timepoint sampled. For urine samples that were enzyme hydrolyzed, 1 mL of urine was diluted with 400  $\mu$ L of  $\beta$ -glucuronidase enzyme (10 000 units/mL, Sigma-Aldrich) in 1.6 M acetate buffer at pH 5.0, containing the internal standard at 50 ng/mL. The samples were heated in an oven for 2 h at 65 °C, allowed to cool to room temperature, and 50  $\mu$ L was injected into the TFC system. Method parameters and calibrators were the same as described above for plasma analysis. For urine samples that did not have enzyme hydrolysis, 1 mL of urine was diluted with 400  $\mu$ L of water containing 50 ng/mL of levorphanol as internal standard. The samples were further diluted with 1.6 mL of 0.6 M phosphate buffer pH 6.5, vortexed briefly to mix, and subjected to solid phase extraction (SPE). The SPE cartridges were Cerex Polychrom Clin II 3cc 35 mg (Cera, Inc. Baldwin Park, CA, USA). The extraction procedure consisted of applying the sample onto the column, rinsing with 3 mL of water, with 2 mL of 1 M acetic acid, with 3 mL of methanol and eluting with 2 mL of 78:20:2 methylene chloride/isopropanol/ammonium hydroxide. The samples were dried under nitrogen and redissolved in 160  $\mu$ L of 5% acetonitrile in water both with 0.2% formic acid. Quantitative analysis of the un-hydrolyzed urine samples was performed on an Exactive Orbitrap mass spectrometer (Thermo Scientific) coupled with a Transcend turbulent flow chromatography system (TFC TLX2 Thermo Scientific). Chromatography employed an ACE 3 C18 10 cm  $\times$  2.1 mm, 3  $\mu$ m, column (Mac-Mod Analytical, Chadds Ford, PA, USA) and a gradient of acetonitrile in water with a constant 0.2% formic acid at a flow rate of 0.4 mL/min. The initial ACN concentration was held at 5% for 0.5 min and ramped up to 95% over 2 min and held for 0.6 min before re-equilibrating for 4.5 min at initial conditions. Detection and quantitation was conducted using full scan accurate mass detection with 50 000 resolution utilizing initial precursor ion for butorphanol [mass to charge ratio 328.22697 ( $m/z$ )] and the internal standard levorphanol (258.18544  $m/z$ ) with a 10 ppm mass window. The response for these accurate mass ions was plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate these analytes. The intraday accuracy (% of nominal concentration) for the urine QC samples was 104 and 88% for 4 and 20 ng/mL, respectively. The interday accuracy was 102 and

95% for 4 and 20 ng/mL, respectively. The intraday precision (% relative standard deviation) was 6 and 8% for 4 and 20 ng/mL, respectively, and 8 and 5% for interday precision, respectively. Urine calibration curves used linear or quadratic equation as best fit with a  $1/X$  weighting. All curves gave correlation coefficients of ( $R^2$ ) of 0.99 or better. The technique was optimized to provide limits of detection of 0.05 ng/mL and limits of quantitation of 0.1 ng/mL.

#### Pharmacokinetic calculations

Nonlinear least square regression analysis was performed on plasma butorphanol concentration vs. time data from the nonhydrolyzed samples, using commercially available software (Phoenix WinNonlin Version 6.2, Pharsight, Cary, NC, USA). Only data points (plasma concentrations) equal to or above the LOQ for the assays were included in the analysis. Based on coefficient of variation, Akaike Information Criterion (Yamaoke *et al.*, 1978), and visual inspection of the residual plots, a two-compartment model ( $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ ) gave the best fit to butorphanol concentration data points from individual animals. Pharmacokinetic parameters for butorphanol are reported as mean ( $\pm$ SD).

## RESULTS

#### Pharmacokinetic analysis and urinary concentrations

Mean plasma concentrations of free butorphanol following intravenous administration of 0.1 mg/kg are depicted in Fig. 1. Butorphanol plasma concentrations were below the limit of detection (LOD) of the assay (0.01 ng/mL) by 48 h post drug administration in all horses studied. The median and range for a number of pharmacokinetic parameters following compartmental analysis are listed in Table 1. The average plasma concentrations of conjugated butorphanol, relative to free butorphanol, are depicted in Fig. 2. Urinary butorphanol concentrations in

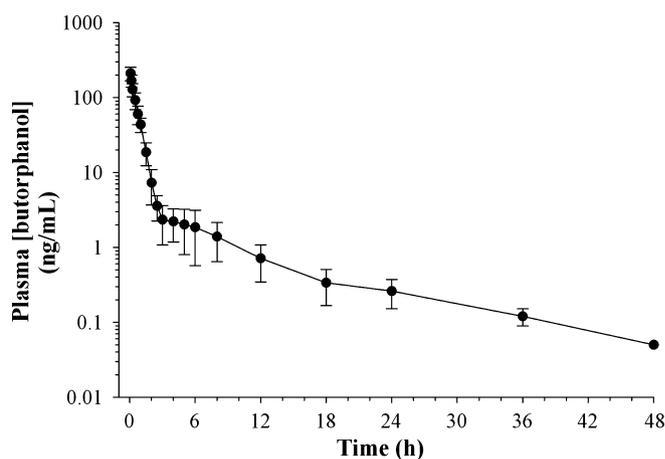


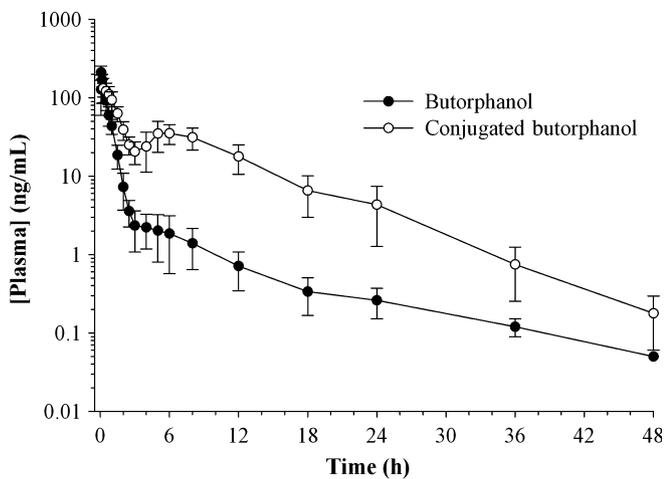
Fig. 1. Mean free plasma butorphanol concentration vs. time curve following intravenous administration of 0.1 mg/kg butorphanol to 10 thoroughbred horses.

**Table 1.** Pharmacokinetic parameters of butorphanol following a single intravenous administration of 0.1 mg/kg of butorphanol to 10 Thoroughbred horses

	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6	Horse 7	Horse 8	Horse 9	Horse 10	Mean ( $\pm$ SD)
A (ng/mL)	245.1	229.0	289.3	250.1	244.5	192.5	298.8	214.6	121.4	260.8	234.6 $\pm$ 50.8
B (ng/mL)	3.3	2.6	7.3	3.5	4.0	1.8	2.5	2.9	1.1	1.7	3.1 $\pm$ 1.7
Alpha (per h)	2.3	1.9	2.0	2.0	1.6	2.0	1.8	2.0	1.1	2.2	1.9 $\pm$ 0.3
Beta (per h)	0.107	0.130	0.122	0.098	0.093	0.112	0.078	0.183	0.163	0.085	0.117 $\pm$ 0.034
AUC (h·ng/mL)	136.9	143.2	201.9	157.9	192.4	11.7	195.0	121.7	112.3	141.5	151.4 $\pm$ 34.2
$t_{1/2\beta}$ (h)	6.5	5.4	5.7	7.1	7.5	6.2	8.9	3.8	4.2	8.1	5.9 $\pm$ 1.5*
K10 (per h)	1.8	1.6	1.5	1.6	1.3	1.7	1.5	1.8	1.1	1.9	1.6 $\pm$ 0.2
K12 (per h)	0.5	0.2	0.5	0.4	0.3	0.2	0.3	0.2	0.1	0.3	0.3 $\pm$ 0.1
K21 (per h)	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1 $\pm$ 0.04
Vdss (L/kg)	1.7	1.1	1.4	1.7	1.5	1.5	1.3	0.9	1.1	1.5	1.4 $\pm$ 0.3
CL (mL/min/kg)	12.2	11.6	8.3	10.6	8.7	14.9	8.6	13.7	14.8	11.8	11.5 $\pm$ 2.5

All values in this table were generated using compartmental analysis.

\*Expressed as harmonic mean  $\pm$  pseudostandard deviation.



**Fig. 2.** Mean plasma free butorphanol and conjugated butorphanol concentration vs. time curves following intravenous administration of 0.1 mg/kg butorphanol to 10 thoroughbred horses.

hydrolyzed samples were still above the LOD of the assay (0.05 ng/mL) in 3/10 samples at 120 h post administration. Individual as well as average urinary butorphanol concentrations for the 10 horses studied are listed in Table 2. Concentrations in Table 2 represent both free butorphanol concentrations as well as butorphanol conjugates; however, free butorphanol concentrations were low (0.31–0.97 ng/mL), were only detected in six of the 10 horses, and only at the first timepoint

sampled (24 h). Plasma and urinary conjugated butorphanol concentrations following intravenous administration of 1 mg and 5 mg of butorphanol (micro doses) are listed in Table 3.

#### Physiologic responses

Eight of 10 horses began to circle in the stall upon administration of butorphanol. Commencement of circling ranged from 2.5 to 15 min post drug administration. Additionally, seven of the eight horses that circled appeared ataxic. One of these horses was easily startled, rearing in the stall in response to uncontrollable external stimuli. For the majority of the horses, circling ceased within 2.5 h of drug administration; however, one horse continued to circle until 5 h post butorphanol. Muscle fasciculations, originating in the pectoral region and spreading to the flank region, were observed in two horses. The average number of steps taken by the horses following administration of butorphanol are depicted in Fig. 3. The number of steps was consistently above pretreatment values starting at 10 and 20 min post drug administration and generally continuing until approximately 3 h post administration. The step count was significantly different from baseline from 50 until 110 min post administration. There was a lag time between the maximal measured plasma concentration and the maximum increase in step count (Fig. 3b), with the maximal increase in step count not occurring until plasma butorphanol concentrations decreased to half of the maximum measured plasma concentration. There

**Table 2.** Urinary concentrations of conjugated butorphanol following intravenous administration of 0.1 mg/kg butorphanol to 10 Thoroughbred horses

Time (h)	(Conjugated butorphanol) (ng/mL)										Average
	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6	Horse 7	Horse 8	Horse 9	Horse 10	
24	–	259.1	52.4	416.5	543.5	770.2	358.3	662.9	1185.3	1322.7	619.0
48	7.9	9.2	2.7	14.8	6.1	99.6	5.7	40.0	14.9	17.6	21.8
72	3.4	1.2	0.6	1.6	0.8	3.4	1.4	1.0	1.8	1.5	1.6
96	<LOD	0.5	0.9	0.1	0.2	–	<LOD	0.7	0.9	0.8	0.5
120	<LOD	<LOD	<LOD	0.3	<LOD	2.7	<LOD	<LOD	<LOD	<LOD	1.5

LOD, limit of detection; –, no sample collected.

**Table 3.** Plasma free butorphanol and urinary conjugated butorphanol concentrations following intravenous administration of 1 and 5 mg (total administered dose) of butorphanol tartrate to a Thoroughbred horse ( $n = 1$  per dose)

Time (h)	1 mg Butorphanol		5 mg Butorphanol	
	Plasma (ng/mL)	Urine (ng/mL)	Plasma (ng/mL)	Urine (ng/mL)
0.08	6.0	–	33.6	–
0.16	4.3	–	22.8	–
0.25	2.9	–	16.6	–
0.5	2.0	–	11.3	–
0.75	1.8	–	7.1	–
1	1.0	1026.4	3.1	2069.1
1.5	0.4	–	1.4	–
2	0.2	476.2	0.8	1049.6
2.5	0.1	–	0.7	–
3	–	389.4	0.4	980.2
4	0.04	540.6	0.4	646.5

–, sample not collected.

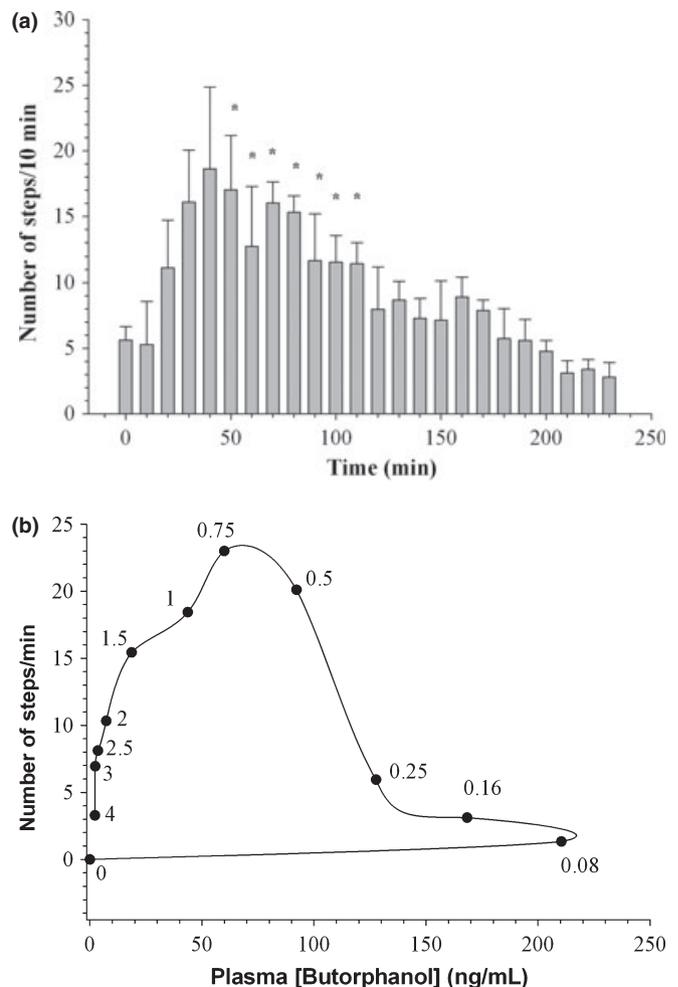
were no significant changes in chin to ground distance, relative to pre-administration, following administration of butorphanol (Fig. 4).

Average changes in heart rate, expressed as change from baseline (prior to butorphanol administration), are shown in Fig. 5a. Heart rate increased (relative to baseline) at the first timepoint measured (2 min) and continued to increase, reaching a maximum at 20 min post administration. Changes in heart rate were significantly different from baseline from 2 min until 2 h post butorphanol administration. There was a lag time between maximal measured plasma butorphanol concentrations and maximum increase in heart rate (Fig. 5b), with the maximum change in heart rate occurring 1 h post butorphanol administration. Prior to butorphanol administration, three horses exhibited a regular atrial–ventricular block. The changes in percent of AV signals blocked per minute prior to and following administration of butorphanol are depicted in Fig. 6.

Gastrointestinal (GI) sounds were decreased in all quadrants in six horses, while they remained unchanged in other four horses. Of the six affected horses, three horses had a noticeable decrease in GI sounds by 10 min, one horse by 15 min and the sixth horse by 2 h post butorphanol administration. GI sounds had returned to baseline by 8 h post administration in five horses, while they remained decreased until 24 h post butorphanol administration in one horse. Feces were noted in the stall of three horses while the remaining seven did not pass any feces during the first 8 h post drug administration. Feces were noted in the stall of all horses by 24 h post administration. Fecal consistency was normal throughout the study for all horses.

## DISCUSSION

Similar to previous studies of both humans (Gaver *et al.*, 1980) and horses (Sellon *et al.*, 2009), a two-compartment model gave the best fit to currently described plasma butorphanol concen-



**Fig. 3.** (a) Number of steps taken with respect to time following intravenous administration of 0.1 mg/kg of butorphanol to 10 thoroughbred horses. The number of steps recorded from the front and hind legs from individual horses were averaged at each timepoint and the number of steps summed over 10 min increments. Data are expressed as mean  $\pm$  SD. \*Indicates statistical differences ( $P < 0.05$ ) from baseline. (b) Number of steps per minute with respect to butorphanol plasma concentrations following intravenous administration of 0.1 mg/kg butorphanol to 10 thoroughbred horses. The time of sampling (h) is noted next to each point. Data are expressed as mean.

tration–time data. The mean terminal elimination half-life for butorphanol in the presently reported study was 5.9 h, which differed significantly from that first reported by Sellon *et al.* (2001). However, the value reported in the current study was only slightly different from that reported in a second study by the same authors (Sellon *et al.*, 2009). In the first study, Sellon *et al.* (2001) reported a terminal elimination half-life of 44 min following intravenous administration of 0.1 mg/kg butorphanol to adult horses. As mentioned previously, the analytical methodology used in that study (Sellon *et al.*, 2001) was much less sensitive compared to that used in the current study (LOQ of 7.8 and 0.02 ng/mL, respectively), likely explaining the difference in the elimination half-life between the two studies. A more sensitive assay allows for detection of drug at lower

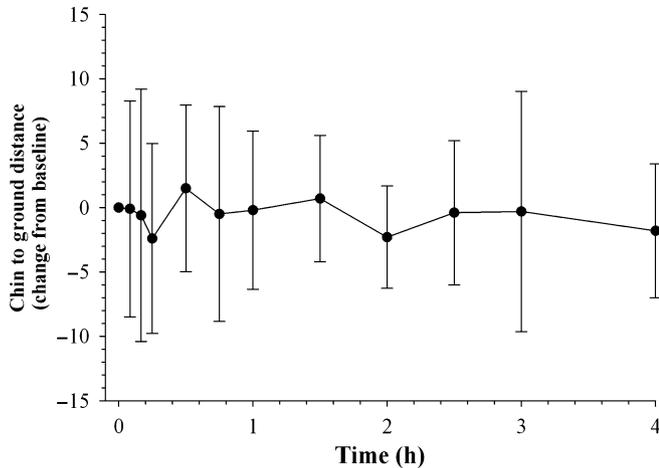


Fig. 4. Change in chin to ground distance (expressed as change from baseline) with respect to time following intravenous administration of 0.1 mg/kg butorphanol to 10 thoroughbred horses. Data are expressed as mean  $\pm$  SD.

concentrations and thus a lower LOQ and potentially for a longer period of time. This circumstance requires less extrapolation of the elimination portion of the curve and likely a more representative elimination half-life as well as a more accurate withdrawal time recommendation. In their more recent study, Sellon *et al.* (2009) used a more sensitive HPLC assay, allowing detection of butorphanol concentrations as low as 1 or 2 ng/mL. The elimination half-life for that study was reported as  $7.8 \pm 5.1$  h following intravenous administration of 0.08 mg/kg of butorphanol, which was in closer agreement with the 5.9 h elimination half-life reported here.

For the purposes of determining an appropriate withdrawal period prior to performance events, it is necessary to have a complete a description of the elimination profile to prevent inadvertent positives. Ideally, this would include sampling until the drug is at or below the LOD of the assay. In the initial study conducted by Sellon *et al.* (2001), plasma butorphanol concentrations in samples collected after 2 h post administration were below the LOD of the assay. However, in their more recent study, the investigators (Sellon *et al.*, 2009) were able to quantitate butorphanol until the final timepoint sampled (6 h post administration). In the study reported here, the low LOQ and LOD along with the longer duration of sample collection allowed for detection of butorphanol in plasma for an average of 30 h (18–48 h (range) post drug administration. Butorphanol was still detectable in the urine of all horses at 72 h post administration and in 7/10 horses at 120 h post administration (the terminal urine collection time), following enzyme hydrolysis.

Results from this study indicate that butorphanol is widely distributed (1.4 L/kg) following intravenous administration, which agrees with reports by Sellon *et al.* (2001, 2009)). However, while the volume of distribution was comparable between the studies, the clearance values were very different. Similar to the half-life of elimination, in their initial study, Sellon *et al.* (2001) reported a much different clearance value compared to their second study (Sellon *et al.*, 2009). In their initial study,

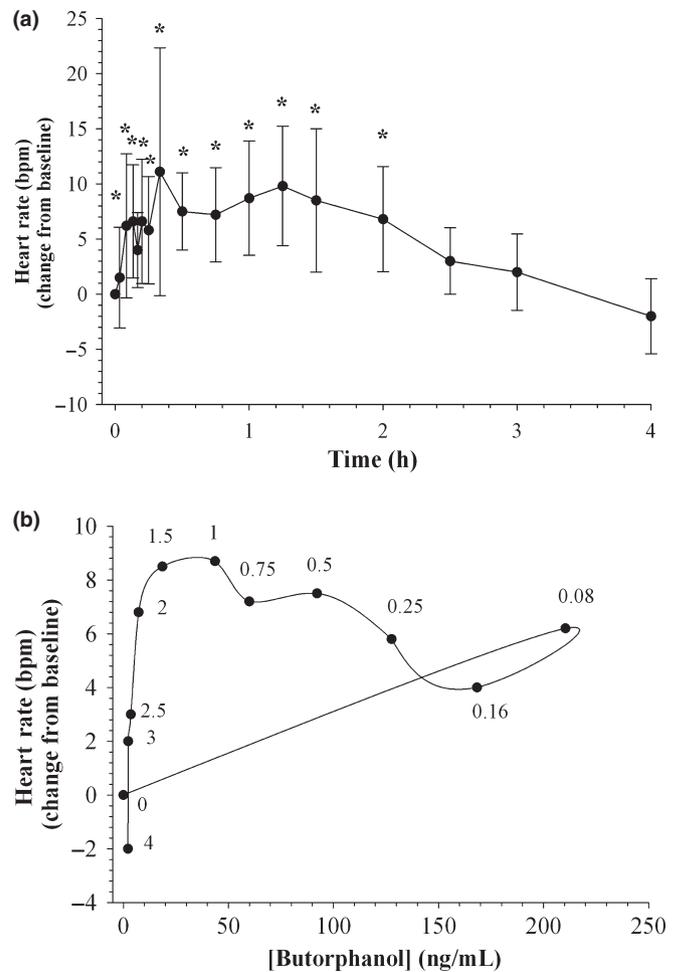


Fig. 5. (a) Change in heart rate (expressed as change from baseline) with respect to time following intravenous administration of 0.1 mg/kg butorphanol to 10 thoroughbred horses. Data are expressed as mean  $\pm$  SD. \*Indicates significant differences ( $P < 0.05$ ) from baseline values. (b) Change in heart rate (expressed as change from baseline) with respect to butorphanol plasma concentrations following intravenous administration of 0.1 mg/kg butorphanol to 10 thoroughbred horses. The time of sampling (h) is noted next to each point. Data are expressed as mean.

Sellon *et al.* (2001) reported a clearance value of  $21.0 \pm 9.48$  mL/kg/min compared to 11.5 and  $4.6 \pm 1.7$  mL/kg/min in the current study and in the second study of Sellon *et al.* (2009). Differences in clearance values between the studies are again likely due to differences in the sensitivity of the analytical analyses used in each study as well as the number of duration of the sampling period. The increased sensitivity of the analytical analyses in the second study of Sellon *et al.* (2009) as well as in the current study allowed for longer detection of plasma butorphanol concentrations and a more accurate calculation of the clearance rate of the drug.

One of the primary goals of the current study was to provide information that could be used to provide a withdrawal time recommendation prior to racing. As such, only horses that met the criteria set forth by the Racing Medication and Testing Consortium for establishment of withdrawal time recommenda-

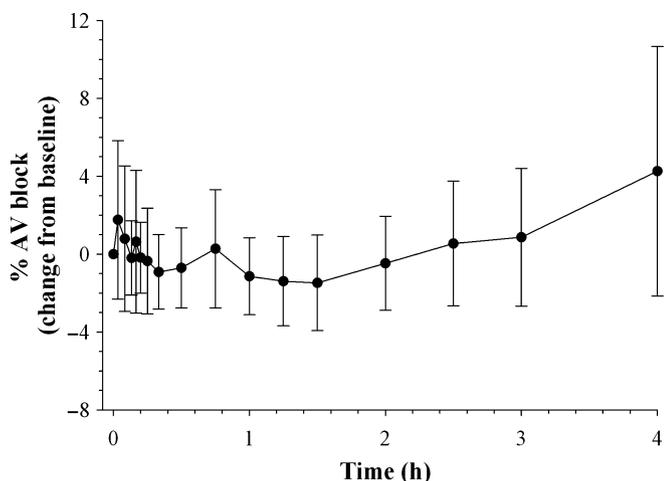


Fig. 6. Percent (%) atrial-ventricular block with respect to time following intravenous administration of 0.1 mg/kg butorphanol to 10 horses. Percent AV block was calculated using the formula  $((\text{atrial beats} - \text{ventricular beats}) / \text{atrial beats}) \times 100$ . Data are expressed as mean  $\pm$  SD.

tions were used. All horses utilized in the current study were young (3–5 years) and within the typical average age range of horses that are racing. In humans, it is well established that with increasing age, physiologic and pathologic changes, including alterations in hepatic metabolism and renal clearance occur that can ultimately affect the pharmacokinetics of a drug. While to the authors' knowledge differences in drug disposition specifically attributable to changes in metabolism and renal clearance have not been documented in the literature for horses, differences in butorphanol pharmacokinetics have been reported between foals and adult horses. Arguedas *et al.* (2008) reported a terminal elimination half-life of 2.1 h in neonatal foals following intravenous administration of 0.05 mg/kg compared to 5.9 h in the current study (horses 3–5 years of age). Similarly, butorphanol clearance appears to vary with age, with a clearance of 31 mL/min/kg reported for foals (Arguedas *et al.*, 2008) compared with 11.5 mL/min/kg in the current study. While it is unlikely that foals would be used to establish withdrawal times for performance horses, the fact remains that age related changes in physiology could affect the disposition of a drug and ultimately determination of an accurate withdrawal time. In addition to age, another important consideration is the fitness status of the animal. Elimination of drugs has been reported to differ between sedentary and fit horses, and for this reason, only Thoroughbred horses that were being actively exercised were used in the current study, enhancing the ability to establish relevant withdrawal times prior to racing.

To the authors' knowledge, there are no published reports describing the identification of butorphanol metabolites following administration to the horse. Whether correctly or incorrectly, it is presumed that butorphanol is metabolized in the horse much the same as in humans. In humans, butorphanol is rapidly and extensively metabolized and eliminated primarily in the urine (72% of the dose) and to a much lesser extent in the feces (14–15% of the dose) (Gaver *et al.*, 1980). Butorphanol is

metabolized by hepatic enzymes and eliminated in the urine as hydroxy-butorphanol (approximately 50% of the dose), free or glucuronide conjugated norbutorphanol (approximately 11% of the dose) or as free butorphanol (approximately 7% of the dose) (Gaver *et al.*, 1980). In the study reported here, butorphanol also appears to be rapidly metabolized and eliminated in the urine. The exact identification of the metabolites was not determined, simply because reference standards are not commercially available; however, based on the results of LC-MS analysis, both with and without beta-glucuronidase, butorphanol appears to be primarily eliminated as a glucuronide conjugated metabolite. Similar to that reported for humans (Gaver *et al.*, 1980), very little of the parent compound was excreted in the urine. Butorphanol and its metabolites were detected in urine samples (following enzyme hydrolysis) for up to 120 h post administration in 3 of the horses sampled. This is similar to the urine butorphanol detection time reported in humans (4–5 days) (Gaver *et al.*, 1980).

In addition to describing the pharmacokinetics of butorphanol at the label dose, in the current study, smaller doses (1 and 5 mg total dose) were also administered and plasma concentrations measured to assess whether a 'sub-therapeutic' dose could be detected in biological samples. The 1 and 5 mg doses were chosen based upon the rumored use of a 'hub' dose prior to racing and both urine and blood samples were collected for up to 4 h post butorphanol administration, based upon the estimated time between administration and collection of regulatory samples post race. At both doses, butorphanol and its conjugated metabolite were above the LOD for the entire sampling period (4 h post drug administration). While more horses and additional timepoints would be necessary to fully characterize the pharmacokinetics as well as to determine the final time of detection post administration, preliminary results generated from this pilot study suggest that attempts at doping using even extremely low doses of drugs prior to competition could be readily detected in both plasma and urine.

Horses appeared to have a bimodal behavioral response following administration of butorphanol, characterized by an initial period (within min) of somnolent ataxia followed shortly thereafter by a more prolonged period of increased locomotion. Similar reactions have been described previously following administration of butorphanol to horses and ponies (Kalpravidh *et al.*, 1984a,b; Nolan *et al.*, 1994; Sellon *et al.*, 2001). Sellon *et al.* (2001) reported that four of seven horses administered butorphanol demonstrated behavioral disturbances including staggering and ataxia immediately upon administration. Similarly, Kalpravidh *et al.* (1984a,b) observed that one of six horses became ataxic within the first 10 min of butorphanol administration and that an additional two horses appeared sedate prior to a period of restlessness. The increase in locomotor activity observed in the current study was characterized by restlessness, incessant circling, and a significant increase in the number of recorded steps. Kalpravidh *et al.* (1984a,b) likewise noted restlessness, shivering, and muscular weakness following intravenous administration of 0.22 mg/kg of butorphanol to ponies. This period of increased activity

(relative to baseline) persisted for nearly 2 h post drug administration, which agreed with the 2 h of increased locomotion reported previously by Kalpravidh *et al.* (1984a,b). These responses are not surprising as central nervous system (CNS) excitation, including an exaggerated increase in locomotor activity, excitement, and restlessness have been associated with high-dose opioid administration in horses (Combie *et al.*, 1981; Carregaro *et al.*, 2007). In the case of butorphanol, it has been hypothesized that the sedative effects are because of binding at the kappa receptor and the increased locomotion because of the activity at the mu receptor (Kammerling *et al.*, 1988; Nolan *et al.*, 1994).

Although results vary, a number of studies have assessed the effects of butorphanol on cardiovascular parameters in the horse and pony (Robertson *et al.*, 1981; Kalpravidh *et al.*, 1984b; Sellon *et al.*, 2001). Robertson *et al.* (1981) reported no change in heart rate following intravenous administration of 0.1, 0.2 and 0.4 mg/kg of butorphanol. However, Kalpravidh *et al.* (1984b) observed an increase in heart rate following intravenous administration of 0.22 mg/kg of butorphanol to ponies. Albeit at a lower dose, in the current study, similar to reports by Kalpravidh *et al.* (1984b), a significant increase in heart rate was noted. This increase in heart rate persisted until 2 h post butorphanol administration. However, of additional significance was the large degree of interindividual variability in the magnitude of the change. The increase in heart rate was slightly delayed with respect to time of butorphanol administration and maximal measured plasma concentrations, suggesting that effects on heart rate may be a result of CNS stimulation. The lag time likely represents the time necessary for drug distribution to the CNS and its action there, in the case of butorphanol, via a second messenger pathway.

Both mu and kappa receptors are present in the gastrointestinal tract (Gintzler & Hyde, 1984), and opioid receptor agonists have been shown to have a profound effect on motility in the horse (Sellon *et al.*, 2001; Boscan *et al.*, 2006). Specifically, butorphanol has been shown to decrease gastrointestinal motility (Kalpravidh *et al.*, 1984a,b; Sellon *et al.*, 2001) in the horse. Although statistical analysis was not performed, based on subjective assessment, gastrointestinal sounds were notably decreased in six horses relative to baseline, following administration of butorphanol. Changes in GI sounds were rapid (within 10–15) which agrees with that reported previously by Sellon *et al.* (2001), whereby mean auscultation scores decreased significantly by 20–60 min post administration.

The current study sought to expand upon current knowledge of the pharmacokinetics of butorphanol utilizing a highly sensitive LC-MS assay to measure plasma and urine concentrations following intravenous administration of 0.1 mg/kg. Pharmacokinetics and detection times are used to determine appropriate withdrawal times prior to competition, which in turn aid in decreasing the incidence of inadvertent positives. Results of the current study, including detection of butorphanol for up to 48 and 120 h for plasma and urine, respectively, suggest that a prolonged withdrawal period prior to racing is warranted. An additional objective of the current study was to

describe some of the physiologic and behavioral effects of butorphanol following administration of the labeled dose and similar to that reported previously, butorphanol administration caused CNS excitation, resulting in a number of behavioral disturbances. Results of this study suggest that the maximum duration of behavioral and cardiac effects is approximately 6 h.

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