Pharmacokinetic and Pharmacodynamic Characteristics of Morphine and its Metabolites in Horses

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Contents

Abstract

Morphine is a potent, effective analgesic that is used widely in both human and veterinary medicine. Metabolism of morphine by UDP glucuronidation conjugation creates two metabolically active glucuronide metabolites: morphine-6 glucuronide and morphine-3 glucuronide. Morphine and its metabolites have been extensively studied in human medicine, however their pharmacokinetics and pharmacodynamics have yet to be explored to the same extent in horses. Such studies would help contribute to more effective opioid use in equine medicine.

Our laboratory previously published a paper identifying M3G and M6G as the major metabolites of morphine in horses. This study also confirmed previous reports of increased motor activity, muscle fasciculation, and flared nostrils following intravenous administration of high doses of morphine (0.2 mg/kg and 0.5mg/kg). Additionally, horses in all dose groups (0.05, 0.1, 0.2 and 0.5 mg/kg) had decreased gastrointestinal activity, a common side effect associated with morphine use. Other side effects associated with morphine use in horses include increased motor activity, increased respiratory rate, increased blood pressure, and muscle fasciculation.

The first aim of this work was to characterize and correlate the pharmacokinetics and selected pharmacodynamics of morphine administration in horses. Additional characterization of the pharmacokinetics and pharmacodynamic effects of morphine and its metabolites would add to existing data describing the relative concentrations of the metabolites of M3G and M6G. A total of ten horses were administered a single intravenous dose of morphine: 0.05, 0.1, 0.2, or 0.5 mg/kg, or saline control and blood samples were collected and analyzed for morphine and metabolites by LC/MS/MS to conduct pharmacokinetic analysis. Pharmacodynamic data in the forms of step count, heart rate and rhythm, gastrointestinal borborygmi, fecal output, packed cell

volume, and total protein was also assessed. Morphine-3 glucuronide (M3G) was the predominant metabolite detected, with concentrations exceeding those of morphine-6 glucuronide (M6G) at all time point. The results also included decreased gastrointestinal motility and increased central nervous excitation with a correlation between increasing doses of morphine, increases in M3G concentrations and adverse effects Findings from this study supported additional studies of administration of purified M3G and M6G to horses to directly characterize pharmacodynamic activity of these metabolites.

The next aim was to characterize and correlate the pharmacokinetics and selected pharmacodynamics of purified M6G in horses. In part one, 3 horses received a single intravenous administration of saline, 0.5 mg/kg M6G and 0.5 mg/kg morphine. Blood samples were collected up to 96-hours post administration, concentrations of drug and metabolites measured, and pharmacokinetics determined. Behavioral and physiologic effects were recorded. In part two of this study, two horses, scheduled to be euthanized for other reasons, were administered 0.5 mg/kg M6G. Blood, CSF and various tissue samples were collected post administration and concentrations of drug determined. The clearance of M6G was more rapid and the volume of distribution at steady state smaller for M6G compared to morphine. A reaction characterized by head shaking, pawing and slight ataxia was observed immediately following administration of both morphine and M6G horses. Following M6G administration, the behaviors subsided rapidly and was followed by a longer period of sedation. Following administration, M6G was detected in the kidney, liver, CSF and regions of the brain. Results of the current student encourage further investigation of M6G as an analgesic in horses.

The final aim was to characterize the invitro metabolism of morphine by determining metabolic enzymes responsible for the formation of M3G and M6G. This was accomplished by

expressing four equine UGT variants: UGT1A1, UGT2A1, UGT2B31 and UGT2B4 Functionality of the enzymes was assessed using 4-methylumbelliferone, testosterone, diclofenac and ketoprofen. Recombinant enzyme, control cells, equine liver microsomes and human UGT2B7 supersomes were then incubated with morphine. Concentrations of metabolites were measured using liquid chromatography- tandem mass spectrometry and enzyme kinetics determined. UGT2B31 metabolized morphine to morphine-3-glucuronide and low concentrations of morphine-6-glucuronide. While UGT2B31 contributes to the glucuronidation of morphine; however, it is probably not the main metabolizing enzyme. These results warrant further investigation of equine UGTs, including expression of additional enzymes and further characterization of UGT2B31 as a contributor to morphine metabolism.

Morphine Use in Horses

Musculoskeletal and soft tissue pain in equids that requires prolonged and potent analgesia is an area that currently lacks a reliable pharmaceutical solution. For many species, the use of opioids offers safe and reliable analgesia for major pain. Morphine use in equine patients is a highly debated topic. Many practitioners argue that the side effects associated with high doses of morphine make the drug unsafe for use in the clinical setting as it poses a risk to both practitioners and the patients. Others will state that the use of morphine along with an effective sedative agent decreases the risks and offers analgesic benefits.

Morphine is a phenanthrene alkaloid belonging to the opioid family of analgesics (Inturrisi, 2002). It is derived from opium poppy seeds and is a full agonist at the opioid mu receptor(Inturrisi, 2002). Opioid receptors are found in peripheral tissues, tissues that make up the ascending pain transmission system and structures that modulate pain in the spinal cord (Guedes, 2017). In humans, morphine undergoes conjugation by UDP glucuronosyltransferase (UGT) resulting in the production of both morphine-3 glucuronide (M3G) and morphine-6 glucuronide (M6G) (Lötsch & Geisslinger, 2001).

In many species, M6G has a high affinity for opioid receptors and has been shown to have analgesic properties (Lötsch & Geisslinger, 2001; Paul et al 1989). Unlike most glucuronide conjugates, M6G appears to cross the blood brain barrier as evidenced by high concentrations in brain tissue following systemic administration to rats (Stain-Texier, et al, 1999; Aasmundstad, et al, 1995). The analgesic properties along with a lesser number of adverse effects compared to morphine administration, make M6G a potentially promising analgesic. It is important to note, however, that following morphine administration to humans, M6G concentrations are much lower than M3G (Hasselstrom et al 1993) .

When administered as a sole agent, it has been suggested that M3G contributes to the neuroexcitatory effects observed with high dose morphine administration (Smith, 2000). Additionally, M3G has been shown to cross the blood brain barrier and has been associated with adverse effects such as hyperalgesia, allodynia, myoclonus, and dependence (Smith, et al, 1990). Recent reports have suggested a toll like receptor (TLR4) and interleukin-1 (IL-1) mediated component to M3G induced effects in the central nervous system (Lewis et al, 2010; Iqbal et al 2020). Blomqvist et al (2020) also found that after acute intrathecal administration of M3G and chronic intrathecal administration of M3G in combination with morphine in Sprague-Dawley rats, there was an increase in substance P and tactile allodynia (Blomqvist et al, 2020). The investigators also discovered that M3G caused antinociceptive cross tolerance to morphine further supporting the idea that M3G exposure may contribute to morphine-induced tolerance and hyperalgesia and have significant effects on the central nervous system (Blomqvist et al, 2020).

The use of morphine in equine patients has perplexed equine practitioners and researchers for decades. In 1979 Tobin and Combie began to explore the effects of morphine in horses with a series of studies describing the pharmacokinetics and effects of varying doses of multiple opioids and stimulants (Tobin et al, 1979; Tobin and Woods, 1979). With regards to morphine, they measured the locomotor response following administration of 0.1, 0.3, 0.6, and 2.4mg/kg and found that at lower doses (0.1mg/kg and 0.3mg/kg) there were rapidly alternating peaks of locomotion, yet with high doses (0.6mg/kg, 2.4mg/kg) there were more prolonged and stable increases in locomotor activity that occurred in a dose dependent manner (Combie et al 1979). They also reported that renal excretion contributed minimally to clearance and that plasma protein binding was found to be about 28.2% at a dose of 0.1mg/kg IV (Combie et al, 1983). In another study, the investigators found that the locomotor stimulation produced by a 2.4mg/kg IV dose of morphine

was reduced by 75% when horses were administered the competitive mu receptor antagonist, naloxone (0.02 mg/kg), suggesting involvement of the mu opioid receptor in eliciting this effect (Combie et al, 1981).

The use of morphine and other opioids in equine practice is often justified as practitioners believe the benefits of strong analgesia outweigh the risk of spontaneous and potentially dangerous locomotion. Many of the studies describing the analgesic effects of morphine assessed regional pain by epidural and intraarticular administration. Natalini and Robinson (2000) used assessment of avoidance threshold to noxious electrical stimulation to evaluate the analgesic effect of 0.1mg/kg epidural morphine (Natanini and Robinson, 2000). The threshold was increased with onset of effect ranging from 4-8 hours and lasting 6-8 hours. The investigators concluded that use would only be indicated in horses with perineal and lumbosacral pain. Sysel and colleagues (1996) administered 0.2mg/kg morphine combined with 0.03mg/kg detomidine epidurally, inducing analgesia in the hind limb of lame horses within 20min post administration (Sysel et al, 1996). Sheehy et al (2001) found that opioid receptors are present on the synovium of equine joints, leading researchers to investigate the analgesic effects of intra-articular (IA) morphine. Lindegard et al (2010) compared IA morphine (0.05mg/kg) to intravenous morphine (0.5mg/kg) and saline in a radiocarpal lipopolysaccharide model of synovitis (Lindegard et al 2010). They found that IA morphine significantly decreased lameness compared to IV morphine, however it did not decrease the visual analogue scale of pain intensity (Lindegard et al 2010). Based on these results, the investigators suggested that IA morphine may be useful as part of an analgesic protocol after arthroscopic surgery to alleviate orthopedic pain (Lindegard et al 2010). Multiple studies have been conducted describing the effects of morphine on healthy synovium and all have concluded that IA morphine

produces mild alterations to healthy synovium and cartilage but were comparable to the changes seen in saline controls (Raekallio et al. 1996; Jaureguito et al. 2002).

The use of morphine in other species such as dogs and cats is often for the relief of major pain associated with surgical procedures. However in horses recovering from surgical procedures, a common complication is post-operative ileus. Morphine is known in many species to decrease gastrointestinal motility which would further exacerbate this complication in equine patients. Two studies have further explored the gastrointestinal effects of morphine in horses (Boscan et al 2006, Figueiredo et al 2012). These studies concluded that doses of 0.05, 0.1 and 0.5mg/kg cause a decrease in borborygmi, propulsive motility and moisture contents in the gastrointestinal tract which predisposes treated horses to ileus and constipation (Boscan et al 2006, Figueiredo et al 2012). Tessier et al (2019) explored the effects of morphine on the gastrointestinal tract with the use of ultrasound and found that 3 doses of 0.1mg/kg IV morphine caused GI depression, distention and hyperphagia (Tessier et al, 2019).

Morphine has demonstrated effects on cardiovascular function at a dose of 0.12mg/kg IV including increasing heart rate and cardiac output, (Muir et al 1978). Figueiredo et al (2012) also explored the cardiovascular effects of systemic morphine administration (0.05 and 0.1mg/kg) and found an immediate increase in heart rate, systemic arterial pressure, diastolic arterial pressure and mean arterial pressure for the first two minutes after administration with greater changes seen in the 0.1mg/kg dose group. Arguably, the most notable side effect associated with systemic morphine administration is the spontaneous increase in locomotor activity. A number of researcher groups have noted an increase in activity following systemic morphine administration (Knych et al, 2014; Bennet and Steffey 2002; Combie et al, 1981).

In conclusion, while some evidence supports continued clinical use of morphine administration alone for both systemic and regional analgesia in equids, reports of undesirable effects necessitate additional study of this drug to improve consistent favorable clinical outcomes.

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Pharmacokinetics and Selected Pharmacodynamics of Morphine and its Active Metabolites in horses after intravenous administration of four doses

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Abstract

The objective of the current study was to describe and characterize the pharmacokinetics and selected pharmacodynamic effects of morphine and its two major metabolites in horses following several doses of morphine. A total of ten horses were administered a single intravenous dose of morphine: 0.05, 0.1, 0.2, or 0.5 mg/kg, or saline control. Blood samples were collected up to 72 hours, analyzed for morphine and metabolites by LC/MS/MS, and pharmacokinetic parameters were determined. Step count, heart rate and rhythm, gastrointestinal borborygmi, fecal output, packed cell volume, and total protein were also assessed. Morphine-3 glucuronide (M3G) was the predominant metabolite detected, with concentrations exceeding those of morphine-6 glucuronide (M6G) at all time points. Maximal concentrations of M3G and M6G ranged from 55.1-504 ng/ml and 6.2-28.4 ng/ml, respectively across dose groups. Plasma concentrations of morphine were best fit by a three-compartment model. The volume of distribution at steady state and systemic clearance ranged from 9.40 – 16.9 L/kg and 23.3 – 32.4 mL/min/kg, respectively. Adverse effects included signs of decreased gastrointestinal motility and increased central nervous excitation. There was a correlation between increasing doses of morphine, increases in M3G concentrations and adverse effects Findings from this study support administration of purified M3G and M6G to horses to directly characterize pharmacodynamic activity of these metabolites.

Keywords: morphine, horse, pharmacokinetics, pharmacodynamics, metabolism

Introduction

Morphine is an effective, potent and reliable analgesic for human and small animal veterinary patients, however, it is less commonly used in equine patients due to unpredictable and often unwanted and potentially dangerous responses (Bennet and Steffey, 2002). Administration of high, and anecdotally therapeutic, doses of morphine to horses are often associated with increases in locomotion and aggression, and a decrease in gastrointestinal function, making its use potentially harmful to equine patients and in the case of behavioral changes to clinicians as well (Bennet and Steffey, 2002, Combie et al 1981, Boscan et al, 2006).

Morphine is classified as a mu opioid receptor full agonist (Inturrisi, 2002). In humans, the parent compound undergoes conjugation by Uridine 5'-diphospho- glucuronosyl transferase (UGT) enzymes producing morphine-3 glucuronide (M3G) and morphine-6 glucuronide (M6G) (Brunk and Delle 1974). Inturrisi and colleagues (2002) suggested that M3G is neuroexcitatory while M6G contributes to the analgesia observed following morphine administration. Although the onset appears to be delayed, systemic administration of purified M6G to mice elicited comparable analgesia to morphine administration with a prolonged effect compared to morphine administration (Paul et al 1989). This is especially noteworthy as most glucuronide conjugates are thought to be inactive.

While the pharmacokinetics of morphine in the horse has been described previously (Combie et al, 1983; Knych et al, 2014), to the best of the authors' knowledge, there is only a single study describing the metabolism of morphine in horses (Knych et al, 2014). Knych and colleagues (2014) identified 2 major metabolites, M3G and M6G, following administration of 4 different doses of morphine to horses. Compared to humans, horses produced almost twice as much M3G but similar concentrations of M6G (Knych et al 2014). Although the study

conducted by Knych and colleagues (2014) was the first to describe the metabolism of morphine in the horse following administration of several doses, results were from a small sample size. Therefore, the goal of the current study was to confirm results of the earlier study and extend knowledge about the metabolism, pharmacokinetics and selective pharmacodynamics of morphine following intravenous administration to healthy horses.

Materials and Methods

Horses

Ten University owned horses (9 thoroughbreds, 1 standardbred) were used. Five mares and 5 geldings aged 4-23 years with an average +/-SD weight of 563.8 ± 41.3 kg were studied. Horses were not administered any medications for a minimum of two weeks prior to the start of the study. Prior to commencement of the study, horses were confirmed healthy by complete blood count, serum biochemistry and physical exam. This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

Instrumentation and drug administration

Horses were randomly assigned to one of five groups: 0.05 mg/kg, 0.1 mg/kg, 0.2 mg/kg, or 0.5 mg/kg morphine sulfate (Hospira, Lake Forest, IL, USA) or 0.9% NaCl (5mL). A 14-guage catheter was aseptically placed in both external jugular veins prior to drug administration. One catheter was used for drug administration, while the contralateral catheter was used for sample collection. Each horse was weighed immediately prior to drug administration. Horses were fasted for 12 hours prior to drug administration and water was available *ad lib* in the 0.05, 0.1 and 0.2 mg/kg dose groups. Water intake was monitored for 8 hours for horses in the 0.5

mg/kg and saline groups. These horses were given 10L of water immediately following dosing and the amount consumed was then measured and recorded every hour. Following determination of water intake at a given time point water levels were restored to 10L. All horses, excluding those in the 0.5 mg/kg dose group, were fed two hours post drug administration. Food was withheld in the 0.5 mg/kg group due to a presumed increased risk of adverse gastrointestinal effects with high dose morphine administration. Nine of 10 horses in this dose group received full feed by eight hours post drug administration while one horse was fed small amounts of soaked feed over a 12 hour period due to concern of colic.

Sample collection

Blood was collected at times 0 (immediately prior to drug administration), 5, 10, 15, 30, and 45 min, and 1,1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 18, 24, 30, 36, 48, and 72 hours post drug administration. Prior to collection of the sample, 10 mL of blood was aspirated from the catheter and discarded. The sample (20 mL) was then collected and placed in EDTA containing blood tubes (Kendall/Tyco Healthcare Mansfield MA, USA). The catheter was subsequently flushed with 5-10 mL of heparinized saline (100 units/mL). After collection of the 24-hour sample, catheters were removed, and the remaining samples were collected by direct venipuncture. After collection, samples were placed on ice until centrifugation at 3000 x g for 10 minutes. Plasma was immediately transferred to cryovials (Phoenix Research Products, Chandler, NC, USA) and stored at -20°C until analysis. Urine samples for determination of morphine and metabolite concentrations were collected at 24, 48, and 72 hours post drug administration by free catch and subsequently stored at -20°C until analysis.

For determination of packed cell volume (PCV) and total protein (TP), blood was aliquoted from the EDTA tubes collected for determination of drug concentrations into

microhaematorcrit tubes. Packed cell volume and TP were determined at 5, 10, 15, 30, and 45 min and 1, 2, 4, 6, and 8 hours post morphine administration. Packed cell volume was measured via microhaematorcrit and TP was measured via refractometer. Both were measured in duplicate and the average of the 2 measurements recorded for each sample.

Drug Concentration Determination

Serum Samples:

Plasma concentrations of morphine, M3G and M6G were measured using a previously validated liquid-chromatography-tandem mass spectrometry (LC/MS/MS) method (Knych et al 2014).

Urine Samples:

Urine calibrators were prepared by dilution of the working standard solutions with drug free equine urine to concentrations of 0.5 to 10,000 ng/mL. Calibration curves, negative control samples, and quality control samples were prepared fresh for each quantitative assay.

Prior to analysis, 1 mL of urine was diluted with 0.1 mL of water containing 0.25 ng/mL of d6-morphine and d3-morphine-3βD-glucuronide internal standards and 2 mL of 0.1M (pH 6) phosphate buffer. The samples were vortexed briefly to mix and subjected to solid phase extraction using CUC18 3cc 200mg Clean-Up Extraction Columns (United Chemical Technologies, Inc., Bristol, PA). Columns were rinsed with 2 mL of methanol and 3 mL of water, samples loaded onto the columns and washed with 3 mL of water prior to elution with 2.5 mL of methanol. Samples were dried under nitrogen in a Zymark TurboVap (McKinley Scientific, Sparta, NJ) at 45 ºC and reconstituted in 150 µL of 5% acetonitrile in water, both with 0.2% formic acid. 20 µL was injected into the LC/MS/MS system. Liquid chromatography

tandem mass spectrometry was used for detection and quantification as described previously by Knych et al (2014).

Pharmacokinetic Calculations:

Pharmacokinetic parameters for morphine and its metabolites were determined using commercially available software (Phoenix WinNonlin Version 8.0, Certara, Princeton, NJ, USA). NCA was performed on serum morphine and metabolite concentrations to assess the basic pharmacokinetics and determine if clearance of morphine appeared to be linear. The area under the curve (AUC) was obtained by using the linear up log down trapezoidal rule then dividing the last plasma concentration by the terminal slope extrapolated to infinity. Determination of pharmacokinetic parameters for the metabolites were as described for the parent compound.

Physiologic Responses and Behavioral Monitoring

Horses were equipped with two Step Monitors (SAM3, Seattle, WA, USA) programmed to count the number of steps taken each minute. Using a Velcro strap, each monitor was fastened to the lateral side of the left lower front leg and right lower rear leg of each horse. All four legs were then wrapped to decrease the likelihood of the horse favoring one leg over another. The number of steps taken was recorded for a minimum of 30 min prior to and 4 hours (8 hours for horses in the 0.5 mg/kg group) post morphine or saline administration.

Horses were also equipped with a Holter monitor (Forrest Medical, East Syracuse, NY, USA) to assess any potential effects of morphine or its metabolites on cardiac parameters. Heart rate and rhythm were recorded continuously for 30 min prior and 4 hours post drug administration (8 hours for horses in the 0.5 mg/kg dose group).

Gastrointestinal sounds were assessed prior to and at 30, and 45 min and 1, 1.5, 2, 2.5, 3, and 4 hours post morphine administration via direct auscultation of all four abdominal quadrants.

Each quadrant was assigned a GI borborygmi score ranging from 0-4 (0 being absent and 4 being increased sounds). Defecation incidence as well as consistency, and fecal ball number were recorded throughout the sampling period. Additional notable physiologic or behavioral observations were recorded throughout the sampling period by both a blinded and non-blinded observer (BDH and HKK not blinded, EPS blinded).

Statistical analysis

Statistical analyses using commercially available software (Stata/IC 13.1, StataCorp LP, TX, USA) were used to determine significant differences in pharmacokinetic and pharmacodynamic parameters for morphine. For pharmacokinetic analysis, differences in parameters between dose groups were assessed. Differences between baseline and each time point and between dose groups were assessed for pharmacodynamic data. Data were analyzed using a mixed effects analysis of variance with the horse as the random effect and time and dose as the fixed effect. Post-hoc comparisons were performed with a Bonferonni multiple comparison adjustment to preserve a nominal significance level of 0.05.

Results

The quality control samples were analyzed in replicates (n=6) for morphine, M3G, and M6G to assess the intraday and interday precision, and accuracy of the assay. Interday and intraday accuracy and precision data for the LC-MS/MS analyses were obtained for morphine, M3G, and M6G (Table 1) and met criterion as defined in the FDA Guidelines for Industry Bioanalytical Method Validation. The lowest calibrator that could be detected with acceptable precision and accuracy was the limit of quantitation (LOQ). The lowest calibrator with a 3:1 signal-to-noise ratio was used to establish the limit of detection (LOD). The resulting LOQ for

morphine, M3G and M6G in blood was 0.25 ng/mL while the LOD was 0.2 ng/mL for all analytes. The LOQ in urine was 0.25 ng/mL for morphine, 0.5 ng/mL for morphine-6β-D-glucuronide and morphine-3 β -D-glucuronide and the LOD was 0.1 ng/mL for morphine and 0.25 ng/mL for both glucuronidated metabolites.

Plasma-concentration time curves (mean ± SD) for all dose groups of morphine are depicted in Figure 1. Pharmacokinetic parameters (mean ± SD) for each dose group are listed in Table 2. There was a significant (p<0.05) difference in clearance between the 0.2 and 0.5 mg/kg dose groups compared to all other groups. The Vd_{ss} and the terminal half-life were not significantly different between dose groups. Morphine-3 glucuronide and M6G were the only metabolites identified following morphine administration. Metabolite plasma-concentration time curves (mean ± SD) for all dose groups are shown in Figure 2 and pharmacokinetic parameters reported in Table 3. Metabolites were detectable as early as 5 minutes post morphine administration with M3G concentrations exceeding M6G concentrations at all time points (Figures 2 and 3).

Urine concentrations of morphine, M3G and M6G at all doses are listed in Table 4. Morphine concentrations in urine were below the LOQ at 72 hours for all horses in the 0.05 mg/kg dose group, six horses in the 0.1 mg/kg dose group, and one horse in the 0.2 mg/kg dose group. Morphine concentrations exceeded the LOQ (0.25 ng/mL) of the assay in all horses in the 0.5 mg/kg dose group at 72 hours (the last time point collected). Morphine-3 glucuronide urine concentrations exceeded the LOQ for all horses in all dose groups at the last time point measured (72 hours). Morphine-6 glucuronide urine concentrations were below the LOQ at 72 hours for all horses in the 0.05 mg/kg dose group, nine horses in the 0.1 mg/kg dose group, five horses in the 0.2 mg/kg dose group and one horse in the 0.5 mg/kg dose group.

In the 0.05 and 0.1 mg/kg dose group, behavioral responses, such as pawing, head shaking, and circling were observed in 7/10 horses immediately following morphine administration. These signs resolved within the first 5 minutes. One horse in the 0.1 mg/kg dose group begun to pace at 15-minutes and continued until 2 hours post drug administration. In the 0.2 mg/kg dose group behavioral changes varied greatly ranging from pawing and head shaking to pacing for up to 2 hours post drug administration. Following administration of 0.5 mg/kg, 6/10 horses began pacing immediately following morphine administration with one horse circling the stall for up to 5 hours post drug administration. Six of 10 horses in the 0.5 mg/kg dose group also demonstrated muscle fasciculations that persisted from 30 minutes to 2 hours.

Heart rate increased significantly, relative to baseline, in the 0.1 mg/kg, 0.2 mg/kg, and the 0.5 mg/kg dose groups. Heart rate also was also significantly increased in the 0.2 mg/kg and 0.5 mg/kg dose group relative to the saline dose group (Figure 3). The degree of atrial ventricular block was significantly reduced relative to baseline in the 0.2 mg/kg and 0.5 mg/kg dose groups. The number of steps was also increased significantly, relative to baseline, in the 0.1 mg/kg, 0.2 mg/kg and the 0.5 mg/kg dose groups (Figure 5). When compared to the saline treatment group, step counts were also increased significantly in the 0.2 mg/kg and 0.5 mg/kg dose groups (Figure 4). Packed cell volume and TP were significantly increased, relative to baseline, in the 0.05 mg/kg dose group (Table 5).

Gastrointestinal borborygmi decreased relative to baseline at all doses studied with the most prolonged decrease noted in the 0.5 mg/kg dose group (Figure 5). Gastrointestinal borborygmi returned to baseline values by 24 hours in all horses. Fecal output was decreased relative to baseline in the 0.5 mg/kg dose group with many horses not passing any feces until 12

hours post morphine administration. In the saline group water intake averaged 9.3L ± 8.7L over an 8-hour period, while the 0.5 mg/kg dose group averaged 3.1L ± 2.7L.

Discussion:

The primary goal of the current study was to confirm and extend current knowledge regarding the metabolism, pharmacokinetics and selected pharmacodynamics of morphine in healthy horses. A preliminary report was published previously, whereby the metabolism and pharmacokinetics of morphine when administered at 4 different doses (0.05 mg/kg, 0.1mg/kg, 0.2 mg/kg, and 0.5 mg/kg) was described, however, the study size was small (n=2/dose group; Knych et al, 2014).

In the current study, total systemic clearance of morphine increased significantly with increasing dose. These results suggest that elimination of morphine is non-linear – namely that clearance increases with dose. This effect was not noted in the preliminary study conducted by Knych and colleagues (2014) likely due to the small sample size in that study (n=2 per dose group). It is possible that the increase in clearance was a result of increased concentrations of free drug as a result of saturation of plasma protein binding sites. However, because morphine plasma protein binding in the horse is low (36%) (Combie et al, 1983) this is unlikely. The most likely explanation is increased hepatic blood flow. Morphine is classified as a high extraction ratio drug making clearance dependent on hepatic blood flow. Morphine administration to horses has been reported to increase heart rate, arterial blood pressure and cardiac output at doses of 0.12 mg/kg (Muir et al, 1978). Although, cardiac output was not measured in the current study, a notable dose dependent increase in heart rate was observed. The increase in heart rate could lead to an increase in cardiac output, an increase in hepatic blood flow and ultimately

increased clearance. While further studies are necessary to determine if this is the case and if so if increased systemic clearance is due to increased hepatic clearance (i.e. a larger contribution by biliary elimination) or hepatic intrinsic activity, similar morphine:M3G and morphine:M6G ratios with increasing doses suggest that there may be an increase in intrinsic clearance. In humans, investigators have hypothesized that morphine undergoes extra-hepatic metabolism (Mazoit et al 1990) based on studies identifying glucuronosyl transferase activity in the kidneys, gastrointestinal tract, and brain (Fisher 2000, Mazoit et al, 1990). Similarly, Knych and colleagues (2014) theorized that morphine undergoes extra-hepatic metabolism in horses, as evidenced by a total systemic clearance that exceeded hepatic blood flow (Knych et al, 2014). However, this theory is confounded in the current study by the suggestion that total systemic clearance exceeding hepatic blood flow may actually be a result of an increase in hepatic blood flow and subsequent increased delivery of drug to metabolic enzymes in the liver. Further studies are necessary to definitively determine if morphine undergoes extra-hepatic metabolism in the horse.

Similar to a previous report in horses (Knych et al, 2014), morphine was primarily eliminated as M3G and M6G. In the current study, glucuronidation of morphine was rapid, with C_{max} for both glucuronidated metabolites occurring by 10 minutes. Interestingly, in humans and rats, M3G and M6G are believed to be active metabolites (Hasselström et al 1993). It has been hypothesized that M3G may have neuroexcitatory properties, especially following administration of high doses of morphine (Smith et al, 2000) and that M6G may be analgesic (Paul et al, 1989). The potential neuroexcitatory effects of M3G reported in other species, may offer a potential explanation for the adverse effects observed in horses after the administration of high doses of morphine. This hypothesis is further supported by the relatively greater concentration of M3G

noted in horses following morphine administration as compared to other species (Knych et al 2014, Faura et al 1998, Di Gregori et al 2012).

In the presently reported study, central nervous system excitation following morphine administration was evidenced by a significant increase in locomotor activity relative to baseline. This is similar to previous reports describing the physiologic effects of morphine following administration to horses (Combie et al, 1981; Knych et al, 2014). In the current study, with increasing doses of morphine, an increase in step count was observed, with the greatest increase in locomotor activity noted at the highest dose (0.5 mg/kg). While a causative effect cannot be definitively determined from the presently reported study, this increase in locomotion was noted in conjunction with increasing metabolite concentrations (Fig 5). While administration of M3G and M6G would be necessary to definitively determine the relationship between metabolite concentrations and the excitatory effects observed in the current study, the authors hypothesize that if these metabolites are active in the horse, that they may be contributing to the locomotor response. It is important to note, however, that external stimuli (i.e. presence of other horses and people in the barn and withholding of food) may also have contributed to the increased locomotion,

Heart rate increased significantly with increasing morphine dose. In humans, it has been hypothesized that morphine administration has a stimulatory effect on the sympathetic nervous system (Hall et al, 1998). It is possible that the increase in heart rate associated with higher doses of morphine observed in the currently reported study is due to increased sympathetic activity. As mentioned previously morphine has been associated with spontaneous locomotion in the horse (Combie et al, 1981, Knych et al, 2014). This may also be responsible for the increase in heart rate seen in this study. Further study is necessary to determine whether the effect of morphine on

heart rate is a direct effect or whether these findings are secondary to an increase in spontaneous locomotion.

Increased sympathetic tone likely also explains the increase in both PCV and TP noted in the current study may. The equine spleen has a large red cell reserve that if stimulated by intense vasoconstriction and concurrent activation of the sympathetic nervous system, can almost double an individual's PCV (Fielding and Magdesian, 2011). However, increases in both PCV and TP may also indicate a dehydrated state. In the current study, horses in the high dose group had a dose dependent increase in locomotion in combination with a decrease in water consumption, which increases the likelihood of dehydration. While it is important to note that environmental factors, such as external temperature, may contribute to dehydration; the balanced crossover design and the various environmental factors including external temperature that presented throughout the study, as well as the consistent increase in PCV in the high dose group suggests that morphine administration is responsible for the increase in PCV and TP.

Gastrointestinal borborygmi and fecal frequency decreased in a dose dependent manner for up to 12 hours post drug administration. This is in agreement with previous studies whereby GI motility decreased following morphine administration (Boscan et al 2006, Figueredo et al 2012). This effect is presumed to be due to activation of opioid receptors in the GI tract, altering motility, secretion, absorption, and blood flow. The clinical implications associated with these effects include an increased risk of impaction and colic (Boscan et al, 2006; Bennett and Steffey, 2002). Due to the increased risk of adverse GI effects, in the current study, horses were fasted prior to and for a period of time after morphine administration. While a previous report described decreased GI motility in non-fasted horses following morphine administration (Boscan

et al, 2006), it is important to note that fasting the horses in the current study may have also contributed in some small measure to the decrease in GI motility.

In conclusion, the higher doses of morphine used in this study (0.2 mg/kg and 0.5 mg/kg) were associated with increased locomotion, decreased gastrointestinal borborygmi, increased heart rate, and elevated PCV. This study sets the stage for additional studies exploring the physiologic responses to morphine administration as well as exploration into the direct pharmacological effects of M3G and M6G.

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Table 1. Accuracy and Precision Values for LC-MS/MS analysis of morphine, M3G, and M6G in equine plasma and urine.

		Concentration (ng/mL)	Intraday Accuracy (% nominal conc)	Intraday precision (% relative SD)	Interday Accuracy (% nominal conc)	Interday Precision (% relative SD)
Plasma						
	Morphine					
		0.75	92.0	6.0	99.0	6.0
		40.0	108	3.0	100	4.0
		600	103	2.0	102	2.0
	M3G					
		0.75	94.0	4.0	89.0	3.0
		40.0	103	2.0	105	3.0
		600	104	3.0	106	3.0
	M6G					
		0.75	97.0	11.0	89.0	7.0
		40.0	105	3.0	100	3.0
		600	104	2.0	104	3.0
Urine						
	Morphine					
		0.75	92.0	8.0		
		7.5	103	4.0		
		250	100	2.0		
	M3G					
		0.75	90.0	4.0		
		250	112	6.0		
		4000	106	5.0		
	M6G					
		0.75	88.0	8.0		
		250	107	4.0		
		4000	94.0	7.0		

M3G, morphine-3-glucuronide; M6G, morphine-6 glucuronide

Table 2. Pharmacokinetic parameters (mean ± SD) for morphine following a single IV administration of morphine sulfate (0.05, 0.1, 0.2, or 0.5 mg/kg) to adult horses (n=10). All values reported were generated using non-compartmental analysis.

Parameters	Dose Group			
	0.05 mg/kg	0.1 mg/kg	0.2 mg/kg	0.5 mg/kg
HL Lambda$_z$ z (h)	8.20 ± 2.52	9.53 ± 5.70	9.68 ± 5.73	10.5 ± 2.76
Vdss (L/kg)	9.45 ± 3.25	10.2 ± 5.08	9.86 ± 3.84	9.40 ± 2.31
CL (mL*min/kg)	25.4 ± 4.55[c,d]	28.5 ± 3.65[d]	31.7 ± 2.09[a]	34.1 ± 4.50[a,b]
$AUC_{0\text{-}inf}$ (h*ng/mL)	34.0 ± 7.60[b,c,d]	59.2 ± 7.0[a,c,d]	106 ± 6.90[a,b,d]	248 ± 31.8[a,b,c]
AUC % Extrap	9.26 ± 3.54	5.91 ± 2.14	3.68 ± 1.38	1.86 ± 0.35
M3G:M $AUC_{0\text{-}inf}$ ratio	16.2 ± 4.04	14.9 ± 2.72	16.1 ± 3.61	15.6 ± 2.06
M6G:M $AUC_{0\text{-}inf}$ ratio	1.13 ± 0.28	1.02 ± 0.22	0.93 ± 0.20	0.84 ± 0.27

HL Lambda$_z$, Terminal half-life; Vdss, Volume of distribution at steady-state; CL, clearance; $AUC_{0\text{-}inf}$, area under the plasma-concentration curve from time 0 to infinity; AUC % Extrap, percentage of area under the curve extrapolated.

a, significantly different ($p < 0.05$) from 0.05 mg/kg; b significantly different ($p < 0.05$) from 0.1 mg/kg; c, significantly different ($p < 0.05$) from 0.2 mg/kg; d, significantly different ($p < 0.05$) from 0.5 mg/kg.

Table 3. Pharmacokinetic parameters (mean ± SD) for A) morphine-3 glucuronide and B) morphine-6 glucuronide respectively following a single IV administration of morphine sulfate (0.05, 0.1, 0.2, or 0.5 mg/kg) to adult horses (n=10). All values reported were generated using noncompartmental analysis.

A)

Parameters	Dose Group			
	0.05 mg/kg	0.1 mg/kg	0.2 mg/kg	0.5 mg/kg
HL Lambda z (h)	8.95 ± 3.83	10.0 ± 3.76	9.84 ± 3.52	7.68 ± 0.49
C_{max} (ng/mL)	104 ± 34.5	171 ± 43.2	307 ± 44.0	696 ± 97.9
T_{max} (h)	0.26 ± 0.09	0.34 ± 0.25	0.33 ±0.25	0.37 ± 0.41
$AUC_{0\text{-}inf}$ (h*h*ng/mL)	546 ± 174	884 ± 190	1700 ± 375	3850 ± 706
AUC % Extrap	0.82 ± 0.29	0.53 ± 0.20	0.29 ± 0.15	0.16 ± 0.05
AUMC (h*h*ng/mL)	40000 ± 1390	6700 ± 1680	12980 ± 3230	31600 ± 7470

B)

Parameters	Dose Group			
	0.05 mg/kg	0.1 mg/kg	0.2 mg/kg	0.5 mg/kg
HL Lambda z (h)	18.6 ± 10.6	22.8 ± 8.35	12.4 ± 3.8	11.0 ± 3.26
C_{max} (ng/mL)	8.6 ± 3.3	13.5 ± 3.88	23.7 ± 8.8	50.5 ± 16.9
T_{max} (h)	0.21 ± 0.05	0.27 ± 0.09	0.20 ± 0.05	0.21 ± 0.05
$AUC_{0\text{-}inf}$ (h*h*ng/mL)	37.7 ± 9.34	58.9 ± 15.5	98.6 ± 24.4	205.0 ± 64.9
AUC % Extrap	18.67 ± 8.49	14.4 ± 6.96	5.76 ± 3.76	2.52 ± 1.09
AUMC (h*h*ng/mL)	650 ± 396	878 ± 313	1000 ± 153	1998± 703

HL $Lambda_z$, elimination half-life; C_{max}, maximal plasma concentration; T_{max}, time to maximal plasma concentration; AUC0-inf, area under the plasma concentration–time curve; AUC% Extrap, percentage of area under the curve extrapolated; AUMC, area under the moment curve.

Table 4. Urine concentrations (mean ± SD) of morphine, morphine-3 glucuronide (M3G) and morphine-6 glucuronide (M6G) following a single IV administration of morphine sulfate (0.05, 0.1, 0.2, or 0.5mg/kg) to adult horses (n=10).

	0.05 mg/kg	0.1 mg/kg	0.2 mg/kg	0.5 mg/kg
Morphine (ng/mL)				
24 hours	6.81 ± 2.72	11.8 ± 5.14	29.4 ± 17.6	130 ± 100
48 hours	0.58 ± 0.38	1.10 ± 0.78	2.48 ± 1.73	8.60 ± 8.90
72 hours	0.13 ± 0.03	0.23 ± 0.16	0.42 ± 0.24	1.31 ± 0.86
M3G (ng/mL)				
24 hours	475 ± 169	896 ± 380	2420 ± 1810	818 ± 537
48 hours	34.7 ± 20.5	83.9 ± 54.4	154 ± 94.0	361 ± 270
72 hours	5.68 ± 2.59	14.0 ± 6.24	19.0 ± 8.59	50.7 ± 35.0
M3G (ng/mL)				
24 hours	16.2 ± 6.41	38.8 ± 18.0	76.3 ± 45.5	261 ± 109
48 hours	1.31 ± 0.61	3.76 ± 3.19	5.60 ± 4.39	8.45 ± 6.74
72 hours	0.29 ± 0	0.56 ± 0.45	0.62 ± 0.47	1.16 ± 0.93

Table 5. Packed cell volume (PCV) and total protein concentration (TP) (mean ± SD) at specified time points following administration of a single dose of saline and a single dose of morphine at 0.05, 0.1, 0.2, and 0.5 mg/kg.

	Saline		0.05 mg/kg		0.1 mg/kg		0.2 mg/kg		0.5 mg/kg	
	PCV	TP	PCV	TP	PCV	TP	PCV	TP	PCV	TP
Baseline	35.5 ± 3.0	6.4 ± 0.5	34.5 ± 3.4	6.2 ± 0.4	34.5 ± 3.4	6.3 ± 0.5	33.6 ± 4.1	6.2 ± 0.4	32.5 ± 5.6	6.3 ± 0.5
5 min	34.2 ± 2.8	6.3 ± 0.5	34.4 ± 3.7	6.2 ± 0.3	34.6 ± 2.5	6.4 ± 0.4	36.3 ± 3.0	6.3 ± 0.4	38.2 ± 2.1	6.4 ± 0.4
10 min	33.6 ± 2.7	6.3 ± 0.5	33.1 ± 3.3	6.1 ± 0.3	33.6 ± 2.6	6.3 ± 0.4	36.0 ± 4.9	6.4 ± 0.5	39.5 ± 3.8	6.5 ± 0.5
15 min	33.5 ± 2.5	6.4 ± 0.5	32.5 ± 3.4	6.2 ± 0.3	33.8 ± 2.6	6.3 ± 0.4	33.3 ± 7.3	6.3 ± 0.5	39.2 ± 4.7	6.5 ± 0.5
30 min	33.3 ± 3.0	6.4 ± 0.6	31.9 ± 2.3	6.2 ± 0.3	32.0 ± 2.7	6.3 ± 0.4	33.1 ± 4.0	6.3 ± 0.4	38.0 ± 6.0	6.4 ± 0.4
45 min	32.9 ± 2.7	6.4 ± 0.5	31.7 ± 2.6	6.2 ± 0.3	32.8 ± 3.0	6.3 ± 0.4	32.8 ± 4.0	6.3 ± 0.4	37.1 ± 4.6	6.4 ± 0.5
1 hour	32.5 ± 1.9	6.4 ± 0.5	32.5 ± 3.3	6.2 ± 0.3	32.4 ± 1.8	6.3 ± 0.4	33.5 ± 3.7	6.3 ± 0.5	36.8 ± 5.1	6.5 ± 0.5
2 hour	32.8 ± 1.6	6.4 ± 0.2	32.9 ± 3.1	6.2 ± 0.4	32.9 ± 2.7	6.4 ± 0.4	33.5 ± 3.8	6.5 ± 0.5	36.5 ± 4.7	6.4 ± 0.5
4 hour	38.5 ± 2.4	7.0 ± 0.4	38.1 ± 3.6	6.7 ± 0.5	37.8 ± 2.8	6.9 ± 0.5	40.2 ± 4.9	7.2 ± 0.2	37.3 ± 4.7	6.5 ± 0.5
6 hour	36.1 ± 2.0	6.6 ± 0.3	36.0 ± 3.2	6.4 ± 0.3	34.8 ± 1.9	6.4 ± 0.5	36.5 ± 2.8	6.7 ± 0.3	38.1 ± 4.1	6.5 ± 0.5
8 hour	35.8 ± 1.3	6.5 ± 0.3	35.1 ± 2.2	6.4 ± 0.3	34.6 ± 1.8	6.4 ± 0.6	37.5 ± 4.8	6.8 ± 0.5	39.8 ± 3.8	6.6 ± 0.3

Figure 1. Morphine plasma concentrations (mean ± SD) over time curve following a single intravenous administration of morphine at 0.05, 0.1, 0.2 or 0.5 mg/kg to adult horses.

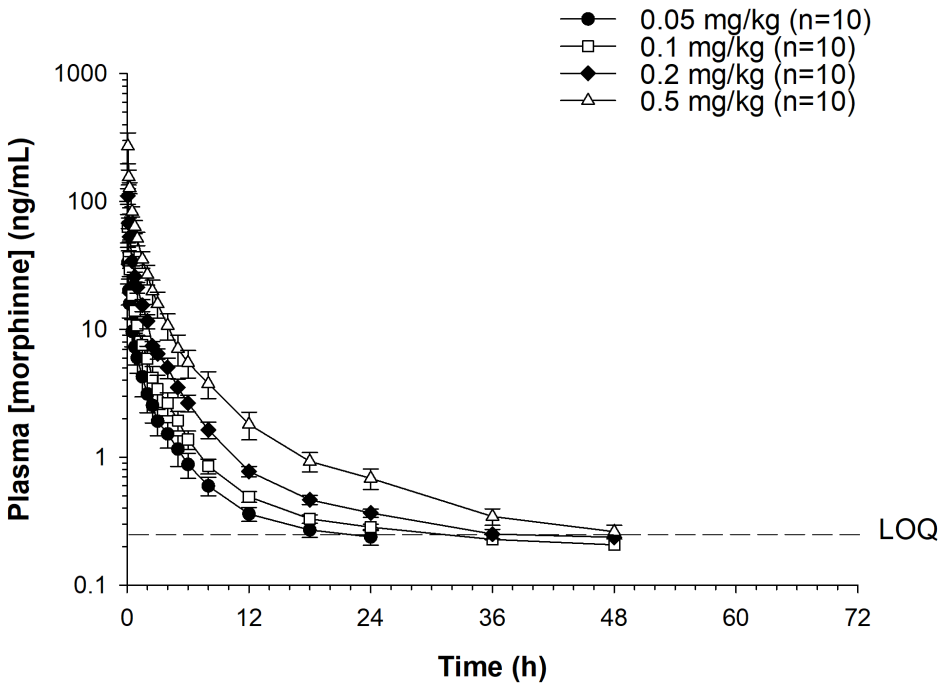
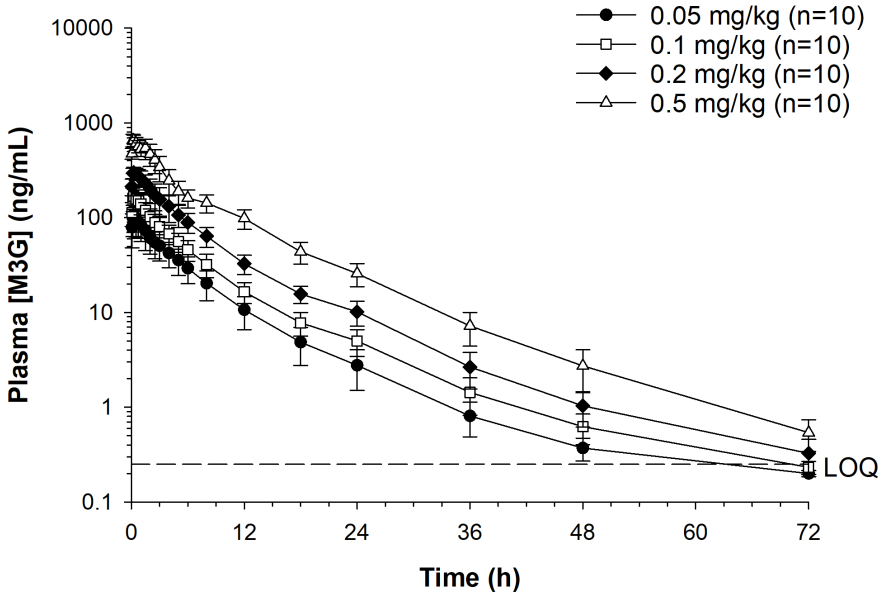


Figure 2. Plasma concentration (mean ± SD) with respect to time curve for A) morphine-3 glucuronide and B) morphine-6 glucuronide respectively following a single administration of morphine at 0.05, 0.1, 0.2 or 0.5 mg/kg IV (n=10 per dose group) to adult horses.



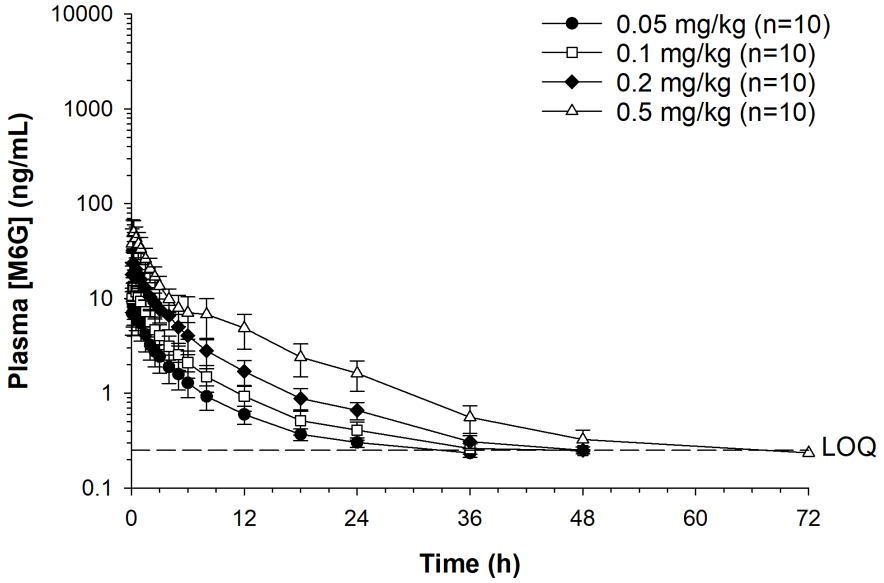


Figure 3. Heart rate (mean ± SD) with respect to time following a single intravenous administration of morphine at 0.05 mg/kg, 0.1mg/kg, 0.2 mg/kg and 0.5 mg/kg.* indicates a significant difference (p<0.05) relative to the saline group

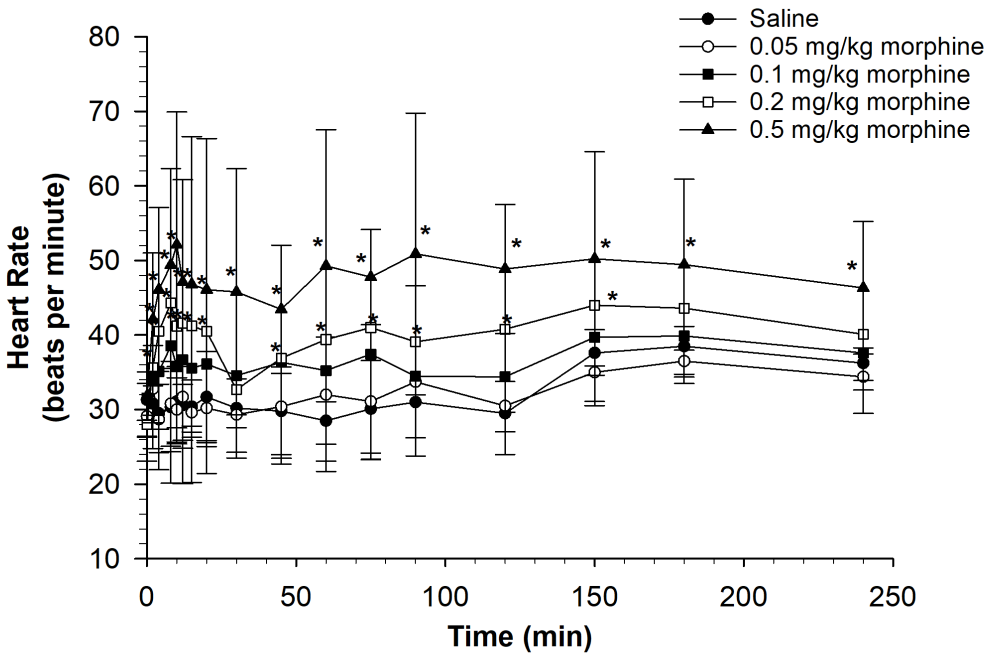


Figure 4. Number of steps shown in bars (mean ± SD) taken with respect to time overlaid on plasma concentrations of morphine, morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G) with respect to time following a single intravenous administration of morphine to adult horses at doses of 0.05mg/kg, 0.1mg/kg, 0.2mg/kg and 0.5mg/kg * indicates a significant difference (p<0.05) relative to the saline group

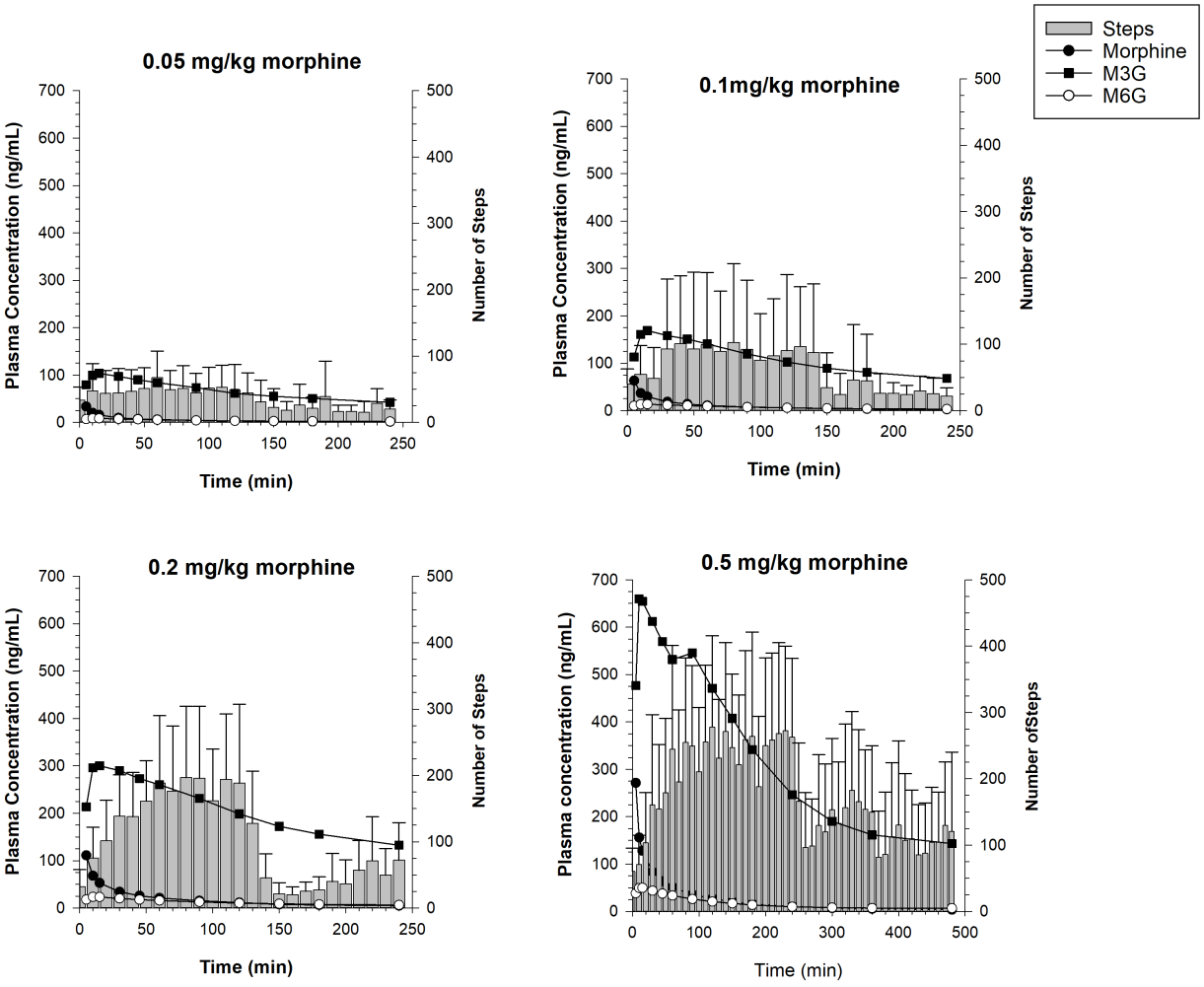
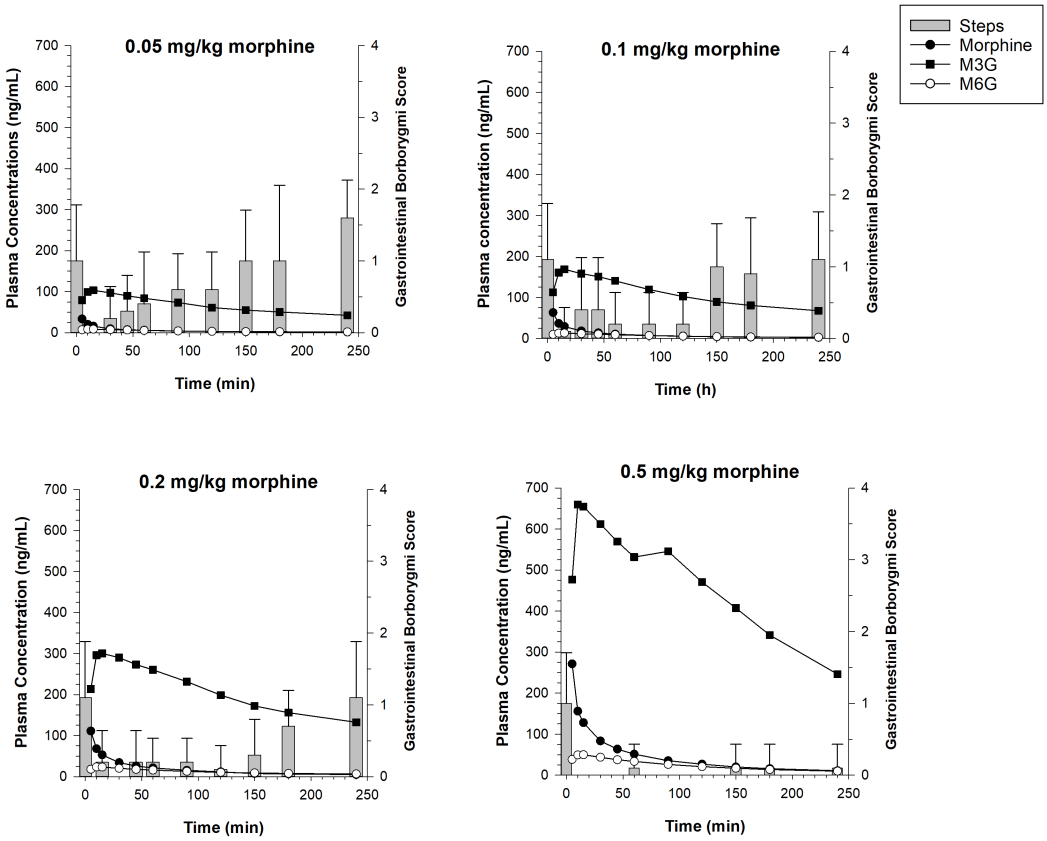


Figure 5. Gastrointestinal score shown as bars (mean ± SD) with respect to time overlaid on plasma concentrations of morphine, morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G) with respect to time following a single intravenous administration of morphine at doses of 0.05mg/kg, 0.1mg/kg, 0.2mg/kg and 0.5mg/kg



Pharmacokinetics, tissue distribution and select physiologic effects of morphine 6-glucuronide (M6G) following intravenous administration to horses

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Abstract

Morphine has demonstrated anti-nociceptive effects in horses, however, administration has been associated with dose-dependent adverse effects. In humans and rats, part of the analgesic effect of morphine has been attributed to the active metabolite, morphine-6-glucuruonide (M6G) and whereas morphine can cause several undesirable effects, M6G has a more favorable safety profile. The objective of this study was to characterize the pharmacokinetics, tissue disposition, behavioral and select physiologic effects of M6G following intravenous administration to a small group of horses. In part one, 3 horses received a single intravenous administration of saline, 0.5 mg/kg M6G and 0.5 mg/kg morphine. Blood samples were collected up to 96-hours post administration, concentrations of drug and metabolites measured, and pharmacokinetics determined. Behavioral and physiologic effects were recorded. In part two of this study, two horses, scheduled to be euthanized for other reasons, were administered 0.5 mg/kg M6G. Blood, CSF and various tissue samples were collected post administration and concentrations of drug determined. The clearance of M6G was more rapid and the volume of distribution at steady state smaller for M6G compared to morphine. A reaction characterized by head shaking, pawing and slight ataxia was observed immediately following administration of both morphine and M6G horses. Following M6G administration, the behaviors subsided rapidly and was followed by a longer period of sedation. Following administration, M6G was detected in the kidney, liver, CSF and regions of the brain. Results of the current student encourage further investigation of M6G as an analgesic in horses.

Introduction

Current pain management for equine patients is limited. While opioids are a frequently and well characterized drug class used for analgesia in other species, their unpredictable and often undesirable side effects limit their use in horses (1). The pharmacodynamics of morphine have been described in horses following intravenous and intramuscular administration (2–4). While it has demonstrated anti-nociceptive effects, (5) intravenous administration has been associated with dose dependent CNS excitatory effects (3,4,6) and unwanted gastrointestinal effects, occurring at what is believed to be a therapeutic dose of approximately 0.2 mg/kg (5).

In humans, morphine undergoes extensive glucuronidation to morphine 3-glucuronide (M3G; 60%) and morphine 6-glucuronide (M6G; 6-10%) (7) In both humans and rats, at least part of the analgesic effects of morphine has been attributed to the M6G metabolite (8). Reports in humans and rats have shown that M6G has a greater affinity for the mu receptor compared to morphine (8,9) and following intrathecal administration, the analgesic potency of M6G is reportedly 100-fold higher than morphine (10,11). Interestingly, although M6G is highly polar, it appears able to cross the BBB as evidenced by studies in rats describing concentrations of the metabolite in brain tissue (12). Whereas morphine can cause a number of unfavorable effects in humans (respiratory depression, nausea and vomiting), M6G has a more favorable safety profile. In contrast to M6G, M3G is believed to be devoid of analgesic properties and does not appear to bind to opioid receptors (14). Furthermore, it has been postulated that M3G antagonizes the analgesic effects of morphine and may have neuroexcitatory effects (15–18).

The metabolism and pharmacokinetics of morphine in the horse have been reported previously (3,4,19). As reported in humans and rats, horses metabolize morphine to M3G and M6G and produce higher concentrations of M3G than humans (3,4). If M6G contributes to the

analgesic effects of morphine in horses and M3G causes excitation, administration of M6G may prove to be an effective analgesic devoid of the excitatory effects observed following morphine administration in horses. Based on this and previous reports in other species, we hypothesized that M6G would be able to enter the CNS following administration of the metabolite to horses. To that end, the objective of this study was to characterize the pharmacokinetics and tissue distribution of M6G and behavioral and select physiologic effects in a small group of horses following intravenous administration.

Materials and Methods

Study 1: Pharmacokinetics and Behavioral and Physiologic Effects Animals

Three healthy university owned thoroughbred geldings (aged 3-8) weighing 510 kg ± 43 (average ± SD) were used for this pilot study. Horses did not receive any medications for a minimum of two weeks prior to the study. A complete blood count, serum biochemistry, and physical exam were performed to confirm the health of the horses. The Institutional Animal Care and Use Committee of the University of California, Davis approved this study (#22516).

Instrumentation and drug administration

This study was conducted in a randomized, 3-way balanced crossover design with a minimum two-week washout between treatments. In each phase, horses were randomly assigned to one of three groups: 0.5 mg/kg morphine sulfate IV, 0.5 mg/kg M6G IV or 5 mL of saline IV.

Dose selection for M6G administration was based on inspection of M6G concentrations following morphine administration (0.5 mg/kg IV) in a previous study conducted by our laboratory (3). Morphine 6-glucuronide powder (Toronto Research Chemical, North York Ontario, Canada) was purchased and subsequently compounded for intravenous administration.

The powder was weighed, dissolved in sterile Lactated Ringers Solution and filter sterilized in a sterile hood. The solution was administered within 20 minutes of mixing.

Each horse was weighed prior to drug administration. Due to the potential for ileus associated with opioid administration in horses, animals were fasted for 12 hours prior to administration of the drug and for 4 hours post administration. Water was available *ad libitum*. A 14-gauge catheter was placed in each jugular vein using aseptic technique prior to drug administration. One catheter was used to for drug administration, while the other was used for sample collection.

Sample Collection

Blood was collected at times 0, 5, 10, 15, 30, 45 minutes, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 18, 24, 36, 48, and 96 hours post drug administration into EDTA blood tubes (Kendall/Tyco Healthcare Mansfield MA, USA) and placed on ice until centrifugation. Catheters were removed after collection of the 24-hour sample with the remaining samples collected via direct venipuncture. Samples were centrifuged at 3000 x g at 4°C for 10 minutes, plasma immediately transferred to cryovials (Phoenix Research Products, Chandler, NC, USA), and samples stored at -20°C. An aliquot of each blood sample was taken at time 0, 5, 10, 15, 30, 45 min, 1, 2, 4, 6, and 8 hours post drug administration for determination of packed cell volume via microhematocrit and total protein via refractometer. Each sample was measured in duplicate with the average recorded for each time point.

Drug Concentration Determination

Concentrations of morphine and metabolites were measured using a previously validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (3,4).

Pharmacokinetic Calculations

Pharmacokinetic parameters for morphine and M6G were determined using non-compartmental analysis and commercially available software (Phoenix WinNonlin Version 8.0, Certara, Princeton, NJ, USA). Non-compartmental analysis was used, as previous studies have demonstrated non-linear elimination of morphine at higher doses (3). The area under curve (AUC) from time 0 to infinity (AUC0→∞) was obtained by using the linear up log down trapezoidal rule, then dividing the last plasma concentration by the terminal slope extrapolated to infinity.

Behavioral and Physiologic Responses

Notable post-drug physiological and behavioral responses were noted and recorded continuously for the first two hours, and then hourly for the next 4 hours. After the initial 6 hours of each study day, direct observations were noted at minimum in the morning and evenings (same time each day) for the next four days.

Step counters and Holter monitors were used as described previously to assess excitatory behavior (3).

To evaluate gastrointestinal behavior, each abdominal quadrant was assigned a GI borborygmi score ranging from 0 to 4 with 0 being absent and 4 being increased sounds. GI scores were assessed prior to and at 30 and 45 minutes, as well as 1, 1.5, 2, 2.5, 3, 4, 5, and 6 hours post drug administration. Defecation frequency and fecal consistency was also recorded throughout the 6-hour sampling period.

Statistical Analysis

Commercially available software (Stata/IC 17.0, StataCorp LP, TX, USA) was used to determine significant differences in pharmacodynamic parameters. Differences between baseline and each time point, and each dose group at each time point, were evaluated using a mixed-effects analysis of variance, with the horse as the random effect and time and dose as the fixed effects. Post-hoc comparisons were accomplished with a Bonferroni multiple comparison adjustment to preserve a nominal significance level of 0.05.

Study 2: Disposition of M6G in tissue

Two horses that were to be euthanized for other reasons (one for neurologic and one for orthopedic reasons) were administered a single IV dose of 0.5 mg/kg of M6G formulated as described in Study 1. Blood samples were collected prior to drug administration, and at 5, 10, 15-30-, 45- and 1-hour post administration. One hour post administration, the horses were euthanized with pentobarbital and blood, cerebral spinal fluid and tissues, including kidney, liver, cerebral cortex, thalamus, caudal brainstem, cerebellum and trigeminal ganglia, were collected and stored at -20°C until processed. Blood samples were processed as described for study 1 above. The Institutional Animal Care and Use Committee of the University of California, Davis approved this study (#22110).

Tissue Drug Concentration Determination:

Approximately 100 mg of tissue (range of 90 to 140 mg) was weighed into tared precellys hard tissue homogenizing vials (Omni International, Kennesaw, GA) and 1 mL of the internal standard (d3-morphine-6BD glucuronide) added. The samples were homogenized twice at 4.5 m/sec for 30 seconds in an Omni Bead Ruptor Elite tissue homogenizer (Omni International), transferred to microcentrifuge tubes and centrifuged at 14,000 rpm (12,753 g) for 5 minutes. The supernatant (500 µL) was dried under nitrogen and reconstituted in 150 µL

of 5% acetonitrile in water with 0.2% formic acid, centrifuged again as before and 20 μL injected into the LC-MS/MS system. The concentrations of morphine, M3G, and M6G were measured by LC-MS/MS as described previously (3,4).

Results

The precision and accuracy of the assay were determined by assaying quality control samples in replicates (n=6) for each analyte. Accuracy and precision were within 10% of the expected value and considered acceptable based on the Food and Drug Administration's guidelines for Bioanalytical Method Development (20). The technique was optimized to provide a LOQ of 0.25 ng/mL and 1 ng/mL and a LOD of approximately 0.1 ng/mL and 0.5 ng/mL in blood and tissues/cerebrospinal fluid.

Concentrations of morphine and metabolites following morphine administration are depicted in Figure 1 and concentrations following M6G administration in Figure 2. Plasma pharmacokinetic parameters for morphine following intravenous administration of 0.5 mg/kg are shown in Table 1 and parameters for M6G following administration of 0.5 mg/kg morphine and 0.5 mg/kg M6G are shown in Table 2. The clearance of M6G was more rapid than morphine, and the volume of distribution at steady state smaller for M6G compared to morphine. The terminal half-life for M6G following administration of morphine was longer compared to the terminal half-life following M6G administration.

A reaction characterized by head shaking, pawing and slight ataxia was observed within the first 5 minutes of M6G administration followed by a longer period of sedation. Following morphine administration, horses also exhibited head shaking and pawing, which was followed by a longer period of these behaviors compared to the M6G group. The number of steps taken per minute following saline, M6G and morphine administration are depicted in Figure 3A, B and C.

Following administration of morphine, the number of steps recorded increased for about 120 minutes (Figure 3B). Following M6G administration, the number of steps initially increased first 10 minutes), relative to baseline, and then subsequently decreased (Figure 3C).

Heart rate was significantly ($p<0.05$) increased relative to baseline from 5 minutes to 3 hours and then again at 5 and 6 hours following M6G administration and from 5 minutes until 6 hours post administration of morphine (Table 3). Packed cell volume and total protein were significantly increased, relative to baseline, at several times post administration in all three dose groups (Table 4).

Gastrointestinal sounds were significantly ($p<0.05$) reduced, relative to baseline, from 30 minutes until 2 hours then again at 3 hours following morphine administration (Table 5). Following M6G administration, gastrointestinal sounds were decreased from 5 minutes to 2 hours and again at 3 hours post administration (Table 5). Fecal output was decreased relative to baseline in the morphine dose group in 2/3 horses for up to 8 hours post drug administration. Fecal output remained consistent, compared to baseline in the saline and M6G dose groups.

In study 2, following a single IV administration of 0.5 mg/kg, M6G was detected in the kidney, liver, CSF, and various regions of the brain (Table 6). The highest concentrations in brain tissue were found in the trigeminal ganglia in both horses (Table 6).

Discussion

Similar to previous studies in horses, whereby M3G was the predominant metabolite following morphine administration, concentrations of M3G far exceeded M6G concentrations in the current study. Following M6G administration, low concentrations of both morphine and M3G were noted. This observation has been reported before in humans (21) whereby investigators theorized that production of M3G and morphine following M6G

administration may be a result of enterohepatic recirculation. Although further study would be necessary to definitively conclude this in horses, this is a possible explanation for the identification of the two compounds following M6G administration in the current study.

The volume of distribution of M6G (1.61-2.24 L/kg) was markedly smaller compared to morphine (6.13-7.30 L/kg) as would be expected based on the polarity and larger molecular weight of M6G. The systemic clearance of morphine was rapid compared to M6G, likely due to a rapid rate of biotransformation of morphine to both M6G and M3G, as has been described for other species (22). For 2/3 horses studied, the terminal half-life of M6G following intravenous morphine administration was longer compared to intravenous administration of M6G. This difference may be attributable to the time it takes the body to metabolize morphine to M6G. This rate of conversion of morphine to M6G may be slower than the elimination of M6G, resulting in a flip-flop effect. In the third horse, the elimination half-life of M6G following morphine administration and following M6G administration were in close agreement. While the reason for the discrepancy between this horse and the other two is not clear, in humans, similar to what was calculated for the third horse, the elimination half-life of M6G following morphine administration and following direct administration of M6G were not different (23). It should also be noted that the number of horses studied was small and additional study, with a larger sample size, would be necessary to draw any definitive conclusions regarding a flip-flop effect. The terminal half-life of M6G, whether following morphine or direct M6G administration is longer than that reported in humans, (23) however, the half-life of M6G following morphine administration is in agreement with previous reports in horses (3,4).

Morphine administration to horses has been associated with a dose-dependent excitatory effect (3,4). As reported previously, in this study, intravenous administration of 0.5

mg/kg of morphine resulted in a prolonged (120 minutes post administration) increase in locomotion and heart rate. Although M6G administration resulted in a brief period (10 minutes) of signs consistent with excitation, this response was transient and was followed by behavior consistent with sedation. Heart rate also increased following administration of M6G but less so in comparison to the increase observed following morphine administration. These results suggest that M6G has less of a central excitatory effect compared to morphine.

Both PCV and total protein increased from baseline following administration of morphine and M6G. This finding is in agreement with our previous report in horses, describing an increase in PCV and total protein following administration of an intravenous dose of 0.5 mg/kg morphine (3) In the previous study, while the authors acknowledged that environmental factors, specifically warm summer temperatures could have led to mild dehydration and subsequent changes in PCV and total protein, they also add further support that this finding is a result of increased sympathetic tone and splenic contraction (3). In extreme circumstances, this response has been shown to nearly double horses' PCV (24,25). While it is important to note that environmental temperatures in the current study were also high and therefore it is not possible to exclude dehydration as a potential explanation for the increases in both PCV and total protein noted in this report, the increase in heart rate observed in both the M6G and morphine dose groups adds further support that these effects are related to drug administration. Furthermore, if changes in PCV and total protein were due to dehydration these changes would be expected to continue to increase with time and both quantitative and qualitative changes in urinary output would be expected, but these were not at least casually noted.

The effects of opioids such as morphine on the gastrointestinal system has been well described in horses. In agreement with previous studies, administration of morphine did appear to decrease GI motility (3,6,26). Interestingly, in the current study, the same effect was seen following administration of M6G. Although adverse effects on the GI tract effects are reportedly less following administration of M6G in humans, (27) this finding in the current report is not completely unexpected given the affinity of M6G for opioid receptors and the knowledge that binding to opioid receptors is thought to alter motility, secretion, absorption, and blood flow in the GI tract. Although it is important to note that fasting the horses in the current study may have also contributed in some small measure to the decrease in GI motility, this was not seen in the saline dose group, suggesting that this effect is related to administration of morphine and M6G.

Reported analgesic effects of M6G suggest that it can cross the blood brain barrier, therefore, in the second part of this study, we sought to describe the tissue distribution of M6G following IV administration. Although highly polar, previous reports conducted in rats describe M6G's ability to cross the blood-brain barrier (BBB) in animals (12,28,29). Morphine-6-glucuronide has been reported M6G in brain tissue and cerebral spinal fluid following subcutaneous administration of 10 mg/kg of M6G to rats (12). Similarly in the current study M6G was detected in homogenates from the occipital, temporal and frontal lobe and the thalamus, cerebellum and brainstem following IV administration, suggesting that it can cross the blood brain barrier in horses.

Since, presumably its hydrophilic nature would prevent diffusion, it has been suggested that the ability of M6G to cross the BBB is the result of transport proteins, such as Oatp2 and GLUT-1 (30,31). An additional hypothesis is that the drug molecule may be able to fold and

mask its polar groups thereby increasing its lipophilicity allowing it to cross the BBB and enter the CNS (32). While M6G appears to be a substrate for some ATP-binding cassette (ABC) transporter multidrug resistance proteins, namely MRP2 and MRP3 (efflux proteins), in *in vivo studies*, it does not appear to be a substrate for P-glycoprotein, the efflux protein present within the BBB (33–35).

Appreciable concentrations of M6G were also found in the trigeminal ganglia in both horses. Notably, entry into the trigeminal ganglia is easier as there is no BBB that must be crossed. The clinical implications of this are not clear. Although there are a large concentration of mu opioid receptors in the trigeminal ganglia reports describing the effectiveness of opioids such as morphine in treating pain conditions associated with the trigeminal ganglia have been inconclusive in humans (36) and not yet reported in horses. Not unexpectedly, since the kidneys represent the primary organ of elimination of both M6G and M3G in other species, (37,38) high concentrations of M6G were found in this organ.

It is important to note the limitations of the current study. As mentioned previously, the number of horses studied was small and only a single dose assessed. While results of the current study provide preliminary information and are supportive of further investigation of M6G, additional study with more horses, additional doses, and an assessment of the effects of this compound on nociception are necessary to assess its clinical feasibility as an analgesic in horses.

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Table 1: Pharmacokinetic parameters (mean ± SD) for morphine following a single IV 382 administration of 0.5 mg/kg to adult horses. All values reported were generated using non-compartmental analysis.

Parameters	0.5 mg/kg Morphine IV		
	Horse 1	**Horse 2**	**Horse 3**
C(0) ng/mL	525.7	376.1	809.2
$Lambda_z$ (1/h)	0.11	0.06	0.06
HL $Lambda_z$ (h)	6.50	11.9	12.9
Vdss (L/kg)	6.13	7.30	6.98
CL (mL/min/kg)	30.4	29.8	30.9
AUC_{0-inf} (h*ng/mL)	274.3	279.3	269.9

C(0), Concentration extrapolated to the origin, $Lambda_z$, terminal slope; HL $Lambda_z$, terminal half-life, Vdss, Volume of distribution at steady-state; CL, Total systemic clearance; AUC_{0-inf}, Area under the plasma-concentration curve from time 0 to infinity.

Table 2. Pharmacokinetic parameters (mean ± SD) for morphine-6 glucuronide (M6G) following a single IV administration of 0.5 mg/kg morphine or 0.5 mg/kg morphine to adult horses. All values reported were generated using non-compartmental analysis.

	Horse 1		**Horse 2**		**Horse 3**	
Treatment	**M6G**	**Morphine**	**M6G**	**Morphine**	**M6G**	**Morphine**
C(0) ng/mL	3675	NA	4998	NA	4416	NA
$Lambda_z$(1/h)	0.205	0.100	0.202	0.078	0.080	0.087
HL $Lambda_z$ (h)	3.39	6.96	3.42	8.99	8.62	8.01
Vdss (L/kg)	2.24	NA	1.61	NA	1.94	NA
CL (mL/min/kg)	2.83	NA	2.18	NA	3.28	NA
AUC_{0-inf} (h*ng/mL)	2925	86.1	3817	80.3	2530	167.4

C(0), Concentration extrapolated to the origin, $Lambda_z$, terminal slope; HL $Lambda_z$, terminal half-life, Vdss, Volume of distribution at steady-state; CL, Total systemic clearance; AUC_{0-inf}, Area under the plasma-concentration curve from time 0 to infinity.

Table 3. Heart rate (beats/min; mean ± SD), following a single IV administration of saline, 0.5 mg/kg morphine-6 glucuronide and 0.5 mg/kg morphine to adult horses.

Time (h)	Saline	0.5 mg/kg M6G	0.5 mg/kg Morphine
0	28.3 ± 4.6	29.3 ± 3.8	27.0 ± 3.0
0.03	29.3 ± 4.7^{bc}	43.7 ± 7.6^{*a}	42.0 ± 4.6^{*a}
0.08	28.3 ± 5.7^{bc}	44.7 ± 8.0^{*a}	43.7 ± 5.0^{*a}
0.13	30.0 ± 3.5 ^{bc}	48.7 ± 0.6^{*a}	53.0 ± 12.8^{*a}
0.17	27.7 ± 6.5^{bc}	47.0 ± 5.0^{*a}	47.0 ± 8.7^{*a}
0.20	31.0 ± 4.6^{c}	45.7 ± 5.9*	48.0 ± 14.7^{*a}
0.25	28.3 ± 8.0^{bc}	44.3 ± 5.5^{*a}	44.3 ± 5.1^{*a}
0.33	32.0 ± 3.6^{bc}	43.0 ± 3.6^{*a}	42.0 ± 8.7^{*a}
0.5	31.0 ± 7.5^{*bc}	42.0 ± 1.0^{*a}	44.0 ± 7.5^{*a}
0.75	28.7 ± 8.0^{c}	38.7 ± 1.5*	49.3 ± 11.9^{*a}
1	30.0 ± 5.6^{bc}	38.3 ± 2.5^{*a}	40.7 ± 2.1^{*a}
1.25	31.7 ± 7.1^{c}	39.0 ± 3.6*	44.0 ± 2.0^{*a}
1.5	33.7 ± 3.1^{*bc}	39.0 ± 2.0^{*ac}	42.7 ± 2.9^{*ab}
2	30.0 ± 8.2^{bc}	36.7 ± 3.5^{*ac}	42.7 ± 2.1^{*ab}
2.5	34.7 ± 4.9^{*bc}	41.3 ± 3.2^{*a}	42.3 ± 3.1^{*a}
3	33.3 ± 3.2^{*bc}	37.7 ± 5.5^{*ac}	41.7 ± 4.7^{*ab}
4	34.0 ± 5.0^{*c}	33.0 ± 10.5^{c}	40.3 ± 8.5^{*ab}
5	42.3 ± 3.8*	37.3 ± 4.5*	40.3 ± 8.0*
6	36.3 ± 2.5*	41.3 ± 2.1*	41.3 ± 8.5*

*,indicates a significant difference ($p < .05$) relative to baseline; [a], indicates significant difference ($p < .05$) from saline group; [b], indicates significant difference ($p < .05$) from M6G group; [c], indicates significant difference ($p < .05$) from morphine group.

Table 4. Packed cell volume (PCV) and total protein concentration (TP) (mean ± SD) at specified time points following administration of a single dose of saline (5 mL), 0.5 mg/kg morphine and 0.5 mg/kg morphine 6-glucuronide (M6G) to 3 horses.

Time	Saline		0.5 mg/kg M6G		0.5 mg/kg Morphine	
	PCV	TP	PCV	TP	PCV	TP
0 min	36.5 ± 0.8	5.9 ± 0.2	34.5 ± 4.6	6.2 ± 0.7	35.9 ± 2.2	6.1 ± 0.5
5 min	34.6 ± 1.0^{*bc}	6.0 ± 0.2*	41.3 ± 3.3^{*a}	6.3 ± 0.8	41.4 ± 1.6^{*a}	6.3 ± 0.6*
15 min	35.0 ± 3.4^{bc}	6.1 ± 0.1*	40.7 ± 4.1^{*a}	6.5 ± 0.7	41.8 ± 1.3^{*a}	6.3 ± 0.5*
30 min	34.1 ± 3.8^{*bc}	6.1 ± 0.1*	37.5 ± 3.5^{*a}	6.4 ± 0.7	38.9 ± 1.7^{*a}	6.4 ± 0.5*
45 min	33.8 ± 3.7^{*c}	6.1 ± 0.2*	36.4 ± 2.8	6.4 ± 0.7	39.7 ± 1.0^{*a}	6.3 ± 0.5*
1 hour	32.8 ± 2.9^{*c}	6.0 ± 0.2*	34.7 ± 4.4	6.4 ± 0.7	37.0 ± 2.2^{a}	6.4 ± 0.6*
2 hours	32.5 ± 2.6^{*c}	6.1 ± 0.2*	32.2 ± 4.0^{c}	6.3 ± 0.6	36.6 ± 2.5^{ab}	6.3 ± 0.3*
4 hours	35.7 ± 2.3^{c}	6.3 ± 0.2*	36.8 ± 4.5^{c}	6.5 ± 0.9*	40.1 ± 2.8^{*ab}	6.5 ± 0.4*
6 hours	39.0 ± 2.5^{*c}	6.3 ± 0.1^{*bc}	41.0 ± 2.1*	6.7 ± 0.5^{*a}	42.5 ± 2.0^{*a}	6.9 ± 0.5^{*a}
8 hours	36.6 ± 1.4^{bc}	6.0 ± 0.2^{*b}	43.1 ± 2.8^{a}	6.7 ± 0.6^{*a}	42.2 ± 1.8^{*a}	6.5 ± 0.5*

*, indicates a significant difference ($p < .05$) relative to baseline; [a], indicates significant difference ($p < .05$) from saline group; [b], indicates significant difference ($p < .05$) from M6G group; [c], indicates significant difference ($p < .05$) from morphine group.

Table 5. Gastrointestinal scores (mean ± SD), following a single IV administration of saline, 0.5mg/kg morphine-6 glucuronide (M6G) and 0.5 mg/kg morphine to 3 horses.

Time (h)	Saline	0.5 mg/kg M6G	0.5 mg/kg Morphine
0	0.7 ± 0.7	1.0 ± 0.0	1.0 ± 0.0
0.25	0.7 ± 0.7[bc]	0.0 ± 0.0[*a]	0.0 ± 0.0[*a]
0.5[†]	1.0 ± 0.0	0.0 ± 0.0[*]	0.0 ± 0.0[*]
0.75	0.7 ± 0.7[bc]	0.0 ± 0.0[*a]	0.0 ± 0.0[*a]
1.0	0.7 ± 0.7	0.3 ± 0.7[*]	0.0 ± 0.0[*]
1.5	0.7 ± 0.7[bc]	0.0 ± 0.0[*a]	0.0 ± 0.0[*a]
2.0[†]	1.0 ± 0.0	0.0 ± 0.0[*]	0.0 ± 0.0[*]
2.5	1.3 ± 0.7	0.7 ± 0.7	0.7 ± 0.0
3.0	1.7 ± 0.0[bc]	0.3 ± 0.7[*a]	0.3 ± 0.7[*a]
4.0	1.3 ± 0.0	1.3 ± 0.0	0.7 ± 0.0
5.0	1.3 ± 0.0	1.3 ± 0.0	1.0 ± 0.7
6.0	1.3 ± 0.0	1.3 ± 0.7	0.7 ± 0.7

[†] Statistical model could not fit data.

*, indicates a significant difference ($p < .05$) relative to baseline; [a], indicates significant difference ($p < .05$) from saline group; [b], indicates significant difference ($p < .05$) from M6G group; [c], indicates significant difference ($p < .05$) from morphine group.

Table 6. Blood (A) and tissue (B) concentrations following intravenous administration of 0.5 mg/kg morphine 6-glucuronide (M6G) to two horses.

A.)

Time (min)	**M6G**		**M3G**		**Morphine**	
	Concentration (ng/mL)					
	Horse 1	Horse 2	Horse 1	Horse 2	Horse 1	Horse 2
0	ND	ND	ND	ND	ND	ND
5	3479.4	2835.0	0.66	2.01	0.81	5.83
10	2907.1	2369.7	0.80	2.47	0.56	5.24
15	3022.3	2009.1	1.04	2.63	0.71	4.57
30	1803.3	1449.3	1.32	2.81	0.39	3.78
45	1277.1	1080.2	1.58	3.01	0.39	2.75
60	953.4	870.4	1.93	3.29	0.38	2.42

B.)

Tissue	M6G		M3G		Morphine	
	Concentration (ng/gram)					
	Horse 1	Horse 2	Horse 1	Horse 2	Horse 1	Horse 2
Kidney	3313.8	2488.4(right) 2683.0 (left)	39.1	37.0 (right) 24.4 (left)	15.5	36.6 (right) 28.3 (left)
Liver	332.8	400.5	2.23	3.88	ND	<LOQ
Cerebral Cortex (occipital lobe)	33.3	11.3	ND	ND	ND	ND
Cerebral Cortex (temporal lobe)	49.3	12.8	ND	ND	ND	ND
Thalamus	32.6	11.7	ND	ND	ND	ND
Caudal Brainstem	23.5	10.2	ND	ND	ND	ND
Cerebral Cortex (Front)	36.7	26.2	ND	ND	ND	ND
Cerebellum	38.6	25.7	ND	ND	ND	ND
Trigeminal Ganglia	348.1	280.8 (right) 304.8 (left)	ND	ND		<LOQ (left) <LOQ (right)

			Concentration (ng/mL)			
Cerebrospinal Fluid	10.8	2.95	ND	ND	ND	ND

ND, not detected

Figure 1. Plasma concentration time curve for morphine, morphine 6-glucuronide (M6G) and morphine 3-glucuronide (M3G) following intravenous administration of 0.5 mg/kg morphine to 3 horses.

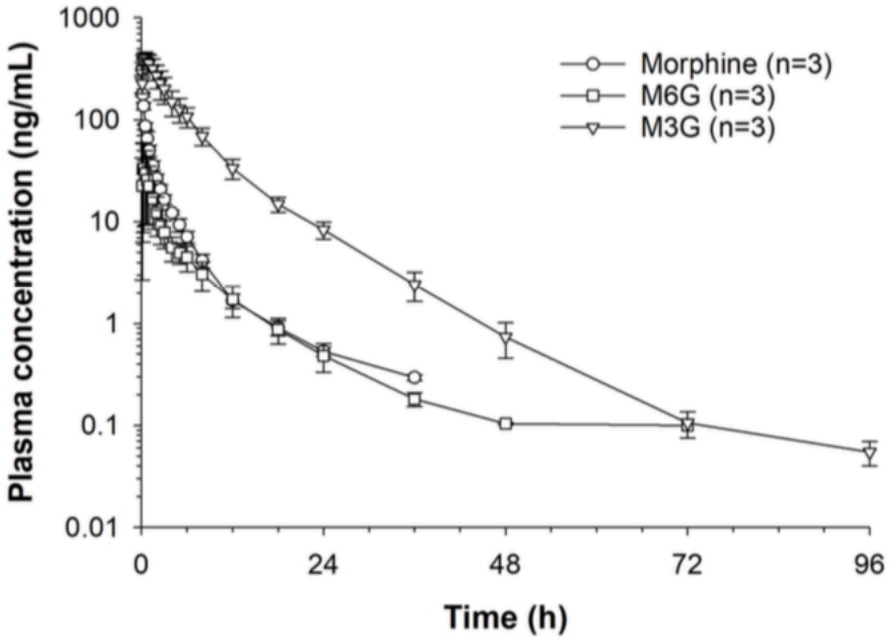


Figure 2. Plasma concentration time curve for morphine, morphine 6-glucuronide (M6G) and morphine 3-glucuronide (M3G) following intravenous administration of 0.5 mg/kg M6G to 3 horses.

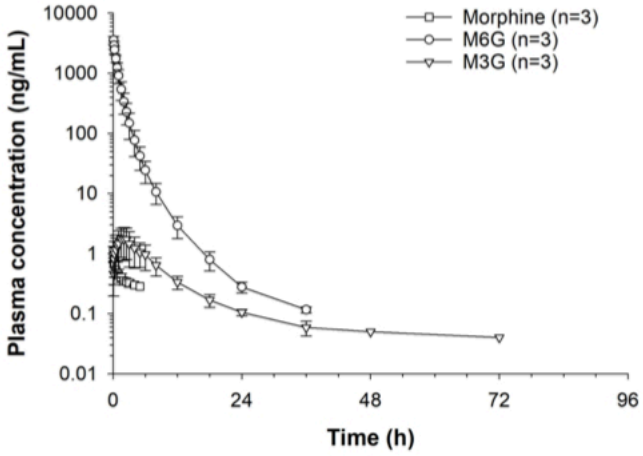
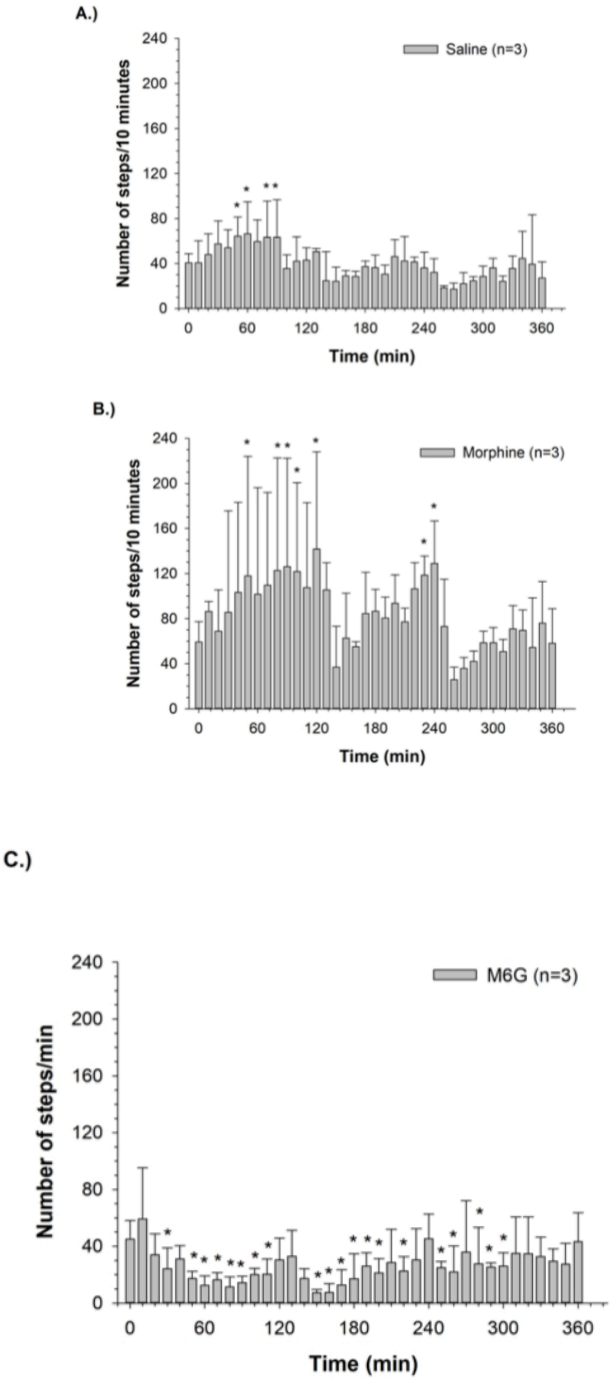


Figure 3. Number of steps shown in bars (mean ± SD) taken with respect to time following a single intravenous administration of (A) saline (B) 0.5 mg/kg morphine 6- glucuronide (M6G) and (C) 0.5 mg/kg morphine to three adult horses. * indicates a significant difference (p<0.05) relative to baseline.



Equine UDP Glucuronosyltransferase 1A1, 2A1, 2B4, 2B31: cDNA cloning, expression, and initial characterization of morphine metabolism

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BDHH- study execution, data analysis, manuscript preparation; RWB: study execution, data analysis; DSM-study execution, data analysis, manuscript preparation; HKK-study design, study execution, data analysis, manuscript preparation.

Conflict of Interest:
The authors have no conflicts to declare.

Abstract

Objective Uridine diphospho-glucuronosyltransferases (UGTs) are membrane bound enzymes that catalyze the conjugation of glucuronic acid onto a diverse set of xenobiotics. Horses efficiently and extensively glucuronidate a number of xenobiotics, including opioids, making UGTs an important group of drug-metabolizing enzymes for the clearance of drugs. Recombinant enzymes have allowed researchers to characterize the metabolism of a variety of drugs. The primary objective was to clone, express and characterize equine UGTs using drugs characterized as UGT substrates in other species. A secondary objective was to characterize the *in vitro* metabolism of morphine in horses.

Study design *In vitro* drug metabolism study using liver microsomes and recombinant enzyme systems.

Animals Liver microsomes and RNA from tissue collected from two Thoroughbred mares euthanized for other reasons.

Methods Based on homology to the human UGT2B7, four equine UGT variants were expressed: UGT1A1, UGT2A1, UGT2B31 and UGT2B4. cDNA sequences were cloned and resulting protein expressed in a baculovirus expression system. Functionality of the enzymes was assessed using 4-methylumbelliferone, testosterone, diclofenac and ketoprofen. Recombinant enzyme, control cells, equine liver microsomes and human UGT2B7 supersomes were then incubated

with morphine. Concentrations of metabolites were measured using liquid chromatography-tandem mass spectrometry and enzyme kinetics determined.

Results 4-methylumbelliferone was glucuronidated by all expressed equine UGTs. Testosterone glucuronide was not produced by any of the expressed enzymes and diclofenac glucuronide and ketoprofen glucuronide were produced by UG2A1 and UGT1A1, respectively. UGT2B31 metabolized morphine to morphine-3-glucuronide and low concentrations of morphine-6-glucuronide.

Conclusion and clinical relevance This is the first successful expression of functional recombinant equine UGTs. UGT2B31 contributes to the glucuronidation of morphine; however, it is probably not the main metabolizing enzyme. These results warrant further investigation of equine UGTs, including expression of additional enzymes and further characterization of UGT2B31 as a contributor to morphine metabolism.

Keywords drug, equine, glucuronide, metabolism, morphine, uridine diphospho-glucuronosyltransferase.

Introduction

Uridine diphosphate glucuronosyltransferases (UGTs) are membrane bound enzymes located on the endoplasmic reticulum. This group of enzymes plays an important role in drug elimination by catalyzing the conjugation of glucuronic acid and increasing the polarity of the substance; thereby aiding drug elimination (Tukey & Strassburg 2000). Substrates for UGTs are frequently metabolites generated from phase I biotransformation reactions, however, in some cases, direct glucuronidation of the parent compound may occur.

More than fifteen variants of UGTs have been identified in humans to date (Rowland et al. 2013; Oda et al. 2015). UGT2B7, one member of the UGT family, has been well studied in humans and plays a large role in metabolism of several important classes of therapeutic substances, including non-steroidal anti-inflammatory drugs (NSAIDs) and opioids (Soars et al. 2003). Morphine, for example, undergoes direct glucuronidation by UGT2B7. It is glucuronidated at the 3 and 6 position to produce two metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). M6G is believed to be a more potent analgesic than morphine (Paul et al. 1989; Osborne et al. 1990) and appears to be predominantly generated by UGT2B7 in humans (Coffman et al. 1997). Although the *in vitro* metabolism of morphine has yet to be described in horses, a similar metabolic profile to that of humans has been described *in vivo* (Knych et al. 2014; Hamamoto-Hardman et al. 2019).

There is limited information describing phase II drug metabolism in horses. A review of the literature shows no reports describing the expression and characterization of an equine recombinant UGT. To that end, the objective of the current study was to clone and express functional equine UGTs and to characterize phase II metabolism in the horse, using morphine as a substrate.

Materials and Methods

Animals

Liver samples used for isolation of ribonucleic acid (RNA) and preparation of liver microsomes were collected from two horses previously euthanized for other studies approved by the Institutional Animal Care and Use Committee of the University of California Davis. Horses were determined to be healthy by physical examination prior to euthanasia.

Reagents

All sequencing primers were synthesized by Integrated DNA Technologies Inc. (IA, USA), WesternDot 625 Goat anti-Rabbit antibody and Bradford reagent were provided by Thermo Fisher Scientific Inc. (NJ, USA). Genomic DNA clean and concentrate kit was purchased from Zymo Research (CA, USA). Restriction digest enzymes were obtained from New England Biolabs Inc. (MA, USA). All reagents used in the bac-to-bac cloning and expression system, NuPage loading dye and transfer buffer and Mark XP ladder were obtained from Invitrogen ThermoFisher Scientific (CA, USA). The UGT2B4 antibody used in the western blot was obtained from ABclonal Technology (MA, USA) and human UGT2B7 supersomes were obtained from Corning (NY, USA). 4-methylumbelliferone (4MU) , acetonitrile (ACN), methanol, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), uridine-diphosphate-glucuronic acid trisodium salt (UDPGA) and magnesium chloride were obtained from Sigma-Aldrich Corp. (MO, USA) and were high performance liquid chromatography (HPLC) grade or better. Morphine sulfate was obtained from Hospira Inc. (IL, USA).

Cloning and expression

A two-step reverse transcription polymerase chain reaction (RT-PCR) was used to amplify PCR products of four UGT variants from equine liver RNA. Complementary DNA (cDNA) was amplified from isolated total liver RNA as described previously (Dimaio Knych & Stanley 2008) using the QuantiTect Reverse Transcription kit (Qiagen, Germany). The UGT enzymes UGT1A1, UGT2A1, UGT2B4 and UGT2B31 were selected from the National Center for Biotechnology Information (NCBI) equine genome database based on greater than 60% homology to human UGT2B7 (Gene ID:7364) (Table 1) and amplified with primers generated from sequences obtained from GenBank (Table 2). cDNA products were cloned directly into the pFastBac1 expression vector (Invitrogen). Plasmids were isolated using Purelink quick plasmid miniprep kit (Thermo Fisher Scientific Inc.). Sequences were confirmed by a commercial sequencing facility (University of California Davis College of Biological Sciences, ^{UC}DNA Sequencing Facility). Verified UGT cDNA sequences were transposed into baculovirus bacmid using a commercially available kit (Bac-to-Bac Baculovirus Expression System, Thermo Fisher Scientific Inc.) and the manufacturer's instructions.

For protein expression, baculovirus stocks were generated by transfecting TriEx Sf9 insect cells (MilliporeSigma, MA, USA) in supplemented media with purified UGT bacmid. Baculovirus stocks were then amplified using TriEx cells in SF-900 III serum free. Fresh TriEx cells were then infected with the baculovirus and used to optimize expression and produce active enzyme. Production of the functional UGT protein was performed using a multiplicity of infection of 2 and protein harvested 72 hours post infection. The cells were harvested and homogenized in homogenization buffer [100 mM KPO_4, pH 7.4, 20% glycerol, 1mM

ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF)] using a Teflon/glass homogenizer (Wheaton, NJ, USA). The homogenized cells were sonicated for 6x cycles (15 seconds followed by a 45 second rest on ice), the preparation spun at 100,000 ***g*** for 1 hour at 4 °C and the supernatant was removed. The pellets were resuspended in buffer (100 mM KPO_4, pH 7.4, 20% glycerol, 1mM EDTA), the pellet containing the protein was subsequently spun at 1000 ***g*** for 1 minute at 4 °C to pellet cellular debris and the resultant supernatant was stored at –80°C.

Preparation of equine liver microsomes

Equine liver microsomes were prepared as described previously (Knych et al. 2019). In summary, the liver sample was collected from an adult female Thoroughbred horse euthanized for a separate study and free of medications. The sample was collected within 20 minutes of the horse being euthanized, kept cold during transport with 0.9% saline, and rinsed and flushed. The sample was then blotted dry then cut into smaller pieces and placed in an ice-cold blender. The sample was homogenized in a homogenization buffer and then subject to a series of centrifugation steps. Microsomes were then stored at –80°C until used.

Verification of product expression and function

Total protein was quantified using the Bradford Assay. To assess whether the desired UGT variant was present, a western blot was performed. Using 80 µg of UGT2A1, UGT1A1, UGT2B4, UGT2B31, liver microsomes, TriEx uninfected cells and Human UGT2B7 Supersomes (Corning) protein were run on a NuPAGE 4-12% Bis-Tris polyacrylamide gel (Invitrogen) 200V for 1 hour, then transferred to a polyvinylidene fluoride (PVDF) membrane at

200 mA for 2 hours before blocking overnight in 3% nonfat milk in tris-buffered saline–Tween (TBS–T). Membranes were then incubated for one hour using a human polyclonal UGT2B4 primary antibody (A4180; Abclonal Technologies) at a concentration of 1:1000 followed by incubation with a Goat anti-Rabbit IgG secondary antibody Western Dot 625 (W10809; Thermo Scientific Inc.) at a concentration of 1:500. The membrane was then scanned using an Alpha Innotech imager with an ethidium bromide filter.

To assess functionality of the expressed UGTs, UGT2A1, UGT1A1, UGT2B4, UGT2B31, liver microsomes, TriEx uninfected cells and human UGT2B7 Supersomes were incubated with 4-methylumbelliferone (4-MU; 50, 100 and 800 μM), testosterone, diclofenac, ketoprofen and morphine (400 μM). Each incubation consisted of 100 mM KPO_4, pH 7.4, 25 mM CHAPS, 50mM UDPGA, 125mM $MgCl_2$, and substrate. Samples were pre-incubated at 37 °C for 5 minutes in a shaking water bath prior to the addition of 20 μL of protein for a final concentration of 50 μg mL^{-1}, after which samples were incubated at 37 °C for 1 hour. Reactions were terminated by the addition of 250 μL of ice-cold ACN and subsequently centrifuged. Concentrations of glucuronidated metabolites were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Metabolism of morphine for kinetic parameter determination

Subsequent to the initial screening assays, morphine was chosen for further study to determine the rate of metabolism of this compound by the recombinant enzymes. Morphine metabolism was determined in 250 μL reaction volumes run in triplicate. All incubations contained 20 μL of recombinant equine UGT protein, liver microsomes, human UGT2B7 Supersomes, or uninfected TriEx cells and 100 mM KPO_4, pH 7.4, 50 mM of UDPGA, 125 mM $MgCl_2$, 50 mM CHAPS and

morphine (0–800 μM). Each reaction was preincubated for 5 minutes at 37 °C prior to the addition of protein. The reactions were then incubated at 37 °C for 1 hour and terminated by the addition of 250 μL of ice-cold ACN. Samples were centrifuged at 1300 ***g*** for 10 minutes and the supernatant transferred to an autosampler vial for analysis.

Morphine incubations with UDP-glucose

Previous studies have demonstrated that UGT enzymes are capable of producing morphine glucoside conjugates when incubated with UDP-glucose (Chau et al. 2014). Incubations were as described earlier for liver microsomes with UDP-glucose (50 μM) in place of the UDPGA.

Quantification of metabolites

All analytical reference standards were obtained from Cerilliant Corporation (TX, USA) as 1 mg mL^{-1} solutions. Working solutions were prepared by dilution of the 1 mg mL^{-1} stock solutions with methanol to concentrations of 0.1, 1, 10 and 100 ng μL^{-1}. Calibrators were prepared by dilution of the working standard solutions with 5% ACN in water with 0.2% formic acid. Calibration curves were prepared fresh for each quantitative assay.

Quantitative analysis was performed on an LTQ XL Orbitrap mass spectrometer (Thermo Scientific) coupled with a Waters Acquity UPLC (Waters Corp., MA, USA). The system was operated in full scan mode at a resolution of 60,000 (M/ΔM, at full width at half maximum of the mass peaks) using positive electrospray ionization [ESI(+)]. The responses were plotted using a 10 ppm mass tolerance for testosterone-glucuronide [mass to charge ratio *(m z^{-1})* 465.2472], diclofenac glucuronide [472.0550+474.0520 *(m z^{-1})*], ketoprofen glucuronide [(453.1112 *(m z^{-1})*] and 4-MUG [353.0871 *(m z^{-1})*] and 20 ppm mass tolerance for the ions M3G and M6G [mass to

charge ratio *(m z^{-1})* 462.17566] and morphine [286.14384 *(m z^{-1})*]. Quanbrowser software (Thermo Scientific) was used to generate calibration curves and quantitate all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves.

The responses were linear and gave correlation coefficients of 0.99 or better for all analytes. For kinetic assays, the technique was optimized to provide a limit of quantitation of 5 ng mL^{-1} for M3G and M6G and 0.5 ng mL^{-1} for morphine. The limit of detection was approximately 3 ng mL^{-1} for M3G and M6G, and 0.3 ng mL^{-1} for morphine.

There is no commercially available morphine glucoside conjugate (M3 glucoside) reference standard and therefore analysis of samples from incubations with UDP glucose was conducted using an LTQ XL Orbitrap mass spectrometer operated in full scan mode and looking for analytes at 448 *(m z^{-1})* as described previously (Chau et al. 2014) to determine presence or absence of this compound. As a further check for the presence of M3 glucoside, data was analyzed using MetWorks Metabolite Identification Software (Thermo Scientific).

Determination of kinetic parameters

For both microsomal and recombinant enzyme reactions, the formation rate (V) of M3G and M6G at the respective substrate concentrations (C) were determined and plotted. Nonlinear regression analysis was used to determine kinetic parameters maximal rate of velocity (V_{max}) and the substrate concentration at 1/2 the maximum velocity (K_m) using the OriginPro software (OriginLab Corporation, MA, USA). For all incubations, the data was fit to the Michaelis-Menten equation:

$$V = \frac{V_{max}[C]}{K_m + [C]}$$

where V is the formation rate, and C is the respective substrate concentrations.
This was determined as the model of best fit based on visual observation of the fitted curve and Akaike's Information Criterion values.

Results

Homology and expression

A CLUSTAL alignment of the NCBI sequences for equine UGT 2B31, 2B4, 1A1, and 2A1 was performed against human UGT2B7 (Fig. 1 & Table 1). UGT2B31 was the most homologous sequence followed by UGT 2B4, UGT2A1 and UGT1A1.

Western blot was used to verify the expression of the desired protein with a primary antibody against human UGT2B4 using a conserved UGT domain to identify the recombinant UGTs. Protein expression was detected in all UGT variants, human UGT2B7 supersomes and equine liver microsomes (Fig. 2). The negative control (uninfected insect cells) did show a faint band, but this was attributed to insect cell proteins.

Each recombinant enzyme was incubated with 4-MU, which is glucuronidated to form 4-MUG, to assess functionality. UGT2A1, 1A1 and 2B31 successfully conjugated 4-MU at 50 μM, 100 μM and 800 μM (Table 3). Low concentrations of conjugated 4-MU were produced by equine UGT2B4 at 800 μM. Uninfected Triex cells did not conjugate 4-MU to 4-MUG. When incubated with 400 μM testosterone, only microsomes were capable of producing testosterone-glucuronide (Table 4). Equine UGT2A1, human UGT2B7 supersomes and equine microsomes were able to produce diclofenac-glucuronide. Ketoprofen-glucuronide was produced in human UGT2B7 supersomes, equine liver microsomes and equine UGT1A1 incubations (Table 4). Both M3G and M6G were produced in incubations with human UGT2B7 supersomes, equine liver

microsomes and equine UGT2B31, although M6G at much lower concentrations than M3G (Table 4).

Metabolic assays

An initial screening assay was conducted to determine which enzymes were capable of metabolizing morphine. Human UGT2B7 supersomes, equine liver microsomes and the four expressed equine UGTs were incubated with morphine for 4 hours. In initial screening incubations, M3G and M6G were detected in equine liver microsomes, recombinant UGT2B31 and human UGT2B7 supersomes. None of the other equine recombinant UGTs conjugated morphine to form either M3G or M6G.

Kinetic assays were conducted in equine liver microsomes, equine UGT2B31 recombinant enzymes and human UGT2B7 supersomes (Fig. 4). Apparent K_m and V_{max} values for morphine metabolism to M3G and M6G are reported in Figure 3. UGT2B31 produced more M3G than the human UGT2B7 supersomes but less than the equine liver microsomes (Fig. 3). M6G production by UGT2B31 and human UGT2B7 supersomes was only seen at the highest concentrations (400 μM and 800 μM) of morphine (Fig. 3).

Discussion

In the current study a number of equine UGTs were cloned, expressed and characterized using compounds known to be UGT substrates in other species. This study is part of an ongoing effort to characterize equine drug metabolism and the first to describe phase II drug metabolism using recombinant UGT enzyme systems in the horse.

The nonspecific UGT probe 4-MU is glucuronidated to 4-MUG by a number of UGT enzymes (Uchaipichat et al. 2004; Lv et al. 2018). In the current study, all expressed recombinant enzymes, with the exception of UGT2B4, were able to metabolize 4-MU to 4-MUG at all substrate concentrations studied (50–800 μM). Only very low concentrations of 4-MUG were produced in incubations with UGT2B4 and only at the highest substrate concentration (800 μM), suggesting it is a low activity enzyme. Testosterone is another compound that is glucuronidated in many species, including horses (Sten et al. 2009). In the current study, testosterone-glucuronide was generated in equine liver microsomal incubations but not found when incubated with the expressed equine recombinant UGT enzymes. Whereas these findings would seem to suggest that testosterone glucuronidation is catalyzed by a UGT enzyme not expressed in the current study, it is important to note that only a single substrate concentration (400 μM) was studied; therefore it is also possible that the expressed enzymes may simply have a low affinity for testosterone.

Earlier reports using recombinant UGTs identified UGT2B7 as the major isoform responsible for the metabolism of morphine to M3G and M6G in humans (Coffman et al. 1997). Subsequently, Soars et al. (2003) suggested, based on sequence identity, that UGT2B31 may be the canine equivalent to human UGT2B7. The investigators also demonstrated that canine UGT2B31 was capable of generating the M3G metabolite in incubations with morphine (Soars et al. 2003). Of the equine UGTs expressed in the current study, only UGT2B31 metabolized morphine to M3G and M6G (albeit at low concentrations for the latter). Following initial screening assays, morphine was selected for further study and determination of V_{max} and K_m. Similar to findings in the initial screening assays, M3G was generated in both equine liver microsomes and recombinant equine UGT2B31 incubations. Notably, the K_m determined in the

UGT2B31 incubations was more than that observed in liver microsome incubations. More recent studies in humans suggest that whereas UGT2B7 may be the primary contributor to morphine glucuronidation, other UGT isoforms may also contribute to the glucuronidation of morphine, including UGT1A1, 1A3, 1A9, 1A10 and 2B4 (Court et al. 2003; Stone et al. 2003; Lv et al. 2018). Results of the current study (differing K_m values between UGT2B31 and liver microsome incubations) suggest that similar to humans, equine UGT2B31 is simply one enzyme contributing to generation of M3G in horses. As a number of other equine recombinant UGT enzymes were expressed in the current study, none of which were capable of morphine glucuronidation, it is possible that an as yet unidentified UGT enzyme contributes to morphine clearance.

Although glucuronidation is the major metabolic pathway for morphine elimination, in humans glucosidation contributes to the elimination of morphine through production of M3 glucoside in liver microsome incubations containing UDP-glucose (Chau et al. 2014). In the current study, similar incubations with equine liver microsomes and morphine in the presence of UDP-glucose did not generate detectable levels of M3 glucoside. These findings suggest that glucosidation does not contribute to the clearance of morphine in horses.

As described for other species, morphine is extensively glucuronidated *in vivo* to M3G and M6G following intravenous administration to horses (Knych et al. 2014; Hamamoto-Hardman et al. 2019). However, studies in horses have demonstrated that concentrations of M3G are approximately 10-fold higher than that observed in humans (Knych et al. 2014; Hamamoto-Hardman et al. 2019). Similar to *in vivo* observations, *in vitro* incubations containing morphine and recombinant UGT2B31 yielded higher concentrations of M3G than incubations containing morphine and human UGT2B7 supersomes.

A notable limitation in the current study was the inability to quantitate enzymatic activity. Although kinetic assays were normalized based on total protein quantification, knowledge of the amount of functional UGT would have provided more accurate characterization of enzymatic activity. Unfortunately, while there are assays available to quantify the amount of active enzyme for recombinant P450s, there are currently no commercially available or validated assays for quantitating UGT activity. Also in this study, recombinant equine UGTs expressed in Triex insect cells were compared with human UGT2B7 supersomes that were expressed in High-five insect cells. While the expression systems are similar, they are not identical; therefore, comparison of findings from human UGT2B7 supersome and the equine UGT2B31 incubations should be done with caution.

Conclusion

In the current study, the first functional equine recombinant UGTs were expressed. These enzymes were incubated with substrates for UGT enzymes in other species to test functionality. Additional enzyme kinetic studies were conducted with morphine to describe the kinetics of M3G and M6G (the two major metabolites produced *in vivo*) formation. Although it was determined that UGT2B31 contributes to the glucuronidation of morphine, probably it is not the main metabolizing enzyme. This study is a foundation for future studies describing phase II metabolism in horses.

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Figure 1 Amino acid alignment of uridine diphospho-glucuronosyltransferases (UGTs). Human UGT2B7 aligned to equine UGT2B31, UGT2B4, UGT2A1, and UGT1A1.

*Conserved amino acid residues among the UGT enzymes.

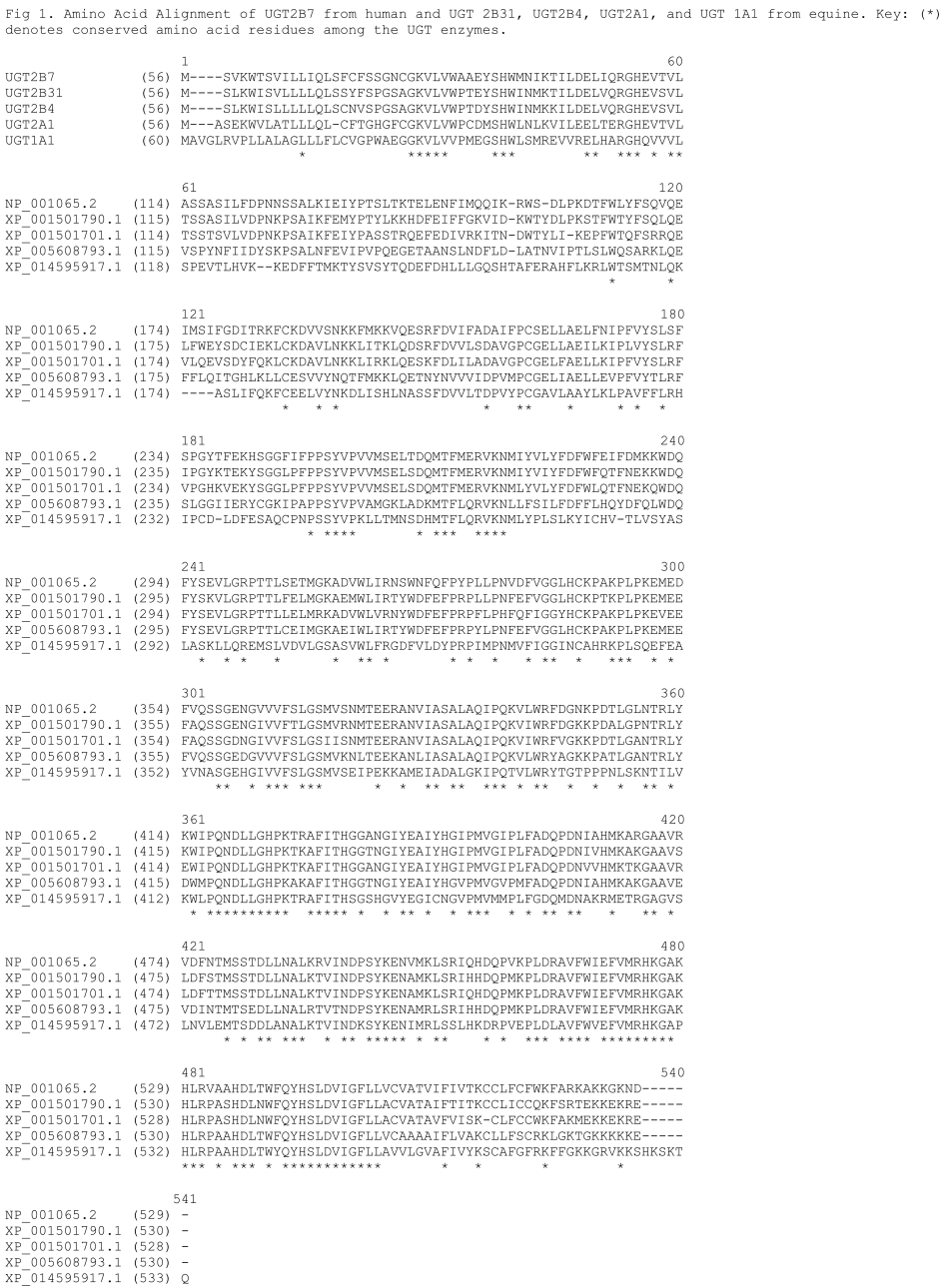

Figure 2 Western Blot of recombinantly expressed equine uridine diphospho-glucuronosyltransferases (UGTs) as well as human UGT2B7, equine liver microsomes and noninfected insect cells. *UGT2A1 harvested at 48 and 72 hours post infection.

Figure 2

*UGT2A1 was harvested at both 48 hours (*48h) and 72 hours (*72h) post infection

Figure 3 Kinetic plots for the determination of the apparent Km and Vmax values for morphine metabolism by equine liver microsomes to (a) morphine-3-glucuronide and (b) morphine-6-glucuronide. Incubations were performed in triplicate (open circle, closed circle, triangle).

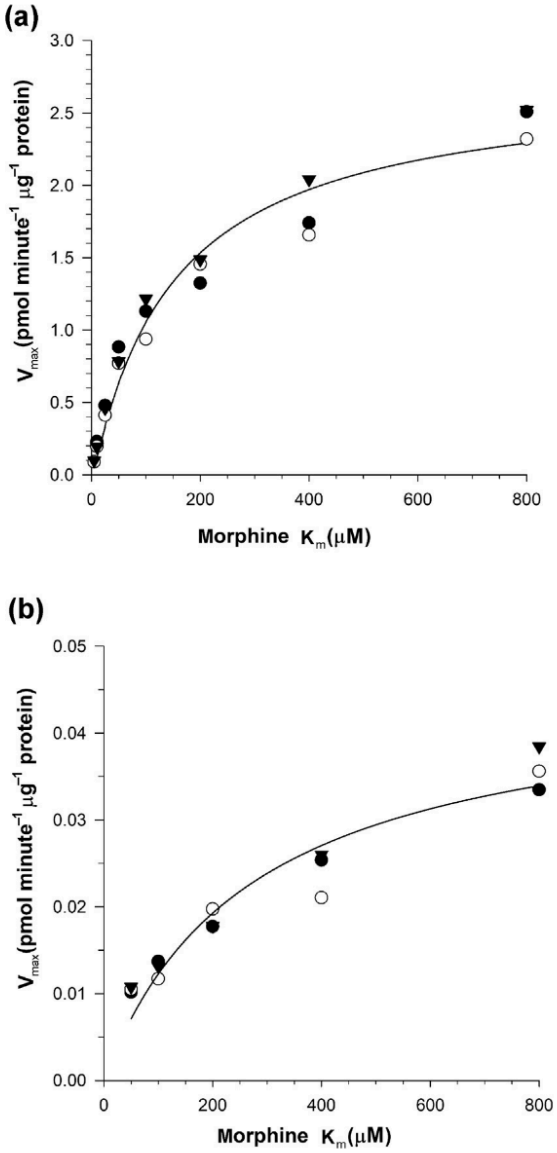

Figure 4 Kinetic plots for the determination of the apparent Km and Vmax values for morphine metabolism by equine recombinant UGT2B31 to (a) morphine-3-glucuronide and (b) morphine-6-glucuronide. Incubations were performed in triplicate (open circle, closed circle, triangle).

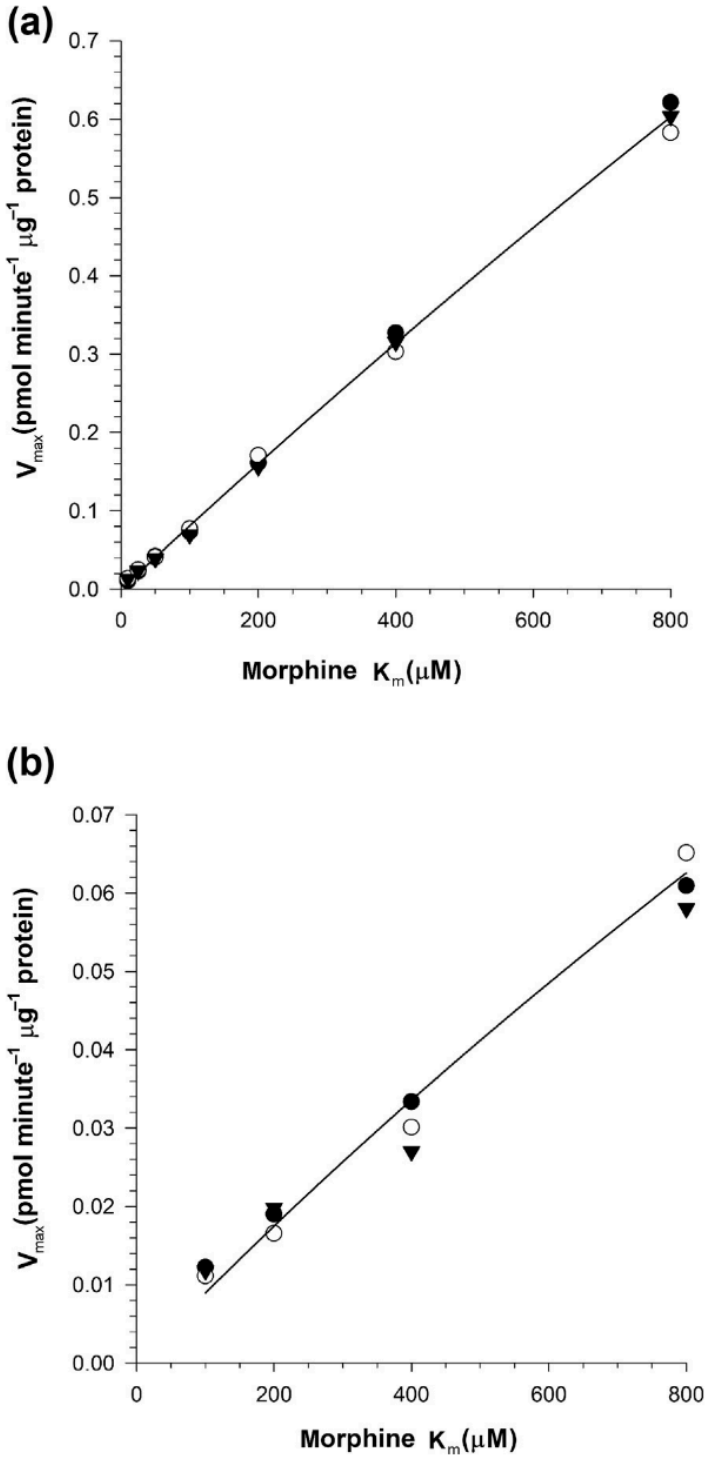

Table 1 Homology of recombinant equine uridine diphospho-glucuronosyltransferases (UGTs) to human UGT2B7.

UGT	Isoform	% Protein residue		
		Identical	Strongly similar	Total
2B31	LOC100066444	76	12	**88**
2B4	-	73	15	**88**
2A	X2	63	17	**80**
1A	X2	44	23	**67**

Table 2 Primers used for expression of equine recombinant uridine diphospho-glucuronosyltransferases (UGTs).

Enzyme	Forward Primer	Reverse Primer
UGT2B31	5'-GGTAGGCCTATGTCTCTGAAATGGATT TCAGTTCTTCTGCTG-3'	5'-GGTCTCGAGCTACTCCTTTTTTCCTTCTTTTCTGTTCTAGAAAAC-3'
UGT2B4	5'-GGTGAATCCACATGTCTCTGAAATGGATTTCACTTCTGC-3'	5'-GGTCTCGAGCTACTCCCTTTTTTCCTTCTTTTCCATTTTTGC-3'
UGT2A1	5'-GGTGGATCCATGGCGTCTGAGAAATGGG-3'	5'GGTCTCGAGGATTGCCCTTTCAGGCCT-3'
UGT1A1	5'-GGTGGATCCAGATGGCTGTGGGACTCCG-3'	5'-GGTCTCGAGTGGTTCACTTCCCACCCACTTC-3'

Table 3 Concentrations (mean ± standard deviation) of 4-methylumbelliferone glucuronide (4-MUG) following incubation of 4-methylumbelliferone (4-MU) with equine recombinant uridine diphospho-glucuronosyltransferases (UGTs), equine microsomes and control cells (uninfected Triex cells).

Enzyme	Concentration of 4-MU (μM)	4-MUG Metabolite (ng mL^{-1})
Human UGT2B7	0	ND
	50	2.8 ± 0.29
	100	6.8 ± 0.29
	800	167.7 ± 21.9
Equine liver microsomes	0	ND
	50	1360.1 ± 40.9
	100	2048.2 ± 86.8
	800	3493.8 ± 21.9
Equine UGT2B31	0	ND
	50	3.8 ± 0.07
	100	5.3 ± 0.09
	800	57.8 ± 4.76
Equine UGT2B4	0	ND
	50	ND
	100	ND
	800	2.6 ± 0.01
Equine UGT1A1	0	ND
	50	16.1 ± 0.46
	100	19.7 ± 2.03
	800	144.8 ± 18.3
Equine UGT2A1	0	ND
	50	4.6 ± 0.05
	100	8.2 ± 1.2
	800	69.6 ± 2.94
Uninfected Triex cells	0	ND
	50	ND
	100	ND
	800	ND

ND, not detected.

Table 4 Concentrations (ng mL^{-1}; mean ± standard deviation) of testosterone-glucuronide and diclofenac-glucuronide following incubation of 800 μM testosterone and diclofenac with equine recombinant uridine diphospho-glucuronosyltransferases (UGTs), equine microsomes and control cells (uninfected Triex cells).

Compound	Human UGT2B7	Equine liver microsomes	Equine UGT2B31	Equine UGT2B4	Equine UGT1A1	Equine UGT2A1	Uninfected Triex cells
Testosterone-glucuronide	ND	268 ± 12.2	ND	ND	ND	ND	ND
Diclofenac-glucuronide	859.3 ± 69.8	7904.7 ± 751.9	ND	ND	ND	455.7 ± 59.7	ND
Ketoprofen-glucuronide	405.6 ± 9.0	707.0 ± 11.3	ND	ND	242.0 ± 10.3	ND	ND
Morphine-3-glucuronide	6.7 ± 0.7	401.4 ± 44.8	69.8 ± 2.7	ND	ND	ND	ND
Morphine-6-glucuronide	2.60 ± 0.24	5.34 ± 0.60	2.76 ± 0.01	ND	ND	ND	ND

ND, not detected.

Table 5 Estimates of kinetic parameters for morphine-3-glucuronide and morphine-6-glucuronide formation in incubations with equine liver microsomes, equine UGT2B31 and human UGT2B7 supersomes.

Parameters	Liver microsomes	UGT2B31	UGT2B7
Morphine-3-glucuronide			
Best fit model	Michaelis-Menten	Michaelis-Menten	Michaelis-Menten
V_{max} (pmol minute^{-1} μg^{-1} protein)	2.90	8.55	0.27
K_m (μM)	152.7	14,233.9	3080.6
Morphine-6-glucuronide			
Best fit model	Michaelis-Menten	NA	NA
V_{max} (pmol minute^{-1} μg^{-1} protein)	0.05	NA	NA
K_m (μM)	110.3	NA	NA

V_{max} = maximal rate of velocity
K_m = substrate concentration at 1/2 the maximum velocity