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# Characterization of the *in vitro* CYP450 mediated metabolism of the polymorphic CYP2D6 probe drug codeine in horses

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ABSTRACT

Despite their widespread popularity as sport and companion animals and published and anecdotal reports of vast difference in drug disposition and pharmacokinetics between individuals, studies describing equine drug metabolism are limited. It has been theorized that similar to humans, members of the CYP2D family in horses may be polymorphic in nature leading to differences in metabolism of substrates. This study aims to build on the limited current knowledge regarding P450 mediated metabolism in horses by describing the metabolism of the polymorphic CYP2D6 probe drug codeine *in vitro*. Codeine, at varying substrate concentrations, was incubated with equine liver microsomes ( ± UDPGA) and a panel of baculovirus expressed recombinant equine P450s. Parent drug and metabolite concentrations were determined using LC-MS/MS. Incubation of codeine in equine liver microsomes generated norcodeine, morphine, codeine glucuronide and morphine 3- and 6- glucuronide. In recombinant P450 assays, the newly described CYP2D82 was responsible for catalyzing the biotransformation of codeine to morphine ($K_m$ of 247.4 μM and a $V_{max}$ of 1.6 pmol/min/pmol P450). CYP2D82 is 80% homologous to the highly polymorphic CYP2D6 enzyme, which is responsible for biotransformation of codeine to morphine in humans. CYP3A95, which shares 79% sequence homology with human CYP3A4 and CYP2D50 catalyzed the conversion of codeine to norcodeine ($K_m$ of 104.1 and 526.9 μM, $V_{max}$ of 2.8 and 2.6 pmol/min/pmol P450). In addition to describing the P450 mediated metabolism of codeine, the current study offers a candidate probe drug that could be used *in vivo* to study the functional implications of polymorphisms in the CYP2D gene in horses.

## 1. Introduction

Codeine is an opioid and a naturally occurring alkaloid used in human medicine for relief of mild to moderate pain, as a cough suppressant and as an anti-diarrheal. In most species, codeine undergoes extensive biotransformation with only 2–7% of the administered dose being excreted as the parent compound [1]. In humans, 5 different metabolites have been reported *in vivo*, including codeine 6-glucuronide (81%), norcodeine (2.2%), morphine (0.56%), morphine 3-glucuronide (2.1%) and morphine 6-glucuronide (0.8%) [2]. The analgesic effects of codeine have been largely attributed to metabolism to morphine, with morphine reportedly ten times more potent than the parent compound [3].

In humans, codeine is metabolized by the CYP450 enzyme, CYP2D6 [4–6]. While constituting only about 2–4% of the total P450 content in the liver, CYP2D6 is responsible for the metabolism of 25–30% of all therapeutic drugs [7]. CYP2D6 is highly polymorphic and notable differences in the biotransformation of codeine to morphine in individuals identified as poor and ultra-rapid metabolizers have been reported [2]. Following codeine ingestion, poor metabolizers have notably lower concentrations of morphine while ultra-rapid metabolizers have measurably higher concentrations that can ultimately result in serious toxicity, including death [2]. While to the best of the authors' knowledge, the metabolism of codeine in the horse has not been studied, it has been theorized, following administration of other proposed CYP2D substrates in horses, that mutations in the equine CYP2D orthologue may also lead to notable differences in the metabolism of CYP2D substrates in horses [8]. Additional CYP2D probe drugs would allow for further characterization of the effects of polymorphisms in the gene that codes for this enzyme in *in vivo* studies. In the current study we hypothesized that, similar to humans, codeine is metabolized to morphine by equine CYP2D enzymes. To that end, the goals of the current study were to (1) add to the limited knowledge of P450-mediated drug metabolism in the horse by characterizing the *in vitro* metabolic profile of codeine and (2) to determine the specific enzymes responsible for the metabolism of codeine, thereby potentially providing data supporting

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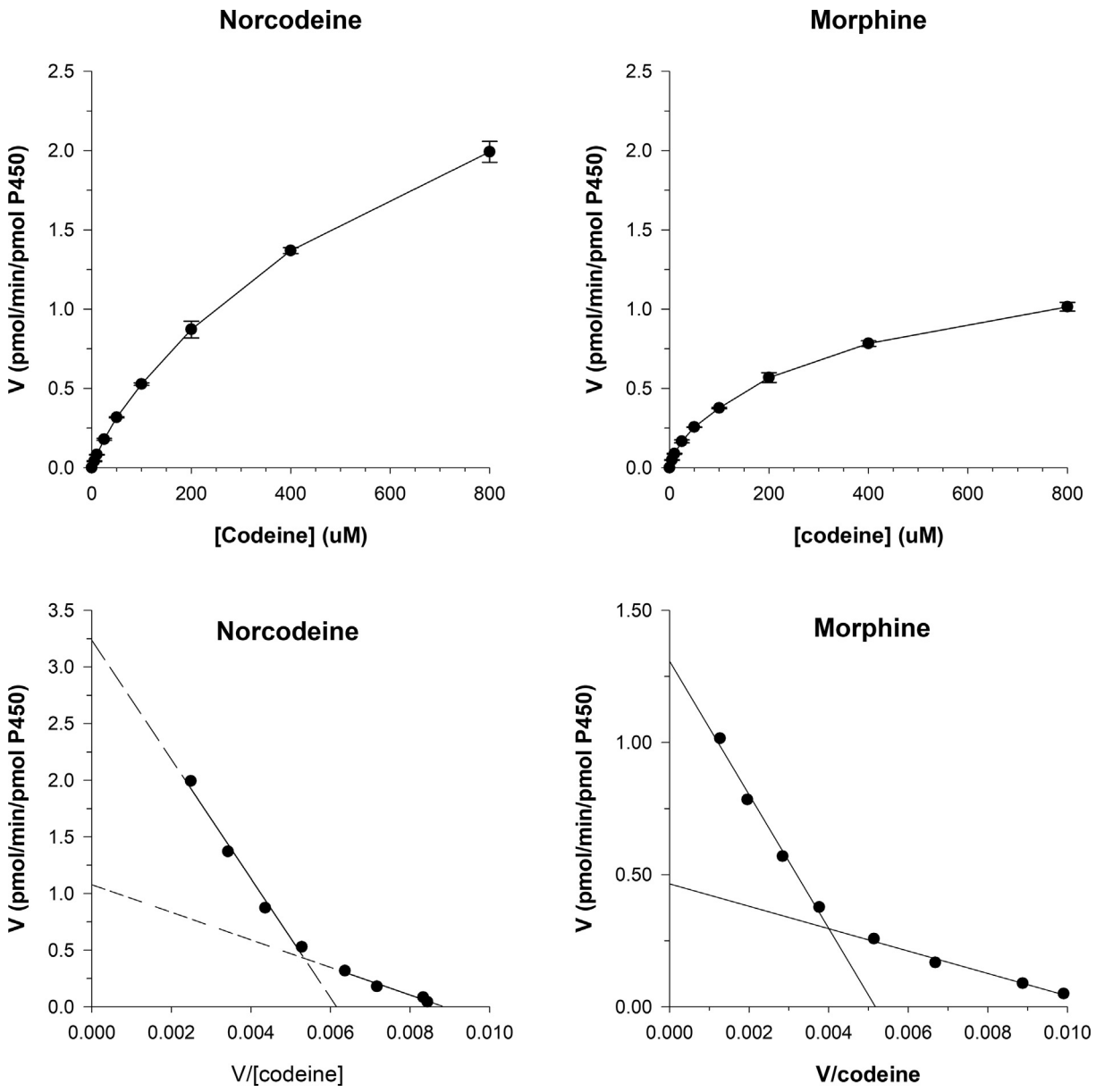


**Fig. 1.** Michaelis-Menten (top) and Eadie Hofstee (bottom) plots for the determination of the apparent $K_m$ and $V_{max}$ values for codeine metabolism to norcodeine and morphine by equine liver microsomes. Values on the Michaelis-Menten plot represent the mean ( ± SD) data from 3 separate incubations at each substrate concentration.

**Table 1**
Estimates of kinetic parameters for norcodeine formation in incubations with equine liver microsomes and several recombinant equine P450s.

| | | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol P450) | $CL_{int}$ |
|---|---|---|---|---|
| Liver microsomes | Enzyme #1 | 926.6 | 3.0 | 0.003 |
| | Enzyme #2 | 196.2 | 1.5 | 0.008 |
| | + **Quinidine** | | | |
| | Enzyme #1 | 946.4 | 2.8 | 0.003 |
| | Enzyme #2 | 284.5 | 1.6 | 0.006 |
| CYP2D82 | | 129.2 | 0.22 | 0.002 |
| | + **Quinidine** | 247.4 | 1.6 | 0.006 |
| CYP3A95 | | 104.1 | 2.8 | 0.027 |
| CYP3A93 | | 381.7 | 0.24 | 0.001 |
| CYP3A94 | | 308.1 | 0.64 | 0.002 |
| CYP2D50 | | 526.9 | 2.6 | 0.005 |

the use of codeine as a probe drug for *in vivo* screening to identify horses that are poor and ultra-rapid metabolizers.

## 2. Materials and methods

### 2.1. Chemicals

Saline (0.9%), Tris-HCl, potassium chloride, potassium phosphate, EDTA, glycerol, dithiothreitol, PMSF, NADPH, CHAPS, cytochrome *c*, cytochrome b5, codeine, quinidine, 5-aminolevulinic acid, ferric citrate and UDPGA were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile and water were purchased from Burdick & Jackson (Muskegon, MS). Methanol was purchased from Thermo Fisher Scientific (Fairlawn, NJ). All solvents were HPLC grade or better.

### 2.2. Preparation of liver microsomes

The liver sample used in the preparation of microsomes was

```
                1                                                          60
CYP2D82     (1) MGLLTGETLGPLAVAVAIFLLLVDLMHRRQRWAPRYPPGPMPLPGLGNLLHVDFQDTVCS
CYP2D50     (1) MGLLTWDKLGPVAVAVAIFLLLVDLMHRRQRWAPRYPPGPMPLPGLGNLLQVDFQDTVSS
CYP2D14     (1) MGLLSGDTLGPLAVALLIFLLLLDLMHRRSRWAPRYPPGPTPLPVLGNLLQVDFEDPRPS
 CYP2D6     (1) MGL---EALVPLAVIVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVDFQNTPYC
                ***     * * **   ***** ****** *** ****** *** ***** ***

                61                                                         120
CYP2D82    (61) FTRLRRRFGDVFSLQLAWTPVVVLNGLAAMREALVHRGEDTSDRPPAVVYEHLGYGPHAE
CYP2D50    (61) FTRLRRRFGDVFSLQLAWTPVVVLNGLAAIREALVHRGEDTSDRPRVPVMEHLGFGPHAE
CYP2D14    (61) FNQLRRRFGNVFSLQQVWTPVVVLNGLAAVREALVYRSQDTADRPPPAVYEHLGYGPRAE
 CYP2D6    (58) FDQLRRRFGDVFSLQLAWTPVVVLNGLAAVREALVTHGEDTADRPPVPITQILGFGPRSQ
                *  ****** *****  ************ *****    ** ***       ** **

                121                                                        180
CYP2D82   (121) GVILARYGRAWREQRRFSLSTLRNFGLGKKSLEQWVTEEASCLCAAFDDQAGRPFSPDAL
CYP2D50   (121) GVVFARYGHTWREQRRFSVSTLRNFGLGKKSLEQWVTQEASYLCAVFADQGGRPFSPDAL
CYP2D14   (121) GVILARYGDAWAEQRRFSLTTLRNFGLGKKSLEQWVTEEASCSCAAFADQAGRPFSPMDL
 CYP2D6   (118) GVFLARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEAACLCAAFANHSGRPFRPNGL
                **  ****  * ******  **** ************ **   ** *    **** *  *

                181                                                        240
CYP2D82   (181) LNKAVTNVIASLTFGRRFEYNDPLFLKLLDLTEDLVKEESGFLRQVLEAIPVLLHIPGVA
CYP2D50   (181) LNKAVSNVIASLTFGRRFDYNDPHFLEILDLTEDILKEQSGFLPQVLNAIPMLLHIPGLV
CYP2D14   (181) LNKAVSNVIASLTFGCRFEYNDPRIIKLLDLTEDGLKEEPNLVRKVVEAVPVLLSIPGLA
 CYP2D6   (178) LDKAVSNVIASLTCGRRFEYDDPRFLRLLDLAQEGLKEESGFLREVLNAVPVLLHIPALA
                * *** ******* * ** * **     ***     **       *  * * ** **

                241                                                        300
CYP2D82   (241) AKVFPGQRAFMAQLDELLAEHRMTRDPTQPPRDLTDAFLDEVAKAKGSPESSFSDDNLRL
CYP2D50   (241) AKVFPGQRAFMAQLDELVAERRMTRDPAQPPRDLTDAFLDEVQKAKGNPESSFNDDNLRL
CYP2D14   (241) ARVFPAQKAFMALIDELIAEQKMTRDPTQPPRHLTDAFLDEVKEAKGNPESSFNDENLRL
 CYP2D6   (238) GKVLRFQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLRI
                  *   * **    ***  *  ** ** **** ** *** *   *** ***** * ***

                301                                                        360
CYP2D82   (301) VVADLFTAGMVTTSTTLAWALLLMILHPDVQRRVQQEVDEVIGQARRPEMGDQACMPFTM
CYP2D50   (301) VVSDLFAAGMVTTSTALAWALLLMILHPDVQRRVQQEIDEVIGQARRPEMGDQARMPFTM
CYP2D14   (301) VVADLFSAGMVTTSTTLAWALLLMILHPDVQRRVQQEIDEVIGQVRRPEMGDQALMPFTV
 CYP2D6   (298) VVADLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQVRRPEMGDQAHMPYTT
                ** *** ******** *** ***************** * **** ********* ** *

                361                                                        420
CYP2D82   (361) AVVHEVQRFGDIIPLGLTHMTSRDIEVQGFLIPKGTTLITNLSSVLKDETVWKKPFRFHP
CYP2D50   (361) AVVHEVQRFGDIAPVGAPHMTSRDIEVQGFLIPKGTTLIPNLSSVLKDETVWKKPFRFHP
CYP2D14   (361) AVVHEVQRFADIVPLGLPHMTSRDIEVQGFHIPKGTTLITNLSSVLKDETVWEKPFRFHP
 CYP2D6   (358) AVIHEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNLSSVLKDEAVWEKPFRFHP
                ** ****** ** * *  ************ ******** ********* ** *******

                421                                                        480
CYP2D82   (421) EHFLDAQGRFVKQEAFMPFSAGRRSCLGEPLARMELFLFFTCLLQRFSFSVPAGQPRPSD
CYP2D50   (421) EHFLDAQGRFVKQEAFMPFSAGRRSCLGEPLARMELFLFFTCLLQRFSFSVPAGQPRPSD
CYP2D14   (421) EHFLDAQGRFVKQEAFIPFSAGRRACLGEPLARMELFLFFTSLLQHFSFSVPAGQPRPSE
 CYP2D6   (418) EHFLDAQGHFVKPEAFLPFSAGRRACLGEPLARMELFLFFTSLLQHFSFSVPTGQPRPSH
                ******** *** *** ******* ***************** *** ****** ******

                481              500
CYP2D82   (481) HGVFGVLVTPSPYQLCAEPR
CYP2D50   (481) HGVFGTLVSPSPYQLCAEPR
CYP2D14   (481) HGVFAFLVTPAPYQLCAVPR
 CYP2D6   (478) HGVFAFLVSPSPYELCAVPR
                ****  ** * ** *** **
```

**Fig. 2.** Amino acid alignment of CYP2D82, CYP2D50, CYP2D14 and CYP2D6. Key: (*) denotes conserved amino acid residues among the CYP enzymes.

collected from an adult female Thoroughbred horse euthanized for a separate study previously approved by the Institutional Animal Care and Use Committee of the University of California at Davis. The horse was determined to be healthy by physical exam prior to euthanasia and had not received any therapeutic medications that had the potential to affect the expression of metabolic enzymes. The liver sample (100–200 g) was collected within 20 min of the horse being euthanized and placed in ice cold 0.9% saline for transport back to the laboratory.

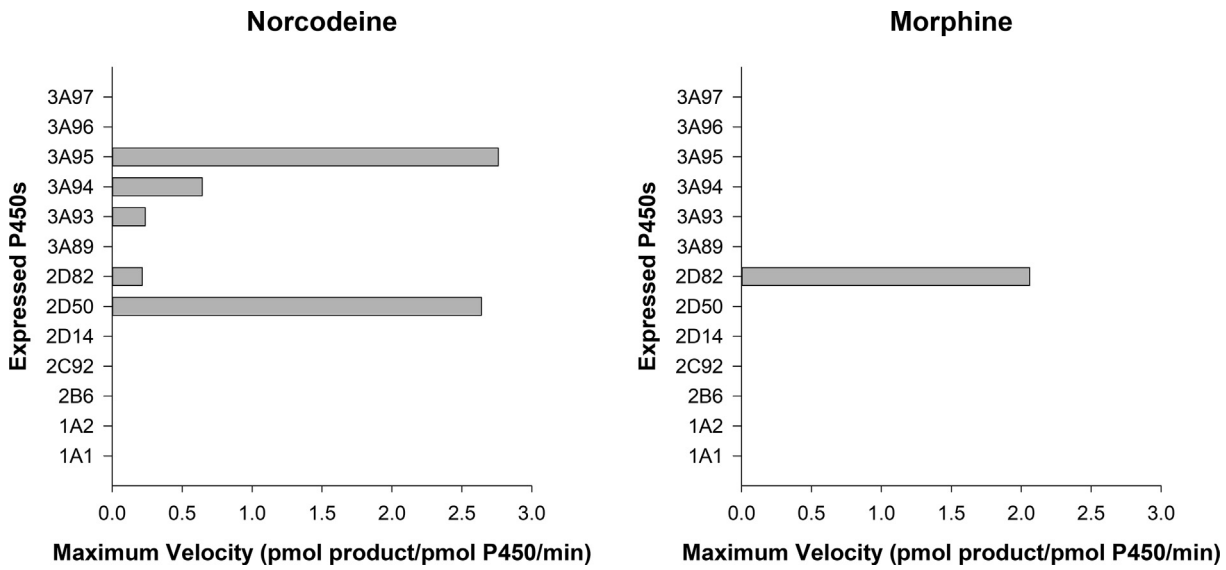


**Fig. 3.** Comparison of the rates of norcodeine and morphine formation (per pmol of P450) by baculovirus expressed P450 enzymes. The maximum velocity of the reactions ($V_{max}$) was calculated using the mean values of 3 incubations at each substrate concentration.

The liver sample was rinsed and flushed with ice cold 0.9% saline using visible arteries, veins or ducts. The sample was blotted dry, weighed, chopped into small pieces and placed in an ice-cold blender. The sample was subsequently homogenized by pulse blending several times, followed by continuous blending on the lowest setting for approximately 20 s, in 3 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM KCl and 2 mM EDTA. The mixture was further homogenized with a motor-driven Teflon/glass tissue grinder. The sample was then subject to a series of differential centrifugation steps. The mixture was first centrifuged at 9000 × *g* for 20 min followed by further centrifugation at 100,000 × *g* for 60 min, following removal of the supernatant and resuspension in the Tris-HCl buffer described above. This centrifugation step was repeated one additional time and the final pellet resuspended by hand, using a teflon/glass homogenizer, in 100 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 20% glycerol and 1 mM dithiothreitol. Protein content was determined using the bicinchoninic acid assay (BCA). P450 levels were determined by obtaining the difference spectra of sodium dithionate-reduced versus CO-bubbled samples at 500–400 nm according to the methods of Omura and Sato [9]. Microsomes were stored at −80 °C until used.

### 2.3. Microsomal incubations

Microsomal incubations were run in triplicate both with and without UDPGA so as to measure both P450 generated and glucuronidated metabolites. A set of "blank" incubations were run in parallel. These reaction mixes were as described below for the other incubations without the addition of NADPH. The incubations consisted of 1 mg/ml protein in 100 mM potassium phosphate buffer (pH 7.4) and 1 mM CHAPS in a total volume of 250 µl. All reactions (buffer + varying concentrations of codeine) were pre-incubated at 37 °C for 5 min prior to initiation of the reaction by the addition of 1 mM NADPH. Reactions were allowed to proceed for 20 min and were terminated by the addition of 250 µl of ice-cold acetonitrile. For reactions containing UDPGA, a final concentration of 5 mM of UDPGA was added to incubations. Codeine metabolism was measured under linear conditions using substrate concentrations ranging from 0 to 800 µM. Triplicate incubations were run at each substrate concentration. The velocity (V), calculated as pmol product/min/pmol of CYP450, at each substrate concentration was determined using the average value of the triplicate reactions. The data was evaluated by plotting V vs V/[Substrate] (Eadie Hofstee plot) and the intercepts calculated by linear regression analysis. Apparent $K_m$ and $V_{max}$ values were calculated from the Eadie Hofstee plot.

Additional incubations were conducted in the presence of the CYP2D6 inhibitor, quinidine (5 µM final concentration). Incubations and kinetic parameter calculations were as described above.

### 2.4. cDNA expressed P450

The cDNA for equine CYP1A1, 1A2, 2B6, 2C92, 2D14, 2D50, 3A89, 3A93, 3A94, 3A95, 3A96 and 3A97 were obtained by amplification of total RNA prepared from equine liver samples and using primers generated from sequences obtained from GenBank. RNA was isolated from previously collected liver samples stored in RNA Later (Ambion, Foster City, CA) and stored at −20 °C. For RNA isolation, liver samples were homogenized in Trizol Reagent (Life Sciences, Carlsbad, CA) and total RNA isolated. The quality and concentration of total RNA was assessed by gel electrophoresis and absorbance of 260 nm, respectively. The PCR products were cloned into an expression vector (pFastBac1; Invitrogen, Carlsbad, CA) and sequences confirmed using a commercial sequencing facility (University of California Davis College of Biological Sciences, University of California DNA Sequencing Facility). An additional sequence designated as CYP2D14-like in GenBank was also amplified as described above using the forward primer sequence (5′- GGTGAATTC ATGGGACTGCTGACCGGG) and reverse (5′- GGTCTCGAGCTAGCGGG GCTCAGCGC). Upon sequencing, the deduced sequence was submitted to the P450 nomenclature committee for name designation and then submitted to GenBank (accession nos. **MK928327**).

The cDNA encoding the pFastBac1/P450 was site specifically transposed into the baculovirus bacmid as described in the manufacturer's protocol (Bac-to-Bac Expression System; Invitrogen, Carlsbad, CA) and the high molecular weight bacmid DNA purified from recombinant clones was used to transfect TriEx Sf9 cells (Millipore Sigma, Burlington, MA) for the production of viral stocks.

Cells grown as suspension cultures in serum-free medium supplemented with L-glutamine were used to optimize expression and produce active enzyme. Production of functional protein was performed using the optimal MOI in the presence of 200 µM 5-aminolevulinic acid and 20 µM ferric citrate. Cell lysates were prepared by pelleting cells at 800 × *g* for 5 min at 4 °C, washing with cold 0.1 M phosphate buffer, pH 7.4, followed by resuspension in 0.1 M phosphate buffer, pH 7.4, with 20% glycerol, 1 mM EDTA, 1 mM PMSF and 1:200 Protease Inhibitor Cocktail Set III; EDTA-free (MilliporeSigma, Burlington, MA). The resuspended pellet was then homogenized using a teflon/glass

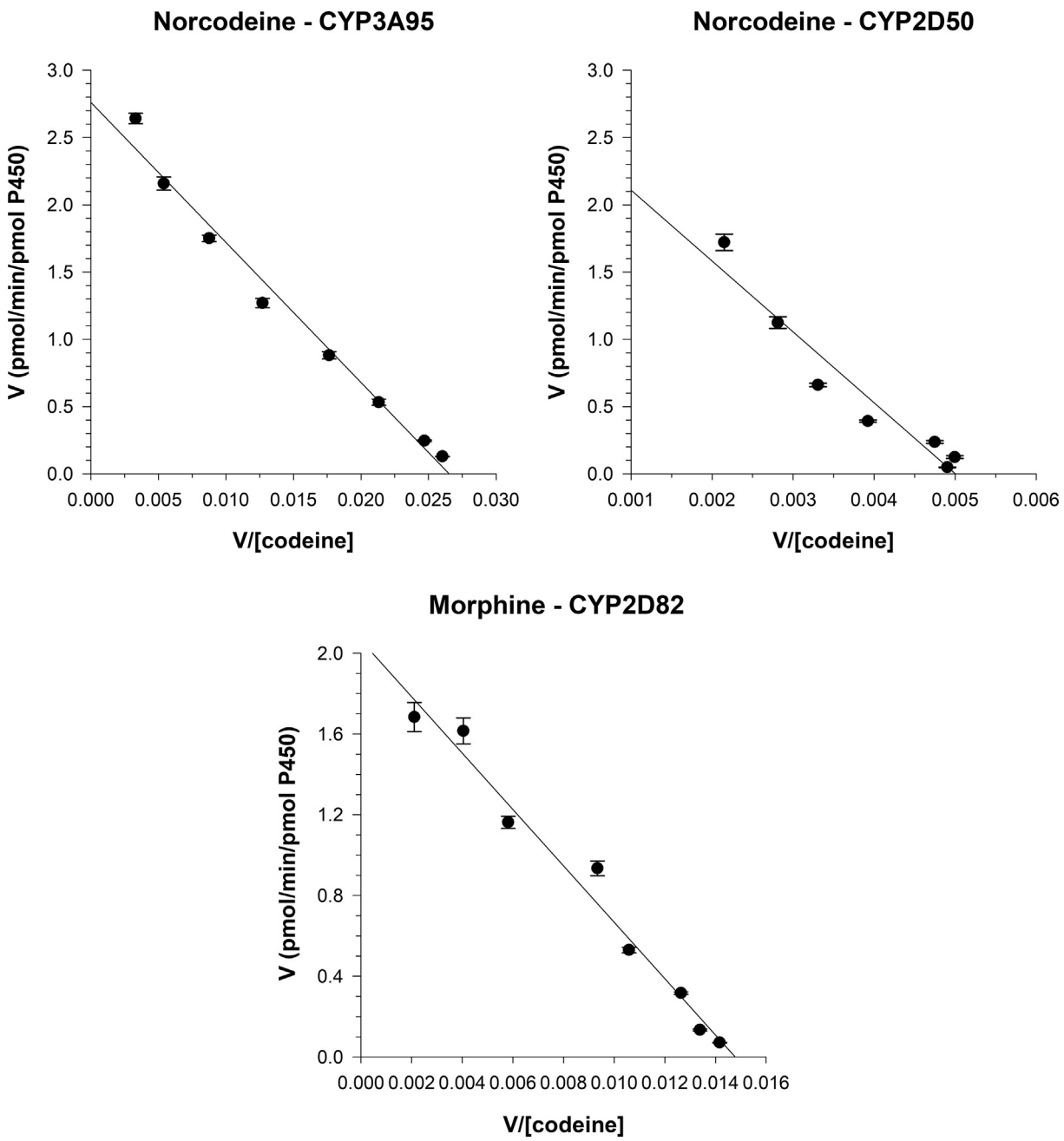


**Fig. 4.** Eadie Hofstee plot for the generation of norcodeine by baculovirus expressed CYP3A95 and CYP2D50 and morphine by baculovirus expressed CYP2D82 following incubation with codeine.

**Table 2**
Estimates of kinetic parameters for morphine formation in equine liver microsomes and CYP2D82.

| | | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol P450) | $CL_{int}$ |
|---|---|---|---|---|
| Liver microsomes | Enzyme #1 | 236.7 | 1.4 | 0.006 |
| | Enzyme #2 | 25.0 | 0.6 | 0.02 |
| | +**Quinidine** | | | |
| | Enzyme #1 | 401.0 | 1.4 | 0.003 |
| | Enzyme #2 | – | – | – |
| CYP2D82 | | 247.4 | 1.6 | 0.007 |
| | + **Quinidine** | 405.6 | 1.5 | 0.004 |

homogenizer and centrifuged at 800 × *g* for 5 min at 4 °C to pellet cellular debris, followed by subsequent centrifugation at 100,000 × *g* for 1 h at 4 °C of the resultant supernatant. The supernatant was discarded, and the pellet resuspended in phosphate storage buffer (0.1 M $KPO_4$, pH 7.4 containing 20% glycerol and 1 mM EDTA) and stored at −80 °C. P450 activity was determined by obtaining the difference spectra of sodium dithionate-reduced vs CO-bubbled samples at 500–400 nm [9].

### 2.5. Reductase expression

The cDNA for equine NADPH Cytochrome P450 reductase was obtained by amplification of total RNA prepared from equine liver samples as described above for recombinant P450s. The cDNA transcripts were generated by reverse transcription with the forward (5′-GGTGA ATTCATGGGGGACTCCAACATGGAC) and reverse (5′-GGTCTCGAGCT

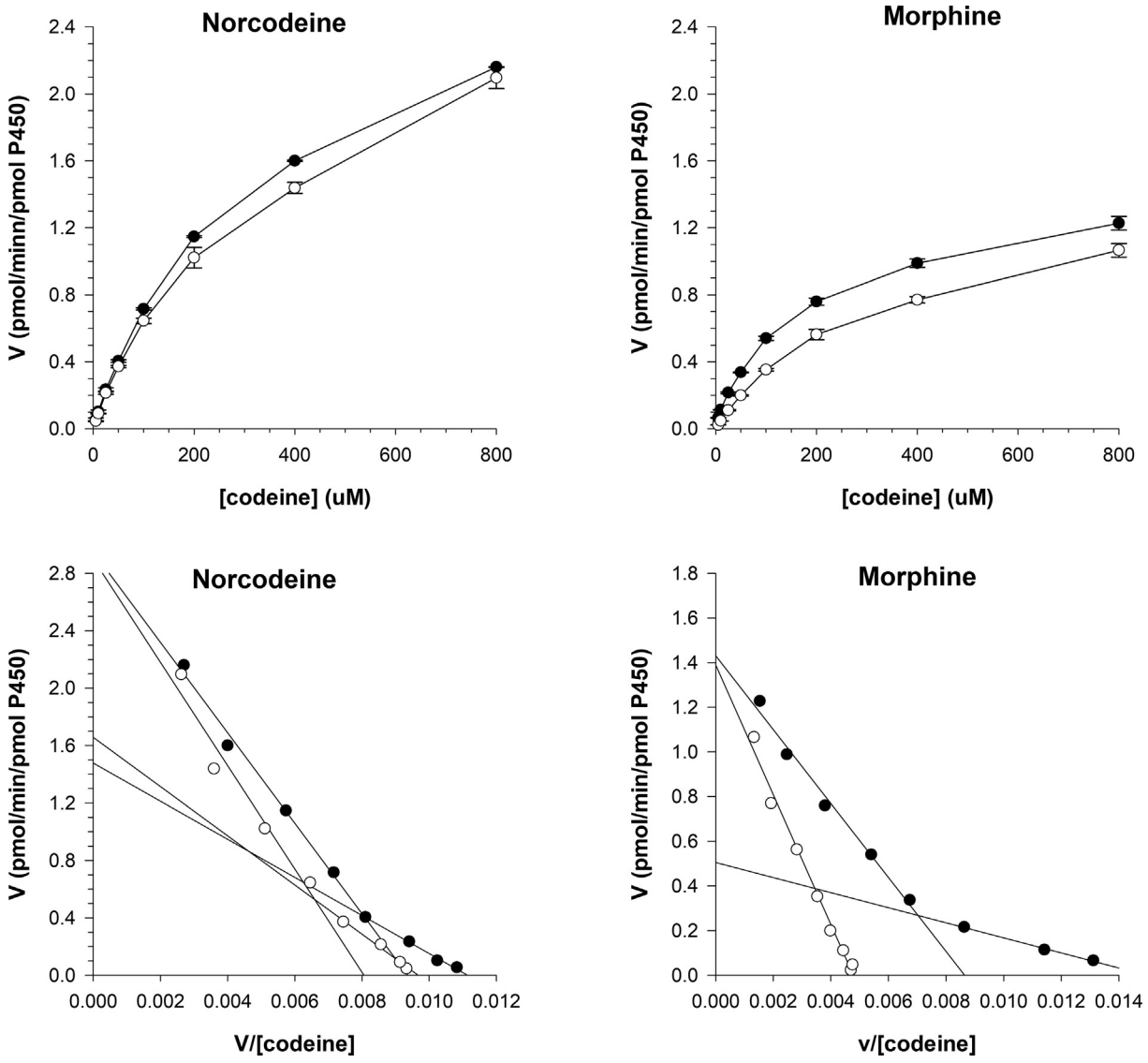


**Fig. 5.** Norcodeine and morphine formation kinetics in equine microsomal incubation studies in the in the absence (filled circles) and presence (open circles) of the CYP2D6 inhibitor, quinidine. Values on the Michaelis-Menten plot represent the mean ( ± SD) data from 3 separate incubations at each substrate concentration.

AGCTCCACACGTCCAGCGA) primers designed against the equine reductase sequence from GenBank (NM_001122655.1). The PCR products were cloned, sequences confirmed, and protein expressed as described above for P450s. Specific activity was determined by the cytochrome *c* reductase assay with one unit being defined as 1 μmol of cytochrome *c* reduced per minute.

### 2.6. Optimization of incubation conditions

Linearity of the assay with respect to time for recombinant P450 enzymes was established and all subsequent kinetic studies were conducted within the linear portion of the rate curve. Additionally, prior to performing kinetic studies, the optimal ratio of recombinant P450 to equine NADPH cytochrome P450 oxidoreductase and cytochrome b5, rHuman (where appropriate; Life Sciences, Carlsbad, CA) was determined. Optimization assays were conducted by adding increasing quantities of equine NADPH cytochrome P450 oxidoreductase to incubations containing recombinant P450, 1 mM CHAPS, 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH and 800 μM codeine in a total volume of 250 μl. The mixture was pre-incubated for 5 min in a 37 °C shaking water bath prior to the addition of recombinant P450. Incubation reactions were terminated by the addition of ice-cold acetonitrile following a 30-minute incubation.

### 2.7. Enzyme activity and kinetics

Codeine metabolism by recombinant P450s was determined in 250 μl reaction volumes, including NADPH CYP450 reductase (amount determined in optimization reactions with individual P450s), cytochrome b5, rHuman (where appropriate), 1 mM CHAPS, 1 mM NADPH, 100 mM potassium phosphate buffer (pH 7.4) and varying codeine concentrations. All reactions were incubated at 37 °C for 5 min prior to initiation of the reaction by the addition of 2.5 pmol of the recombinant P450 and then allowed to proceed for 30 min. Blank samples consisted of uninfected TriEx cell homogenate in place of recombinant P450. Reactions were subsequently terminated by the addition of 250 μl of ice-cold acetonitrile. The rate of the reaction was measured under linear conditions, Eadie Hofstee plots created and Km and Vmax calculated as described previously for liver microsomes. Additional incubations with the CYP2D6 inhibitor, quinidine, and CYP2D82 were conducted as described above for other recombinant P450s.

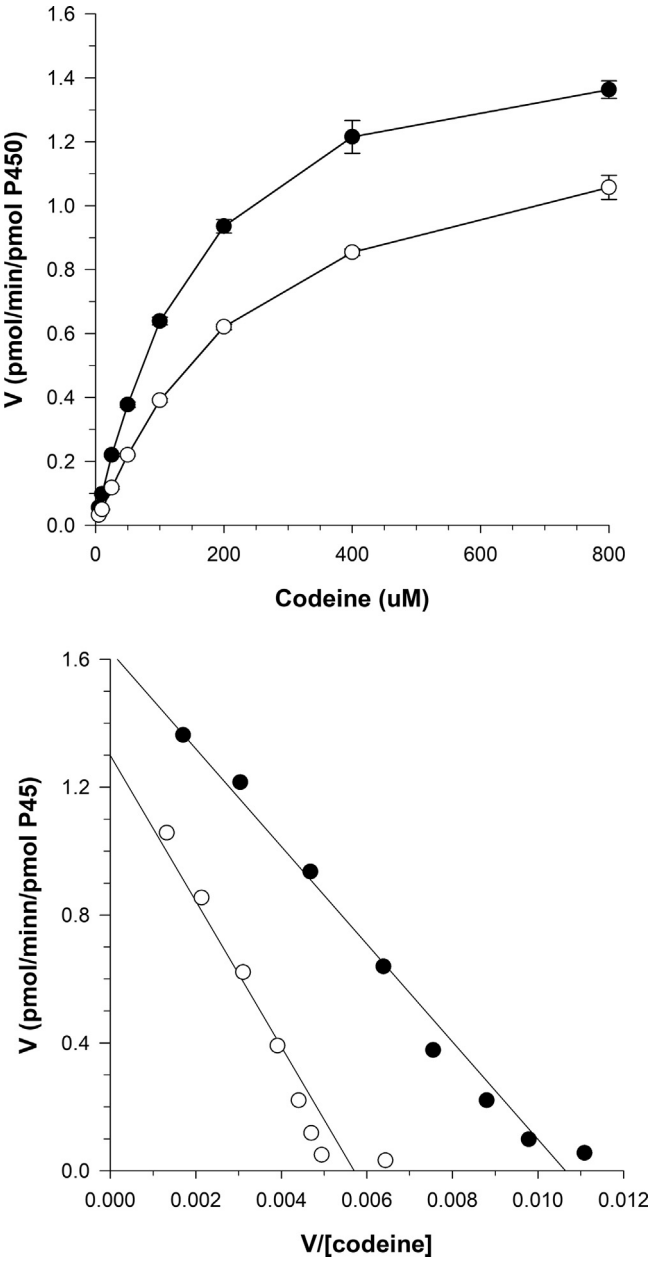


**Fig. 6.** Morphine formation kinetics in equine CYP2D82 incubation studies in the absence (filled circles) and presence (absence) of the CYP2D6 inhibitor, quinidine. Values on the Michaelis-Menten plot represent the mean ( ± SD) data from 3 separate incubations at each substrate concentration.

### *2.8. Determination of codeine and metabolite concentrations in liver microsomal and recombinant P450 reactions*

The analytical reference standards for codeine, codeine-6β-D-glucuronide, morphine, morphine-3β-D-glucuronide, morphine-6β-D-glucuronide, and norcodeine were obtained from Cerilliant (Round Rock, TX). The six analytes were combined into one solution and and working solutions prepared by dilution of the 1 mg/mL or 0.1 mg/mL stock solutions with methanol to concentrations of 0.1, 1, and 10 ng/μL. Calibrators were prepared by dilution of the working standard solutions with 5% ACN in water, with 0.2% formic acid, to concentrations from 0.5 to 1000 ng/mL. Calibration curves were prepared fresh for each quantitative assay.

Quantitative analysis was performed on an LTQ XL Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) coupled with a Waters Acquity UPLC (Milford, MA). The system was operated at a resolution of 60,000 using positive electrospray ionization (ESI(+)). The spray voltage was set at 4300 V, sheath gas and auxillary gas were 40 and 10 respectively (arbitrary units), and capillary temperature was 350 °C. Chromatography employed an Eclipse-XDB-Phenyl 2x150mm, 5 μm column (Agilent Technologies, Inc., Santa Clara, CA) and a linear gradient of ACN in water with a constant 0.2% formic acid, at a flow rate of 0.4 ml $min^{-1}$. The initial ACN concentration was held at 3% for 0.2 min, ramped to 35% over 7.8 min, ramped to 80% over 0.2 min before re-equilibrating for 3.3 min at initial conditions.

Detection and quantification were conducted using full scan accurate mass from 150 to 575 *(m/z)*, with a resolution of 60,000. The responses were plotted using a 20 ppm data window for the ions: morphine (mass to charge ratio 286.14384 *(m/z)*), M3G (462.17566 *(m/z)*), M6G (462.17566 *(m/z)*), C6G (476.19147 *(m/z)*), codeine (300.15917 *(m/z)*), and norcodeine (286.14377 *(m/z)*). 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 ($R^2$) of 0.99 or better. The technique was optimized to provide a limit of quantitation of 0.5 ng/mL for all analytes except morphine-6β-D-glucuronide which was 1 ng/mL. The limit of detection was approximately 0.1 ng/mL for all analytes, and 0.5 ng/mL morphine-6β-D-glucuronide.

## 3. Results

### *3.1. Kinetics of codeine metabolism in equine liver microsomes*

Incubation of codeine with equine liver microsomes generated the metabolites norcodeine and morphine. In addition to the phase 1 metabolites listed above, the glucuronidated metabolites, codeine 6-glucuronide and morphine 3-glucuronide were observed in microsomal incubations containing UDPGA. The Michaelis-Menton and Eadie-Hofstee plots for norcodeine and morphine generated in the microsomal incubations are depicted in Fig. 1. Examination of Eadie-Hofstee plots and subsequent calculation of a $K_m$ and $V_{max}$ (Table 1) for both norcodeine and morphine revealed biphasic plots suggesting that 2 enzymes (high affinity, low $K_m$) and low affinity (high $K_m$), are involved in both reactions.

### *3.2. Cloning and sequencing of equine CYP2D82*

The sequence amplified using primers generated based on the sequence for CYP2D14-like was submitted to the CYP450 Nomenclature Committee and was given the designation CYP2D82. The sequence showed a homology of 94% to CYP2D14, 89% to equine CYP2D50 and 80% to human CYP2D6 (Fig. 2).

### *3.3. cDNA expressed equine P450s*

The major phase 1 metabolites identified in equine microsomal incubations with codeine were morphine and norcodeine. Recombinant baculovirus-expressed equine P450s were used to examine which P450 isoforms were responsible for generating each metabolite. The oxidative activity of individual equine P450s in forming morphine and norcodeine, normalized for P450 content, are shown in Fig. 3. CYP3A95 and CYP2D50 demonstrated the highest rates for conversion of codeine to norcodeine. Several other P450s, including CYP2D82, CYP3A93 and CYP3A94 were capable of generating norcodeine but the velocity of the reaction was much slower. Of all the recombinant P450s tested, CYP2D82 was the only P450 that generated morphine in incubations with codeine. The Eadie-Hofstee plots for the major P450s are depicted in Fig. 4 and kinetic parameters listed in Tables 1 and 2.

### 3.4. Inhibition assays with the CYP2D6 inhibitor quinidine

In order to ascertain which of the 2 kinetic components reflected CYP2D mediated metabolism in microsomal incubations, additional incubations with the CYP2D6 inhibitor, quinidine were included. Biphasic kinetics were observed for norcodeine generation in microsomal incubations including quinidine (Fig. 5). The $K_m$ for the high affinity (low $K_m$) enzyme increased in the presence of quinidine while $V_{max}$ remained similar to microsomal incubations without quinidine (Table 1). The low affinity (high $K_m$) enzyme appeared unaffected by the addition of quinidine to the microsomal incubations.

Microsomal incubations including quinidine demonstrated uni-enzyme kinetics with respect to morphine generation (Fig. 5) with the activity of the high affinity (low $K_m$), lower velocity enzyme completely inhibited. The activity of the low affinity (high $K_m$), high velocity enzyme persisted; however, the $K_m$ for morphine formation in the presence of quinidine increased compared to incubations without the CYP2D6 inhibitor while $V_{max}$ was similar between the 2 incubations (Table 1). Since CYP2D82 appeared to be the primary enzyme responsible for generation of morphine in recombinant incubations, additional CYP2D82 incubations in the presence of quinidine were conducted (Fig. 6). In these incubations, the $K_m$ and $V_{max}$ were similar to values generated in microsomal incubations with quinidine for the low affinity (high $K_m$), high velocity enzyme (Table 1).

## 4. Discussion

The current study describes the *in vitro* metabolism of codeine by hepatic microsomal enzymes and expressed P450s from horses, including identification of the specific P450 isoforms responsible for biotransformation. Similar to studies reported in humans [10,11], 2 phase 1 metabolites, norcodeine and morphine, were generated in microsomal and recombinant P450 incubations. Norcodeine and morphine are also the major phase 1 metabolites observed in *in vivo* pharmacokinetic studies in horses (anecdotal report).

Knowledge of the specific enzyme(s) responsible for metabolizing therapeutic compounds is important in the prediction of drug-drug interactions. Concurrent administration of compounds that rely on the same enzymes for metabolism and ultimately drug clearance has the potential to alter the clearance of one or all concurrently administered drugs. Tools for identifying specific enzymes include recombinantly expressed enzymes, microsomal incubations with known inhibitors of specific enzymes or microsomal incubations with inhibitory antibodies. To the best of the authors' knowledge, in contrast to the extensive knowledge base in human 2D polymorphisms and inhibitors only limited data are available in veterinary medicine to characterize potential inhibitors. Studies have been undertaken to determine the P450 inhibitory potential of different compounds in animal species. Furthermore, there are no commercially available or published studies describing the use of P450 inhibitory antibodies in animal species.

In the current study, baculovirus expressed recombinant equine P450 enzymes were utilized in an attempt to identify the specific isoforms responsible for codeine metabolism in the horse. The selection of specific P450s for expression and subsequent codeine incubations was based on the P450s known to metabolize the vast majority of therapeutic compounds in humans [12]. Similar to reports in humans, whereby a member of the CYP3A family is responsible for catalyzing the conversion of codeine to norcodeine, in the current study, a CYP3A enzyme (CYP3A95) appears to be a major contributor to this reaction in the horse. However, in contrast to reports in humans, a second P450, a member of the CYP2D family (CYP2D50) appears to be as efficient in carrying out this reaction in horses [2]. Of the recombinant equine enzymes studied, the newly assigned CYP2D82 was the only enzyme shown to generate morphine following incubation with codeine. Based on sequence homology, CYP2D82 is an orthologue to the human enzyme, CYP2D6 (80% homology) which in humans is responsible for catalyzing the reaction that generates morphine from codeine [4–6]. Despite being highly homologous to CYP2D82, CYP2D14 and CYP2D50 were not capable of generating morphine following incubation with codeine, suggesting that a high degree of sequence homology is not necessarily indicative of similar substrate specificity. Additionally, although microsomal incubations demonstrated biphasic kinetics, suggesting that 2 enzymes catalyze the codeine to morphine reaction, incubations with recombinant P450s, in addition to members of the CYP2D family described above, failed to identify the second enzyme responsible for this reaction.

P450 inhibition studies were subsequently conducted in an attempt to determine whether CYP2D82 was the high or low affinity enzyme identified in microsomal incubations. Selection of quinidine, an established CYP2D6 inhibitor was based on the high degree of sequence homology between the equine enzyme and CYP2D6. Additionally, it has been suggested that quinidine decreases the metabolic activity of the equine enzyme, CYP2D50, also highly homologous to CYP2D82, in equine microsomal incubations [13]. Kinetic parameters for the low affinity (high $K_m$), high velocity enzyme in microsomal incubations with quinidine were similar to those generated in CYP2D82 incubations with quinidine suggesting that CYP2D82 was the low affinity enzyme identified in microsomal incubations without quinidine. While the identity of the high affinity, low velocity enzyme observed in microsomal incubations was not determined in recombinant P450 incubations, it is interesting to note that this enzyme did not appear when quinidine was added to the microsomal incubations. Although a number of P450s were tested, including 3 members of the CYP2D family (CYP2D14, CYP2D50 and CYP2D82), it is possible that there are additional, as of yet unidentified members of the CYP2D family in the horse that are inhibited by the CYP2D6 inhibitor quinidine. It is also possible that this second enzyme is a member of a different P450 family altogether and that quinidine is not a specific CYP2D inhibitor.

In human medicine, there are countless reports describing polymorphisms, or sequence variations, in genes coding for CYP2D6 and the functional implications with respect to drug metabolism [14]. In the case of codeine and mutations in the CYP2D6 gene, increased production of morphine has been reported in individuals classified as ultra-rapid metabolizers which has led to toxicity and in some cases death. Experimentally, understanding the functional implications of these mutations requires administration of a probe drug (a known selective substrate for a particular enzyme) followed by determination of parent drug and metabolite concentrations at various time post administration [15]. As codeine appears to be highly selective for CYP2D82, this would appear to be a good probe drug candidate for identification/determination of the functional implications of mutations in the CYP2D82 gene in horses.

While this study adds to knowledge regarding drug metabolism in horses, it is important to note that there is much left to be investigated. In the current study, a well-established inhibitor of CYP2D6 was utilized and while data from a single previous study as well as the current study suggests that quinidine may be an effective inhibitor of members of the CYP2D6 family in horses, additional characterization of the inhibitory potential of this compound is necessary. Of equal importance in metabolic capabilities is the relative amounts of the individual P450 enzymes. While kinetic parameters are important in determining the metabolic capabilities of individual enzymes, the relative amount of each enzyme is also an important determinant of the overall contribution of individual enzymes to the clearance of a particular compound. This has yet to be determined in the horse as we are only beginning to identify and characterize individual P450 enzymes in this species.

In the current study, we describe the metabolic pathway for codeine in the horse, including identification of specific P450s involved. In addition, a new member of the CYP2D family, CYP2D82, which appears to be an orthologue to the highly polymorphic CYP2D6 enzyme in humans, was identified and its contribution to codeine metabolism elucidated. Based on the results of the presently reported study, much

like in humans, codeine may prove effective as an *in vivo* probe drug in identifying polymorphisms in genes that code for members of the CYP2D family.

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## Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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