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Complementary DNA cloning, functional expression and characterization of a novel cytochrome P450, CYP2D50, from equine liver

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ABSTRACT

Members of the CYP2D family constitute only about 2–4% of total hepatic CYP450s, however, they are responsible for the metabolism of 20–25% of commonly prescribed therapeutic compounds. CYP2D enzymes have been identified in a number of different species. However, vast differences in the metabolic activity of these enzymes have been well documented. In the horse, the presence of a member of the CYP2D family has been suggested from studies with equine liver microsomes, however its presence has not been definitively proven. In this study a cDNA encoding a novel CYP2D enzyme (CYP2D50) was cloned from equine liver and expressed in a baculovirus expression system. The nucleotide sequence of CYP2D50 was highly homologous to that of human CYP2D6 and therefore the activity of the enzyme was characterized using dextromethorphan and debrisoquine, two isoform selective substrates for the human orthologue. CYP2D50 displayed optimal catalytic activity with dextromethorphan using molar ratios of CYP2D50 to NADPH CYP450 reductase of 1:15. Although CYP2D50 and CYP2D6 shared significant sequence homology, there were striking differences in the catalytic activity between the two enzymes. CYP2D50 dextromethorphan-*O*-demethylase activity was nearly 180-fold slower than the human counterpart, CYP2D6. Similarly, rates of formation of 4-hydroxydebrisoquine activity were 50-fold slower for CYP2D50 compared to CYP2D6. The results of this study demonstrate substantial interspecies variability in metabolism of substrates by CYP2D orthologues in the horse and human and support the need to fully characterize this enzyme system in equids.

1. Introduction

Cytochrome P450 monooxygenases (CYP450s) are a superfamily of hemeproteins responsible for metabolizing a vast array of drugs, environmental pollutants and endogenous compounds. CYP450 enzymes enable a functional group to be introduced or unmasked in a compound, generating a more polar intermediate, which is more readily eliminated. CYP450s have been well characterized in many laboratory animal species as well as in humans. In humans, more than 50 individual CYP450s have been identified. Of these 50, only six have been shown to play a major role in metabolism and subsequent clearance of drugs. These include members of the CYP1, CYP2 and CYP3 families. CYP2D enzymes are particularly important in drug metabolism as they are responsible for metabolism of 20–25% of commonly prescribed drugs, including antiarrythmics, β-adrenoreceptor antagonists, neuroleptics and tricyclic antidepressants and because there are

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several functionally significant polymorphisms in the human CYP2D6 gene [1].

The most common variants of human CYP2D6 result in very high activity (as a result of gene duplication), in altered affinity for some substrates or in no activity which results from inactive splice variants or point mutations that result in an unstable product. Some of the variants have low penetrance in the human population (1–2%) while others such as some of the point mutations are present in more than half of Asians studied. The functional importance of these variants combined with the relatively high penetrance in the human population means that doses of those therapeutic agents with a narrow therapeutic index must be adjusted to obtain appropriate therapeutic levels. Polymorphic expression of CYP2D is of particular importance because of the wide array of therapeutic compounds metabolized by this enzyme. A decrease or complete inability to metabolize CYP2D6 substrates presents the potential for adverse drug reactions as a result of an inability to metabolize and clear the administered compound from the body.

Although there is considerable experimental evidence supporting the functional importance of CYP2D6 and its variants in both humans and animals used for human drug development, little is known about this protein in other species. Therapeutic agents used in equine medicine are those that are effective in other species and dose regimens are established on a trial and error basis. The studies reported here are part of a long term effort to systematically evaluate the metabolic capabilities of the drug metabolizing enzymes in equids.

Although there have been a limited number of studies using equine liver microsomes [2–4], these provide little information about individual equine CYP450s, specifically which enzymes are responsible for the metabolism of specific drugs. Human CYP450s have been well characterized and various *in-vitro* systems, such as recombinant cytochrome P450 enzymes expressed in *E. coli* or insect cells, have been developed to study the metabolism of therapeutic agents. To date, there are no reports of equine recombinant CYP450 enzymes.

Studies with equine liver microsomes have suggested the presence of a member of the CYP2D family in the horse, based on metabolic activity with the human isoform selective substrate dextromethorphan [2]. In this study, equine liver microsomes demonstrated more than 20 times the metabolic turnover of dextromethorphan as compared to human liver microsomes. However, the only definitive way to determine whether CYP2D is responsible for metabolism of dextromethorphan in the horse, or if there are multiple members of this family playing a role in its metabolism, is through the use of a recombinant enzyme. Accordingly, in this study, a member of the CYP2D family was cloned, sequenced, expressed and characterized with respect to human CYP2D6 selective substrates.

2. Materials and methods

2.1. Chemicals

Superscript III One Step RT-PCR Kit, Novex mini-gel system, 10% Bis-Tris precast gels, NuPage loading dye and NuPage transfer buffer (20×) were from Invitrogen (Carlsbad, CA). BsrGI restriction enzyme was purchased from New England Biosciences (Ipswich, MA). All sequencing primers were synthesized by IDT (Coralville, IA). TOPO and pDEST8 cloning vectors, Cellfectin reagent, *Spodoptera frugiperda* (Sf9) and High Five cells and human recombinant CYP2D6 were from Invitrogen (Carlsbad, CA). Kaleidoscope Prestained Standards were from Biorad (Hercules, CA). Human CYP2D6 antibody was purchased from ABCAM (Cambridge, MA) and Alexa Fluor secondary antibody was purchased from Invitrogen (Carlsbad, CA). NADPH, CHAPS, cytochrome *c*, dextromethorphan, debrisoquine and dextrorphan were obtained from Sigma (St. Louis, MO). 4-Hydroxydebrisoquine was purchased from US Biological (Swampscott, MS). 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. Animals

Liver samples were collected from two horses (one female Tennessee Walker and one male Paint Horse). Samples were collected from horses being euthanized for other studies previously approved by the Institutional Animal Care and Use Committee of the University of California at Davis. Both horses were determined to be healthy by physical examination prior to euthanasia.

2.3. RNA isolation

Liver samples were collected, cut into approximately 3 mm cubes and stored at −20 °C in RNA Later (Ambion, Foster City, CA) until processed. Sections weighing approximately 100 mg were cut into small pieces, homogenized directly in Trizol Reagent (Invitrogen, Carlsbad, CA) and total RNA isolated from each horse. The quality of total RNA was checked by gel electrophoresis. Concentrations were measured by absorbance at 260 nm.

2.4. Cloning of equine CYP2D50

The cDNA for equine CYP2D50 was obtained by amplification of total RNA prepared from liver samples from two individual horses. The cDNA transcripts were generated by reverse transcription using the Superscript III One Step RT-PCR kit with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA) with the forward (5′-CACCATGGGGCTGCT GACCTGG-3′) and reverse (5′-AGGACCTGATCCTGAGGAGGCTGCT-3′) primers designed against predicted CYP2D sequences from the equine genome project (GI:149743367). The PCR products were cloned into pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA) and the two DNA sequences confirmed using an automated sequencer (ABI 3730, Applied Biosystems, Foster City, CA). The deduced sequence was submitted to the P450 nomenclature committee for name designation and then to GenBank (EU190996).

2.5. Expression of CYP2D50 in insect cells

The cDNA insert encoding equine CYP2D50 was directionally cloned into the multiple cloning site of pDEST8 (Invitrogen,

Carlsbad, CA). The integrity and orientation of the pDEST8/2D50 clone was verified by forward sequencing. The cDNA encoding CYP2D50 from pDEST8/2D50 was site specifically transposed into the baculovirus genome as described in the manufacturer's protocol (Bac-to-Bac Expression System; Invitrogen, Carlsbad, CA). The high molecular weight genomic DNA was purified from recombinant clones and used to transfect Sf9 cells for the production of baculovirus stocks.

Baculovirus stocks were amplified in Sf9 cells and the viral titer was determined using the BacPAK Baculovirus Rapid Titer Kit (BD Biosciences Clontech; Palo, Alto, CA). High Five cells (Invitrogen, Carlsbad, CA), grown as monolayers in serum-free medium supplemented with L-glutamine were used to optimize expression and produce active enzyme. Multiplicity of infection (MOI) and time to collection of cells were determined by infecting at an MOI of 0.1, 1, 5 and 10 pfu/cell and using post infection harvest times of 24, 48 and 72 h. The product was verified by Western blot analysis. Production of functional protein was performed using the optimal MOI in the presence of 300 μM 5-aminolevulinic acid (ALA) and 200 μM ferric citrate (FC). Cell lysates were prepared by pelleting cells at 1000 × *g* for 5 min at 4 °C followed by resuspension in 0.1 M phosphate buffer, pH 7.4, with 0.25 M sucrose, 1 mM EDTA and 0.5 mM PMSF. The resuspended pellet was then centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was discarded and the pellet resuspended in phosphate storage buffer (0.1 M KPO_4, pH 7.4 containing 20% glycerol) and stored at −80 °C. P450 levels were determined by obtaining the difference spectra of sodium dithionate-reduced vs CO-bubbled samples at 500–400 nm according to the methods of Omura and Sato [5].

2.6. Reductase purification

NADPH cytochrome P450 reductase was purified from equine liver microsomes as described by Yasukochi and Masters [6]. Specific activity was determined by the cytochrome *c* reductase assay with one unit being defined as 1 μmol of cytochrome *c* reduced per minute.

2.7. Optimization of CYP2D50 metabolism

Earlier work has demonstrated the importance of optimizing conditions of incubation for studies examining the kinetics of substrate metabolism catalyzed by recombinant proteins [7]. Accordingly, prior to conducting enzyme kinetic studies, the ratio of recombinant CYP2D50 to equine NADPH cytochrome P450 oxidoreductase was determined by adding increasing quantities of equine NADPH cytochrome P450 oxidoreductase to the incubation. Incubations contained 5 pmol of recombinant CYP2D50, 1 mM CHAPS, 100 mM potassium phosphate buffer, pH 7.4 and 1 mM dextromethorphan in a total volume of 250 μl. Incubation vessels were incubated for 2 min in a 37 °C shaking water bath prior to the addition of 1 mM NADPH. Following an additional 15 min incubation, all reactions were terminated by the addition of ice cold acetonitrile. Reaction conditions for recombinant CYP2D6 were according to the manufacturer's protocol with the addition of 1 mM CHAPS. Linearity was established with protein and with time for equine and human recombinant enzymes and all subsequent kinetic studies were conducted within the linear portion of the rate curve.

2.8. Enzyme activity and kinetics

Dextromethorphan metabolism by equine recombinant CYP2D50 and equine liver microsomes was carried out in 250 μl reaction volumes. Recombinant assays included 2.5 pmol of recombinant CYP2D50 or CYP2D6, 37.5 pmol NADPH CYP450 reductase (rCYP2D50), 1 mM CHAPS, 100 mM potassium phosphate buffer (pH 7.4) and varying substrate concentrations. Microsomal incubations consisted of 1 mg/ml protein in 100 mM potassium phosphate buffer (pH 7.4). All reactions were incubated at 37 °C for 2 min prior to initiation of the reaction by the addition of 1 mM NADPH. Reactions were allowed to proceed for either 15 min (rCYP2D50 and rCYP2D6) or 5 min (liver microsomes). Reactions were terminated by the addition of 250 μl of acetonitrile containing levallorphan as the internal standard. Reaction rates were measured under linear conditions to obtain values for K_m or V_{max} using a varying number of substrate concentrations ranging from 0 to 400 μM (rCYP2D50 and rCYP2D6) or 0 to 1000 μM (liver microsomes). The data was evaluated by plotting velocity (*V*) vs *V*/[Substrate] (Eadie Hofstee plot) and the intercepts calculated by linear regression analysis. Debrisoquine hyroxylase activity measurements were carried out for 10 min with saturating substrate concentrations (1000 μM) and activity calculated as pmol product/min/pmol CYP450.

2.9. Sample analysis

The liquid chromatography–mass spectrometry (LC–MS) system consisted of an Aquity UPLC system (Waters, Milford, MA), an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and an HPLC column (HSS T3 2.1 mm × 50 mm, 1.8 μm, Waters, Milford, MA). UPLC was performed using a gradient of 3% B to 90% B over 6.0 min (solvent A, H_2O with 0.2% formic acid; solvent B, acetonitrile with 0.2% formic acid), followed by an immediate return to initial conditions that were maintained for 3.0 min with a flow rate of 400 μl/min. The UPLC gradient system started with 10% B and linearly increased to 80% B in 15 min, followed by an increase to 100% B in 3 min prior to column re-equilibration. All experimental data were acquired using external calibration 5 days prior to data acquisition, and all collected mass scan data were recorded in the centroid mode in order to minimize data file sizes.

2.9.1. MS of dextromethorphan and analogues – quadrupole-linear ion trap analyzer

High-resolution/accurate mass measurement of fragment ions of dextrophan in MS^2 and MS^3 experiments was accomplished using a solution at 10 μg/ml in acetonitrile:-water (1:1, v/v) with 0.2% formic acid. The protonated precursor ion at *m*/*z* 258 was isolated at a width of 1.5 u and dissociated at a normalized collision energy value of 35%. In the case of MS^3, the second precursor at *m*/*z* 201 was dissociated at 35% of the normalized collision energy. Thirty product ion mass spectra were recorded with the Orbitrap from 50 to 265 *m*/*z* at a resolution of 60,000.

2.9.2. MS of debrisoquine and analogues – quadrupole-linear ion trap analyzer

All reference standards were dissolved in methanol at concentrations of 1 mg/ml and analyzed by electrospray ionization (ESI) and collision-induced dissociation (CID) on a LTQ Orbitrap XL at room temperature. High-resolution/accurate mass measurement of fragment ions of 4-hydroxydebrisoquine in MS^2 and MS^3 experiments was accomplished using a solution at 10 μg/ml in acetonitrile:water (1:1, v/v) with 0.2% formic acid. The protonated precursor ion at *m/z* 192 was isolated at a width of 1.5 u and dissociated at a normalized collision energy value of 37%. In the case of MS^3, the second precursor at *m/z* 174 was dissociated at 35% of the normalized collision energy. Thirty product ion mass spectra were recorded with the Orbitrap from 50 to 200 *m/z* at a resolution of 60,000.

The calculation of chemical formulae from the accurate measurement of *m/z* values and data processing of EIC were carried out using the Qual Browser of Xcaliber 2.0.7 (Thermo Fisher Scientific, San Jose, CA). High-resolution EICs of dextromethorphan and debrisoquine metabolites were obtained by processing the full-scan MS data set using potential dextromethorphan and debrisoquine metabolite ions with 5 ppm mass tolerance. The potential dextromethorphan and debrisoquine metabolite ions were calculated from a default metabolite list in MetWorks 1.1 (Thermo Fisher Scientific).

3. Results

3.1. Cloning and sequencing of equine CYP2D50

RT-PCR products from two different horses were generated. The consensus sequence was 1503 base pairs. The sequence was submitted to the CYP450 Nomenclature Committee and was given the novel designation of CYP2D50. The deduced amino acid sequence of CYP2D50 (accession no. EU0190996) showed a homology of 80% to cattle CYP2D14 and CYP2D43. The amino acid sequence alignment of CYP2D50 with that of human CYP2D6 (Fig. 1) revealed that CYP2D50 is 77% homologous to CYP2D6.

```
                1                                                    60
2D50      (1)   MGLLTWDKLGPVAVAVAIFLLLVDLMHRRQRWAPRYPPGPMPLPGLGNLLQVDFQDTVSS
2D6       (1)   ---MGLEALVPLAVIVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVDFQNTPYC
                (1)        * **** ****************** ********** **** *

               61                                                   120
2D50     (61)   FTRLRRRFGDVFSLQLAWTPVVVLNGLAAIREALVHRGEDTSDRPRVPVMEHLGFGPHAE
2D6      (58)   FDQLRRRFGDVFSLQLAWTPVVVLNGLAAVREALVTHGEDTADRPPVPITQILGFGPRSQ
                (61) *  ********************************  ******** **    *****

              121                                                   180
2D50    (121)   GVVFARYGHTWREQRRFSVSTLRNFGLGKKSLEQWVTQEASYLCAVFADQGGRPFSPDAL
2D6     (118)   GVFLARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEAACLCAAFANHSGRPFRPNGL
                (121) **  ****  ************** ************ *** *** **   **** *  *

              181                                                   240
2D50    (181)   LNKAVSNVIASLTFGGRFDYNDPHFLEILDLTEDILKEQSGFLPQVLNAIPMLLHIPGLV
2D6     (178)   LDKAVSNVIASLTCGRRFEYDDPRFLRLLDLAQEGLKEESGFLREVLNAVPVLLHIPALA
                (181) * *********** * ** * ** ** ****    *** ****  **** * *******

              241                                                   300
2D50    (241)   AKVFPGQRAFMAQLDELVAERRMTRDPAQPPRDLTDAFLDEVQKAKGNPESSFNDDNLRL
2D6     (238)   GKVLRFQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLCI
                (241)  **   ****  *****  * *** ********** *** ** ***************

              301                                                   360
2D50    (301)   VVSDLFAAGMVTTSTALAWALLLMILHRDVQRRVQQEIDEVIGQARRPEMGDQARMPFTM
2D6     (298)   VVADLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQVRRPEMGDQAHMPYTT
                (301) ****** ******** *** ******* ***************** ********* ** *

              361                                                   420
2D50    (361)   AVVHEVQRFGDIAPVGAPHMTSRDIEVQGFLIPKGTTLITNLSSVLKDETVWKKPFRFHP
2D6     (358)   AVIHEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNLSSVLKDEAVWEKPFRFHP
                (361) ************ * *  ************ ****************** ** *******

              421                                                   480
2D50    (421)   EHFLDAQGRFVKQEAFMPFSAGRRSCLGEPLARMELFLFFTCLLQRFSFSVPAGQPRPSD
2D6     (418)   EHFLDAQGHFVKPEAFLPFSAGRRACLGEPLARMELFLFFTSLLQHFSFSVPTGQPRPSH
                (421) ******** *** *** ************************ *** ****** ******

              481             500
2D50    (481)   HGVFGTLVSPAPYQLCAEPR
2D6     (478)   HGVFAFLVTPSPYELCAVPR
                (481) ****  ** * ** *** **
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Fig. 1 – Amino acid alignment of CYP2D50 from equine and CYP2D6 from human liver. Key: (*) denotes conserved amino acid residues among the CYP enzymes.

3.2. Expression of CYP2C92 in insect cells

An entry vector containing CYP2D50 from one of the horses was used in the Bac-to-Bac system to generate a recombinant bacmid construct. The bacmid DNA was then used to transfect Sf9 cells to generate a recombinant baculovirus, which was further amplified by an additional two rounds to make a high titer baculovirus stock for expression studies. The expression of full-length recombinant CYP2D50 by Sf9 cells was assessed using Western blot analysis. Protein expression was apparent at 72 h post infection in Sf9 cells from the third round of amplification. Western blot analysis was also used to optimize MOI and harvest times after infection in High Five cells that were infected with amplified baculovirus stock. Expression levels were determined to be the greatest at an MOI of 10 and accordingly a MOI of 10 pfu/cell was chosen for subsequent expression studies. There was a noticeable difference in expression levels between a harvest time of 24, 48 and 72 h post infection with maximum expression being apparent at 72 h post infection. Easily detectable quantities of CYP with a soret maximum at 452 nm were observed following the addition of ferric citrate and ALA to the medium.

3.3. Optimization of dextromethorphan metabolism

Prior to conducting kinetic studies, optimal incubation conditions for dextromethorphan metabolism by recombinant CYP2D50 were established. The optimal ratio of CYP2D50 to NADPH cytochrome P450 reductase was determined in incubations containing 5 pmol of CYP2D50 and increasing amounts of reductase (1:1–1:20). The rate of dextromethorphan metabolism increased up to a ratio of 1:15 (Fig. 2). A decrease in substrate turnover was seen after 1:15 and therefore a ratio of 1:15 was used for all subsequent metabolism studies. The optimal CHAPS concentration of 1 mM was determined in a previous study.

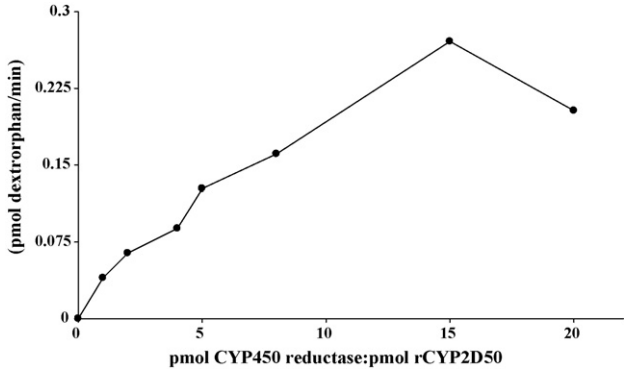

Fig. 2 – Effect of increasing amounts of NADPH CYP450 reductase on the rate of dextromethorphan metabolism by recombinant CYP2D50. Incubations containing 5 pmol of recombinant CYP2D50, 1 mM CHAPS, 100 mM potassium phosphate buffer, pH 7.4, 1 mM dextromethorphan and varying amounts of equine liver reductase in a total volume of 250 μl were incubated at 37 °C for 20 min. Values are the mean of two separate incubations.

3.4. Kinetic properties

Kinetic parameters (K_m and V_{max}) for the metabolism of dextromethorphan by recombinant CYP2D50 were established with the optimized system described previously. For comparison, incubations of dextromethorphan with human CYP2D6 recombinant enzyme were performed according to the manufacturer's protocol with the addition of 1 mM CHAPS. Incubations for dextromethrophan metabolism were performed for 5 min (liver microsomes) or 15 min (CYP2D50 or CYP2D6) using substrate concentrations ranging from 0 to 1000 μM (liver microsomes) or 0 to 400 μM (CYP2D50 and CYP2D6). The data was evaluated by plotting velocity (*V*) vs *V*/[Substrate] (Eadie Hofstee plot) and the intercepts calculated by linear regression analysis. The K_m value for CYP2D6 is nearly five times lower when compared with the equine orthologue, CYP2D50 (Fig. 3A and B). Similarly the maximal rates of substrate metabolism in the incubations containing the human recombinant CYP2D6 were more than 180-fold higher (Table 1) than CYP2D50. The calculated V_{max} value for dextromethorphan-*O*-demethylase activity in equine liver microsomes was 27.6 pmol/min/pmol CYP450. In addition to dextrorphan, a hydroxylated metabolite was detected in incubations with CYP2D50 and CYP2D6. Dextromethorphan-*O*-demethylation was the predominant reaction in liver microsome incubations, however low concentrations of additional metabolites were detected including a hydroxylated, a di-demethylated, and a methylated product.

Debrisoquine is another isoform selective substrate for human CYP2D6, and was tested as a possible substrate for recombinant CYP2D50. For this experiment, saturating concentrations of debrisoquine (1000 μM) were incubated with both CYP2D50 and CYP2D6 and samples removed following a 10 min incubation. CYP2D50 activity was minimal (nearly 50-fold less) as compared to CYP2D6. Liver microsomes exhibited minimal debrisoquine 4-hydroxylase activity with a calculated V_{max} of 1.41 pmol/min/pmol CYP450. In addition to 4-hydroxydebrisoquine, a ketone metabolite was detected in incubations with CYP2D50. Products detected in equine liver microsomal incubations included 4-hydroxydebrisoquine, a dehydrogenated product, a reduced hydroxy and a di-hydroxy metabolite.

3.5. Sample analysis

For incubations with dextromethorphan, mass accuracy of less than 2 ppm was obtained with external calibration for all selected fragment ions allowing for the assignment of a single elemental composition given candidate compositions encompassing $C^{17}H^{24}O^{1}N^{1}$ at nominal precursor *m*/*z* of 258.1848 corresponding to the *O*-desmethyl product. The full-scan MS data set contained rich information on protonated molecules of dextromethorphan metabolites with high mass accuracy. For debrisoquine samples, mass accuracy of less than 2 ppm was obtained with external calibration for all selected fragment ions allowing for the assignment of a single elemental composition given candidate compositions encompassing $C^{10}H^{14}O^{1}N^{3}$ at nominal precursor *m*/*z* of 192.1133 corresponding to the hydroxylated product. The full-scan MS

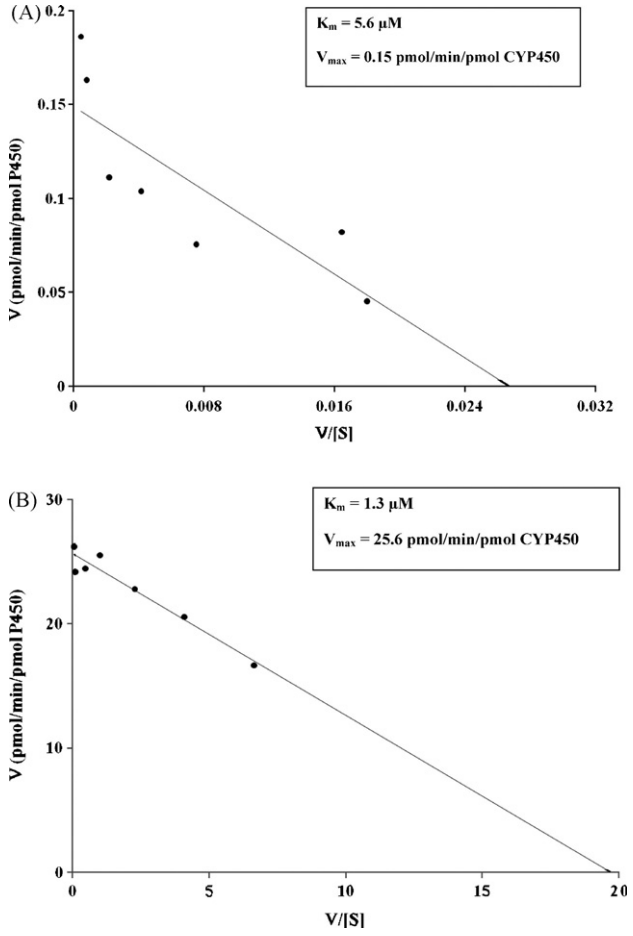

Fig. 3 – (A) Eadie Hofstee plot for the determination of the apparent K_{max} and V_{max} values for dextromethorphan metabolism by equine recombinant CYP2D50. Incubations contained 2.5 pmol of CYP2D50, 37.5 pmol of CYP reductase, NADPH and dextromethorphan (0, 2.5, 5, 10, 25, 50, 200 and 400 μM) and were incubated for 15 min at 37 °C. Values are the mean from data obtained from two separate incubations at each substrate concentration. (B) Double reciprocal plot for the determination of the apparent K_{max} and V_{max} values for dextromethorphan metabolism by human recombinant CYP2D6. Incubations contained 2.5 pmol of CYP2C9, 2.5 pmol of CYP reductase, NADPH and dextromethorphan (0, 2.5, 5, 10, 25, 50, 200 and 400 μM) and were incubated for 15 min at 37 °C. Values are the mean from data obtained from two separate incubations at each substrate concentration.

data set contained rich information on protonated molecules of debrisoquine metabolites with high mass accuracy.

4. Discussion

Due to their large size, the expense associated with maintenance and drug administration as well as limited access to research animals, performing drug metabolism studies in the horse, metabolism data from other species must frequently be extrapolated for equine veterinary use. However, it is important to note that interspecies differences in *in-vitro* metabolism as well as substrate specificity have been well documented. In this study, we have cloned and identified a member of the CYP2D family in the horse, given the novel designation CYP2D50. In humans, CYP2D6 constitutes only about 2–4% of total CYP450s, however, it is responsible for the metabolism of a broad range of commonly prescribed therapeutic compounds. Through studies utilizing equine liver microsomes and isoform selective substrates for CYP2D6, it has been suggested that a member of the CYP2D family is present in the horse liver that may be capable of metabolizing similar substrates to that of CYP2D6 [2]. However, the only definitive way of determining precisely which enzyme is responsible for the metabolism of a given drug and which metabolites a specific enzyme produces, is by constructing a recombinant enzyme. To that end, in the present study, CYP2D50 cDNA was cloned and expressed in insect cells in order to characterize and compare enzymatic activity to the human orthologue, CYP2D6.

The amino acid sequence of equine CYP2D50 is 77% homologous to that of human CYP2D6. However when the catalytic properties of the two enzymes were compared with respect to *O*-demethylation of dextromethrophan and debrisoquine 4-hydroxylation, two markers of CYP2D6 activity, some distinct differences in activity were observed. Dextromethorphan-*O*-demethylase activity levels for CYP2D6 were nearly 180 times higher than those observed for CYP2D50. Similar to the findings for CYP2D50 and CYP2D6 reported here, there are a number of other reports of interspecies differences in dextromethorphan-*O*-demethylase activity for members of the CYP2D family. The rate of dextromethorphan-*O*-demethylation by human CYP2D6 has been shown to be 10- and 12-fold higher in comparison to CYP2D17 (cynomologous monkey) [8] and CYP2D15 (dog) [9], respectively. LC–MS analysis of CYP2D50 and CYP2D6 incubations, performed in full-scan mode, revealed that in addition to dextrorphan, both species produce a hydroxylated product, albeit at low concentrations relative to dextrorphan. This suggests that the substrate, dextromethorphan, orients in the same manner in both the human and equine recombinant enzyme and that the other metabolites of dextromethorphan observed in equine liver microsomal incubations likely arise as a result of metabolism by enzymes other than CYP2D50.

4-Hydroxydebrisoquine activity proved to be very different between the human and horse orthologues as well. Full enzyme kinetic studies were not performed with debrisoquine as very little metabolite was detected in incubations with CYP2D50. Following a 10 min incubation at saturating substrate concentrations, CYP2D6 demonstrated a nearly 50-fold higher rate of production of the 4-hydroxydebrisoquine metabolite as compared to CYP2D50. Furthermore, liver microsomes proved to have very low debrisoquine 4-hydroxylase activity with a V_{max} of 1.4 pmol/min/pmol CYP450. In addition, the K_m value was very high and the V_{max}/K_m was calculated at 0.03, indicating highly inefficient debrisoquine 4-hydroxylase activity. The results from equine liver microsome incubations suggests that the horse CYP450s are either very inefficient at metabolizing debrisoquine or that the horse may produce metabolites other than the 4-hydoxy product. LC–MS analysis revealed three additional metabolites in liver micro-

Table 1 – Catalytic activities of recombinant CYP2D50, CYP2D6 and equine liver microsomes with two CYP2D substrates

Substrate	Apparent K_m (μM)	V_{max} (pmol/min/pmol CYP450)	V_{max}/K_m [d]
Dextromethorphan			
rCYP2D50[a]	5.60	0.15	0.03
rCYP2D6[a]	1.30	25.6	19.7
ELM[b]	0.24	27.5	114.5
Debrisoquine			
rCYP2D50[a]	ND[c]	0.11	ND[c]
rCYP2D6[a]	ND[c]	5.4	ND[c]
ELM[b]	48.0	1.4	0.03

[a] rCYP2D50, rCYP2D6: average (n = 2 incubations).
[b] ELM: equine liver microsomes, average (n = 3, duplicate determinations).
[c] ND = not determined.
[d] Expressed as min^{-1} μmol^{-1}.

some incubations and one additional product in the CYP2D50 incubations. Based on signals at m/z of 174.013, 194.1288 and 208.1081 for the dehydrogenated, the reduced hydroxyl and the di-hydroxy metabolite, respectively and assuming that the ionization efficiencies were similar to 4-hydroxydebrisoquine, we found that the formation of these additional products occurred at far lower rates as compared to the rate of formation of the primary product, 4-hydroxydebrisoquine. These findings suggest that 4-hydroxydebrisoquine is the major product of debrisoquine metabolism in the equine species, however, metabolism of this compound by equine CYP450s appears to be very slow.

Two members of the CYP2D family have been identified in the marmoset, CYP2D19 and CYP2D30 [10]. Even though both enzymes are members of the CYP2D family, they demonstrated very different catalytic capabilities. In incubations with debrisoquine, CYP2D30 exhibited a debrisoquine 4-hydroxylase activity of 10.8 pmol/min/pmol CYP450. However CYP2D19 yielded non-detectable levels of the 4-hydroxydebrisoquine metabolite. It is possible that similar to the marmoset, there are other CYP2D variants in the horse capable of higher rates of dextromethorphan-O-demethylation and debrisoquine 4-hydroxylation. In the current study two horses were evaluated and only one member of the CYP2D family was cloned. This however, does not rule out the existence of additional members of this family in the horse. It is possible that the discovery of only one member of the CYP2D family in the horse can be attributed to the methodology used in this study. RT-PCR was employed to amplify the CYP2D gene and primers were designed based on the Equine Genome Project predicted sequence for the equine orthologue of CYP2D6. This methodology would not allow for amplification of sequences that are not highly homologous in the region in which the primers anneal. Further work screening for CYP2D variants in a cDNA library from equine liver is underway.

In humans, CYP2D6 is highly polymorphic, with nearly 50 allelic variants identified thus far [1]. Individuals carrying this polymorphic gene, can exhibit either low or very high hepatic clearance of drugs that are substrates for CYP2D6 which can result in therapeutic failure or adverse reactions. CYP2D transcript variants have also been identified in other species. In the dog, investigators have isolated two full-length transcript variants of CYP2D15 as well as a third non-functional splice variant devoid of exon 3 [11]. Both full-length variants demonstrated enzymatic activity with the isoform selective substrate, but showed very different rates of catalytic activity. It is possible that one or more transcript variants may exist in the horse and that this may explain why the CYP2D50 enzyme described here does not metabolize dextromethorphan very efficiently as compared to the human enzyme. Equine liver microsomes used in this study demonstrated significant dextromethorphan-O-demethylase activity indicating that the horse is capable of metabolizing dextromethorphan at high rates. Additional studies are required to determine whether another enzyme is capable producing the dextrorphan metabolite or whether the activity demonstrated by the liver microsomes can be attributed to CYP2D50 variants. The recently released Equine Genome Project has identified four additional CYP2D sequences, which appear to be highly homologous. Based on a nucleotide sequence homology of 98% with CYP2D50, it is highly likely that these sequences are transcript variants of CYP2D50 and that one of the variants may have higher catalytic activities with dextromethorphan and/or debrisoquine.

In this study, we have established the presence of a member of the CYP2D family in the horse that is capable of metabolizing isoform selective substrates for CYP2D6, albeit at dramatically different rates from the human orthologue. Further investigation, including characterizing the substrate specificities of other equine CYP450 isoforms, is required before dextromethorphan-O-demethylase and debrisoquine 4-hydroxylase activities can be assigned to CYP2D50. Furthermore, additional studies are necessary to determine the role of CYP2D50 in the metabolism of other therapeutic agents commonly administered to the horse. CYP2D50's role in the metabolism of endogenous compounds also warrants further investigation. In the pig, CYP2D25 is involved in the metabolism of vitamin D3 [12] and has the ability to reduce N-hydroxylated compounds [13]. The current study is only the first step in characterizing the metabolism of compounds by members of the CYP2D family in the horse.

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