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Developmental Expression of the Major Human Hepatic CYP3A Enzymes

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Abbreviations: PNA, post-natal age; EGA, estimated gestational age (given in days); PMI, post-mortem interval; DHEA, dehydroepiandrosterone; 16α-OH-DHEA, 16-alpha-hydroxydehydroepiandrosterone; 7β-OH-DHEA, 7-beta-hydroxydehydroepiandrosterone; Cl_{int}, intrinsic clearance; LC-MS, liquid

chromatography-mass spectrometry; APCI, atmosphere pressure chemical ionization; ANOVA, analysis of variance; P450, cytochrome P450

Abstract

The human cytochrome P4503A forms show expression patterns subject to developmental influence. CYP3A7 and CYP3A4 are generally classified as the major fetal and adult liver forms, respectively. However, characterization of CYP3A4, -3A5, and -3A7 developmental expression has historically been confounded by the lack of CYP3A isoform-specific antibodies or marker enzyme activities. Therefore, the objective of this study was to characterize the developmental expression of hepatic CYP3A forms from early gestation to 18 years of age using up to 212 fetal and pediatric liver samples. Based on immunoquantitation, CYP3A5 protein expression was found to be highly variable, generally independent of age, and more frequently observed for African-American individuals. For differentiation of CYP3A4 and -3A7 levels, dehydroepiandrosterone (DHEA) metabolite patterns for expressed CYP3A forms were characterized and used for simultaneous quantitation of protein levels within liver microsome samples. The major metabolite formed by CYP3A4, 7β-hydroxy-dehydroepiandrosterone, was identified based on co-chromatography and mass spectra matching with the authentic standard. Kinetic analysis showed a 34-fold greater intrinsic clearance of 7β-hydroxy-dehydroepiandrosterone by CYP3A4 versus -3A7, while CYP3A7 showed the highest 16α-hydroxy-dehydroepiandrosterone intrinsic clearance. Metabolite profiles for the expressed enzymes were fit to a multiple response model and CYP3A4 and -3A7 levels in fetal and pediatric liver microsome samples were calculated. Fetal liver microsomes showed extremely high CYP3A7 levels (311-158 pmol/mg protein), and significant expression through 6 months postnatal age. Low CYP3A4

expression was noted for fetal liver (≤10 pmol/mg), with mean levels increasing with postnatal age.

The cytochromes P450 (P450) are a gene superfamily of hemoproteins that catalyze the oxidation of lipophilic substrates to more water soluble products (Guengerich et al., 1998). The large interindividual variability observed in the clinical pharmacokinetics and/or efficacy of certain drugs can often be attributed to P450 genetic polymorphisms and the resultant impact on expression, regulation, and/or substrate specificity. For adults, the two primary determinants of the expression level of a particular P450 form are genotype and induction status. However, several in vitro studies have shown that P450s are not uniformly expressed from the prenatal period to adulthood and that ontogenesis is a highly variable process. Many of these differences are of direct significance for pediatric clinical practice, as the rate of P450-dependent metabolic clearance of drugs such as midazolam (Burtin et al., 1994) and theophylline (Hendeles and Weinberger, 1983) cannot be simply extrapolated from body weight adjustments or from data on P450 expression and regulation in adults.

The human CYP3A forms have been extensively studied from several perspectives. They are collectively the most abundant P450s, have the largest number of drug substrates, and illustrate many of the aforementioned issues of expression, polymorphism, and clinical impact (Wrighton et al., 2000;Guengerich, 1999). CYP3A4 is the predominant form in adults, comprising 10-50% of total hepatic P450 in addition to substantial intestinal levels (Shimada et al., 1996;Paine et al., 1997). CYP3A5 levels in liver and intestine may exceed those of CYP3A4 in some individuals, however expression is subject to the polymorphism dictated by the frequency of the *CYP3A5*1* allele (Kuehl et al., 2001;Lin et al., 2002). By comparison, substantially less is known about the role of CYP3A7 in human drug metabolism. Originally isolated from and

historically regarded as a 'fetal' hepatic P450 form (Wrighton and Vandenbranden, 1989), CYP3A7 has more recently been shown to be expressed in some adult liver and intestine samples (Burk et al., 2002). In addition, given that CYP3A7 has shown unique activity among the 3A subfamily for endogenous substrates such as retinoids (Marill et al., 2002) and that expression may be influenced by various nuclear receptors (Burk et al., 2002), greater definition of the role of CYP3A7 in human metabolism is warranted.

Investigations on the developmental expression of human hepatic CYP3A forms at the level of protein and enzyme activity have been hampered by logistical and technical problems. First, the number of fetal and pediatric liver samples analyzed within any single investigation has been small; thus preventing statistical analysis among age groups in many cases (Tateishi et al., 1997;Blanco et al., 2000). In addition, the quality of the tissue sample (particularly fetal specimens) may be compromised by substantial post-mortem intervals (PMI) prior to tissue collection, and therefore could be a contributing factor in reports of low P450-dependent enzyme activity. Accurate immunoquantitation of hepatic CYP3A4 and –3A7 has historically been unsuccessful due to the lack of CYP3A7-specific antibody preparations, and probes for activity or chemical inhibitors have been largely non-specific. Therefore, the primary objective of this study was to characterize the developmental expression of CYP3A forms using the largest set of fetal and pediatric liver samples assembled to date. To address this issue, we developed a novel approach that includes a multiple regression model for the contribution of CYP3A4 and –3A7 forms to the formation of specific hydroxylated metabolites of the steroid dehydroepiandrosterone (DHEA). The results show distinct patterns of developmental expression for CYP3A4, -3A5, and –3A7.

Methods and Materials

Materials. Expressed human CYP3A4, -3A7, -3A5, -1A2, -1B1, -2B6, -2C8, -2C9, -2C18, -2C19, -2D6, -2E1, -4A11, -4F2, -4F3A, -4F3B, polyclonal antibody raised against a P4503A5 peptide (WB-3A5 antiserum), polyclonal antibody raised against a P4503A4 peptide (recognizing both CYP3A4 and CYP3A7 by immunoblot) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from BD-Gentest (Woburn MA). For expressed CYP3A forms, the ratio of cytochrome b_5 to P450 content was provided by the manufacturer: CYP3A4, 8.2 nmol/nmol; CYP3A5, no coexpression of b_5; CYP3A7, 1.4 nmol/nmol. The corresponding cytochrome *c* reductase activities (μmol/min/mg protein) were CYP3A4, 2.10; CYP3A5, 0.62; and CYP3A7, 1.10. Hybond-P™ polyvinylidene difluoride membranes and chemiluminescence reagents (ECL™) used for western blot analysis were purchased from Amersham Pharmacia Biotech (Arlington Heights IL). NADPH and progesterone were obtained from Sigma-Aldrich (St. Louis MO), and DHEA, 7α-hydroxy-, 7β-hydroxy-, 11α-hydroxy-, 11β-hydroxy-, 16α-hydroxy-, 16β-hydroxy-, 7-oxo-, and 11-oxo-DHEA standards were obtained from Steraloids, Inc. (Newport RI). Reagents for gel electrophoresis and immunodetection were purchased from Bio-Rad Laboratories (Hercules CA). All other reagents and materials were procured from common commercial sources at the highest grade available.

Human liver microsome samples. Fetal and pediatric liver samples were obtained and microsomal fractions prepared as described previously (Koukouritaki et al., 2002). Tissue samples from individuals with disease processes that could involve liver damage were excluded from the sample set. Given that gender identification was not uniform for

all samples and histories of drug administration were not available, these parameters were not considered in the evaluation of CYP3A expression. Pooled human liver microsomes were obtained from Xenotech LLC (Kansas City KS) and individual adult human liver microsomes were obtained from the Pharmacia liver microsome bank. Information on marker P450 enzyme activities for these samples was provided by the manufacturer or has been previously published (Wienkers et al., 1996).

Immunoquantitation of microsomal CYP3A5. Proteins from human liver microsomes (3 μg total protein) and preparations containing cDNA-expressed CYP3A forms were separated by gel electrophoresis (10% acrylamide, 1.0 mm thickness) and transferred to polyvinylidene difluoride membranes. The membranes were blocked, and then incubated with the primary antibody (CYP3A5 antiserum) at a 1:1000 dilution. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and proteins were detected by chemiluminescence. Following film development, the optical density of the immunoreactive protein band was determined, and liver microsome CYP3A5 protein content quantitated by linear regression using a standard curve of 0.005 – 0.1 pmol expressed 3A5 included on each blot. The lowest standard of expressed enzyme did not represent the limit of detection; therefore some samples with lower enzyme concentrations were quantifiable. The reproducibility of immunoquantitation between blots was demonstrated by an average correlation coefficient of 0.96 for the CYP3A5 standard curves (n=12). In addition, a pooled human liver microsome sample was included with each blot as a positive control, and the average (± SEM) CYP3A5 protein level determined for this sample was 3.11 ± 0.25 pmol/mg (n=12).

DHEA metabolite identification and DHEA hydroxylase assays. Profiles of DHEA metabolites were generated from incubations containing expressed CYP3A4, -3A5, -3A7, or human liver microsomes. These incubations contained 100 μM DHEA, 0.5 mg/ml human liver microsomes (0.2 ml incubation volume), 100 pmol/ml expressed P450 (0.3 ml incubation volume), and buffer concentrations given below. Incubations were conducted for 20 min at 37°C in the presence of NADPH (1 mM final conc.), stopped by the addition of a 0.25 volume of cold acetonitrile, centrifuged, and the supernatant removed for analysis. For quantitation of two of the hydroxylated DHEA metabolites, 16-alpha-hydroxydehydroepiandrosterone (16α-OH-DHEA) and 7-beta-hydroxydehydroepiandrosterone (7β-OH-DHEA), incubations with human liver microsomes (100 μg/ml) or prepared mixtures of expressed P450s (0-50 pmol/ml) were performed using a 0.20 ml reaction mixture containing 50 μM DHEA and 1.0 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4) with 2.5 mM $MgCl_2$ /0.05 mM EDTA. For kinetic determinations of DHEA hydroxylation by individual expressed CYP3A forms, incubations contained substrate concentrations of 1-200 μM and 10 pmol/ml of expressed enzyme. Samples were preincubated at 37°C for 3 min, reactions were initiated by NADPH addition and continued for 20 min. Reactions were terminated with 50 μl acetonitrile containing 5 μM progesterone as an internal standard for quantitation by liquid chromatography-mass spectrometry (LC-MS).

Following sample centrifugation, LC-MS analysis was carried out using a Perkin-Elmer (Norwalk, CT) series 200 autosampler, Agilent (Palo Alto, CA) 1100 LC pump system, and a ThermoFinnigan (San Jose, CA) TSQ7000 mass spectrometer equipped with an atmosphere pressure chemical ionization (APCI) source. Different LC-MS

procedures were used depending on whether the objective was qualitative (metabolite profiling and identification) or quantitative analysis. These two methods utilized different chromatrography columns and mobile phases, however, the APCI source parameters were the same, including a 450^{o}C vaporizer temperature, a 200^{o}C capillary temperature, and a 4.5 μA corona discharge current. Nitrogen was used as both the sheath gas (at 80 psi) and as auxiliary gas (at 20 arbitrary units of the manufacturer). The qualitative verification of DHEA metabolites against standards was performed using a SymmetryShield^{TM} (Waters, Milford, MA) 2.1 x 150 mm RP8 (5μ) column. A 30 μl aliquot of each sample was injected onto the column and eluted using a mobile phase consisting of water containing 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.25 ml/min. A linear gradient of 22.5% B to 27.5% B over 25 min was followed by another gradient of 27.5% B to 90% B over the next 5 min, and finally the mobile phase was held at 90% B for 10 min. The *m/z* acquisition range with positive APCI was 200-400 Daltons.

For the quantitative analysis of specific DHEA metabolites, 50 μl of each sample was injected onto a Symmetry® (Waters) 2.1 x 50 mm C8 (5μ) column. The mobile phase consisted of 95:5:0.2 (v:v:v) water:methanol:acetic acid (mobile phase A) and methanol (mobile phase B). A linear gradient of 25% B to 75% B over 15 min was carried out at a flow rate of 0.5 ml/min. The metabolites were detected by monitoring in-source fragment $[MH\text{-}H_2O]^+$ ions (*m/z* 287) that are the most abundant ions observed in APCI-MS for the hydroxylated DHEA metabolites. The metabolites were quantified by comparing the peak area of each analyte to that of the internal standard, progesterone (MH^+, m/z 315).

DHEA model development and validation. A multiple regression model was developed to predict the concentrations of CYP3A4 and -3A7 from the measured 16α-OH and 7β-OH metabolite concentrations. Incubations of DHEA with varying amounts of expressed CYP3A4 and -3A7 were performed followed by the quantitation of the 7β- and 16α-OH metabolites. Based on this data, a model was generated in MATLAB (The Mathworks, Inc., Natick MA) using the PLS Toolbox (Eigenvector Research, Inc., Manson WA). The measured amounts of the 16α-OH and 7β-OH metabolites were imported into Excel (Microsoft Corp., Redman WA) and then exported to MATLAB via Excel Link (The Mathworks, Inc., Natick MA). A partial least squares regression model with polynomial inner relations, polyPLS, was used to calculate the concentration (pmol/ml) of CYP3A4 and -3A7 based on the metabolite data. Modifications were made to the polyPLS functions in the PLS Toolbox in order to force the model through the origin. The predicted CYP3A enzyme concentrations for pediatric and adult liver microsomes were then converted to specific content (pmol/mg protein) based on the protein concentrations for the respective DHEA hydroxylase incubations. Approximately 5% of the values for CYP3A4 or –3A7 were negative, and these were reported as 0 pmol/mg.

Statistical analysis. To enable a statistical comparison of CYP3A5 development expression, the data were divided into age categories defined by regression tree analysis (S-Plus V4.5, Insightful Corp., Seattle WA). The regression tree was pruned based on node deviance, yielding a final tree with 8 terminal nodes, as shown in Table 1. The data was then analyzed using Kruskal-Wallis one-way analysis of variance (ANOVA)(Unistat V5.0, London UK). Pairwise comparisons were calculated using Fishers LSD on the

mean ranks for the categories and a cut off of $p \leq 0.01$ was used to assess significant differences. Correlation analysis was performed using GraphPad Prism V3.0 (San Diego CA).

Results

Immunoquantitation of CYP3A forms. Due to the well-documented overlapping substrate specificity of human CYP3A forms (Williams et al., 2002;Kenworthy et al., 1999), initial efforts to distinguish the developmental expression of CYP3A4, -3A5, and-3A7 focused on form-specific immunoquantitation. Figure 1 illustrates the sensitivity and selectivity of an antibody to CYP3A5. Given the detection of CYP3A5 protein below the lowest standard of 5 fmol expressed protein and the consistent linearity of the standard curves, CYP3A5 levels were quantitated from ~0.1 to 33 pmol/mg. Specificity was demonstrated by the lack of cross-reactivity of the antibody with CYP3A4 or -3A7 (lanes 16 and 17). Immunoreactive CYP3A5 was detected in liver microsomes selected from both prenatal (lanes 6-10) and postnatal (lanes 11-15) categories, and results for the immunoquantitation of CYP3A5 levels for 212 individual fetal and pediatric liver microsome samples are shown in Figure 2. Age categories were defined by regression tree analysis (8 nodes/categories, Table 1), following the removal of samples where CYP3A5 protein was not detected. CYP3A5 protein was clearly expressed and quantitated in approximately one-half of the fetal liver microsome samples. For all samples, 46% (97/212) showed undetectable CYP3A5 protein. In general, no change in 3A5 protein expression with age was observed up to the 341 – 430 day group. However, by Kruskal-Wallis one-way ANOVA analysis, significant differences ($p \leq 0.01$) were observed between the 341- 430 day group and the following groups; 144-221 day, 298-341 day, 430-981 day, and 981- 3668 day. In addition, average CYP3A5 protein levels were significantly different between the highest age group and all other groups except the 341-430 day group. If samples where CYP3A5 protein was not detected were

included in the overall analysis, no age-dependent change in CYP3A5 expression was observed up to 9 years of age (data not shown). CYP3A5 genotype analysis was not performed for any of the liver tissue samples, and therefore the dependence of these results on genetic factors (i.e. high frequency of *CYP3A5*3/*3* genotype within certain age groups) compared with a regulatory factor related to age cannot be determined. It is worth noting that the percentage of African Americans in the highest age group was 33% (4/12, Table 1), the lowest percentage of any of the eight age categories. Overall, fetal and pediatric liver samples were characterized by large interindividual variability, with levels ranging from 0-25 pmol 3A5/mg protein (highest levels measured for a sample with PNA of 339 days). This range of immunodetectable 3A5 protein is lower than similar analyses of adult liver samples (Lin et al., 2002;Tateishi et al., 1999), and suggests that the *CYP3A5*1/*1* genotype associated with high CYP3A5 protein expression is not represented within this sample set.

Information on ethnic background was available for the majority of the tissue donors. The association of ethnicity with CYP3A5 protein levels is shown in Figure 3. As in Figure 2, only samples where CYP3A5 protein was detected were included, and the age categories are the same as those used for Figure 2 and Table 1. Due to the limited representation of many ethnic groups, only Caucasian, African-American, and Hispanic populations were included and statistical comparisons between groups were not performed. Taking into account that certain age categories were represented by a single Caucasian or Hispanic donor sample, the general trend was for African Americans to have the highest mean CYP3A5 protein content within each age category.

DHEA metabolite profiling and identification. In contrast to the successful analysis of CYP3A5 by western blot, similar approaches for CYP3A4 and -3A7 failed due to the lack of selectivity of the commercially available antibody (manufacturer's data, BD-Gentest) and the nearly identical mobility of these enzymes during gel electrophoresis. Therefore, an alternative approach of using CYP3A form-specific enzyme activities followed by calculation of the associated CYP3A4 and –3A7 protein levels in human liver microsomes was investigated. The hydroxylation of DHEA at the 16-alpha position has been shown to be catalyzed by CYP3A7, and to be correlated with immunodetectable CYP3A protein levels in fetal liver (Kitada et al., 1987). In addition, a major but unidentified CYP3A-dependent hydroxylated metabolite of DHEA formed by *adult* human liver microsomes was recently described (Fitzpatrick et al., 2001). Because of the potential application of CYP3A-mediated DHEA hydroxylation to the characterization of CYP3A4 and –3A7 developmental expression, definitive profiling and identification of DHEA metabolites was pursued. Due to the poor ionization of steroids using the electrospray ionization technique, APCI was evaluated for metabolite identification. First, the major site of DHEA hydroxylation by pediatric liver microsomes was confirmed as the 16-alpha position, based on retention time (~18 min, Figure 4A and 4C) and mass spectra matching with the authentic standard. As shown in Figure 5, the spectral patterns of hydroxy-DHEA analytes were distinctive in relative abundance of protonated molecular ions, fragment ions formed by the loss of one or two water molecules from protonated molecular ions, and ion-solvent adducts. Figure 4 (panels B and C) illustrates that the major DHEA metabolite produced by incubations with adult human liver microsomes had an identical retention time (9.15 min) as that of the 7β-OH-

DHEA standard. In addition, the MS fragmentation pattern for the 7β-OH-DHEA metabolite and standard were characteristic and identical in that acetonitrile adducts for the molecular ion and the m/z 287 fragment were observed along with greater abundance of the $[MH-H_2O]^+$ ion relative to the $[MH-2H_2O]^+$ ion (Figure 5, panels A and B).

The ability for expressed human P450 forms to catalyze the 16α- and 7β-hydroxylation of DHEA was also examined (Figure 6). Of the sixteen P450 forms examined, CYP3A7 was clearly shown to be the dominant DHEA 16α-hydroxylase. The low levels of this activity measured for CYP1B1 and CYP2C19 indicate that these forms are unlikely to make a significant contribution relative to the levels or intrinsic clearance for CYP3A7. DHEA 7β-hydroxylation was catalyzed primarily by CYP3A forms. Finally, the metabolite profiles produced from incubations of DHEA with expressed CYP3A4 and CYP3A7 were similar to those obtained for adult and fetal liver microsome incubations, respectively (data not shown).

The production of significant amounts of 16α-OH-DHEA by expressed CYP3A4 negated the use of this enzyme activity as a direct measure of CYP3A7 expression when enzyme incubations were conducted at a substrate concentration of 50 μM DHEA. In an attempt to define substrate concentrations that might impart selectivity for DHEA hydroxylation by CYP3A4 and CYP3A7, kinetic analyses were performed. For expressed CYP3A4, -3A5, and –3A7, the rates of 7β- and 16α-hydroxylation with increasing concentrations of DHEA were consistent with single enzyme kinetics (data not shown). The resultant kinetic parameters (Table 2) definitively show 7β-OH DHEA formation to be catalyzed primarily by CYP3A4, with a 34-fold greater intrinsic clearance (Cl_{int}) for CYP3A4 compared with CYP3A7. In contrast, the apparent K_m

values for CYP3A4- and CYP3A7-catalyzed DHEA 16α-hydroxylation were similar, thus contributing to only a 3-fold greater Cl_{int} for CYP3A7-mediated DHEA 16α-hydroxylation compared with CYP3A4. These results dictated that a means of modeling or otherwise accounting for the involvement of each enzyme in the respective DHEA hydroxylase activities would be required. These kinetic analyses also demonstrated a negligible contribution by CYP3A5 to the formation of these DHEA metabolites. Finally, this conclusion is supported by an additional experiment where high levels of expressed CYP3A5 (50 pmol/mg or twice the highest protein level measured for the pediatric samples) were added to incubations of pooled human liver microsomes and DHEA. The formation of the 7β- and 16α-hydroxy metabolites increased only 4.0% and 2.2%, respectively, compared with incubations where expressed CYP3A5 was not added (data not shown).

Determination of CYP3A4 and –3A7 protein levels. In order to quantitate the involvement of CYP3A4 and –3A7 in DHEA hydroxylation, incubations of DHEA with varying amounts of each expressed enzyme were performed followed by the quantitation of the 7β- and 16α-OH metabolites. Because some degree of nonlinearity was observed in the amount of each metabolite formed with respect to amounts of expressed CYP3A4 and -3A7, the simultaneous solution to linear regressions was not possible (data not shown). Instead, a nonlinear multivariate regression model was applied to calculate the amounts of CYP3A4 and –3A7 from the known amount of expressed enzyme and the measured DHEA metabolite levels. For each enzyme-metabolite pair, the correlation coefficient of the nonlinear fit was greater than 0.99. To assess the validity of the model, an independent set of incubations of DHEA with mixtures of expressed CYP3A4 and

–3A7 were performed, amounts of 16α- and 7β-hydroxy DHEA were measured, and CYP3A4 and -3A7 content were calculated. Table 3 shows the results of this validation. The data from both sets of incubations was used to determine the dynamic range of the regression. Across the dynamic range of model for the concentrations of prepared enzyme, the accuracy of prediction was 92% for CYP3A4 and 95% for CYP3A7. Generally the accuracy of prediction increased with the level of enzyme (≥70% accuracy at ≥5 pmol/ml), most likely due to reduced cumulative errors introduced by factors such as enzyme dilutions and limits of detection for the analytical methods. An additional evaluation of this method for measurement of CYP3A4 protein levels was conducted using adult human liver microsome samples. Based on measurements of DHEA 7β- and 16α-hydroxylase activities in these samples followed by calculation of CYP3A4 levels, a highly significant correlation was found between previously determined CYP3A4–marker testosterone 6β-hydroxylase activity and CYP3A4 protein content (n= 9 adult human liver microsome samples, $r=0.98$, $p<0.001$, Figure 7).

A subset of 77 fetal and pediatric liver microsome samples was then selected for DHEA hydroxylase activity measurements and calculation of CYP3A4 and –3A7 protein levels. The number of individual samples per age category ranged from 4 (217-287 EGA) to 16 (3-6 months PNA). Calculated protein levels for these samples are shown in Figure 8. For the category approximating the second trimester (94-168 days EGA), average CYP3A7 levels of 311 pmol/mg protein were calculated. A substantial decrease in CYP3A7 levels was observed with increasing EGA, with average CYP3A7 levels decreasing to 201 pmol/mg for the 217-287 EGA category and 158 pmol/mg for the premature birth samples (EGA<280, birth). Given the predominance of CYP3A7

expression in fetal liver microsome samples as determined by the DHEA hydroxylase method, immunodetectable levels of CYP3A7 were determined in a subset of these samples using the commercially available antibody to CYP3A4/7. Levels of CYP3A7 were determined to be 271 pmol/mg by the DHEA hydroxylase activity method and 200 pmol/mg by immunoblot analysis (n=11, data not shown). These values were not statistically different by t-test analysis, thus providing further validation of the DHEA hydroxylase activity method. Also, significant levels of CYP3A7 for up to six months PNA were measured. For groups from 1 year to 15 years PNA, CYP3A7 protein levels were extremely low (<5 pmol/mg protein). Interestingly, both a sample set of individual adult liver microsomes (N=8, Figure 8) and a pooled human liver microsome sample (pool of 30 samples, data not shown) had similar CYP3A7 protein levels of 7-12 pmol/mg. For the individual adult liver microsome samples, two donors with calculated levels of 41 and 32 pmol/mg significantly impacted the average, whereas only very low levels could be measured for the other samples. This finding of significant CYP3A7 expression in 2 of 9 adults (22%) is consistent with the elevated CYP3A7 mRNA levels observed by other investigators for 15% of individuals (Koch et al., 2002).

Figure 9 shows a composite of CYP3A expression in fetal and pediatric liver samples. For 10 of 11 fetal liver samples, CYP3A7 levels as a percentage of total 3A ranged from 87-100%, demonstrating the pronounced influence of CYP3A7 levels in fetal liver on total CYP3A expression. In contrast to the sharp decline in CYP3A7 levels from early gestation to infancy, CYP3A4 levels show a slow increase with age, and the contribution of CYP3A4 to total CYP3A content is highly variable (Figures 8 and 9). For example, for the 0-6 month PNA categories, CYP3A4 content ranged from 1-10 pmol/mg protein,

or <1 to 36% of total hepatic CYP3A content. The data also suggest a difference in CYP3A4 expression between the oldest pediatric category (11 pmol/mg CYP3A4, N=8) and an adult human liver microsome value (45.8 pmol/mg, N=8).

Discussion

Despite recent advances in the characterization of molecular determinants of interindividual variability in the expression of CYP3A forms (Kuehl et al., 2001;Bertilsson et al., 2001;Burk et al., 2002), the ontogeny of individual CYP3A forms and the resultant impact on pediatric clinical pharmacology remain poorly understood (de Wildt et al., 1999;Leeder and Kearns, 2002). Although in vivo studies aimed at phenotyping both children and adults for total CYP3A provide useful clinical information, there is debate as to the preferred substrate(s) for measuring individual CYP3A forms in vivo (Lowry et al., 2003;Streetman et al., 2000). Thus a reductionist approach would suggest that in vitro techniques are best suited to the measurement of these highly related forms. Previous studies on the developmental expression of CYP3A4, -3A5, and -3A7 have been limited by three primary logistical and technical issues. First, such studies have typically relied on only 5-20 individual fetal and pediatric samples, thus necessitating broad age groupings and limited statistical analysis (Tateishi et al., 1997;Shimada et al., 1996;Blanco et al., 2000). The present study utilized the largest number of fetal and pediatric liver samples described to date for P450 ontogeny studies, providing advantages such as smaller age groupings, data division by regression tree analysis, and adequate samples for a non-statistical consideration of ethnicity. Secondly, tissue quality is an important, but largely undocumented variable for in vitro studies. Due to the difficulty in obtaining human tissues, factors such as donor hepatic disease or PMI may not be considerations for sample rejection. The sample quality for the present study is attested to by the sample selection process (Koukouritaki et al., 2002) or lack of correlation of PMI with CYP2D6-dependent dextromethorphan O-

demethylation (J. Stevens et al., manuscript in preparation). Also, the current study showed significantly higher fetal DHEA 16α-hydroxylase activity (average of 5.74 nmol/min/mg, n=11) compared with a range of ~0.3 to 0.8 nmol/min/mg protein previously reported for 34 fetal liver microsome samples (Lacroix et al., 1997). The final issue is the historical inability to differentiate individual CYP3A forms using marker activities or immunoquantitation, thus limiting investigators to making conclusions about 'total CYP3A' (Tateishi et al., 1997;Shimada et al., 1996). The development of a novel method for discriminating between CYP3A4 and CYP3A7 activities and protein content based on the regioselective metabolism of DHEA was an important methodological development, and is discussed later in context with the developmental expression of these enzymes.

CYP3A5 expression was found to be generally independent of age. CYP3A5 protein was not detected in approximately one-half of the samples, regardless of donor age. The finding of CYP3A5 in fetal liver is consistent with the reported detection of this protein in 5 of 9 fetal samples (Hakkola et al., 2001). In fact, early studies on CYP3A5 suggested that this enzyme is expressed in a greater percentage of children compared with adults (Wrighton et al., 1990;Wrighton et al., 1989), however, the broad age range (0-19 years) and the small sample size (n=17) precluded a detailed analysis for change in expression with age. Our results for the oldest pediatric group suggest that lower 3A5 levels were observed compared with all other age groups with the exception of the 341-430 day age group. The genotype analysis necessary to validate this finding was beyond the scope of this work.

The role of ethnicity in CYP3A5 expression was also examined. Although a statistical analysis was not possible, a trend for higher hepatic CYP3A5 levels with African-American donors compared with Hispanic and Caucasian donors was observed in 4 of 8 age categories, with single observations for Hispanics or Caucasians only slightly higher than average levels for African Americans in 3 other age groups. These results represent the first report of an ethnic component to CYP3A5 expression in fetal and pediatric populations, and are consistent with the study of Kuehl et al. (2001) where African Americans (sample ages undefined) were found to have greater frequency of 3A5 expression and higher average protein levels, with both measurements correlated with *CYP3A5*1* allele frequency. Given the significant, albeit drug-dependent, role of CYP3A5 in first-pass metabolism, additional studies are required on the clinical impact of ethnic-dependent differences in hepatic and extrahepatic CYP3A5 expression.

Due to the close link between the validity of conclusions on the developmental expression of CYP3A4 and –3A7 and the methodology involved, DHEA metabolite identification and multivariate model development were carefully documented. DHEA, like other endogenous steroids such as testosterone and progesterone (Waxman et al., 1991), may undergo P450-dependent catalysis to form multiple hydroxylated isomers. DHEA 16-alpha hydroxylation has been previously attributed to CYP3A7 (Kitada et al., 1987), thus identification of a CYP3A4-dependent metabolite could allow differentiation of these forms from a single incubation. Several analytical approaches were used to identify 7β-OH-DHEA as the major metabolite formed by expressed CYP3A4 and adult human liver microsomes[1]. Kinetic studies showed DHEA 7-beta hydroxylation to be highly specific for CYP3A4; however, it was necessary to apply a multivariate regression

model to differentiate the relative contributions of CYP3A7 and –3A4 to DHEA 16α-hydroxylation.

The calculation of P450 specific content based on activity determinations for the corresponding expressed P450 could be influenced by at least three factors. First, activity can be effected by levels of P450 reductase and cytochrome b_5 (Guengerich, 1983;Yamazaki et al., 2002). Commercial preparations of expressed P450s may contain high ratios of these enzymes relative to P450, and the implications for scaling of expressed P450 data to human liver microsomes (Venkatakrishnan et al., 2001;Venkatakrishnan et al., 2000) have been extended to differences in expression systems and marker activities (Nakajima et al., 2002). This later investigation found that differences in P450 reductase or cytochrome b_5 levels with a baculovirus system did not significantly influence predictions for several P450 forms based on a relative activity factor (RAF) approach. In fact, 9-fold differences in the ratio of expressed b_5:CYP3A4 did not affect the RAF calculation using marker testosterone 6β-hydroxylase activity. Thus the model developed for this study to calculate levels of CYP3A4 and CYP3A7 may contain variability in that a) expressed CYP forms used to develop the model may have high activity due to optimized accessory proteins, resulting in an underestimation of liver microsomal CYP3A4 or –3A7 specific content, or b) the ratio of reductase and cytochrome b_5 to each CYP3A protein likely changes with development and may affect the resulting calculation of specific content. The second consideration is for inter-sample variability in the amount of catalytically active enzyme. Low DHEA hydroxylase activity may be attributable to poor sample quality rather than low enzyme levels; however, all analyses suggest uniformly high liver sample quality. Finally, some samples

may contain single nucleotide polymorphisms for CYP3A4 or –3A7 that result in decreased DHEA hydroxylase activity and a concomitant underestimation of the corresponding protein content. Despite these considerations, the validity of the model and resulting measurements of CYP3A4 and –3A7 are supported by the accuracy in predicting the specific content for expressed enzymes, the high correlation of predicted CYP3A4 content and testosterone 6β-hydroxylase activity (Figure 7), and the comparable levels of CYP3A7 protein in a set of fetal samples using both the DHEA hydroxylase activity approach and immunoquantitation.

Several important comparisons with published data can be made based on the CYP3A4 and –3A7 developmental expression patterns shown in Figures 8 and 9. CYP3A4 protein levels increased very gradually during the first 6 months of age, and levels for the 5-15 year age group were lower compared with adults. These results are in contrast to those of Lacroix et al. (1997), who reported CYP3A4 levels in children ≥ 4 yrs of age equal to those of adults (Lacroix et al., 1997). Interestingly, there is both clinical and preclinical data suggesting that changes in enzyme expression for children 5-15 years of age may be associated with growth hormone or hormonal changes related to sexual maturation (Cheung et al., 1996;Liddle et al., 1998). For CYP3A7, average protein amounts for the fetal age categories were 311 and 201 pmol/mg, considerably higher than the 100 pmol/mg value estimated by Shimada (Shimada et al., 1996;Kitada et al., 1987). Also, CYP3A7 content decreased with EGA and PNA, in contrast to the Lacroix (1997) report showing no gestational age-related decrease in CYP3A7 and even an increase in DHEA 16α-hydroxylase activity in neonates. Although measurements of total spectrally detectable P450 were not feasible for the current study, it is reasonable to attribute the

majority of fetal P450 to CYP3A7. Given the role of this enzyme in the metabolism of retinoic acid, developmental changes have important implications for the clearance of this endogenous substrate and possible protection from teratogenic effects (Hirschfeld, 1996). This data points to the need for more investigation of CYP3A7-dependent clearance of pharmaceuticals in premature infants and neonates.

In conclusion, we have presented a comprehensive account of the developmental expression of human hepatic CYP3A forms, including novel methodology for the calculation of CYP3A4 and -3A7 levels based on DHEA hydroxylase activities. Delineation of contributing factors for CYP3A regulation and expression should aid in understanding and possibly predicting variability in drug clearance in pediatric patients.

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Footnotes

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[1]This finding has recently been confirmed using a GC/MS method (Dr. Russell Prough, personal communication).

Figure legends

Figure 1. Western blot analysis of CYP3A5 protein in human liver microsomes and expressed P450 forms. Lane 1 contains insect control microsomes, and lanes 2-5 contain 0.005, 0.01, 0.05, and 0.1 pmol expressed CYP3A5, respectively. Lanes 6-10 contain liver microsomal protein from the prenatal groups, and lanes 11-15 include samples from the postnatal age groups (1 μg of total protein per analysis). Lanes 16 and 17 contain 0.1 pmol of expressed CYP3A4 and 3A7, respectively. Electrophoresis and immunodetection using an antibody to CYP3A5 were conducted as described in Materials and Methods.

Figure 2. Box and whisker plots showing levels of immunodetectable CYP3A5 determined for fetal and pediatric liver microsome samples. Only samples where CYP3A5 protein was detected are represented, and the number of non-expressors per age group is given in parentheses. The eight age categories (given in days), were defined by regression tree analysis, and are also shown in Table 1. Protein levels (pmol CYP3A5/mg microsomal protein) were quantitated as described in Materials and Methods. The bottom and top of the boxes represent the 25^{th} and 75^{th} percentiles respectively. The solid horizontal line within each box represents the median value. The horizontal dash within each box represents the mean value. The upper whisker is equal to the minimum of (i) the 75^{th} percentile plus 1.5 times the distance between the 25^{th} and 75^{th} percentiles and (ii) the maximum observation.

Figure 3. Analysis of hepatic CYP3A5 protein levels by age and ethnicity, African American, ■; Caucasian, ●; Hispanic, ▲. Age in given in days, and only samples where CYP3A5 protein was detected are represented. The size of the symbols represents the number of observations, with a range of 1 observation (for example, Caucasian, age group <144 days) to 10 (African American, age group 981<age<3668). The age categories (Table 1) are the same groupings determined by regression tree analysis and applied to Figure 2.

Figure 4. LC-MS metabolite profiles resulting from incubations of pediatric (panel A) and adult (panel B) liver microsomes with DHEA. The microsome sample used for the pediatric analysis had a PNA of 0, and the adult sample represents a pooled (N=15) mixed-gender preparation. The separation of eight commercially available hydroxy-DHEA or oxo-DHEA standards is shown in panel C. The y-axis represents the relative abundance for the total ion current. These incubations contained 100 μg of microsomal protein, 100 μM DHEA, and were conducted in the presence of NADPH for 20 min. The gradient elution and MS conditions are described in Materials and Methods.

Figure 5. LC-MS analysis of DHEA metabolites. Panels A, C, and D represent spectra of analytes generated from incubations of DHEA with human liver microsomes (see Figure 4). Hydroxylated DHEA metabolites (m/z $[MH]^+$ = 305) were separated using the qualitative procedure described in Materials and Methods. Panel B shows the spectra for the commercially available 7β-OH-DHEA standard. Positive ion APCI was used for introduction and fragmentation of the analytes, with a *m/z* acquisition range of 200-400 Daltons.

Figure 6. Comparison of the formation of 7β-OH-DHEA (solid bars) and 16α-OH-DHEA (open bars) by expressed P450s. Incubations were performed at 100 pmol/ml and 50 μM DHEA under conditions described previously. DHEA hydroxylase activity was below the limit of detection for the following expressed P450 forms; 1A2, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, 4A11, 4F2, 4F3A and 4F3B.

Figure 7. Correlation of predicted CYP3A4 protein levels with testosterone 6β-hydroxylase activity for nine adult human liver microsome samples. The multivariate regression model described in Results was used to predict the concentrations of CYP3A4 from the amounts of 7β-OH- and 16α-OH-DHEA formed during incubations with the same microsome samples and 50 μM substrate. The correlation coefficient ($r=0.98$) was statistically significant at the $p<0.001$ level.

Figure 8. Age-dependent expression of CYP3A4 and -3A7. Hydroxy DHEA levels were measured from incubations of human liver microsomes (20 μg), with DHEA (50 μM) for 20 min. These values were used to calculate levels (pmol/mg protein) of CYP3A4 (solid bars) and –3A7 (open bars) protein, using the previously described multivariate regression model. Age categories are different than those described in Table 1, and were generated based on a sufficient number of observations per category (range of n=4 to 16, total number of samples = 86).

Figure 9. Levels of CYP3A4, -3A5, and –3A7 protein as a function of age. CYP3A5 protein levels (hatched bars) were determined by immunoquantitation (Figures 1 and 2)

and values reflect all samples. CYP3A4 (solid bars) and –3A7 (open bars) protein levels were determined from the relative amounts of hydroxylated DHEA metabolites generated from microsomal incubations and subsequent calculations by multivariate regression analysis.

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Table 1. Age category definitions for fetal and pediatric liver microsome samples. The divisions were determined by regression tree analysis of the CYP3A5 protein levels as described in Materials and Methods, and only considering liver microsome samples with detectable levels of CYP3A5 protein. Age range is defined as gestational age for fetal samples or the total of postnatal age and 280 days (estimated gestational age). A total of 212 individual samples were analyzed for immunodetectable CYP3A5 levels.

Age Range (days)	**Description (approx.)**	**N**	**N = African American**	**Node Deviance**
Age < 144	1st/early 2nd Trimester	12	8	151.1
144 < Age < 221	Late 2nd Trimester	16	7	483.2
221 < Age < 298	Late 2nd trimester to infancy	16	8	395.7
298 < Age < 341	1 to 2 months	13	5	272.1
341 < Age < 430	3 to 5 months	20	10	269.1
430 < Age < 981	5 months to 2 years	10	7	416.3
981 < Age < 3668	2 to 9 years	16	10	280.5
3668 < Age	9+ years	12	4	41.64

Table 2. Kinetic parameters determined for DHEA 7β- and 16α-hydroxylation by expressed CYP3A forms. Activities determined for DHEA concentrations of 1-200 μM were fit to a one-site Michaelis Menten equation as described in Materials and Methods. Intrinsic clearance is defined as the quotient of V_{max} and apparent K_m.

Expressed CYP	DHEA Metabolite	K_m (μM)	V_{max} (nmol/min/nmol)	Cl_{int} (ml/min/nmol)
3A4	7β-OH	42.3	17.9	0.423
3A7	7β-OH	23.8	0.298	0.0125
3A5	7β-OH	187	2.45	0.0134
3A4	16α-OH	10.0	4.33	0.433
3A7	16α-OH	12.0	15.8	1.32
3A5	16α-OH	218	0.391	0.0018

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Table 3. Levels of 7β- and 16α-hydroxy DHEA formed from incubations of various concentrations of expressed CYP3A4 and –3A7. Each incubation of expressed enzyme(s) was performed in duplicate at a final concentration of 50 μM DHEA. The production of hydroxylated DHEA metabolites was measured by LC-MS, and these amounts were then applied to a multivariate regression method in order to predict the concentrations of each enzyme. Details on incubation conditions, metabolite quantitation, and calculation of predicted enzyme concentrations are described in Materials and Methods.

Prepared concentration[a] (pmol/ml)		**Metabolite produced (nmol)**		**Predicted concentration (pmol/ml)**	
CYP3A4	**CYP3A7**	**7β-OH DHEA**	**16α-OH DHEA**	**CYP3A4**	**CYP3A7**
0.0	1.0	0.000	0.0210	0.0	0.3
0.0	2.5	0.000	0.131	0.1	1.7
0.0	5.0	0.011	0.304	0.4	3.9
0.0	7.5	0.014	0.612	0.6	7.8
0.0	9.0	0.014	0.556	0.5	7.1
0.0	10.0	0.015	0.775	0.6	9.8
0.0	40.0	0.041	3.542	-0.6	40.6
1.0	0.0	0.018	0.014	0.3	0.1
2.5	0.0	0.074	0.035	1.1	0.1

5.0	0.0	0.248	0.093	3.8	0.1
7.5	0.0	0.432	0.156	6.6	0.1
9.0	0.0	0.540	0.191	8.2	0.0
10.0	0.0	0.605	0.220	9.2	0.1
40.0	0.0	2.767	1.125	40.4	0.1
9.0	1.0	0.555	0.279	8.45	1.07
7.5	2.5	0.450	0.373	6.95	2.74
5.0	5.0	0.293	0.511	4.68	5.22
2.5	7.5	0.129	0.695	2.32	8.30
1.0	9.0	0.060	0.719	1.30	8.94
40	10	2.494	1.987	35.93	10.64
10	40	0.709	3.939	8.15	40.21

[a]The first 14 rows of data are from the calibration data set, and the lower 7 rows are data from the validation data set.

Figure 1

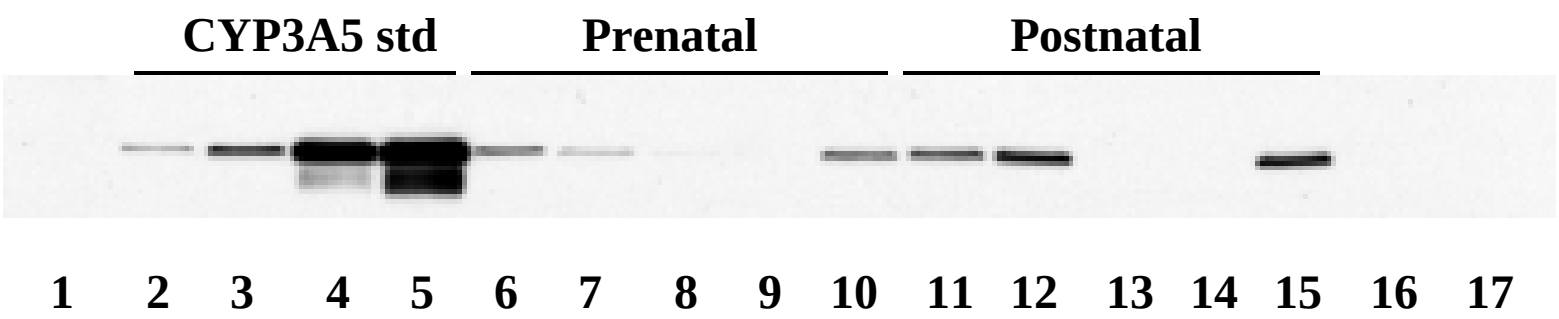

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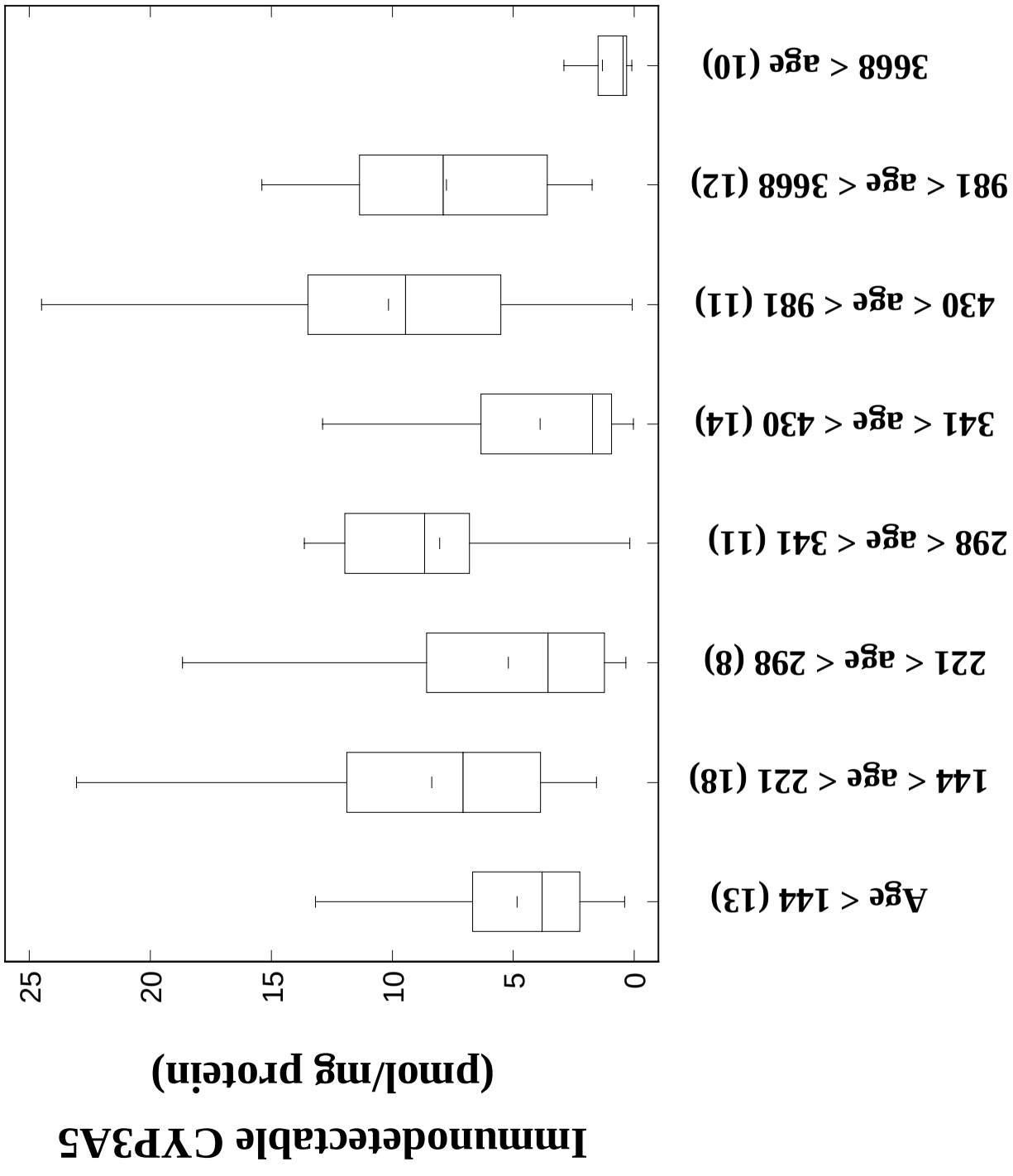
Figure 2



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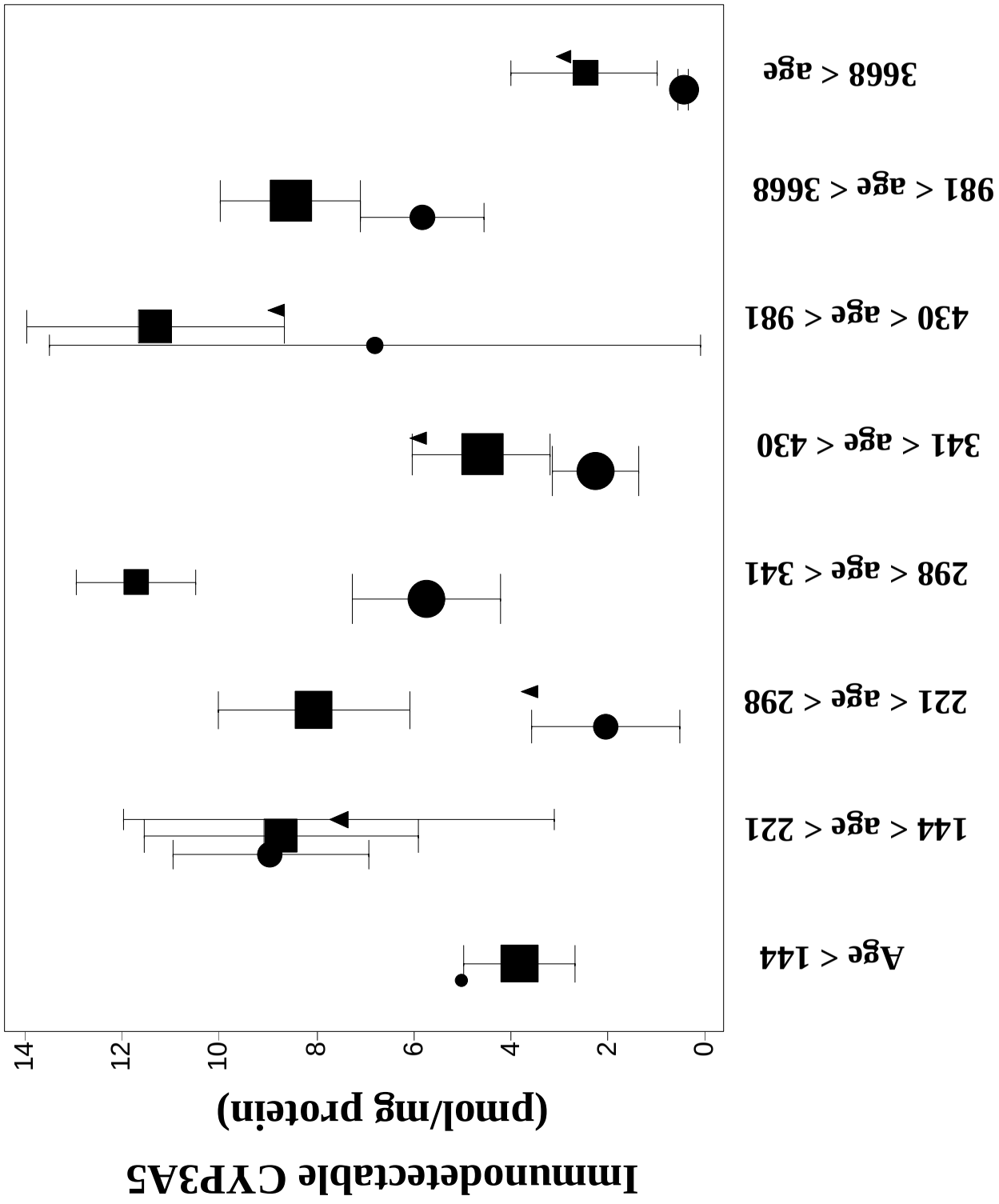


Figure 3

Figure 4

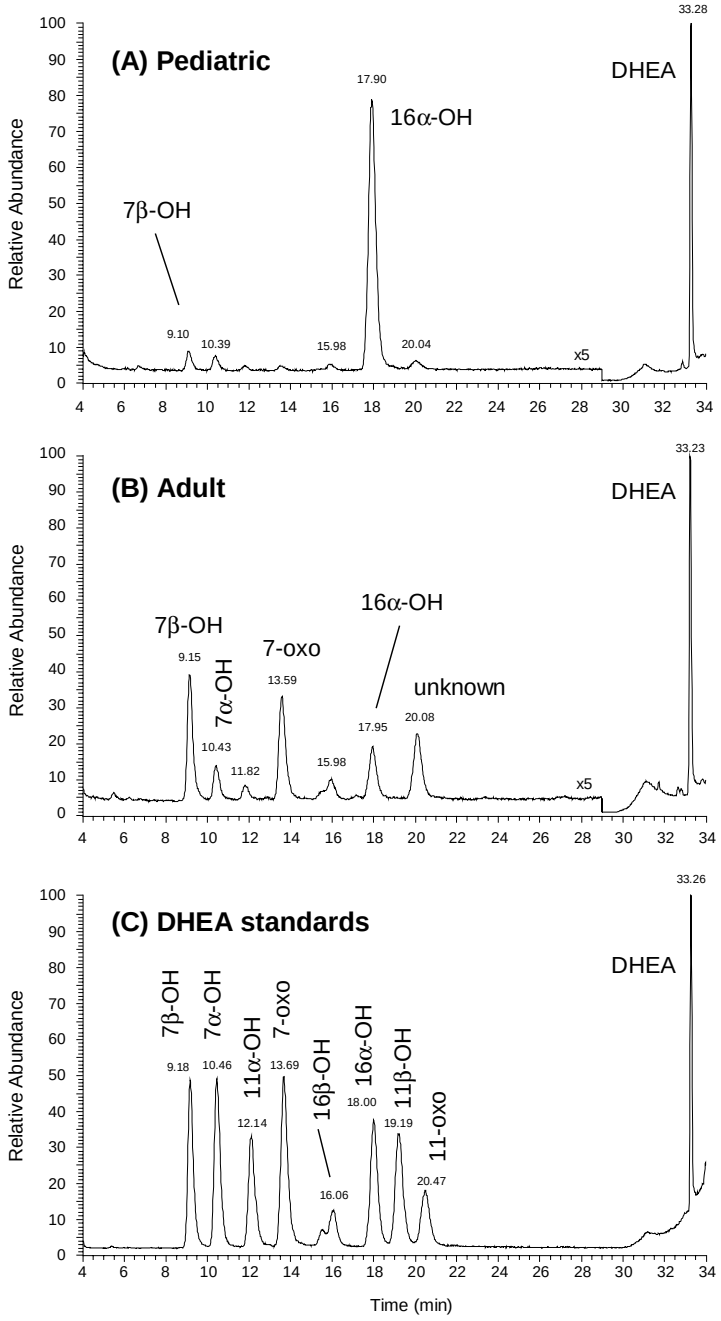


Figure 5

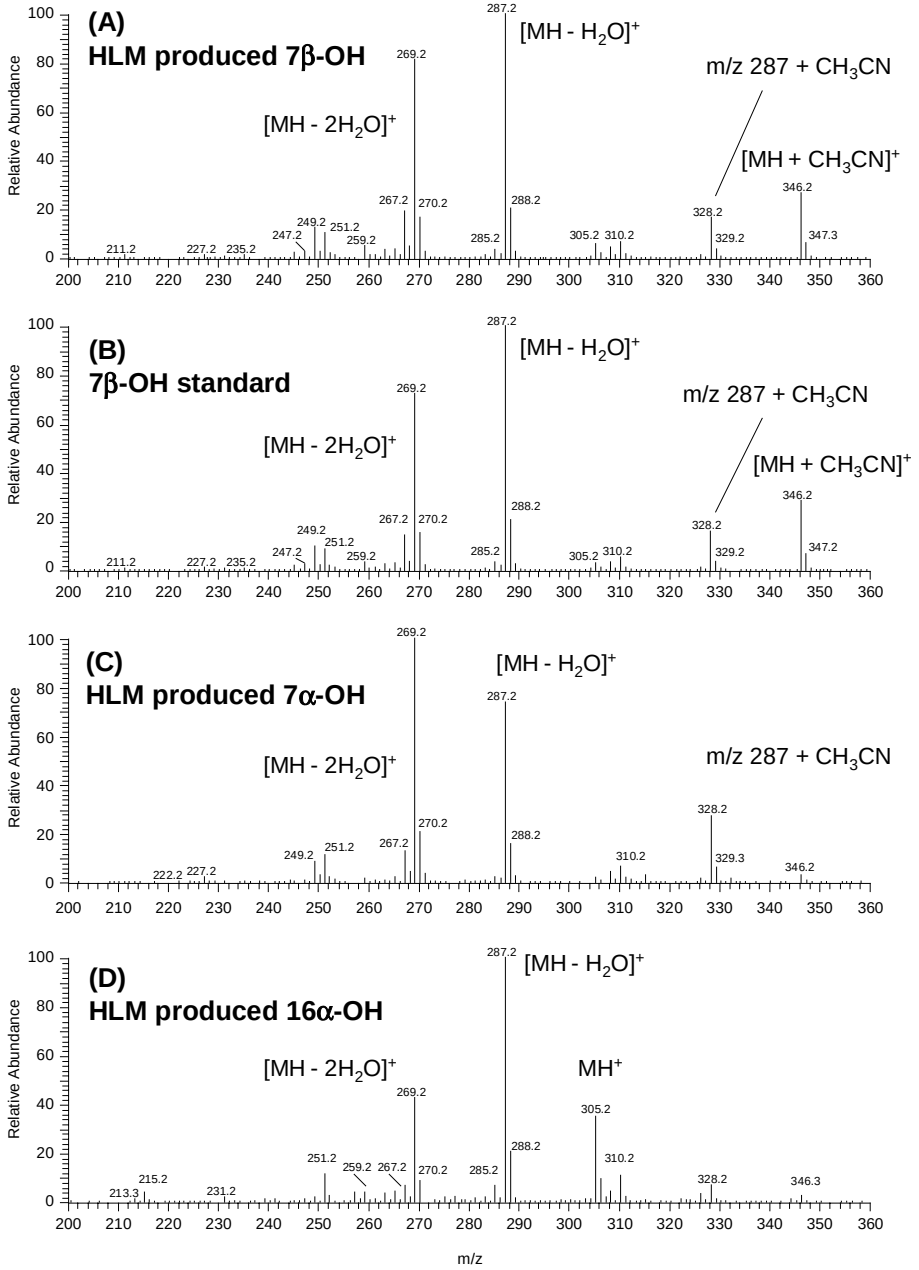


Figure 6

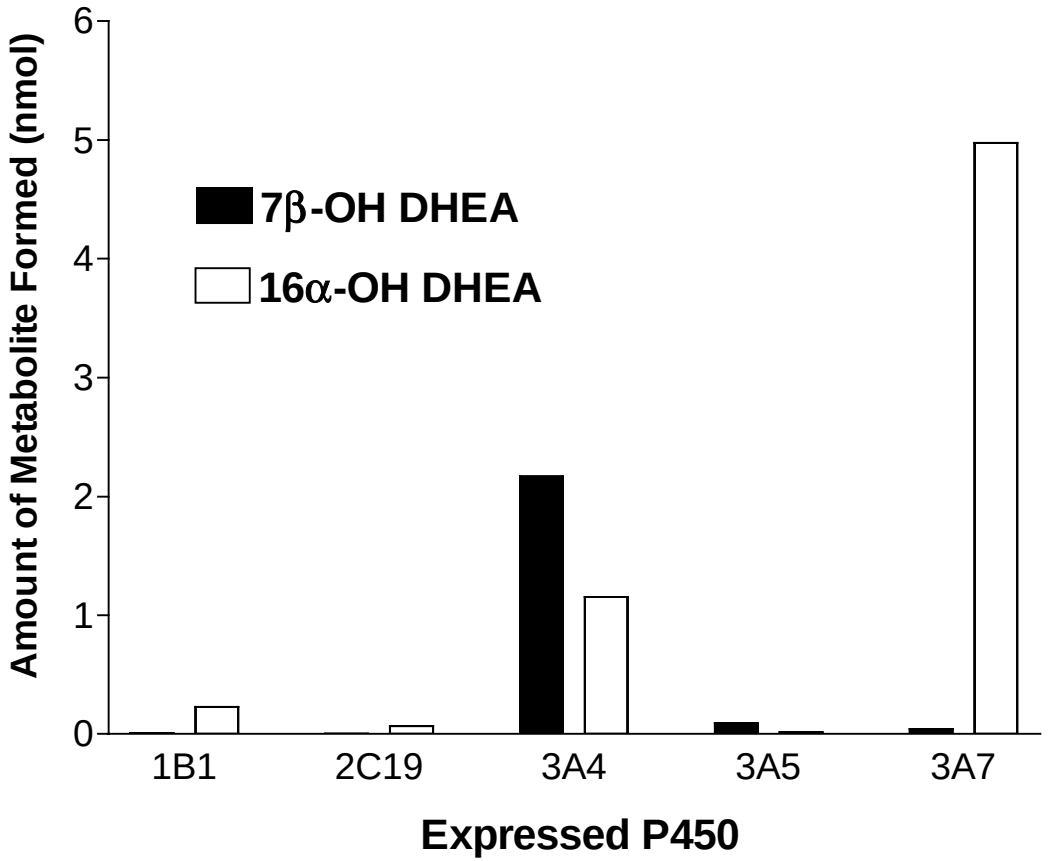


Figure 7

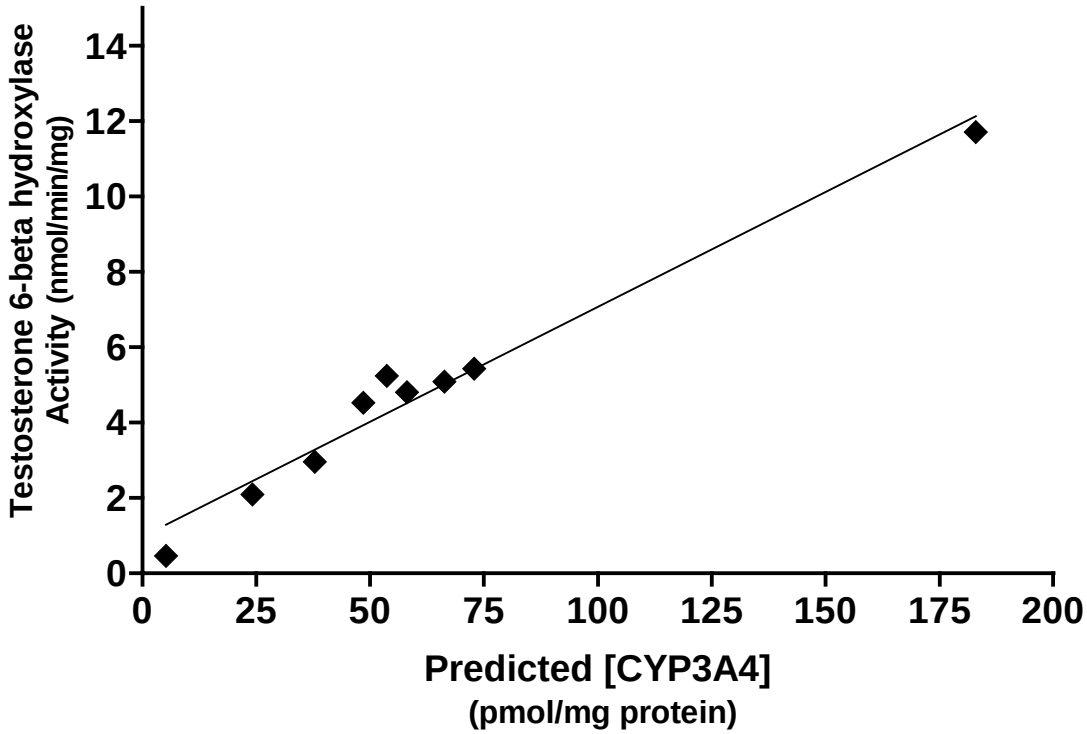
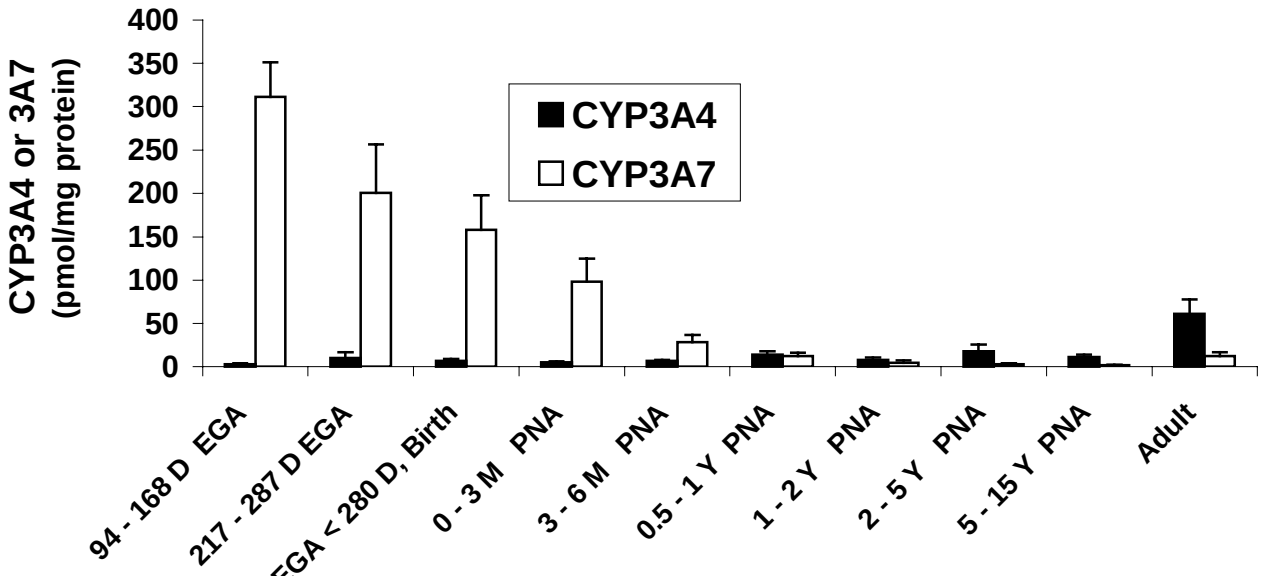


Figure 8



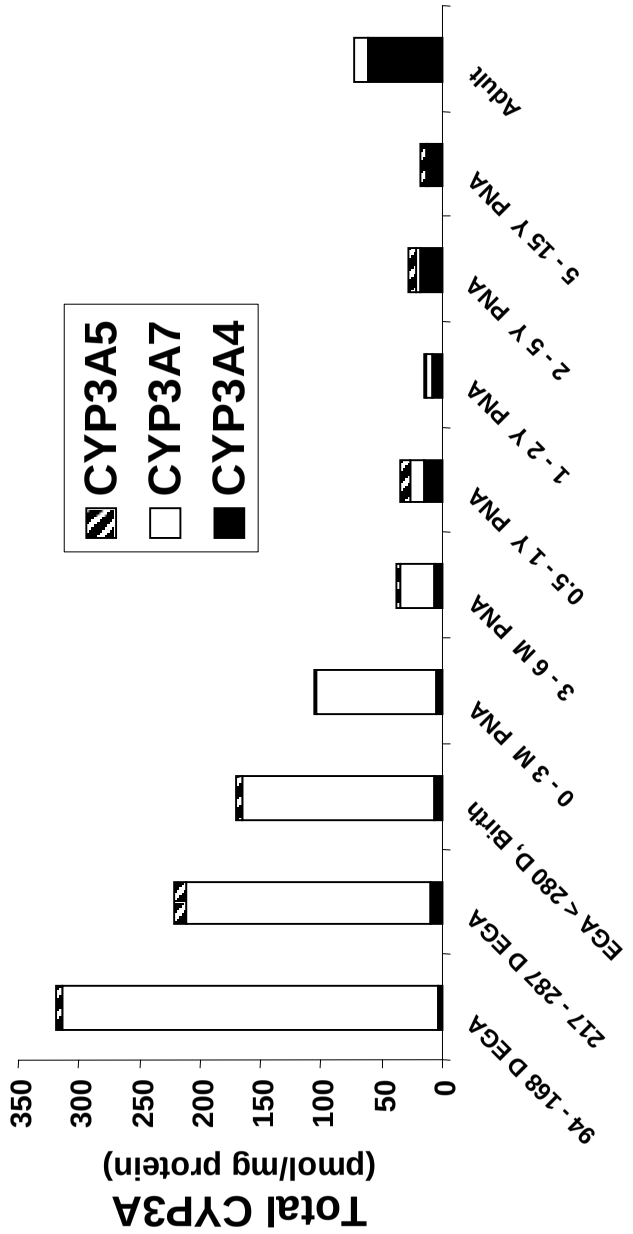


Figure 9