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# Identification of Human Hepatic Cytochrome P450 Enzymes Involved in the Biotransformation of Cholic and Chenodeoxycholic Acid

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**ABSTRACT:**

**3α,7α,12α-Trihydroxy-5β-cholan-24-oic (cholic) and 3α,7α-dihydroxy-5β-cholan-24-oic (chenodeoxycholic) acids are the predominant hepatic and biliary bile acids of most mammalian species including humans. Cholic and chenodeoxycholic acids are synthesized from cholesterol and accumulate in the liver during cholestasis. Biotransformation by hepatic cytochrome P450 (P450) enzymes represents a potentially effective pathway for elimination of these lipid-soluble bile acids. We developed a liquid chromatography/mass spectrometry-based assay to identify and quantify the human hepatic microsomal metabolites of cholic acid and chenodeoxycholic acid, and using a panel of human recombinant P450 enzymes, we determined the P450 enzymes involved. Incubation of cholic acid with human hepatic microsomes and NADPH produced a single metabolite, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic (3-dehydrocholic) acid. Of the recombinant P450 enzymes tested, only CYP3A4 catalyzed 3-dehydrocholic acid formation. Similar experiments with chenodeoxycholic acid revealed the formation of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid and 3α,6α,7α-trihydroxy-5β-cholan-24-oic (γ-muricholic) acid as major metabolites and 3α-hydroxy-7-oxo-5β-cholan-24-oic (7-ketolithocholic) acid and cholic acid as minor metabolites. Among the human recombinant P450 enzymes examined, CYP3A4 exhibited the highest rates of formation for 7α-hydroxy-3-oxo-5β-cholan-24-oic acid and γ-muricholic acid from chenodeoxycholic acid. Formation of 7-ketolithocholic acid and cholic acid from chenodeoxycholic acid has not been reported previously and could not be attributed to any of the recombinant P450 enzymes tested. In conclusion, the predominant pathway for the biotransformation of both cholic and chenodeoxycholic acids in human hepatic microsomes was oxidation at the third carbon of the cholestane ring. This study highlights a major role for CYP3A4 and suggests a possible route for the elimination of these two bile acids.**

Cholic acid and chenodeoxycholic acid comprise approximately 70% of hepatic and biliary bile acids in humans (Hofmann, 2002; Ridlon et al., 2006). Cholic acid and chenodeoxycholic acid are biosynthesized from cholesterol in the liver through a tightly regulated multienzyme pathway that includes several cytochrome P450 (P450) enzymes such as CYP7A1, CYP7B1, CYP8B1, and CYP27A1 (Norlin and Wikvall, 2007). In the liver of healthy adults, the cholic acid concentration is reported to be 14 to 21 nmol/g liver, whereas the chenodeoxycholic acid concentration is 23 to 31 nmol/g liver (Fischer et al., 1996; Setchell et al., l997). During cholestasis, a pathophysiological condition that results from obstruction of bile flow and is a common manifestation of liver diseases (Hofmann, 2002), cholic acid and chenodeoxycholic acid levels are elevated to 120 and 85 nmol/g liver, respectively (Fischer et al., 1996).

Hepatic bile acids provide the primary stimulus for canalicular bile flow and facilitate the excretion of excess hepatic cholesterol into the bile (Hofmann, 1999). Bile acids function as highly effective emulsifiers in the small intestine, facilitating the solubilization and absorption of dietary lipids and lipid-soluble nutrients and the elimination of phospholipid and cholesterol (Hofmann, 1999, 2002). In addition, bile acids serve as signaling molecules in the liver (Chiang, 2002; Makishima, 2005) and even play a role in normal liver regeneration (Huang et al., 2006). More specifically, chenodeoxycholic acid and its analogs, and to a lesser extent cholic acid, have been identified as farnesoid X receptor (FXR) agonists (Parks et al., 1999; Ellis et al., 2003; Fiorucci et al., 2005; Rizzo et al., 2005). FXR, a nuclear transcription factor, regulates expression of several genes involved in

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**ABBREVIATIONS:** cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; P450, cytochrome P450; FXR, farnesoid X receptor; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; γ-muricholic acid, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid; LC/MS, liquid chromatography/mass spectrometry; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholan-24-oic acid; isolithocholic acid, 3β-hydroxy-5β-cholan-24-oic acid; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholan-24-oic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid; murideoxycholic acid, 3α,6β-dihydroxy-5β-cholan-24-oic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic acid; 3-dehydrocholic acid, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid, also known as 3-oxo-cholic acid; 3-ketocholanoic acid, 3-oxo-5β-cholan-24-oic acid, also known as 3-oxo-cholan-24-oic acid; 6-ketolithocholic acid, 3α-hydroxy-6-oxo-5β-cholan-24-oic acid; 7-ketolithocholic acid, 3α-hydroxy-7-oxo-5β-cholan-24-oic acid.

bile acid biosynthesis and transport (Grober et al., 1999). At high concentrations, chenodeoxycholic acid binds and activates FXR, leading to down-regulation of the biosynthetic enzymes CYP7A1 and CYP8B1 and up-regulation of proteins involved in bile salt trafficking, including bile acid export pump (BSEP/ABCB11) and multidrug resistance related proteins (MDR3/B4 and MRP2/C2) (Grober et al., 1999). This feedback mechanism helps maintain bile acid homeostasis and provides protection against bile acid toxicity. The role of this autoregulatory mechanism in providing protection against bile acid toxicity was shown with FXR gene knockout mice, which have impaired resistance to bile acid–induced hepatotoxicity (Sinal et al., 2000).

Biotransformation is an additional process, besides bile acid synthesis and transport, for regulating bile acid levels in the liver. Bile acids are subject to multiple metabolic biotransformations in hepatocytes, including conjugation with taurine, glycine, glucuronic acid, and sulfate, as well as P450-mediated oxidation, whereas in the colon, bile acids undergo dehydroxylation, deconjugation, and hydroxylation reactions catalyzed by bacterial enzymes. Although much is known about bile acid hydroxylation catalyzed by bacterial systems, knowledge of bile acid hydroxylation by hepatic P450 enzymes is limited. Hydroxylation increases hydrophilicity and introduces additional functional sites for glucuronide and sulfate conjugation, thereby facilitating excretion of the bile acids. The relatively few in vivo and in vitro studies that identified hydroxylated metabolites of cholic and chenodeoxycholic acids reported different metabolite profiles. Tetrahydroxylated metabolites of cholic acid, namely, 3$\alpha$,6$\alpha$,7$\alpha$,12$\alpha$-tetrahydroxy-5$\beta$-cholanoic acid and 1$\xi$,3$\alpha$,7$\alpha$,12$\alpha$-tetrahydroxy-5$\beta$-cholanoic acid, were identified in the urine of a patient with biliary cirrhosis (Bremmelgaard and Sjövall, 1980). In contrast, 3-dehydrocholic acid was the sole metabolite found when cholic acid was incubated with recombinant human CYP3A4 (Bodin et al., 2005). Similarly, $\gamma$-muricholic acid was the only product identified when chenodeoxycholic acid was incubated with human liver microsomes or with recombinant CYP3A4 (Araya and Wikvall 1999). Because $\gamma$-muricholic acid was found in urine from healthy individuals and patients with intrahepatic cholestasis (Almé and Sjövall, 1980; Bremmelgaard and Sjövall, 1980; Shoda et al., 1990), 6$\alpha$-hydroxylation of chenodeoxycholic acid was proposed to be a major hydroxylation pathway in humans (Setchell et al., 1997; Araya and Wikvall, 1999). However, a more recent study reported that $\gamma$-muricholic acid was a major but not the predominant metabolite formed from chenodeoxycholic acid when incubated with recombinant CYP3A4 (Bodin et al., 2005).

In the present study, we investigated the biotransformation of cholic acid and chenodeoxycholic acid by human hepatic microsomes using a liquid chromatography/mass spectrometry (LC/MS)–based assay. Metabolites of cholic acid and chenodeoxycholic acid were identified; metabolite formation was quantified; and kinetic parameters associated with metabolite formation were determined. Using a panel of human recombinant P450 enzymes, we also identified the P450 enzymes involved in metabolite formation.

## Materials and Methods

**Chemicals and Reagents.** Chenodeoxycholic acid, cholic acid, deoxycholic acid, hyodeoxycholic acid, isolithocholic acid, lithocholic acid, $\alpha$-muricholic acid, $\beta$-muricholic acid, $\gamma$-muricholic acid, murideoxycholic acid, ursodeoxycholic acid, 3-dehydrocholic acid, 3-ketocholanoic acid, 6-ketolithocholic acid, and 7-ketolithocholic acid were purchased from Steraloids Inc. (Newport, RI). 1$\alpha$,3$\beta$,7$\alpha$,12$\alpha$-Tetrahydroxy-5$\beta$-cholan-4-oic acid and 3$\alpha$,6$\beta$,7$\beta$,12$\alpha$-tetrahydroxy-5$\beta$-cholan-4-oic acid were gifts from Dr. Lee R. Hagey (University of California, San Diego, CA). 7$\alpha$-Hydroxy-3-oxo-5$\beta$-cholan-24-oic acid was custom-synthesized by Steraloids Inc. specifically for this study. The identity and purity of 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid were confirmed by Steraloids Inc. Bile acid standards were dissolved in methanol as 1-mg/ml stock solutions and stored at −4°C. Additional dilutions were made in methanol for the biotransformation assay. Cholesterol, 25-hydroxycholesterol, and cholestanol were purchased from Sigma Inc. (Oakville, ON, Canada). Stock solutions of cholesterol, 25-hydroxycholestrol, and cholestanol (10 mM) were dissolved in acetone and stored at room temperature. Proadifen (SKF-525A · HCl) was provided by Dr. T.K.H. Chang (University of British Columbia, Vancouver, BC, Canada). Pooled human liver microsomes were purchased from Xenotech (Lenexa, KS). Baculovirus-insect cell control microsomes containing expressed human P450-oxidoreductase and baculovirus-insect cell microsomes containing expressed human P450 enzymes (BD Supersomes enzymes), coexpressed with human P450-oxidoreductase or with human P450-oxidoreductase and human cytochrome $b_5$, were purchased from BD Biosciences (Oakville, ON, Canada). High-performance liquid chromatography–grade chemicals and solvents were purchased from Fisher Scientific (Ottawa, ON, Canada).

**Cholic Acid and Chenodeoxycholic Acid Biotransformation Assays.** Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 3 mM magnesium chloride, 0.5 mg of human hepatic microsomal protein, 1 mM NADPH, and varying concentrations (1–800 $\mu$M) of either cholic acid or chenodeoxycholic acid, in a final volume of 1 ml. After preincubation for 10 min at room temperature, reactions were initiated with NADPH and allowed to proceed for 30 min at 37°C. Reactions were terminated with 8 ml of dichloromethane/isopropanol (80:20 v/v). A fixed amount (0.4 $\mu$g) of murideoxycholic acid, which was the internal standard, was then added to each sample. Sample extraction, evaporation, and reconstitution in preparation for analysis by LC/MS were carried out as described previously (Deo and Bandiera, 2008). Reaction mixtures that were devoid of substrate, NADPH, or microsomes, as well as reaction mixtures that contained defined concentrations of authentic bile acid standards, were routinely included in each assay.

Assay conditions were tested using pooled human microsomes to ensure that substrate and cofactor concentrations were saturating and that product formation was linear with respect to incubation time (1–60 min) and protein concentration (0.25–2 mg/ml of reaction mixture). To determine whether metabolite formation was P450-mediated, preliminary experiments were conducted with carbon monoxide–treated hepatic microsomes or heat-denatured microsomes or by replacing NADPH with NADH or by adding SKF-525A.

Incubations with human recombinant P450 enzymes, instead of human hepatic microsomes, were also carried out. Reaction mixtures contained 30 pmol of each recombinant P450 enzyme (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, or CYP4A11) or, in the case of insect cell control microsomes and reductase control, an equivalent amount of protein (0.15 mg).

**Analytical Methods.** Formation of chenodeoxycholic acid and cholic acid metabolites was analyzed by LC/MS using a procedure described previously for lithocholic acid metabolites (Deo and Bandiera, 2008), with the following modifications. Chenodeoxycholic acid and cholic acid metabolites were detected using a Waters Acquity Ultra Performance Liquid Chromatograph System (UPLC, Waters, Milford, MA) consisting of a Binary Solvent Manager and Sample Manager and connected to a Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters). The MS was operated in single ion recording mode using negative electrospray ionization with 600 l/h desolvation gas and 52 l/h cone gas, respectively, a source temperature of 100°C, and capillary and cone voltages of 3 kV and 20 V, respectively. Waters MassLynx v4.1 software (Waters) was used for data acquisition. Metabolites were identified by comparison of their retention times and mass to charge ratios ($m/z$) with those of authentic standards. A mixture of 17 bile acid standards was prepared. Under these conditions, 1$\alpha$,3$\beta$,7$\alpha$,12$\alpha$-tetrahydroxy-5$\beta$-cholan-4-oic acid (molecular mass 424.57) and 3$\alpha$,6$\beta$,7$\beta$,12$\alpha$-tehydroxy-5$\beta$-cholan-24-oic acid (molecular mass 424.57) typically eluted at 4 and 5 min, respectively, and were monitored at $m/z$ 423. $\alpha$-Muricholic acid (molecular mass 408.57), $\beta$-muricholic acid (molecular mass 408.57), $\gamma$-muricholic acid (molecular mass 408.57), and cholic acid (molecular mass 408.57) eluted at 12, 13, 15, and 16 min, respectively, and were monitored at $m/z$ 407. 3-Dehydrocholic acid (molecular mass 406.58) eluted at 13 min and was monitored at $m/z$ 405. Murideoxycholic acid (molecular mass 392.57), ursodeoxycholic acid (molecular mass 392.57), hyodeoxycholic acid (molecular mass 392.57), chenode-

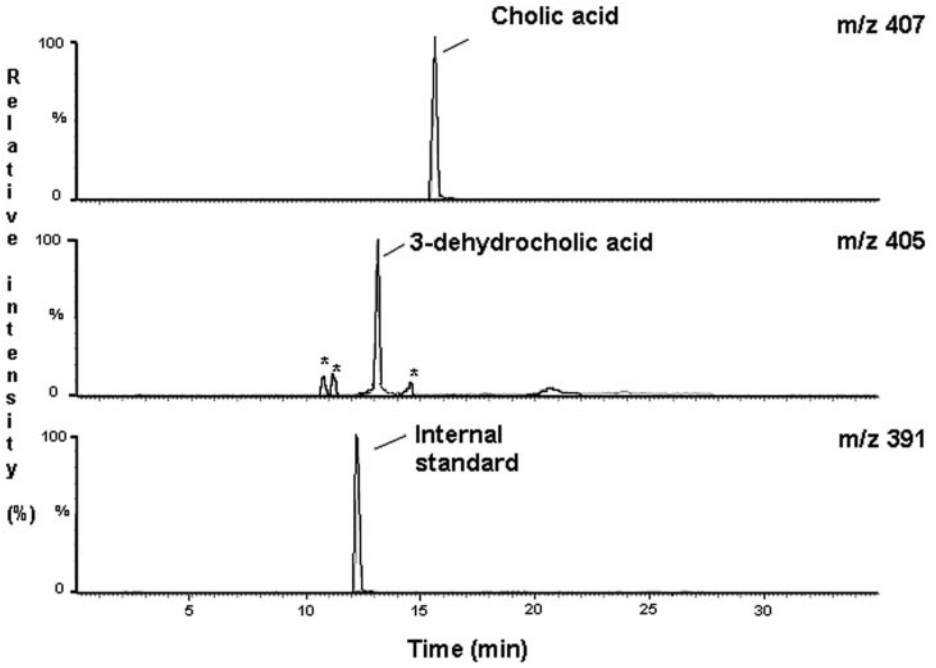


FIG. 1. Representative LC/MS chromatogram showing metabolites of cholic acid extracted from a standard reaction mixture after a 30-min incubation of human hepatic microsomes (0.5 mg) with 100 $\mu$M cholic acid and 1 mM NADPH. Metabolite identification was performed by cochromatography and spiking with authentic standards. In the above figure, the internal standard (murideoxycholic acid, *m/z* 391) eluted at 12 min; 3-dehydrocholic acid eluted at 13 min; and cholic acid (*m/z* 405) eluted at 16 min, respectively. Peaks denoted by * indicate peaks that were present in controls and blanks and are not metabolites.

oxycholic acid (molecular mass 392.57), and deoxycholic acid (molecular mass 392.57) eluted at 11, 15, 17, 20, 20.5 min, respectively, and were monitored at *m/z* 391. 6-Ketolithocholic acid (molecular mass 390.6), 7-ketolithocholic acid (molecular mass 390.6), and 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid (molecular mass 390.6) eluted at 15, 16, and 17 min, respectively, and were monitored at *m/z* 389. Isolithocholic acid (molecular mass 376.57) and lithocholic acid (molecular mass 376.57) eluted at 23 and 26 min, respectively, and were monitored at *m/z* 375. 3-Ketocholanoic acid (molecular mass 374.56) eluted at 25 min and was monitored at *m/z* 373. All the ion channels were routinely scanned with the help of this standard mixture for identification of metabolites during initial experiments. Metabolites were quantified from calibration plots of the peak area ratio of authentic standard and internal standard plotted against the concentration of the authentic standard.

**Data Analysis and Calculation of Enzyme Kinetic Parameters.** Data were analyzed using the SigmaPlot Enzyme Kinetics Module (version 1.1; Systat Software Inc., Richmond, CA). Metabolite formation as a function of substrate concentration was analyzed by nonlinear regression analysis, and apparent $K_m$, $K'$, and $V_{max}$ values were generated using the Michaelis-Menten (eq. 1) or the Hill equation (eq. 2).

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

$$v = \frac{V_{max} \times [S]^n}{K' + [S]^n} \quad (2)$$

where $v$ is initial velocity of the reaction, $V_{max}$ is the maximal velocity, [S] is the substrate concentration, $K'$ is the Hill dissociation constant, $n$ is the Hill coefficient representing cooperativity of the reaction, and $K_m$ is the Michaelis-Menten constant. Several criteria such as sum of squares, S.D. of residuals, for each equation, Akaike information criterion, and visual inspection of the fit were considered in selecting the most appropriate model for each data set (Tracy and Hummel, 2004).

## Results

**Biotransformation and Kinetic Analysis of Cholic Acid Metabolites.** Incubation of cholic acid with human liver microsomes yielded a single metabolite identified as 3-dehydrocholic acid by comparison with authentic standards (Fig. 1). A microsomal protein concentration of 0.5 mg/ml and an incubation time of 30 min were found to be within the linear range with respect to product formation and were used in subsequent experiments. Formation of 3-dehydrocholic acid evaluated over a range of substrate concentrations (1–800 $\mu$M) exhibited unsaturable kinetics (Fig. 2). Higher cholic acid concentrations were not used routinely because distorted peak profiles as a result of column overload were observed at concentrations greater than 800 $\mu$M. Apparent kinetic parameters were determined using eq. 1 (Fig. 2). The inset in Fig. 2 shows the Eadie-Hofstee plot (velocity versus velocity/substrate concentration).

**Biotransformation and Kinetic Analysis of Chenodeoxycholic Acid Metabolites.** Incubation of chenodeoxycholic acid with human liver microsomes yielded two major metabolites identified as 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid and $\gamma$-muricholic acid and two

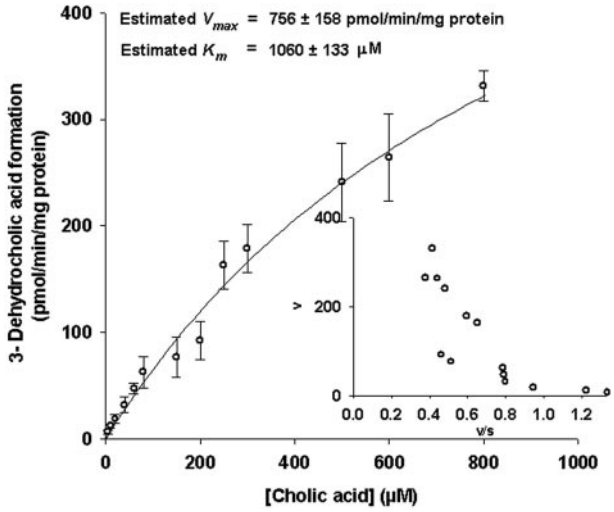


FIG. 2. Enzyme kinetic profile of 3-dehydrocholic acid formation by human hepatic microsomes. Metabolite formation (activity) was plotted as a function of substrate concentration following 30-min incubation with human liver microsomes (0.5 mg). Data points are the mean ± S.E.M. of at least three separate experiments. Data were fitted to a one-enzyme Michaelis-Menten model. Lines represent rates modeled by nonlinear regression analysis. Kinetic parameters were calculated using eq. 1. The inset depicts the Eadie-Hofstee plot. Error bars are not shown on the insets to avoid obscuring the data points, which represent mean values.

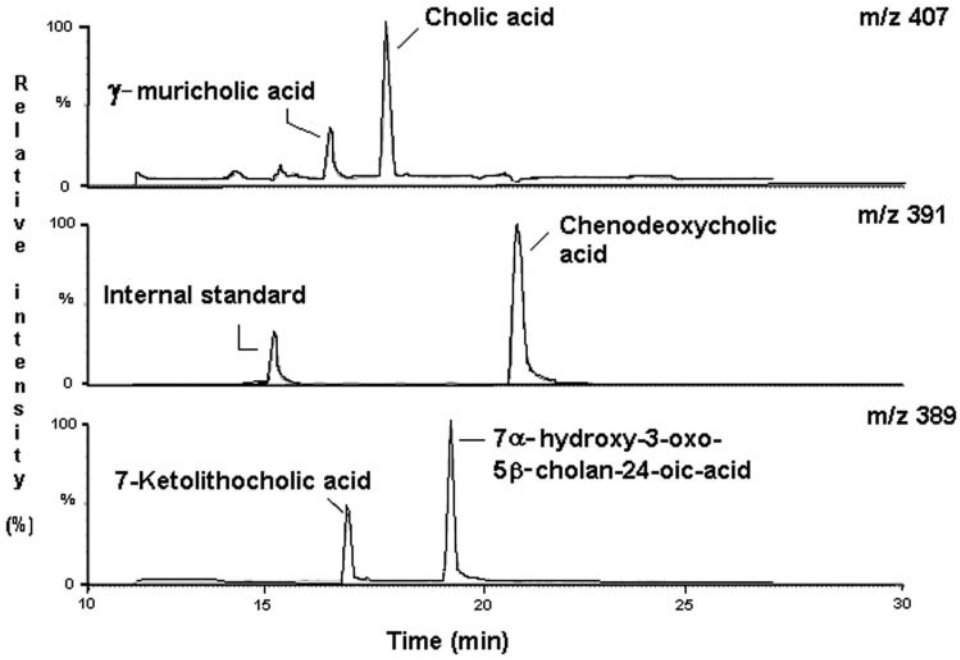


FIG. 3. Representative LC/MS chromatogram showing metabolites of chenodeoxycholic acid extracted from a standard reaction mixture after a 30-min incubation of human hepatic microsomes (0.5 mg) with 100 $\mu$M chenodeoxycholic acid and 1 mM NADPH. Metabolite identification was performed by cochromatography and spiking with authentic standards. In the above figure, the internal standard (murideoxycholic acid, *m/z* 391) eluted at 15.5 min. γ-Muricholic acid (*m/z* 407) and cholic acid (*m/z* 407) eluted at 16.5 and 18 min, respectively. 7-Ketolithocholic acid (*m/z* 389) and 7α-hydroxy-3-oxo-5β-cholan-24-oic acid (*m/z* 389) eluted at 18 and 18.5 min, respectively.

minor metabolites identified as 7-ketolithocholic acid and cholic acid (Fig. 3). Identification of metabolites was confirmed by comparing retention times and spiking with authentic standards. An incubation time of 30 min and a microsomal protein concentration of 0.5 mg/ml were found to be optimal for all four chenodeoxycholic acid metabolites and were used in subsequent experiments.

Formation of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid and γ-muricholic acid was not observed with reaction mixtures that were devoid of substrate, NADPH, or microsomes. However, chromatographic peaks with the same *m/z* values and retention times as 7-ketolithocholic acid and cholic acid were detected when chenodeoxycholic acid was incubated with boiled microsomal preparations or when human liver microsomes or NADPH was omitted from the reaction mixture. We observed that the 7-ketolithocholic acid and cholic acid peaks were approximately 2 to 3 times larger when substrate was incubated with both NADPH and human liver microsomes, and peak areas increased with increasing microsomal protein concentration or increasing incubation time. Moreover, addition of carbon monoxide or SKF-525A (1 mM) to the reaction mixtures inhibited formation of all four metabolites. Taken together these data indicate that 7-ketolithocholic acid and cholic acid were hepatic microsomal metabolites, as well as contaminants of chenodeoxycholic acid. Hence, quantification of 7-ketolithocholic acid and cholic acid formation necessitated subtraction of peak area ratios for 7-ketolithocholic acid and cholic acid obtained with blank reaction mixtures from peak area ratios measured with complete reaction mixtures.

Metabolite formation was evaluated over a range of substrate concentrations (1–800 $\mu$M). A chenodeoxycholic acid concentration of 500 $\mu$M was found to be saturating for all four metabolites. Plots of metabolite formation versus substrate concentration showed that hepatic microsomal formation of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid, 7-ketolithocholic acid, and cholic acid followed Michaelis-Menten kinetics (Fig. 4, A, C, and D, respectively), whereas formation of γ-muricholic acid followed sigmoidal kinetics (Fig. 4B). The inset in Fig. 4B shows the Eadie-Hofstee plot (velocity versus velocity/substrate concentration), typical of homotropic positive cooperativity and suggestive of substrate autoactivation (Tracy and Hummel, 2004).

Apparent kinetic parameters for 7α-hydroxy-3-oxo-5β-cholan-24-oic acid, cholic acid, and 7-ketolithocholic acid were determined using eq. 1 (Fig. 4, A, C, and D). Apparent $K'$ and $V_{max}$ values for γ-muricholic acid formation were determined using eq. 2 (Fig. 4B). Rates of hepatic microsomal metabolite formation, expressed as apparent $V_{max}$, show that 7α-hydroxy-3-oxo-5β-cholan-24-oic acid would be the predominant metabolite of chenodeoxycholic acid with human liver microsomes at high substrate concentrations.

**Biotransformation Studies with Human Recombinant P450 Enzymes.** The contribution of individual P450 enzymes to cholic acid and chenodeoxycholic acid biotransformation was evaluated using a panel of 12 human recombinant P450 enzymes. Initial experiments were conducted to determine P450 concentrations that would ensure linearity of product formation with incubation time. For cholic acid and chenodeoxycholic acid biotransformation, an incubation time of 30 min and human recombinant P450 enzyme concentration of 30 pmol P450/ml were found to be optimal at a substrate concentration of 500 $\mu$M.

Conversion of cholic acid to 3-dehydrocholic acid was catalyzed solely by CYP3A4 (Fig. 5A). Formation of 3-dehydrocholic acid by recombinant CYP3A4, evaluated over a range of substrate concentrations, gave a sigmoidal kinetic profile exhibiting saturation at 500 $\mu$M (Fig. 6). Kinetic parameters for 3-dehydrocholic acid formation by recombinant CYP3A4 were determined using eq. 2 (Fig. 6).

With chenodeoxycholic acid as the substrate, the most active enzyme catalyzing conversion of chenodeoxycholic acid to 7α-hydroxy-3-oxo-5β-cholan-24-oic acid was CYP3A4 (Fig. 5B). CYP3A5 and several other P450 enzymes catalyzed 7α-hydroxy-3-oxo-5β-cholan-24-oic acid formation at much lower rates. CYP3A4 was the only enzyme that mediated formation of γ-muricholic acid (Fig. 5B). Formation of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid by recombinant CYP3A4, evaluated over a range of substrate concentrations (1–600 $\mu$M), exhibited typical Michaelis-Menten kinetics (Fig. 7A). Formation of γ-muricholic acid by recombinant CYP3A4 exhibited a sigmoidal kinetic profile as was observed with human liver microsomes (Fig. 7B). Kinetic parameters obtained for formation of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid and γ-muricholic acid were determined using eq. 1 and eq. 2, respectively.

In contrast, conversion of chenodeoxycholic acid to 7-ketolithocholic acid and cholic acid was not catalyzed by the human recom-

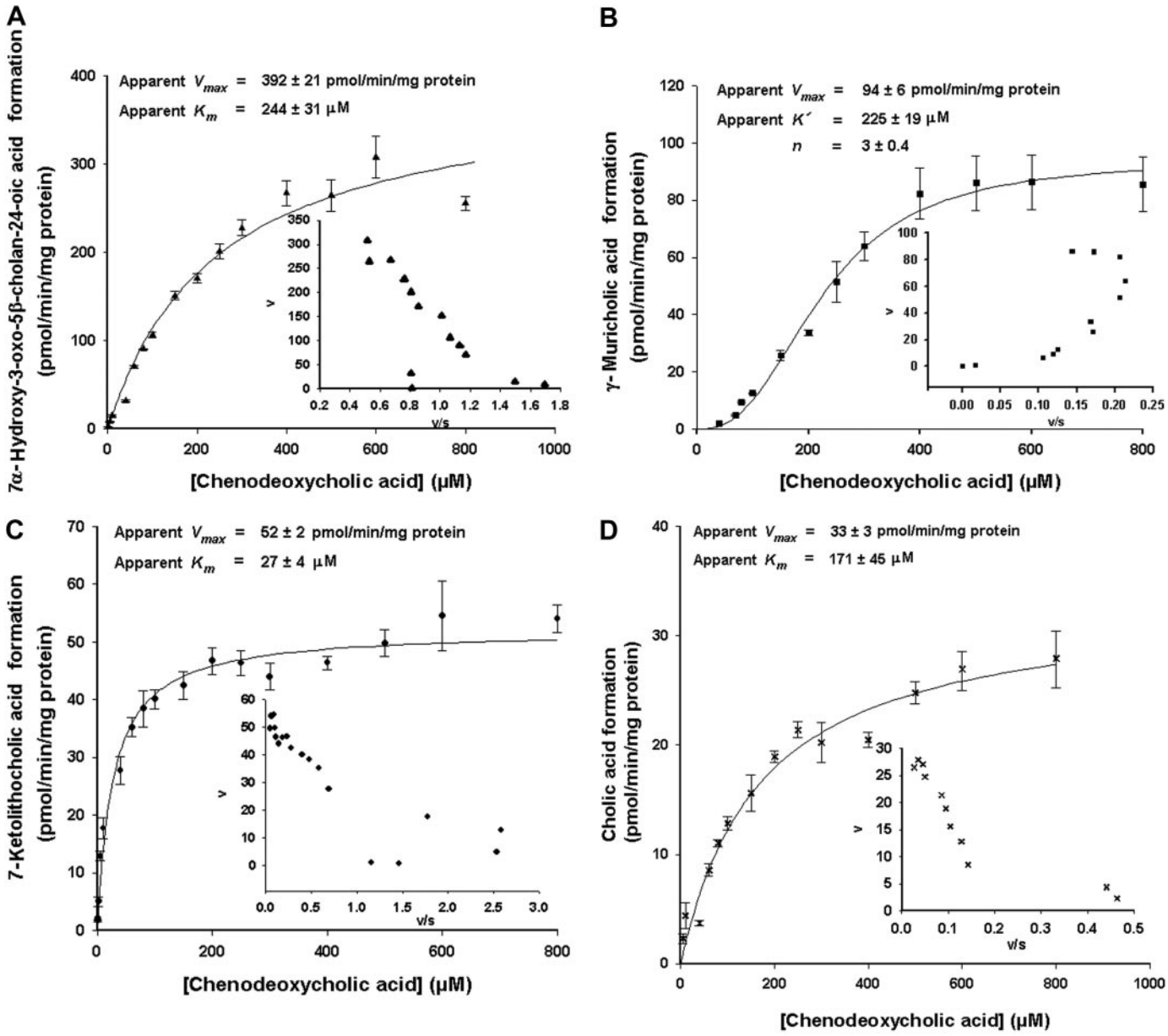


FIG. 4. Enzyme kinetic profiles of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid (A), γ-muricholic acid (B), 7-ketolithocholic acid (C), and cholic acid (D) formation by human hepatic microsomes. Metabolite formation (activity) was plotted as a function of substrate concentration following 30-min incubation with human liver microsomes (0.5 mg). Data points are the mean ± S.E.M. of at least three separate experiments. Lines represent rates modeled by nonlinear regression analysis of the data. Kinetic parameters for 7α-hydroxy-3-oxo-5β-cholan-24-oic acid, 7-ketolithocholic acid, and cholic acid formation were calculated using eq. 1. Kinetic parameters for γ-muricholic acid formation were calculated using eq. 2. The insets depict Eadie-Hofstee plots. Error bars are not shown on the insets to avoid obscuring the data points, which represent mean values.

binant P450 enzymes tested. Formation of 7-ketolithocholic acid involves oxidation of the 7α-hydroxy group of chenodeoxycholic acid. Steroid ring oxidation at the 7-position occurs in the conversion of cholesterol to 7α-hydroxycholesterol, which is catalyzed by CYP7A1, and in the conversion of 25-hydroxycholesterol to 7α-hydroxylated oxysterols, which is catalyzed by CYP7B1 (Martin et al., 1993; Schwarz et al., 2001). To determine whether CYP7A or CYP7B enzymes are involved in 7-ketolithocholic acid formation, chenodeoxycholic acid was incubated with human liver microsomes and NADPH in the presence of cholesterol (a CYP7A1 substrate) and 25-hydroxycholesterol (a CYP7B1 substrate) and cholestanol (a nonspecific CYP7A and CYP7B inhibitor) (Martin et al., 1993). Addition of increasing concentrations (100, 200, 250, and 300 μM) of cholesterol, hydroxycholesterol, or cholestanol to a saturating chenodeoxycholic acid concentration (500 μM) did not alter formation of 7-ketolithocholic acid or cholic acid (data not shown).

## Discussion

Biotransformation of cholic acid by human liver microsomes generated a single metabolite, 3-dehydrocholic acid, as determined by LC/MS. By comparison, chenodeoxycholic acid produced two major metabolites, 7α-hydroxy-3-oxo-5β-cholan-24-oic acid and γ-muricholic acid, and two minor metabolites, 7-ketolithocholic acid and cholic acid. Oxidation at the C-3 position was the predominant biotransformation pathway for both cholic acid and chenodeoxycholic acid. Hydroxylation at the 6α position was the next most important biotransformation pathway for chenodeoxycholic acid. Both reactions were catalyzed almost exclusively by CYP3A4.

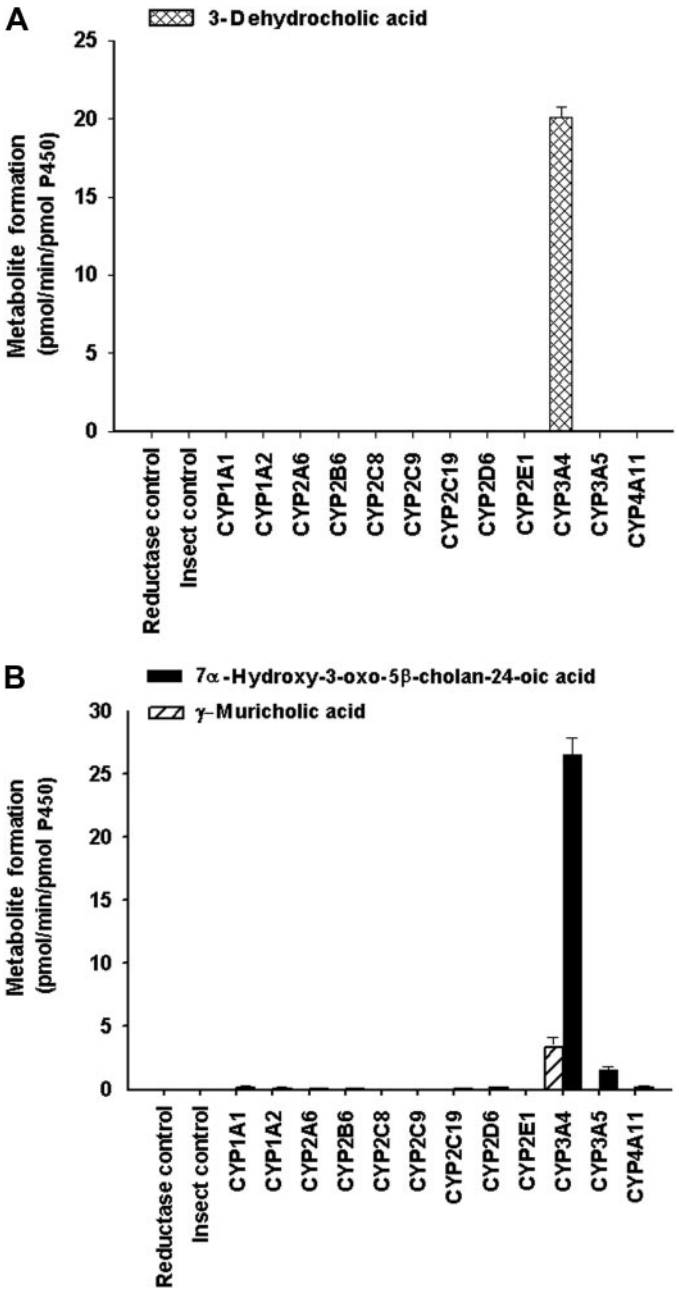


FIG. 5. Comparison of 3-dehydrocholic acid formation from cholic acid (A) and 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid and $\gamma$-muricholic acid formation from chenodeoxycholic acid (B) by a panel of recombinant human P450 enzymes. Metabolite formation (activity) was measured following 30-min incubation of cholic acid or chenodeoxycholic acid (500 $\mu$M) with baculovirus-insect cell microsomes containing expressed human P450 enzymes (30 pmol). Plots show the mean values $\pm$ S.E.M. of triplicate determinations.

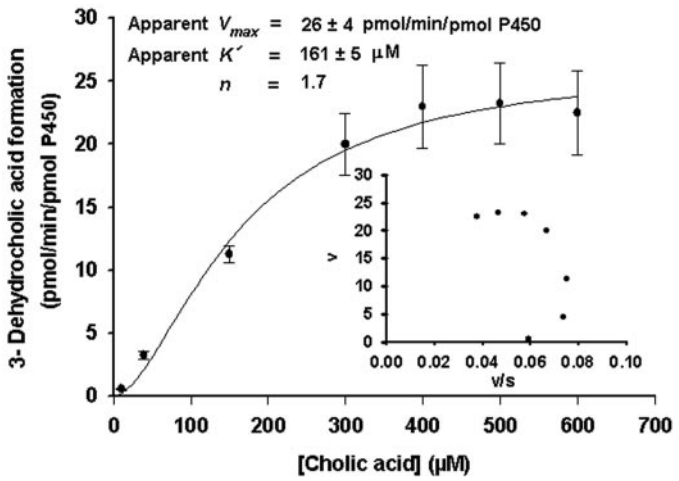


FIG. 6. Enzyme kinetic profile of 3-dehydrocholic acid formation from cholic acid by recombinant human CYP3A4. Metabolite formation (activity) was plotted as a function of substrate concentration following 30-min incubation with recombinant CYP3A4 (30 pmol). Data points are the mean $\pm$ S.E.M. of triplicate determinations. Lines represent rates modeled by nonlinear regression analysis of the data. Kinetic parameters for 3-dehydrocholic acid formation by recombinant CYP3A4 were calculated using eq. 2. The inset depicts the Eadie-Hofstee plot. Error bars are not shown on the inset to avoid obscuring the data points, which represent mean values.

Limited information is available regarding cholic acid and chenodeoxycholic acid hydroxylation by human liver microsomes, and the information that exists is largely derived from analyses of urinary and fecal metabolites and more recently from in vitro incubations performed with recombinant CYP3A4. We initially expected cholic acid to be converted, at least partially, to tetrahydroxylated metabolites because urinary tetrahydroxy metabolites were identified in a patient with primary biliary cirrhosis who was given labeled cholic acid i.v. (Bremmelgaard and Sjövall, 1980). Consequently, we scanned for but did not detect molecular ions (*m/z* 423) corresponding to tetrahydroxylated metabolites of cholic acid. Instead, oxidation of cholic acid by human liver microsomes yielded a single metabolite, 3-dehydrocholic acid, and was catalyzed by CYP3A4. This result is consistent with a previous study that identified 3-dehydrocholic acid as the only product formed when cholic acid was incubated with recombinant CYP3A4 (Bodin et al., 2005). Oxidized bile acid metabolites containing a keto group at C-3 are found in human feces and are thought to result from bacterial metabolism in the colon (Ridlon et al., 2006). Evidence that hepatic oxidation of cholic acid at the C-3 position occurs in vivo is provided by a report that 3-oxo-bile acids are present, at low levels, in bile from human fetal gallbladder samples (Setchell et al., 1988).

The present study is the first to identify 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid as the predominant hepatic microsomal metabolite of chenodeoxycholic acid. This metabolite was detected as a major chromatographic peak by LC/MS, but the retention time and molecular ion *m/z* ratio of the peak did not correspond to those of the commercially available bile acid standards. Based on its chromatographic characteristics, we predicted the chemical structure of the metabolite and had 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid custom-synthesized by Steraloids Inc. The retention time and molecular ion *m/z* ratio of 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid matched that of the major metabolite peak, and its identification was established. As was the case with cholic acid, the predominant biotransformation pathway for chenodeoxycholic acid in human liver microsomes was CYP3A4-catalyzed oxidation at the C-3 position. The physiological significance of the conversion of chenodeoxycholic acid to 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid, or of cholic acid to 3-dehydrocholic acid, is unknown as this reaction does not produce an increase in hydrophilicity of either metabolite. Trace amounts of 7$\alpha$-hydroxy-3-oxo-5$\beta$-cholan-24-oic acid have been reported in human fetal gallbladder bile samples (Setchell et al., 1988), indicating that this metabolite is formed in vivo, as well as in vitro.

$\gamma$-Muricholic acid, a 6$\alpha$-hydroxylated bile acid, is found at low levels in bile, serum, and urine of healthy adult humans (Almé and Sjövall, 1980; Shoda et al., 1989; Wietholtz et al., 1996) but is a major bile acid component in human fetal bile (Setchell et al., 1988). Two in vitro studies (Araya and Wikvall, 1999; Bodin et al., 2005) identified $\gamma$-muricholic acid as a CYP3A4-mediated metabolite of chenodeoxy-

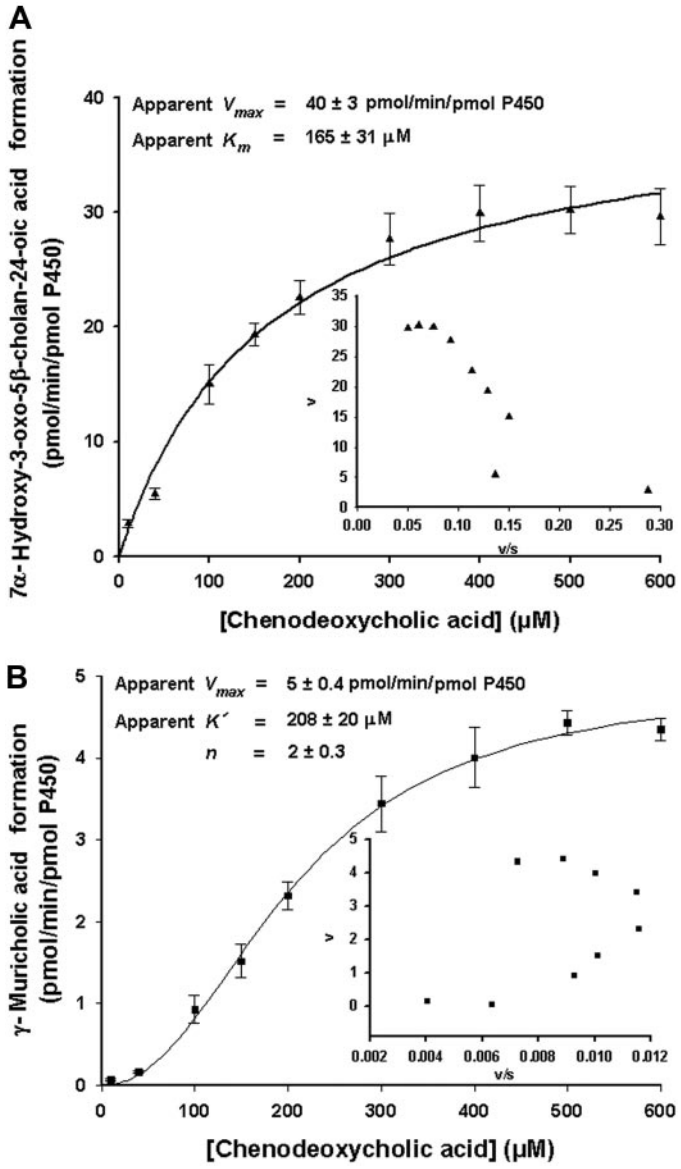


FIG. 7. Enzyme kinetic profiles of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid (A) and γ-muricholic acid (B) formation from chenodeoxycholic acid by recombinant human CYP3A4. Metabolite formation (activity) was plotted as a function of substrate concentration following 30-min incubation with recombinant CYP3A4 (30 pmol). Data points are the mean ± S.E.M. of triplicate determinations. Lines represent rates modeled by nonlinear regression analysis of the data. Kinetic parameters for 7α-hydroxy-3-oxo-5β-cholan-24-oic acid formation by recombinant CYP3A4 were calculated using eq. 1. Kinetic parameters for γ-muricholic acid formation by recombinant CYP3A4 were calculated using eq. 2. The insets depict Eadie-Hofstee plots. Error bars are not shown on the insets to avoid obscuring the data points, which represent mean values.

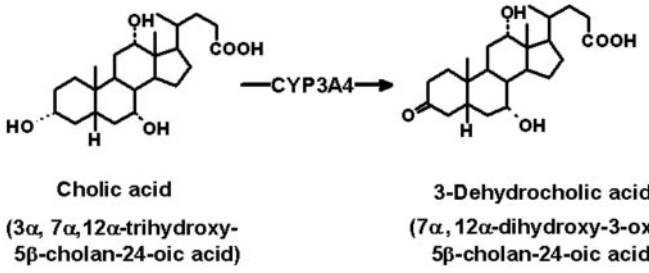


FIG. 8. Scheme showing P450-mediated cholic acid biotransformation by human hepatic microsomes. Results of the present study suggest that CYP3A4 is the only enzyme involved in 3-dehydrocholic acid formation.

cholic acid. The present study corroborates the role of CYP3A4 in γ-muricholic formation. Excretion of γ-muricholic acid is increased in patients with hepatobiliary disease and in pregnant women with cholestasis (Summerfield et al., 1976; Bremmelgaard and Sjövall, 1980; Nakashima et al., 1990; Shoda et al., 1990), indicating that CYP3A4-catalyzed 6α-hydroxylation of chenodeoxycholic acid is up-regulated in cholestasis.

Our study identified 7-ketolithocholic acid and cholic acid as contaminants of chenodeoxycholic acid and as minor metabolites of chenodeoxycholic acid biotransformation by human hepatic microsomes. These two bile acids were not previously reported to be metabolites of chenodeoxycholic acid. 7-Ketolithocholic acid is considered to be a major intermediate in the conversion of chenodeoxycholic acid to ursodeoxycholic acid in human colon (Fromm et al., 1983), and its formation is known to be catalyzed by bacterial 7-hydroxysteroid dehydrogenases of resident intestinal microflora (Ridlon et al., 2006). Reduction of 7-ketolithocholic acid to chenodeoxycholic acid and ursodeoxycholic acid by human liver enzymes has been shown (Fromm et al., 1983; Amuro et al., 1989). We did not observe ursodeoxycholic acid formation in the present study. Formation of 7-ketolithocholic acid by human liver microsomes has not been reported previously. However, hepatic synthesis of 7-ketolithocholic acid has been shown in guinea pig, a species in which 7-ketolithocholic acid constitutes approximately 30 to 35% of the total biliary bile acid pool (Tint et al., 1990). None of the panel of recombinant P450 enzymes tested catalyzed formation of 7-ketolithocholic acid. In addition, formation of 7-ketolithocholic acid did not appear to be catalyzed by CYP7A and CYP7B enzymes because its formation was not affected by competitive inhibitors of CYP7A1 or CYP7B1 (i.e., cholesterol, 25-cholesterol, or cholestanol). Formation of 7-ketolithocholic acid can possibly be mediated by non-P450 enzymes, but the identity of these enzymes remains unknown.

Conversion of chenodeoxycholic acid to cholic acid involves 12α-hydroxylation. This reaction was also not catalyzed by any of the recombinant P450 enzymes tested and was not inhibited by competitive inhibitors of CYP7A1 or CYP7B1 (i.e., cholesterol, 25-cholesterol, or cholestanol). A hepatic microsomal P450 enzyme that was not examined in this study is CYP8B1. CYP8B1, also known as sterol 12α-hydroxylase, catalyzes the 12α-hydroxylation of 3-oxo-7α-hydroxy-4-cholestene, which is an intermediate step in the multienzyme pathway leading to cholic acid formation from cholesterol (Russell and Setchell, 1992). CYP8B1 has relatively broad substrate specificity in vitro and has been shown to hydroxylate chenodeoxycholic acid at the 12α position (Andersson et al., 1998). We speculate that CYP8B1 may be responsible for the formation of cholic acid from chenodeoxycholic acid observed in the present study, but we were unable to assess the involvement of CYP8B1 because recombinant CYP8B1 or an inhibitory antibody to CYP8B1 was not commercially available.

The physiological significance of hepatic P450-catalyzed cholic acid and chenodeoxycholic acid biotransformation in vivo is unknown. Cholic and chenodeoxycholic acid concentrations of approximately 120 and 85 μM, respectively, have been reported in the liver of patients with chronic cholestasis (Fischer et al., 1996). At these concentrations, the rate of formation of 3-dehydrocholic acid from cholic acid and the rate of formation of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid from chenodeoxycholic acid would be approximately 50 to 75 and 75 to 100 pmol/min/mg protein, respectively, as determined by our biotransformation assay. The lower apparent $K_m$ value associated with 7-ketolithocholic acid formation by human liver microsomes (27 μM, Fig. 4C) suggests that this metabolite may be prefer-

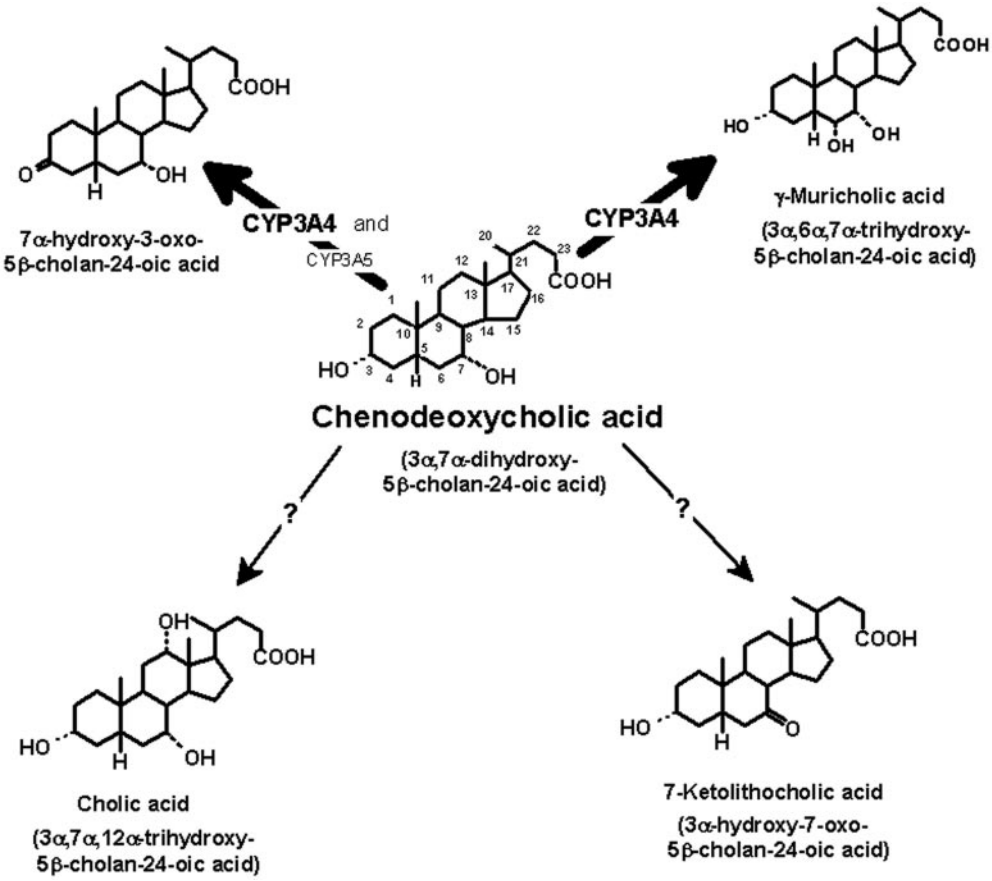


FIG. 9. Scheme showing chenodeoxycholic acid biotransformation by human hepatic microsomes. Results of the present study suggest that formation of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid and γ-muricholic acid was mediated mainly by CYP3A4. Enzymes involved in the formation of 7-ketolithocholic acid and cholic acid have not been determined.

entially formed at physiological concentrations of chenodeoxycholic acid in vivo.

The present study focused on the contribution of P450 enzymes in the biotransformation of cholic acid and chenodeoxycholic acid by human hepatic microsomes. P450-mediated oxidation probably represents a relatively minor pathway for bile acid elimination compared with conjugation reactions catalyzed by phase 2 enzymes such as sulfotransferases, glucuronosyl transferases, and amino acid transferases. Based on the results obtained, a scheme for biotransformation of cholic acid and chenodeoxycholic acid in human hepatic microsomes is proposed in Fig. 8 and Fig. 9, respectively. The results provide compelling evidence that cholic acid and chenodeoxycholic acid can serve as physiological substrates of CYP3A4. CYP3A4 is the most abundant P450 enzyme in human adult liver and small intestine (Guengerich, 1995; Paine et al., 2006) and plays a major role in the oxidation of a large number of structurally diverse xenobiotics and physiological compounds, including steroid hormones and bile acids (Hrycay and Bandiera, 2008). CYP3A4 expression is subject to regulation by the pregnane X receptor and constitutive androstane receptor, which are activated by several drugs and other xenobiotics. Induction of CYP3A4 by treatment with drugs such as phenobarbital, rifampicin, and phenytoin or, alternately, inhibition of CYP3A4 activity by antifungal agents or macrolide antibiotics can affect cholic and chenodeoxycholic acid biotransformation, in addition to resulting in clinically significant drug interactions.

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