CYP3A specifically catalyzes 1β-hydroxylation of deoxycholic acid: Characterization and enzymatic synthesis of a potential novel urinary biomarker for CYP3A activity

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Characterization and synthesis of 1β-OH-DCA as a biomarker for CYP3A activity

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CDCA-24-G, chenodeoxycholic acid-24-acyl-β-D-glucuronide; DCA, deoxycholic acid; DCA-3-S, deoxycholic acid 3-sulfate; DDI, drug-drug interaction; DHEA, dehydroepiandrosterone; G-CA, glycocholic acid; G-DCA, glycodeoxycholic acid; G-CDCA, glycochenodeoxycholic acid; GDH, glucose dehydrogenase; G-1β-OH-DCA, 1β-hydroxydeoxycholic acid glycine conjugate; 1β-OH-DCA, 1β-hydroxydeoxycholic acid, (1β,3α,5β,12α)-1,3,12-trihydroxycholan-24-oic acid; [2H4]-1β-OH-DCA, (1β,3α,5β,12α)-1,3,12-trihydroxy(2,2,4,4-2H4)-cholan-24-oic acid; P450, cytochrome P450; T-CA, taurocholic acid; T-CDC, taurochenodeoxycholic acid.
Abstract

The endogenous bile acid metabolite 1β-hydroxy-deoxycholic acid (1β-OH-DCA) excreted in human urine may be used as a sensitive CYP3A biomarker in drug development reflecting in vivo CYP3A activity. An efficient and stereospecific enzymatic synthesis of 1β-OH-DCA was developed using a Bacillus megaterium (BM3) cytochrome P450 (P450) mutant and its structure was confirmed by NMR spectroscopy. A [2H4]-labelled analogue of 1β-OH-DCA was also prepared. The major hydroxylated metabolite of deoxycholic acid (DCA) in human liver microsomal incubations was identified as 1β-OH-DCA by comparison with the synthesized reference analyzed by UPLC-HRMS. Its formation was strongly inhibited by CYP3A inhibitor ketoconazole. Screening of 21 recombinant human cytochrome P450 (P450) enzymes showed that with the exception of extrahepatic CYP46A1, the most abundant liver P450 subfamily CYP3A, including CYP3A4, 3A5 and 3A7, specifically catalyzed 1β-OH-DCA formation. This indicated that 1β-hydroxylation of DCA may be a useful marker reaction for CYP3A activity in vitro. The metabolic pathways of DCA and 1β-OH-DCA in human hepatocytes were shown to be predominantly via glycine and to a lesser extent via taurine and sulfate conjugation. The potential utility of 1β-hydroxylation of DCA as a urinary CYP3A biomarker was illustrated by comparing the ratio of 1β-OH-DCA:DCA in a pooled spot urine sample from six healthy control subjects to a sample from one patient treated with carbamazepine, a potent CYP3A inducer; 1β-OH-DCA:DCA was considerably higher in the patient vs. controls (ratio 2.8 vs. 0.4). Our results highlight the potential of 1β-OH-DCA as a urinary biomarker in clinical CYP3A DDI studies.
Introduction

Drug-drug interactions (DDIs) are a major concern in drug development and clinical practice. The likelihood and magnitude of inhibition and/or induction of drug metabolizing enzymes is an integral part of the assessment of new drug candidates. When the potential for a DDI is identified in preclinical studies, further evaluation of the risk of DDI is often required in clinical investigations. Such studies are most often carried out using drug probe compounds, which are selectively metabolized by the affected enzyme. The vast majority of clinical DDI studies investigate the effect on cytochrome P450 (P450) 3A. A sensitive probe drug for CYP3A is midazolam which is commonly used to assess inhibition or induction of this enzyme. Sensitive endogenous biomarkers, especially urinary biomarkers if applicable, would have great utility, avoiding unnecessary drug exposure and invasive sampling. The 6β-hydroxycortisol to cortisol ratio (6β-hydroxycortisol ratio) in urine has been suggested as an endogenous marker for CYP3A4 activity (Galteau and Shamsa, 2003). The intra- and inter-individual variation of this ratio is large however and can only be used when the subjects are their own controls. Its specificity for CYP3A activity is also debated (Galteau and Shamsa, 2003). The urinary ratios of 6β-hydroxycortisone to cortisone and 7β-hydroxy-dehydroepiandrosterone (7β-hydroxy DHEA) to DHEA have also been suggested to be useful indicators of CYP3A4 activity, but this observation needs to be validated in vivo (Shin et al., 2013). One newly developed endogenous biomarker for CYP3A is the blood levels of 4β-hydroxycholesterol (Kanebratt et al., 2008; Bjorkhem-Bergman et al., 2013). The elimination half-life of 4β-hydroxycholesterol is about 17 days, resulting in stable plasma concentrations within subjects (Diczfalusy et al., 2009) but this excludes 4β-hydroxycholesterol as a marker for rapid changes in CYP3A4 activity. A sensitive new biomarker, preferably
measureable in urine spot samples, with a fast response to change in CYP3A activity would thus be of great benefit for the assessment of DDIs and phenotyping in humans.

Concentrations of the bile acid metabolite 1β-hydroxy-deoxycholic acid (1β-OH-DCA) were observed to increase significantly in the urine of patients taking the known CYP3A4 inducer carbamazepine (Bodin et al., 2005). This study also showed that the conversion of deoxycholic acid (DCA) to 1β-OH-DCA was catalysed in vitro by recombinant CYP3A4 and by human liver microsomes though no data was presented on the specificity of this transformation in terms of the contribution of additional enzymes from the CYP3A family or other CYPs (Bodin et al., 2005). We reasoned that it may be possible to use 1β-OH-DCA as a urinary biomarker to measure CYP3A induction and inhibition in clinical drug–drug interaction studies rather than using a conventional invasive plasma biomarker or probe drug.

The metabolite 1β-OH-DCA is not available commercially. A nine step chemical synthesis (Tohma et al., 1986) and a microbial synthesis (Carlström et al., 1981) of 1β-OH-DCA have been reported previously. Bacterial P450s have shown promise in the synthesis of drug metabolites (Gillam and Hayes, 2013) and we thought it may be possible to synthesize 1β-OH-DCA enzymatically using a commercially available bacterial mutant P450.

The aim of the present study was to investigate the 1β-hydroxylation of DCA by human liver microsomes and to assess the contribution of individual CYPs using human recombinant P450 enzymes and chemical inhibitors. To facilitate this work a library of Bacillus megaterium P450 mutants was screened using DCA as a substrate and a suitable enzyme was identified which produced the major hydroxylated DCA.
metabolite formed in HLM. The aim was to generate sufficient quantities of this hydroxylated metabolite for full structural characterization by NMR spectroscopy. A stable isotope labelled analogue $[^2H_4]-1\beta$-OH-DCA, required for bioanalytical method development was also synthesized using similar methodology. An ultra-high performance liquid chromatography/high resolution mass spectrometry (UPLC/HRMS) based assay was used for metabolite identification and quantification. The potential application of $1\beta$-hydroxylation of DCA as a urinary CYP3A biomarker was illustrated by comparison of two spot human urine samples, one pooled sample from healthy subjects as a control and one from a patient treated with carbamazepine, a CYP3A inducer.
Materials and Methods

Chemicals and Reagents

Deoxycholic acid (DCA), cholic acid (CA), chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (G-CDCA), glycocholic acid (G-CA), taurochenodeoxycholic acid (T-CDCA), taurocholic acid (T-CA), NADPH, β-glucuronidase/arylsulfatase (*Helix pomatia*) and choloylglycine hydrolase from *Clostridium perfringens* (*C. welchii*) were obtained from Sigma-Aldrich (Dorset, UK). Deoxycholic -2,2,4,4-d₄ acid (Lot No. M403P52, 99 atom % D) was purchased from CDN isotopes, Quebec, Canada. Deoxycholic acid 3-sulfate (DCA-3-S) and ketoconazole were obtained from Compound Management, AstraZeneca Gothenburg (Sweden). Chenodeoxycholic acid-24-acyl-β-D-glucuronide (CDCA-24-G) was purchased from Toronto Research Chemicals (Toronto, Canada). Pooled human liver microsomes (HLM) were purchased from BD Gentest (Woburn, MA) and recombinant human CYPs 1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 3A7, 4A11, 4F2, 4F3, 17A1 and 46A1 were obtained from Cypex Ltd (Dundee, Scotland, UK). Pooled human hepatocytes were purchased from Celsis-In Vitro Technologies (Baltimore, MD, USA). Microcyp™ screening plates (MCYP-0343), purified P450 enzyme (MCYP0029) and glucose dehydrogenase (GDH) were purchased from Codexis (Redwood City, CA, USA). Acetonitrile (LC–MS grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (purity 98-100%) were purchased from Merck KGaA, Germany. All other chemicals and solvents were of the highest quality available commercially.
Spot human urine samples

A spot human urine sample originated from a reported clinical study in which all patients were on monotherapy with carbamazepine for at least 1 year and, apart from epilepsy, were healthy (Bodin et al., 2005) (approved by the local Human Ethics committee). Control urine was prepared by pooling 2 ml of spot urine samples collected from six healthy subjects.

Screening for BM3 P450 catalysed conversion of deoxycholic acid

The screening of DCA against the Codexis Microcyp 96-well screening plate A (MCYP-0343) was performed as follows: DCA (20 mg, 0.05 mmol) was dissolved in acetonitrile (1.0 ml) and added to a solution of MCYP-RXN buffer (46.6 mL, 0.802 g) in deionized water (28.1 ml). The MCYP-RXN buffer contained glucose (30 mM), NADP+ (1.2 mM), glucose dehydrogenase (0.6 mg/ml) in potassium phosphate buffer (120 mM). The solution was adjusted to pH 8.0 with NaOH (0.1 M). Aliquots (0.25 ml) were pipetted into each enzyme well and the solution shaken at 30°C at 250 rpm in an orbital shaker. An aliquot of reaction mixture (10 µL) was removed from each well of the 96-well screening plate and diluted with 90 µL of mobile phase A (0.1% formic acid in water). The mixture was centrifuged using a benchtop centrifuge at 1200 × g then transferred to either a 96-well Nunc plate or to glass injection vials (Waters) prior to UPLC/HRMS analysis. LC/MS data was processed using Metabolynx (Waters) software. Hit enzymes were identified on the basis of forming a ‘+16 Da’ (monooxidised) metabolite of DCA with retention time 3.22 min.

Enzymatic synthesis of 1β-OH-DCA and [2H₄]-1β-OH-DCA using MCYP0029

Preparation of 1β-OH-DCA; (1β,3α,5β,12α)-1,3,12-trihydroxycholan-24-oic acid.
To a 250 mL conical flask cooled in an ice/water bath was added phosphate buffer (0.12 M, 95 mL, pH 8), D-glucose (0.45 g, 2.5 mmol), NADP⁺ (74.3 mg, 0.1 mmol) and glucose dehydrogenase (50 mg). Deoxycholic acid (39.2 mg, 0.1 mmol) was dissolved in MeOH (100 µL) and added to the reaction flask. MicroCYP0029 (151 mg, 100 nmol) was dissolved in phosphate buffer (0.12 M, 5 mL, pH 8) and the solution added to the reaction flask. The flask was shaken in an orbital shaker at 200 rpm, 30°C and the reaction monitored by UPLC/HRMS. After 16 hrs the reaction was quenched with cold acetonitrile (100 mL) and the solution was allowed to stand in a refrigerator at 4°C for 30 mins after which the contents transferred to Falcon tubes (4 × 50 mL) and centrifuged (4000 × g, 4°C) for 15 mins. The supernatents were decanted off, pooled and concentrated on a rotary evaporator. After removal of the organics the aqueous was split between two Oasis HLB SPE cartridges (2 × 6 cc, 200 mg). Each cartridge was washed with H₂O (1 × 5 mL) and eluted with acetonitrile (1 × 5 mL). The organic eluates were pooled, concentrated to dryness under an N₂ stream and the crude residue purified by mass directed preparative HPLC.

Preparation of [²H₄]-1β-OH-deoxycholic acid, (1β,3α,5β,12α)-1,3,12-trihydroxy(2,2,4,4-²H₄)cholan-24-oic acid.

The [²H₄]-1β-OH-deoxycholic acid was prepared using the same methodology as that used for the unlabelled analogue. Deoxycholic-2,2,4,4-d₄ acid (39.3 mg, 0.1 mmol) was used as substrate.

Mass directed preparative HPLC purification of 1β-OH-DCA and [²H₄]-1β-OH-DCA

The residue from the concentrated SPE eluate was dissolved in DMSO (1 mL) and purified by mass directed preparative HPLC using a Waters Sunfire C₁₈ ODB column.
Mobile phases were 0.1 M NH$_4$OAc (Solvent A) and acetonitrile (Solvent B). Gradient was 10% B increasing to 50% over 20 mins. Flow rate was 60 mL/min. A Waters ZQ mass spectrometer was used for mass detection. 1β-OH-DCA eluted between 11.0-11.8 mins. Fractions containing the targeted mass of m/z 407, corresponding to mono-oxidised DCA were pooled, the organics were removed on a rotary evaporator and the aqueous residue was subjected to SPE on a Waters Oasis HLB cartridge (500 mg). The cartridge was washed with water (3 mL), and allowed to dry under vacuum and then eluted with MeOH (3 mL). The MeOH eluate was concentrated to dryness yielding a white solid (10.5 mg) with mass of m/z 407.2799 (Figure 1).

The [2H$_4$]-1β-OH-DCA was purified using identical methodology and yielded (13.4 mg) as a white solid with a mass of m/z 411.3060 (Figure 1).

**NMR characterization of 1β-OH-DCA and [2H$_4$]-1β-OH-DCA**

NMR spectra were recorded on a Bruker 600 MHz AVANCE III system equipped with a 5 mm QCI Cryoprobe using standard Bruker pulse sequences. Experiments were run in deuterated methanol (CD$_3$OD) at 25°C. Chemical shifts are referenced relative to the residual methyl signal in CD$_3$OD set to 3.31 ppm (1H) or 47.8 ppm (13C). NMR assignments of 1β-OH-DCA and [2H$_4$]-1β-OH-DCA were based on 1H and 13C chemical shifts and 2D correlations via 1H-1H COSY, ROESY and 1H-13C HSQC, 1H-13C HMBC experiments.

**Incubation of DCA in HLM**

Incubation of DCA in HLM was performed in 96-well plates in a shaking incubator at 37°C. The incubation mixture contained DCA (at 2 and 20 µM, respectively), 1 mM NADPH, and HLM at a protein concentration of 0.5 mg/mL in potassium phosphate buffer.
buffer (0.1 M, pH 7.4). The total incubation volume was 50 µL/well. All incubations were performed in duplicate. Following 5 min sample preincubation at 37°C, the reaction was initiated by the addition of NADPH and was stopped by adding 100 µL of ice-cold acetonitrile after 60 min. The reaction mixture was then centrifuged at 4,000 × g for 20 min, 4 °C, and an aliquot of 50 µL of supernatant was diluted with an equal volume of water. The resulting mixture was analysed by UPLC/HRMS.

To investigate the effect of the CYP3A inhibitor ketoconazole on the hydroxylation of DCA, similar incubation conditions were conducted in HLM with or without co-incubation of ketoconazole at concentrations of 0.1, 1 and 10 µM. The reaction was initiated by the addition of NADPH and was stopped by the addition of ice-cold acetonitrile after 30 min. All samples were prepared for UPLC/HRMS analysis as described previously.

**Incubation of DCA in a panel of 21 recombinant human CYPs**

A panel of 21 recombinant human CYPs (CYPs 1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 3A7, 4A11, 4F2, 4F3, 17A1 and 46A1; 100 pmol/mL) were screened to investigate their catalytic activity on the 1β-hydroxylation of DCA. The incubation conditions were similar to those in HLM. In brief, the incubation mixture contained 100 pmol/mL of recombinant human CYP, 2 or 20 µM of DCA, and 1 mM NADPH in potassium phosphate buffer (0.1 M, pH 7.4). For the purpose of metabolite characterization, an incubation time of 60 min was used.

**Incubation of DCA and 1β-OH-DCA in human hepatocytes**

DCA and 1β-OH-DCA were incubated in human hepatocytes separately. Each incubation (50 µL/well) contained 1 million cells per mL and test compound, DCA or 1β-OH-DCA, respectively, at a concentration of 4 µM, in 96-well flat bottomed
polyethylene plates at 37°C, 95% air, 5% CO₂ in saturated humidity. After 10 min pre-incubation at 37°C, the test compound was added to start the reaction. At 0, 40 and 120 minutes, the reactions were stopped by addition of 150 µL of ice-cold acetonitrile and kept on ice for at least 20 min before centrifugation at 4000 × g and 4°C for 20 min. The supernatants were analyzed by UPLC/HRMS. The effect of ketoconazole on DCA metabolite formation was also examined with and without the addition of ketoconazole (3 µM) in hepatocyte incubations with DCA for 4 hours.

**Determination of 1β-OH-DCA and DCA in human urine**

The analyses of 1β-OH-DCA and DCA in human urine were performed using a modified method originally described by Bodin et al. (Bodin et al., 2005). To 200 µL of urine, an aliquot of 200 µL of 0.1 M sodium phosphate buffer (pH 5) was added followed by the addition of 5 µL of β-glucuronidase/arylsulfatase and 5 µL of choloylglycine hydrolase (2 U/µL), respectively. The mixture was incubated overnight at 37°C. After the enzymatic hydrolysis of glucuronides and amino acid conjugates, an aliquot of 50 µL of the reaction mixture was quenched with acetonitrile (150 µL, containing 200 nM [²H₄]-1β-OH-DCA as internal standard) and centrifuged at 10 000 × g for 10 min. The supernatant was separated and diluted with an equal volume of water for UPLC/HRMS analysis. A second aliquot of incubation mixture (150 µL) was quenched with acetonitrile (150 µL containing 200 nM [²H₄]-1β-OH-DCA as internal standard) and centrifuged at 4 000 × g for 20 min. An aliquot of 150 µL of the supernatant was separated and evaporated under nitrogen to a reduced volume of less than 50 µL. The remaining liquid was acidified by adding formic acid (0.1% in H₂O, 50 µL). The resulting mixture was loaded onto an Oasis MAX 96-well µElution Plate (2 mg sorbent per well, 30 µm particle size, Waters) pre-conditioned with methanol and water. Loaded cartridges were then successively washed with 0.2 mL.
water and eluted with 150 µL isopropanol-acetonitrile (6:4, v/v). The eluate containing bile acids was then solvolyzed. To the eluate, an aliquot of 100 µL acetone and 40 µL of hydrochloric acid (1 M) was added and incubated for 1 hour at 37°C. After 1 hour, the solvent was evaporated and the residue reconstituted in 0.5 mL NaOH (4 M) and 1.2 mL isopropanol and incubated overnight at 70 °C. The incubated mixtures were then neutralized with the addition of formic acid (200 µL, 98%) at the end of the solvolysis and the solvent evaporated to a volume of less than 300 µL. The remaining aqueous phase was diluted with 200 µL of 0.1% fomic acid and a second extraction was performed on an Oasis MAX 96-well μElution Plate using the same method described previously. The eluate was then evaporated under nitrogen and the residue was dissolved in 100 µL 50% acetonitrile solution prior to UPLC/HRMS analysis. Calibration standards were constructed by spiking serial concentrations (16 to 5000 nM) of DCA and 1β-OH-DCA into 0.1 M sodium phosphate buffer (pH 8). The standard solutions were then subjected to sample preparation in parallel with urine samples including enzyme hydrolysis and solvolysis. Reference solutions in sodium phosphate buffer (pH 5) containing 2 µM of G-CDCA, T-CDCA, G-CA, T-CA, CDCA-24-G or DCA-3-S were subjected to enzymatic hydrolysis using a mixture of β-glucuronidase/arylsulfatase and choloylglycine hydrolase to monitor the hydrolysis of bile acid amide, glucuronide and/or sulfate conjugates.

**UPLC/HRMS analysis**

To separate and identify the hydroxylated metabolites of DCA, a Synapt G2 Q-TOF mass spectrometer equipped with an ACQUITY UPLC system (Waters Corp.) was used. The UPLC/HRMS system was operated by MassLynx software (version 4.1, Waters Corp.). UPLC separations were performed on an Acquity UPLC BEH C18 column (2.1×100 mm, 1.7 µm; Waters, Milford, MA). Mobile phase A was 0.1% formic
acid aqueous solution and mobile phase B was acetonitrile. The initial mobile phase
was 90:10 A-B, and was transitioned via a linear gradient to 10:90 A-B over 6 min.
The flow rate was 0.5 mL/min and the total run time was 7 minutes. The column oven
and autosampler were set at 45 °C and 8 °C, respectively. The UPLC eluent was
introduced into the Q-TOF mass spectrometer with an electrospray ionization source
(ESI) in negative mode. Specific mass spectrometric source conditions were as
follows: capillary voltage of 0.5 kV, sample cone voltage of 40 V, desolvation
temperature and source temperature were of 550 and 150 °C, respectively. HRMS
and MS^E scan functions were programmed with two independent collision energies
(CE). The HRMS spectra were obtained with a low collision energy, where the
transfer CE and trap CE were 0 eV and 4 eV, respectively. The MS^E spectra were
acquired with a high collision energy, where the transfer CE and trap CE were 30 eV
and were ramped from 15 to 35 eV, respectively. Data were acquired over the range
m/z 100–1200. In the analysis, extracted ion chromatograms (XIC) at m/z 391.285
(DCA), m/z 407.280 (1β-OH-DCA) and m/z 411.305 ([2H₄]-1β-OH-DCA) were
generated with a mass tolerance of 10 mDa. Reference standards were analyzed in
parallel for definitive confirmation. Quantification of the 1β-OH-DCA and DCA was
performed using TargetLynx. The concentrations of bile acids were calculated based
on the peak area ratio of each compound to the internal standard and compared to
the calibration curves. Mass responses for standards of DCA, 1β-OH DCA, CA, G-
CA, T-CA, CDCA, G-CDCA and T-CDCA were similar by the UPLC-HRMS method
used. This allowed a semiquantitative assessment of the relative abundance of DCA
and 1β-OH-DCA and their conjugated metabolites in hepatocytes to be made by
comparison of peak areas. The metabolite identification of DCA and 1β-OH-DCA in
different samples was performed using Metabolynx (Waters) software.
Results

Enzyme synthesis and structural characterization of 1\(\beta\)-OH-DCA.

Deoxycholic acid was screened against a library of commercially available BM3 P450 mutants looking for formation of its major oxidized metabolite (M3) which had been identified in HLM and rCYP3A4 incubations. Chromatographic retention time and mass spectral data for M3 were known allowing a focussed screen to be performed. Only BM3 mutants forming M3 in good conversion and ideally as a single metabolic product were of interest. Of the 90 mutants screened only MCYP0027 and MCYP0029 gave >35% conversion to a +16 Da metabolite which co-chromatographed (retention time 3.22 mins) with the desired major HLM metabolite based on semi-quantitative estimates of parent compound consumed and M3 formed (Figure 2). The enzyme MCYP0029 was selected for scale up due to better conversion (ca 50-60%) and a simpler metabolite profile. Interestingly a wide range of mono-oxidized metabolites of DCA were also produced by the enzyme library with retention times at 3.06, 3.59, 3.68, 3.73 and 3.98 mins. The identity of these metabolites have not been investigated further.

A weighable quantity of M3 (which we reasoned was 1\(\beta\)-OH-DCA) was required for structural confirmation by NMR. Larger quantities of both 1\(\beta\)-OH-DCA and [\(\text{H}_4\)]-1\(\beta\)-OH-DCA were also required for use as bioanalytical standards in forthcoming clinical DDI studies. Therefore scale up experiments using MCYP0029 and either DCA or deoxycholic -2,2,4,4-\(d_4\) acid as substrate, were performed at 0.1 mmol scale and monitored by UPLC/HRMS. After work up and purification by mass directed preparative LC the colourless solids were characterized by NMR spectroscopy.
NMR spectroscopy confirmed that the identity of M3 was indeed 1β-OH-DCA. The NMR assignments of 1β-OH-DCA and [2H₄]-1β-OH-DCA were compared to the fully assigned ¹H and ¹³C spectra of DCA as shown in Table 1. Numbering below follows the IUPAC approved atom numbering of steroids. Some marker groups of DCA are the OH substituted positions CH(12); 3.96, 72.8 (¹H, ¹³C chemical shift in ppm) and CH(3); 3.53, 71.3, methyl groups CH₃(18); 0.71, 12.0, CH₃(19); 0.93, 22.5 and CH₃(21); 1.01, 16.4. CH₂(1) is found at 0.98(dt, J=14.2, 14.2, 3.4)/1.78, 35.2 ppm.

The equatorial (β) ¹H signal from CH(12) appeared as a triplet with 2 small J-couplings (1.8, 1.8 Hz) to the axial and equatorial CH₂(11) protons. The axial (β) ¹H signals from CH(3) appeared as a triplet of triplets with two large trans-couplings and two smaller cis-couplings (11.1, 11.1, 4.6, 4.6 Hz). ¹H NMR spectra of 1β-OH-DCA and [2H₄]-1β-OH-DCA are shown in Figure 3. ¹³C NMR spectra of 1β-OH-DCA and [2H₄]-1β-OH-DCA are shown in Supplemental Data Figure S1.

In 1β-OH-DCA the signals assigned to CH₂(1) in DCA are missing and a new CH signal appeared at  δ 3.82, 72.9 ppm indicating hydroxylation at this position. The assignment of this signal to CH(1) was supported by COSY cross-peaks to CH₂(2), CH₃(19) (Supplemental Data Figure S2), ROESY cross peaks indicated its close proximity to CH₂(2), CH₃(19) and CH₂(11eq (α)) at 1.43 ppm (Figure 4) as well as C-H long-range correlations to C(10), CH₂(2), CH₃(19). The ¹H coupling constants (t, J~2.6, 2.6 Hz) to CH₂(2) were consistent with an equatorial configuration of the proton i.e. OH in the axial (β)-position.

It should be noted that the axial (β) ¹H signal from CH(3) which appeared in DCA as a well resolved triplet of triplets, were not as well resolved in 1β-OH-DCA though, again, two large and two small couplings were observed. This difference was
explained by the fact that, in 1-β-OH-DCA, the resonances from the axial(α) CH(4) and CH(5) appear at approximately the same shift resulting in 2nd order J-coupling effects on CH(3).

The NMR data of [2H₄]-1β-OH-DCA, deuterated at positions C(2) and C(4), confirmed the findings of 1β-OH-DCA discussed above. As expected ¹H signals from CH(1); 3.81, 72.8 and CH(3); 3.96, 65.6 now lacked J-couplings and appeared as singlets (Table 1). Notably the ¹³C signals of C(2) and C(4) broadened and disappeared in the spectrum due to quadrupolar coupling to ²H and lack of Overhauser effect to the directly bonded ¹H. Also notable were the ²H induced isotope shifts, δᵢ, observed in the ¹³C spectrum of [2H₄]-1β-OH-DCA compared to 1β-OH-DCA, which decreased with the number of bonds from the deuterated carbons; δᵢ, C(3);0.22, C(5);0.18, C(6);0.04, C(1);0.09, C(10);0.06, C(9);0.01 ppm (Supplemental Data Figure S3).

1β-Hydroxylation of DCA in human liver microsomes and the inhibitory effect of CYP3A inhibitor ketoconazole.

Incubations of DCA with HLM in the presence of NADPH resulted in four hydroxylated metabolites (M1 to M4, m/z 407.280) with M3 as the most abundant one (Figure 2). A further oxidized metabolite (-2 Da) was also found in the incubation. The profiles of the hydroxylated metabolites of DCA when incubated at 20 μM (data not shown) were similar to those incubated with 2 μM of DCA (Figure 2). Metabolites M3 and M4 were identified as 1β-OH-DCA and CA, respectively, by comparison with the synthesized and commercially available reference standards. In comparison with the control samples, the formation of 1β-OH-DCA formation was significantly inhibited by ketoconazole by 37%, 93% and 100% at ketoconazole concentrations of 0.1, 1, and
10 µM, respectively, when incubated at 2 µM of DCA (Figure 5), and by 22%, 89% and 98%, respectively, when incubated at 20 µM of DCA.

**P450 enzymes involved in the 1β-hydroxylation of DCA.**

To identify the specific P450s that catalyzed DCA 1β-hydroxylation, a panel of 21 recombinant human CYP isozymes was screened and compared to the profiles obtained in human liver microsomes. The same conclusion was drawn from incubations at both 2 and 20 µM of DCA. Of the 21 recombinant human CYPs tested, CYP3A4 and 3A7 showed the highest catalytic activity of 1β-OH-DCA formation and CYP3A5 and CYP46A1 showed much less activity towards this reaction. Only trace amounts of 1β-OH-DCA were detected in CYP2C8 incubations and none was detected in the incubation of the other CYPs investigated (Figure 6).

**Metabolite profiles of DCA and 1β-OH-DCA in human hepatocytes.**

Both DCA and 1β-OH-DCA were rapidly metabolized in human hepatocytes. No parent DCA was detected after a 40-min incubation and the glycine conjugate (G-DCA) was the predominant metabolite formed. Additional minor metabolic pathways were also detected including direct taurine conjugation and the combination of G-DCA with oxidation and sulfation (Figure 7). Similar metabolic pathways were also detected in incubations of 1β-OH-DCA. After 40 min incubation, the remaining parent 1β-OH-DCA accounted for 44% of the 0 min sample and the direct glycine conjugate (G-1β-OH-DCA) was the only metabolite detected. With a longer incubation time of up to 2 hours, low levels of the direct taurine conjugate and the metabolites derived from the combination of desaturation and sulfation of G-1β-OH-DCA were also detected. It is noteworthy that the MS peak corresponding to the G-1β-OH-DCA in 1β-OH-DCA incubations was also detected in hepatocyte incubations of DCA, where
the MS peak area of the G-1β-OH-DCA was approximately 11% of the peak area of G-DCA after 2 hours incubation of DCA. Furthermore, the formation of the G-1β-OH-DCA was totally abolished in the co-incubation of DCA with ketoconazole in human hepatocytes. However, no significant differences were observed on the formation of amidated conjugates (glycine and taurine conjugates) of these two bile acids with or without coincubation with ketoconazole.

**Determination of 1β-OH-DCA and DCA in human urine samples.**

Urine samples from healthy subjects and from a patient on monotherapy with carbamazepine were prepared by enzymatic deconjugation followed by chemical solvolysis. Table 2 shows the concentrations of 1β-OH-DCA, DCA and their ratios expressed as 1β-OH-DCA/DCA in urine samples following enzyme hydrolysis with and without solvolysis. After enzyme hydrolysis and chemical solvolysis the ratio of 1β-OH-DCA to DCA in the urine (ratio of 2.8) from a carbamazepine-treated patient was 7-fold higher than that in the control urine (ratio of 0.4). Urine samples following only enzymatic hydrolysis also showed that the ratio of 1β-OH-DCA/DCA was 7-fold higher in patient urine (ratio of 1.3) than that in the control sample (ratio of 0.2) (Figure 8). The efficiency of the enzyme catalyzed deconjugation was estimated by monitoring the disappearance of the reference conjugates and the formation of their corresponding free bile acids after incubation with the enzymes. The β-glucuronidase/arylsulfatase catalyzed hydrolysis of glucuronides and choloylglycine hydrolase catalyzed hydrolysis of the amidated conjugates (glycine and taurine conjugates) of bile acids was determined to be complete by UPLC analysis. However, the desulfation of the reference compound DCA-3-S was poor with only low levels of deconjugated DCA formed, indicating the poor catalytic activity of the
arylsulfatase to the desulfation reaction of these sulfated bile acids under the experimental conditions.

Discussion

A prerequisite for using an endogenous metabolite as a sensitive biomarker for probing a particular enzyme activity in humans is that the metabolite is formed selectively by the enzyme of interest. The current study clearly shows that the formation of 1β-OH-DCA from DCA is predominantly catalyzed by CYP3A enzymes. The only other CYP enzyme investigated that produced a significant amount of this metabolite was CYP46A1 which is a cholesterol metabolizing enzyme located in the brain (Dutheil et al., 2009). Since the level of bile acids in the brain is very low (Bron et al., 1977) the contribution of CYP46A1 to the overall production of 1β-OH-DCA in humans should be minor thereby making the formation of 1β-OH-DCA from DCA a specific biomarker for CYP3A activity.

The finding that CYP3A7 has a high capacity to produce 1β-OH-DCA is intriguing. CYP3A7 is mainly a fetal form of CYP3A that is down regulated after birth (Kitada et al., 1985). A high capacity of the fetal liver to metabolize DCA and specifically form its 1β-hydroxylated metabolite was described by Gustafsson et al. (Gustafsson et al., 1985). CYP3A7 is very active in the fetal liver, peaks during the first weeks after birth and then progressively declines. At the same time CYP3A4 levels begin to rise reaching 30-40% of adult levels after one month (Lacroix et al., 1997). There are known substrate specificity differences between CYP3A7 and CYP3A4, e.g. the catalytic efficiency of CYP3A7 to metabolize midazolam is much lower than for CYP3A4 and CYP3A5 (Williams et al., 2002). The specificity of CYP3A to form 1β-OH-DCA from DCA could also be developed into a useful in vitro tool for phenotyping
in human liver microsomes and human hepatocytes, keeping in mind the extensive
glycine conjugation of both DCA and 1β-OH-DCA observed in human hepatocytes.

Human P450s metabolise the majority of current small molecule therapeutics (Rendic
and Guengerich, 2015). Their use for preparative biotransformation is limited
primarily due to poor stability. Bacterial P450s continue to show promise for the
synthesis of drug metabolites (Gillam and Hayes, 2013) with mutant enzymes from
*Bacillus megaterium* being particularly thoroughly investigated (Sawayama et al.,
2009; Reinen et al., 2015). Both protein and process engineering approaches
continue to enhance the possibilities of using these diverse catalysts at scale (Kiss et
al., 2015; Venkataraman et al., 2015). The synthesis of rare and unusual
endogenous metabolites with engineered P450s is less well explored. In this study
we have successfully synthesized at mg scale both unlabelled 1β-OH-DCA and [2H₄]-
1β-OH-DCA using a BM3 mutant P450, in one step with high stereo and regio-
selectivity.

Bile acids constitute a large family of steroids carrying a carboxyl group in the side
chain. DCA is a secondary bile acid and mainly excreted in urine as conjugates, the
sulfated and amidated metabolites and their combinations being the major ones
(Alme et al., 1977; Bathena et al., 2015a; Bathena et al., 2015b). In this study, we
have established that the 1β-hydroxylation of DCA is specifically catalyzed by CYP3A
and therefore has potential to be used in the clinic as a biomarker reflecting CYP3A
activity in DDI investigations. To be able to evaluate the applicability of this metabolic
reaction as a urinary CYP3A biomarker, establishing a robust and sensitive analytical
method to determine total 1β-OH-DCA and DCA concentrations was critical. One
approach to achieve this goal was to cleave the conjugates of these two bile acids,
enzymatically and chemically, releasing the free bile acids for quantification (Bodin et al., 2005; Kakiyama et al., 2014). Using this approach, the quantification of the non-conjugated 1β-OH-DCA and DCA by LC/MS is rather straightforward with access to the reference standards. The disadvantage of this method is that the sample preparation steps are very time consuming and the chemical cleavage reaction may be incomplete or give side reactions. Another approach is to directly measure concentrations of free bile acids and their corresponding amidated and sulfated conjugates to obtain the total concentrations of bile acids. However, this approach suffers from the lack of access to some of the reference standards for accurate quantification. In this study, we modified a reported sample preparation method (Bodin et al., 2005). In addition to the reported enzyme hydrolysis by β-glucuronidase, we have used choloylglycine hydrolase to deconjugate the predominant amidated conjugates of 1β-OH-DCA and DCA in urine, followed by solvolysis to complete desulfation. The urinary concentrations of DCA after enzymatic hydrolysis and solvolysis were in the range of that reported for total DCA concentrations in urine (Bathena et al., 2015a). The concentration ratio of 1β-OH-DCA/DCA was used as a marker for CYP3A activity instead the individual concentration. Using this method, the impact of variable DCA levels, as a substrate for 1 β-hydroxylation, can be minimized. Our results showed the ratio of 1β-OH-DCA/DCA was 7-fold higher in the urine from a patient with elevated CYP3A activity than that in the control sample. It is interesting to note that in performing only the mild enzymatic hydrolysis without solvolysis, the ratio of 1β-OH-DCA/DCA in the patient urine was again 7-fold higher than that in the control, though the concentrations of DCA and 1β-OH-DCA obtained were lower than those in samples treated by an additional chemical solvolysis step. This observation indicated that the improved
enzymatic deconjugation method may be robust enough to provide data that differentiates between ratios of 1β-OH-DCA/DCA observed with differing levels of CYP3A activity.

In this study, the relative contribution of CYP3A4 versus CYP3A5 to DCA 1β-hydroxylation has not been studied in detail and needs further investigation. However, screening data from 21 rCYPs showed that 1β-OH-DCA formation by rCYP3A4 was approximately 50- and 10-times higher than rCYP3A5 at DCA concentrations of 2 and 20 μM, respectively, and highlights the major contribution of CYP3A4 compared with CYP3A5. The role of active transport of 1β-OH-DCA and its conjugates into urine has not been fully explored. Future studies validating 1β-OH-DCA as a CYP3A biomarker need to consider possible effects of inhibitors and inducers on transporter functions as possible confounding factors.

The value of using 1β-OH-DCA as a urine biomarker for CYP3A activity needs to be further studied in a large set of clinical samples to understand its variability over time and intersubject differences. It is clear that if the analysis of spot urine samples reveals human CYP3A activity, this would provide an obvious advantage in clinical DDI studies. Urine sampling over an extended period of time could be avoided, as is the case when using the biomarker 6β-hydroxycortisol. The 6β-hydroxycortisol/cortisol ratios exhibit large diurnal variability in urinary levels thereby necessitating urine collection over a long period of time to obtain samples representing CYP3A activity (Shin et al., 2013).

The dynamic range of a biomarker should be large enough to clearly display effects on CYP3A activity both by enzyme inhibitors and inducers. Ideally the change in 1β-OH-DCA in spot urine should reflect the changes in CYP3A activity in subjects.
treated with inhibitors or inducers. A carefully designed clinical study is needed to optimize treatment and sampling schedules to evaluate this biomarker. Generally potent inhibitors and inducers of cytochrome CYP3A are excluded from further development based on preclinical evaluation. However, some compounds with the potential for clinically relevant drug interactions do advance into human clinical studies and some are eventually approved for use in patients. A useful biomarker should be able to reliably detect and estimate the level of changes in CYP3A activity by weak to moderate inhibitors and inducers. A disadvantage of using 4β-hydroxycholesterol as a biomarker is the small dynamic range which is affected by CYP3A inhibition. After 14 days treatment by the strong CYP3A inhibitor ketoconazole, plasma 4β-hydroxycholesterol levels decreased by a maximum of 13% while oral midazolam levels increased 11-fold (Kasichayanula et al., 2014) highlighting the low dynamic range of this biomarker.

The plasma biomarker 4β-hydroxycholesterol was previously found to be sensitive to the CYP3A5 genotype (Diczfalusy et al., 2008). Based on the results from expressed CYP enzymes the contribution of CYP3A5 to the formation of 1β-OH-DCA is probably minor but should be investigated in subjects representing the CYP3A5 slow and extensive metabolising genotypes.

In conclusion we have shown that 1β-hydroxydeoxycholic acid is specifically formed by CYP3A4/5/7 and may have potential as an in vivo CYP3A biomarker, replacing drug probes currently used. A sensitive UPLC/HRMS method was established and an efficient BM3 P450 mutant catalysed synthesis generated sufficient amounts of the metabolite required for ongoing clinical studies.
Acknowledgements

We thank Dr. Ralf Nilsson for fruitful scientific discussions about the analysis and metabolism of bile acids.
Authorship Contributions

Participated in research design: Hayes, Li, Diczfalusy and Andersson

Conducted experiment: Hayes, Grönberg and Li

Performed data analysis: Hayes, Grönberg and Li

Wrote or contributed to the writing of the manuscript: Hayes, Li, Grönberg and Andersson
References


Diczfalusy U, Kanebratt KP, Bredberg E, Andersson TB, Bottiger Y, and Bertilsson L (2009) 4beta-hydroxycholesterol as an endogenous marker for CYP3A4/5


Footnotes

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Martin A. Hayes and Xue-Qing Li contributed equally to this work.
LEGENDS FOR FIGURES

Figure 1. MS spectra of 1β-OH-DCA (upper panel) and [2H₄]1β-OH-DCA (lower panel) formed by Codexis MCYP0029.

Figure 2. Extracted MS ion chromatograms of DCA and its hydroxylated metabolites formed following incubation of DCA in human liver microsomes (HLM), recombinant CYP3A4 and Codexis MCYP00029. Mass tolerance was 10 mDa and MS was operated under negative ionization mode. The start concentrations of DCA in incubations were 2 µM in HLM and CYP3A4, and 10 µM in Codexis MCY0029. XIC – extracted ion chromatogram.

Figure 3. ¹H NMR spectra of 1β-OH-DCA (upper trace) and [2H₄]-1β-OH-DCA (lower trace).

Figure 4. 2D-ROESY spectrum of 1β-OH-DCA showing correlations from CH(1) to CH₂(2', 2''), CH₂(11' (α), 11'' (β)) and CH₃(19).

Figure 5. Effect of ketoconazole on the formation of 1β-OH-DCA following incubation of DCA (2 µM) in human liver microsomes for 60 min.

Figure 6. Catalytic activities of DCA 1β-hydroxylation in a panel of 21 recombinant human CYPs and in HLM following 60 min incubation. Results are the average of duplicates.

Figure 7. Proposed major metabolic pathways of DCA.

Figure 8. Representative extracted MS chromatograms (XIC) of 1β-OH-DCA (m/z 407.280) (A) and DCA (m/z 391.285) (B) in the spiked standard sample (1 µM, enlarged 4 times on the y-axis), the pooled control urine and the urine from a patient with carbamazepine treatment following sample preparation by enzymatic deconjugation and chemical solvolysis. Mass tolerance was 10 mDa.
Table 1. $^1$H and $^{13}$C NMR data for $\beta$-OH-DCA, $[^2H_4]$-1$\beta$-OH-DCA and DCA.

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Chemical shifts are reported relative to the residual methanol signal set to 3.31 ppm ($^1$H) and 48.7 ppm ($^{13}$C). Chemical shifts for overlapping $^1$H signals are taken from relevant 2D experiments. $^1$H coupling constants for selected signals are obtained from 10$^5$ $^1$H-spectra. Abbreviation: s (singlet), d (doublet), t (triplet), dt (doublet of triplet), tt (triplet of triplet), m (multiplet), br (broad), n.a. (not applicable), n.d. (not determined).
Table 2 Concentrations of 1β-OH-DCA and DCA determined in a control urine and a urine sample from a patient treated with carbamazepine. Urine samples were treated using enzymatic hydrolysis with and without chemical solvolysis.

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<th>Urine</th>
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<th>DCA (nM)</th>
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</table>
Figure 1

- \([\text{M-H}]^-\) for 1\(\beta\)-OH-DCA (\(C_{24}H_{39}O_5\))
  - Mass: 407.2799

- \([\text{M-H}]^-\) for 1\(\beta\)-OH-DCA (\(C_{24}[^{2}\text{H}_4]H_{35}O_5\))
  - Mass: 411.3060

- Mass spectrum showing peaks at 408.2833, 409.2758, 412.3084, and 413.3109
Figure 3
Figure 4
Figure 5

% 1\beta-OH-DCA Formation vs Control

Control, Ketoconazole 0.1 µM, Ketoconazole 1 µM, Ketoconazole 10 µM, No NADPH
Figure 6
Figure 7
Figure 8