Molecular and Protein characterization of Human cytochrome P450 expressed Recombinant E.Coli and its application in the synthesis of Tramadol metabolite

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ABSTRACT

Drug metabolites standards are required for drug metabolic studies like pharmacological, toxicological and clinical trials. However, synthesis of metabolite standards by chemical methods is considerably difficult. Biotransformation of drugs by recombinant enzymes is an alternative method to chemical synthesis. Cytochrome P450 enzymes are known to metabolize many chemical compounds. Several studies have been done to study the expression of human cytochrome enzyme in microbial system. The aim of this study is to evaluate the potential of microbial expressed human CYP450 enzyme to synthesize drug metabolites. In the present study, human CYP450 enzyme is expressed in *E. coli* and its protein characterization was studied. Molecular characterization of the enzyme was performed for the presence of the plasmid in the recombinant culture. The protein was characterized by estimating its concentration and determining its molecular weight using SDS-PAGE. The molecular weight of the isolated protein ranged from 35-90 kDa. Further, the ability of the recombinant culture in the biotransformation of drugs was tested and it was found to efficiently convert Tramadol into its metabolites. Preparative scale of Tramadol metabolites were synthesized and purified by column chromatography and characterized by Mass chromatography. The fraction was found to contain didesmethylated metabolite. Thus, from this study, it was proved that human CYP450 enzyme expressed in recombinant *E. coli* can be used for the bioconversion of drug metabolites.

Keywords: Cytochrome P450, *E. coli*, Metabolites, Tramadol HCl, Recombinant enzyme

INTRODUCTION

Metabolism is a term used to describe all chemical reactions involved in maintaining living state of the cells and the organism. Metabolism comprises anabolism, which involves a sequence of chemical reactions that synthesizes molecules from smaller units, usually requiring input of energy (ATP) in the process and catabolism, which includes the metabolic pathways that breaks down
molecules into smaller units and release energy. Drugs are exogenous substances, which form a subgroup under xenobiotics.

Cytochromes P450 constitute a superfamily of enzymes involved in the metabolism of xenobiotics (Anzenbacher et al., 2001). The electrons are supplied by NADPH-CYP450 reductase, which transfers electrons from NADPH to CYP450. CYP450 enzymes can be induced or inhibited by many drugs and substances resulting in drug interactions in which one drug enhances the toxicity or reduces the therapeutic effect of another drug (Buck et al., 1997; Vail et al., 2005). CYP450 catalyzing lα-hydroxylation of 25-hydroxyvitamin D3 was purified from pig liver mitochondria. It was also found to catalyze the 27-hydroxylation of 25-hydroxyvitamin D3 and 25-hydroxylation of vitamin D3 (Axen et al., 1994). A CYP450 cDNA was prepared from liver cDNA by polymerase chain reaction methods and several variants with modified 5'-termini were constructed (Gillam et al., 1994). Heterologous expression of CYP105D1 in E. coli phosphate-limited medium resulted in the abundant synthesis of recombinant enzyme that was translocated across the bacterial inner membrane and processed to yield authentic, heme-incorporated P450 within the periplasmic space (Kaderbhai et al., 2001). In this contribution, an update of human CYP450 enzymology and pharmacogenetics is given with particular emphasis on CYP1B1, CYP2B6, CYP2E1 and CYP3As (Ingelman-Sundberg, 2004). A biotechnological approach using human CYP isozymes heterologously expressed in fission yeast was developed for the synthesis of drug metabolites (Peters et al., 2009).

In another study, human CYP3A4 was recombinantly expressed by surface display in a gram-negative bacterium (Schumacher et al., 2012). Nardo and Gilardi (2012) optimized the production of metabolites of human enzymes cytochrome P450, P450 BM3 from Bacillus megaterium. The most important polymorphic CYPs were found to be 1A2, 2D6, 2C9 and 2C19. Thirty-four common allele variants of CYPs in Caucasians led to altered enzyme activity. 199 non-synonymous SNPs of CYPs with frequencies were found over one percent in 1000 individual genomes (Preissner et al., 2013; Bozina et al., 2009). In a recent study, it was found that the expression level of CYP3A4 when coexpressed with cytochrome b5 (cyt b5) in E. coli was 20-60% higher than that when it was expressed alone over an extended period (48-72 h) (Dong et al., 2013). In another study, it was found that several optimizations are required for the sufficient expression of CYP450s at the desired cellular localization (Fujii et al., 2009).

Tramadol ((±) cis-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl) cyclohexanol hydrochloride) is used to treat moderate to severe pain. The molecular formula and molecular mass of Tramadol are C_{16}H_{25}NO_{2}.HCl and 299.84 respectively. Tramadol hydrochloride is a white to off-white, crystalline, odorless powder with a melting point between 180-184°C. The principal metabolic pathways, O- and N-desmethylation, involve CYP450 isoenzymes viz., 2D6, 2B6 and 3A4, respectively (Lintz et al., 1998). The primary metabolites of Tramadol viz., O-desmethy tramadol and N-desmethyltramadol can be further metabolized to three additional secondary metabolites namely,
N,N-didesmethyltramadol, N,N,O-tridesmethyltramadol and N,O-didesmethyltramadol. Approximately 10-30% of Tramadol is excreted unchanged in the urine (Ardakani et al., 2007).

It has an analgesic efficacy and potency ranging between weak opioids and morphine (Raffa et al., 1992). Pharmacokinetic studies have shown that tramadol is rapidly and almost completely absorbed after an oral administration (Kolars et al., 1994). However, its mean absolute bioavailability is only 65-70% due to the first-pass hepatic metabolism (Gibson et al., 1996). Some of the common metabolites of Tramadol are Tramadol HCl, O-desmethyl Tramadol Hydrochloride, O-desmethyl tramadol glucuronide, N,O-didesmethyl Tramadol O-β-D-Glucuronide, Tramadol n-oxide, N-desmethyl, O-desmethyl Tramadol, N-desmethyl O-desmethyl Tramadol HCl, N,N-didesmethyl tramadol N,N-didesmethyl Tramadol HCl and N-desmethyl tramadol (+)-N-desmethyl Tramadol HCl.

The objective of the present study is biotechnological synthesis of Tramadol metabolites using human CYP450 expressed in recombinant E. coli and to evaluate its characterization.

**METHODS**

**Transformation of Cloned CYP P450 gene containing Plasmid Vector in E.coli DH5α**

The human pCMV6 genes were mostly isolated directly from human cDNA libraries. The libraries were constructed using the pCMV6 mammalian expression vector (size- 5.9 kb) (Esser et al., 1996) and the Cyt P450 cDNA insert was cloned unidirectionally using a linker-based strategy with EcoR1 on the 5’ end and Xho1 on the 3’ end of the insert. The linker contributes an adopter sequence between the EcoR1 site and the start of the insert cDNA and should not be considered part of the endogenous cDNA insert sequence (vector-GAATTCGGCAGG-cDNA insert). The insert was cloned into the EcoR1 and Sal1 sites of the pCMV6-xl expression vector, destroying the jfSal1 site on the 3’ end of the insert. The cDNA can be removed from the plasmid using Not1. The plasmid was gifted from Refsyn Biosciences Pvt Ltd, Puducherry. An overnight culture of fresh E.coli DH5α was inoculated in a sterile LB broth and kept for overnight incubation at 37°C at about 120 rpm. This was used for heat shock transformation by chemical method. The transformed culture was plated on LB plates with ampicillin and kanamycin as selection markers.

**Plasmid isolation and electrophoresis of Recombinant E. Coli culture**

Plasmid was isolated from an overnight recombinant culture of E. coli by the method of Birnboim and Doly (1979). The isolated plasmid was confirmed by agarose gel electrophoresis.

**Extraction and Characterization of Recombinant E. Coli culture**

**Total Protein Extraction**

Pellet bacterial cells by centrifugation at 8000 rpm for 10 min. Transfer the supernatant to another tube carefully, making sure not to disturb the insoluble pellet. Resuspend the supernatant with ice cold acetone. Incubate it in ice for 10min. Centrifuge the supernatant at 8000 rpm for 10 min. Collect the pellet and add Phosphate buffer saline to the pellet and vortex it. Centrifuge it at 8000 rpm for 10 min and collect the pellet. The concentration of protein in the pellet was estimated by the standard method.

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of Lowry et al. (1951).

Separation of Proteins by Sodium Dodecyl Polyacrylamide Gel Electrophoresis

7.5% of resolving gel and 4.5% of stacking gel were used to prepare the polyacrylamide gel. After the gel has polymerized, the plates were kept in the electrophoresis chamber (Genei) and tightened with leveling screws. The tank was completely filled with TRIS Glycine buffer and the protein samples were loaded in the respective lanes. Power supply was given at a voltage about 80V and the electrophoresis continued till the dye front reached 0.5 cm above the gel. The gel was stained in Coomassie Brilliant Blue for about 2 to 3 hours. Destaining was done until the dye was completely removed off from the gel or until the protein bands appeared.

Screening of Drugs for the conversion of metabolites using Recombinant E.Coli culture

Drugs which were reported to be metabolized by the human enzyme CYP450 and present in the liver and gut cells were considered for the screening process. Drugs such as Tramadol, Aripiprazole, Diclofenac, Risperidone, Escitalopram and Pioglitazone were subjected to microbial breakdown under controlled conditions. Each drug was added to the individual recombinant culture for inducing metabolite formation and incubated for 120 hours. Overnight recombinant culture was used for the addition of drugs. The formation of drugs metabolites were screened at regular time intervals of 24 hours by using Thin layer chromatography (TLC). After elution in mobile phase, the compounds were visualized under UV (254 nm). After incubation for 5 days, the compounds were extracted thrice with ethyl acetate. The organic layer was separated and dried under anhydrous sodium sulphate. The organic layer was concentrated under Roteva (Superfit) and the crude material was reanalyzed by TLC using the same mobile phase.

Preparative scale Synthesis of Tramadol Metabolites

The reaction setup was done for the preparative scale synthesis of Tramadol metabolites. The recombinant culture was inoculated and incubated overnight. 20 mg of Tramadol was added to all flasks. The control flask was used as recombinant culture without drug. The flasks were kept in shaker for further 3 days. After 3 days, the crude material was extracted and analysed by TLC.

Column Purification of Tramadol crude material

The crude material was dissolved in 5ml of chloroform. 1gm of silica gel (from column chromatography 60-120 mesh) was added to the crude material. The mixture was heated gently using a water bath until the silica gel becomes free flowing. A cylindrical glass column (1.5x15 mm) was taken and plugged in a small piece of cotton. The column was mounted on the stand. 20 g of fresh silica gel (from column chromatography 60-120 mesh) was taken in a 250ml beaker. 100ml of chloroform was added into the beaker and stirred well using a glass rod to make slurry of the silica. The slurry was transferred into the column. A conical flask was kept below the mounted column and the excess solvent was drained out. The stop cock was closed when the level of the solvent reached just above the settled silica gel. The adsorbed crude material slurry was poured into the solvent layer above the silica gel in the packed column. The cotton was kept above the compound slurry. The
column was filled with chloroform and the elution was continued. Each eluent was collected separately in test tubes. The column chromatography was continued until the spot separates and increased the polarity with methanol the polarity was increased using 100% chloroform: methanol at a ratio of 90:10, 80:20 and 70:30 etc. Individual fractions were monitored by TLC. Same fractions were mixed and concentrated using roteva at 40°C under vacuum pressure.

**Characterization of purified fractions**

The purified material was analyzed by MASS by using Shimadzu-LCMS with APCI and ESI probes; LC-2010EV model to determine the molecular weight.

**RESULTS AND DISCUSSION**

**Molecular characterization of Recombinant E.Coli culture Using Agarose gel electrophoresis (AGE)**

The recombinant *E.coli* DH5α culture containing CYP450 plasmid was grown in the antibiotic resistant media which showed pale white, pin headed colonies (Figure 1). The plasmid was isolated and electrophoresed by using AGE. The DNA

![Figure 1. Transformed colonies culture in broth (A) & plate (B) &](image1)

![Figure 2. Stages of Plasmid isolation from Broth, Pellet & Plasmid in TE buffer](image2)

![Figure 3. AGE of recombinant *E. coli* ; (A) and (B) Lanes 1, 2,3,4,7 & 8 - without marker (C) and (D) Lanes 2, 4 & 6 - sample; Lane 3 – Ladder](image3)

Many studies for the expression of CYP 450 enzyme in E.Coli. Uchida et al., (2015) have earlier expressed CYPs (CYP81E11, CYP81E12, and CYP81E18) from soya bean in *E. coli* and have used them for flavonoid biosynthesis. Also, CYP51 from another plant system, *Mycosphaerella graminicola* was expressed in *E. coli* (Price et al., 2015). In addition, the CYP450 isoform 356A1 (CYP356A1) was expressed in oyster *Crassostrea gigas* recently (Silva et al., 2015). In this study also the recombinant culture were produced and characterized.
Characterization of protein in recombinant culture using SDS-PAGE

The molecular weight of CYP450 enzymes was found to range between 45 and 60 kDa. In this study, the concentration of protein in the recombinant culture was 3.14 mg/ml. The SDS-PAGE was developed and the protein bands were compared with standard markers and these showed the molecular weight of 97, 66, 43 & 30 kDa respectively (Figure 4 and Figure 5). In a study by Gangl et al., the membrane-targeting ability of CYP450 in the chloroplast of green alga *Chlamydomonas reinhardtii* was studied by PAGE analysis. Lin et al., (2002) used PAGE analysis to study the mechanism based inactivation of CYP4503A4 by 17α-ethynylestradiol. Similarly, the interaction between membrane-embedded portions of CYP450 2B4 and CYP450 b, was studied by PAGE analysis (Jecmen T et al., 2015). In another recent study, morphinan biosynthesis in opium poppy using a P450-oxidoreductase fusion protein was studied using PAGE (Winzer et al., 2015).

![Figure 4. Protein analysis by Lowry’s Method; Figure 5. Molecular weight Determination of protein in the recombinant E. coli culture](image)

<table>
<thead>
<tr>
<th>M.W. Values</th>
<th>Lane 1</th>
<th>Lane 2</th>
<th>Lane 3</th>
<th>Lane 4</th>
<th>Lane 5</th>
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Screening of Drugs using Recombinant E.Coli culture

Single colony was obtained from the transformed colonies. The culture was screened for the transformation of drugs like Risperidone, Diclofenac sodium, Tramadol HCl, Aripiprazole, Pioglitazone and escitalopram by Thin Layer Chromatography. These drugs were metabolized by CYP450 enzymes. Reaction screening was observed for 5 days (Figure 6). In a study by Kulig et al., (2015), the role of P450 fusion library of heme domains from *Rhodococcus jostii* RHA1 to metabolise Imipramine was studied. In another study by Wustrow et al., (2012), the in vitro identification of CYP450 isozymes involved in the N-demethylation of nortilidine to bisnortilidine was studied. Similarly, Leppert et al., (2011) studied the role of CYP2D6 in the metabolism of opioids. In another study by Chan et al., (2013), the hepatic clearance of compounds metabolized by CYP450 was studied using a novel hepatocyte model. Jaakkola et al., (2006) analysed the metabolism of Pioglitazone by CYP2C8 and CYP3A4 in vitro. Cytochromes mediated metabolism of Escitalopram...
S-Citalopram) has also been studied earlier (Moltke et al., 2001). Metabolic activation of Diclofenac by human CYPs has been earlier studied by Shen et al., (1999) and Bort et al., (1999) respectively. In overall screening Tramadol showed the highest conversion in TLC analysis. Preparative scale syntheses were done for the tramadol conversion.

**Figure 6. Screening of different drugs for their bioconversion using recombinant E. coli by Thin Layer Chromatography**


**Preparative scale synthesis of Tramadol HCl metabolites**

The preparative scale synthesis of Tramadol HCl was performed to get sufficient level of compound for further characterization (Figure 7 and 8).

**Column Purification of crude Tramadol compound**

The extracted compound was purified by column chromatography using chloroform and methanol solvents. The compounds showed best separation in chloroform: methanol ratio of 97:3 (Figure 9).

**Figure 7. Recombinant culture before & after Inoculation**  **Figure 8. TLC of Tramadol**

**Figure 9. Column purification of Tramadol crude sample & Crude sample slurry**
Mass Analysis of Purified fractions

Three different fractions were selected and the second fraction was analysed by Mass spectroscopy (Collins et al., 1993). The second fraction showed a molecular mass of 271.9 and was found to match either with N, N-didesmethyl tramadol HCl or N, O-didesmethyl tramadol HCl (Figure 10). The further characterization has to be continued by NMR.

Figure 10. MASS analysis of purified Tramadol compound

In a recent study by Yilmaz et al., (2015) tramadol and its metabolite (O-desmethyl tramadol) were analysed in human urine by gas chromatography-mass spectrometry. Similarly, Qiao et al., (2014) evaluated the key Licorice constituents which interact with CYP450 by LC/MS/MS profiling. In another study by Ceccato et al., (2000), tramadol and its main metabolite O-desmethyl tramadol were estimated in the human plasma by liquid chromatography-tandem mass spectrometry. Also, Vazzana et al., (2015) have analysed the pharmacokinetics, pharmacodynamics, adverse side effects, co-administration of drugs and new drug delivery systems of Tramadol hydrochloride.

CONCLUSION

The results of the present work have shown that human cytochrome p450 gene can be cloned in pCMV expression vector and expressed using a prokaryotic model -E. coli and can be used in the metabolism of Tramadol HCl.

REFERENCES


membrane-embedded portions of cytochromes P450 2B4 and b5. Methods


CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests regarding the publication of this paper.