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Facile Scale-up Biocatalytic Production of P-450 Metabolites Using a Novel Recombinant Human Catalytic System

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ABSTRACT

Classical chemical synthesis of milligram quantities of drug metabolites, which are needed for FDA-mandated metabolites in safety testing (MIST) studies, is often difficult and expensive. Alternative biotechnological approaches that typically use either liver microsomes or recombinant human drug-metabolizing enzymes also present multiple challenges. This work describes a new recombinant human P450 catalytic system that retains high catalytic activity in the presence of millimolar substrate and solvent concentrations. This system is comprised of a semipermeable matrix that contains a specific unmodified human P450 isoform, human P450-oxidoreductase, glucose-6-phosphate dehydrogenase (for NADPH recycling), catalase (which removes excess peroxide generated during the catalytic cycle), as well as required metal ions and cofactors. A catalyst suspension of 20 mg/mL in 100 mM, pH 7.4 phosphate buffer containing 2.0 mM $NADP^+$, and 5.0 mM glucose-6-phosphate was found to be optimal for the isoforms studied. CYP 2C9 was found to tolerate up to 6% (v/v) DMSO with no loss of activity and 20% DMSO with ~ 50% loss in activity. CYP 3A4 activity was only slightly reduced in the presence of up to 3% (v/v) DMSO and *N,N*-DMF. Methanol was found to be deleterious to both isoforms. The reaction rate of a stirred suspension was significantly faster than that obtained by shaking in microplates due to an increase in oxygen transfer. Post-reaction metabolite extraction of the catalyst with isopropanol or acetone was found to be superior to methanol or acetonitrile. Starting with 1.0 mM testosterone, human CYP3A4 produced 0.15 mM 6-β-hydroxytestosterone, and human CYP2C9 produced 0.59 mM 4'-hydroxydiclofenac in a single three-hour reaction cycle. Higher yields can be obtained using multi-cycle reactions.

METHODOLOGY

Reactions were performed in a total volume of 1 to 3 mL in pH 7.4 phosphate buffer containing 0.5 mM testosterone or 1.0 mM diclofenac, 3.0 mM $NADP^+$ and 5.0 mM glucose-6-phosphate. Testosterone and diclofenac were added as concentrated stocks solutions in DMSO and water, respectively. In a 16 mm by 100 mm borosilicate glass test tube, CypExpress™ was suspended in the reaction buffer containing substrate and cofactors, and a Teflon stir bar was used to agitate the reaction for three hours in a 37˚C incubator. At the end of the reaction, the suspensions were quenched with organic solvent and centrifuged at 23,100 x g for 10 minutes to provide a clear solution. HPLC analysis was performed with a 5µ, 250 mm by 4.6 mm Phenomenex Luna or a 5µ, 250 mm by 4.6 mm Phenomenex Kinetex EVO C-18 column. Spectra were collected using a photodiode array detector and the data compiled using Dionex Chromeleon software.

SOLVENT TOLERANCE

Most organic compounds of pharmacological interest are poorly soluble in aqueous media. Methanol has been used as a co-solvent to help solubilize these hydrophobic drugs and make them accessible to enzymes, and has been typically been limited to 1.0% (v/v) to prevent damaging the enzyme.

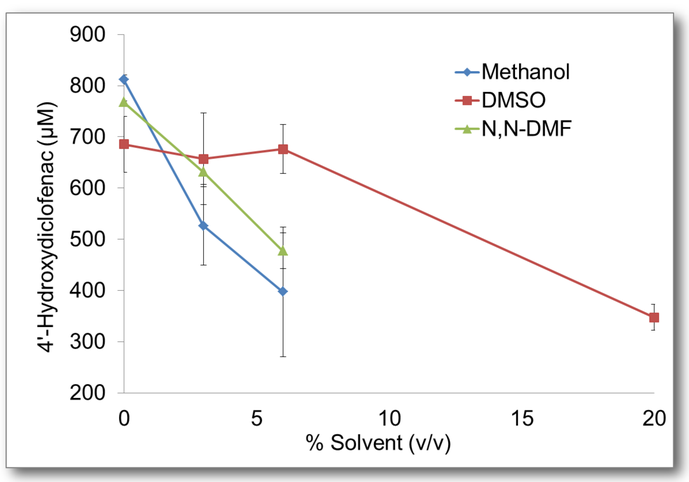

Figure 1. Effect of organic solvents on the production of 4'-hydroxydiclofenac by CypExpress™. This reaction was not affected significantly by DMSO, while *N,N*-DMF and methanol resulted in a decreased rate of metabolite production.

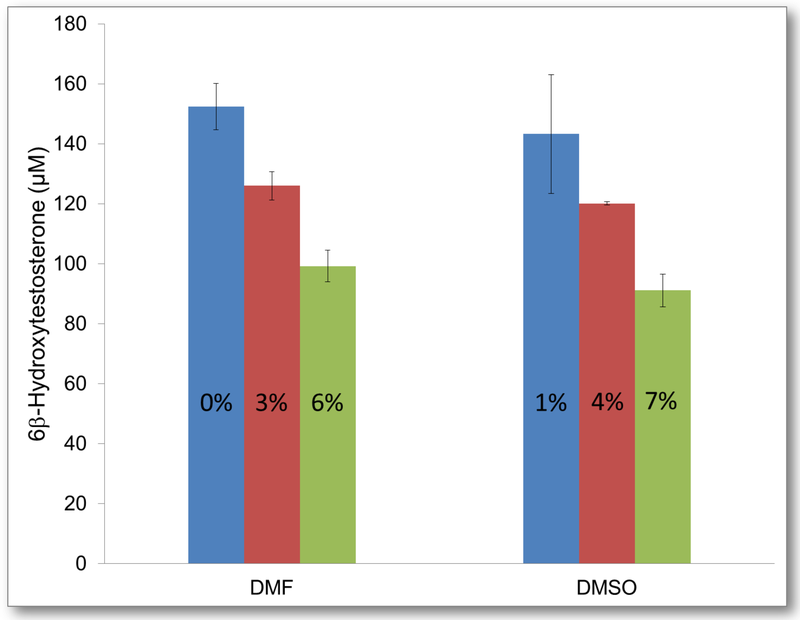

Figure 2. The effect of organic solvents on the CypExpress™ 3A4 catalyzed production of 6β-Hydroxytestosterone. Both DMSO or *N,N*-DMF were well tolerated, whereas methanol was detrimental to this catalyst.

OPTIMIZING PRODUCT ISOLATION

Extracting metabolites from reactions utilizing microsomal P450 catalysts can be difficult and may not allow for a clean biphasic extraction. The unique properties of CypExpress™ should allow for facile removal from the reaction suspension, with a liquid/liquid extraction being an attractive method for isolating metabolites post-reaction. It was determined that adding acetone or isopropanol to the crude reaction mixture removed the most metabolites from the CypExpress™ solids.

BIPHASIC SOLVENT EXTRACTION

Performing a simple liquid/liquid extraction with ethyl acetate/hexanes (50/50) provided an facile route to removing metabolites from the reaction mixture, and is especially applicable to large-volume reactions.

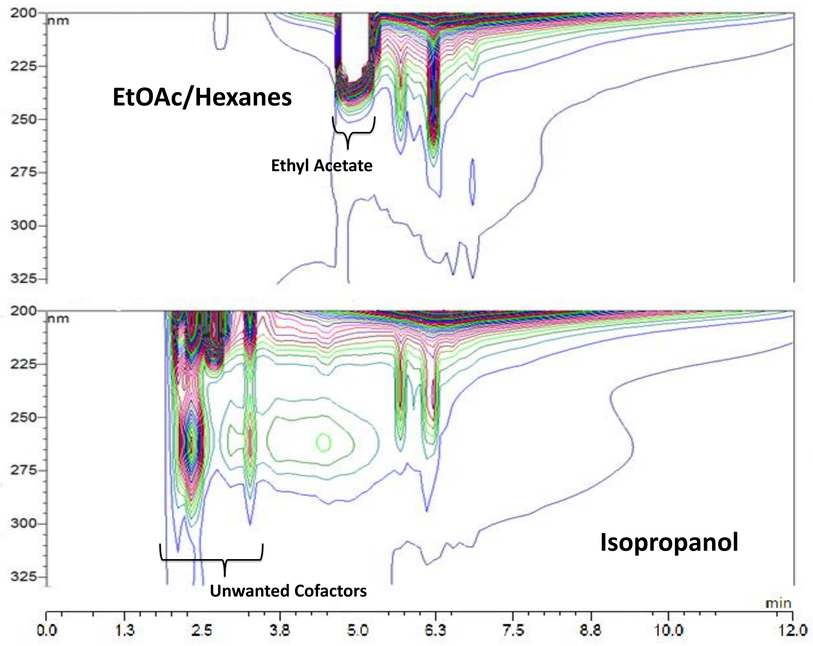

Figure 3. A topographical plot of a 3A4/testosterone reaction mixture extracted with ethyl acetate/hexanes or isopropanol. The biphasic mixture did not extract the CypExpress™ cofactors from the final reaction mixture, providing a cleaner chromatogram.

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EFFECT OF EXTRACTION SOLVENT ON YIELD

A potentially useful property of CypExpress™ is its ability to absorb drug substrates from solution, thereby increasing the proximal concentration to the P450. Following metabolism of irbesartin by CypExpress™ 2C9, several water-miscible solvents were evaluated for their ability to extract metabolites from the reaction system. The number of metabolite peaks was highest with isopropanol and acetone. Isopropanol is especially attractive for compatibility with polystyrene microplates in high-throughput systems.

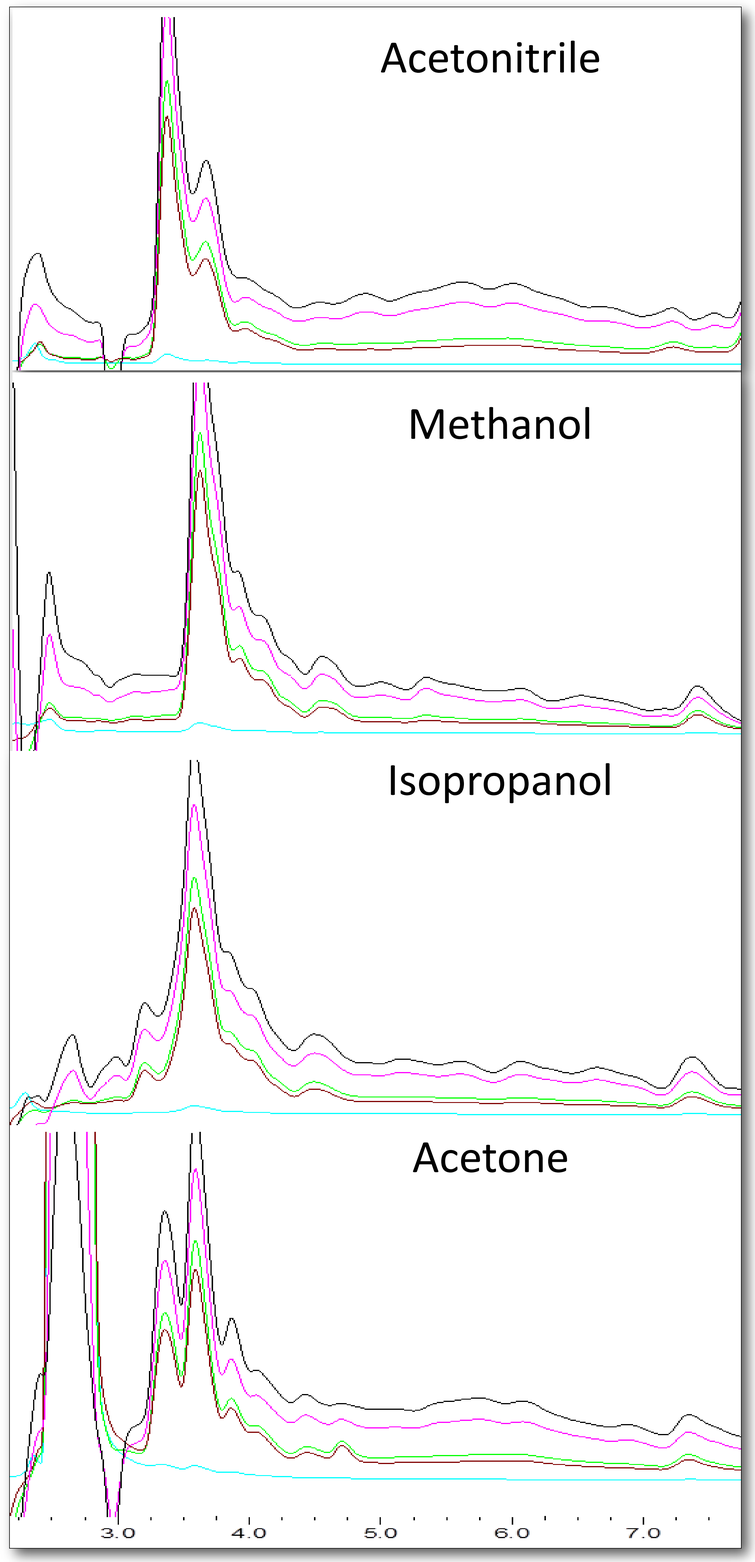

Figure 4. Various organic solvents were used to extract reaction metabolites from the CypExpress™ reaction system following catalysis.