A Novel, Robust Recombinant Human P450 Biocatalytic System

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ABSTRACT

Cytochromes P450 play major roles in the metabolism of many drug candidates, and various methods have been used to identify and synthesize P450 metabolites of new drug candidates. The large-scale preparation of milligram quantities of drug metabolites synthetically, which are needed for FDA-mandated metabolites in safety testing (MIST) studies, is a particularly lengthy and problematic step in pharmaceutical development. Available P450 systems (including microsomes and recombinant P450s) suffer from several drawbacks, including:

- the requirement for an NADPH regeneration system
- inactivation by solvents needed to solubilize the substrate
- a relatively short catalytic lifespan
- low concentrations of some P450s (microsomes)
- high cost (most recombinant P450s)

We have characterized a robust P450 Catalytic System (PCS) comprised of:
- an unmodified, recombinant human cytochrome P450 (CYP)
- an unmodified, recombinant human oxidoreductase
- $NADP^+$ and NADPH recycling system
- antioxidants

These components are all encased in a semipermeable structure that greatly stabilizes the PCS and increases its usable lifetime. Using FDA-recommended substrates, we have examined • temperature stability for storage, shipping and best reaction yields • reaction kinetics, including the ability to perform multiple reaction cycles • compatibility with solvents used to dissolve drug candidates – with little or no loss of activity using 3% (v/v) DMSO or DMF • extraction solvents, and • substrate concentrations up to 1 mM. Although optimal parameters did vary among substrates and specific P450s, reaction conditions for the systems studied thus far were very robust, providing for significantly higher yield production of drug metabolites than other P450 biocatalytic systems.

METHODOLOGY

Reactions were performed in one- to three-milliliter volumes with 1.0 mM substrate, 3.0 mM $NADP^+$ and 5.0 mM glucose-6-phosphate in pH 7.4 phosphate buffer. Testosterone or diclofenac was added as a concentrated stock solution in dimethylsulfoxide. In a 16 mm by 100 mm borosilicate glass test tube, CypExpress was suspended in the reaction buffer containing substrate and cofactors with a Teflon stir bar in a 37˚C incubator. For the oxygen study, 96, 48, 24, 12 and 6-well plates were used with reaction volumes of 200, 400, 500, 1,000 and 2,000 µL, respectively. The plates were shaken at a rate of 800 rpm. At the end of the reaction time, 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.

CO-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). *CypExpress can tolerate up to 6% DMSO without significant loss of catalysis*, with *N,N*-DMF also being an acceptable solvent.

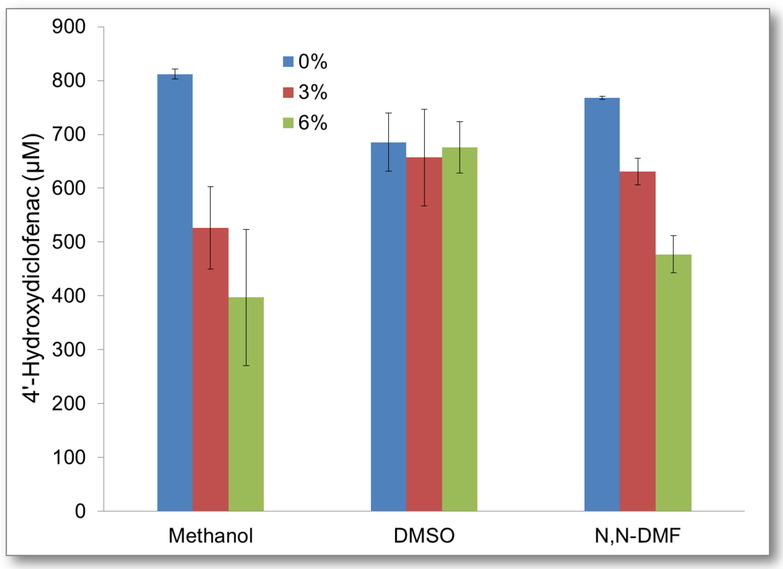

Figure 1. Conversion of diclofenac to 4'-hydroxydiclofenac with CypExpress 2C9 was not significantly affected by DMSO, while methanol was somewhat inhibitory towards catalysis.

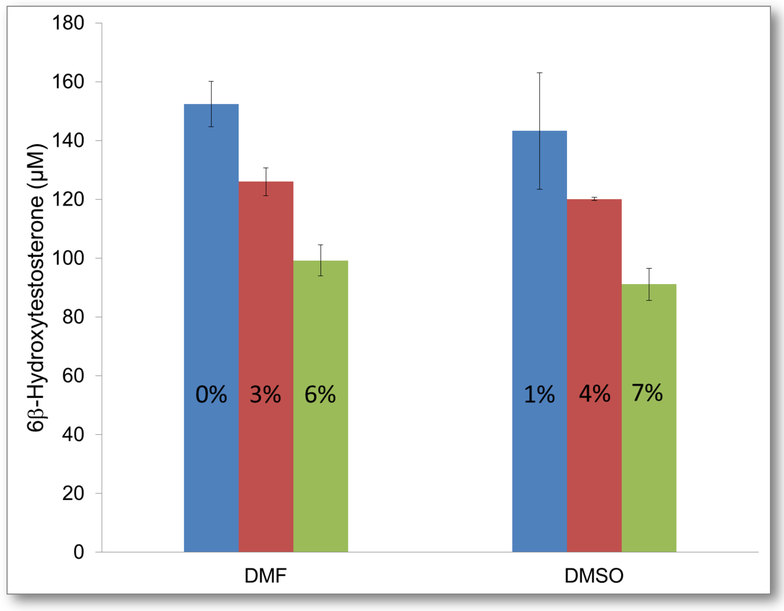

Figure 2. Conversion of testosterone to 6β-hydroxytestosterone with CypExpress 3A4. Methanol was found to be detrimental to the reaction and so was not used as a co-solvent.

HEATED STABILITY STUDY

Unlike recombinant proteins or liver microsomes, CypExpress is a very robust catalyst that can be handled at room temperature for an extended period of time. Storing the dry powder at varied temperatures and time intervals showed its remarkable stability at room temperature, with the conversion of testosterone to the 6β-hydroxylated metabolite used as an indicator for activity. CypExpress is stable for years when stored at -80˚C.

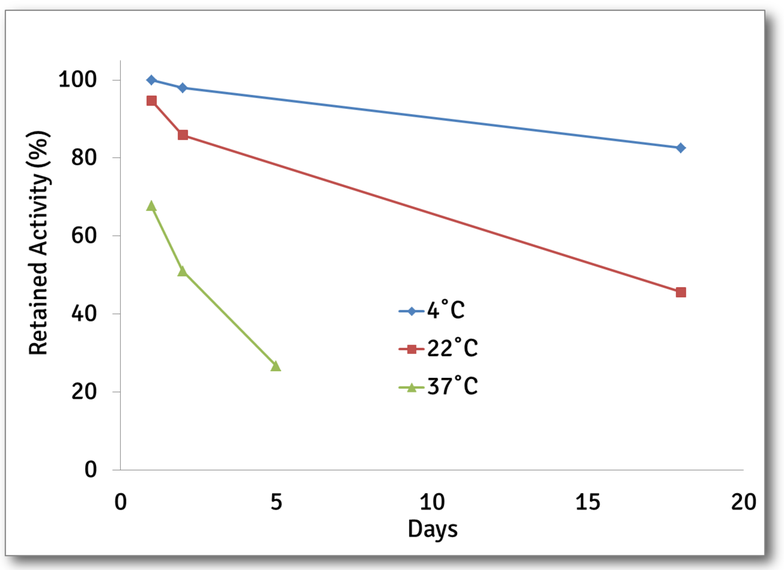

Figure 3. The retained catalytic activity for CypExpress 3A4 was determined at four temperatures over a period of 18 days.

OXYGEN REQUIREMENTS

Molecular oxygen is necessary for the P450 catalytic cycle. Performing the conversion of testosterone to the 6β-hydroxy derivative in various microplate well sizes showed minor variation in the amount of metabolite produced for the 3A4/testosterone. Shaking in flasks as well as using traditional stirring methods for larger reaction volumes produces similar conversions.

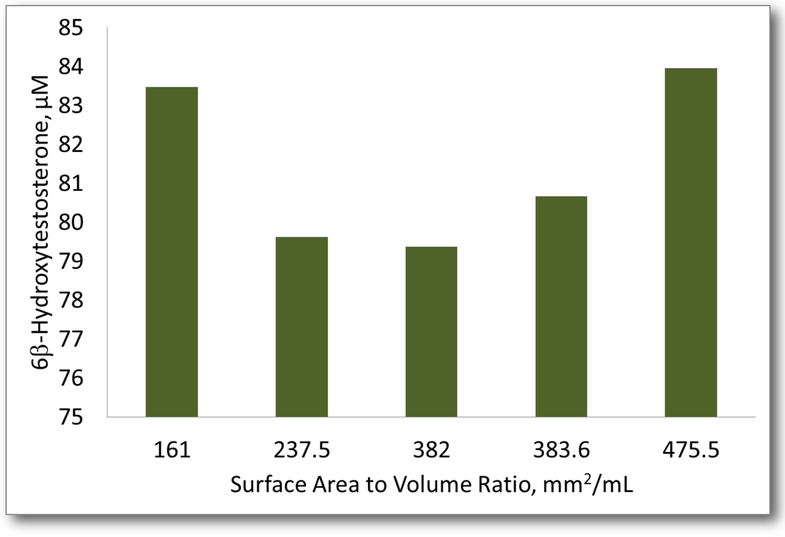

Figure 4. The size of the well did not affect the conversion of testosterone to 6β-hydroxytestosterone with CypExpress 3A4.