Utilizing a Novel Biocatalytic Human P450 System for the Multi-Cycle Production of Metabolites

Enrique Martinez[2], Shuvendu Das[1], Denis Callewaert[2] and Mani Subramanian[1]

(1) Department of Chemical & Biochemical Engineering, The University of Iowa, Coralville, IA,

(2)Oxford Biomedical Research, Inc., Rochester Hills, MI

ABSTRACT

The large-scale preparation of drug metabolites is typically a lengthy and problematic step in pharmaceutical development. We have characterized a robust P450 Catalytic System (PCS), which contains unmodified recombinant human cytochrome P450 (CYP) and oxidoreductase enzymes. The engineered inclusion of cofactors and antioxidants within its semipermeable structure greatly stabilizes the PCS and increases its usable lifetime. The present study focused on evaluating the ability of PCS expressing different isoforms to absorb and sequester substrate and generate additional product in multiple reaction cycles. After incubation at 30°C with 500 µM substrate for an initial 4 hour reaction cycle, the PCS was pelleted *via* low speed centrifugation and resuspended in fresh buffer containing glucose-6-phosphate but no additional substrate, and incubated for an additional 4 hours (cycle 2). The substrate and product concentrations in the reaction supernatant and that sequestered within the PCS system after each reaction cycle, were determined by reversed phase HPLC. The second cycle produced significant amounts of additional product.

Isoform	Substrate	Cycle 1 Yield, µM	Cycle 2 Yield, µM
1A2	Phenacetin	43	28
2C9	Diclofenac	68	308
2C19	Mephenytoin	43	95
2D6	Dextromethorphan	68	89
3A4	Testosterone	47	46

The PCS retained activity for multiple reaction cycles performed at 30° - 37°C with substrate concentrations ranging from 200 – 1,000 µM. Similar results were obtained for reaction volumes ranging from 200 µL to 400 mL, with the larger scale reactions typically yielding several milligrams of product. After the first reaction cycle, the PCS could be stored at 4°C overnight without significant loss of activity.

INTRODUCTION

CypExpress™ exhibits several characteristics for improved P450 metabolite production, including:

- The ability to absorb and retain hydrophobic substrates from the reaction mixture.
- Retention of catalytic activity for several reaction cycles, thereby increasing overall yield.
- Easily removed from the reaction mixture *via* low speed centrifugation for metabolite analysis.
- A higher total product yield for multi-cycles vs. a longer single cycle reaction.
- Room-temperature handling and freeze/thaw stability.

METHODOLOGY

Testosterone/Dextromethorphan multi-cycle runs: 20 g of *CypExpress*™ was pre-washed with 50 mM potassium phosphate buffer pH 7.5, centrifugation at 3,000 x *g* for 5 min, then re-suspended in the same buffer containing 12 mM Na-G6P and 500 or 1,000 µM of substrate in baffled flasks. The reaction mixtures were shaken at 30°C and 225 rpm in rotary orbital shaker for the specified times. On the second day, the pellet was removed from 4°C storage and re-suspended in fresh reaction buffer containing only G6P and incubated for an additional five hours. Aliquots were taken at the indicated intervals for HPLC analysis. Diclofenac multi-cycle runs: 44 mg of *CypExpress*™ 2C9 was added to 5.0 mL of 100 mM potassium phosphate buffer pH 7.4 containing 1.0 M G6P and 1.3 mM $NADP^+$ with 1.0 mM diclofenac, disodium salt. After the first five hour cycle, the suspension was pelleted via centrifugation at 23,100 x *g* for 10 minutes and the pellet re-suspended in the same buffer. The second, third, and fourth cycles were run for 18, 5 and 18 hours, respectively.

OPTIMIZATION

As each substrate is unique, the user should determine the following before scaling-up the reaction:

- Amount of retained substrate and metabolite after the first cycle.
- Difference in the product yield obtained by adding additional substrate to each reaction cycle using the substrate retained in the *CypExpress*™.
- Conversion rate for each substrate based on its solubility or ionic charge.
- Washing of *CypExpress* ™ can be skipped if the metabolites are well separated in the HPLC profile.

TESTOSTERONE MULTI-CYCLE EXPERIMENT

To distinguish between these two possibilities, a 400 mL reaction was run in which 500 µM testosterone was incubated in 100 mM phosphate buffer with *CypExpress*™ 3A4 at 30°C. At the specified time intervals aliquots of the suspension were removed, extracted and analyzed by HPLC. After 4 hours, the reaction mixture was centrifuged at 16,000 x *g*, and the pellet stored overnight.

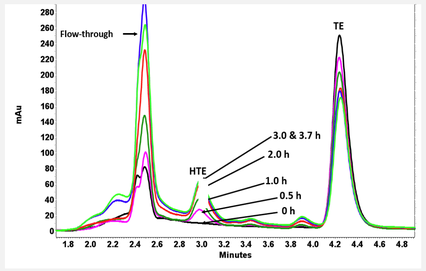

Figure 1. Time-resolved HPLC analysis of testosterone metabolites produced by *CypExpress*™ 3A4 in **Cycle 1**.

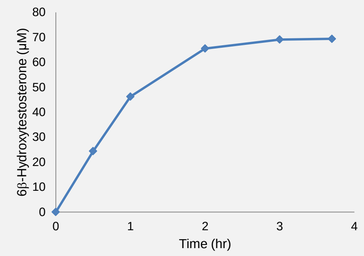

Figure 2. Kinetics of 6β-hydroxytestosterone production in **Cycle 1**.

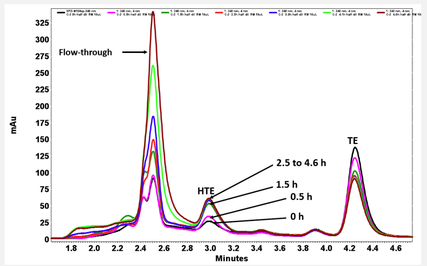

Figure 3. HPLC analysis of *CypExpress*™ 3A4/testosterone metabolites produced from a second reaction cycle.

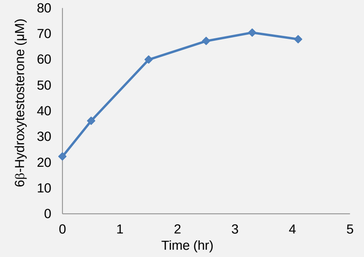

Figure 4. Kinetics of 6β-hydroxytestosterone production in the **2nd cycle**.

DICLOFENAC MULTI-CYCLE EXPERIMENT

Diclofenac, an excellent P450-2C9 substrate, was converted to 4'-hydroxydiclofenac in high yield with a *CypExpress*™ 2C9 loading of 100 mg/mL. However, to examine whether lower levels of *CypExpress*™ could efficiently produce large quantities of product, diclofenac was incubated with 20 mg/mL of *CypExpress*™ 2C9 for a total of four reaction cycles. A cost-comparable quantity of rat liver microsomes were used to compare total yield for both systems, shown below in **Figure 5**.

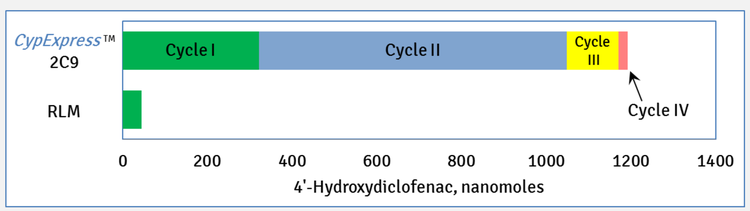

Figure 5. A 20 mg/mL *CypExpress*™ 2C9 loading was used to produce 27-times more 4'-hydroxydiclofenac than rat liver microsomes, which could only be used for a single cycle.

DEXTROMETHORPHAN MULTI-CYCLE EXPERIMENT

High conversions have been obtained with *CypExpress*™ 2C9 and dextromethorphan. Unlike testosterone and diclofenac, the majority of dextrorphan was obtained in the first cycle **(Figure 6)**, with the second cycle producing approximately 10% of the first **(Figure 7)**. Note the starting concentration for the second cycle showing the product absorbed by the *CypExpress*™ system.

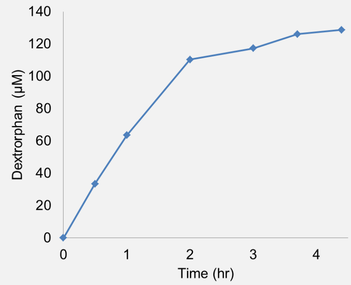

Figure 6. Kinetics of dextrorphan production in **Cycle 1**.

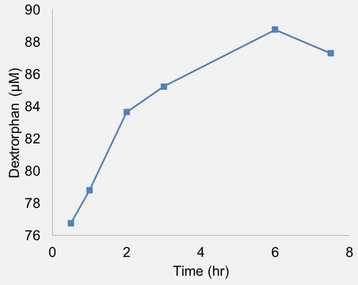

Figure 7. Kinetics of dextrorphan production in **Cycle 2**.

SUMMARY

- *CypExpress*™ retains catalytic activity over multiple cycles and can be used to prepare P450 metabolites in high yield.
- Certain substrates, such as testosterone, accumulate in *CypExpress*™ 3A4 and allow for additional cycles to be run with only the addition of cofactors.
- High turnover substrates such as diclofenac can be efficiently metabolized using lower *CypExpress*™ 2C9 loadings.
- Dextromethorphan at 1.0 mM required only one cycle to produce a significant quantity of dextrorphan, with the second cycle yielding significantly less product and being unnecessary.
- Small-scale pilot reactions should be run to optimize *CypExpress*™ loading, substrate concentrations, number of cycles run and buffer type.

Oxford Biomedical Research

SUPERIOR SCIENCE. RELIABLE RESULTS.

For more information contact:

martinez@oxfordbiomed.com