

Rapid identification and production of metabolites using *CypExpress*TM 2D6, 3A4 and 2C9

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ABSTRACT

Every drug in development needs to be fully characterized with respect to all the metabolites produced in humans. A wide range of methods has been developed to rapidly screen drug candidates for metabolism by cytochromes P450. However, production and purification of metabolites in quantities sufficient for Metabolite in Safety Test (MIST) studies is presently a challenging, time-consuming and expensive process — whether the preparative-scale synthesis of metabolites is performed using classical chemical synthesis, or liver microsomes. These technologies often have low yields, multiple byproducts, and microsome preparations suffer from lot-to-lot variability — and the requirement for large numbers of animals. Biocatalytic tools involving recombinant human P450s expressed in various hosts have been available for several years and can be very useful for metabolite identification. However, these systems are typically only stable for short periods, and require addition of NADPH or a system to generate NADPH, so that their use to generate milligram quantities of metabolites can prove extremely expensive.

We have developed a novel system, *CypExpress*TM; in which human liver CYPs are co expressed with human NADPH-P450 oxidoreductase in *Pichia* cells. Using proprietary processes, these cells are grown in large-scale fermenters, harvested and dried to a stable semi-permeable powder that retains very high levels of P450, NADPH-P450 oxidoreductase and NADPH-regenerating activity. *CypExpress*TM should prove invaluable for large-scale preparation of drug metabolites, production of reference standards, drug-drug interaction analysis, and rapid identification of human drug metabolites. *CypExpress*TM CYP2D6, CYP3A4 and CYP2C9 products are already available. All three of these products mimic the human liver reactions of corresponding CYPs when tested using recommended standard substrates for each CYP.

The present studies are focused on (a) the utility of *CypExpress*TM to scale up production of specific metabolite(s) — including the ability to recycle *CypExpress*TM for higher efficiency production of metabolites, (b) optimization of catalytic parameters for this system vs. traditional biocatalytic systems, and (c) direct comparison of the yields obtained using *CypExpress*TM, commercial liver microsomes. *CypExpress*TM 2C9 catalyzed conversion of diclofenac (DN) to 4-hydroxydiclofenac (HDN) in a 400 mL reaction generated 53.6 mg of HDN from 59.2 mg of DN in only 1.5 hours. In two-cycles of a 200 mL reaction containing 14.4 mg of testosterone (TE), *CypExpress*TM 3A4 catalyzed the production of 7.01 mg of 6 β -Hydroxytestosterone (HTE) in 3.0 hours. In two-cycles of a 200 mL reaction containing 27 mg of dextromethorphan, *CypExpress*TM 2D6 catalyzed production of 7.5 mg dextromethorphan. Reaction conversion rates and product formation in reactions catalyzed by *CypExpress*TM 3A4, 2C9, and 2D6 were directly compared to reactions catalyzed by rat liver microsomes. *CypExpress*TM is a very efficient, cost-effective, and less complex alternative for scale-up production of specific metabolites.

METHODOLOGY

Pilot-scale reactions: Typical reactions were run in one- or five-milliliter volumes with glucose-6-phosphate and NADP⁺ concentrations at 2.5 and 3.3 mM, respectively. A pH 7.4 phosphate buffer solution was prepared with these co-factors and the *CypExpress* catalysts were added and vortexed to make a suspension. This was then stirred with a Teflon stir bar in a heated incubator set to 37°C and allowed to react uncovered for a prescribed length of time after a concentrated solution of substrate was added to initiate the reaction. *CypExpress* catalysts can tolerate up to 4% (v/v) of dimethylsulfoxide or N,N-dimethylformamide to help with solubilizing hydrophobic substrates. After the reaction had come to completion, an equal volume of HPLC buffer containing 79% methanol, 20% water and 1.0% acetic acid was added to the reaction to stop the catalysis and precipitate any proteins. Centrifugation at 23,100 x g yielded a clear solution that was analyzed by HPLC using a five micron 250 mm by 4.6 mm Phenomenex Luna C-18 column with a flow rate of 0.6 mL/min and a photodiode array detector.

Scale-up of metabolite synthesis: Large Scale (400 mL) reaction conditions, suitable for product isolation, were carried out under the following conditions: 20 g of the appropriate *CypExpress*TM reagent was pre-washed by centrifugation at 3,000 x g for 5 min, then resuspended in 50 mM KPi buffer pH 7.5, containing a final concentration of 12 mM NaG6P and 250-500 μ M of Substrate in baffled flasks. The reaction mixtures were incubated at 30°C and 225 rpm in rotary orbital shaker, for the specified times. Reaction product yields were calculated based on HPLC analyses as for the pilot-scale reactions.

RESULTS

Scale-up of Metabolite Synthesis

<i>CypExpress</i> Isoform	Cycles	Substrate	Reaction	Product	Yield
2C9	1 x 1.5 hr	Diclofenac	59.2 mg in 400 mL	4'-hydroxydiclofenac	53.6 mg (90.5%)
2D6	2 x 3 hr	Dextromethorphan	27 mg in 200 mL	Dextrophan	7.5 mg (27.8%)
3A4	2 x 3 hr	Testosterone	14.4 mg in 200 mL	6 β -Testosterone	7.01 mg (48.7%)

COMPARATIVE PERFORMANCE

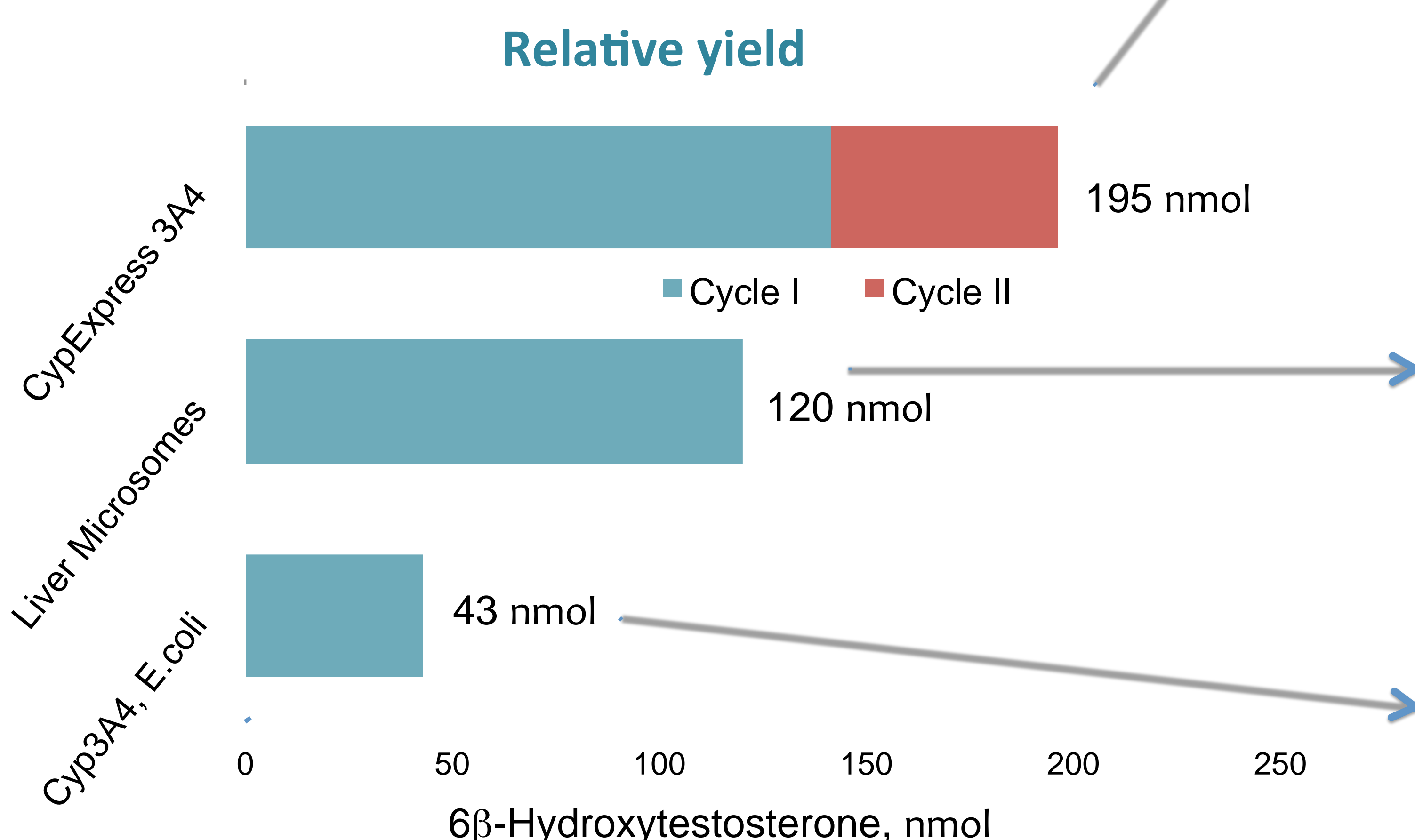
Several methods are presently used to (a) to screen for metabolites of drug candidates, (b) perform "P450 phenotyping", (c) identify metabolites of drug candidates, and (d) synthesize specific metabolites at a scale sufficient to perform MIST studies.

Among the most well characterized and most employed systems employed for these various *in vitro* drug metabolism studies are rat liver microsome preparations and recombinant human cytochromes P450, most commonly expressed in baculoviral-infected insect cells or (in N-terminal modified forms) in *E. coli*.

The utility of *CypExpress*TM was therefore directly compared to two commercial systems that have been widely employed: rat liver microsome and recombinants expressed in *E. coli*. All studies were performed under at 37°C under optimal conditions specified by the manufacturer.

Critical parameters: Key considerations in selection and use of any *in vitro* drug metabolism system include STABILITY, easy of use, lot-to-lot consistency, **SIGNAL/NOISE** (to allow for optimal identification an isolation of metabolites, **YIELD** and convenience of scale-up, and (of course) **COST** (including cofactors and NADPH regeneration).

Using equivalent amounts of *CypExpress*TM and systems purchased from standard vendors, results obtained for P3403A4 metabolism of Testosterone are presented here.

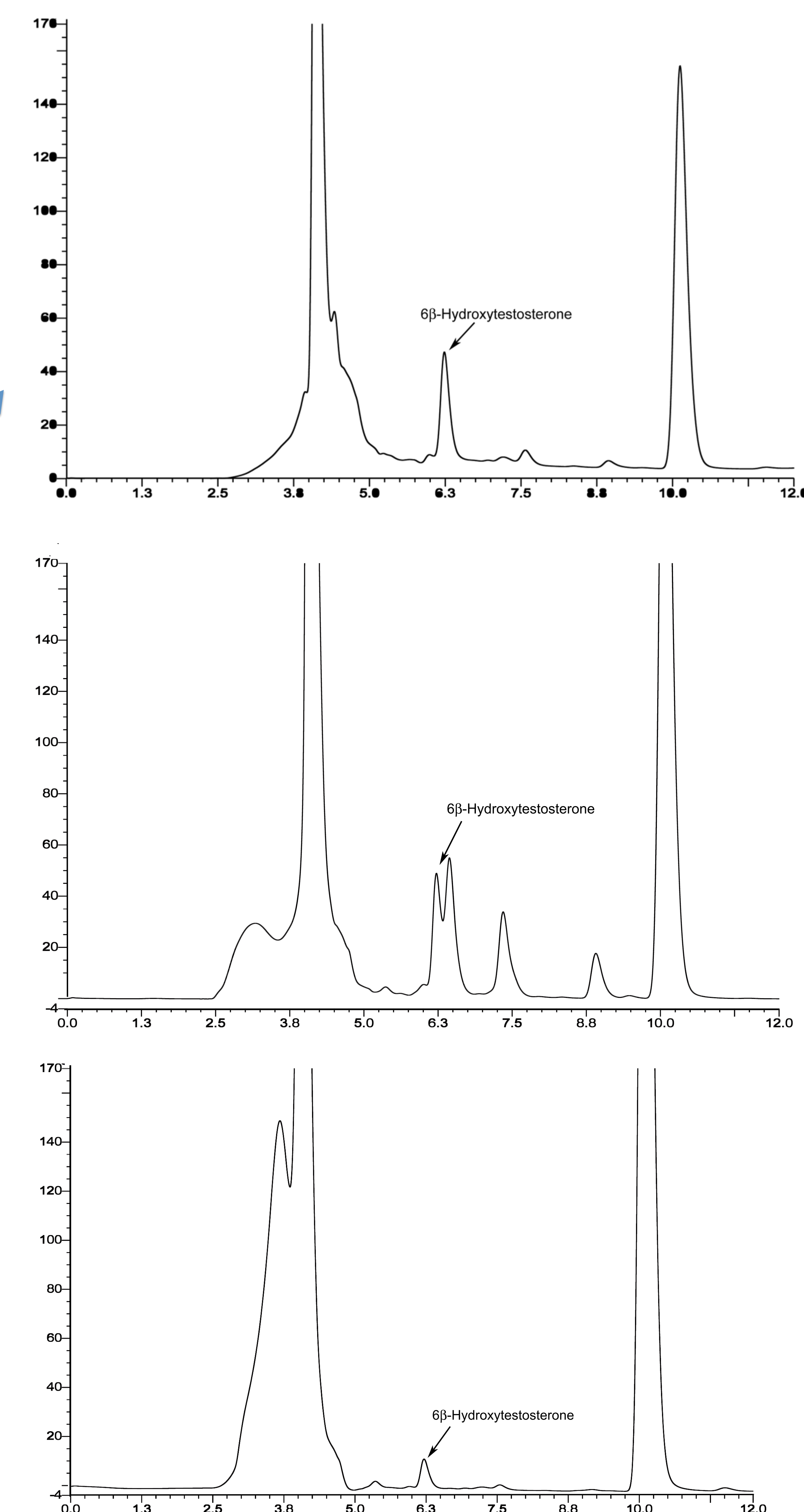


Testosterone (500 μ M) was incubated in 100 mM phosphate buffer for 5 hrs (cycle 1) with quantities of *CypExpress*3A4 (top), rat liver microsomes (Sigma, center), and *E. coli* membranes containing rec. human CYP3A4 (Xenotech, bottom) of equal value based on list price. Following the reaction, the samples were processed under recommended conditions and analyzed by HPLC. In the case of *CypExpress*3A4, the yield in cycle 1 represents the product obtained in the supernatant after low speed centrifugation; and a second cycle was performed by resuspending the pellet in fresh buffer, NADP⁺ and G6P.

SUMMARY OF *CypExpress*TM PERFORMANCE

- Provided as a very stable dry powder
- High catalytic activity
- Efficient catalysis, even at room temperature
- Excellent for screening & metabolite ID
- High signal/noise for HPLC metabolite profiling
- Easy and rapid metabolite identification
- High yield scale up & isolation of metabolites
- Facile large-scale metabolite production
- Excellent yields – easy scale-up to mg quantities
- Active over multiple catalytic cycles
- **LOW COST**

RELATIVE METABOLITE PURITY



System	Isoform	Needs G6PDH	Recommended Temp	Reaction Buffer	Conditions Time	Cost	Unwanted Byproducts	Ease of use	Metabolite Yield/\$
Microsomes	Multiple	YES	37°C	Phosphate	30 min	\$\$	Many	+/-	++
Recombinants									
Bacterial	specific	YES	37°C	Phosphate	30 min	\$\$	Few	+/-	+
Baculoviral	specific	YES	37°C	Phosphate	30 min	\$\$\$\$	Few	-	+/-
<i>CypExpress</i> TM	specific	NO	20°C to 37°C	Phosphate, Tris or Other	Min to days	\$	Few	+++	++++