

CASE STUDY

CypExpress™ 3A4 Catalyzed Conversion of Testosterone (TE) to 6 β -Hydroxytestosterone (HT)

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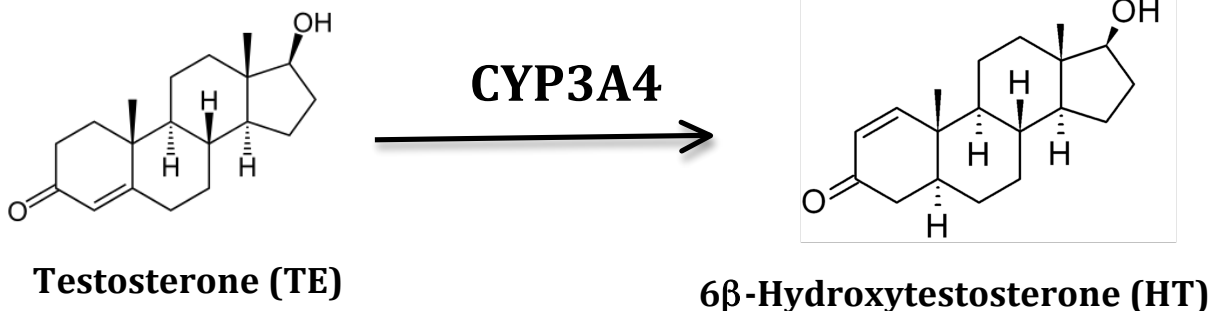
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Introduction: These studies were performed using the FDA-recommended substrate Testosterone to evaluate the utility of CypExpress3A4 for drug metabolism and disposition studies.



Reaction Conditions:

Metabolite Identification in 24-well microplates: 250-500 μM, 20-100 mg/mL CypExpress3A4, 50 mM K_P_i buffer pH 7.5, 30°C, 600 rpm in microplate shaker, Reaction volume 200 μL, Reaction time 2 to 4 h

Pilot-scale reactions were performed in a total volume of 1.0 mL in a 20x150 mm glass tube using the following final concentrations:

Substrate = 20 to 500 μM TE (500 μM for scale-up). Unless otherwise specified, TE was added to the reaction mixture as a concentrated solution in DMSO).

CypExpress 3A4 = 100 mg/mL

Buffer = 50 – 100 mM K_P_i, or Tris-HCl (as specified in tables), pH 7.5

NADP⁺, G6P = NONE ADDED for 1st cycle; see details for additional cycles

G6PDH and Mg⁺⁺ = NONE ADDED. Contained in CypExpress™ system.

The 20 x 150 mm reaction tube was placed in a tilted position on a shaker platform at 30°C, and agitated by rotation at 225 rpm for 3.0 h.

At the end of the reaction period, either

A. The entire suspension was extracted and subjected to HPLC (figure 1).

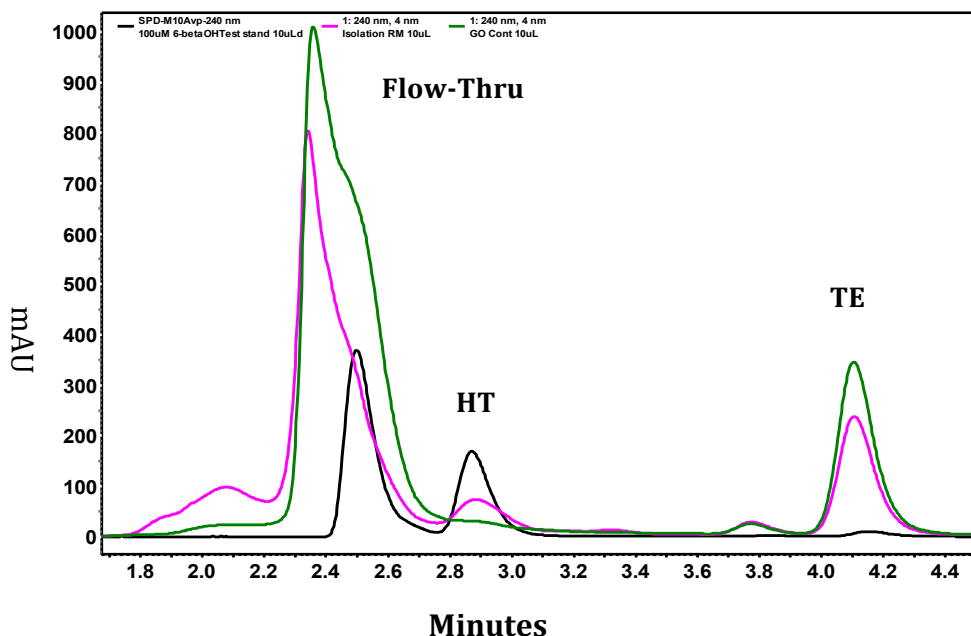
Or

B. CypExpress3A4 was pelleted by centrifugation at 14,000 x g.

a. The supernatant from the first cycle was analyzed by HPLC.

b. The pellet – was resuspended in fresh buffer containing G6P, but no additional substrate, and incubated for a second reaction cycle. After which the entire suspension was extracted and subjected to HPLC.

Conversion of TE to HT in a single reaction cycle: CypExpress™ 3A4 (100 mg/mL) catalyzed conversion of 200 μM Testosterone (TE) to 6β-Hydroxytestosterone (HT) in a single 3 hr. reaction cycle in 500 μM TE, in 50 mM KPi of pH 7.5 at 30°C was investigated by RP-HPLC after extraction of the entire reaction mixture with HPLC Mobile Phase containing 79% (v/v) methanol, 1% (v/v) acetic acid, and 20% (v/v) water in 1:1 (v/v) ratio. The results are shown in figure 1 in which the black line represents the HT standard, the light blue line represents results obtained using a vector control (no 3A4), and the red line represents results obtained using CypExpress3A4.

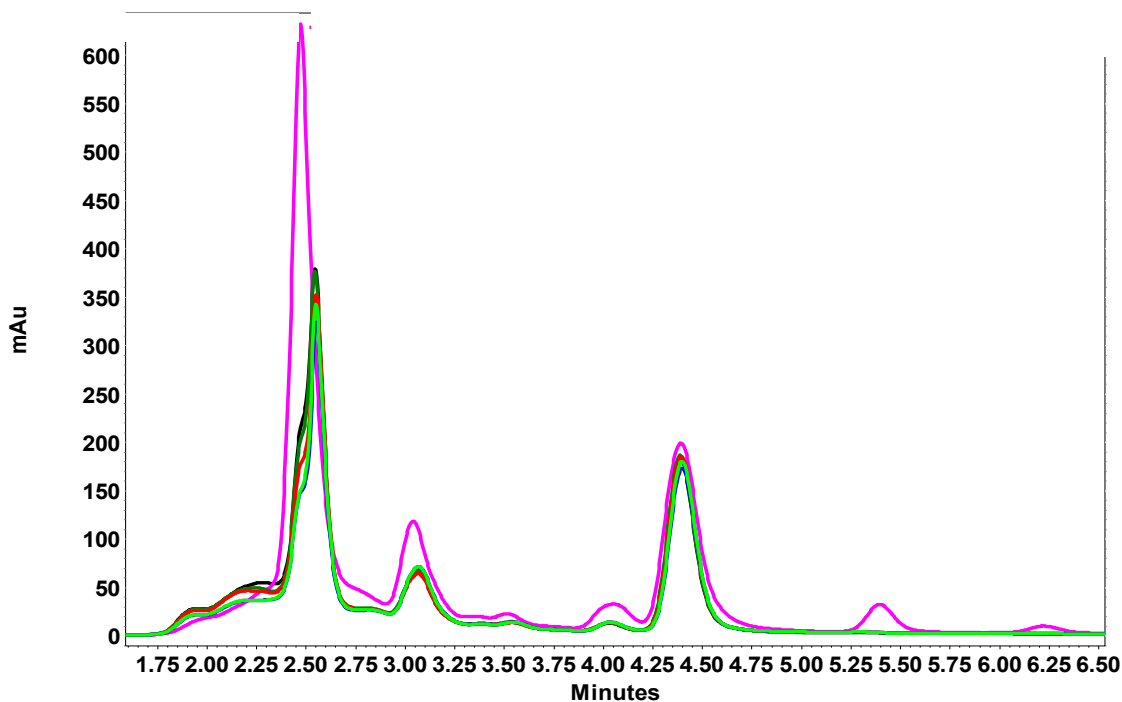


Yield of HT from TE in a single reaction cycle: Using the conditions stated above (500 μM TE, 100 mg/mL CypExpress3A4, 30°C, 3 hr., 50 mM Pi pH 7.5), the yield of HT in a single reaction cycle was:

$$87.9 \mu\text{M} = 17.6\% \text{ conversion}$$

Comparison of the catalytic efficiency and product purify for pre-washed vs. unwashed CypExpress3A4: All conditions were identical to those in the first set of experiments, except that in a parallel experiment the CypExpress3A4 was pre-washed by (a) suspending in reaction buffer and allowed to stand for various periods of time (see graph and chart) followed by (b) centrifugation at 14,000 x g, (c) removing the supernatant, and (d) resuspending the CypExpress3A4 in fresh reaction buffer (G6P)? . Results comparing the HPLC profiles and the catalytic efficiency are presented in Figure 2 and in Table 1.

Figure 2. HPLC analysis of TE \rightarrow HT catalyzed by unwashed CypExpress3A4 (light blue) and CypExpress3A4 following washing for 5 (dark red), 10 (dark blue), 15 (dark green) or 20 min (light green).



<i>CypExpress 3A4</i> wash	μM of TE	Reaction hrs	μM of HT*
None	500	3	73.62
5 min	500	3	72.67
10 min	500	3	68.98
15 min	500	3	81.25
20 min	500	3	80.84

CONCLUSION: Pre-washing CypExpress reduces the size of the flow-thru peak comprised of very hydrophilic components, but does not significantly alter the catalytic efficiency of CypExpress.

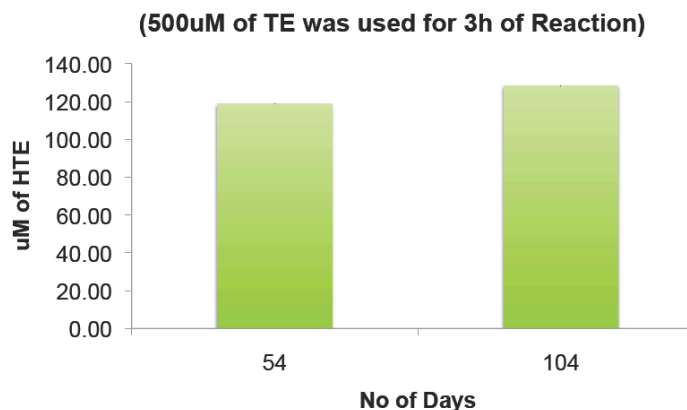
Applications of CypExpress for P450 profiling & metabolite identification:

Investigations into P450-mediated metabolism of new chemical entities, including determination of the metabolism (if any) catalyzed by specific P450 isoforms and the identification of the metabolite(s) produced is typically performed in microplates followed by HPLC-MS analysis. The suitability of CypExpress for such applications was investigated using CypExpress3A4 (100 mg/mL) conversion of TE to HT in 50 mM phosphate buffer pH 7.5. The conversion of various concentrations of TE to HT in a 200 μ L reaction volume in 24 well microplates incubated with shaking for 3 hr at 30°C is shown in Table 2:

Sample	TE (μ M)	Reaction Time (h)	HT (μ M)
1	50	3.0	39.79
2	100	3.0	48.53
3	150	3.0	61.96
4	250	3.0	83.03
5	500	3.0	118.59
6	1000	3.0	146.37

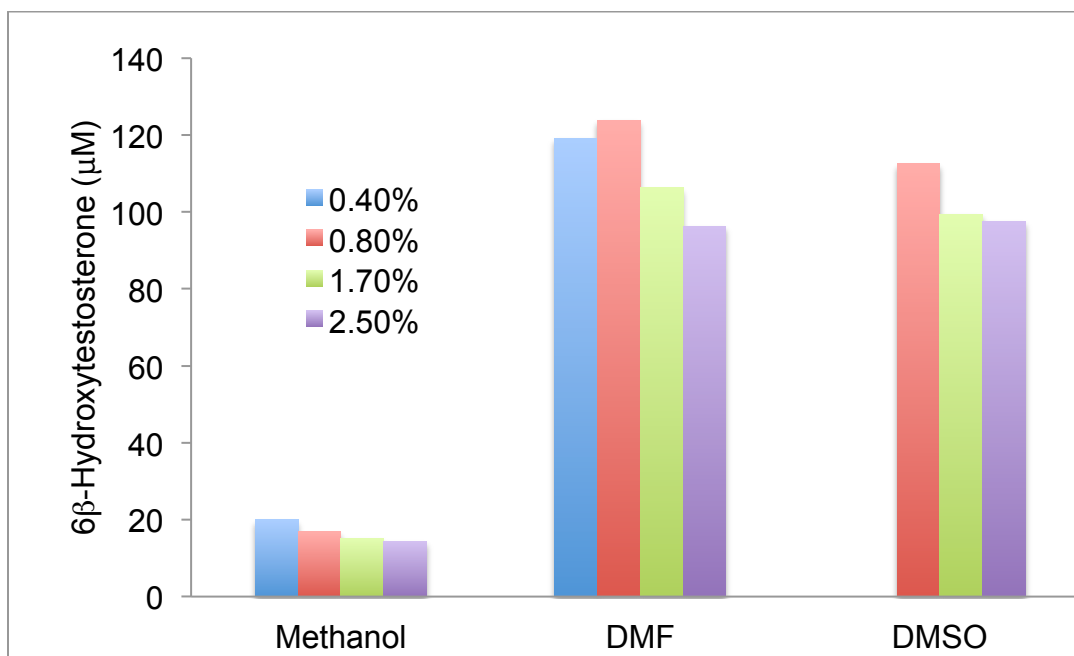
CONCLUSION: CypExpress is well suited for microplate reactions. **NOTE:** for longer reaction times it is important to restrict evaporation, e.g. by performing in a humidified environment.

Stability of CypExpress3A4 stored at -80°C: CypExpress is a novel *in vitro* P450 catalytic system with multiple components (recombinant human 3A4, recombinant human NADP oxidoreductase, cofactors, G6PDH, and antioxidants in an eukaryotic matrix). Hence, it was important to fully characterize various aspects of the system and its activity. The stability upon storage under various conditions was evaluated. Not unsurprisingly, it proved very stable when stored as a dry powder at -80°C (figure 3):



Although not recommended, we have also observed that dry CypExpress powder can be stored at room temperature for up to 3 weeks, and that CypExpress suspensions in buffer can be frozen and thawed up to four times without demonstrable loss of activity.

Effects of solvents: Testosterone and many pharmaceutically active substances are poorly soluble in aqueous solutions, so they are typically added to *in vitro* P450 reaction systems as a concentrated solution in a water-miscible solvent. The general guidance has been that the final concentration of that solvent in the *in vitro* P450 reaction system should be kept below 1%. For CypExpress, we investigated the effect of the solvent used to dissolve TE on the conversion of TE to HT. The results obtained are shown in figure 4:



CONCLUSIONS: As has been reported for other *in vitro* P450 catalytic systems, the use of methanol as a solvent should be avoided. However, at least for this 3A4 system with TE as substrate, CypExpress activity is minimally inhibited by concentrations of DMSO and DMF up to at least 2.5%.

Effect of buffer on the production of HT from TE: The production of HT in Tris and Phosphate buffers was compared (Table 3):

Buffer	TE (μM)	Reaction Time (h)	HT (μM)
KPi, 100 mM	500	3.0	105.06
Tris-HCl, 100 mM	500	3.0	140.17

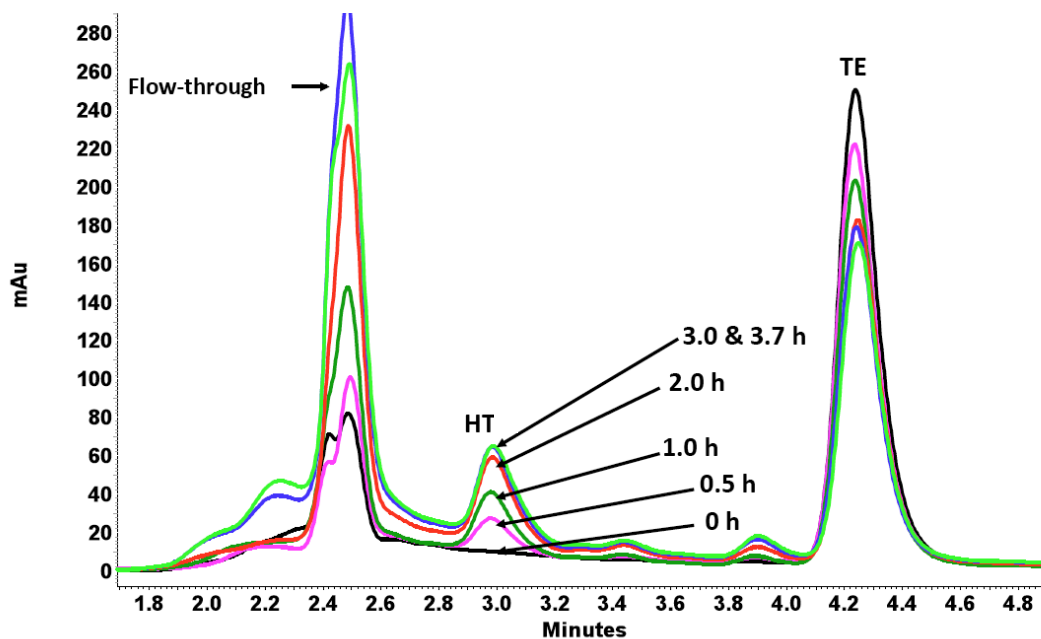
CONCLUSION: Although high levels of conversion of TE to HT were obtained in reactions catalyzed by CypExpress3A4 in both buffers, for this isoform and this substrate the conversion Tris-HCl buffer is more efficient than the same reaction performed in KP_i

Increased yields for the production of HT from TE by CypExpress3A4 in multiple reaction cycles:

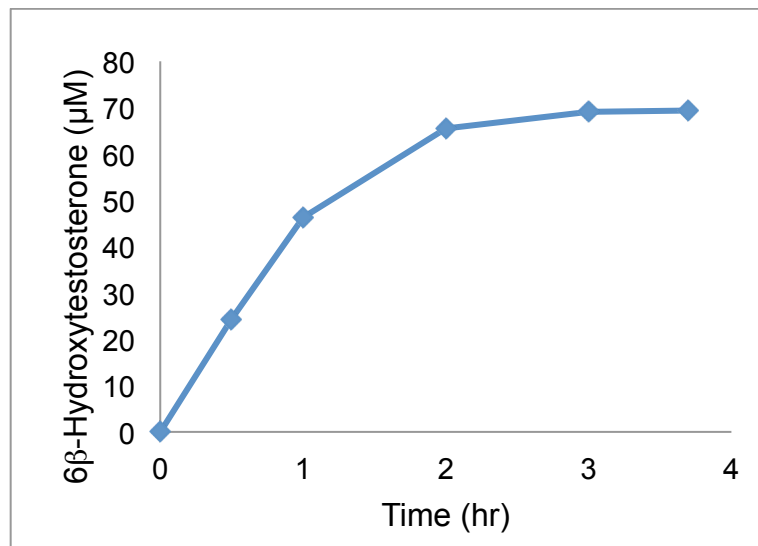
Rationale: Studies of the stability and activity of CypExpress systems expressing various recombinant human P450 enzymes have shown that:

- Significant CypExpress activity is retained after a single reaction cycle
- CypExpress typically adsorbs large quantities of relatively hydrophobic substrates from the reaction mixture, but releases most of the more hydrophobic product(s) into the buffer.
- Low speed centrifugation after one reaction cycle pellets CypExpress, which contains significant quantities of unreacted substrate and some product.
- Typically, the total product yield is greater for multi-cycle reactions vs. longer incubations for a single cycle.
- Additional reaction cycles convert substantial amounts of the retained substrate to product – thereby increasing overall metabolite yield.

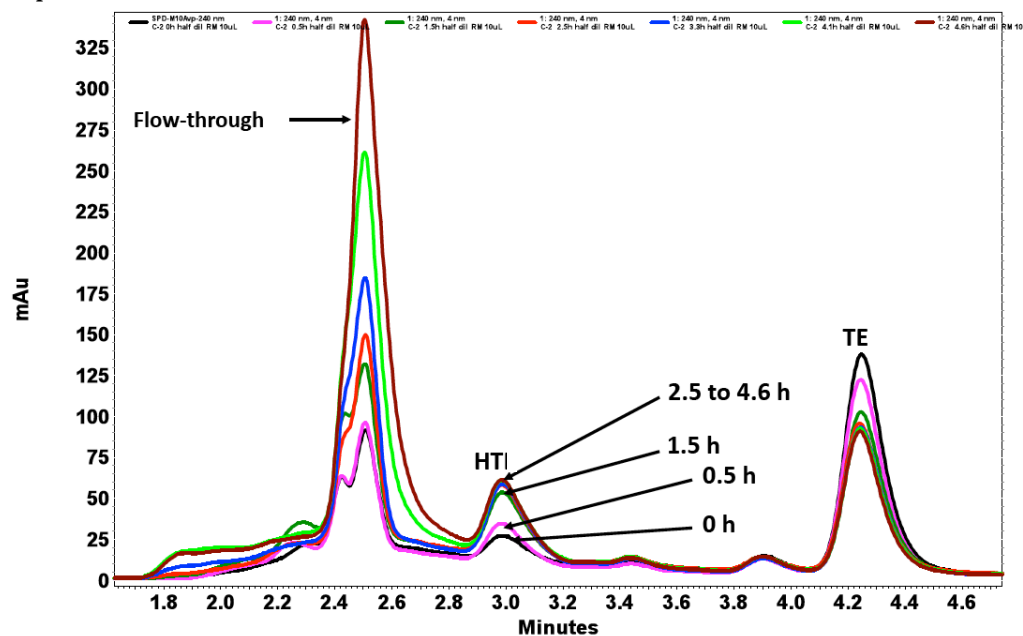
Single cycle scale-up synthesis of TE: A 200 mL reaction was performed using a TE concentration of 250 μ M and withdrawing aliquots at different time intervals to follow the time course of the reaction. HPLC analysis of the production of HT from TE (figure 5) indicates that the reaction rate slows appreciably after ~ 2hr.



different time intervals in this experiment is shown in Figure 6 below.



Scale-up production of TE in a second catalytic cycle: A 200 mL reaction was performed using a TE concentration of 250 μM. After a 3 hr. first reaction cycle, the CypExpress was pelleted, and the supernatant harvested. The pelleted CypExpress was stored overnight at 4C, then resuspended in fresh buffer containing G6P but no additional NADP+ or TE substrate. This suspension was incubated at 30°C and aliquots withdrawn at different time intervals to follow the time course of the reaction by HPLC. The results obtained (figure 6) support the production of substantial additional TE for at least an additional 2.5 hr.



Discussion: CypExpress3A4 retains activity after several hours and the yields of metabolite in scale-up can be significantly increased by multiple reaction cycles.