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CASE STUDY

CypExpress™ 2C19 Catalyzed Conversion of Mephenytoin (MP) to 4-Hydroxymephenytoin (HMP)

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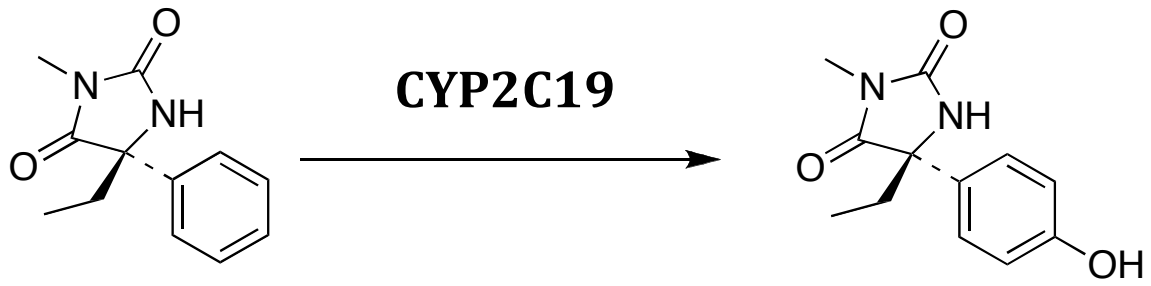
Introduction: These studies were performed using the FDA-recommended substrate Mephenytoin to evaluate the utility of CypExpress2C19 for drug metabolism and disposition studies.



***S*-Mephenytoin (MP)** ***S*-4-Hydroxymephenytoin (HMP)**

Reaction Conditions: 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 MP (500 µM for scale-up). Unless otherwise specified, MP was added to the reaction mixture as a concentrated solution in DMSO

CypExpress 2C19 = 100 mg/mL

Buffer = 50 – 100 mM KP_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.

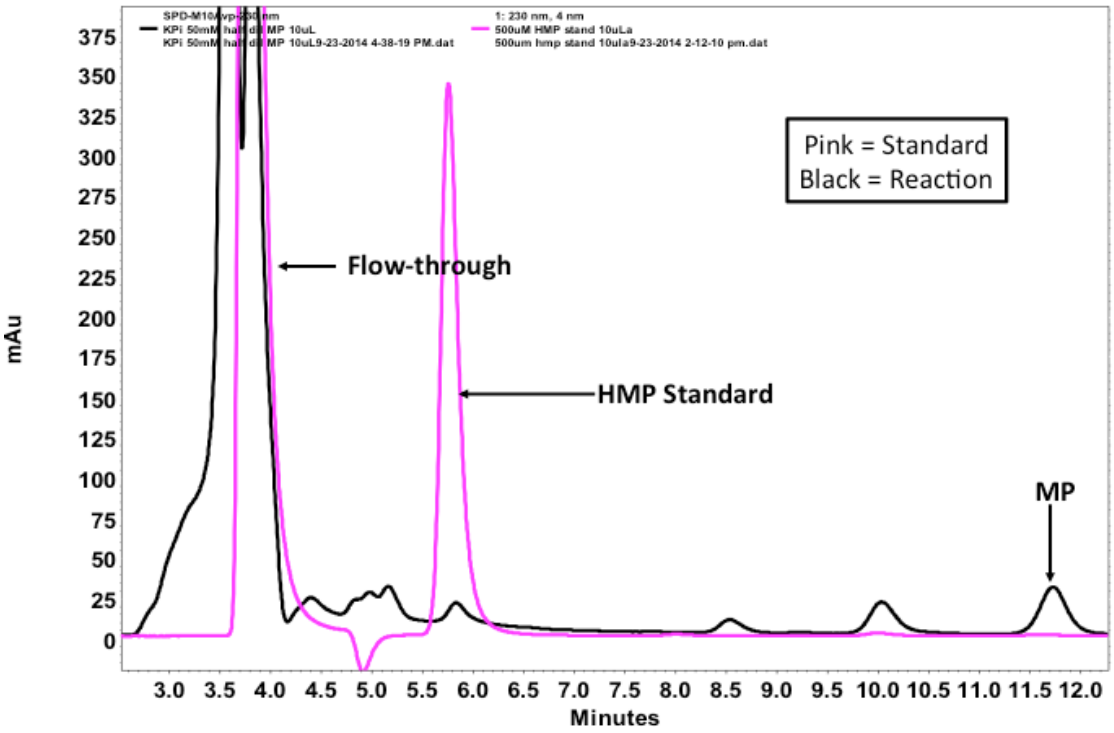
At the end of the reaction period, either

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

   Or

B. CypExpress2C19 was pelleted by centrifugation at 6,000 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 MP to HMP in a single reaction cycle: CypExpress™ 2C19 (100 mg/mL) catalyzed conversion of 200 µM Mephenytoin (MP) to *S*-4-Hydroxymephenytoin (HMP) in a single 3 hr. reaction cycle in 500 µM MP, in 50 mM KP_i of pH 7.5 at 30°C was investigated by RP-HPLC after extraction of the entire reaction mixture with HPLC Mobile Phase containing 15% (v/v) Methanol, 84.5% (v/v) Water, and 0.5% (v/v) Acetic acid. The results are shown in figure 1.

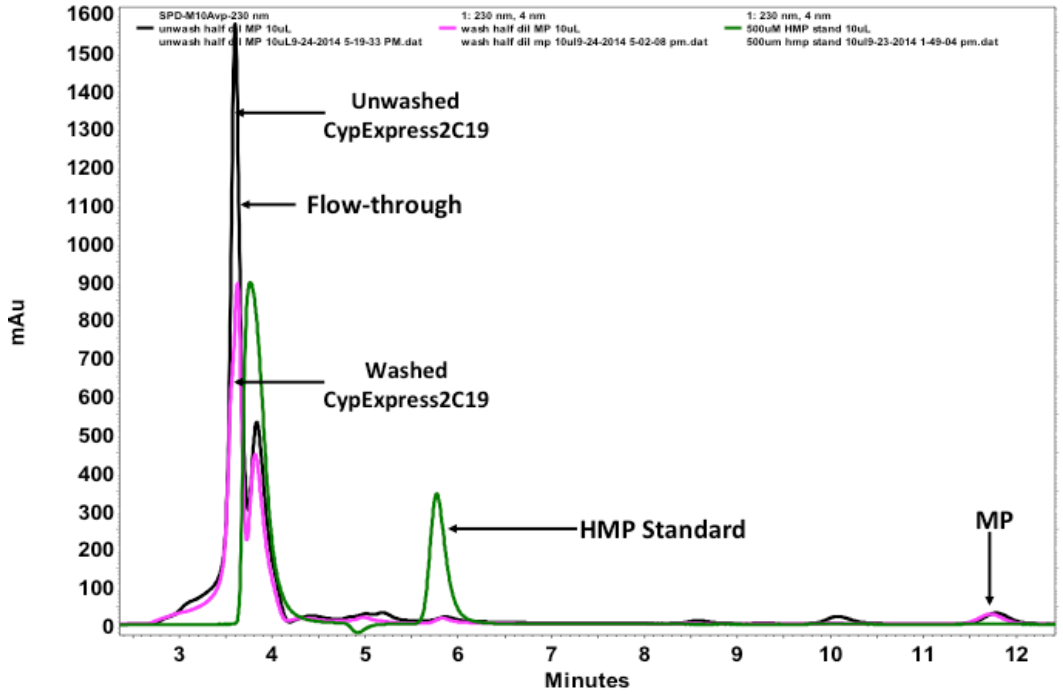


Yield of MPH from MP in a single reaction cycle: Using the conditions stated above (200 µM PH, 100 mg/mL CypExpress2C19, 30°C, 4 hr., 50 mM Pi pH 7.5), the average yield of MPH in a single reaction cycle were:

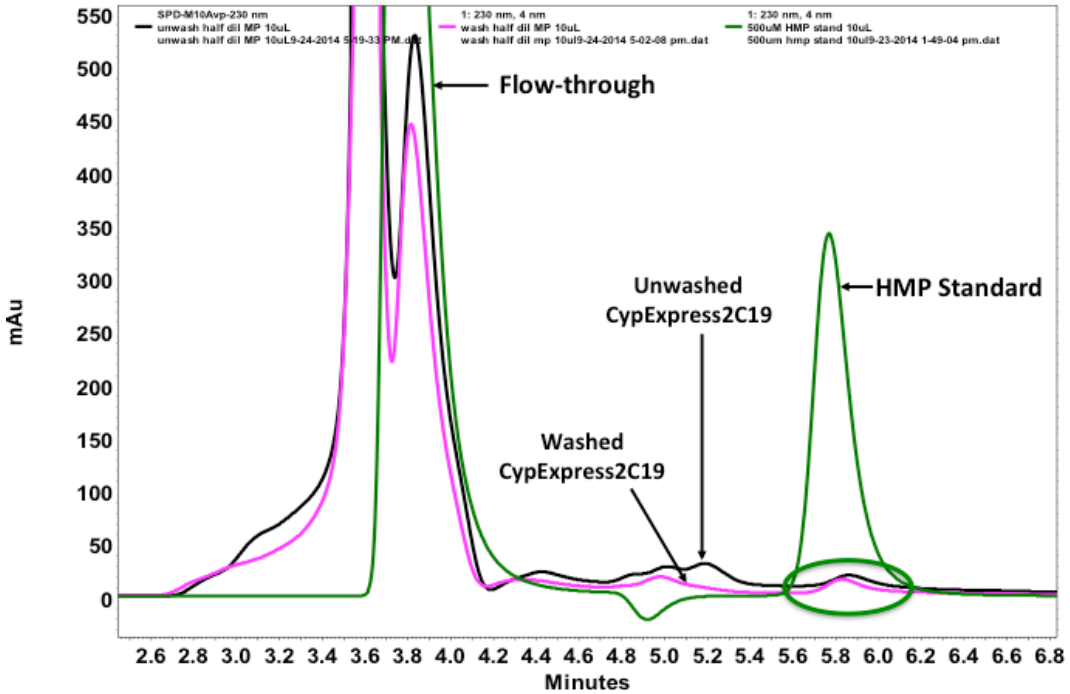
42.76 µM = 8.6% conversion

Comparison of the catalytic efficiency and product purify for pre-washed vs. unwashed CypExpress2C19: All conditions were identical to those in the first set of experiments, except that in a parallel experiment the CypExpress was pre-washed by (a) suspending in reaction buffer, (b) centrifugation at 6,000 x g, (c) removing the supernatant, and (d) resuspending the CypExpress2C19 in fresh reaction buffer . Results comparing the HPLC profiles and the catalytic efficiency are presented in Figure 2 and 3 and in Table 1.

Figure 2. HPLC analysis of MP -> HMP catalyzed by washed vs. unwashed CypExpress2C19



The HMP product peak is more clearly visible by "zooming" the HPLC to the first 7 min as shown in figure 3.



CypExpress 2C19	µM of MP	Reaction hrs	µM of HMP
Washed	500	4	45.33*
Unwashed	500	4	36.07

*The integration of the peak area may not be accurate due to broad peak area.

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.

Effect of buffer on the production of HMP from MP: Other than the buffer, all conditions were identical to those in the first set of experiments. The CypExpress 2C19 catalyzed reaction for this substrate proceeds equally well in 50 mM Tris or Phosphate.

Buffer	MP (µM)	Reaction Time (h)	HMP (µM)
Tris-HCl, 50 mM	500	4.0	41.54
Tris-HCl, 100 mM	500	4.0	33.15
KPi, 50 mM	500	4.0	42.76
KPi ,100 mM	500	4.0	32.11

CONCLUSIONS:

- Increasing buffer strength did not increase the yield of HMP; rather it decreased the yield
- Tris-HCl buffer is equally efficient for the CYP2C19 catalyzed bioconversion of MP to HMP compared to KP_i

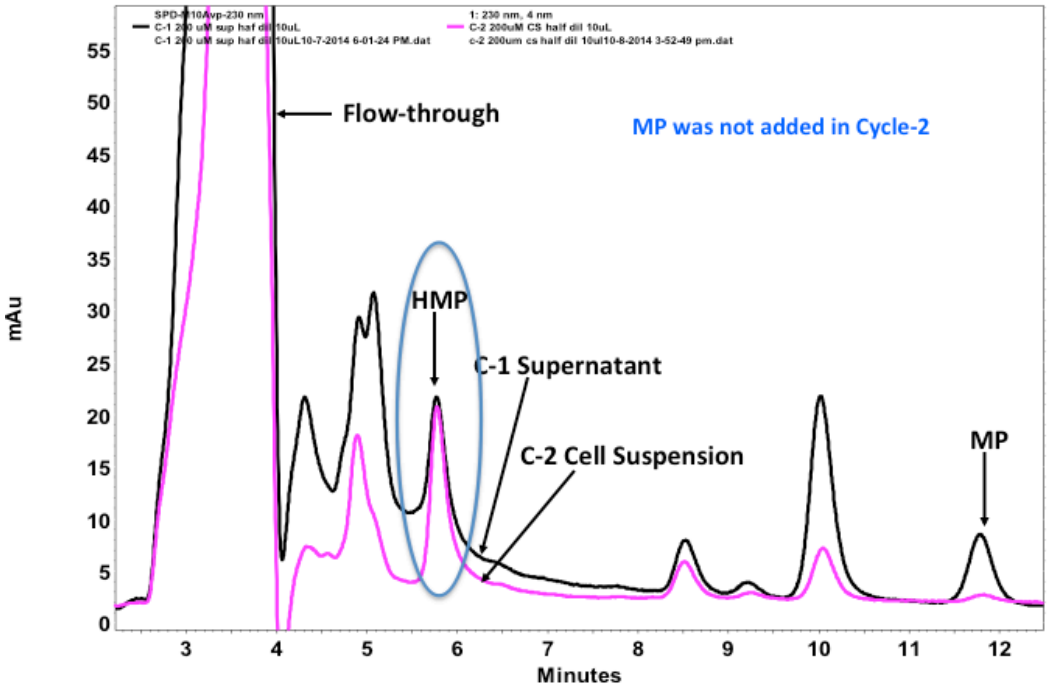
Increased yields for the production of HMP from MP by CypExpress in multiple reaction cycles:

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 containing significant quantities of unreacted substrate as well as some product.
- Typically, the total product yield is greater for multi-cycle reactions vs. longer incubations for a single cycle. This may be due to inhibition of some P450 reactions by substrate or product in long incubation periods.
- Additional reaction cycles convert substantial amounts of the retained substrate to product – thereby increasing overall metabolite yield.

Conditions: Multi-cycle production of HMP from MP by CypExpress2C19. The reactions conditions were generally identical to those in the first set of experiments. Initial MP concentrations of 200 µM and 500 µM were used to determine relative catalytic efficiency as a function of substrate concentration.

HPLC profile: In Figure 4 below, the *black curve* is the analysis of a sample of the supernatant after cycle 1. The *blue curve* is the results obtained following extraction of the entire suspension following the second cycle.



Yield: The HMP yields obtained following the first and second cycles are shown in table 2.

Reaction Sample	µM of MP	Reaction hrs.	µM of HMP
C-1 Supernatant	200	4	40.83
C-1 Supernatant	500	4	43.64
C-2 Cell Suspension	200 in Cycle-1 No addition in C-2	4	53.07**
C-2 Cell Suspension	500 in Cycle-1 No addition in C-2	4	95.53**

*After Cycle-1, cell pellet was stored at 4°C over night before start of Cycle-2
**Centrifugation of an aliquot following the 2nd cycle confirmed that majority of the HMP following the 2nd cycle was present in the supernatant.

Summary: For 200 µM MP, two reaction cycles effected 47% conversion to HMP.
For 500 µM MP, two reaction cycles effected 28% conversion to HMP.

The apparently very significant increase in HMP yields from 500 µM MP in a second reaction cycle suggest that high substrate concentrations may inhibit this reaction. To confirm the results obtained in this experiment, it was repeated in duplicate. The results are shown in table 3.

Reaction Sample	µM of MP	Reaction hrs.	µM of HMP
C-1 Supernatant	500	4	36.07
C-1 Supernatant	500	4	31.77
C-2 Cell Suspension	500 in Cycle-1 No addition in C-2	4	94.75
C-2 Cell Suspension	500 in Cycle-1 No addition in C-2	4	77.84

Discussion: The data presented support the adsorbtion of MP during the first reaction cycle and, after centrifugation to remove unabsorbed MP and the HMP produced during the first cycle, CypExpress 2C19 catalyzes significant additional production of HMP in a second cycle. These data are consistent with the inhibition of CypExpress2C19 at high concentrations of the substrate MP.

