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CASE STUDY

CypExpress™ 1A2 Conversion of Phenacetin to Acetaminophen

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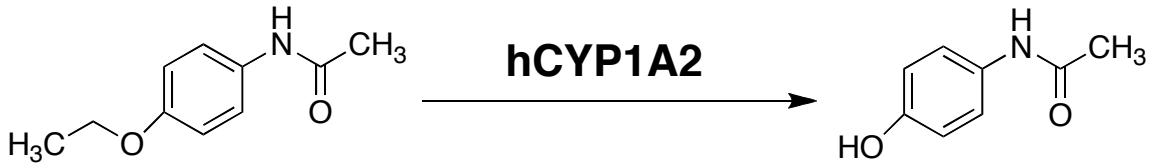
Introduction: These studies were performed using the FDA-recommended substrate Phenacetin to evaluate the utility of CypExpress1A2 for drug metabolism and disposition studies.



Phenacetin (PH) **Acetaminophen (AC)**

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 = 200 or 500 µM PH. Unless otherwise specified, PH was added to the reaction mixture as a concentrated solution in DMSO

CypExpress 1A2 = 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 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.

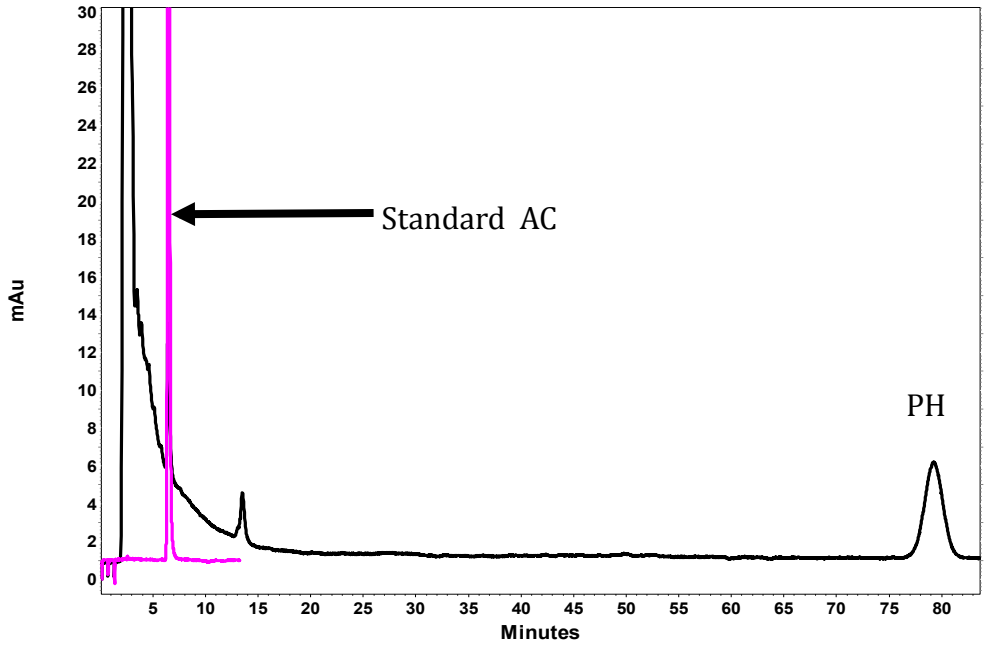
At the end of the reaction period, either

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

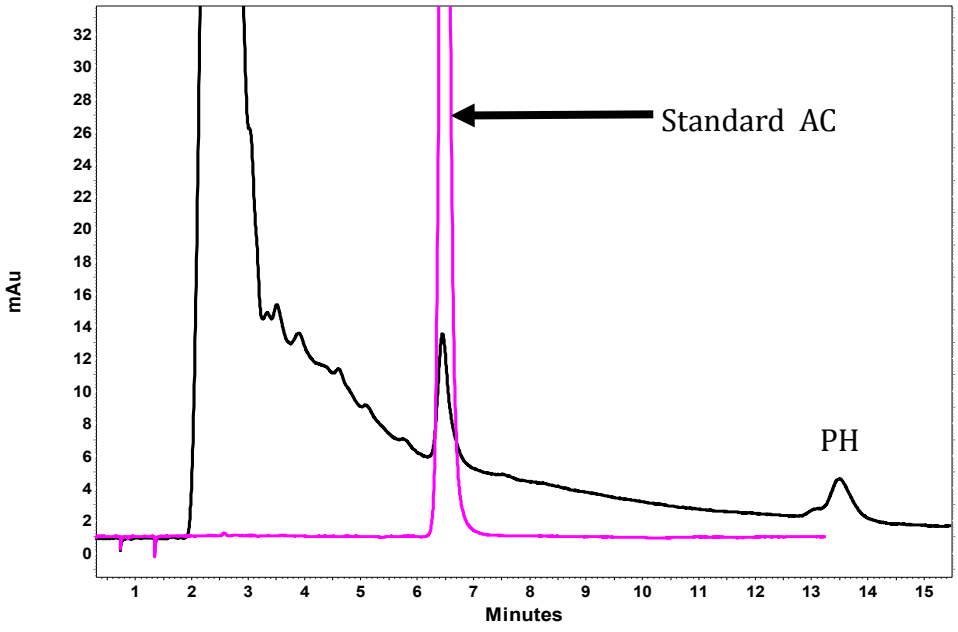
   Or

B. CypExpress1A2 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 PH to AC in a single reaction cycle: *CypExpress* 1A2 (100 mg/mL) catalyzed conversion of 200 µM Phenacetin (PH) to Acetaminophen (AC) in a single 3 hr. reaction cycle in 200 µM PH, 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.



The AC product peak is more clearly visible by "zooming" the HPLC to the first 15 min as shown in figure 2.



Yield of AC from PH in a single reaction cycle: The average of two different sets of reactions using the conditions stated above (200 µM PH, 100 mg/mL CypExpress1A2, 30°C, 3 hr., 50 mM Pi pH 7.5) were:

39.06 µM ± 7.93 = 19.5% conversion

Effect of buffer on the production of AC from PH: Other than the buffer, all conditions were identical to those in the first set of experiments. Although the reaction proceeds very well in Tris or Phosphate, the reactivity of CypExpress 1A2 for this substrate is somewhat greater in phosphate buffer.

Buffer	PH (µM)	Reaction Time (h)	AC (µM)
Tris-HCl, 50 mM	200	3.0	35.19
Tris-HCl, 100 mM	200	3.0	32.53
KPi, 50 mM	200	3.0	41.98
KPi ,100 mM	200	3.0	41.89

Increased yields for the production of AC from PH 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 AC from PH by CypExpress1A2. The reactions conditions were generally identical to those in the first set of experiments. Initial PH concentrations of 200 µM and 500 µM were used to determine relative catalytic efficiency as a function of substrate concentration.

HPLC profiles: In Figure 3 below, the red curve is the analysis of a sample of the supernatant after cycle 1. The black curve is the results obtained for a sample of the supernatant from the second cycle. The AC product peaks more clearly visible by "zooming" the HPLC to the first 9 min as shown in figure 4.

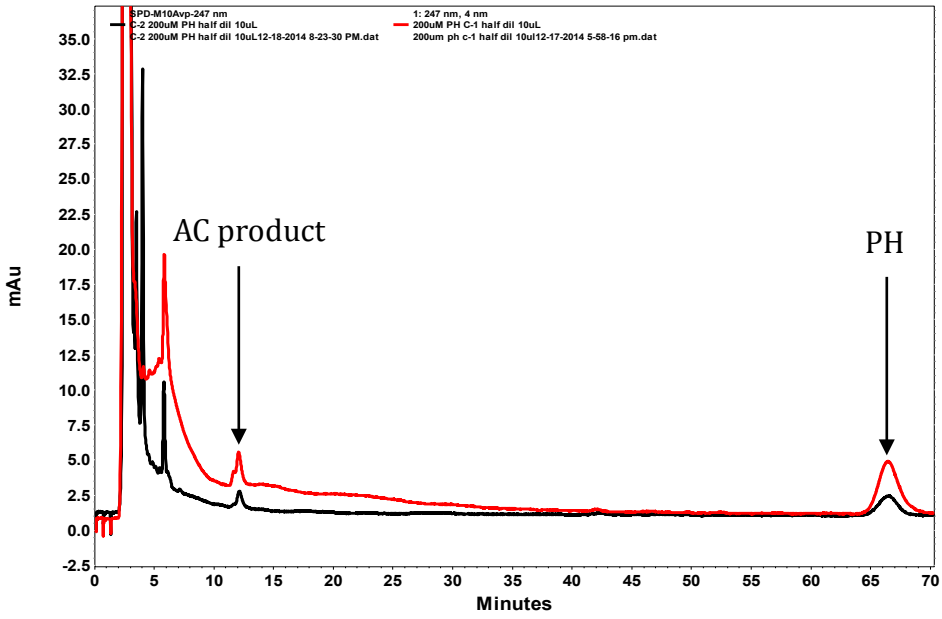
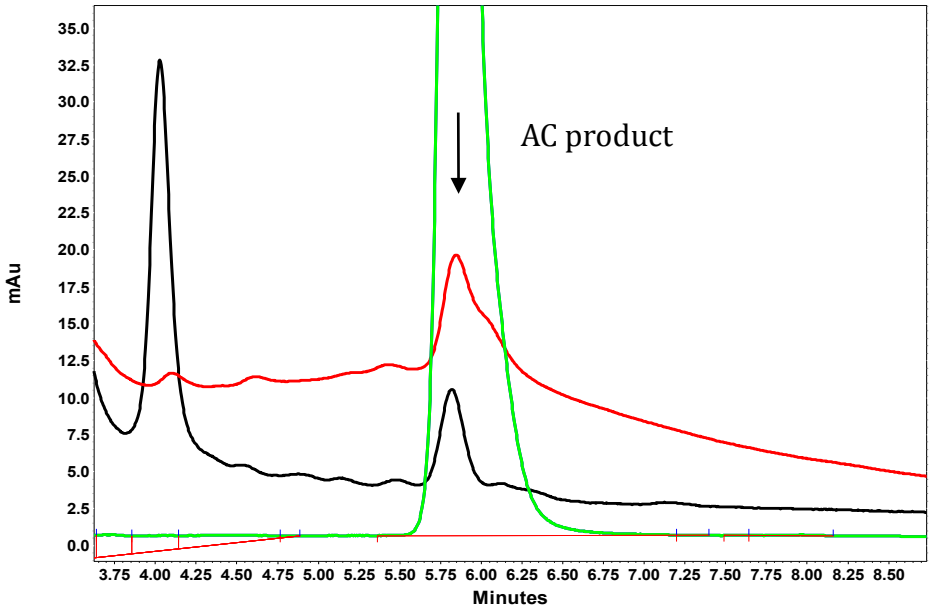


Figure 4:



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

Reaction Sample	µM of PH	Reaction hrs.	µM of AC ± SD
C-1 Supernatant	200	4	**36.53 ± 3.97
C-1 Supernatant	500	4	**43.60 ± 0.87
C-2 Cell Suspension	200 in Cycle-1 No addition in C-2	4	18.18 ± 0.17
C-2 Cell Suspension	500 in Cycle-1 No addition in C-2	4	28.16 ± 0.03

*After Cycle-1, cell pellet was stored at 4°C over night before start of Cycle-2

**One unknown peak was partially co-eluted with AC peak. Hence, determination of the peak area was done by manually splitting of the partially overlapped peak.

Summary: For 200 µM PH, two reaction cycles effected 27.4% conversion to AC.

For 500 µM PH, two reaction cycles effected 14.4% conversion to AC.

