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CASE STUDY

CypExpress™ 2D6 Catalyzed Conversion of Dextromethorphan (DOM) to Dextrorphan (DOH)

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CypExpress™

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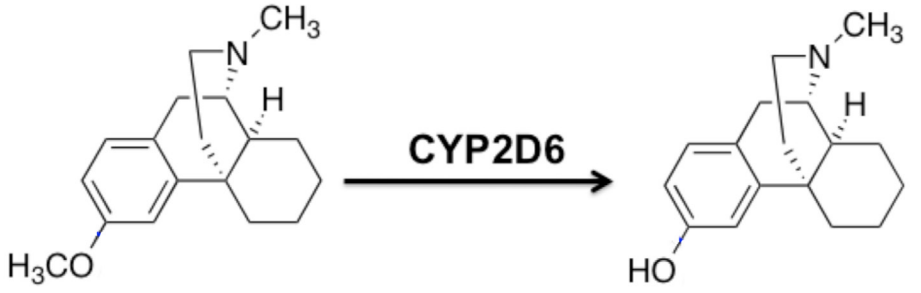
Introduction: These studies were performed using the FDA-recommended substrate Dextromethorphan to evaluate the utility of CypExpress2D6 for drug metabolism and disposition studies.



Dextromethorphan (DOM) **Dextrorphan (DOH)**

Reaction Conditions:

Metabolite Identification in 24-well microplates: 500 – 1,000 µM DOM, 20-100 mg/mL CypExpress2D6, 50 mM KP_i buffer pH 7.5, 30°C, 200-600 rpm in microplate shaker, Reaction volume 200 µL, Reaction time 3 to 4 h or as specified.

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 = 500 to 1,000 µM DOM.

CypExpress 2D6 = 100 mg/mL

Buffer = 50 mM KP_i 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.

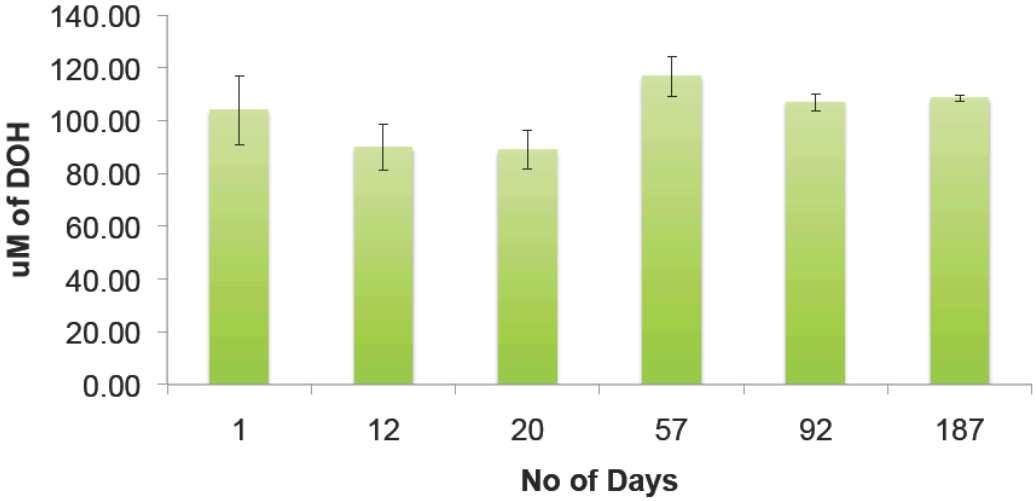
At the end of the reaction period, either

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

   Or

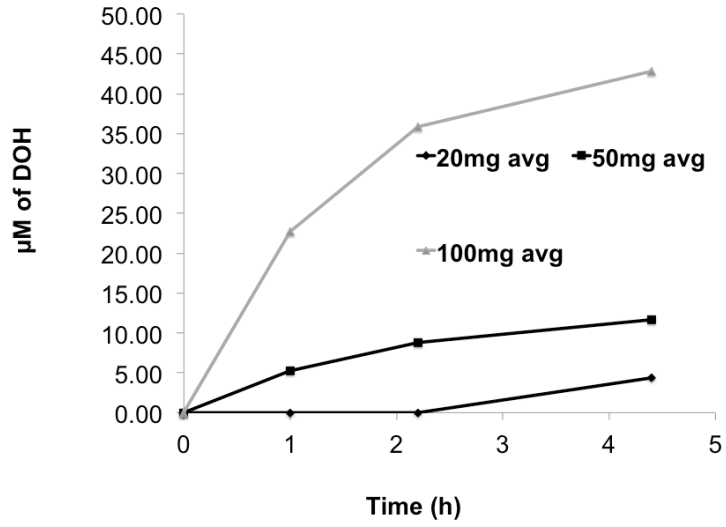
B. CypExpress2D6 was pelleted by centrifugation at 14,000 x g.
   a. The supernatant from the first cycle was analyzed by HPLC.
   b. The pellet – was resuspended in fresh buffer containing G6P, but no additional substrate, and incubated for a second reaction cycle. After which the entire suspension was extracted and subjected to HPLC.

Stability of CypExpress2D6 stored at -80°C: CypExpress is a novel *in vitro* P450 catalytic system with multiple components (recombinant human 2D6, 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 1):

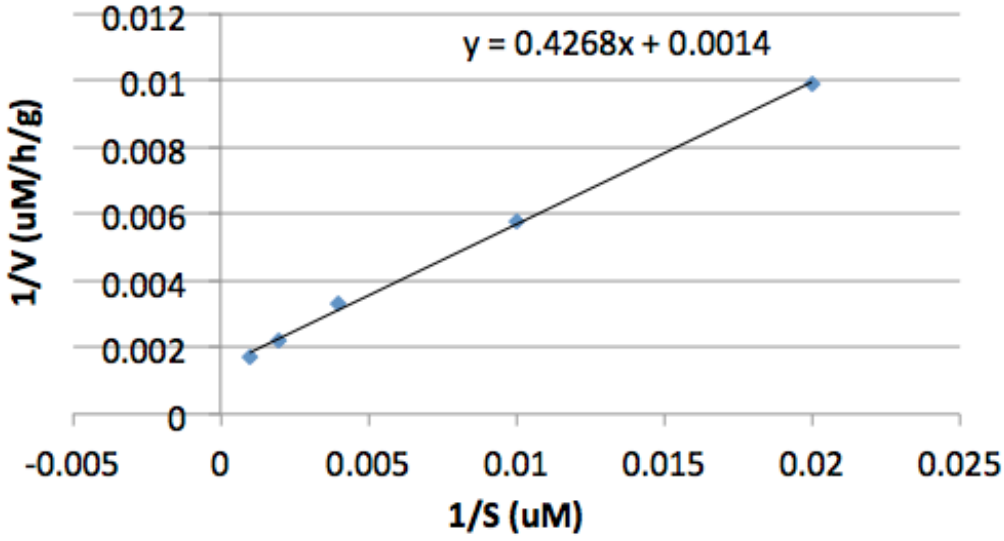


Although not recommended, we have also observed that dry CypExpress powder can be stored at room temperature for up to 3 weeks, and that CypExpress suspensions in buffer can be frozen and thawed up to four times without demonstrable loss of activity.

Dose-dependent conversion of DOM to DOH by CypExpress2D6 in 24 well microplates: 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 CypExpress2D6 (100 mg/mL) conversion of DOM to DOH in 50 mM phosphate buffer pH 7.5. The production of DOH from 500 µM DOM by different concentrations of CypExpress2D6 at varying incubation times (30°C) was examined (figure 2). Although the reaction rate slows with time, the greatest product yield was obtained using 100 mg/mL of CypExpress2D6 for 3-4 hrs. This is a convenient time for high-throughput microplate studies.



Determination of Km and Vmax of CypExpress2D6 for DOM: Km and Vmax values were obtained for CypExpress2D6 (100 mg/mL) incubated for 4 hrs at 30°C with with DOM ranging from 50 – 1,00 µM. A Lineweaver-Burke plot of the results thus obtained is presented in figure 3, and gave a Km of 304 µM and a Vmax of 714 µM/hr/gram of CypExpress. However, as shown in Figure 2 and Table 1 (below), at 100 mg/mL CypExpress 2D6, the reaction slows significantly after 2 hr.

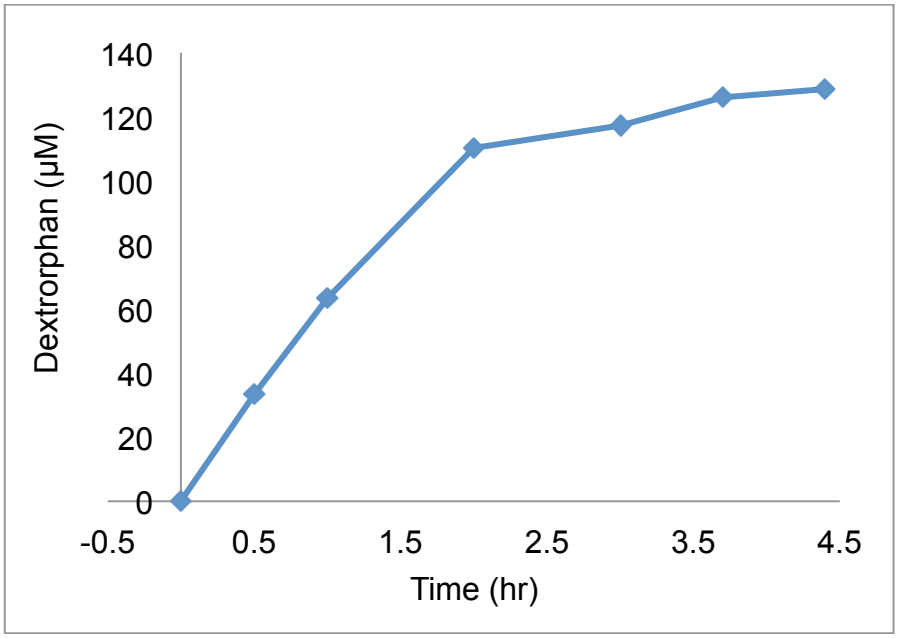


The data presented in Table 1 also demonstrate the reproducibility of these microplate studies.

CypExpress 2D6	µM of DOM	Reaction hrs	µM of DOH
100 mg/mL	250	2	54.6 ± 1.7
100 mg/mL	500	2	67.1 ± 2.2
100 mg/mL	250	4	64.5 ± 0.0
100 mg/mL	500	4	78.5 ± 1.0

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.

Scale-up conversion of DOM to DOH in a single reaction cycle: The rate of conversion 500 µM DOM to DOH by CypExpress™ 2D6(100 mg/mL) at 30°C in a of a 200 mL reaction volume 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 4:



The HPLC profiles of aliquots of the reaction mixture taken at these time intervals are presented in Figure 5:

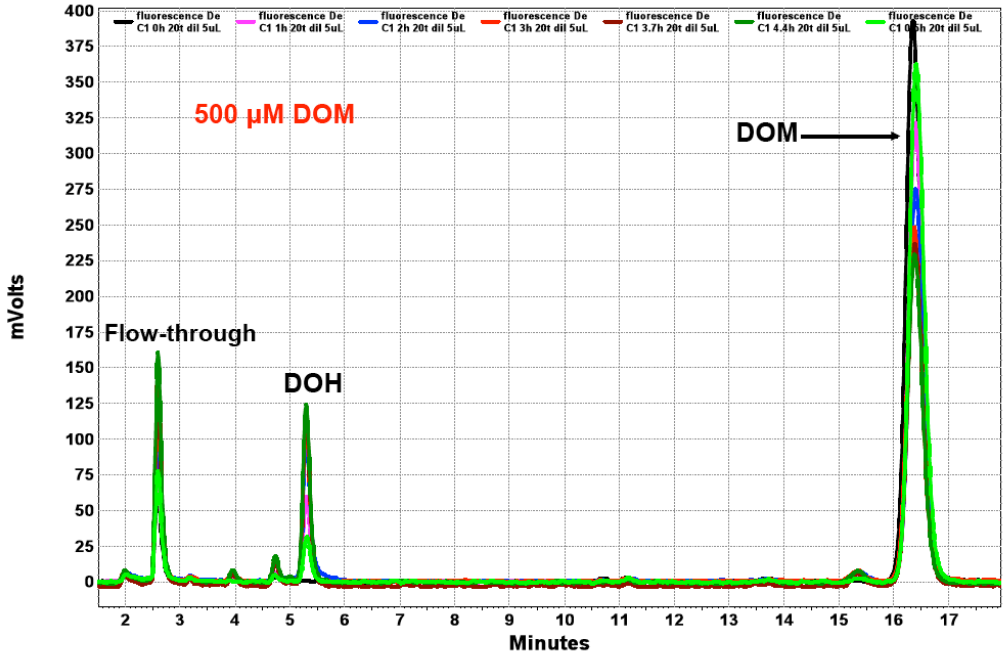


Figure 6 zooms in on the peak obtained for DOH production

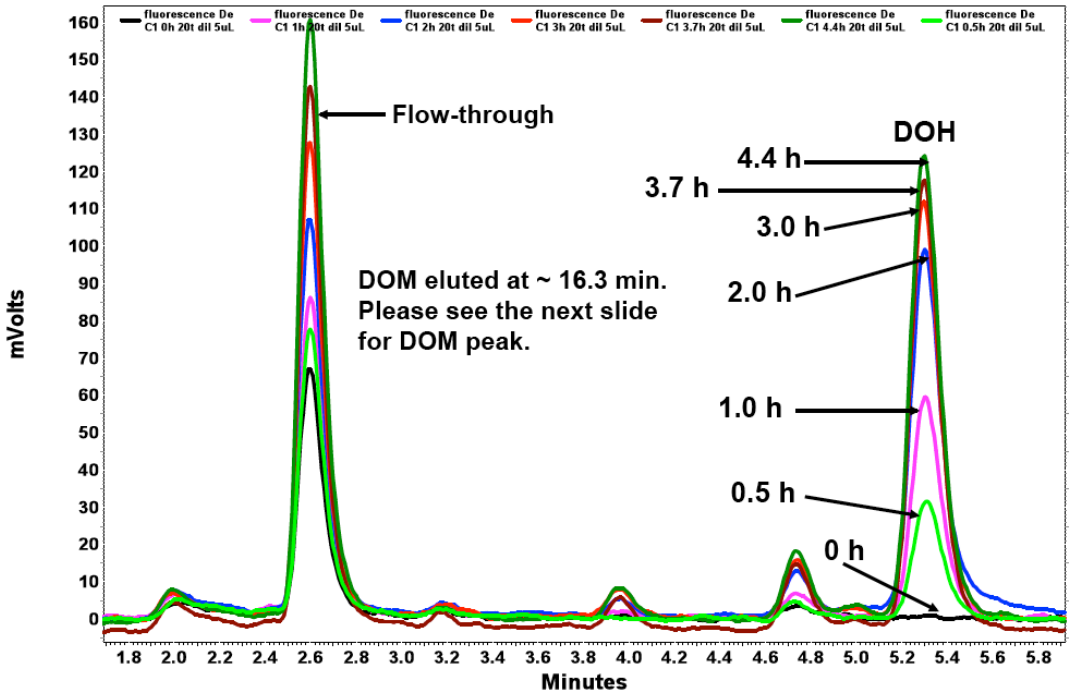
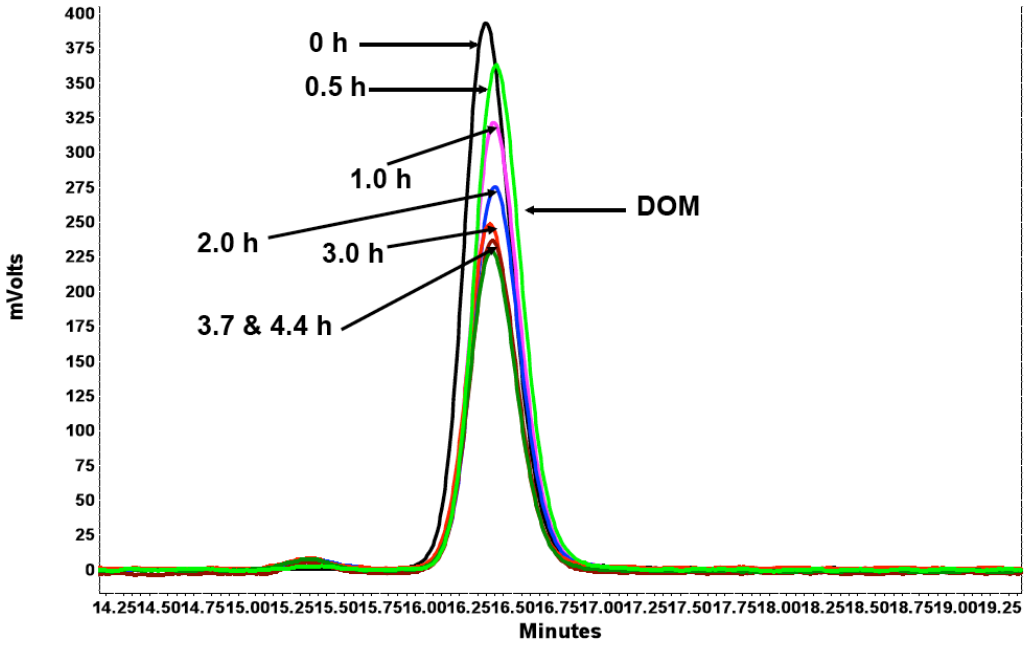


Figure 7 zooms in on the time-dependent diminution of the peak obtained for the substrate DOM



Yield of DOH from DOM in a single reaction cycle: Using the conditions stated above (500 µM DOM, 100 mg/mL CypExpress2D6, 30°C, 3 hr., 50 mM Pi pH 7.5), the yield of 5.65 DOM in a single reaction cycle (extracted reaction suspension). If the CypExpress is centrifuged, the quantity of DOH recoverable from the supernatant fraction was 3.0 mg.

Increased yield for the production of DOH from DOM by CypExpress2D6 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.

Scale-up production of DOH in a second catalytic cycle: A 200 mL reaction was performed using a DOM concentration of 500 µM. After a 4 hr. first reaction cycle, the CypExpress was pelleted, and the supernatant harvested. The pelleted CypExpress was stored overnight at 4°C, then resuspended in fresh buffer containing G6P but no additional NADP+ or DOM 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 8) supports:

a) The retention by CypExpress of significant quantities of unreacted DOM substrate
b) Retention of significant amounts of the DOH product
c) Retained catalytic activity of the resuspended CypExpress (see insert),
d) However, unlike some other CypExpress systems (see 3A4/Testosterone case study) production of some additional DOH from the retained DOM was relatively low.

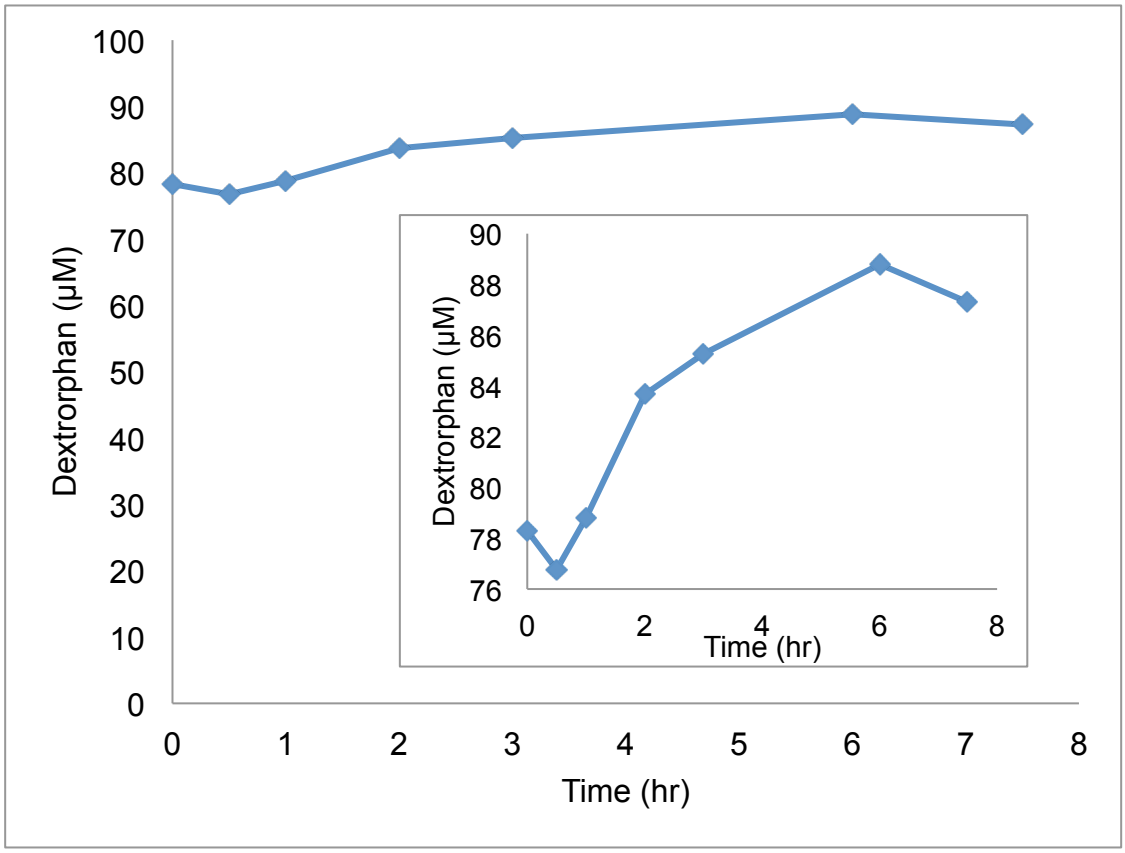
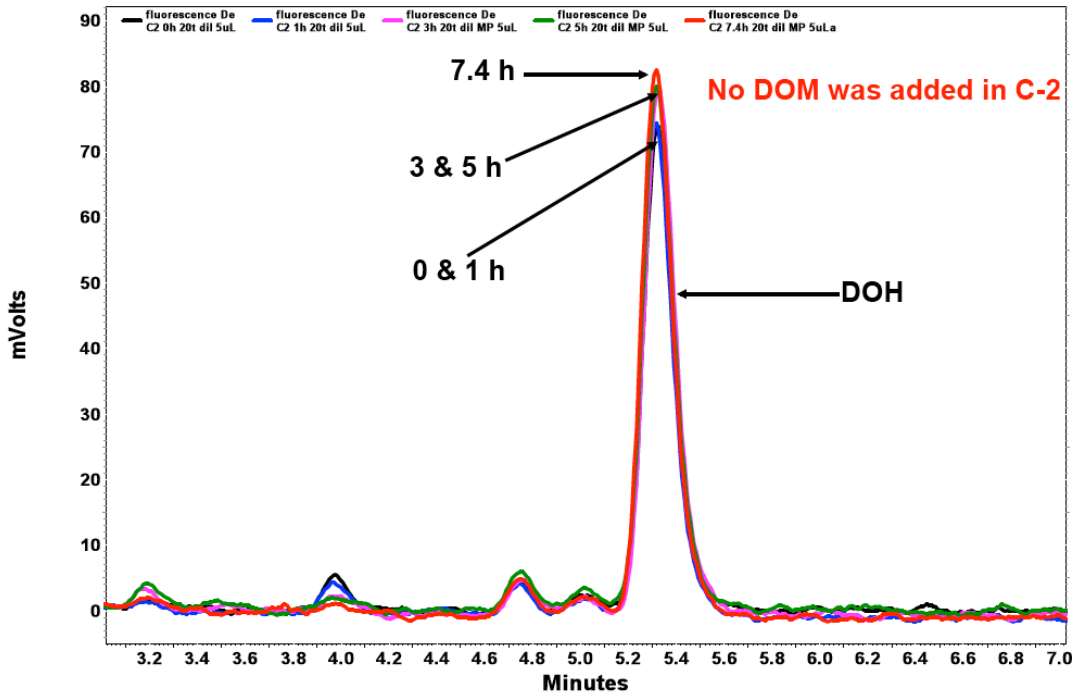


Figure 9: HPLC analysis of the samples withdrawn at specified times from the second CypExpress2D6 reaction cycle.



Discussion: CypExpress2D6 retains activity after several hours. However, relatively little DOH was synthesized from the DOM retained by the CypExpress2D6 after an initial reaction cycle. Simple extraction of the pellet yields about the same amount of DOH as was released into the supernatant during the initial reaction cycle.

The total yield obtained from 27 mg of DOM substrate in two reaction cycles was 13.2 mg, or ~ 50% conversion of the substrate in this 200 mL reaction mixture.