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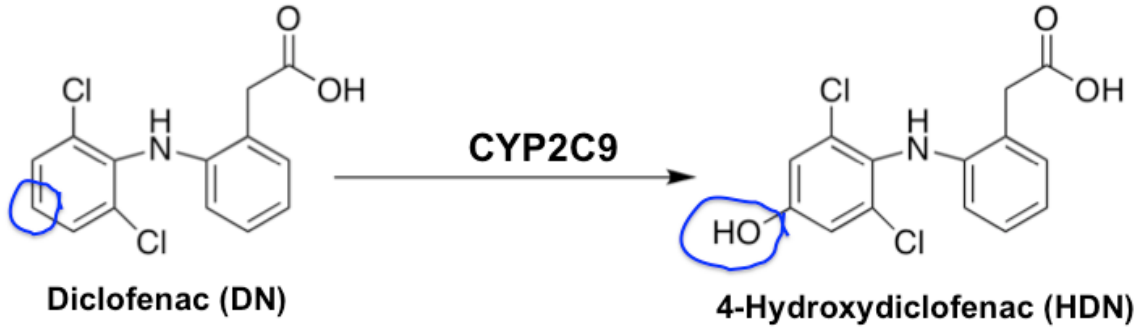
CASE STUDY

CypExpress™ 2C9 Catalyzed Conversion of Diclofenac (DN) to to 4-Hydroxydiclofenac (HDN)

Shuvendu Das,[1] Enrique Martinez,[2] and Mani Subramanian[1]
[1]Center for Biocatalysis and Bioprocessing, University of Iowa
[2] Oxford Biomedical Research, Inc.

P.O. Box 522 • Oxford, MI 48371 • tel: 800.692.4633 • fax: 248.852.4466 • hppt://www.oxfordbiomed.com

Introduction: These studies were performed using the FDA-recommended substrate Diclofenac to evaluate the utility of CypExpress2C9 for drug metabolism and disposition studies.



Diclofenac (DN) 4-Hydroxydiclofenac (HDN)

Reaction Conditions:

Metabolite Identification in 24-well microplates: 250-1000 µM diclofenac, 20-100 mg/mL CypExpress2C9, 50 mM KP_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 MP (500 µM for scale-up)

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

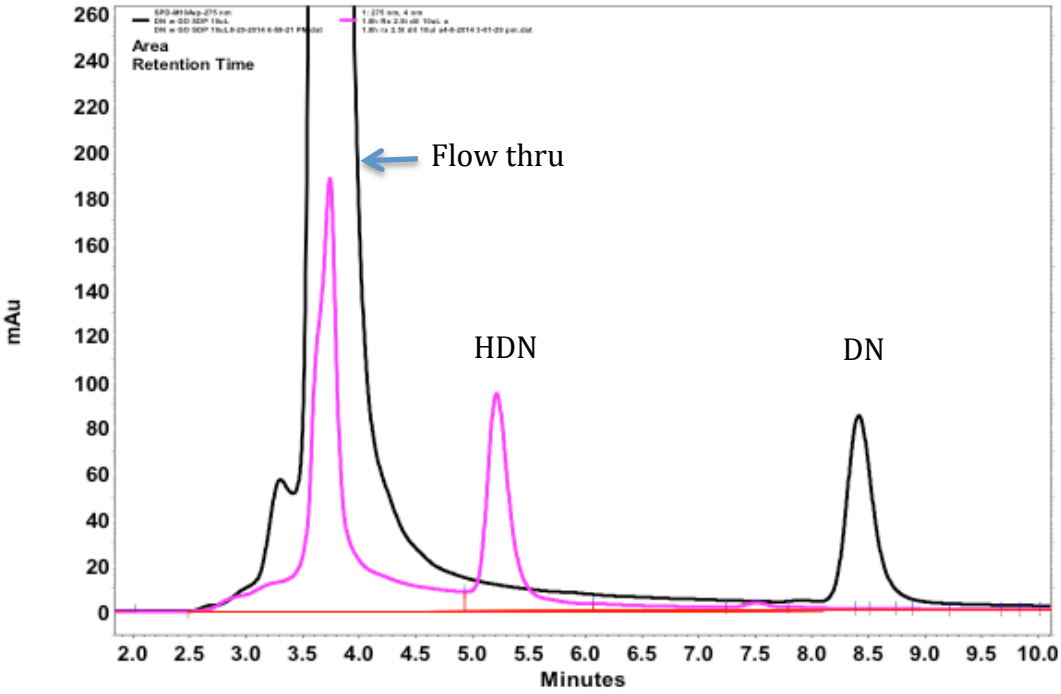
B. CypExpress2C19 was pelleted by centrifugation at 6,000 x g.
   a. The supernatant from the first cycle was analyzed by HPLC.
   b. The pellet – was resuspended in fresh buffer containing G6P, but no additional substrate, and incubated for a second reaction cycle. After which the entire suspension was extracted and subjected to HPLC.

Large Scale (200 mL) Reaction Conditions for Product Isolations: 250-500 µM Diclofenac, 20 g washed CypExpress2C9, 50 mM KP_i buffer pH 7.5, final concentration of NaG6P 12 mM, 30°C, 225 rpm in rotary orbital shaker, Reaction volume 200 mL, Reaction time 2 to 4 h

Conversion of DN to HDC in 24-well microplates: CypExpress™ 2C9 (100 mg/mL) rapidly catalyzed conversion of Diclofenac (DN) to 4-Hydroxydiclofenac (HDN) in a single 2 hr. reaction cycle. The conversion of 500 µM DN to HDN 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. Typical results are shown in Table 1.

NO.	Sampling Time	µM of HDN[1]
1	0 h Sample	0.00
2	0.5 h Sample	94.63
3	2.0 h Sample	332.66

The HPLC profile of the sample taken after 2 hr incubation with CypExpress2C9 (blue curve) is shown in Figure 1 along with results obtained for a vector control (devoid of recombinant human CYP2C9) shown in black.



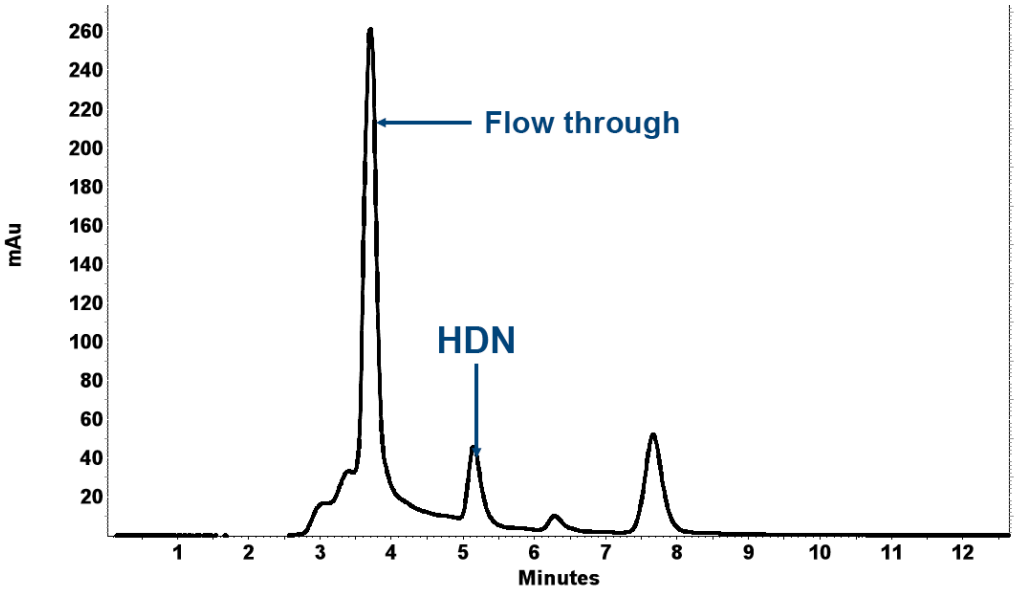
The results clearly show the very specific formation of HDN from DN catalyzed by CypExpress2C9

Conversion of DN to HDN in a single reaction cycle: Large scale (80 mL) CypExpress™ 2C9 (100 mg/mL) catalyzed conversion of 500 µM Diclofnac (DN) to 4-Hydroxydiclofenac (HDN) was performed in 500 µM MP, in 100 mM KP_i of pH 7.5 at 30°C. Results obtained for aliquots that were extracted and subjected to HPLC after a single reaction cycle are presented in Table 2:

No.	Sample	µM of DN	Reaction hrs	µM of HDN
1	Supernatant	500	3	67.91
2	Cell Suspension	500	4	187.91

Discussion: Under these conditions, 37.6% of the DN was converted to HDN. Most of the HDN was retained in the CypExpress pellet after the 3 hr. incubation period, with about 1/3 of the total HDN found in the supernatant.

HPLC Analysis of the reaction product: Results obtained for RP-HPLC after extraction with HPLC Mobile Phase containing 15% (v/v) Methanol, 84.5% (v/v) Water, and 0.5% (v/v) Acetic acid of an aliquot of the suspension after the first cycle are shown in figure 2.

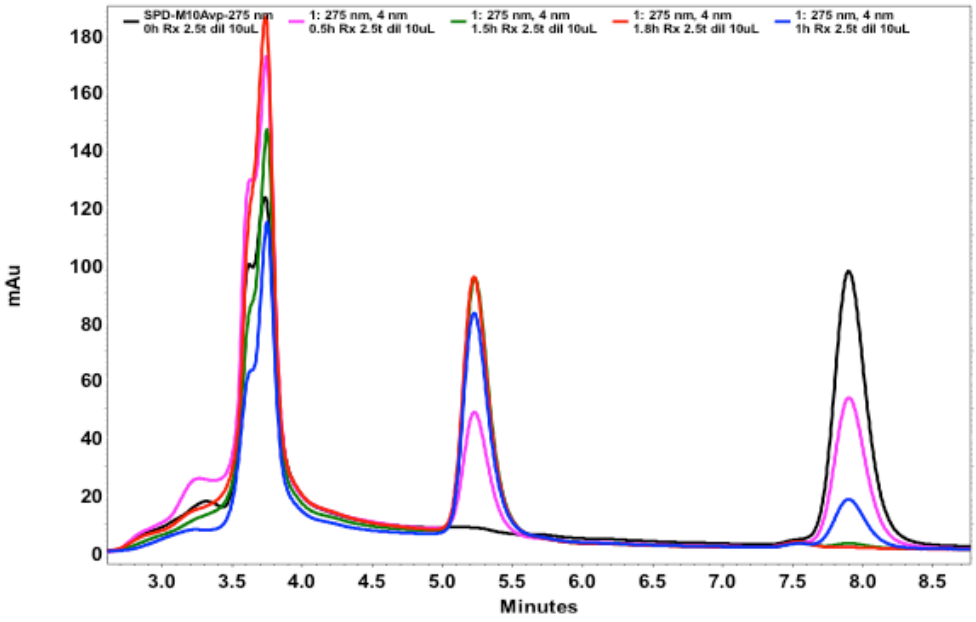


Second cycle production of HDN: The ability of CypExpress2C9 to catalyze further generation of HDN from DN that was absorbed during the first reaction cycle was investigated. To distinguish catalysis from release of product formed during the first reaction cycle, the CypExpress pellet from the first reaction cycle was resuspended in buffer + G6P. An aliquot was removed (T=0) extracted and subjected to HPLC. The remainder of the suspension was incubated, without adding additional DN, for an additional (2^{nd}) 3 hr. reaction cycle. Results are shown in Table 3:

2nd Cycle Sample	Reaction Sample	µM of HDN
0 hr	Resuspended CypExpress (after cycle 1)	160.98
3 hr	Extracted CypExpress suspension	308.74
3 hr	CypExpress supernatant	113.83

Discussion: In addition to the large quantity of HDN that is retained by CypExpress2C9 after one reaction cycle, a even larger quantity of the DN substrate is also retained. Incubation of this CypExpress suspension for an additional 3 hr. yields significant amounts of additional HND product. The total yield for 2 cycles of this large scale CypExpress catalyzed reaction = 68 µM (Cycle 1 supernate) + 309 µM (extracted Cycle 2 suspension) = 376 µM DNH, which is a yield of 75% from the initial 500 µM DN.

HPLC analysis of the kinetics of HDN generation from DN by CypExpress2C9: To address whether comparable amounts of HDN may produced simply by incubation DN with CypExpress for longer periods (vs. multiple reaction cycles) the kinetics of HDN production in a single 400 mL reaction (one cycle) were investigated. Aliquots of the reaction suspension were removed at 0, 0.5, 1, 1.5 and 1.8 hr, extracted with HPLC Mobile Phase containing 15% (v/v) Methanol, 84.5% (v/v) Water, and 0.5% (v/v) Acetic acid, and subjected to RP-HPLC. Results are presented in figure 3.



Discussion: These results clearly show the rapid production of HDN is the first hour of the reaction, and slower production thereafter – as the DN substrate (peak at 8 min) is depleted .

