DICLOFENAC 4'-HYDROXYLASE ASSAY

This assay measures P450-dependent oxidation of the oral anti-inflammatory agent diclofenac (Voltaren®) (**DCF)** to its 4'-hydroxylated metabolite 4-hydroxydiclofenac (**4-OH DCF**). High affinity DCF 4'-hydroxylation is catalyzed exclusively by CYP2C9, a member of the CYP2C P450 enzyme subfamily present in human liver.

I. REAGENTS NEEDED

A) 100 mM Potassium Phosphate buffer, **pH 7.4**, at room temperature.

B) 2.5 mM DCF
MW = 318.1
Prepare by dissolving 10 mg DCF in 1.0 ml MeOH. Dilute 40 µl of this solution with 460 µl MeOH to give 2.5 mM DCF. Add 20 ul 2.5 mM DCX per ml assay buffer to give final conc of 50 µM.

C) 10 mM NADPH
Prepare by dissolving 4.7 mg in 0.5 ml Buffer A – enough for 20 assay tubes.

D) 2N HCl

E) Ethyl Acetate

F) Methanol

G) 0.25 mM 4-OH DCF
[MW = 312.2]

II. PROCEDURE

1. Pipette appropriate amount of Reagent A containing 50 µM DCF into 1.5 ml Eppendorf-type microfuge tubes, keeping in mind that the final assay volume is **0.25 ml**. Place tubes in an ice bath.

2. Add human liver microsomes (0.1 - 0.2 nmol microsomal P450) to the tubes.

3. Add 25 µl of Reagent C to the appropriate tubes, vortex, and place tubes in shaking water bath at 37°C. **DO NOT ADD** Reagent C to incubation tubes that will be used as blanks or for standards.

4. Terminate reactions after **15 min** by adding 50 ul of Reagent D, vortexing well, and place on ice.

5. After all reactions have been stopped, add 1.0 ml of Reagent E to each tube, and vortex the tubes for 5 min using the Multiple Vortexer.

6. Centrifuge tubes at 10,000 rpm for 5 min in the Heraeus Microfuge to separate the organic and aqueous layers. Transfer 0.70 ml of the organic (upper) phase to a 10 x 75 mm disposable glass tubes, and evaporate sample to dryness at room temp using the N2-EVAP nitrogen evaporator.

7. Add 60 µl of Reagent F to each tube, cover with a plastic cap, vortex briefly, and sonicate for 5 min in a bath-type sonicator to completely resolubilize residues. Process samples by HPLC as described below or store @ -20°C until HPLC analysis can be performed.

☞**NOTE A** - Assay blanks contain all components except NADPH (Reagent C). Standard curves are constructed by adding 5, 10, 20 and 30 µl of Reagent G (equivalent to 1,25, 2.5, 5.0 and 7.5 nmol 4-OH DCF) to the assay tubes with NADPH OMITTED, and performing assay as described above.

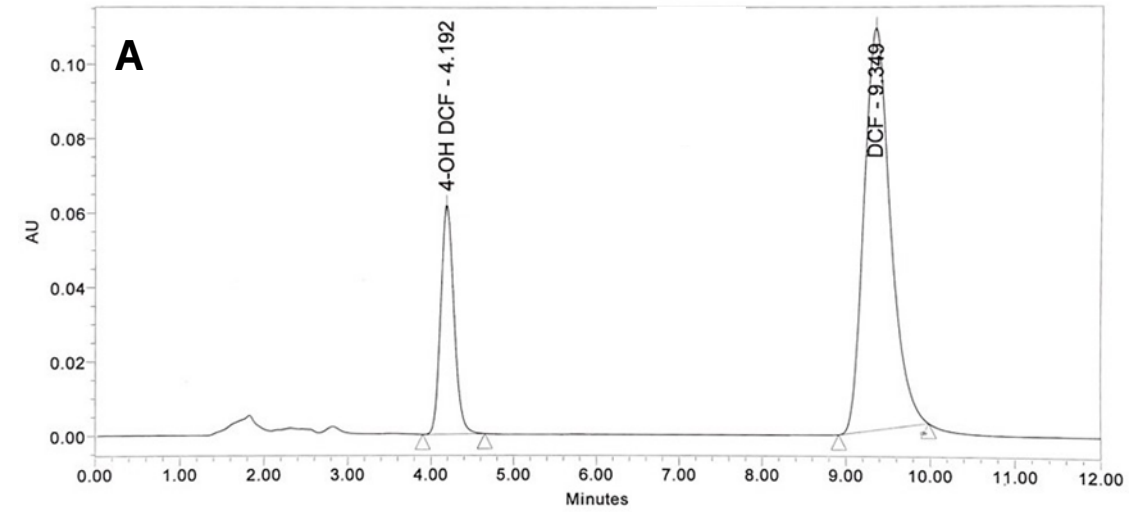
III. HPLC ANALYSIS CONDITIONS FOR DCF and 4-OH DCF

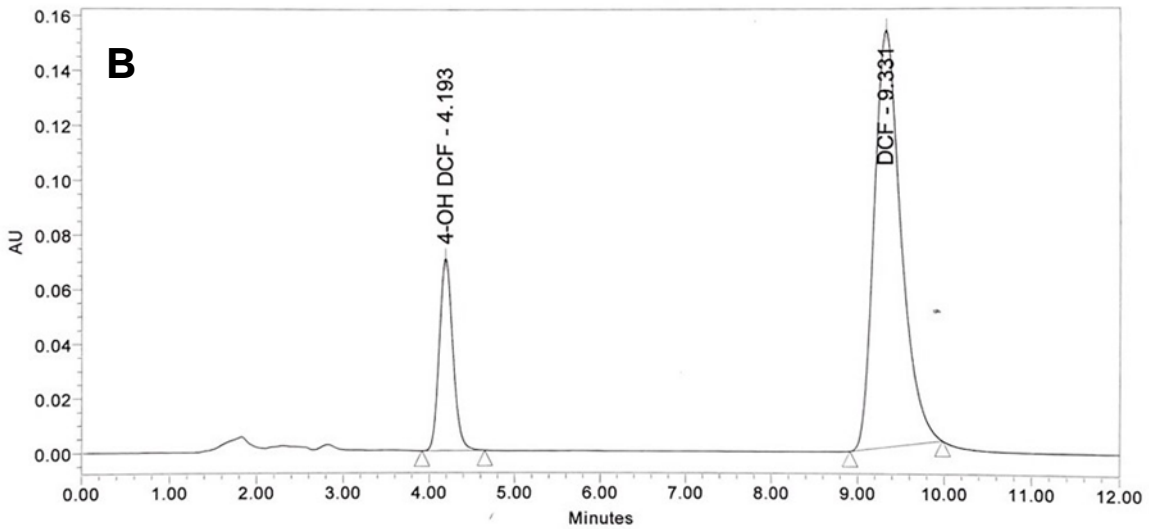
Column:	**Phenomenex Kinetex (4.6 x 150 mm) w/ CrudCatcher Guard**
Mobile Phase:	**Solvent A** – 0.05% Phosphoric Acid
	Solvent B – ACN
Flow Rate:	0.75 ml/min
Column Temp:	Ambient
Sample Temp:	Ambient
Peak Detection:	282 nm
Run Time:	12 min (isocratic)
Injection Volume:	**30 µl**

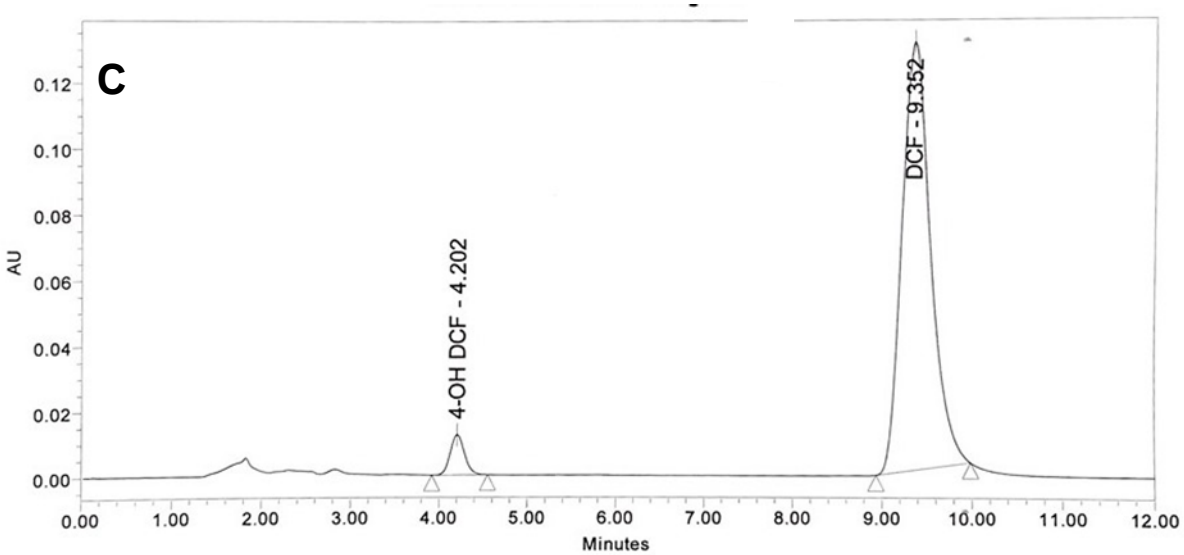
(min)	(A %)	(B %)	(ml/min)	(gradient)
Initial	50	50	0.75	N/A

Using these HPLC conditions, 4-OH DCF elutes at **4.2 min** while the parent compound DCF elutes at **9.7 min** (see attached chromatograms).

DICLOFENAC – CYP2C9







HPLC analysis of diclofenac 4'-hydroxylation by human liver microsomes. Diclofenac (DCF; 50 µM) was incubated with human liver microsomes (0.1 nmol aggregate P450) for 15 min at 37°C in the presence of NADPH and immune-specific IgG or control (preimmune) IgG. DCF was separated from the metabolite 4'-hydroxydiclofenac (4-OH DCF) by reversed-phase HPLC performed with a Waters Alliance 2690 HPLC system equipped with a UV/Vis detector. 4-OH DCF and DCF were resolved using a Phenomenex Kinetex C18 column (4.6 mm x 15 cm, 5 mm particle size). The mobile phase was a 1:1 mixture of 0.05% phosphoric acid and acetonitrile using a flow rate of 0.75 ml/min, and the column eluate was monited at 282 nm. Under these conditions, 4-OH DCF and DCF gave retention times of 4.2 and 9.7 min, respectively; the total sample analysis time was 12 min. 4-OH DCF formation was quantified by comparison of peak areas with those of known analytical standards.

a) DCF + liver microsomes + NADPH + Preimmune IgG
b) DCF + liver microsomes + NADPH + Anti-CYP1A2 IgG
c) DCF + liver microsomes + NADPH + Anti-CYP2C9 MaB