

## Enzyme Assays

### CYP2C9: Assay Conditions for Diclofenac 4'-Hydroxylation

*Diclofenac 4'-hydroxylation is a low  $K_m$ , high turnover assay for human CYP2C9. The apparent  $K_m$  for the CYP2C9-catalyzed reaction is 2-3  $\mu\text{M}$ . The turnover number (per unit cDNA-expressed CYP2C9\*1 is over 40  $\text{min}^{-1}$ ). When performing kinetic analyses near the apparent  $K_m$ , protein concentration and incubation time should be chosen to avoid excessive metabolism of substrate. BD Biosciences supplies 4'-hydroxydiclofenac metabolite standard (Cat. no. 451443 and 451743) for quantitation of the assay results.*

#### Solutions

- I. 0.4 mM Diclofenac in 0.1 M Tris pH 7.5, 1:5 dilution of Cat. no. 451202
- II. 20 mg/ml Glucose 6-phosphate, 20 mg/ml NADP, 13.3 mg/ml  $\text{MgCl}_2 \cdot \text{H}_2\text{O}$ , (Cat. no. 451220)
- III. 40 U/ml Glucose 6-phosphate dehydrogenase in 5 mM sodium citrate (tribasic), (Cat. no. 451200)
- IV. 0.1 M Tris pH 7.5, 1:5 dilution of Cat. no. 451202
- V. 94% Acetonitrile, 6% glacial acetic acid

#### Incubation Conditions (for 1 ml Final Volume)

- |                        |  |
|------------------------|--|
| 50 $\mu\text{l}$       | Solution II.   |
| 10 $\mu\text{l}$       | Solution III.  |
| 250 $\mu\text{l}$      | Solution I. (Final concentration 100 $\mu\text{M}$ , a saturating concentration) |
| xx $\mu\text{l}$       | Enzyme. (human liver microsomes or cDNA-expressed)                               |
| 690 - xx $\mu\text{l}$ | Solution IV.   |

Mix and prewarm to 37°C all solutions except enzyme. Initiate incubation with the addition of enzyme. After the desired incubation time, stop the reaction by the addition of 200  $\mu\text{l}$  of solution V and cool on ice. Centrifuge 12000 x g for 4 minutes to precipitate protein. Analyze the supernatant for product formation by HPLC separation with UV detection. Recommended range of injection volumes - 10 to 150  $\mu\text{l}$ .

#### HPLC Conditions

Mobile Phase A: 30% Acetonitrile, 70% water, 1 mM Perchloric acid (See Note 1)

Mobile Phase B: 100% Methanol

Gradient: Initial conditions: 30% B with a linear gradient to 100% B over 20 minutes

Column: Nucleosil C18, 4.6 x 250 mm, 5  $\mu\text{m}$  particle size (see Note 2)

Temperature: 50°C (see Note 3)

Flow Rate: 1 ml/min

Detector: Absorbance at 280 nm

Retention Times: 4'-Hydroxydiclofenac (Cat. no. 451443 and 451743), 11 minutes; Diclofenac, 15 minutes

#### Note 1

Diclofenac and 4'-hydroxydiclofenac are being chromatographed at acidic pH with the carboxylic acid protonated. Separation can also be achieved at slightly alkaline pH (pH 7.4) when the carboxylic acid is ionized, however, column life may be adversely affected. This is the same mobile phase used for the bufuralol 1'-hydroxylase assay.

#### Note 2

4'-hydroxydiclofenac and diclofenac are easily separated and most C18 columns should be adequate for the purpose. However, some adjustment in mobile phase gradient conditions may be desired.

#### Note 3

Column temperature can range from room temperature to 50°C. The use of a controlled, elevated temperature provides greater reproducibility in retention times and lower column back pressures.

#### Reference

J. Chromatography (1985) 338, 151; J. Chromatography (1990) 528, 487; Life Sciences (1993) 52, 29; J. Chromatography (1993) 620, 158.