DEVELOPMENT AND VALIDATION OF A HIGH-THROUGHPUT RADIOMETRIC CYP2C9 INHIBITION ASSAY USING TRITIATED DICLOFENAC

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ABSTRACT:

A rapid and sensitive radiometric assay for assessing the potential of drugs to inhibit cytochrome P450 (P450) 2C9 in human liver microsomes is described. In contrast to the conventional diclofenac 4′-hydroxylation assay, the new method does not require high performance liquid chromatography (HPLC) separation and mass spectrometry. The assay is based on the release of tritium as tritiated water that occurs upon CYP2C9-mediated 4′-hydroxylation of diclofenac labeled with tritium in the 4′ position. The radiolabeled product is separated from the substrate using 96-well solid-phase extraction plates. The reaction is NADPH-dependent, and sensitive to CYP2C9 inhibitors and inhibitory monoclonal antibodies, but not to inhibitors of or antibodies against other P450 enzymes. Competition experiments using tritiated and unlabeled diclofenac indicated that CYP2C9-mediated diclofenac 4′-hydroxylation exhibits positive cooperativity and no significant kinetic isotope effect or NIH shift. IC₅₀ values for 18 structurally diverse chemical inhibitors were not significantly different from those determined in the diclofenac 4′-hydroxylation assay, using HPLC-tandem mass spectrometry. All the steps of the new assay, namely, incubation, product separation, and radioactivity counting, are performed in 96-well format and can be automated. This assay thus represents a high-throughput version of the classic diclofenac 4′-hydroxylation assay, which is one of the most widely used methods to assess the potential for CYP2C9 inhibition of new chemical entities.

The main drug-metabolizing system in mammals is cytochrome P450 (P450), a family of microsomal enzymes present predominantly in the liver. When a drug that is metabolized by a particular P450 is coadministered with an inhibitor of that same enzyme, changes in its pharmacokinetics can occur, which can give rise to adverse effects (Bertz and Granneman, 1997; Lin and Lu, 1998). It is therefore important to predict and prevent the occurrence of clearance changes due to metabolic inhibition. During the drug discovery process, it is a practical challenge posed by these assays is that they require HPLC separation of the reaction product from the substrate, followed by UV or mass spectrometric detection. This renders the assays relatively laborious, time-consuming, and not ideally suited for screening the large number of compounds typically required in an industrial drug discovery setting.

Several alternative assays, amenable to high-throughput screening, have been introduced in the past several years. These assays are based on the use of fluorogenic (Crespi and Stresser, 2000) substrates, eliminating the need for HPLC separation. One of the most widely used fluorogenic substrates is 7-methoxy-4-(trifluoromethyl)-coumarin (Crespi and Stresser, 2000). Even though fluorometric CYP2C9 assays are rapid, easy to perform, and amenable to automation, they suffer from a number of limitations, such as the absence of CYP2C9-selective probes, the need to use recombinant enzyme rather than HLMs, imperfect correlation of IC₅₀ values with those determined using classical substrates (Cohen et al., 2003), and fluorescence interference by many test compounds. On the other hand, O-methyl-14C-labeled naproxen, which has been used to probe CYP2C9 activity in HLMs, is not a selective CYP2C9 sub-

ABBREVIATIONS: P450, cytochrome P450; HLM, human liver microsome; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; IC₅₀, concentration of drug required to inhibit activity by 50%; SA, specific radioactivity; S₅₀, concentration of substrate at which 50% of maximal activity is observed.
strate, with CYP1A2 and CYP2C19 contributing significantly to
O-demethylation (Moody et al., 1999).

For these reasons, a non-HPLC assay based on the use of a classical
and selective CYP2C9 substrate such as diclofenac would be highly
desirable. Hydroxylation of diclofenac at the 4’ position is accompa-
nied by release of the corresponding hydrogen as water. When this
position is labeled with tritium, CYP2C9-mediated hydroxylation
generates radiolabeled water, which can easily be separated from
the unreacted substrate. We recently reported the development of a
high-throughput CYP3A4/5 assay based on the use of \[^{1}H\]testosterone
and separation of the tritiated water product on 96-well solid-phase
extraction plates (Di Marco et al., 2005). In the present paper, we
describe the synthesis of diclofenac labeled with tritium in the 4’
position, the development of a radiometric assay using this substrate,
and the study of reaction kinetics in the presence and absence of
CYP2C9 inhibitors. We show that the new assay accurately measures
the activity of CYP2C9 and the potency of CYP2C9 inhibitors and
represents a high-throughput version of the conventional diclofenac
4’-hydroxylation assay.

Materials and Methods

Materials. Oasis HLB 96-well extraction plates and vacuum manifold
were purchased from Waters (Milford, MA). Pooled human liver microsomes
were obtained from BD Gentest (Woburn, MA). Other chemicals were purchased
from Sigma-Aldrich (Milano, Italy).

Synthesis of \[^{4}\text{H}]\text{Diclofenac.} The synthetic route is schematically
described in Fig. 1. Synthesis of diclofenac using the Ullman reaction has been
described (Moser et al., 1990; Satoh et al., 1993; Oza et al., 2002). The Ullman
reaction was modified to produce 2-[(2,6-dichloro-4-bromophenyl)amino]
phenylacetic acid (I), which, in the presence of tritium and a palladium catalyst,
was then converted to diclofenac labeled in the 4’ position.

Synthesis of 2-[(2,6-dichloro-4-bromophenyl)amino]phenylacetic acid (I)
as was follows. 2-Biphenyl acetic acid (21.3 mg, 0.08 mmol) was added to
a mixture of 2,6-dichloro-4-bromobenzoic acid (78 mg, 0.32 mmol), anhydrous
potassium carbonate (33.6 mg, 0.24 mmol), and activated copper powder (2.25
mg, 0.035 mmol) in N-methylpyrrolidone (0.5 ml). The reaction mixture was
heated at 135°C for 22 h with stirring while water was distilled off through a
descending condenser. The color of the reaction mixture changed to brown-
black. The hot reaction mixture was treated with hot water and filtered through
Celite (World Minerals, Santa Barbara, CA). The crude product was purified
by reserve-phase HPLC (Luna Phenyl Hexyl 250 × 10 mm column, Phenome-
nex, Torrance, CA), water containing 0.1% trifluoroacetic acid/acetonitrile
(50:50; flow rate 4 ml/min, UV = 254 nm, retention time = 23–24 min). The
required fractions were collected and passed through Sep-Pak C-18, followed
by eluting with 10 ml of ethanol to yield 5 mg of 2-[(2,6-dichloro-4-
bromophenyl)amino]phenylacetic acid (I).

Synthesis of \[^{4}\text{H}]\text{Diclofenac from compound I was as follows. 2-[(2,6-
Dichloro-4-bromophenyl)amino]phenylacetic acid (I) (5 mg) was stirred with
tritium gas using catalyst 10% Pd/C (5 mg) in dimethylformamide (1 ml) for
1 h. The reaction mixture was filtered and coevaporated with ethanol (two
times, 10 ml) to remove any exchangeable tritium. The crude product
was purified by using a semipreparative HPLC column [Luna Phenyl Hexyl, 250 ×
10 mm column, water containing 0.1% trifluoroacetic acid/acetonitrile (55:45),
flow rate 4 ml/min, UV = 254 nm, retention time = 20-21 min] to yield
\[^{4}\text{H}]\text{diclofenac} [10 mCi, SA = 22.7 Ci/mmol, as determined by liquid
chromatography/mass spectrometry (LC/MS)]. LC/MS: 296 (M)\(^+\), 298 (M +
2)\(^+\). Radiochemical purity was >98%.

Radiometric CYP2C9 Assay. Reactions were carried out in 96-well con-
cial microtiter plates (Corning Glassworks, Corning, NY) containing \[^{4}\text{H}]
diclofenac (0.05–0.1 µCi), unlabeled diclofenac (10 µM, except otherwise
noticed), pooled HLMs (125 µg/mL, except otherwise noted), and 0.1 M potas-
sium phosphate buffer, pH 7.6, in a final volume of 100 µL. Inhibitors were
added to the reaction mixture from stock solutions in dimethyl sulfoxide/
acetonitrile/water (35:25:40, v/v), giving final solvent concentrations of 0.7%
dimethyl sulfoxide and 0.5% acetonitrile. Controls without inhibitors con-
tained an equivalent amount of vehicle. After preincubation for 10 min at
37°C, reactions were started by addition of an NADPH-regenerating system
containing 1 mM NADPH, 5 mM glucose 6-phosphate, 3 mM MgCl\(_2\), and 1
U/ml glucose-6-phosphate dehydrogenase. Assays were conducted for 10 min
at 37°C, reactions were started by addition of an NADPH-regenerating system
containing 1 mM NADPH, 5 mM glucose 6-phosphate, 3 mM MgCl\(_2\), and 1
U/ml glucose-6-phosphate dehydrogenase. Assays were conducted for 10 min
at 37°C and stopped by addition of HCl to a final concentration of 0.1 N. Plates
were then centrifuged for 10 min in a microplate rotor, and supernatants were
loaded on a preconditioned 10-mg Oasis HLM 96-well plate. Vacuum was
applied and the flow-through was collected in the collection plate. Then, 75 µL
of water was added, vacuum was applied again, and the wash was collected
into the same plate. Pooled flow-through and water wash were transferred into
scintillation vials and counted in a β-scintillation counter. Alternatively, ali-
quots of this mixture were counted in 96-well scintillation plates using a
TopCount scintillation counter (PerkinElmer Life and Analytical Sciences,
Boston, MA). For the calculation of enzyme activity, product counts were
corrected by subtraction of counts obtained in control incubations performed in
the absence of NADPH-regenerating system. Oasis plates were regenerated by
washing with 5 ml of methanol and 5 ml of water and were reused for up to
5 times.

Calculation of the Apparent Rate of Formation of Unlabeled Product
from Tracer Competition Experiments and Determination of the Kinetic
Tritium Isotope Effect. At low values of substrate conversion, such as those
observed in the present experiments, \( \frac{V}{K} \), the kinetic isotope effect on the \( V/K \) ratio, is given by (Northrop, 1982):

\[
\frac{V}{K} = \frac{SA_0}{SN_A}
\]

where \( SA_0 \) is the initial specific radioactivity of labeled substrate, and \( SN_A \) is the specific radioactivity of product.

If diclofenac 4'-hydroxylation is not subject to a significant NIH shift and tritium is not retained in 4'-hydroxydiclofenac upon hydroxylation of the radiolabel, the tritiated water product from \([4'\text{-}^3\text{H}]\)diclofenac is formed stoichiometrically with 4'-hydroxydiclofenac. When assays are performed using a fixed amount of \([4'\text{-}^3\text{H}]\)diclofenac and varying concentrations of unlabeled substrate, it can be shown (Di Marco et al., 2005) that \( v' = \frac{V}{K} = vS/SA_p \), where \( v' \) is the apparent rate of formation of unlabeled product, \( v \) is the velocity of formation of unlabeled product, and \( vS \) is the velocity of formation of tritiated water.

When \( v' \) is plotted against the substrate concentration, \( S \), and fitted to the Hill equation, \( V_{max} \), \( V_{50} \), the substrate concentration at 50% of \( V_{max} \), and \( n \), the Hill coefficient, can be derived:

\[
v' = \frac{V_{max} \times S^n}{V_{50}^n + S^n}
\]

where \( V_{max} = \frac{V_{max}(V/K)}{S_{50}} \), i.e., the apparent maximal rate of product formation. If \( v \) and \( V_{max} \) are determined independently (by quantification of the 4'-hydroxydiclofenac product), these data can also be used to calculate the kinetic isotope effect \( \frac{V}{K} \).

**Quantification of 4'-Hydroxydiclofenac.** Aliquots of the assay mixture and of metabolite standard curves were analyzed by HPLC-MS/MS using an Agilent HP 1100 liquid chromatograph equipped with a CTC Analytics (Zwischen, Switzerland) PAL autosampler. Chromatography was performed on an Xterra MS C18 column (4.6 mm x 5 cm; 5 μm; Waters) at a flow rate of 2 ml/min, using a mobile phase consisting of a mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) (linear gradient 0–0.5 min, 10% B; 3.0 min, 90% B; 3.5 min, 90% B; 3.6 min, 10% B; the system was equilibrated for 1.4 min at 10% B before the next injection). The eluate was diverted to waste for the first minute and then to a Sciex API-3000 (Applied Biosystem/MDS Sciex, Foster City, CA) triple quadrupole mass spectrometer with a Turbo IonSpray ionization source operated in the positive ion mode. Analysis was performed by nonlinear regression analysis using Analyst Quantitation Wizard software version 1.2 (Applied Biosystems, Foster City, CA).

**Results**

**Separation of Radiolabeled Diclofenac and Tritiated Water Using 96-Well Solid-Phase Extraction Plates.** When a solution of assay buffer containing labeled diclofenac (from 10⁴ to 10⁶ dpm) and stopping solution was applied to 96-well extraction plates containing 10 mg of Oasis sorbent, over 99.8% of the radioactivity was retained on the plate. The labeled diclofenac could be recovered by eluting with methanol. As previously reported (Di Marco et al., 2005), tritiated water was not retained on the Oasis plates under the same conditions and was recovered quantitatively in the combined void volume and aqueous wash.

**Formation of Tritiated Water from \([4'\text{-}^3\text{H}]\)Diclofenac in HLMs.** When \([4'\text{-}^3\text{H}]\)diclofenac was incubated with HLMs in the presence of an NADPH-regenerating system, tritiated water was formed in a time-dependent manner and increased with the concentration of HLMs (Fig. 2). Product formation increased linearly with time for up to 30 min at an HLM concentration of 0.125 mg/ml (correlation coefficient, \( r^2 = 0.979 \)) but was not linear at higher HLM concentra-

**Fig. 2.** Dependence of tritiated water formation from \([4'\text{-}^3\text{H}]\)diclofenac on incubation time and concentration of human liver microsomes. Experiments were conducted at the indicated concentrations of HLMs. Product formation was expressed as a percentage of total radioactivity. Each point is the mean ± half-range of duplicate determinations.

**Effect of P450 Inhibitors and Anti-P450 Antibodies.** To confirm that CYP2C9 mediates product formation, reactions were performed in the presence or absence of furafylline (CYP1A2 inhibitor; Bourrie et al., 1996) and monoclonal antibodies that are inhibitors of CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 (Mei et al., 1999; Shou et al., 2000). As shown in Fig. 3, none of these agents significantly affected formation of tritiated water in HLMs, with the exception of the anti-CYP2C9 and anti-CYP2C19 monoclonal antibodies. The CYP2D6 inhibitor quinidine (10 μM) reduced product formation by less than 25% (data not shown). The antibody directed against CYP2C9 inhibited the reaction by more than 80%. The monoclonal antibody against CYP2C19 is known to cross-react with CYP2C9 (data not shown). These results confirmed that the assay was specific for detecting CYP2C9 activity.

**Competition between Radiolabeled and Unlabeled Diclofenac.** The effect of unlabeled diclofenac on the formation of tritiated water in HLMs is depicted in Fig. 4A. The curve displays a "low dose hook;" i.e., product formation rate increased with increasing concentration of unlabeled substrate, reached a peak at a diclofenac concentration of ~3 μM, and then decreased.

Since \([4'\text{-}^3\text{H}]\)diclofenac is used as an isotopic tracer, the formation rate of tritiated water (\( v' \)) is representative of that of unlabeled product, namely, water derived from 4'-hydroxylation of diclofenac (which is formed stoichiometrically with 4'-hydroxydiclofenac). The apparent formation rate of unlabeled product, \( v' \), is defined as \( v' \)
enacted in the presence of a fixed amount of [3H]-diclofenac and varying

S

regression analysis. Inset, magnification of the initial part of the curve (Materials and Methods)

about the dependence on substrate concentration of the unlabeled

v

and Vmax values are reported under

FIG. 3. Effect of P450 inhibitors and anti-P450 monoclonal antibodies on tritiated

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Enzyme activity is expressed as a percentage of untreated controls and represents

the average ± half-range of duplicate determinations.

DICLOFENAC/H2O),

3-hydroxy-

apparent kinetics of formation of unlabeled product, determined from isotopic

duplicate determinations. The experiment was repeated twice with similar results. B,

[3H]-diclofenac. Experiments were

conducted in the presence or absence of 0.4 mg/ml antibodies, or 30 μM furafylline.

[44x618 to 295x738]

the radiometric assay were almost identical to those determined in the

HPLC-mass spectrometric assays. Tritiated water formation and for-

mations. The experiment was repeated 3 times with similar results. Average S50

and 3H isotope.

TABLE 1

Comparison between IC50 values in radiometric vs. conventional assays

The effect of inhibitors on the formation rate of tritiated water (radiometric) and 4'-

hydroxydiclofenac (conventional) was determined in the same reaction mixture. 4'-

Hydroxydiclofenac was quantified using liquid chromatography-MS/MS. IC50 values were

calculated from full inhibition curves with at least eight concentration points. Data are mean

values ± half-range, n = 2, except for sulfaphenazole (S.E.M., n = 5, conventional assay, or

n = 9, radiometric assay).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 Radiometric/Conventional</th>
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<tbody>
<tr>
<td></td>
<td>μM</td>
</tr>
<tr>
<td>Sulphaphenazole</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.36 ± 0.07</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>Miconazole</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Progestrone</td>
<td>25.2 ± 3.1</td>
</tr>
<tr>
<td>α-Naphthothalone</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>4.8 ± 1.2</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>27.7 ± 6.9</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>54.8 ± 8.1</td>
</tr>
<tr>
<td>Phenybutazone</td>
<td>41.4 ± 6.5</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>43.8 ± 10.4</td>
</tr>
<tr>
<td>Ketocozazole</td>
<td>7.3 ± 0.8</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>37.0 ± 5.5</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Mibefradil</td>
<td>40.0 ± 5.0</td>
</tr>
<tr>
<td>S-Warfarin</td>
<td>14.6 ± 2.3</td>
</tr>
</tbody>
</table>

product, even if the latter is not measured directly (see Materials and Methods). As depicted in Fig. 4B, the curve of v’ versus S could be fitted to the Hill equation, with S50 = 6.8 ± 1.0 μM, n = 1.15 ± 0.05, and V’max = 1.5 ± 0.1 nmol/min/mg (average ± half-range from two independent experiments, each performed in duplicate). The Hill coefficient was slightly greater than 1, suggesting weak positive cooperativity. Indeed, at low substrate concentrations, a sigmoidal relationship was observed between v’ and S, as depicted in the inset of Fig. 4B.

The kinetics of 4'-hydroxydiclofenac formation is depicted in Fig. 4C. The reaction had a S50 of 6.2 ± 0.9 μM, Vmax of 1.3 ± 0.3 nmol/min/mg protein, and Hill coefficient of 1.1 ± 0.1 (average ± SEM, n = 3). Note that the ratio between V’max and Vmax is 0.9, indicating the absence of a significant kinetic isotope effect or NIH shift of the 3H isotope.

Kinetics of Inhibition by CYP2C9 Inhibitors. IC50 values of 18 compounds were compared in the radiometric versus conventional HPLC-mass spectrometric assays. Tritiated water formation and formation of the unlabeled reaction product 4'-hydroxydiclofenac were determined in the same reaction mixture. The 4'-hydroxydiclofenac was quantified by HPLC coupled to triple quadrupole mass spectrometric analysis. IC50 values are summarized in Table 1. IC50 values in the radiometric assay were almost identical to those determined in the conventional assay. Linear regression analysis, excluding the two compounds with IC50 > 30 μM, resulted in a line with a slope of 1.09 and a correlation coefficient (r²) of 0.938 (Fig. 5). These results demonstrate that the radiometric assay provides a reliable measurement of the potency (IC50) of CYP2C9 inhibitors.

Discussion

The release of tritium that accompanies hydroxylation of a substrate has been used to measure the activity of P450 enzymes (Draper et al., 1998; Di Marco et al., 2005; and references therein). We recently reported the development of a high-throughput CYP3A4/5 assay based on the separation of the tritiated water product on 96-well solid-phase extraction plates (Di Marco et al., 2005). The results of the
present study show that this procedure can be used to assay the activity of CYP2C9, using [4-3H]diclofenac as substrate. The new radiometric assay is very sensitive, with a signal to noise ratio greater than 30; requires short incubation times, and low amounts of tracer (~100,000 dpm) and microsomal protein (<15 μg); is performed in 96-well format throughout the incubation, product separation, and scintillation counting steps; and is amenable to automation. The assay represents a high-throughput radiometric version of the classical diclofenac 4'-hydroxylation assay and is suitable for rapid screening of the inhibitory potential of investigational drugs.

Formation of tritiated water from [4-3H]diclofenac in HLMs is mediated almost exclusively by CYP2C9, as shown by its sensitivity to chemical and antibody inhibitors of this enzyme and the lack of inhibition by chemical inhibitors of CYP1A2 and CYP2D6, and inhibitory monoclonal antibodies against CYP2A6, CYP2D6, and CYP3A4/5. The finding that the apparent velocity of formation of tritiated water was very similar to that of 4'-hydroxydiclofenac indicates that the reaction occurs in the absence of a significant kinetic isotope effect or NIH shift of the 3H isotope. To our knowledge, these effects have not been studied before for CYP2C9-mediated diclofenac hydroxylation. Substrate competition experiments showed that the kinetics of [4-3H]diclofenac hydroxylation deviate slightly from typical Michaelis-Menten kinetics, with a Hill coefficient of ~1.2, indicating weak positive cooperativity. Even though positive cooperativity has not been previously reported for diclofenac hydroxylation, other CYP2C9 substrates such as dapsone and naloxone have been shown to exhibit non-Michaelian kinetics (Korzekwa et al., 1998). Heterotropic enzyme activation has also been described for CYP2C9, suggesting that, similarly to CYP3A4 (Shou et al., 2001), CYP2C9 when probed with diclofenac (IC50 = 22 μM), but not when probed with the fluorescent substrate 7-methoxy-4-(trifluoromethyl)-coumarin. As expected, warfarin inhibited tritiated water formation in the present radiometric assay, with an IC50 value of 15 μM. On the basis of the poor correlation between fluorometric and conventional assays, Cohen et al. (2003) recommended that screening with fluorogenic probes should be followed up by studies with conventional substrates. The present assay, which combines the advantages of speed and high throughput, and the use of a conventional substrate, should prove to be a valuable tool for rapidly determining the potential of compounds to inhibit CYP2C9 in a drug discovery setting.

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