DEVELOPMENT AND VALIDATION OF A HIGH-THROUGHPUT RADIOMETRIC CYP3A4/5 INHIBITION ASSAY USING TRITIATED TESTOSTERONE

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ABSTRACT:
A rapid and sensitive radiometric assay for assessing the potential of drugs to inhibit cytochrome P450 (P450) 3A4/5 in human liver microsomes is described. In contrast to the conventional testosterone 6β-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 CYP3A4/5-mediated 6β-hydroxylation of testosterone labeled with tritium in the 6β position. The radiolabeled product is separated from the substrate using 96-well solid-phase extraction plates. Using commercially available [1,2,6,7,3H]testosterone as substrate, we demonstrated that the reaction is NADPH-dependent, and sensitive to CYP3A4/5 inhibitors and a CYP3A4/5-specific inhibitory monoclonal antibody, but not to inhibitors of or antibodies against other P450 enzymes. The method was further improved by synthesis of testosterone specifically tritiated in the 6β position, which displayed greatly improved conversion rate with an ensuing increase in assay sensitivity. Competition experiments using tritiated and unlabelled testosterone indicated that CYP3A4/5-mediated 6β-hydroxylation exhibits positive cooperativity and a modest kinetic isotope effect. IC_{50} values for more than 40 structurally diverse chemical inhibitors were not significantly different from those determined in the testosterone 6β-hydroxylation assay, using HPLC-tandem mass spectrometry analysis. 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 classical testosterone 6β-hydroxylation assay, which is the most widely used method to assess the potential for CYP3A4/5 inhibition of new chemical entities.

The pharmacokinetic and toxicokinetic properties of pharmaceuticals depend in great part on their biotransformation by drug-metabolizing enzymes. The main drug-metabolizing system in mammals is cytochrome P450 (P450), a family of microsomal enzymes present predominantly in the liver. Multiple P450 enzymes catalyze the oxidation of chemicals of endogenous and exogenous origin, including drugs, steroids, prostanoids, eicosanoids, fatty acids, and environmental toxins (Ioannides, 1996). When a drug that is metabolized by a particular P450 enzyme is coadministered with an inhibitor of that same enzyme, changes in its pharmacokinetics can occur, which can give rise to adverse effects (Bertz and Grammeman, 1997; Lin and Lu, 1998; Thummel and Wilkinson, 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 routine practice in the pharmaceutical industry to assess the P450 inhibition potential of drug candidates to exclude potent inhibitors from further development (Lin and Lu, 1998; Crespi and Stresser, 2000; Riley, 2001).

CYP3A4/5 is the most abundant P450 in human liver and is involved in the metabolism of about 50% of drugs used in human therapy (Guengerich, 1999). Inhibition of CYP3A4/5 activity can give rise to clinically significant and potentially life-threatening drug interactions (Thummel and Wilkinson, 1998). Several assay methods are currently used for determining the potential of drug candidates to inhibit CYP3A4/5 activity, and each of these methods presents distinct advantages and disadvantages. The most widely used method is the testosterone 6β-hydroxylation assay, which is specific for enzymes of the CYP3A family (CYP3A4/5) (Waxman et al., 1988; Maenpaa et al., 1993; Wang et al., 1997; Yamazaki and Shimada, 1997). According to recent surveys conducted by reviewers in the Center for Drug Evaluation and Research of the United States Food and Drug Administration, the testosterone 6β-hydroxylation assay represents the most commonly used probe reaction in support of new drug applications (Yuan et al., 1999, 2002). The practical challenge posed by this assay is that it requires HPLC separation of the reaction product from the substrate, followed by UV or mass spectrometric detection. This renders the assay relatively laborious, time-consum-

ABBREVIATIONS: P450, cytochrome P450; HLM, human liver microsome; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography-mass spectrometry; DMSO, dimethyl sulfoxide; NCE, new chemical entity; MS/MS, tandem mass spectrometry; IC_{50}, concentration of drug required to inhibit activity by 50%; S_{50}, concentration of substrate at which 50% of maximal activity is observed.
ing, and not ideally suited for screening the large number of compounds typically required in an industrial drug discovery setting.

Several alternative CYP3A4/5 assays, suitable for high-throughput screening, have been introduced in the past several years. These assays are based on the use of fluorogenic (Crespi and Stresser, 2000) or radiolabeled (Zhang and Thomas, 1996; Moody et al., 1999) substrates, eliminating the need for HPLC separation. One of the most widely used fluorogenic substrates is 7-benzoxo-4-trifluoromethylcoumarin (Crespi and Stresser, 2000). Because this probe is not a specific substrate for CYP3A4/5, the assay cannot be run with HLMs, but requires the use of recombinant enzyme. Alternative fluorometric assays, which use CYP3A-specific substrates and can therefore be performed with HLMs, have been described (Charet et al., 1999; Stresser et al., 2002). Even though fluorometric assays are rapid, easy to perform, and amenable to automation, they suffer from a number of limitations. First, the use of recombinant P450 instead of HLMs, which contain the full complement of P450 enzymes, may give rise to differences in inhibitory potency because test compounds may be subject to metabolism by more than one enzyme, leading to different rates of substrate depletion or formation of inhibitory metabolites. Even when this concern is eliminated by the use of a CYP3A-specific substrate and HLMs, the assay procedure, adapted it to a high-throughput 96-well format, and studied reaction kinetics in the presence and absence of P450 inhibitors. The method was further optimized by synthesis of testosterone labeled with tritium in only the 6β position. In the present report, we describe that the new assay accurately measures the potency of CYP3A4/5 inhibitors and represents a high-throughput version of the classical testosterone 6β-hydroxylation assay.

Materials and Methods

Materials. Oasis HLB extraction plates and vacuum manifold were purchased from Waters (Milford, MA). Pooled human liver microsomes were obtained from BD Gentest (Woburn, MA). Other materials and methods were purchased from American Radiolabeled Chemicals (Saint Louis, MO). Testosterone-3-ethyleneacetal was purchased from Steraloids (Newport, RI). Other chemicals were purchased from Sigma-Aldrich (Milan, Italy). The synthetic scheme is described in Fig. 1.

Synthesis of [6β,3H]Testosterone. The synthetic scheme is described in Fig. 1. 5β,6β-Epoxy-17β-hydroxyandrost-3-one, 3-Ethyleneacetal (2), and 5α,6α-Epoxy-17β-hydroxyandrost-3-one, 3-Ethyleneacetal (3) (Campbell et al., 1958; Liston and Toft, 1969). To a stirring solution of 0.85 g of testosterone, 1.3 ml of acetic anhydride, 0.05 ml of water, and 0.15 ml of pyridine were added. After 14 h, the solution was washed with 1 N sodium hydroxide solution (5 ml) and neutralized with 1 N hydrochloric acid. The product was filtered and dried to yield 190 mg of product. LC/MS: *M* + 338 (100%).

Fig. 1. Synthesis of [6β,3H]Testosterone.
layer was washed with saturated sodium bicarbonate (5 ml), water (two times, 5 ml), and dried over anhydrous sodium sulfate. The crude product (5, 10 mCi) was dissolved in methanol (5 ml) and water (2 ml), and treated with 0.1 N sodium hydroxide (0.1 ml). The solution was stirred at room temperature for 16 h and then treated with acetic acid (0.1 ml). The solution was evaporated to half-volume on a rotary evaporator. The crude product was purified by reversed-phase HPLC (Luna Phenyl Hexyl column (Phenomenex, Torrance, CA), water containing 0.1% trifluoroacetic acid/acetonitrile, 55:45, UV = 254 nm, flow rate 4 ml/min, retention time = 10.5 min). The combined fractions were passed through Sep-Pak C-18, which was further washed with ethanol (10 ml) to yield 2.2 mCi (11%) of [6°H]testosterone (6). The specific activity of this tracer was 1.6 Ci/mmol as calculated by LC/MS. Tritium NMR confirmed this tracer was 1.6 Ci/mmol as calculated by LC/MS. Tritium NMR confirmed that the tritium label is not retained in the hydroxylated reaction product (however, see Discussion), the tritiated water product from [6°H]testosterone is formed stoichiometrically with 6°-hydroxytestosterone. When assays are performed using a fixed amount of [6°H]testosterone and varying concentrations of unlabeled testosterone, the velocity of formation of unlabeled product, v, is given by

\[ v = \frac{v^*}{SA_0} \]

where \( v^* \) is the velocity of formation of tritiated water. Substituting from eq. 2, we obtain:

\[ v = v^* \times \frac{(V/K)}{SA_0} \]

Defining \( v' \) as the velocity of formation of unlabeled product divided by the kinetic isotope effect, i.e.,

\[ v' = v/v^* \]

we obtain

\[ v' = v^*/SA_0 \]

\( v' \), the apparent formation rate of unlabeled product, can thus be calculated. When plotted against the substrate concentration, \( S \), and fitted to the Hill equation (eq. 7), \( V'_m \), \( S_{50} \), the substrate concentration at 50% of \( V'_m \), and \( n \), the Hill coefficient, can be derived.

\[ v' = \frac{V'_m \times S^n}{S_{50} + S} \]

where

\[ V'_m = V_m/(V/K), \]

i.e., the apparent maximal rate of product formation.

Quantification of 6°-Hydroxytestosterone and 2β-Hydroxytestosterone. Aliquots of the assay reaction mixture and of metabolite standard curves were analyzed by HPLC-MS/MS using an Agilent HP1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a CTC Analytics (Zwingen, Switzerland) PAL autosampler. Chromatography was performed on an XTerra MS C18 column (4.6 mm × 5 cm; 5 μm; Waters) at a flow rate of 1 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 for 4 min from 5 to 100% B; 2 min at 100% B). The eluate was diverted to waste for the first minute and then to a Sciex API-2000 (Applied Biosystems/MDS Sciex, Foster City, CA) triple quadrupole mass spectrometer with a Turbo IonSpray ionization source operated in the positive ion mode. Metabolites and internal standard were detected and identified using the transition m/z 351.0→269.1. Metabolite concentrations were determined by weighted linear least-squares regression analysis, using Analyst Quantitation Wizard software version 1.4 (Applied Biosystems, Foster City, CA).

Curve Fitting. Curve fitting of enzyme kinetics data to the Hill equation or to the Michaelis-Menten equation was performed using nonlinear regression analysis using Xlfit 4.0 (IDBS, Guildford, UK).

Results

Separation of Radiolabeled Testosterone and Tritiated Water Using 96-Well Solid-Phase Extraction Plates. When a solution of assay buffer containing purified [1,2,6,7-3°H]testosterone (from 10⁵ to 10⁷ dpm) and stopping solution was applied to 96-well extraction
plates containing 10 or 30 µg of Oasis resin, greater than 99.9% of the radioactivity was retained on the plate. The tracer could be recovered by elution with methanol (data not shown). Only 0.045 ± 0.006% (average ± S.E.M., n = 7) of radioactivity eluted in the combined flow-through and aqueous wash. For [6β-3H]testosterone, 0.13 ± 0.1% (average ± S.E.M., n = 3) of radioactivity eluted in the aqueous fraction. In contrast, tritiated water (from 102 to 105 dpm) was not retained on the extraction plates under the same conditions. In both 10-µg and 30-µg plates, recovery of tritiated water eluted in the combined flow-through and the 100-µl or 400-µl aqueous wash, respectively, was quantitative (94 ± 6%, average ± S.D., n = 6). Recoveries of radiolabeled testosterone and water were not affected by the presence of unlabeled testosterone at concentrations up to 600 µM (data not shown). For chronological reasons, experiments with [1,2,6,7-3H]testosterone and [6β-3H]testosterone were performed using 30-µg and 10-µg Oasis plates, respectively.

Formation of Tritated Water from [1,2,6,7-3H]Testosterone and [6β-3H]Testosterone in HLMs. When [1,2,6,7-3H]testosterone was incubated with HLM in the presence of an NADPH-regenerating system, tritiated water was formed in a time-dependent manner (Fig. 2A). At HLM concentrations of 0.05, 0.1, and 0.25 mg/ml, the reaction was linear for up to 20 min, with conversion rates of 0.006%/min, 0.011%/min, and 0.024%/min, respectively (correlation coefficients, r², of 0.990, 0.963, and 0.963, respectively). Product formation at 10 min increased linearly with the concentration of microsomes up to a protein concentration of 0.5 mg/ml. Formation of tritiated water was dependent on NADPH (see below). Similar results were obtained using [6β-3H]testosterone as substrate (Fig. 2B). The main differences between the two substrates were the signal to noise ratios and conversion rates. We define signal to noise ratio as the ratio between the two tracers, it is more appropriate to compare their clearance, rather than conversion rates. At substrate concentrations of 10 and 60 µM, clearance for [1,2,6,7-3H]testosterone was 0.74 and 1.1 µl/min/mg and that for [6β-3H]testosterone was 5.9 and 10.5 µl/min/mg, respectively.

Effect of P450 Inhibitors and Anti-P450 Antibodies. To confirm that product formation is mediated by CYP3A4/5, reactions were performed in the presence or absence of a series of selective chemical inhibitors (Bourrie et al., 1996; Eagling et al., 1998) or monoclonal antibodies (Mei et al., 1999; Shou et al., 2000). Chemical inhibitors chosen were furafylline (1A2 inhibitor), coumarin (2A6), sulfaphenazole (2C9), quinidine (2D6), diethylthiocarbamate (2E1), and ketoconazole (3A4/5). Monoclonal antibodies used were inhibitors of CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5. As shown in Fig. 3, none of these agents significantly affected formation of tritiated water in HLMs, with the exception of ketoconazole and the anti-CYP3A4/5 monoclonal antibody, which inhibited the reaction by over 90%. Taken together, these results indicate that formation of tritiated water from [1,2,6,7-3H]testosterone in HLMs is mediated by CYP3A4/5.

Kinetic Isotope Effect. CYP3A4/5-mediated loss of tritium from [1,2,6,7-3H]testosterone may occur as a consequence of hydroxylation at either the 6β or 2β positions (Waxman et al., 1988; Yamazaki and Shimada, 1997). It was recently reported that 1β-hydroxytestosterone is another minor hydroxylation product formed by CYP3A4/5 (Krauser et al., 2004). The activity of testosterone 2β-hydroxylase in the batch of HLMs used in the present experiments was ∼11% of that of the 6β-hydroxylation (data not shown). The 2β-hydroxylase activity was not determined. The formation of both 6β- and 2β-hydroxylated metabolites was mediated by CYP3A4/5, since it was completely inhibited by 10 µM ketoconazole over the entire substrate concentration range (data not shown). The relative proportions of label in the 2β and 6β positions of [1,2,6,7-3H]testosterone are not known, and it cannot, therefore, be determined from which position the tritium loss occurred.

Following CYP3A4/5-mediated hydroxylation of [6β-3H]testosterone, tritium can be lost only from the 6β position. To determine whether tritium substitution gives rise to a kinetic isotope effect, the rate of formation of tritiated water was compared with the rate of formation of 6β-hydroxytestosterone in the same reaction mixture. Considering that the hydrogen lost upon hydroxylation of testosterone is formed stoichiometrically with 6β-hydroxysterosterone, the specific radioactivity of the tritiated water product can be calculated by dividing product counts by the amount of 6β-hydroxytestosterone formed (Table 2). Together with the conversion rate of tritiated tracer and its known initial specific radioactivity, this information can be used to calculate the kinetic isotope effect on V/K. As shown in Table 2, the V/K is 2.3, which is equal to the substrate/product specific radioactivity ratio.

Competition between Radiolabeled and Unlabeled Testosterone. The effect of unlabeled testosterone on the formation of tritiated water in HLM is depicted in Fig. 4A. The curve displays a "low dose
presence or absence of 0.4 mg/ml antibodies, or of the following compounds: 30

time points was between 2 and 30 min, using four different concentrations of HLMs. 6

percentage of untreated controls and represents the average

radioactivity of the tracer. The dependence of rate of unlabeled product,

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

probe," i.e., product formation rate increased with increasing concen-

tration (S) can be used to obtain information about the dependence on

substrate concentration of the unlabeled 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 \( S_{50} = 98 \pm 18 \) mg, \( n = 1.4 \pm 0.2 \), and \( V′_{max} = 1.3 \pm 0.1 

nmol/min/mg (average ± S.E.M., \( n = 3 \)). The Hill coefficient was

greater than 1, suggesting positive cooperativity. Indeed, at low sub-

strate concentrations, a sigmoidal relationship was observed between

v′ and S, as shown in the inset of Fig. 4B.

The kinetics of 6β-hydroxytestosterone formation is depicted in

Fig. 4C. The reaction had a \( S_{50} \) of 65 ± 17 mg, \( V′_{max} \) of 3.4 ± 0.4

nmol/min/mg protein, and Hill coefficient of 1.5 ± 0.2 (average ±

S.E.M., \( n = 5 \)). Note that the ratio between \( V′_{max} \) and \( S_{50} \) is 2.6,

which is close to the kinetic isotope effect on V/K, as expected from

the definition of \( S_{50} \) (see Materials and Methods). The formation of

tritiated water and of 6β-hydroxytestosterone was completely inhib-

ited by ketoconazole, confirming that both products are formed via

CYP3A4/5-mediated metabolism.

Similarly, substrate competition between a fixed concentration of

[1,2,6,7-3H]testosterone and increasing concentrations of unlabeled

testosterone displayed a low-dose hook, and tritiated water formation

from this substrate was completely inhibited by ketoconazole (Fig.

4D). Since neither the specific radioactivity of the tracer nor the

proportion of product coming from the 2β and 6β positions are

known, the formation rates of the corresponding unlabeled products

cannot be determined from isotopic dilution studies.

Kinetics of Inhibition by CYP3A4/5 Inhibitors. The effect of

known inhibitors of CYP3A4/5 on the rate of formation of tritiated

water from [1,2,6,7-3H]testosterone and [6β-3H]testosterone is shown in

Fig. 5. IC50 values are summarized in Table 3. With

[1,2,6,7-3H]testosterone, inhibition experiments were carried out in

the presence of two different concentrations of unlabeled testos-

terone, 10 and 60 mg. No significant differences (<3.5-fold) in

IC50 values were observed at the two different substrate concen-

trations. IC50 values determined with the two different tracers were

very similar, with differences of less than 2-fold. To confirm that

IC50 values obtained with the radiometric assay reflect those of the

conventional assay, the effect of the inhibitors on tritiated water

and 6β-hydroxytestosterone formation was determined in the same

reaction mixture. As shown in Table 3, almost identical IC50 values

were obtained.

P450 inhibitors tested in these experiments had to be dissolved in

organic solvents. Since inhibitory effects of solvents on

CYP3A4/5 activity have been reported (Chauret et al., 1998; Hickman et al., 1998), we assessed the effect of two different concentrations of DMSO on the release of tritiated water from

[6β-3H]testosterone and on IC50 values of selected inhibitors.

DMSO inhibited product formation, with about 70% and 50% of

control activity remaining at solvent concentrations of 0.3% and

0.7% (v/v), respectively. However, DMSO had no effect on the

TABLE 1

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<th>10 µM</th>
<th>Conversion Rate</th>
<th>60 µM</th>
<th>Conversion Rate</th>
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<td></td>
<td>n</td>
<td>SN</td>
<td>%/min/mg</td>
<td>n</td>
</tr>
<tr>
<td>[1,2,6,7-3H]Testosterone</td>
<td>3</td>
<td>5.3 ± 1.0</td>
<td>0.37 ± 0.05</td>
<td>9</td>
</tr>
<tr>
<td>[6β-3H]Testosterone</td>
<td>3</td>
<td>18 ± 9</td>
<td>5.9 ± 1.9</td>
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TABLE 2

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<td>Average ± S.D.</td>
<td>2.32 ± 0.14</td>
<td>2.34 ± 0.15</td>
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IC50 values of ethynylestradiol, ketoconazole, miconazole, bromocriptine, nicardipine, nifedipine, quinidine, and verapamil (data not shown). Inhibition assays can therefore be performed using DMSO concentrations between 0.3 and 0.7%, as long as care is taken to include solvent controls, and activity data are expressed relative to these controls.

FIG. 4. Reaction kinetics in human liver microsomes. A, effect of unlabeled testosterone on tritiated water formation from [6β-3H]testosterone. Experiments were conducted in the presence of a fixed amount of [6β-3H]testosterone and varying concentrations of unlabeled testosterone. Product formation was assessed in the absence (filled symbols) or presence (open symbols) of 10 μM ketoconazole. Each point is the mean ± S.E.M. from three separate experiments. B, apparent kinetics of formation of unlabeled product, determined from isotopic dilution studies. The apparent rate of formation of unlabeled product (6β-hydroxytestosterone/H2O), v', was calculated from the data depicted in panel A, as described under Materials and Methods. Data were fitted to the Hill equation by nonlinear regression analysis. Inset, magnification of the initial part of the curve (5 < 12 μM). C, kinetics of formation of 6β-hydroxytestosterone. The product was quantified using HPLC-MS/MS analysis. Data were fitted to the Hill equation by nonlinear regression analysis. Each point is the mean ± S.E.M. from five separate experiments. D, effect of unlabeled testosterone on tritiated water formation from [1,2,6,7-3H]testosterone in human liver microsomes. Product formation was assessed in the absence (filled symbols) or presence (open symbols) of 10 μM ketoconazole. Results are average ± half-range, n = 2.

FIG. 5. Effect of known CYP3A4/5 inhibitors on tritiated water formation from [1,2,6,7-3H]testosterone in human liver microsomes. Data were fitted to a four-parameter logistic equation by nonlinear regression analysis. IC50 values are summarized in Table 3. Data are average ± half-range from duplicate experiments.
Comparison with the Conventional Testosterone 6β-Hydroxylation Assay for a Large Number of Compounds. IC<sub>50</sub> values for 39 structurally diverse NCEs from different Merck programs were determined in the radiometric assay using [6β-<sup>3</sup>H]testosterone and compared with data that had been previously generated using the conventional testosterone 6β-hydroxylation assay. The results of this comparison are depicted in Table 4. Three compounds had IC<sub>50</sub> values >100 μM in both assays. Six compounds had IC<sub>50</sub> >100 μM in one assay and IC<sub>50</sub> >45 μM in the other assay. One compound had IC<sub>50</sub> <0.4 μM in one assay and 0.2 μM in the other assay. The IC<sub>50</sub> values of the remaining 29 compounds differed less than 4.2-fold between the two assays, and in 25 of these (86%), they differed less than 3-fold. For the compounds with measurable IC<sub>50</sub> values in both assays, linear regression analysis of the IC<sub>50</sub> data in the radiometric assay versus IC<sub>50</sub> in the conventional assay resulted in a line with a slope of 1.09 and a correlation coefficient, r<sup>2</sup>, of 0.562 (Fig. 6A). The point that deviated most from the trend line corresponded to a weak inhibitor (compound 28) with a less than 3-fold difference in IC<sub>50</sub> values between the two assays. When this compound was excluded from the analysis, the r<sup>2</sup> increased to 0.757 (Fig. 6B).

Discussion
The release of tritium that accompanies hydroxylation of a substrate has been used to measure the activity of cytochrome P450 enzymes (Daly, 1970; Hayakawa and Udenfriend, 1973; Thompson and Siiteri, 1974; Reed and Ohno, 1976; Tomaszewski et al., 1976; Osawa and Coon, 1987). Draper et al. (1998) described a CYP3A assay procedure based on this principle, in which tritiated water generated from CYP3A-mediated metabolism of [1,2,6,7-<sup>3</sup>H]testosterone was separated from the unreacted substrate by charcoal extraction. Several features of the procedure described by these authors render it less than ideal for high-throughput screening, and we therefore modified and adapted the method for this purpose. The present procedure, which is amenable to automation, allows the assays to be run in 96-well format throughout the incubation and extraction steps and collection of the reaction product for scintillation counting in multwell plates. The following modifications were introduced. First, the assay volume was reduced from 500 μl to 200 μl and the microsome concentration from 0.4 mg/ml to 0.25 mg/ml, with a final amount of microsomes of 50 μg instead of 200 μg. Second, incubations were performed in 96-well plates. Finally, tritiated water was separated from [1,2,6,7-<sup>3</sup>H]testosterone using 96-well solid-phase extraction plates containing Oasis reverse phase resin.

Previous studies with [1,2,6,7-<sup>3</sup>H]testosterone showed that the release of tritium-labeled water from [1,2,6,7-<sup>3</sup>H]testosterone is catalyzed by recombinant CYP3A4/5 at a 6-fold higher rate than by recombinant P450s 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, and 2E1 (Draper et al., 1998). NADPH-dependent metabolism of unlabeled testosterone in HLM occurred mainly (>75%) by 6β-hydroxylation, and the rate of release of tritiated water from [1,2,6,7-<sup>3</sup>H]testosterone corre-
lated with that of testosterone 6β-hydroxylation in HLM from 12 different donor livers. On the basis of these findings, Draper et al. (1998) proposed that the NADPH-dependent release of tritium from [1,2,6,7-3H]testosterone in HLM could be used as a probe reaction for CYP3A4/5 activity (Draper et al., 1998). Convincing validation of this claim would come from the demonstration that the reaction can be completely inhibited by specific CYP3A4/5 inhibitors, but not by inhibitors of other P450 enzymes. The only inhibitor used in the study by Draper et al. (1998) was ketoconazole and an anti-CYP3A4/5 monoclonal antibody potently inhibited release of tritiated water from [1,2,6,7-3H]testosterone in HLM, demonstrating that this reaction is mediated almost exclusively (>95%) by CYP3A4/5. Further proof for this notion comes from the results of a more detailed analysis of the inhibitory potencies of a series of CYP3A4/5 inhibitors. Ketoconazole, nifedipine, bromocriptine, miconazole, nicardipine, and the proprietary compound A inhibited release of tritiated water from [1,2,6,7-3H]testosterone with IC₅₀ values that were not significantly different from those for inhibition of testosterone 6β-hydroxylation, measured by LC-MS/MS in the same reaction mixture. Taken together, these results demonstrate that the present method faithfully measures the activity of microsomal CYP3A4/5-mediated hydroxylation of testosterone and that it can be used to analyze the inhibitory potencies of investigational drugs.

Draper et al. (1998) previously reported that water release from [1,2,6,7-3H]testosterone and 6β-hydroxylation exhibited similar Kₘ and Vₘₐₓ values, based on the assumption that the reaction of the tritiated tracer proceeds without any isotopic effect, and that formation of 1 Eq of (unlabeled) 6β-hydroxytestosterone corresponds to formation of 1 Eq of tritiated water. However, as discussed below, the assumption that hydroxylation of the tritiated tracer is not subject to a kinetic isotope effect is not valid, and it is not possible, therefore, to determine molar reaction velocities using commercially available [1,2,6,7-3H]testosterone. To calculate the velocity of product formation from the tritiated tracer, it would be necessary to know the specific activity of radiolabel present in the 6β position. For commercial [1,2,6,7-3H]testosterone, this information is not available. According to the information supplied by the vendor, ~20% of the label is in the 1α position, 19% in 2α, 20% in 6α, 25% in 7β, 10% in (1β plus 7β), and less than 6% in positions 2β and 6β, but the exact proportion of label present in the 6β position is not known.

Aside from the impossibility to determine kinetic reaction parameters, the use of commercial [1,2,6,7-3H]testosterone as a CYP3A probe presents some additional drawbacks. Because of the low amount of label in the 6β position, it is necessary to use quite high amounts of radioactive substrate (0.5–1 x 10⁶ dpm/assay) to obtain adequate product counts. This obviously has a significant impact on the cost of the assay and renders it difficult to miniaturize the product-counting procedure, for example, using 96-well scintillation counters. Furthermore, it cannot be formally excluded that tritium loss from positions other than the 6β position contributes to product formation. The availability of testosterone specifically labeled in the 6β position represents a significant advantage with regard to these issues. When using this novel tracer, the assay volume could be further reduced to 100 μl and the amount of microsomes to 25 μg, and reasonable product counts (2000–4000 dpm) could be obtained with as little as 100,000 dpm/assay of substrate probe. The signal to noise ratio was significantly improved to 25-fold. The 10-fold higher clearance rate of [6β-3H]testosterone relative to that of [1,2,6,7-3H]testosterone is slightly less than expected from the relative proportions of label in the 6β position (100% versus ≤6%). However, as pointed out above, the exact proportion of label is not known for the commercial tracer, and it cannot be excluded that it is slightly higher than the estimate provided by the supplier. Formation of tritiated water from [6β-3H]testosterone was NADPH-dependent and inhibited by a series of CYP3A5 inhibitors with IC₅₀ values that were not significantly different from those obtained with [1,2,6,7-3H]testosterone. This improved assay thus represents a high-throughput radiometric version of the classical testosterone 6β-hydroxylation assay and should be suitable for rapid screening of the inhibitory potential of investigational drugs, as discussed further below.

Using [6β-3H]testosterone, for which the specific radioactivity is known, as substrate probe allows determination of the kinetic isotope effect, 3V/K. The large disparity between the concentrations of triti-
ated and unlabeled substrate makes it impossible to measure their separate reaction velocities. When trace concentrations of a tritiated substrate are used to measure reaction velocities in the presence of competing unlabeled substrate, it is possible to measure the kinetic isotope effect on the V/K ratio (not on the individual values of V_max and K_m) (Northrop, 1982). By measuring formation of tritiated water and 6β-hydroxytestosterone in the same reaction mixture, the specific activity of the tritiated water product can be calculated and compared with the initial specific radioactivity of the substrate, and these values can be used to derive 1/V/K. The kinetic isotope effect on 1/V/K was 2.3-fold. It was previously reported that 6β-hydroxylation of deuterated testosterone proceeds in the absence of a kinetic isotope effect (Bjorkhem, 1972). This is most likely due to the difference between C–D and C–T bond energies. It cannot be excluded that the modest difference between the formation rates of tritiated water and 6β-hydroxytestosterone involves abstraction of the 6α-hydrogen with partial retention of the isotopic label in the hydroxylated product, as reported for P450cam-mediated oxidation of camphor, where either the exo or the endo hydrogen is abstracted, but rebound occurs only on the exo side (Gelb et al., 1982). Independently of the mechanism, the inhibitory potencies of competitive CYP3A inhibitors are not affected, as demonstrated by the observation that IC50 values for inhibition of tritiated water formation were not significantly different from those for 6β-hydroxytestosterone formation and were not modified by 6-fold isotopic dilution of the substrate probe.

When reaction velocity is measured in the presence of a fixed amount of radiolabeled substrate and increasing concentrations of unlabeled substrate, product counts are expected to decrease when the concentration of unlabeled substrate becomes sufficiently high to compete with binding of the tracer to the active site of the enzyme. As depicted in Fig. 4A, at low concentrations of unlabeled testosterone, the formation of tritiated water is actually enhanced as unlabeled substrate increases, and starts to decrease only at high concentrations (>40 μM) of unlabeled substrate. This effect, called low dose hook, is characteristic for positively cooperative ligand displacement interactions (De Lean and Rodbard, 1979). The reason for the increased formation of tritiated product is that at low substrate concentrations, the reaction velocity of a positively cooperative enzyme increases more than dose proportionally with increasing substrate concentration. When the apparent formation rate of unlabeled product was calculated from the isotopic dilution experiment and plotted against the substrate concentration, a sigmoidal relationship was observed, confirming that the reaction displays positive cooperativity. Data could be fitted to the Hill equation, with a Hill coefficient of 1.4 and a S_50 of 98 μM. Measurement of 6β-hydroxytestosterone formation under the same conditions demonstrates that reaction kinetics of unlabeled testosterone indeed displays positive cooperativity, with a Hill coefficient of 1.5 and a S_50 of 65 μM. The differences between the values of S_50 and the Hill coefficient (n) obtained in the radiometric versus LC-MS/MS assays were not statistically significant (p > 0.1). A Hill coefficient of 1.3 has previously been reported for CYP3A4/5-mediated 6β-hydroxylation of testosterone (Ueng et al., 1997). The good agreement between kinetic parameters obtained from the tracer competition (isotopic dilution) data with those determined by direct measurement of unlabeled product reinforces the notion that formation of tritiated water from [6β-3H]testosterone in HLM is mediated by the same mechanism as formation of 6β-hydroxytestosterone. A low dose hook was also observed using [1,2,6,7,1H]testosterone as substrate. It should be noted that hyperbolic (nonsigmoidal) kinetics have been reported for this tracer (Draper et al., 1998). The most likely explanation for this apparent discrepancy is that the low dose hook was observed in the present studies only at substrate concentrations between 0.4 and 20 μM, whereas Draper et al. (1998) studied reaction kinetics at substrate concentrations greater than 14 μM.

To validate the use of the new assay as a screening method for CYP3A4/5 inhibition, we determined IC50 values for a large number of structurally diverse investigational compounds from different Merck programs and compared the results with those obtained in the conventional testosterone 6β-hydroxylation assay (with product quantification by LC-MS/MS). The results of this analysis indicate that IC50 values obtained with the new radiometric assay are very similar to those of the conventional assay. IC50 values differed less than 4-fold in every case, and for 86% of the compounds, less than 3-fold. Most importantly, not a single compound of the 39 tested would have been misclassified as either a strong or a weak inhibitor based on the results of the radiometric assay.

Several authors have recommended that CYP3A4/5 inhibition should be assessed using more than one substrate (Stresser et al., 2000; Yuan et al., 2002; Cohen et al., 2003). Even though testosterone is not necessarily a better predictor of drug interactions than other substrates, it is one of the most commonly used probes for CYP3A4/5 inhibition studies. A detailed comparison for a large number of compounds of IC50 values obtained with a fluorogenic probe versus testosterone revealed that the correlation between these assays was relatively poor (Cohen et al., 2003). Results obtained in our laboratory support the same conclusion (data not shown). The authors of this study recommended that screening with fluorogenic probes should be followed up by studies with conventional substrates, such as testosterone. The present assay, which combines the advantages of speed, 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 CYP3A4/5 in a drug discovery setting. Using appropriately radiolabeled substrates, the assay principle should be adaptable to other P450 enzymes. In the accompanying paper (Di Marco et al., 2005), we describe the synthesis of [4′-3H]diclofenac and the development of a high-throughput CYP2C9 assay using this substrate.

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