Fully automated analysis of activities catalysed by the major human liver cytochrome P450 (CYP) enzymes: assessment of human CYP inhibition potential

G. C. MOODY, S. J. GRIFFIN, A. N. MATHER†, D. F. McGINNITY and R. J. RILEY*
Department of Physical & Metabolic Sciences and † Medicinal Chemistry Department, Astra Charnwood, Loughborough LE11 5RH, UK

Received 20 May 1998

1. Fully automated inhibition screens for the major human hepatic cytochrome P450s have been developed and validated. Probe assays were the fluorometric-based ethoxyresorufin O-deethylation for CYP1A2 and radiometric analysis of erythromycin N-demethylation for CYP3A4, dextromethorphan O-demethylation for CYP2D6, naproxen O-demethylation for CYP2C9 and diazepam N-demethylation for CYP2C19. For the radiometric assays > 99.7% of 14C-labelled substrate was routinely extracted from incubations by solid-phase extraction.

2. Furafylline, sulphaphenazole, omeprazole, quinidine and ketoconazole were identified as specific markers for the respective CYP1A2 (IC50 = 6 μM), CYP2C9 (0.7 μM), CYP2C19 (6 μM), CYP2D6 (0.02 μM) and CYP3A4 (0.2 μM) inhibition screens.

3. For the radiometric methods, a two-point IC50 estimate was validated by correlating the IC50 obtained with a full (seven-point) assay (r2 = 0.98, p < 0.001). The two-point IC50 estimate is useful for initial screening, while the full IC50 method provides more definitive quantitation, where required.

4. IC50 determined for a series of test compounds in human liver microsomes and cytochrome P450 cDNA-expressed enzymes were similar (r2 = 0.89, p < 0.001). In particular, the CYP1A2, CYP2D6 and CYP3A4 screens demonstrated the flexibility to accept either enzyme source. As a result of incomplete substrate selectivity, expressed enzymes were utilized for analysis of CYP2C9 and CYP2C19 inhibition. Good agreement was demonstrated between IC50 determined in these assays to IC50 published by other laboratories using a wide range of analytical techniques, which provided confidence in the universality of these inhibition screens.

5. These automated screens for initial assessment of P450 inhibition potential allow rapid determination of IC50. The radiometric assays are flexible, sensitive, robust and free from analytical interference, and they should permit the identification and eradication of inhibitory structural motifs within a series of potential drug candidates.

Introduction

The co-prescribing of medications can lead to a plethora of complex drug-drug interactions that may delay the establishment of optimized individual therapy or culminate in serious (and even occasionally life-threatening) adverse reactions (Honig et al. 1993, Ahmad and Wolfe 1995). An in vitro investigation of the potential for a given drug to inhibit the biotransformation of co-medications can aid the (retrospective) interpretation of drug interactions observed clinically and may even help predict which drugs are likely to cause problems when co-prescribed with the test compound of interest (Peck et al. 1993). Human liver microsomes (HLM) and expressed human drug-metabolizing enzymes have been shown to be valuable tools for...
in such studies and the use of human material avoids extrapolation of data obtained with animal tissue to the situation in man. Since the majority of marketed drugs are metabolized by cytochrome P450 (CYP) enzymes (Bertz and Granneman 1997), it has now become commonplace to characterize the CYP inhibition potential to respond to requests from regulatory authorities (Peck et al. 1993) and to market safer drugs with fewer side effects, predictable pharmacokinetic properties and quantifiable drug–drug interactions.

The now routine use of combinatorial chemistry and high throughput screens in drug discovery programmes generates much larger numbers of chemically diverse compounds (Rodrigues 1997). Most assays for the routine analysis of drug interaction involving human CYP enzymes rely on labour- and equipment-intensive sample extraction and HPLC or LC-MS analysis. Automated assays capable of handling these increased numbers would offer the opportunity to use human CYP inhibition potential as a criterion for compound progression. Moreover, a generic method to monitor drug–drug interactions involving human CYP enzyme inhibition would enable rapid screening and eradication of this undesirable property prior to marketing.

Inhibition screens utilizing fluorescent assays (Crespi et al. 1997) and LC-MS methods (Ayrton et al. 1998) have recently been reported. However, these techniques can be prone to interference and/or quenching which limits their general applicability. The in vitro assays for each CYP isoform employed in this study were chosen based on factors including compatibility for automation, robustness and reproducibility. Radiometric in vitro methods have been described for reactions catalysed by CYP2E1 (Yang et al. 1991), CYP2D6 (Bloomer et al. 1992, Rodrigues et al. 1994), CYP1A2 (Bloomer et al. 1995, Rodrigues et al. 1997), CYP2C9 (Rodrigues et al. 1996) and CYP3A4 (Riley and Howbrook 1998).

This communication now describes the development of rapid, sensitive, automated assays for human CYP enzymes using both fluorescent analysis and methodology employed clinically for several breath tests: N-demethylation of [14C]-radiolabelled probe substrates and measurement of the resultant [14C]-formaldehyde/formic acid. Data comparing CYP inhibition in these assays with an array of values documented in the open literature using different analytical methodologies are provided to validate this approach. These assays should prove valuable in the rapid establishment of databases and, in association with other emerging technologies, subsequent molecular models with which to predict and minimize human CYP interaction potential.

Materials and methods

Chemicals

All chemicals and reagents used were of the highest available commercial grade. Apigenin, α-naphthoflavone, 7-ethoxycoumarin, diazepam, theophylline, miconazole, caffeine, sulphaphenazole, clotrimazole, 1,7-dimethylxanthine, piroxicam, mexiletine, quinine, ibuprofen, tolbutamide, propafenone, quinidine, propafenone, lobeline, pentazocine, clozapine, paroxetine, chloroquine, debrisoquine, bromocryptine, dihydroergotamine, troleandomycin, nifedipine, erythromycin, diethylthiocarbamate, diltiazem, dextromethorphan, naproxen, S-mephenytoin, ethoxyresorufin, resorufin and β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) were purchased from Sigma Chemical Co. (Poole, UK). Warfarin, 4-methylimidazole, phenytoin, primaquine, 4-methylpyrazole, hydroquinidine, haloperidol, cimetidine and verapamil were purchased from Aldrich Chemical Co. Ltd (Gillingham, UK). Furafylline was purchased from Ultrasine Chemicals (Manchester, UK). Dimethylsulphoxide (DMSO) and acetonitrile were purchased from Fisher Scientific (Loughborough, UK) and methanol was purchased from Romil Ltd (Cambridge, UK).
Omeprazole, flucanazole and ketoconazole (purity > 99%) were synthesized at Astra Charnwood (Loughborough, UK). The $[^{14}C]$ labelled probe substrates [$N$-methyl-$[^{14}C]$ diazepam (specific radioactivity 55 mCi/mmol, chemical purity 98.5%, radiochemical purity 99.4%), [O-$[^{14}C]$] dextromethorphan (specific radioactivity 56 mCi/mmol, chemical purity 99.8%, radiochemical purity 99.6%) and [O-$[^{14}C]$] naproxen (specific radioactivity 55 mCi/mmol, chemical purity > 99%, radiochemical purity > 99.8%) were made by alkylation of the respective des-methyl compound with $[^{14}C]$ methyl iodide (specific radioactivity 55 mCi/mmol), purchased from Amersham International (Aylesbury, UK), in dimethylformamide (naproxen and dexamethasone) or DMSO (dextromethorphan) in the presence of potassium hydroxide. The products were purified by reverse-phase HPLC and stored in ethanol before use. [$N$-$[^{14}C]$] erythromycin (specific radioactivity 55 mCi/mmol, radiochemical purity > 97%) was purchased from DuPont NEN (Stevenage, UK).

**Source of cytochrome P450**

The LINK consortium, a collaboration between UK academia and industry, provided human CYP2D6, CYP2C9 and CYP1A2, individually co-expressed with human NADPH-cytochrome P450 reductase in *Escherichia coli*. The strategy for expression of CYP2C9 and CYP1A2 was identical to that described for CYP2D6 (Pritchard et al. 1998). For optimal expression, the first eight codons of each CYP cDNA were replaced with eight codons of the bovine 17α-hydroxylase sequence to generate the constructs 17α-CYP2D6 (Pritchard et al. 1998), 17α-CYP2C9 and 17α-CYP1A2. Expression of the recombinant proteins and preparation of the respective *E. coli* membranes were carried out in our laboratory essentially as described for CYP2D6 (Pritchard et al. 1998).

Microsomes prepared from human lymphoblastoid cells co-expressing recombinant human NADPH-cytochrome P450 reductase and human CYP2C9, CYP2D6 and CYP3A4 were purchased from Gentest Corp. (Woburn, MA, USA). Microsomes prepared from insect cells infected with a baculovirus containing the cDNA for human CYP2C19 and rabbit NADPH-cytochrome P450 reductase were purchased from PanVera Corp. (Madison, WI, USA). Pooled human liver microsomes (HLM; batch numbers 217, 219 and 220) were purchased from IIAM (Leicester, UK). Cytochrome P450 contents were estimated spectrally by the method of Omura and Sato (1964). Protein concentrations were measured using the Randox Laboratories Ltd (Crumlin, UK) protein kit based on pyrogallol red complexing with protein in an acid environment containing molybdate ions (Watanabe et al. 1986).

**Manual enzyme assay**

Ethoxyresorufin O-deethylation (EROD) (Riley et al. 1995), dextromethorphan O-demethylation (Rodríguez et al. 1994), naproxen O-demethylation (Rodríguez et al. 1996) and erythromycin N-demethylation (Zhang and Thomas 1996, Riley and Howbrook 1998) assays were used as probe reactions for CYP1A2, CYP2D6, CYP2C9 and CYP3A4 respectively and based on methods previously described. [$[^{14}C]$]-diazepam N-demethylation has been developed specifically as a probe assay for CYP2C19 (Ono et al. 1996b, Jung et al. 1997).

The isoform selectivity of these probe substrate reactions was investigated in incubations with *E. coli* membranes expressing CYP1A2, CYP2C9 and CYP2D6 or baculosomes expressing CYP2C19 (all at 50 pmol P450/ml) in 100 mM potassium phosphate buffer, pH 7.4, in a final volume of 200 μl. Microsomes from human B-lymphoblasts expressing CYP3A4 were incubated also at 50 pmol P450/ml but in 1× TSE buffer (50 mM Tris-acetate, pH 7.6, 250 mM sucrose, 0.25 mM EDTA) in a final volume of 200 μl. Each radiometric assay included 0.05–1 μCi [$^{[14]}$C]-naproxen, [$^{[14]}$C]-dextromethorphan, [$^{[14]}$C]-diazepam or [$^{[14]}$C]-erythromycin. An appropriate amount of cold substrate was added to give final substrate concentrations of dextromethorphan (30 μM), diazepam (100 μM), naproxen (2 mM) and erythromycin (300 μM). All final concentrations were chosen to select Vmax conditions for each substrate.

For the fluorometric assay, ethoxyresorufin was used at a final concentration of 3 μM to reflect the Vmax conditions for CYP1A2. The probe substrate in ethanol was aliquoted and the solvent evaporated under a stream of nitrogen, before addition of protein. All incubations were carried out at 37 °C and reactions were started, after preincubation for 2 min, with the addition of NADPH (1 mM). Incubation times were 15 min for EROD, 10 min for dextromethorphan O-demethylation and diazepam N-demethylation, 30 min for naproxen O-demethylation and 5 min for erythromycin N-demethylation. Reactions were terminated by the addition of 50 μl ice-cold trichloroacetic acid (10% w/v). Solid-phase extraction (SPE) was carried out using disposable Supelclean Envi-Carb 1 ml cartridges (Supelco, Bellefonte PA, USA). Cartridges were conditioned with methanol (2×1 ml) and water (4×1 ml). All of the quenched incubation was applied to the column and eluted with 2×500 μl aliquots water. The resulting volume was transferred to vials containing 7 ml UltimaGold liquid scintillation cocktail (Packard Instrument Co., Pocelona, UK) and samples counted for 2 min using a Packard 2200CA Tri-Carb Liquid Scintillation Analyser.

Furafylline, sulphaphenazonine, quindine, omeprazole and ketoconazole were used to determine inhibitor selectivity against reactions associated with CYP1A2, CYP2C9, CYP2D6, CYP2C19 and CYP3A4, respectively, in both expressed CYPs and HLM. The final concentration of diagnostic inhibitors used for all inhibitor selectivity assays were > 10 times their reported Ki; furafylline, 20 μM...
Figure 1. Layout of the radiometric inhibition assays on the robotic sample processor. The schematic representation of the robotic sample processor shows four 96-well plates. The sample rack contained primary stocks of test compounds which were diluted to seven secondary stocks in the predilution rack, using dimethylsulphoxide and 100 mM phosphate buffer, pH 7.4 (from reagent reservoirs labelled DMSO and P buffer respectively). The reagent rack contained aliquots of 10 mM NADPH (N) and 10% (w/v) trichloroacetic acid (T). Aliquots of protein-substrate mixture were taken from a glass vial situated proximal to the destination rack, a heated aluminium block (37 °C) controlled by a temperature probe (point of insertion indicated by arrow) and thermostat.

(Clarke et al. 1994); sulphaphenazole, 20 μM (Back et al. 1988, Baldwin et al. 1995); quinidine, 10 μM (Wu et al. 1993); omeprazole, 50 μM (Ko et al. 1997); and ketoconazole, 1 μM (Baldwin et al. 1995). The probe substrates were incubated at or near their K_m for the respective CYP isoform: ethoxyresorufin, 0.6 μM; naproxen, 109 μM; dextromethorphan, 5 μM; diazepam, 19 μM; and erythromycin, 59 μM, since under these conditions the experimentally derived IC_50 should have been within 2-fold of the reported K_i (Cheng and Prusoff 1973). The amount of expressed CYP and HLM used in each respective assay was: 20 pmol CYP1A2/ml and 0.5 mg protein/ml HLM for EROD; 70 pmol CYP2C9/ml and 0.5 mg protein/ml HLM for naproxen O-demethylation; 40 pmol CYP2D6/ml and 0.5 mg protein/ml HLM for dextromethorphan O-demethylation; 50 pmol CYP2C19/ml and 0.3 mg protein/ml HLM for diazepam N-demethylation; 50 pmol CYP3A4/ml and 0.7 mg protein/ml HLM for erythromycin N-demethylation. All other assay conditions including the incubation times and the subsequent sample treatment were as described for the substrate selectivity assays.

Automated radiometric inhibition assays

The fully automated CYP inhibition screen was performed by a robotic sample processor (RSP) (Genesis RSP 150, Tecan, Reading, UK). All of the inhibition assays performed by the RSP were programmed by the user and are not default programmes supplied with the hardware. Copies of the program developed are available from the corresponding author upon request.

All reactions were conducted under conditions shown to be linear with respect to time and protein concentration. Figure 1 displays the layout of the components for the radiometric assays which includes; the sample rack, 300 μl of test compounds were aliquoted in 1.2 ml 96-well polypropylene tubes; the predilution rack, for dilution of test compounds (for more than six compounds a second predilution rack was required); the reagent rack containing NADPH and trichloroacetic acid to start and stop the reaction respectively; the destination rack, an aluminium block heated to 37 °C using a digi-visc hot plate (IKA...
Labortechnik, Staufen, Germany) for incubations; and dimethylsulphoxide / acetonitrile and 100 mM phosphate buffer, pH 7.4, reagent reservoirs. The appropriate amount of probe substrate (in ethanol) was dispensed manually into a glass vial and the solvent evaporated under a steady stream of nitrogen, before addition of protein. Incubations contained 100 µl protein and probe substrate mixture (0.1–0.5 mg/ml final protein concentration), 10 µl compound at different stock concentrations, 70 µl phosphate buffer and reactions were started by the addition 20 µl NADPH (10 mM) giving a final volume of 200 µl. Control incubations from which NADPH had been omitted and in which vehicle replaced inhibitor were also included. Incubations were conducted for 15 min and the reactions were quenched by the addition of 50 µl trichloroacetic acid (10% w/v).

**Full IC₅₀ determination**

A maximum of 10 compounds per 96-well plate were screened. The dilution of test compounds for a full (seven) - point IC₅₀ determination by the RSP were programmed as follows: the primary stock of each compound (e.g. 5 mM) was prepared manually in DMSO or acetonitrile and serially diluted by the RSP (using DMSO or acetonitrile) to give six secondary solutions (e.g. 5 mM to 20 µM). Each of these secondary solutions was further diluted 1:5 in 100 mM phosphate buffer, pH 7.4, to generate tertiary solutions (e.g. 1 mM to 4 µM). Finally 10 µl of each of the tertiary solutions were spiked into the incubation mix (200 µl total volume to give final concentrations e.g. 50 to 0.2 µM). An incubation containing vehicle alone allowed calculation of control activity. The final organic solvent concentration was 1% (v/v) in all incubations, which demonstrated minimal inhibition (data not shown). All data reported have been calculated with respect to a vehicle control. Between all dilutions the RSP’s pipette tips were programmed to flush with 2 ml water.

**Two-point IC₅₀ determination**

A maximum of 18 compounds, in duplicate, per 96-well plate can be screened per robot. The primary stock (e.g. 5 mM) of each compound was diluted by the RSP to generate duplicates of a high and a low concentration secondary solution (e.g. 1 mM and 100 µM). Of each secondary solution, 10 µl was spiked into the incubation mixture to give final concentrations of 50 and 5 µM respectively (these concentrations have worked well in this laboratory for initial screening). Control activities were determined by spiking 10 µl solvent vehicle into an incubation mixture.

The two-point assay IC₅₀ determination fitted the data to a variation of the expression:

\[ V = \frac{V_o}{1 + (I/IC_{50})^x} - b, \]

where \( V \) = velocity or % control activity, \( V_o \) = control activity, \( s \) = slope factor and \( b \) = background (uninhibitable) activity.

Assuming, \( s = 1 \) and \( b = 0 \), this relationship simplified to give:

\[ IC_{50} = \frac{I(100 - I_s)}{I_s}, \]

where \( I \) = inhibitor concentration, \( I_s = \% \) inhibition at \( I \), as described previously (Zomorodi and Houston 1996). Experience showed that this was valid when \( I_s \) was between 20 and 80%—furthermore, in practice one value of \( I \) satisfied this in any one run under the conditions outlined. The appropriate concentrations of inhibitor were used to cover the reported IC₅₀ range.

**Solid phase extraction**

Fully automated positive pressure solid-phase extraction was performed by the RSP controlled by GenSPE software (Labstar Software Ltd, Strathaven, UK). SPE columns were conditioned as described earlier with two 1-ml methanol aliquots and two 1-ml water aliquots. Of the quenched incubation, 225 µl was applied to the column and eluted with 2 x 300 µl aliquots water and 1 ml air. The resulting 825 µl was transferred to vials containing 5 ml liquid scintillation cocktail and samples counted using the liquid scintillation analyser as described above.

**Automated ethoxyresorufin O-deethylation inhibition assay**

Seven compounds, including furafylline (positive control) at six concentrations, can be screened in duplicate per 96-well plate per run. Test compounds (e.g. 5 mM) in DMSO were diluted in water by the RSP, giving a range of concentrations (e.g. 250 to 1 µM) with the DMSO constant at 5% (v/v). Stocks were diluted 1:10 into the incubation to give 25 to 0.1 µM (in duplicate) of each test compound. Each incubation contained 60 µl NADPH (1.6 mM), 100 µl protein (0.1–0.5 mg/ml final concentration) to give 15 pmol enzyme/ml, 20 µl test compound in 5% DMSO, and 20 µl ethoxyresorufin (6 µM) in 2%
DMSO was added to start the reaction. Thus the final concentration of DMSO in the incubation was 0.7%. An incubation containing DMSO alone allowed calculation of control activity. Production of resorufin ($\lambda_{ex}$ 544 nm, $\lambda_{em}$ 590 nm) was measured over 15 min (33 readings) on a fluorescence plate reader ($f_{max}$; Molecular Devices Co. Sunnyvale, CA, USA). All data represent means from at least duplicate determinations.

Data analysis
Data was transferred into a Microsoft Excel (Microsoft Co., Seattle, USA) spreadsheet and then manipulated by non-linear regression analysis with the Win-NonLin (Scientific Consulting Inc., Cary, NC, USA) software package to calculate $IC_{50}$.

Results
This paper demonstrates the development and validation of automated screens for inhibition of the major human hepatic CYPs, which allow a rapid determination of $IC_{50}$. Figure 1 is a schematic representation of the components for the automated radiometric assays controlled by the robotic sample processor (RSP). Table 1 summarizes the experimental conditions adopted routinely and the control parameters obtained for the five probe assays used in the CYP inhibition screens. The substrate concentrations used were at or near the apparent $K_m$, determined for each respective assay. For the radiometric assays, quantitative recovery of total incubated radioactivity was achieved, thus demonstrating negligible loss of [$^{14}$C]-HCHO over the course of the incubations (data not shown). In addition, > 99.7% of [$^{14}$C]-labelled substrate was routinely retained by the automated SPE procedure which provided almost quantitative recovery of [$^{14}$C]-HCHO, as detailed previously (Riley and Howbrook 1998). Control (uninhibited) activity, % retention of [$^{14}$C]-substrate and $IC_{50}$ for standard inhibitor served as quality control parameters for each individual assay (table 1).

Substrate selectivity
Figure 2a and e demonstrates that, under $V_{max}$ conditions for the major (high-affinity) isofoms, EROD and erythromycin N-demethylation are specific reactions for CYP1A2 and CYP3A4 respectively, with other isoforms catalysing < 35% of their activity. As figure 2b demonstrates, the O-demethylation of naproxen (2 mM) was catalysed not only by CYP2C9 but also by CYP2C19 (≈ 80% of CYP2C9 activity) and CYP1A2 (≈ 50% of CYP2C9 activity). Diazepam (100 μM) was N-demethylated by CYP2C19 and CYP3A4 at approximately equal rates under the conditions used (figure 2c). Dextromethorphan was O-demethylated by CYP2D6, CYP2C9 (≈ 30% of CYP2D6 activity) and CYP2C19 (figure 2d). Interestingly, in these incubations, the rate of O-demethylation catalysed by CYP2C19 (4.5 min⁻¹) was double that observed with CYP2D6 (2.2 min⁻¹). Further work also showed that this reaction was inhibited > 80% by omeprazole (50 μM) indicating that this reaction was likely CYP2C19-dependent and not the result of indirect catalysis by futile cycling (data not shown).

Inhibitor selectivity
The O-deethylation of ethoxyresorufin (0.6 μM) in HLM and in E. coli membranes expressing CYP1A2 was inhibited markedly (> 80%) by the CYP1A2 inhibitor, furafylline (figure 3a). Ketoconazole, sulphaphenazole and quinidine, specific potent inhibitors of human CYP3A4, CYP2C9 and CYP2D6 respectively,
Table 1. Summary of experimental conditions for automated cytochrome P450 inhibition screens.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final substrate concentration (μM)</th>
<th>μCi 14 C substrate/ incubation</th>
<th>CYP source</th>
<th>pmol CYP/ incubation</th>
<th>Incubation time (min)</th>
<th>Control activity (pmol/min/μmol)</th>
<th>% retention of 14 C-substrate</th>
<th>IC₅₀ for standard inhibitor (μM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethoxyresorufin</td>
<td>0.6</td>
<td>–</td>
<td>E. coli</td>
<td>3</td>
<td>15</td>
<td>0.4±0.1</td>
<td>–</td>
<td>6±2</td>
<td>15</td>
</tr>
<tr>
<td>Naproxen</td>
<td>109</td>
<td>0.1</td>
<td>E. coli</td>
<td>14</td>
<td>15</td>
<td>1.5±0.1</td>
<td>99.70±0.01</td>
<td>0.7±0.1</td>
<td>3</td>
</tr>
<tr>
<td>Diazepam</td>
<td>19</td>
<td>0.1</td>
<td>baculosomes</td>
<td>8</td>
<td>15</td>
<td>0.3±0.06</td>
<td>99.98±0.02</td>
<td>6±2</td>
<td>4</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>5</td>
<td>0.05</td>
<td>E. coli</td>
<td>4</td>
<td>15</td>
<td>0.8±0.4</td>
<td>99.81±0.18</td>
<td>0.03±0.01</td>
<td>7</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>59</td>
<td>0.1</td>
<td>HLM</td>
<td>0.5#</td>
<td>15</td>
<td>119±7*</td>
<td>99.84±0.01</td>
<td>0.26±0.02</td>
<td>5</td>
</tr>
</tbody>
</table>

Control activities, % retention of 14 C-substrate and IC₅₀ values for standard inhibitors are given as the mean of n separate experiments with the standard deviation from the mean. # mg/mL, *pmol/min/mg protein.

Standard inhibitors: furfurylline (ethoxyresorufin O-deethylation), sulfaphenazole (naproxen O-demethylation), omeprazole (diazepam N-demethylation), quinidine (dextromethorphan O-demethylation) and ketoconazole (erythromycin N-demethylation).

Table 2. Comparison of IC₅₀ determined in our laboratory with literature Kᵢ/IC₅₀ for CYP1A2.

<table>
<thead>
<tr>
<th>E. coli IC₅₀ (μM)</th>
<th>HLM IC₅₀ (μM)</th>
<th>IC₅₀ (μM)</th>
<th>Kᵢ (μM)</th>
<th>HLM</th>
<th>CYP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthoflavone</td>
<td>0.02</td>
<td>0.02</td>
<td>–</td>
<td>0.002</td>
<td>√</td>
<td>Pastrakuljic et al. (1997)</td>
</tr>
<tr>
<td>Apigenin</td>
<td>1.1</td>
<td>0.5</td>
<td>–</td>
<td>0.36</td>
<td>√</td>
<td>Pastrakuljic et al. (1997)</td>
</tr>
<tr>
<td>7-Ethoxycoumarin</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>–</td>
<td>√</td>
<td>Tassaneeyakul et al. (1993)</td>
</tr>
<tr>
<td>Furfurylline</td>
<td>6</td>
<td>6</td>
<td>–</td>
<td>3</td>
<td>√</td>
<td>Bourrie et al. (1996)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>11</td>
<td>49</td>
<td>13</td>
<td>–</td>
<td>√</td>
<td>Baldwin et al. (1995)</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>78</td>
<td>96</td>
<td>–</td>
<td>101-135</td>
<td>√</td>
<td>Rost et al. (1995)</td>
</tr>
<tr>
<td>Theophylline</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>–</td>
<td>200-1000*</td>
<td>√</td>
<td>Rost et al. (1995)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>–</td>
<td>800</td>
<td>√</td>
<td>Tassaneeyakul et al. (1992)</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>&gt;250</td>
<td>1260</td>
<td>&gt;125</td>
<td>–</td>
<td>√</td>
<td>Chang et al. (1994)</td>
</tr>
<tr>
<td>1,7-Dimethylxanthine</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>110</td>
<td>–</td>
<td>√</td>
<td>Brosen (1995)</td>
</tr>
</tbody>
</table>

*Km (μM).
Figure 2. Selectivity of probe substrate catalysis by CYP1A2, 2C9, 2C19, 2D6 and 3A4. The activity (turnover number) of the specific isoform for the chosen probe reaction is represented as 100% and the respective activities of the other isoforms are relative to this rate: (1) EROD (turnover number for CYP1A2 = 0.4 min⁻¹), (2) naproxen O-demethylation (CYP2C9 = 6.8 min⁻¹), (3) diazepam N-demethylation (CYP2C19 = 0.8 min⁻¹), (4) dextromethorphan O-demethylation (CYP2D6 = 2.2 min⁻¹) and (5) erythromycin N-demethylation (CYP3A4 = 1.3 min⁻¹). The source of cytochrome P450 were E. coli membranes over-expressing CYP1A2, 2C9 and 2D6 respectively, insect baculosomes over-expressing CYP2C19 and a human B-lymphoblastoid cell line over-expressing CYP3A4. Data are means of triplicate determinations.
Figure 3. Inhibitor selectivity of probe substrate catalysis by CYP1A2, 2C9, 2C19, 2D6 and 3A4. The control activity of the isoforms for the respective probe reaction: (1) CYP1A2-EROD, (2) CYP2C9-naproxen O-demethylation, (3) CYP2C19-diazepam N-demethylation, (4) CYP2D6-dextromethorphan O-demethylation and (5) CYP3A4-erythromycin N-demethylation, in both HLM (clear bars) and cDNA-expressed cell lines (hatched bars) is represented as 100%. E. coli membranes over-expressing CYP1A2, CYP2C9 and CYP2D6, insect baculosomes over-expressing CYP2C19 and human B-lymphoblasts over-expressing CYP3A4 were used. Substrate concentration was at or near the respective Keq (as reported in the Materials and methods) and the diagnostic inhibitors ketoconazole (K), sulphinpyrazone (S), furafylline (F), omeprazole (O) and quindine (Q) were added at concentrations ≥10 times their reported Keq (as reported in the Materials and methods). In the presence of each respective inhibitor, catalytic activities are shown as a percentage relative to the control activity in both HLM and cDNA-expressed CYPs. Data are means of duplicate determinations representative of up to three experiments.
Figure 4. Comparison of the two-point $IC_{50}$ determination against the full (seven)-point $IC_{50}$ determination for the radiometric inhibition assays. Both the two- and full-point $IC_{50}$ determinations were carried out as described in the Materials and methods. The data represent $IC_{50}$ determined for *E. coli* membranes over-expressing CYP2C9 (□) and CYP2D6 (○), insect baculosomes over-expressing CYP2C19 (●) and a human B-lymphoblastoid cell line over-expressing CYP3A4 (△). Several probe compounds per CYP (from tables 2–6), covering a wide range of $IC_{50}$, are shown. For the two-point determination; 0.5 and 5 μM were used for inhibitors with $IC_{50} \leq 0.5$ μM; 5 and 50 μM for inhibitors with $IC_{50} = 0.5–250$ μM; 50 and 500 μM for inhibitors with $IC_{50} \geq 250$ μM. The solid line depicts a linear regression analysis of the data ($r^2 = 0.98$, $p < 0.001$).

had no significant effect on this activity at concentrations ≥ 10 times their reported $K_i$. Omeprazole (a high-affinity CYP2C19 substrate) inhibited EROD activity in *E. coli* membranes expressing CYP1A2 only weakly (by ~ 50%) but exhibited no effect in HLM.

Naproxen O-demethylase activity in *E. coli* membranes expressing CYP2C9 was inhibited > 90% by the CYP2C9 inhibitor sulphaphenazole (figure 3b). Minimal inhibition (< 20%) was observed with the other diagnostic inhibitors in *E. coli* membranes expressing CYP2C9. When the CYP1A2 component of naproxen O-demethylation was abolished in HLM by the inclusion of α-naphthoflavone (1 μM), sulphaphenazole was equally effective as an inhibitor of this reaction in HLM and the *E. coli* membranes expressing CYP2C9. In addition, in the presence of α-naphthoflavone, none of the other CYP inhibitor probes afforded any significant inhibition of the remaining CYP2C9-dependent activity in HLM at concentrations ≥ 10 times their reported $K_i$ (data not shown).

As anticipated, diazepam N-demethylation activity in baculosomes-expressing CYP2C19 was inhibited (85%) by the CYP2C19 substrate omeprazole (50 μM) (figure 3c). Negligible inhibition of expressed CYP2C19 was observed with the other diagnostic CYP inhibitors. In HLM, diazepam N-demethylation was inhibited by both omeprazole (65% at 50 μM) and ketoconazole (80% at 1 μM), a reflection of the contribution towards diazepam N-demethylation of both CYP2C19 and CYP3A4 in HLM. None of the other CYP inhibitor probes afforded any significant inhibition of diazepam N-demethylation activity in HLM.

Dextromethorphan O-demethylation in HLM and in *E. coli* membranes expressing CYP2D6 was selectively and almost completely inhibited by the CYP2D6 inhibitor quinidine (figure 3d). Omeprazole inhibited dextromethorphan O-demethylation in both *E. coli* membranes expressing CYP2D6 and HLM (by 50 and 30% respectively). Ketoconazole, sulphaphenazole and furafylline had no effect on this activity under identical conditions.
Figure 5. Inhibition of probe reactions by diagnostic inhibitors in HLM and cDNA-expressed CYPs. The control activity of the isoforms for the probe reaction: (1) CYP1A2-EROD, (2) CYP2C9-naproxen O-demethylation, (3) CYP2C19-diazepam N-demethylation, (4) CYP2D6-dextromethorphan O-demethylation and (5) CYP3A4-erythromycin N-demethylation, in the absence of inhibitor was represented as 100%. A full $IC_{50}$ determination used the appropriate diagnostic inhibitors, CYP1A2-furafylline CYP2C9-sulphaphenazole, CYP2C19-omeprazole, CYP2D6-quinidine and CYP3A4-ketoconazole, in HLM (□), and the respective cDNA-expressed cell line, E. coli membranes expressing the respective CYP (■), insect baculosomes expressing CYP2C19 (△) and lymphoblastoid B cells expressing CYP3A4 (▲). Data shown are from one representative experiment (see table 1 for variation).
Table 3. Comparison of $IC_{50}$ determined in our laboratory with literature $K_{i}/IC_{50}$ for CYP2C9.

<table>
<thead>
<tr>
<th></th>
<th>$IC_{50}$ (µM)</th>
<th>$IC'_{50}$ (µM)</th>
<th>$IC_{50}$ (µM)</th>
<th>$K_{i}$ (µM)</th>
<th>HLM</th>
<th>CYP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miconazole</td>
<td>0.5</td>
<td>–</td>
<td>4</td>
<td>0.9</td>
<td>–</td>
<td>√</td>
<td>Back et al. (1988)</td>
</tr>
<tr>
<td>Sulphaphenazole</td>
<td>0.7</td>
<td>0.5</td>
<td>1.5</td>
<td>2.5</td>
<td>–</td>
<td>√</td>
<td>Back et al. (1988)</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>2.5</td>
<td>–</td>
<td>√</td>
<td>Back et al. (1988), Newton et al. (1995)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>5</td>
<td>–</td>
<td>42</td>
<td>16.5, 24</td>
<td>–</td>
<td>√</td>
<td>Tracy et al. (1997)</td>
</tr>
<tr>
<td>Warfarin</td>
<td>14</td>
<td>–</td>
<td>74</td>
<td>27</td>
<td>–</td>
<td>√</td>
<td>Back et al. (1988)</td>
</tr>
<tr>
<td>4-Methylimidazole</td>
<td>18</td>
<td>–</td>
<td>71</td>
<td>90</td>
<td>–</td>
<td>√</td>
<td>Back et al. (1988)</td>
</tr>
<tr>
<td>α-naphthoflavone</td>
<td>46</td>
<td>13</td>
<td>6</td>
<td>0.5, 42</td>
<td>–</td>
<td>√</td>
<td>Chang et al. (1994), Newton et al. (1995)</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>52</td>
<td>–</td>
<td>201</td>
<td>–</td>
<td>50*</td>
<td>√</td>
<td>Mancy et al. (1995)</td>
</tr>
<tr>
<td>Primaquine</td>
<td>–</td>
<td>86</td>
<td>15</td>
<td>80</td>
<td>–</td>
<td>√</td>
<td>Back et al. (1988)</td>
</tr>
<tr>
<td>4-Methylpyrazole</td>
<td>79</td>
<td>–</td>
<td>166</td>
<td>900</td>
<td>–</td>
<td>√</td>
<td>Back et al. (1988)</td>
</tr>
<tr>
<td>Quinine</td>
<td>96</td>
<td>–</td>
<td>481</td>
<td>353</td>
<td>–</td>
<td>√</td>
<td>Newton et al. (1995)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>195</td>
<td>145</td>
<td>361</td>
<td>–</td>
<td>53*</td>
<td>√</td>
<td>Back et al. (1988)</td>
</tr>
</tbody>
</table>

*K$_{i}$ (µM).
### Table 4. Comparison of $IC_{so}$ determined in our laboratory with literature $K_i/IC_{so}$ for CYP2C19.

<table>
<thead>
<tr>
<th></th>
<th>Baculosomes $IC_{so}$ (µM)</th>
<th>HLM $IC_{so}$ (µM)</th>
<th>Literature $IC_{so}$ (µM)</th>
<th>$K_i$ (µM)</th>
<th>HLM CYP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>8</td>
<td>14</td>
<td>–</td>
<td>2</td>
<td></td>
<td>Wienkers et al. (1996)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>10</td>
<td>4</td>
<td>28</td>
<td>–</td>
<td></td>
<td>Baldwin et al. (1995)</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>84</td>
<td>75</td>
<td>–</td>
<td>–</td>
<td></td>
<td>Wienkers et al. (1996)</td>
</tr>
<tr>
<td>S-mephenytoin</td>
<td>88</td>
<td>250</td>
<td>–</td>
<td>60</td>
<td></td>
<td>Baldwin et al. (1995)</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>319</td>
<td>370</td>
<td>&gt;100</td>
<td>–</td>
<td></td>
<td>Wienkers et al. (1996)</td>
</tr>
<tr>
<td>Warfarin</td>
<td>&gt;1000</td>
<td>&gt;2000</td>
<td>–</td>
<td>320</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3e demonstrates that erythromycin N-demethylation was inhibited by ketoconazole (1 µM) in the CYP3A4 cDNA-expressing cell line and HLM (by 100 and 90%, respectively). Sulphaphenazole, furafylline and quinidine had little or no effect on this activity using either source. Omeprazole (also a low-affinity CYP3A4 substrate) exhibited 50% inhibition at 50 µM in HLM, but generated a 4-fold stimulation in this activity in the CYP3A4 cDNA-expressing cell line.

### Automated $IC_{so}$ determinations of test compounds for the major human CYPs

For the radiometric assays, automated $IC_{so}$ determination methods based on using either two (two-point $IC_{so}$) or seven (full $IC_{so}$) concentrations of the marker compound were developed. Figure 4 demonstrates an excellent correlation ($r^2 = 0.98, p < 0.001$) between $IC_{so}$ determined by both methods for all the radiometric assays and validates the use of the two-point determination for the initial screening of compounds which may require even higher throughput.

Figure 5a–e defines the inhibition of CYP1A2, 2C9, 2C19, 2D6 and 3A4 by the diagnostic probe inhibitors furafylline, sulphaphenazole, omeprazole, quinidine and ketoconazole respectively. For CYP1A2, 2C9, 2D6 and 3A4 the inhibition curves generated in HLM and cell lines expressing each individual CYP compare well. For CYP2C19, although an $IC_{so} = 6$ µM was determined for omeprazole in baculosomes expressing CYP2C19, inconsistent profiles were observed in HLM and were not included in figure 5c.

Tables 2–6 compare $IC_{so}$ for a series of test compounds obtained in this laboratory in HLM and cloned CYP expression systems to previously published values. Some of these compounds, generally those with low $IC_{so}$, represent known specific inhibitors of the relative isofoms while others, with higher $IC_{so}$, may be poor competitive substrates or act as negative controls. For CYP1A2, CYP2D6 and CYP3A4, both sources of enzyme generated remarkably similar values. For the limited data-set obtained for CYP2C19 (a reflection of the available literature data) there remains a good correlation between the two enzyme sources. However, for CYP2C9 a consistent 3–4-fold higher $IC_{so}$ was determined using HLM compared with expressed CYP2C9. This is demonstrated more clearly in figure 6, which shows a significant correlation ($r^2 = 0.89, p < 0.001$) between the $IC_{so}$ for compounds
Table 5. Comparison of $IC_{50}$ determined in our laboratory with literature $K_i$ for CYP2D6.

<table>
<thead>
<tr>
<th></th>
<th>E. coli $IC_{50}$ (μM)</th>
<th>B-Lymphoblastoids $IC_{50}$ (μM)</th>
<th>HLM $IC_{50}$ (μM)</th>
<th>Literature</th>
<th></th>
<th></th>
<th></th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinidine</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.005, 0.03, 0.1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>Wu et al. (1993), Ching et al. (1995),</td>
</tr>
<tr>
<td>Hydroquinidine</td>
<td>–</td>
<td>–</td>
<td>0.01</td>
<td>0.01</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Kerry et al. (1994)</td>
</tr>
<tr>
<td>Propafenone</td>
<td>0.13</td>
<td>0.16</td>
<td>0.09</td>
<td>0.07</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Ching et al. (1995)</td>
</tr>
<tr>
<td>Lobeine</td>
<td>0.15</td>
<td>0.22</td>
<td>0.12</td>
<td>0.03</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Wu et al. (1993)</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>1.2</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Wu et al. (1993)</td>
</tr>
<tr>
<td>Pentazocine</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>0.4</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Fonse-Pfister and Meyer (1988)</td>
</tr>
<tr>
<td>Quinine</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Wu et al. (1993)</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Wu et al. (1993)</td>
</tr>
<tr>
<td>Mexiletine</td>
<td>17</td>
<td>23</td>
<td>23</td>
<td>18</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Broly et al. (1990)</td>
</tr>
<tr>
<td>Clozapine</td>
<td>–</td>
<td>–</td>
<td>29</td>
<td>4</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Fischer et al. (1992)</td>
</tr>
<tr>
<td>Sparteine</td>
<td>–</td>
<td>–</td>
<td>85</td>
<td>45</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Dayer et al. (1989)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>17</td>
<td>39</td>
<td>21</td>
<td>127</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Halliday et al. (1995)</td>
</tr>
<tr>
<td>Debrisoquine</td>
<td>46</td>
<td>134</td>
<td>58</td>
<td>25</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Dayer et al. (1989)</td>
</tr>
</tbody>
</table>
Table 6. Comparison of $IC_{50}$ determined in our laboratory with literature $K_i/IC_{50}$ for CYP3A4.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Erythromycin</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-Lymphoblastoids</td>
<td>HLM</td>
</tr>
<tr>
<td></td>
<td>$IC_{50}$ ($\mu$M)</td>
<td>$IC_{50}$ ($\mu$M)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.17</td>
<td>0.26</td>
</tr>
<tr>
<td>Bromocryptine</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Dihydroergotamine</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>32</td>
<td>48</td>
</tr>
<tr>
<td>Verapamil</td>
<td>–</td>
<td>76</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>–</td>
<td>79</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>–</td>
<td>132</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>72</td>
<td>218</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>–</td>
<td>350</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>–</td>
<td>1000</td>
</tr>
</tbody>
</table>

All literature $K_i$ and $IC_{50}$ are determined from human liver microsomal incubations.

$^{\dagger}$Pichard et al. (1990).


$^{\#}$Zhao and Ishizaki (1997).
Figure 6. Comparison of $IC_{50}$ determined in cDNA-expressed CYPs and HLM for a series of compounds. The data represent $IC_{50}$ tabulated in tables 2–6 for the five different isoforms CYP1A2 (■), CYP2C9 (□), CYP2C19 (●), CYP2D6 (○) and CYP3A4 (∆). For CYP2C9 and CYP2D6 (tables 3 and 5 respectively), where more than one cDNA-expression system was used, the figure reflects only $IC_{50}$ determined from *E. coli* membranes expressing the respective isoform. The solid line depicts a linear regression analysis of the data ($r^2 = 0.89, p < 0.001$). The dashed line depicts unity. Two obvious outliers in the plot are: (1) α-naphthoflavone and (2) omeprazole.

obtained in HLM and different cloned CYP expression systems. Both α-naphthoflavone (1) and omeprazole (2) generated divergent $IC_{50}$ in the two enzyme sources.

Discussion

With the advent of combinatorial chemistry and parallel synthesis methodologies, the pharmaceutical industry now has the potential to generate large numbers of compounds for progression to high throughput screening techniques. To deal with this increased throughput, *in vitro* drug metabolism models are increasingly being used, in conjunction with classical *in vivo* methods, to assess the extent and route of metabolism of these compounds, as well as screening for inducers and inhibitors of xenobiotic-metabolizing enzymes. This paper describes the development and validation of fully automated medium throughput assays with which to assess inhibition of the major human hepatic CYPs.

Most routine CYP assays require HPLC or LC-MS analysis and are therefore time consuming and labour- or equipment-intensive. More recently, fluorescent assays (Crespi et al. 1997) and LC-MS methods (Ayrton et al. 1998) have been reported which, in theory, offer enhanced throughput over these traditional methods. However, these techniques, particularly fluorescence analysis, can be prone to interference and/or quenching which limits their general applicability since the spectroscopic properties of the majority of compounds and their metabolites may not be defined at an early stage of the research process.

The fluorescent and radiometric *in vitro* assays for each CYP isoform employed in this study were chosen based on factors including compatibility for automation, robustness and reproducibility. The radiometric assays offered many advantages over fluorescence-based assays from a consideration of selectivity and solubility of substrates together with the use of marketed drugs with well-defined metabolic pathways. In addition, the extremely low turnover of some fluorescence probe substrates by individual isoforms (e.g. 0.004 min$^{-1}$ for 3-cyano-7-ethoxycoumarin with CYP2C9; Crespi et al. 1997) may potentially result in preferential metabolism
of test compounds, resulting in a change in the apparent $IC_\text{app}$ over time. Therefore, although the radiometric assays required a sample clean-up phase not necessary in the direct fluorometric analyses, this was offset by the factors discussed above and the selectivity afforded by this approach.

The absolute requirement for probe substrate specificity for each of the respective isoforms was obviated with the use of cloned CYP enzymes in some assays. The radiometric assays included [$^1$C]-erythromycin N-demethylation for CYP3A4 (Zhang and Thomas 1996, Riley and Howbrook 1998), [$^1$C]-naproxen O-demethylation for CYP2C9 (Rodrigues et al. 1996) and [$^1$C]-dextromethorphan O-demethylation for CYP2D6 (Rodrigues et al. 1994). In addition [$^1$C]-diazepam N-demethylation was developed in our laboratory as a novel assay for CYP2C19 activity. For the radiometric assays > 99.7% of 14C labelled substrate was routinely extracted by the SPE procedure marking a significant improvement in sensitivity over previously reported radiometric assays (95%, Zhang and Thomas 1996; 90%, Rodrigues et al. 1996).

Our results confirm that out of the five major human hepatic CYPs, CYP1A2 catalyses the majority of EROD with negligible contribution (~10% of CYP1A2 activity) from CYP2C19. Indeed, any CYP2C19 component to this reaction in HLM will be even smaller when one considers the relative abundance of these two isoforms (Shimada et al. 1994, Ono et al. 1996b). As predicted EROD catalysed by expressed CYP1A2 and HLM was significantly inhibited by the CYP1A2 specific inhibitor furafylline (Tassaneeyakul et al. 1993, Tassaneeyakul et al. 1994, Sesardic et al. 1990), but not by ketoconazole, sulphaphenazole or quinidine. Omeprazole has been reported to only weakly inhibit human CYP1A2 (Zomorodi and Houston 1997) and did indeed inhibit EROD by ~50% using E. coli membranes expressing CYP1A2. However, no inhibition was observed in pooled HLM, possibly as a result of efficient metabolism of omeprazole by CYP2C19 and CYP3A4 in HLM or non-specific binding of omeprazole to another component in HLM.

Naproxen O-demethylation was catalysed by two low-affinity isoforms CYPs 2C19 ($K_m \sim 3 \text{ mm}$) and 1A2 ($K_m$—not determined) as well as the high affinity isoform CYP2C9 ($K_m \sim 300 \mu M$), a result in broad agreement with reaction kinetics reported by Rodrigues et al. (1996) and Miners et al. (1996) using both HLM and expressed CYPs. In contrast, Tracy et al. (1997) using a B-lymphoblastoid cell line (Gentest Corp.) expressing individual P450 isoforms concluded that only CYPs 1A2, 2C8 and 2C9 are involved in naproxen O-demethylation and not CYP2C19. Our data would indicate a role for CYP2C19 as the low-affinity component identified by Rodrigues et al. (1996) in HLM. In addition, naproxen O-demethylation activity was markedly inhibited in HLM by the CYP2C9 inhibitor sulphaphenazole after the minor CYP1A2 contribution to this activity is abolished with the addition of α-naphthoflavone.

Using the respective cloned CYPs, diazepam was N-demethylated by a high-affinity isoform, CYP2C19, but also by the low-affinity CYP3A4 ($K_m = 1.8 \text{ mm}$; Ono et al. 1996b), a result consistent with several studies using HLM (including Yasumori et al. 1993, Andersson et al. 1994) and cDNA-expressed CYPs (Jung et al. 1997). This was confirmed by the inhibitory action of the diagnostic CYP2C19 probe omeprazole in both baculosomes expressing CYP2C19 and HLM, and by the inhibitory effect of ketoconazole, solely in HLM.

It has long been established that dextromethorphan O-demethylation is mediated predominantly by CYP2D6 (Kupfer et al. 1984, Dayer et al. 1989).
Dextromethorphan N-demethylation, a minor metabolic pathway (Schmider et al. 1997), is catalysed by several CYP enzymes including CYP3A4 (Jacqz-Aigrain et al. 1993, Gorski et al. 1994, Ducharme et al. 1996, Jones et al. 1996, Schmider et al. 1997). However, reports that dextromethorphan O-demethylation is multiphasic in vitro (Kronbach et al. 1987, Kerry et al. 1993, 1994, Schmider et al. 1997) indicates the involvement of more than one isoform with different affinities, but the identity of these isoforms has yet to be firmly established. Our results confirm the high affinity component to be CYP2D6 and suggest roles for two low-affinity components, CYP2C19 and CYP2C9. There are reports of CYP2C9 involvement in dextromethorphan O-demethylation (Ono et al. 1996a). The apparent $K_m$ for dextromethorphan O-demethylation in CYP2D6 and CYP2C19 were determined to be $\sim 5$ and $90 \mu M$ respectively. The $90 \mu M$ value is consistent with $K_m$ for the low-affinity component in HLM (Kerry et al. 1994, Schmider et al. 1997). Collectively, these data verify a preliminary communication which has intimated a role for CYP2C19 as the low-affinity dextromethorphan O-demethylase (Van Moltke et al. 1998). In the 5–10% of Caucasians that are classed as ‘poor metabolizers’ due to the polymorphic expression of CYP2D6, CYP2C9 and CYP2C19 may represent the residual dextromethorphan O-demethylation pathway.

Erythromycin N-demethylation is known to be catalysed by the CYP3A family (Cook et al. 1993) and results presented here confirm that the contribution from the other major human hepatic CYPs is minimal, particularly when one considers their relative levels of expression (Shimada et al. 1994). As predicted, the CYP3A4 probe ketoconazole, selectively inhibits erythromycin N-demethylation in both HLM and the CYP3A4 cDNA-expressed cell line. The moderate inhibition of erythromycin N-demethylation in HLM observed with omeprazole is consistent with this drug being an alternative substrate for CYP3A4 ($K_m = 50 \mu M$ for sulphone formation; Andersson et al. 1993, Yamazaki et al. 1997). Omeprazole appeared to stimulate this activity with the cDNA-expressed cell, presumably as a result of positive cooperativity similar to that observed for other CYP3A4 ligands (Ueng et al. 1997).

For the radiometric based assays described herein, the basis for the two-point $IC_{50}$ determination has been outlined (see Materials and methods) and this method was validated by correlating the $IC_{50}$ obtained from the two-point and full (seven) -point assays (figure 4). Using the two- and full-point $IC_{50}$ methods, a maximum of 18 and 10 compounds per plate can be screened per run, respectively. Thus in this laboratory, the two-point assay is used to expedite an initial screen of a series of compounds. Theoretically, using typical test compound concentrations of 5 and $50 \mu M$ in the two-point assay, $IC_{50}$ between 0.5 and $200 \mu M$ can be estimated routinely. A subset of compounds generating $IC_{50}$ below a predetermined concentration can then be screened using the full $IC_{50}$ method, which generates further confidence in the data and ideally allows any structural features underlying CYP inhibition to be identified and designed out from the series of potential developmental drug candidates.

HLM are the most widely used in vitro drug metabolizing source and have been used to assess the potential of compounds to inhibit CYP enzymes (Pichard et al. 1990, Gascon and Dayer 1991, Zhao and Ishizaki 1997). The availability of heterologously expressed CYP isoforms have markedly aided the identification of individual isoforms responsible for metabolism of the compound of choice (Aoyama et al. 1990, Guengerich et al. 1993, Ha et al. 1995, Yang et al. 1998) and are now being applied to the development of CYP inhibition screens (Crespi et al. 1997).
The high isoform specificity of erythromycin N-demethylation and EROD allowed the flexibility to employ either pooled HLM or cloned CYP as the enzyme source for development of the inhibition screens. Furthermore, from a consideration of the reaction kinetics using a two-enzyme model, it was evident that at a substrate concentration of 5 μM, the contribution of CYP2C19 to dextromethorphan O-demethylation would be negligible. This was confirmed in experiments with quinidine which almost completely abolished dextromethorphan O-demethylation in both HLM and expressed CYP2D6, thus demonstrating both enzyme sources may be utilized for this activity. However for naproxen O-demethylation, and diazepam N-demethylation, incomplete substrate selectivity was overcome with the use of cloned enzymes in the inhibition screens.

These data demonstrate that for CYP1A2, CYP2D6 and CYP3A4, both sources of enzyme generate similar IC₅₀ and so the assays remain flexible enough to employ either HLM or subcellular fractions from CYP cDNA-expressed cells.

For CYP2C9, 3–4-fold higher IC₅₀ values were determined using HLM compared with expressed CYP2C9, probably as a result of the contribution of CYP1A2 (and CYP2C19?) to this reaction in HLM. For CYP2C19, only a limited data set was obtained which reflects in part the paucity of information concerning the structure-activity relationship of this polymorphic isoform. It is of interest to note that despite the relatively good correlation between the two enzyme sources with the five human CYP isoforms, in general higher IC₅₀ were observed with HLM compared with cDNA-expressed systems. This has been observed by other groups (Crespi and Penman 1997) and is probably a result of some contribution to probe substrate catalysis by other CYP isoforms in HLM and possibly to binding of the test compound to components in HLM that are absent or present at lower levels in the respective expressed system. One or more of these explanations may account for the high IC₅₀ determined for omeprazole in HLM compared with cDNA-expressed CYP2C19.

In contrast, α-naphthoflavone has a lower apparent IC₅₀ in HLM than in CYP2C9 cDNA-expressed E. coli membrane. This divergence can be rationalized as naproxen O-demethylation is catalysed by both CYP1A2 and CYP2C9 in HLM and as α-naphthoflavone is a very potent CYP1A2 inhibitor, this will result in lower IC₅₀ in HLM.

Intuitively, it may appear preferable to use cDNA-expressed CYPs as the enzyme source for inhibition assays as opposed to the multi-isoform HLM, to predict accurately isoform-specific CYP inhibitors from a series of test compounds. Perceived advantages of using expressed enzymes over HLM may be exploited in inhibition assays (where substrates with incomplete specificity may be employed) and for the direct, effective identification of the human CYP enzyology of the biotransformation of drugs. However, caveats such as catalysis to an inhibitory metabolite in HLM by a CYP other than that present in the expression system must be considered.

The general agreement between IC₅₀ determined for a range of marker compounds in both expressed systems and HLM, in this study, with IC₅₀ and/or Ki published by other laboratories generates confidence in the universality of these automated inhibition screens. These data are particularly encouraging when one considers the potential interlaboratory variability arising from the use of different substrate concentrations, probe assays and enzyme source (table 6; Boobis et al. 1998).
In conclusion, this paper demonstrates the development and validation of automated screens for the initial assessment of inhibition of the major human hepatic CYPs, allowing a rapid determination of $IC_{50}$ for a large number of compounds. The assays developed are fully automated, flexible yet sensitive and with the potential exception of the CYP1A2 EROD assay, free from analytical interference. These assays may generate data suitable for the elucidation of inhibitory structural motifs within a series of compounds to design out this undesirable feature. In lieu of achieving no CYP inhibition, a detailed inhibition profile may help predict potential clinical drug-drug interactions and to prioritize clinical studies. Finally, as increasing numbers of compounds are screened, a comprehensive database of the proclivity of compounds to inhibit CYP will enable the determination of more accurate and predictive molecular models together with complementary emerging technologies.

Acknowledgements

We acknowledge support for this project from the BBSRC, UKDTI and the UK-LINK consortium of pharmaceutical companies: Astra, Glaxo-Wellcome, Janssen Pharmaceutica, Lilly, Novo Nordisk, Parke-Davis, Pfizer, Roche Products, Sanofi-Winthrop, Servier, Smith-Kline Beecham, Wyeth-Ayerst and Zeneca. We also thank Dr Marco Skrinjar of Astra Draco for the gift of [O-methyl-14C]dextromethorphan.

References


CHENG, Y. and PRUSSOFF, W. H., 1973, Relationship between the inhibition constant (k.) and the concentration of an inhibitor that causes a 50% inhibition (I_{50}) of an enzymatic reaction. Biochemical Pharmacology, 22, 3099–3108.


JUNG, F., RICHARDSON, T. H., RAUCY, J. L. and JOHNSON, E. F., 1997, Diazepam metabolism by cDNA-expressed human 2C P450s: identification of P450C18 and P450C19 as low K_{m} diazepam N-demethylases. Drug Metabolism and Disposition, 25, 133–139.

KERRY, N. L., SOMOVOY, A. A., BOCHNER, F. and MIKUS, G., 1994, The role of CYP2D6 in primary and


Rost, K. L., Fuhr, U., Ziegler, M., Bohnemeyer, H. and Roots, I., 1995, Omeprazole not only inducers but also inhibits CYP1A2 in man. In 9th International Conference on Cytochrome P450, P., -6, p. 47.


Yamazaki, H., Inoue, K., Shaw, P. M., Cheivos, W. J., Guengerich, P. and Shimada, T., 1997, Different contributions of cytochrome P450 2C19 and 3A4 in the oxidation of omeprazole by human liver microsomes: effects of contents of these two forms in individual human samples. Journal of Pharmacology and Experimental Therapeutics, 283, 434-442.


Zhao, X. J. and Ishizaki, T., 1997, Metabolic interactions of selected antimalarial and non-antimalarial drugs with the major pathway (3-hydroxylation) of quinine in human liver microsomes. British Journal of Clinical Pharmacology, 44, 505-511.
