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

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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 ^{14}C-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 (IC_{50} = 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 IC_{50} estimate was validated by correlating the IC_{50} obtained with a full (seven-point) assay (r^2 = 0.98, $p < 0.001$). The two-point IC_{50} estimate is useful for initial screening, while the full IC_{50} method provides more definitive quantitation, where required.

4. IC_{50} determined for a series of test compounds in human liver microsomes and cytochrome P450 cDNA-expressed enzymes were similar (r^2 = 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 IC_{50} determined in these assays to IC_{50} 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 IC_{50}. 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

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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 [^{14}C]-radiolabelled probe substrates and measurement of the resultant [^{14}C]-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-dimethlyxanthine, piroxicam, mexiletine, quinine, ibuprofen, tolbutamide, propranolol, quinidine, propafenone, lobeline, pentazocine, clozapine, sparteine, chloroquine, debrisoquine, bromocryptine, dihydroergotamine, troleandomycin, nifedipine, erythromycin, diethyldithiocarbamate, 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 Ultrafine Chemicals (Manchester, UK). Dimethylsulphoxide (DMSO) and acetonitrile were purchased from Fisher Scientific (Loughborough, UK) and methanol was purchased from Romil Ltd (Cambridge, UK).

Omeprazole, fluconazole and ketoconazole (purity $\geqslant 99\%$) 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*-methyl-^{14}C] dextromethorphan (specific radioactivity 56 mCi/mmol, chemical purity 99.8%, radiochemical purity 99.6%) and [*O*-methyl-^{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 diazepam) or DMSO (dextromethorphan) in the presence of potassium hydroxide. The products were purified by reverse-phase HPLC and stored in ethanol before use. [*N*-methyl-^{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 (Rodrigues *et al*. 1994), naproxen *O*-demethylation (Rodrigues *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$\times$ 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 0.05–0.1 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 reflect V_{max} conditions for each substrate.

For the fluorometric assay, ethoxyresorufin was used at a final concentration of 3 μM to reflect the V_{max} 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$\times$ 1 ml) and water (4$\times$ 1 ml). All of the quenched incubation was applied to the column and eluted with 2$\times$ 500 μl aliquots water. The resulting volume was transferred to vials containing 7 ml UltimaGold liquid scintillation cocktail (Packard Instrument Co., Pangbourne, UK) and samples counted for 2 min using a Packard 2200CA Tri-Carb Liquid Scintillation Analyser.

Furafylline, sulphaphenazole, quinidine, 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 $\geqslant 10$ times their reported K_i; furafylline, 20 μM

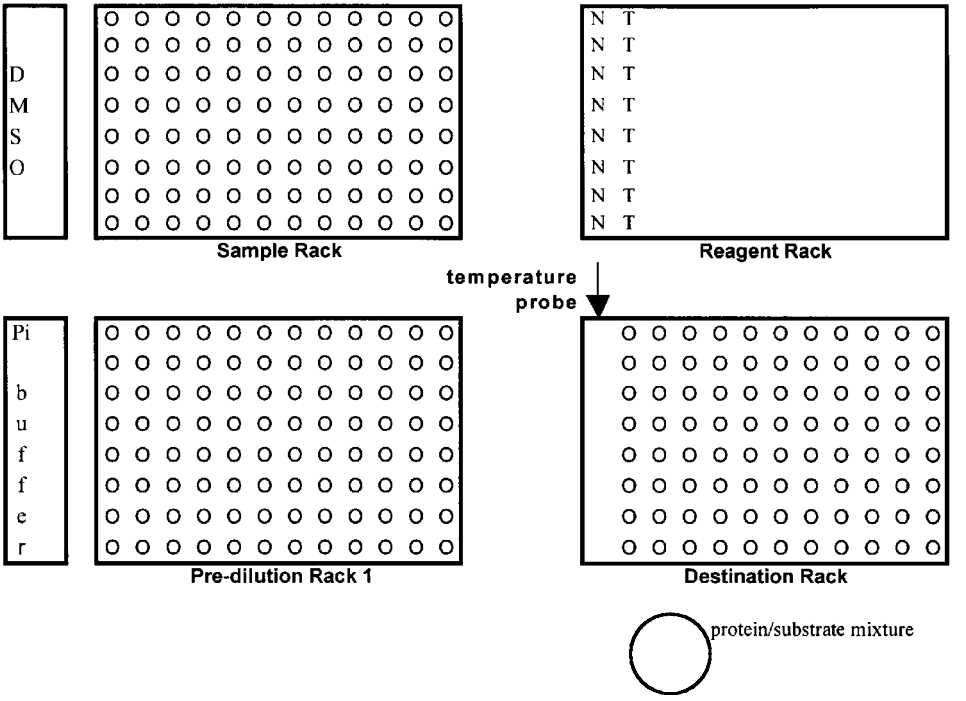

Figure 1. Layout of the radiometric inhibition assays on the robotic sample processor. The schematic representation of the robotic sample processor shows four 96-well plates. The sample rack contained primary stocks of test compounds which were diluted to seven secondary stocks in the predilution rack, using dimethylsulphoxide and 100 mM phosphate buffer, pH 7.4 (from reagent reservoirs labelled DMSO and P_i buffer respectively). The reagent rack contained aliquots of 10 mM NADPH (N) and 10% (w/v) tricholoroacetic 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 IC_{50} *determination*

A maximum of 10 compounds per 96-well plate were screened. The dilution of test compounds for a full (seven) -point IC_{50} 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 IC_{50} *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 IC_{50} determination fitted the data to a variation of the expression:

$$V = \frac{V_o}{1 + (I/IC_{50})^s} - b,$$

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:

$$IC_{50} = \frac{I(100 - I_x)}{I_x}.$$

where I = inhibitor concentration, I_x = % inhibition at I, as described previously (Zomorodi and Houston 1996). Experience showed that this was valid when I_x 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 IC_{50} 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× 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 (λ_{ex} 544 nm, λ_{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) isoforms, 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 ($\sim 80\%$ of CYP2C9 activity) and CYP1A2 ($\sim 50\%$ 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 ($\sim 30\%$ 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.

Substrate	Final substrate concentration (μM)	μCi ^{14}C substrate/ incubation	CYP source	pmol CYP/ incubation	Incubation time (min)	Control activity (pmol/min/ pmol)	% retention of ^{14}C-substrate	IC_{50} for standard inhibitor (μM)	n
Ethoxyresorufin	0.6	–	*E. coli*	3	15	0.4± 0.1	–	6± 2	15
Naproxen	109	0.1	*E. coli*	14	15	1.5± 0.1	99.70± 0.01	0.7± 0.1	3
Diazepam	19	0.1	baculosomes	8	15	0.3± 0.06	99.98± 0.02	6± 2	4
Dextromethorphan	5	0.05	*E. coli*	4	15	0.8± 0.4	99.81± 0.18	0.03± 0.01	7
Erythromycin	59	0.1	HLM	0.5#	15	119± 7*	99.84± 0.01	0.26± 0.02	5

Control activities, % retention of ^{14}C-substrate and IC_{50} 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: furafylline (ethoxyresorufin *O*-deethylation), sulfaphenazole (naproxen *O*-demethylation), omeprazole (diazepam *N*-demethylation), quinidine (dextromethorphan *O*-demethylation) and ketoconazole (erythromycin *N*-demethylation).

Table 2. Comparison of IC_{50} determined in our laboratory with literature K_i/IC_{50} for CYP1A2.

	E. coli IC_{50} (μM)	HLM IC_{50} (μM)	Literature				
			IC_{50} (μM)	K_i (μM)	HLM	CYP	Reference
α-Naphthoflavone	0.02	0.02	–	0.002		√	Pastrakuljic *et al*. (1997)
Apigenin	1.1	0.5	–	0.36		√	Pastrakuljic *et al*. (1997)
7-Ethoxycoumarin	4	8	3	–	√		Tassaneeyakul *et al*. (1993)
Furafylline	6	6	–	3	√		Bourrie *et al*. (1996)
Ketoconazole	11	49	13	–	√		Baldwin *et al*. (1995)
Omeprazole	78	96	–	101–135	√		Rost *et al*. (1995)
Theophylline	>200	>200	–	200–1000*	√		Rost *et al*. (1995)
Caffeine	>200	>200	–	800		√	Tassaneeyakul *et al*. (1992)
Diethyldithiocarbamate	>250	1260	>125	–		√	Chang *et al*. (1994)
1,7-Dimethlyxanthine	>300	>300	110	–		√	Brøsen (1995)

*Km (μM).

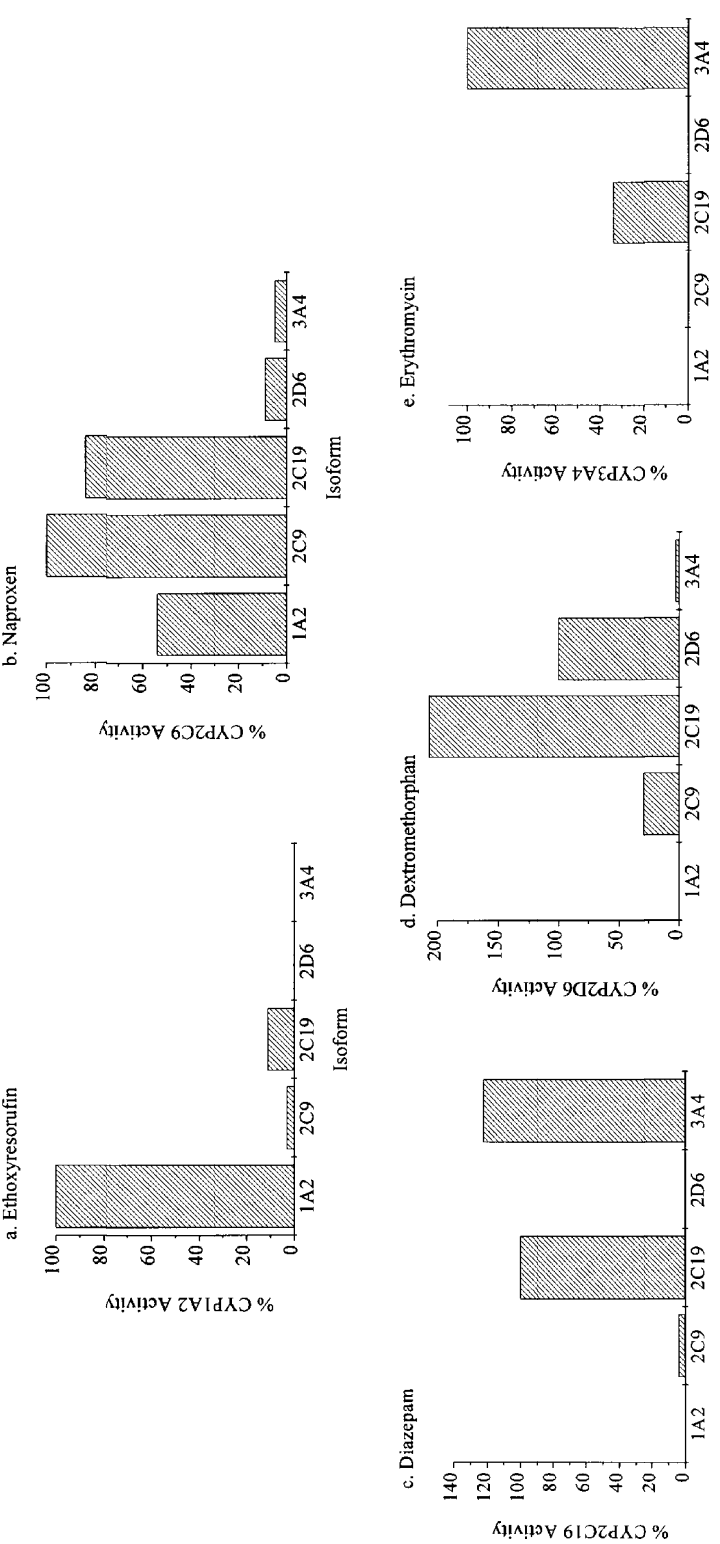

Figure 2. Selectivity of probe substrate catalysis by CYP1A2, 2C9, 2C19, 2D6 and 3A4. The activity (turnover number) of the specific isoform for the chosen probe reaction is represented as 100% and the respective activities of the other isoforms are relative to this rate: (1) EROD (turnover number for 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.

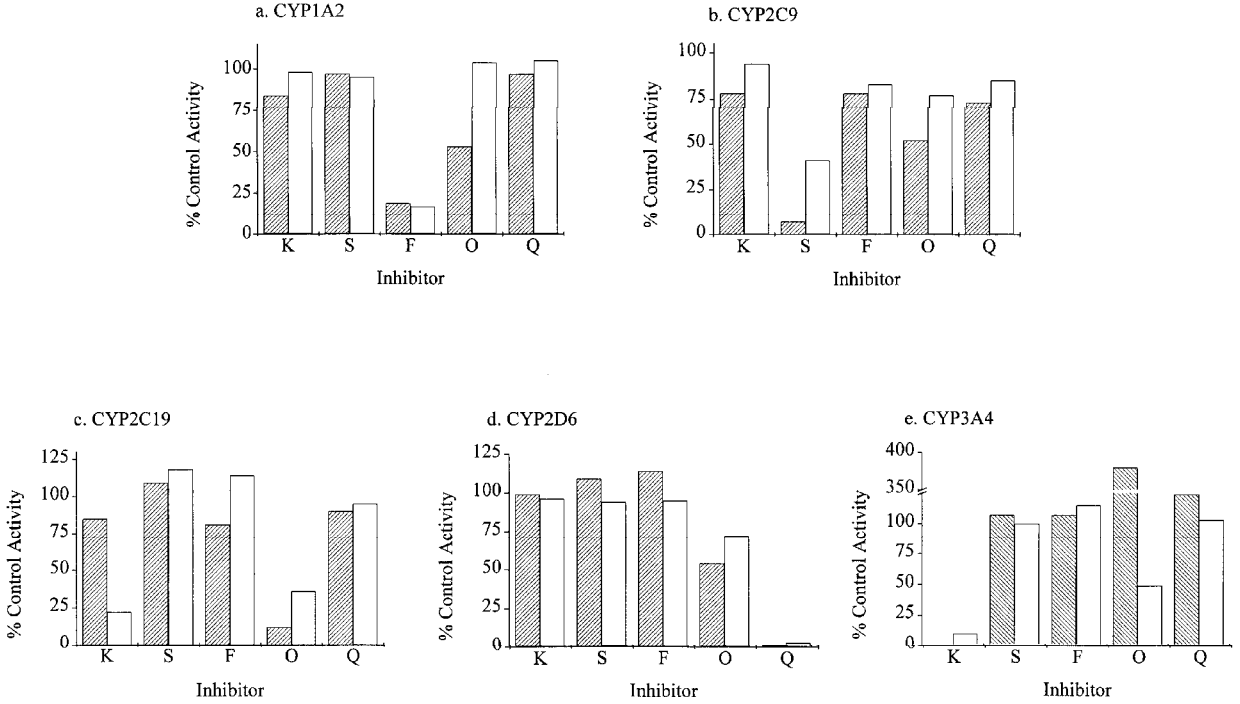

Figure 3. Inhibitor selectivity of probe substrate catalysis by CYP1A2, 2C9, 2C19, 2D6 and 3A4. The control activity of the isoforms for the respective probe reaction: (1) CYP1A2-EROD, (2) CYP2C9-naproxen *O*-demethylation, (3) CYP2C19-diazepam *N*-demethylation, (4) CYP2D6-dextromethorphan *O*-demethylation 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 K_m (as reported in the Materials and methods) and the diagnostic inhibitors ketoconazole (K), sulphaphenazole (S), furafylline (F), omeprazole (O) and quinidine (Q) were added at concentrations $\geqslant$ 10 times their reported K_i (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.

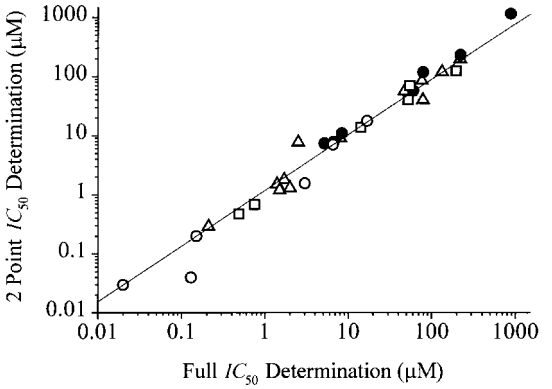

Figure 4. Comparison of the two-point IC_{50} determination against the full (seven) -point IC_{50} determination for the radiometric inhibition assays. Both the two- and full-point IC_{50} determinations were carried out as described in the Materials and methods. The data represent IC_{50} determined 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 $IC_{50} \leqslant 0.5$ μM; 5 and 50 μM for inhibitors with IC_{50} = 0.5–250 μM; 50 and 500 μM for inhibitors with $IC_{50} \geqslant 250$ 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 $\geqslant 10$ times their reported K_i. Omeprazole (a high-affinity CYP2C19 substrate) inhibited EROD activity in *E. coli* membranes expressing CYP1A2 only weakly (by $\sim 50\%$) 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 $\geqslant 10$ 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*-demethylase 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.

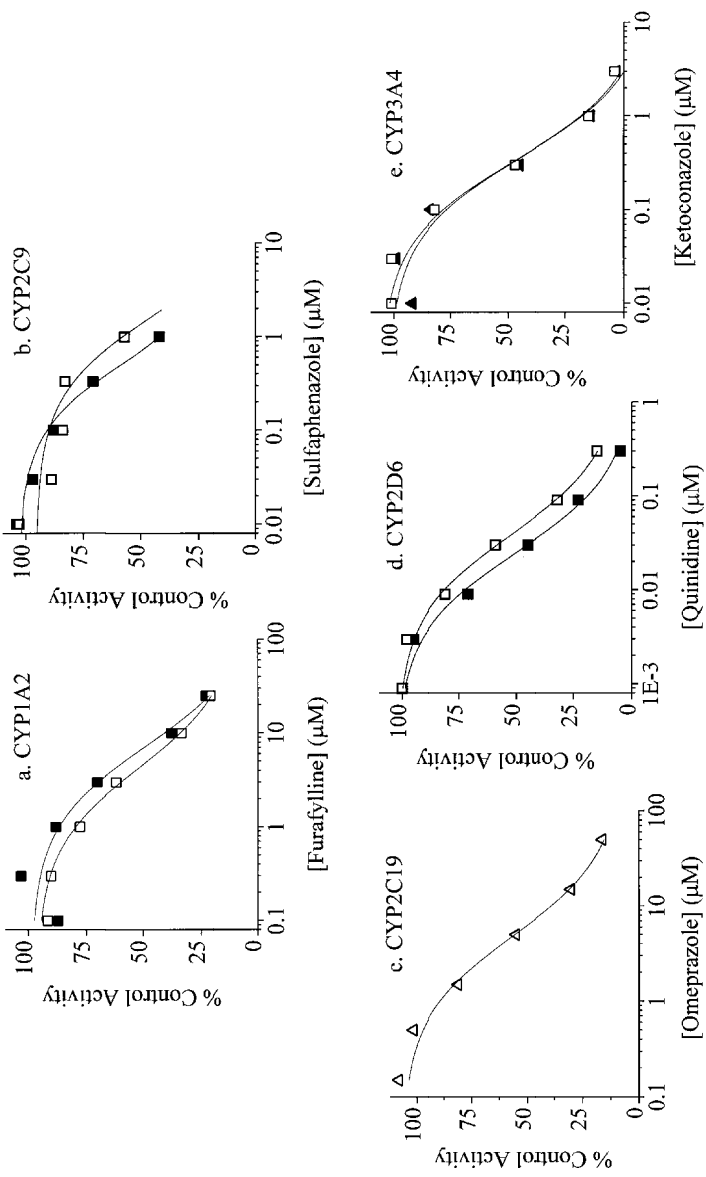

Figure 5. Inhibition of probe reactions by diagnostic inhibitors in HLM and cDNA-expressed CYPs. The control activity of the isoforms for the probe reaction: (1) CYP1A2-EROD, (2) CYP2C9-naproxen *O*-demethylation, (3) CYP2C19-diazepam *N*-demethylation, (4) CYP2D6-dextromethorphan *O*-demethylation and (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.

	E. coli IC_{50} (μM)	B-Lymphoblastoids IC_{50} (μM)	HLM IC_{50} (μM)	Literature				
				IC_{50} (μM)	K_i (μM)	HLM	CYP	Reference
Miconazole	0.5	–	4	0.9	–	√		Back *et al*. (1988)
Sulphaphenazole	0.7	0.5	1.5	–	0.3		√	Mancy *et al*. (1996)
Clotrimazole	–	–	6	2.5	–	√		Back *et al*. (1988)
Ketoconazole	5	–	42	16.5, 24	–	√		Back *et al*. (1988), Newton *et al*. (1995)
Warfarin	14	–	74	–	27	√		Tracy *et al*. (1997)
4-Methylimidazole	18	–	71	90	–	√		Back *et al*. (1988)
α-napthoflavone	46	13	6	0.5, 42	–	√	√	Chang *et al*. (1994), Newton *et al*. (1995)
Piroxicam	52	–	201	–	50*	√		Mancy *et al*. (1995)
Phenytoin	55	–	158	–	23	√		Doecke *et al*. (1991)
Primaquine	–	86	15	80	–	√		Back *et al*. (1988)
4-Methylpyrazole	79	–	166	900	–	√		Newton *et al*. (1995)
Quinine	96	–	481	353	–	√		Back *et al*. (1988)
Ibuprofen	195	145	361	–	53*	√		Mancy *et al*. (1995)
Tolbutamide	291	–	778	–	128, 106	√		Tracy *et al*. (1997), Doecke *et al*. (1991)

* K_m (μM).

Table 4. Comparison of IC_{50} determined in our laboratory with literature K_i/IC_{50} for CYP2C19.

	Baculosomes IC_{50} (μM)	HLM IC_{50} (μM)	Literature				
			IC_{50} (μM)	K_i (μM)	HLM	CYP	Reference
Omeprazole	6	150	–	9		√	Flockhart (1995)
Fluconazole	8	14	–	2	√		Wienkers *et al.* (1996)
Ketoconazole	10	4	28	–	√		Baldwin *et al.* (1995)
Phenytoin	84	75	–	–			
S-mephenytoin	88	250	–	60	√		Wienkers *et al.* (1996)
Sulfaphenazole	319	370	>100	–	√		Baldwin *et al.* (1995)
Warfarin	>1000	>2000	–	320	√		Wienkers *et al.* (1996)

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_{50} *determinations of test compounds for the major human CYPs*

For the radiometric assays, automated IC_{50} determination methods based on using either two (two-point IC_{50}) or seven (full IC_{50}) concentrations of the marker compound were developed. Figure 4 demonstrates an excellent correlation (r^2 = 0.98, $p < 0.001$) between IC_{50} 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_{50} = 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_{50} 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_{50}, represent known specific inhibitors of the relative isoforms while others, with higher IC_{50}, 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_{50} 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_{50} for compounds

Table 5. Comparison of IC_{50} determined in our laboratory with literature K_i for CYP2D6.

	E. coli IC_{50} (μM)	B-Lymphoblastoids IC_{50} (μM)	HLM IC_{50} (μM)	Literature			
				K_i (μM)	HLM	CYP	References
Quinidine	0.03	0.02	0.02	0.005, 0.03, 0.1	√	√	Wu *et al.* (1993), Ching *et al.* (1995), Kerry *et al.* (1994)
Hydroquinidine	–	–	0.01	0.01	√	√	Ching *et al.* (1995)
Propafenone	0.13	0.16	0.09	0.07		√	Wu *et al.* (1993)
Lobeline	0.15	0.22	0.12	0.03	√		Wu *et al.* (1993)
Haloperidol	3	4	3	1.2	√		Fonne-Pfister and Meyer (1988)
Pentazocine	–	–	2	0.4	√		Wu *et al.* (1993)
Quinine	4	8	8	4	√		Wu *et al.* (1993)
Dextromethorphan	7	6	8	5	√		Broly *et al.* (1990)
Mexiletine	17	23	23	18		√	Broly *et al.* (1990)
Clozapine	–	–	29	4	√		Fischer *et al.* (1992)
Sparteine	–	–	85	45	√		Dayer *et al.* (1989)
Chloroquine	17	39	21	127		√	Halliday *et al.* (1995)
Debrisoquine	46	134	58	25	√		Dayer *et al.* (1989)

Table 6. Comparison of IC_{50} determined in our laboratory with literature K_i/IC_{50} for CYP3A4.

	Erythromycin		Literature			
			Cyclosporin A[ξ]	Midazolam*		Quinine[#]
Substrates	B-Lymphoblastoids IC_{50} (μM)	HLM IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)
Ketoconazole	0.17	0.26	0.7	–	0.1	0.03
Bromocryptine	–	3	8	40	–	–
Dihydroergotamine	–	3	23	7	–	–
Troleandomycin	4	8	10	180	–	29
Nifedipine	32	48	10	–	5	38
Verapamil	–	76	24	100	–	64
Omeprazole	–	79	–	–	–	18
Erythromycin	–	132	75	–	148	61
Diltiazem	72	218	63	–	–	127
Chloroquine	–	350	–	150	–	>200
Cimetidine	–	1000	–	550	–	97

All literature K_i and IC_{50} are determined from human liver microsomal incubations.
[ξ] Pichard *et al*. (1990).
* Gascon and Dayer (1991).
# Zhao and Ishizaki (1997).

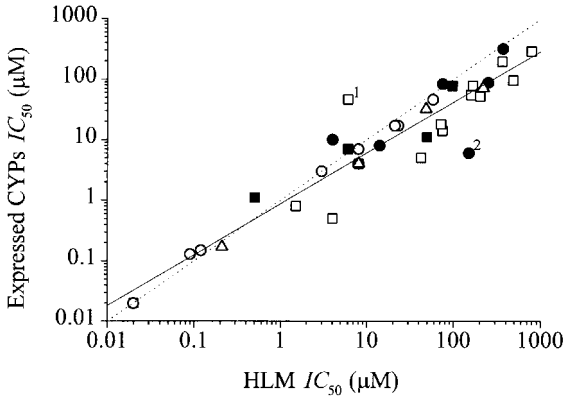

Figure 6. Comparison of IC_{50} determined in cDNA-expressed CYPs and HLM for a series of compounds. The data represent IC_{50} tabulated in tables 2–6 for the five different isoforms CYP1A2 (■), CYP2C9 (□), CYP2C19 (●), CYP2D6 (○) and CYP3A4 (△). For CYP2C9 and CYP2D6 (tables 3 and 5 respectively), 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-napthoflavone and (2) omeprazole.

obtained in HLM and different cloned CYP expression systems. Both α-napthoflavone (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_{50} 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 [^{14}C]-erythromycin *N*-demethylation for CYP3A4 (Zhang and Thomas 1996, Riley and Howbrook 1998), [^{14}C]-naproxen *O*-demethylation for CYP2C9 (Rodrigues *et al*. 1996) and [^{14}C]-dextromethorphan *O*-demethylation for CYP2D6 (Rodrigues *et al*. 1994). In addition [^{14}C]-diazepam *N*-demethylation was developed in our laboratory as a novel assay for CYP2C19 activity. For the radiometric assays $> 99.7\%$ of ^{14}C 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 ($\sim 10\%$ 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 $\sim 50\%$ 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 ($K_m \sim 3$ mM) and 1A2 (K_m—not determined) as well as the high affinity isoform CYP2C9 ($K_m \sim 300$ μM), 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 ($K_m = 1.8$ 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 ~ 5 and 90 μM respectively. The 90 μM 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 μM 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 μM in the two-point assay, IC_{50} between 0.5 and 200 μM 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 IC_{50} 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 IC_{50} 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 IC_{50} 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 IC_{50} determined for omeprazole in HLM compared with cDNA-expressed CYP2C19.

In contrast, α-naphthoflavone has a lower apparent IC_{50} 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 IC_{50} 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 enzymology 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 IC_{50} determined for a range of marker compounds in both expressed systems and HLM, in this study, with IC_{50} and/or K_i 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.

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References

Ahmad, S. R. and Wolfe, S. M., 1995, Cisapride and torsades de pointes. *Lancet*, 345, 508.

Andersson, T., Miners, J. O., Veronese, M. E. and Birkett, D. J., 1994, Diazepam metabolism by human liver microsomes is mediated by both *s*-mephenytoin hydroxylase and CYP3A isoforms. *British Journal of Clinical Pharmacology*, 38, 131–137.

Andersson, T., Miners, J. O., Veronese, M. E., Tassaneeyakul, W., Tassaneeyakul, W., Meyer, U. A. and Birkett, D. J., 1993, Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. *British Journal of Clinical Pharmacology*, 36, 521–530.

Aoyama, T., Korzekwa, K., Nagata, K., Gillette, J., Gelboin, H. V. and Gonzalez, F. J., 1990, Estradiol metabolism by complementary deoxyribonucleic acid-expressed human cytochrome P450s. *Endocrinology*, 126, 3101–3106.

Ayrton, J., Plumb, R., Leavens, W. J., Mallett, D., Dickins, M. and Dear, G. J., 1998, Application of a generic fast gradient liquid chromatography tandem mass spectrometry method for the analysis of cytochrome P450 probe substrates. *Rapid Communications in Mass Spectroscomy*, 12, 217–224.

Back, D. J., Tjia, J. F., Karbwang, J. and Colbert, J., 1988, *In vitro* inhibition studies of tolbutamide hydroxylase activity of human liver microsomes by azoles, sulphonamides and quinolines. *British Journal of Clinical Pharmacology*, 26, 23–29.

Baldwin, S. J., Bloomer, J. C., Smith, G. J., Ayrton, A. D., Clarke, S. E. and Chenery, R. J., 1995, Ketoconazole and sulfaphenazole as the respective selective inhibitors of P4503A and 2C9. *Xenobiotica*, 25, 261–270.

Bertz, R. J. and Granneman, G. R., 1997, Use of *in vitro* and *in vivo* data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clinical Pharmacokinetics*, 32, 210–258.

Bloomer, J. C., Clarke, S. E. and Chenery, R. J., 1995, Determination of P4501A2 activity in human liver microsomes using [3-^{14}C-methyl]caffeine. *Xenobiotica*, 25, 917–927.

Bloomer, J. C., Woods, F. R., Haddock, R. E., Lennard, M. S. and Tucker, G. T., 1992, The role of cytochrome P4502D6 in the metabolism of paroxetine by human liver microsomes. *British Journal of Clinical Pharmacology*, 33, 521–523.

Boobis, A. R., McKillop, D., Robinson, D. T., Adams, D. A. and McCormick, D. J., 1998, Interlaboratory comparison of the assessment of P450 activities in human hepatic microsomal samples. *Xenobiotica*, 28, 493–506.

Bourrie, M., Meunier, V., Berger, Y. and Fabre, G., 1996, Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalysed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics*, 277, 321–332.

Broly, F., Libersa, C., Lhermitt, M. and Dupuis, B., 1990, Inhibitory studies of mexiletine and dextromethorphan oxidation in human liver microsomes. *Biochemical Pharmacology*, 39, 1045–1053.

Brøsen, K., 1995, Drug interaction and the cytochrome P450 system. *Clinical Pharmacokinetics*, 29 **(suppl. 1), 20–25.**

Chang, T. K. H., Gonzalez, F. J. and Waxman, D. J., 1994, Evaluation of triacetyloleandomycin, α-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics*, 311, 437–422.

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

Ching, M. S., Blake C. L., Ghabrial, H., Wynne Ellis, S., Lennard, M. S., Tucker, G. T. and Smallwood, R. A., 1995, Potent inhibition of yeast-expressed CYP2D6 by dihydroquinidine, quinidine, and its metabolites. *Biochemical Pharmacology*, 50, 833–837.

Clarke, S. E., Ayrton, J. C. and Chenery, R. J., 1994, Characterization of the inhibition of P4501A2 by furafylline. *Xenobiotica*, 24, 517–526.

Cook, C. S., Hauswald, C., Oppermann, J. A. and Schoenhard, G. L., 1993, Involvement of cytochrome P4503A in the metabolism of potassium canrenoate to an epoxide: mechanism of inhibition of the epoxide formation by spiranalactone and its sulfur-containing metabolite. *Journal of Pharmacology and Experimental Therapeutics*, 266, 1–7.

Crespi, C. L., Miller, V. P. and Penman, B. W., 1997, Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Annals of Biochemistry*, 248, 188–190.

Crespi, C. L. and Penman, B. W., 1997, Use of cDNA-expressed human cytochrome P450 enzymes to study potential drug-drug-interactions. *Advances in Pharmacology*, 43, 171–188.

Dayer, P., Leemann, T. and Striberni, R., 1989, Dextromethorphan *O*-demethylation in liver microsomes as a prototype reaction to monitor cytochrome P450 db_1 activity. *Clinical Pharmacology and Therapeutics*, 45, 34–40.

Doecke, C. J., Veronese ,M. E., Pond, S. M., Miners, J. O., Birkett, D. J., Sansom, L. N. and McManus, M. E., 1991, Relationship between phenytoin and tolbutamide hydroxylations in human liver microsomes. *British Journal of Clinical Pharmacology*, 31, 125–130.

Ducharme, J., Abdullah, S. and Wainer, I. W., 1996, Dextromethorphan as an *in vivo* probe for the simultaneous determination of CYP2D6 and CYP3A activity. *Journal of Chromatography B*, 678, 113–128.

Fischer, V., Vogels, B., Maurer, G. and Tynes, R. E., 1992, The antipsychotic clozapine is metabolised by the polymorphic human microsomal and recombinant cytochrome P450 2D6. *Journal of Pharmacology and Experimental Therapeutics*, 260, 1355–1360.

Flockhart, D. A., 1995, Drug interactions and the cytochrome P450 system. *Clinical Pharmacokinetics*, **29(suppl. 1), 45–52.**

Fonne -Pfister, R. and Meyer, U. A., 1988, Xenobiotic and endobiotic inhibitors of cytochrome P450 db1 function, the target of the debrisoquine/sparteine type polymorphism. *Biochemical Pharmacology*, 37, 3829–3835.

Gascon, M.-P. and Dayer, P., 1991, *In vitro* forecasting of drugs which may interfere with the biotransformation of midazolam. *European Journal of Clinical Pharmacology*, 41, 573–578.

Gorski, J. C., Jones, D. R., Wrighton, S. A. and Hall, S. D., 1994, Characterisation of dextromethorphan *N*-demethylation by human liver microsomes: contribution of the cytochrome P450 3A subfamily. *Biochemical Pharmacology*, 48, 173–182.

Guengerich, F. P., Gillam, E. M. J., Ohmori, S., Sandhu, P., Brian, W. R., Sari, M. and Iwasaki, M., 1993, Expression of human cytochrome P450 enzymes in yeast and bacteria and relevance to studies on catalytic specificity. *Toxicology*, 82, 21–37.

Ha, H. R., Chen, J., Freiburghaus, A. U. and Follath, F., 1995, Metabolism of theophylline by cDNA-expressed human cytochromes P450. *British Journal of Clinical Pharmacology*, 39, 321–326.

Halliday, R. C., Jones, B. C., Smith, D. A., Kitteringham, N. R. and Park, B. K., 1995, An investigation of the interaction between halofantrine CYP2D6 and CYP3A4: studies with human liver microsomes and heterologous enzyme expression systems. *British Journal of Clinical Pharmacology*, 40, 369–378.

Honig, P. K., Wortham, D. C., Zamani, K., Conner, D. P., Mullin, J. C. and Cantilena, L. R., 1993, Terfenadine-ketoconazole interaction: pharmacokinetic and electrocardiographic consequences. *Journal of the American Medical Association*, 269, 1513–1518.

Jones, D. R., Gorski, C., Haehner, B. D., O'Mara, E. M. and Hall, S. D., 1996, Determination of cytochrome P450 3A4/5 activity *in vivo* with dextromethorphan *N*-demethylation. Clinical Pharmacology and Therapeutics, 60, 374–384.

Jacqz-Aigrain, E., Funck-Brentano, C. and Cresteil, T., 1993, CYP2D6 and CYP3A-dependent metabolism of dextromethorphan in humans. *Pharmacogenetics*, 3, 197–204.

Jung, F., Richardson, T. H., Raucy, J. L. and Johnson, E. F., 1997, Diazepam metabolism by cDNA-expressed human 2C P450s: identification of P4502C18 and P4502C19 as low K_m diazepam *N*-demethylases. *Drug Metabolism and Disposition*, 25, 133–139.

Kerry, N. L., Somogyi, A. A., Bochner, F. and Mikus, G., 1994, The role of CYP2D6 in primary and

secondary oxidative metabolism of dextromethorphan: *in vitro* studies using human liver microsomes. *British Journal of Clinical Pharmacology*, 38, 243–248.

Kerry, N. L., Somogyi , A. A., Mikus, G. and Bochner , F., 1993, Primary and secondary oxidative metabolism of dextromethorphan: *in vitro* studies with female Sprague-Dawley and dark agouti rat liver microsomes. *Biochemical Pharmacology*, 45, 833–839.

Ko, J.-W., Sukhova , N., Thacker , D., Chen , P. and Flockhart , D. A., 1997, Evaluation of omeprazole and lansoprazole as inhibitors of cytochrome P450 isoforms. *Drug Metabolism and Disposition*, 25, 853–862.

Kronbach , T., Mathys , D., Gut, J., Catin , T. and Meyer , U. A., 1987, High-performance liquid chromotographic assays for bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase and dextromethorphan *O*-demethylase in microsomes and purified cytochrome P450 isozymes of human liver. *Annals of Biochemistry*, 162, 24–32.

Kupfer , A., Schmid , B., Preisig , R. and Pfaff , G., 1984, Dextromethorphan as a safe probe for debrisoquine hydroxylation polymorphism. *Lancet*, ii, 517–518.

Mancy, A., Broto , P., Dijols , S., Dansette , P. M. and Mansuy , D., 1995, The substrate binding site of human liver cytochrome P4502C9: an approach using designed tienilic acid derivatives and molecular modelling. *Biochemistry*, 34, 10365–10375.

Mancy, A., Dijols , S., Poli , S., Guengerich , F. P. and Mansuy , D., 1996, Interaction of sulfaphenazole derivatives with human liver cytochromes P450 2C: molecular origin of the specific inhibitory effects of sulfaphenazole on CYP2C9 and consequences for the substrate binding site topology of CYP2C9. *Biochemistry*, 35, 16205–16212.

Miners , J. O., Coulter , S., Tukey , R. H., Veronese , M. E. and Birkett , D. J., 1996, Cytochromes P450, 1A2 and 2C9 are responsible for the human hepatic *O*-demethylation of *R*- and *S*-naproxen. *Biochemical Pharmacology*, 51, 1003–1008.

Newton , D. J., Wang, R. W. and Lu, A. Y. H., 1995, Cytochrome P450 inhibitors: evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition*, 23, 154–158.

Omura , T. and Sato , R., 1964, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry*, 239, 2370–2378.

Ono, S., Hatanaka , T., Hotta , H., Satoh , T., Gonzalez, F. J. and Tsutsui , M., 1996a, Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica*, 26, 681–693.

Ono, S., Hatanaka , T., Miyazawa , S., Tsutsui , M., Aoyama , T., Gonzalez, F. J. and Satoh , T., 1996b, Human liver microsomal diazepam metabolism using cDNA-expressed cytochrome P450s: role of CYP2B6, 2C19 and the 3A subfamily. *Xenobiotica*, 26, 1155–1166.

Pastrakuljic , A., Tang, B. K., Roberts , E. A. and Kalow , W., 1997, Distinction of CYP1A1 and CYP1A2 activity by selective inhibition using fluvoxamine and isosafrole. *Biochemical Pharmacology*, 53, 531–538.

Peck, C. C., Temple , R. and Collins , J. M., 1993, Understanding consequences of concurrent therapies. *Journal of the American Medical Association*, 269, 1550–1552.

Pichard , L., Fabre , I., Fabre , G., Domergue , J., Aubert , B. S., Mouard , G. and Maurel , P., 1990, Cyclosporin A drug interactions: screening for inducers and inhibitors of cytochrome P450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug Metabolism and Disposition*, 18, 595–606.

Pritchard , P. M., Glancey , M. J., Blake , J. A. R., Gilham , D. E., Burchell , B., Wolf , C. R. and Friedberg , T., 1998, Functional co-expression of CYP2D6 and human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Pharmacogenetics*, 8, 33–42.

Riley , R. J. and Howbrook , D., 1998, *In vitro* analysis of the activity of the major human hepatic CYP enzyme (CYP3A4) using [*N*-methyl ^{14}C]erythromycin. *Journal of Pharmacology and Toxicological Methods*, 38, 189–193.

Riley , R. J., Lambert , C., Cooper , A. E., Richmond , H., Hall , M., Jordan , M. C., Logan , C. J. and Clark , B., 1995, Induction of rodent hepatic drug-metabolizing enzyme activities by the novel anticonvulsant remacemide hydrochloride. *Drug Metabolism and Disposition*, 23, 922–928.

Rodrigues , A. D., 1997, Preclinical drug metabolism in the age of high throughput screening: an industrial perspective. *Pharmaceutical Research*, 14, 1504–1510.

Rodrigues , A. D., Kukulka , M. J., Roberts , E. M., Ouellet , D. and Rodgers , T. R., 1996, [*O*-methyl ^{14}C] naproxen *O*-demethylase activity in human liver microsomes: evidence for the involvement of cytochrome P4501A2 and P4502C9/10. *Drug Metabolism and Disposition*, 24, 126–136.

Rodrigues , A. D., Kukulka, M. J., Surber , B. W., Thomas , S. B., Uchic , J. T., Rotert , G. A., Michel , G., Thome -Kromer , B. and Machinist , J. M., 1994, Measurement of liver microsomal cytochrome P450 (CYP2D6) activity using [*O*-methyl ^{14}C] dextromethorphan. *Annals of Biochemistry*, 219, 309–320.

Rodrigues , A. D., Surber , B. W., Yao, Y., Wong , S. L. and Roberts , E. M., 1997, [*O*-ethyl ^{14}C] phenacetin *O*-deethylase activity in human liver microsomes. *Drug Metabolism and Disposition*, **25, 1097–1100.**

ROST, K. L., FUHR, U., ZAIGLER, M., BOHNEMEIER, H. and ROOTS, I., 1995, Omeprazole not only inducers but also inhibits CYP1A2 in man. In 9th International Conference on Cytochrome P450, P_{pol}-6, p. 47.

SCHMIDER, J., GREENBLATT, D. J., FOGELMAN, S. M., VON MOLTKE, L. L. and SHADER, R. I., 1997, Metabolism of dextromethorphan *in vitro*: involvement of cytochromes P450 2D6 and 3A3/4, with a possible role of 2E1. *Biopharmacology and Drug Disposition*, 18, 227–240.

SESARDIC, D., BOOBIS, A. R., MURRAY, B. P., MURRAY, S., SEGURA, J., DE LA TORRE, R. and DAVIES, D. S., 1990, Furafylline is a potent and selective inhibitor of cytochrome P4501A2 in man. *British Journal of Clinical Pharmacology*, 29, 651–663.

SHIMADA, T., YAMAZAKI, H., MIMURA, M., YUKIHARU, I. and GUENGERICH, F. P., 1994, Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics*, 270, 414–423.

TASSANEYAKUL, W., BIRKETT, D. J., VERONESE, M. E., MCMANUS, M. E., TUKEY, R. H. and MINERS, J. O., 1994, Direct characterisation of the selectivity of furafylline as an inhibitor of cytochromes P450 1A1 and 1A2. *Pharmacogenetics*, 4, 281–284.

TASSANEEYAKUL, W., BIRKETT, D. J., VERONESE, M. E., MCMANUS, M. E., TUKEY, R. H., QUATTROCHI, L. C., GELBOIN, H. V. and MINERS, J. O., 1993, Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2. *Journal of Pharmacology and Experimental Therapeutics*, **265, 401–407.**

TASSANEEYAKUL, W., MOHAMED, Z., BIRKETT, D. J., MCMANUS, M. E., VERONESE, M. E., TUKEY, R. H., QUATTROCHI, L. C., GONZALEZ, F. J. and MINERS, J. O., 1992, Caffeine as a probe for human cytochromes P450: validation using cDNA-expression, immunoinhibition and microsomal kinetic and inhibitor techniques. *Pharmacogenetics*, 2, 173–183.

TRACY, T. S., MARRA, C., WRIGHTON, S. A., GONZALEZ, F. J. and KORZEKWA, K. R., 1997, Involvement of multiple cytochrome P450s in naproxen *O*-demethylation. *European Journal of Clinical Pharmacology*, 52, 293–298.

UENG, Y.-F., KUWBARA, T., CHUN, Y.-J. and GUENGERICH, F. P., 1997, Cooperativity in oxidations catalysed by cytochrome P450 3A4. *Biochemistry*, 36, 310–381.

VAN MOLTKE, L. L., GREENBLATT, J. G., GRASSI, J. G., GRANDA, B. W., SCHMIDER, J., HARMATZ, J. S. and SHADER, R. I., 1998, Reevaluation of the specificity of dextromethorphan as an index substrate. *Clinical Pharmacology and Therapeutics*, 63, 227.

WATANABE, N., KAMEI, S., OHKUBO, A., YAMANAKA, M., OHSAWA, S., MAKINO, K. and TOKUDA, K., 1986, Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer. *Clinical Chemistry*, 32, 1551–1554.

WIENKERS, L. C., WURDEN, C. J., STORCH, E., KUNZE, K. L., RETTIE, A. E. and TRAGER, W. F., 1996, Formation of (*R*)-8-hydroxywarfarin in human liver microsomes: a new metabolic marker for the (*S*)-mephenytoin hydroxylase, P4502C19. *Drug Metabolism and Disposition*, 24, 610–615.

WU, D., OTTON, S. V., MORROW, P., INABA, T., KALOW, W. and SELLERS, E. M., 1993, Human hepatic cytochrome P450 2D6-like activity in nonhuman primates: catalytic characterization *in vitro*. *Journal of Pharmacology and Experimental Therapeutics*, 266, 715–719.

YAMAZAKI, H., INOUE, K., SHAW, P. M., CHECOVICH, 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.

YANG, C. S., PATTEN, C. J., ISHIZAKI, H. and YOO, J. S. H., 1991, Induction, purification and characterisation of cytochrome P450IIE. *Methods in Enzymology*, 206, 595–603.

YANG, T. J., SHOU, M., KORZEKWA, K. R., GONZALEZ, F. J., GELBOIN, H. V. and YANG, S. K., 1998, Role of cDNA-expressed human cytochromes P450 in the metabolism of diazepam. *Biochemical Pharmacology*, 55, 889–896.

YASUMORI, T., NAGATA, K., YANG, S. K., CHEN, L.-S., MURAYAMA, N., YAMAZOE, Y. and KATO, R., 1993, Cytochrome P450 mediated metabolism of diazepam in human and rat: involvement of human CYP2C in *N*-demethylation in the substrate concentration-dependent manner. *Pharmacogenetics*, 3, 291–301.

ZHANG, X.-J. and THOMAS, P. E., 1996, Erythromycin as a specific substrate for cytochrome P4503A isozyme and identification of a high-affinity erythromycin *N*-demethylase in adult female rats. *Drug Metabolism and Disposition*, 24, 23–28.

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.

ZOMORODI, K. and HOUSTON, J. B., 1996, Diazepam-omeprazole inhibition interaction: an *in vitro* investigation using human liver microsomes. *British Journal of Clinical Pharmacology*, 42, 157–162.

ZOMORODI, K. and HOUSTON, J. B., 1997, Selectivity of omeprazole inhibition towards rat liver cytochromes P450. *Xenobiotica*, 27, 49–58.