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High-throughput inhibition screening of major human cytochrome P450 enzymes using an in vitro cocktail and liquid chromatography–tandem mass spectrometry

Samuel A. Testino, Jr. [a,*], Gabor Patonay [b]

[a] *Bioanalytical Department, Solvay Pharmaceuticals, Inc., 901 Sawyer Road, Marietta, GA 30062, USA*
[b] *Department of Chemistry, Georgia State University, Atlanta, GA 30303, USA*

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Abstract

A method has been developed for the high-throughput inhibition screening of the major human cytochrome P450 (CYP) enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) using an in vitro substrate cocktail and liquid chromatography–tandem mass spectrometry (LC–MS-MS). A cocktail consisting of the selective substrates phenacetin (CYP1A2), tolbutamide (CYP2C9), omeprazole (CYP2C19), bufuralol (CYP2D6), and midazolam (CYP3A4) was incubated with human liver microsomes. The metabolic reactions were terminated with methanol containing dextrorphan as an internal standard. Following centrifugation, the supernatant was analyzed by LC–MS-MS employing a fast gradient. The concentrations of the substrate metabolites—paracetamol, 4-hydroxytolbutamide, 5-hydroxyomeprazole, 1′-hydroxybufuralol, and 1′-hydroxymidazolam—in each sample were determined by LC–MS-MS in a single assay. The method was validated by incubating known CYP inhibitors (furafylline, CYP1A2; sulfaphenazole, CYP2C9; *s*-mephenytoin, CYP2C19; quinidine, CYP2D6; and troleandomycin, CYP3A4) with the individual substrates they were known to inhibit and with the substrate cocktail. IC50s (μM) determined using the substrate cocktail were in good agreement with those obtained with individual substrates (furafylline, 2.9 vs. 2.0; sulfaphenazole, 0.75 vs. 0.72; *s*-mephenytoin, 170 vs. 180; quinidine, 0.17 vs. 0.24; troleandomycin, 2.6 vs. 3.2) and with previously reported values in the literature.
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1. Introduction

Cytochrome P450 (CYP) enzymes are heme-thiolate proteins that are responsible for the oxidative metabolism of a wide variety of xenobiotics. They comprise a superfamily of related

* Corresponding author. Tel.: +1-770-578-5832; fax: +1-770-565-3311
E-mail address: tony.testino@solvay.com (S.A. Testino, Jr.).

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enzymes that are grouped into families and subfamilies based on similarities in amino acid sequences. The five major human CYP enzymes responsible for the metabolism of xenobiotics are CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. It is estimated that these five CYP enzymes are responsible for approximately 99% of CYP-mediated drug metabolism [1]. The determination of the CYP enzymes responsible for the metabolism of new chemical entities (NCEs) and the identification of interactions with a specific CYP isozyme (e.g. inhibition of that isozyme) can aid in predicting clinical drug interactions. In vitro methods are commonly used to determine the CYP inhibitory potential of NCEs. A compound being evaluated is co-incubated with a known substrate for a specific CYP enzyme. The effect of the test compound on the metabolism of the substrate is then determined. The concentration of the substrate metabolite has most commonly been measured using HPLC with UV or fluorescence detection [2].

The increased flux of NCEs into drug discovery due to combinatorial chemistry and high-throughput screening techniques has placed an increased demand for speed and efficiency on the CYP inhibition screening methodologies. To this end, novel high-throughput liquid chromatography–mass spectrometry (LC–MS) [3–5] and fluorescence [6] assays have been developed. Recently, a substrate ‘cocktail’ strategy, in which a mixture of CYP substrates are added in a single human microsomal incubation and the metabolites of the substrates determined in a single assay by LC–MS, has been utilized [7–9].

We report here a method employing an in vitro substrate cocktail and liquid chromatography–tandem mass spectrometry (LC–MS-MS) for the high-throughput inhibition screening of the major human CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4). No post-incubation extraction or concentration procedures are necessary. Validation of the method using known CYP selective inhibitors is described.

2. Experimental

2.1. Chemicals

Phenacetin, tolbutamide, midazolam, paracetamol, phenylmethylsulfonyl fluoride (PMSF), butylated hydroxytoluene (BHT), Sigma Ultra grade potassium chloride, EDTA, HEPES, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, glycerol, and magnesium chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Bufuralol, 4-hydroxytolbutamide, 1′-hydroxybufuralol, and 1′-hydroxymidazolam were purchased from GenTest Corporation (Woburn, MA). Omeprazole and 5-hydroxyomeprazole were a kind gift from Kjell Andersson, Ph.D. of Astra Hässle (Mölndal, Sweden). Formic acid, GC-MS grade methanol (Burdick and Jackson), and UV grade acetonitrile (Burdick and Jackson) were purchased from VWR Scientific Products (Suwanee, GA). Bovine serum albumin and other materials for determining microsomal protein content were obtained from BioRad (Hercules, CA).

2.2. Isolation of hepatic microsomes

Human liver microsomes were prepared using a differential centrifugation method as follows: human livers rejected for transplant were received fresh from the International Institute for the Advancement of Medicine (Exton, PA) in less than 36 h post-clamp time or frozen from the Association of Human Tissue Users (Tucson, AZ). The liver tissue was minced and rinsed in a 1.15% (w/v) KCl solution. The tissue was weighed, washed in 3 volumes of a homogenization buffer (100 mM Tris acetate, 1 mM EDTA, 100 mM KCl, 20 μM BHT, pH 7.4) and then homogenized in a motorized homogenizer (Omni International, Atlanta, GA) with two 20 s bursts. The homogenate was centrifuged at approximately 10 000 × *g* for 30 min at 4 °C. The supernatant (s9) was transferred to fresh centrifuge tubes and then centrifuged at approximately 100 000 × *g* for 70 min at 4 °C. The pellet was washed once in 1.5 volumes of a buffer containing 100 mM tetrapotassium pyrophosphate, 1 mM EDTA, and 20 μM BHT (adjusted to pH 7.4 with 6 M HCl) by

resuspending and homogenizing in a glass dounce homogenizer. The resuspended pellet was centrifuged again at $100\,000 \times g$ for 70 min at 4 °C. The resulting pellet was resuspended in a storage buffer (10 mM Tris acetate, 1 mM EDTA, 100 μM PMSF, and 20% glycerol, pH 7.4) using the glass dounce homogenizer, aliquoted (1 ml) into screw-cap cryovials, and stored at −80 °C until use. Microsomal protein content was determined using the Bradford method (BioRad, Hercules, CA) [10].

2.3. Microsomal Incubations

Incubation mixtures (500 μl) contained HEPES buffer (50 mM HEPES, 15 mM $MgCl_2$, 0.1 mM EDTA, pH 7.6), an NADPH regeneration system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 IU glucose 6-phosphate dehydrogenase), hepatic microsomes (0.5 mg protein), inhibitor, and substrate or substrate cocktail (final concentration of 10 μM phenacetin, 100 μM tolbutamide, 10 μM omeprazole, 10 μM bufuralol, and 10 μM midazolam using 5 μl of a stock prepared in 50/50 (v/v) acetonitrile/water). After addition of inhibitor, the samples were pre-incubated for 4 min prior to addition of substrate or cocktail. Following a 20 min incubation at 37 °C, the reactions were terminated with 250 μl of methanol containing 5 μM dextrorphan to be used as an internal standard (IS). The samples were vortexed briefly, placed on ice for approximately 15 min, and centrifuged at $13\,000 \times g$ for 5 min. The supernatant from each sample was then transferred to a separate vial for LC–MS-MS analysis.

2.4. LC–MS-MS conditions

The samples were analyzed on a HP1100 Series Liquid Chromatograph (Hewlett-Packard, Palo Alto, CA) interfaced to a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer (ThermoQuest, San José, CA). An aliquot (15 μl) from each sample was injected onto a Phenomenex Luna C18(2) column (50 mm × 2.0 mm, 5 μ particle size). The flow rate was 1 ml/min. Mobile phase A was acetonitrile and mobile phase B was 0.1% (v/v) formic acid in water. Mobile phase A was linearly ramped from 0 to 50% in 3 min, held at 50% for an additional 0.5 min, and then immediately stepped back down to 0% for reequilibration. Total run time was 5.5 min. After 1.5 min, the LC eluent was diverted from waste to the mass spectrometer fitted with an electrospray ionization (ESI) source and operated in the positive ion mode. The LC flow was split so that approximately 150 μl/min entered the mass spectrometer. The needle voltage was set to 4.5 kV and the sheath and auxiliary gas flows to 80 and 20 (arbitrary units), respectively. The capillary heater temperature was maintained at 325 °C and the source manifold at 70 °C. The argon gas pressure in the collision cell was approximately 2 mTorr. For quantitation, the mass spectrometer was operated in the selected reaction monitoring (SRM) mode to monitor for metabolites of each substrate with the dwell time set to 0.1 s for each reaction.

2.5. IC50 determinations

Control samples (with no inhibitor) were assayed in each analytical run. The amount of metabolite in each sample (relative to the control samples) was plotted versus concentration of the inhibitor present. A sigmoid-shaped curve was fitted to the data and IC50s calculated using Prism (GraphPad Software, Version 3.02).

3. Results and discussion

3.1. Substrate selection

The structures of the substrates, metabolites, and IS utilized in the assay are shown in Fig. 1. The substrates to be used in the substrate cocktail were determined based on (1) their solubility in the solvent system chosen for the cocktail (50/50 (v/v) acetonitrile/water), (2) the sensitivity of their metabolites by electrospray LC–MS, and (3) their specificity for the CYP enzyme for which they were primarily metabolized.

A solvent system was required that would solubilize the substrates and minimally affect CYP metabolism. Many organic solvents (e.g. methanol, ethanol, DMSO) significantly inhibit

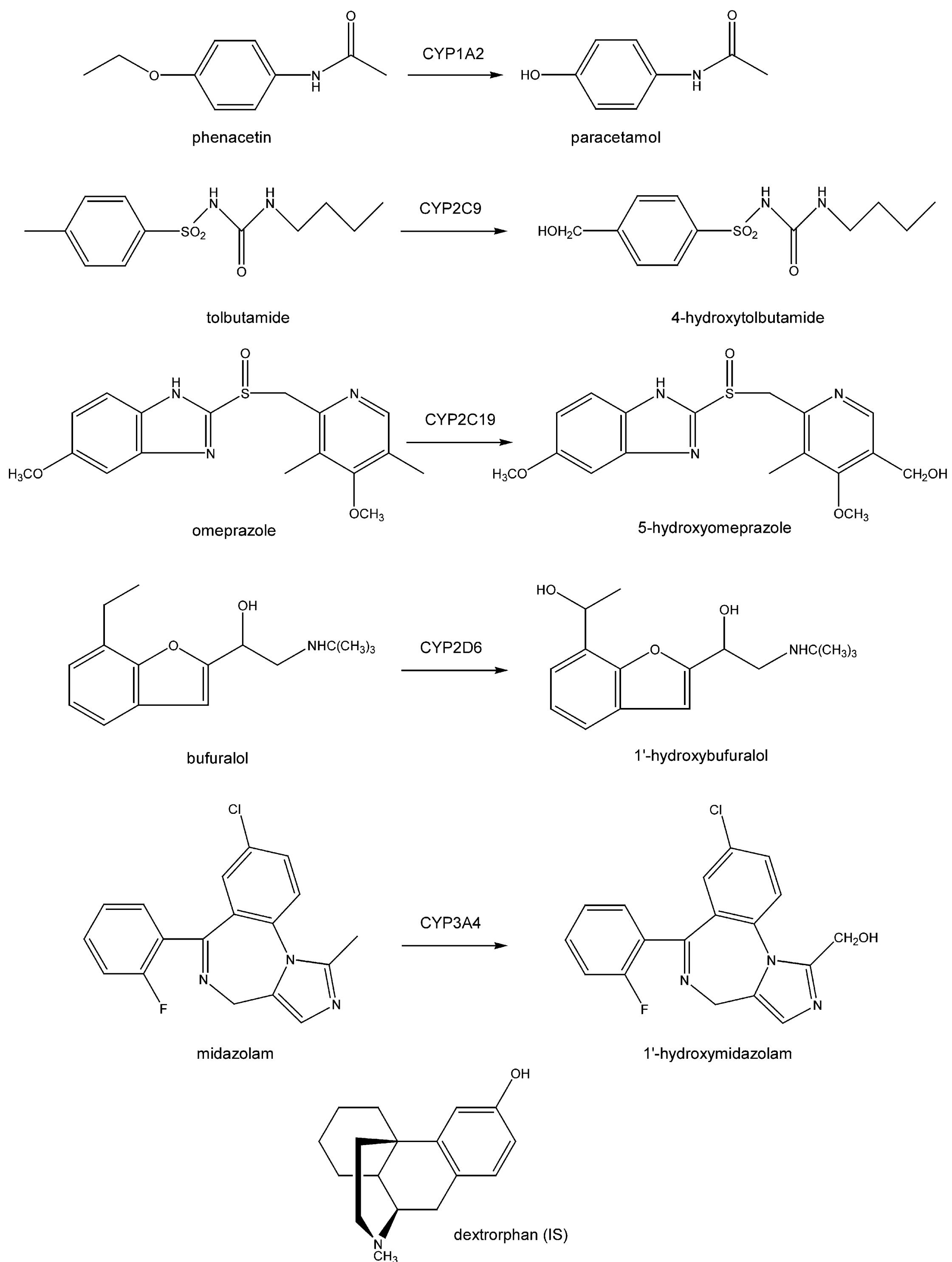

Fig. 1. Structures of substrates, metabolites, and IS.

CYP metabolism, even at low concentrations [11,12]. Acetonitrile, on the other hand, has been shown to have a small effect on the CYP enzymes being investigated at concentrations less than 1% (v/v) [13]. A mixture of 50/50 (v/v) acetonitrile/water (resulting in 0.5% (v/v) acetonitrile in the incubated samples) was found to satisfactorily dissolve a mixture of the substrates at concentrations (1–10 mM) necessary for the cocktail spiking solution.

In a previous CYP substrate cocktail method [9], *s*-mephenytoin was used as a CYP2C19 substrate. However, it is a low-turnover substrate and, using positive ion ESI, its 4-hydroxymephenytoin metabolite is not very sensitive. Using this methodology, the metabolite could not be detected in incubated samples unless a timely solvent evaporation procedure to concentrate the samples was employed. To avoid the need for post-incubation sample concentration, the utility of another CYP2C19 substrate, omeprazole, was investigated. CYP2C19 specifically catalyzes the hydroxylation of omeprazole to 5-hydroxyomeprazole. This metabolite was easily detectable by positive ion ESI with direct injection of the sample immediately following incubation. The good sensitivity of 5-hydroxyomeprazole, and the other substrate metabolites, obtained using positive ion ESI permitted the direct analysis of samples following incubation without the need for post-incubation extraction and/or concentration.

Once substrates with adequate solubility and MS sensitivity for each of the five major CYP enzymes were identified, their potential to inhibit other CYP enzymes was evaluated. The inhibition potential of the substrates was assessed by comparing the response for the metabolites in incubations of each single substrate to the response for the same metabolites formed in incubations with the substrate cocktail. Concentrations of the substrates were then adjusted so that inhibition of the CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) was minimized. The inhibition of each CYP enzyme was less than 20% at substrate concentrations in the final cocktail (10 μM phenacetin, 100 μM tolbutamide, 10 μM omeprazole, 10 μM bufuralol, and 10 μM midazolam; data not shown).

3.2. Method development

Positive ion ESI MS and MS/MS spectra were obtained for the metabolites of each of the substrates in the substrate cocktail and the IS, dextrorphan. The intensity of a selected product ion in the MS/MS spectrum of each compound was optimized by varying the MS/MS collision energy. The SRM transitions and optimum collision energies determined for each metabolite and IS are listed in Table 1.

Reconstructed SRM chromatograms from the analysis of a human liver microsomal sample incubated with the substrate cocktail are shown in Fig. 2. The retention times for paracetamol, 1′-hydroxybufuralol, dextrorphan (IS), 5-hydroxyomeprazole, 1′-hydroxymidazolam, and 4-hydroxytolbutamide were approximately 3.0, 3.4, 3.4, 3.7, 4.1, and 4.5 min, respectively. The specificity of the mass spectrometer allowed for a fast LC gradient to be employed. There were no interferences at the retention times of interest in any metabolite SRM channel from other substrates or metabolites.

The chromatography was optimized to adequately separate the 4- (RT 3.9 min) and 1′-

Table 1
Selected reaction monitoring parameters

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)
Paracetamol	152	110	22
4-hydroxytolbutamide	287	89	46
5-hydroxyomeprazole	362	214	14
1′-hydroxybufuralol	278	186	30
1′-hydroxymidazolam	342	297	25
Dextrorphan (IS)	258	157	44

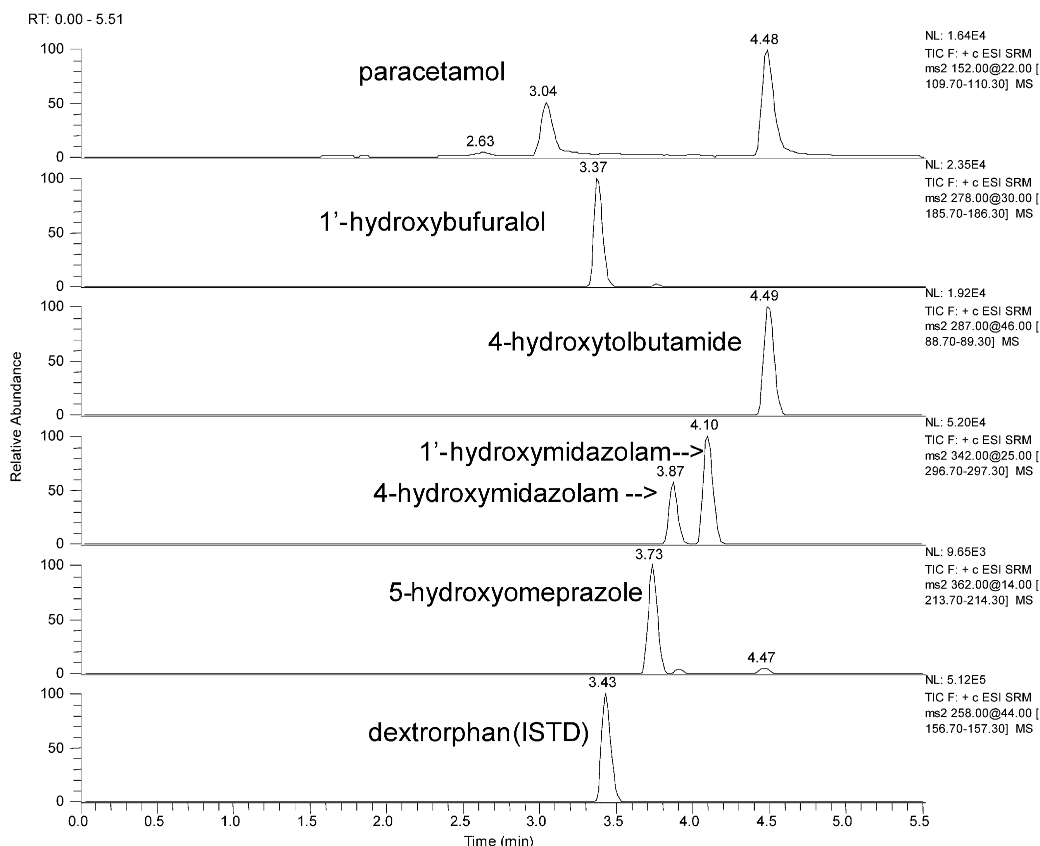

Fig. 2. Reconstructed SRM chromatograms from the analysis of a human liver microsomal sample incubated with the substrate cocktail.

hydroxylated (RT 4.1 min) metabolites of midazolam. Other methods have not shown separation of the two metabolites or only monitored the 1′-hydroxylated metabolite [7–9]. Midazolam, is, however, regioselectively hydroxylated by members of the CYP3A family (CYP3A4, CYP3A5, and fetal CYP3A7) with the ratio of the 1′- and 4-hydroxylated metabolites indicative of the CYP3A forms present [14]. The monitoring of both metabolites makes this assay useful for the study of these multiple CYP3A isozymes.

3.3. IC50 determinations of known CYP inhibitors

The method was validated by incubating known CYP inhibitors (furafylline, CYP1A2; sulfaphenazole, CYP2C9; *s*-mephenytoin, CYP2C19; quinidine, CYP2D6; and troleandomycin, CYP3A4) with the individual substrate they were known to inhibit and with the substrate cocktail. The inhibition curves obtained from these experiments are shown in Fig. 3. The best-fit *log IC50* value and associated standard deviation determined from the data are displayed on each graph. The IC50s calculated from these data are listed in Table 2. The ranges of previously reported IC50 values for each inhibitor are also shown in Table 2.

The IC50s determined with the individual substrates using this methodology were in good agreement with the range of previously reported values in the literature. In addition, the IC50s determined using the substrate cocktail also agreed with the literature values and with the values determined using the individual substrates. This

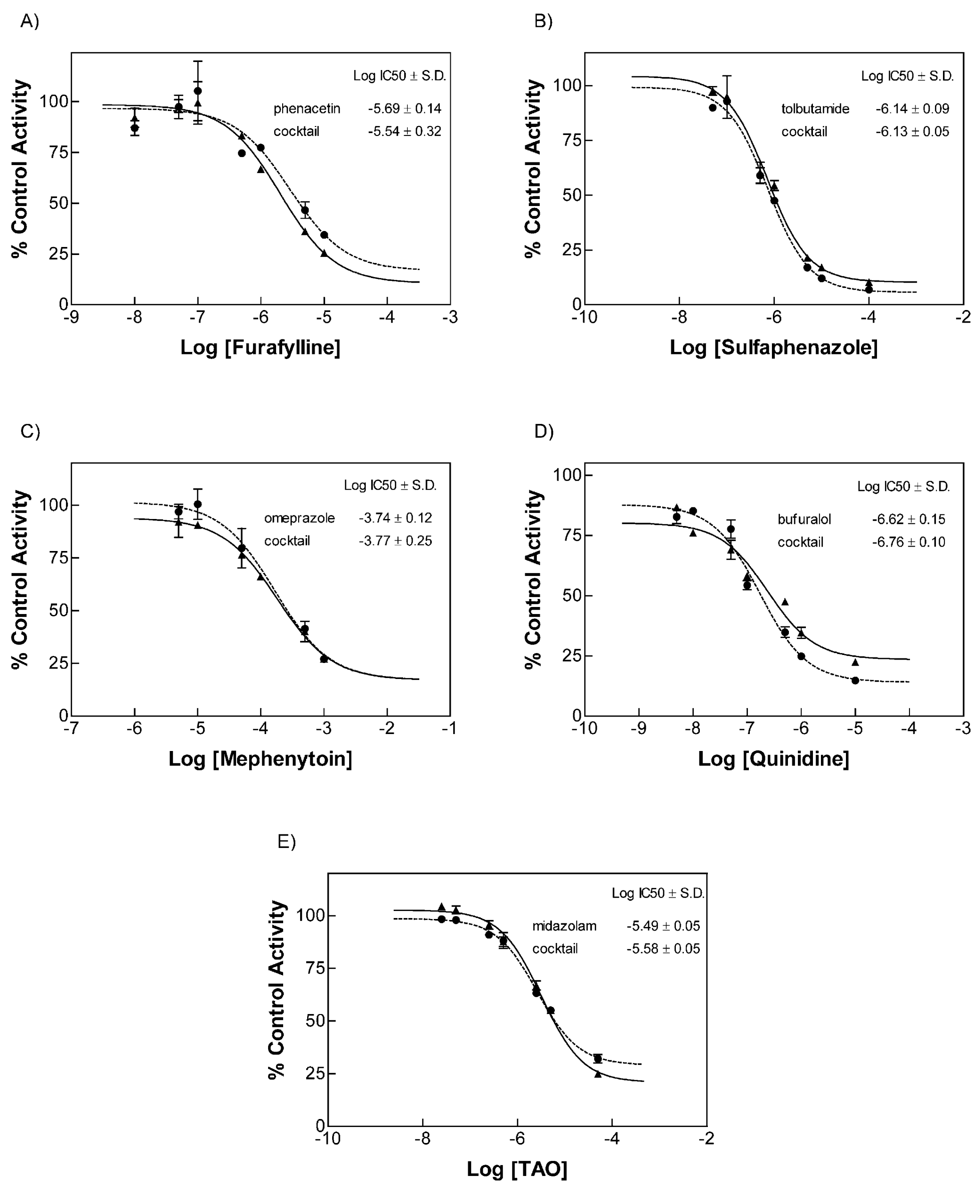

Fig. 3. Inhibition curves obtained using individual substrates and the substrate cocktail. Each inhibitor was incubated in separate experiments with a single CYP substrate (▲, –) or the substrate cocktail (●, ---). Each point is the mean of duplicate experiments. (A) Inhibition of phenacetin *O*-deethylation by furafylline, (B) inhibition of tolbutamide 4-hydroxylation by sulfaphenazole, (C) inhibition of omeprazole 5-hydroxylation by *s*-mephenytoin, (D) inhibition of bufuralol 1′-hydroxylation by quinidine, and (E) inhibition of midazolam 1′-hydroxylation by troleandomycin.

demonstrates that IC50s of CYP inhibitors can be accurately determined using the substrate cocktail instead of five separate individual substrate incubations, thus saving a tremendous amount of time in the NCE CYP inhibition screening process.

4. Conclusions

A method has been developed for the high-throughput inhibition screening of the major human CYP enzymes (CYP1A2, CYP2C9,

Table 2
Comparison of IC50s obtained using cocktail and individual substrate incubations

		IC50 (μM)		
CYP enzyme	Inhibitor	Single substrate	Cocktail	Literature
1A2	Furafylline	2.0	2.9	0.5–6 [15–18]
2C9	Sulfaphenazole	0.72	0.75	0.5–1.5 [15,16,19,20]
2C19	s-mephenytoin	180	170	162–250 [15,21]
2D6	Quinidine	0.24	0.17	0.02–0.22 [15,16,22]
3A4	Troleandomycin	3.2	2.6	1.96–8 [15,23,24]

CYP2C19, CYP2D6, and CYP3A4) using an in vitro substrate cocktail and LC–MS-MS. The sensitivity and selectivity of the mass spectrometer obviates the need for post-incubation extraction or concentration procedures and helps eliminate possible interferences from compounds evaluated using this methodology. The IC50s of selective CYP inhibitors (furafylline, CYP1A2; sulfaphenazole, CYP2C9; *s*-mephenytoin, CYP2C19; quinidine, CYP2D6; and troleandomycin, CYP3A4) determined using the substrate cocktail were in good agreement with those obtained with individual substrates and with previously reported values in the literature. The method has been utilized in our laboratory for the rapid determination of the CYP inhibition potential of NCEs, and is also useful for the characterization of CYP enzyme activity in human liver microsomal preparations and for the evaluation of the induction of CYP enzymes in in vitro systems (e.g. hepatocytes) by NCEs as well.

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