

HIGH-THROUGHPUT INHIBITION SCREENING OF FIVE MAJOR HUMAN CYTOCHROME P450 ENZYMES USING AN *IN-VITRO* SUBSTRATE COCKTAIL

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Introduction

One of the main reasons for the failure of a drug in preclinical and clinical trials is the ability of the drug to inhibit specific P450 enzymes and cause unwanted drug to drug interactions with concomitant medication.

Summary

A method has been developed for the high-throughput inhibition screening of the five major human cytochrome P450 enzymes using an *in-vitro* substrate cocktail and analysis by 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 dextrophan as an internal standard.

Following centrifugation, the supernatant was analysed by LC-MS/MS employing a fast gradient. The concentrations of the substrate metabolites – acetaminophen, 4-hydroxytolbutamide, 5-hydroxyomeprazole, 1-hydroxybufuralol and 1-hydroxymidazolam – in each sample were determined by LC-MS/MS.

The method was validated by incubating known CYP inhibitors (furylline, CYP1A2; sulfaphenazole, CYP2C9; tranlycypromine, CYP2C19; quinidine, CYP2D6 and troleandomycin, CYP3A4) with the individual substrates they are known to inhibit and with the substrate cocktail. IC₅₀ values (µM) determined using the substrate cocktail were in good agreement with those obtained with individual substrates and with previously reported values in the literature.

Method

Incubation mixtures contained HEPES buffer (50 mM HEPES, 15mM MgCl₂, 0.1mM EDTA, pH 7.6), an NADPH regeneration system (1mM NADPH, 10mM glucose-6-phosphate and 1 IU glucose-6-phosphatase), pooled human liver microsomes (0.5mg protein), inhibitor and substrate or substrate cocktail. Substrate cocktail final concentrations of 10µM phenacetin, 100µM tolbutamide, 10µM omeprazole, 10µM bufuralol and 10µM midazolam added to incubation samples using 5µL of a cocktail stock mix in 50/50 (v/v) acetonitrile/water.

After addition of inhibitor, the samples were pre-incubated for 4 minutes prior to addition of substrate or substrate cocktail.

Following a 20 minute incubation at 37°C, the reactions were terminated with 250µL of ice cold methanol containing 5µM dextrophan as an internal standard.

The samples were vortexed briefly and centrifuged at approximately 13000 x g for 5 minutes. The supernatant from each sample was then transferred to a deep well 96 well plate for LC-MS/MS analysis.

LC-MS/MS conditions

The samples were analysed on a Waters 2795 Alliance HT HPLC system interfaced with a Micromass Quattro Micro mass spectrometer. A 20µL aliquot of each sample was injected onto a Phenomenex MercuryMS Luna C18(2) column (3µM, 20 x 2.0mm) using the gradient conditions:-

The initial flow rate was 0.5mL/min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 100% acetonitrile.

Mobile phase B was linearly ramped from 0% to 50% in 3 minutes, held at 50% for 0.5 minutes then immediately stepped back down to 0% at a flow rate of 0.75mL/min for reequilibration. Total run time was 4.5 minutes.

Multiple reaction monitoring of the +ve substrate metabolite ions with specific collision product ions was monitored using Micromass MassLynx 3.5 software as in Table 1.

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 the concentration of the inhibitor present.

IC₅₀ values calculated using Prism GraphPad software, version 3.02.

Table 1. Multiple Reaction Monitoring of 6 mass pairs

Compound	Parent ion	Collision ion
Acetaminophen	152.02	110.04
Dextrophan (IS)	258.47	157.21
1'-hydroxybufuralol	278.40	186.20
4'-hydroxytolbutamide	287.21	88.90
1'-hydroxymidazolam	342.30	203.20
5'-hydroxyomeprazole	362.42	214.24

Results

Figure 1. Chromatograms of substrate metabolites with internal standard.

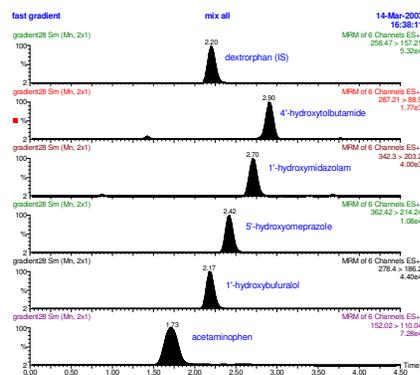
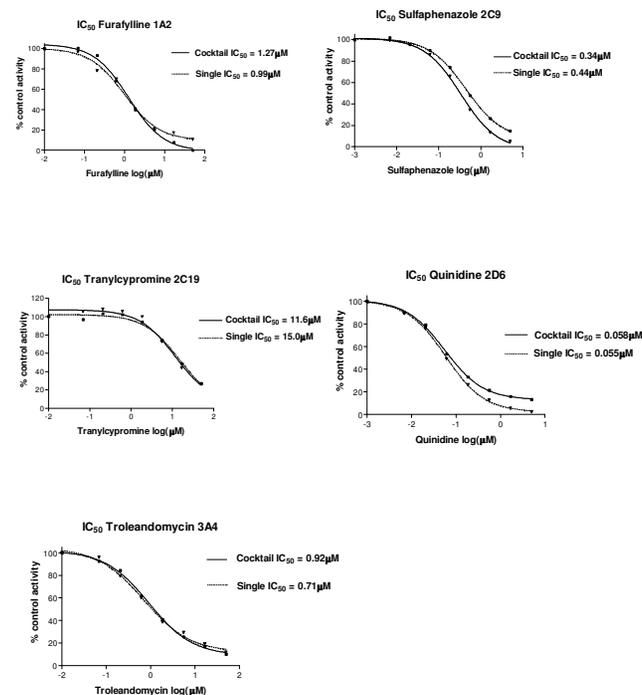


Figure 2. IC₅₀ values for inhibitors using single and cocktail substrate mix.



Conclusions

A method has been developed for the high-throughput inhibition screening of the major human CYP enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4.

The sensitivity and selectivity of the mass spectrometer negates the need for post-incubation extraction or purification of the metabolites produced.

The IC₅₀ values of selective CYP inhibitors determined using single substrates and the substrate cocktail mix are in good agreement with reported values in the literature. (Data not shown).

This method has been utilised in our laboratory for the rapid determination of the CYP inhibition potential of NCE's, and is also useful for the characterisation of CYP enzyme activity in human liver microsomal preparations and for the evaluation of the induction of CYP enzymes in *in vitro* systems.