Cocktail Analysis of CYP Inhibition using MS/MRM Technology and Recombinant Proteins

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Abstract

Detailed CYP inhibition profiles are now routinely required for the registration of novel molecular entities. Current enhanced throughput assay formats (radiometric or fluorescent analysis) may have limitations *e.g.* single enzyme analysis, potential for fluorescent interference and 'non-drug' substrates are often utilised.

This method uses a cocktail of substrates (phenacetin, diclofenac, S-mephenytoin, bufuralol and midazolam) and a pool of recombinant CYP enzymes (CYP1A2, 2C9, 2C19, 2D6 and 3A4) in an attempt to limit interactions between substrates and hepatic microsomal CYPs and associated reductases.

Kinetic analysis of the individual enzymes yielded apparent Km values of 25, 2, 20, 9 and 3 μM, respectively, in agreement with literature values. Selectivity of the substrates for each isoform was also demonstrated under the conditions chosen.

IC_{50} determinations with α-naphthoflavone (0.04 μM), sulfaphenazole (0.26 μM) tranylcypromine (9 μM), quinidine (0.04μM), and ketoconazole (0.01μM) were very similar for individual (sSsCYP) and pooled enzymes (cScCYP).

This validated method offers high throughput CYP inhibition profiling with advantages over current methods.

Abbreviations : sSsCYP = single substrate single CYP; cScCYP = combined substrates combined CYPs

Introduction

There has been a considerable increase in publications in recent years regarding the use of mass spectrometry for assessing drug/drug interactions *in vitro*[1,2,3]. This is partly due to technological advances in both the sensitivity of instruments and software improvements. Mass spectrometry may offer advantages over more traditional methods especially regarding throughput and 'ease of use'.

We describe our findings with single/pooled recombinant CYP enzymes with selective substrates and inhibitors.

Materials and Methods

E.Coli CYP isoforms expressing relevant CYPs and human reductase were purchased from CYPex.

Bufuralol (2D6), Diclofenac (2C9), S-mephenytoin (2C19), Midazolam (3A4), Phenacetin (1A2) and their respective metabolites were purchased from Ultrafine Chemicals.

Time and protein linearity experiments were performed with each recombinant CYP and their respective substrates at an assumed Km. Full Vmax/Km values were then determined both for single and combined isoforms.

Substrate selectivity was checked against each CYP isoform and protein concentrations were adjusted in the pooled experiments to minimise interferences.

Incubation conditions

Stop time = 10min (1:1 MeOH / Water)

Protein concentration 3A4,2C9,2D6 (5pmol/ml), 1A2 (15pmol/ml), 2C19 (2.5pmol/ml)

Incubation volume = 200μl

Samples were shaken, chilled at -20°C for 2h, spun at 3,500rpm for 15min and transferred to vials for analysis.

Analytical conditions

30μl of sample was injected onto a C30 column at 1.2ml/min – total run time = 2.5min. HPLC was performed with a Waters Alliance 2790 coupled to a triple quadrupole Quattro Ultima (Micromass) running in MRM mode (5 MRMs simultaneously - dwell time = 0.2s)

Results Enzyme Kinetics

Midazolam (3A4), and to some extent diclofenac (2C9), displayed substrate inhibition at higher concentration of substrates. This data was fitted using a one site Michaelis-Menten model with autoinhibition[4]. Generally, Km values did not change drastically for single substrate / single isoform vs. multi-substrate / 5 isoform. The velocity substrate plots for midazolam and bufuralol demonstrating non-classical and classical Michaelis Menten kinetics are given in figure 1.

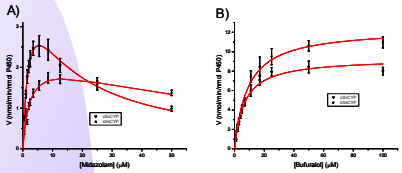

Figure 1 *velocity/substrate plots for A) midazolam and B) bufuralol sSsCYP and cScCYP*

Enzyme Inhibition

The IC_{50} values were determined for five selective probe inhibitors (quinidine (2D6), sulphaphenazole (2C9), tranylcypromine (2C19), ketoconazole (3A4), α-napthoflavone (1A2)) both with single isoforms and as a cocktail of all 5 isoforms.

The values obtained are given in table 1. To further validate the method, 13 literature compounds were screened at single isoforms (MS) and as a cocktail of all 5 isoforms (MS) (figure 2). The MS cocktail IC_{50} values were then compared with the traditional radiometric/fluorescent values (figure 2). Good correlations for both graphs were observed.

Further validation included screening in-house NCEs, incorporating a wide range of physicochemical properties. The data was plotted vs. the traditional radiometric/fluorometric values generated with E.Coli expressed CYPs (figure 3).

Inhibitor/rCYP Isoform	IC50 by MS (μM)	Literature IC50 (μM)
alpha-napthoflavone/1A2	0.042 +/- 0.012 (n=15)	0.035 +/- 0.020
Sulphaphenazole/2C9	0.26 +/- 0.08 (n=16)	0.57 +/- 0.17
Tranylcypromine/2C19	8.9 +/- 3.1 (n=16)	
Quinidine/2D6	0.040 +/-0.019 (n=16)	0.031 +/- 0.017
Ketoconazole/3A4	0.011 +/- 0.007 (n=16)	0.19 +/- 0.19

Table 1 *Generated IC_{50} values vs. literature values*

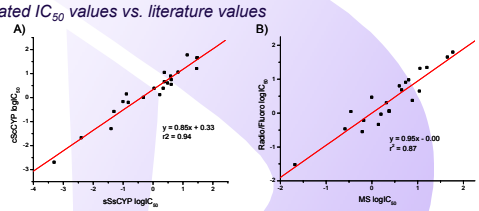

Figure 2 *IC_{50} comparisons between A) sSsCYP and cScCYP and B) cScCYP MS and sSsCYP Radiometric / fluorometric assays. NB, many data points could not be represented graphically as IC_{50}s > 50μM were observed for certain compound / isoform combinations in both sSsCYP and cScCYP*

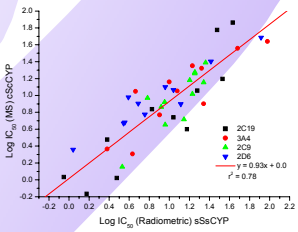

Figure 3 *cScCYP MS data vs. sSsCYP radiometric data for in-house NCEs NB data does not include CYP1A2*

Conclusion

- **Enzyme kinetics established for selective substrates with *E.coli* expressed 2D6, 2C9, 2C19, 3A4, 1A2 enzymes**
- **Comparison with traditional radiometric/fluorometric IC_{50} determinations validate approach for literature and in-house NCEs**

References

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