Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 92–99

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

An automated, high-throughput, 384 well Cytochrome P450 cocktail IC_{50} assay using a rapid resolution LC–MS/MS end-point

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ARTICLE INFO

Article history:
Received 26 February 2008
Received in revised form 1 May 2008
Accepted 9 May 2008
Available online 17 May 2008

Keywords:
Cytochrome P450
Drug–drug interactions
High-performance liquid chromatography
Human liver microsomes

ABSTRACT

The current study focused on the development of an automated IC_{50} cocktail assay in a miniaturized 384 well assay format. This was developed in combination with a significantly shorter high pressure liquid chromatography (HPLC) separation and liquid chromatography–mass spectrometry (LC–MS/MS) run-time; than those currently reported in the literature. The 384-well assay used human liver microsomes in conjunction with a cocktail of probe substrates metabolized by the five major CYPs (tacrine for CYP1A2, diclofenac for CYP2C9, (S)-mephenytoin for CYP2C19, dextromethorphan for CYP2D6 and midazolam for CYP3A4). To validate the usefulness of the automated and analytical methodologies, IC_{50} determinations were performed for a series of test compounds known to exhibit inhibition across these five major P450s. Eight compounds (sertraline, disulfuram, ticlopidine fluconazole, fluvoxamine, ketoconazole, miconazole, paroxetine, flunitrazepam) were studied as part of a cocktail assay, and against each CYPs individually. The data showed that the IC_{50}s generated with cocktail incubations did not differ to a great extent from those obtained in the single probe experiments and hence unlikely to significantly influence the predicted clinical DDI risk. In addition the present method offered a significant advantage over some of the existing cocktail analytical methodology in that separation can be achieved with run times as short as 1 min without compromising data integrity. Although numerous studies have been reported to measure CYP inhibition in a cocktail format the need to support growing discovery libraries not only relies on higher throughput assays but quicker analytical run times. The current study reports a miniaturized high-throughput cocktail IC_{50} assay, in conjunction with a robust, rapid resolution LC–MS/MS end-point offered increased sample throughput without compromising analytical sensitivity or analyte resolution.

1. Introduction

Over the past decade there has been a significant increase in the productivity of chemistry departments in pharmaceutical companies; enabling the production of thousands of new chemical entities (NCE) for *in vitro* screening. Consequently, there is an increasing demand to profile more *in vitro* absorption, distribution, metabolism and excretion (ADME) and physicochemical properties of these newly synthesized compounds early during a discovery program [1–3]. This can result in a demand upon *in vitro* groups to implement innovative, automated and high-throughput technologies; in order to provide the discovery project scientists ADME data in a more rapid manner, without compromising on quality. A variety of different approaches and automated platforms have been developed and ADME assays have been adapted to operate at relatively high capacities [1]. The use of high-throughput liquid chromatography–mass spectrometry (LC–MS) has accelerated the development of ADME assays in recent years and can be configured in micro-plate formats established in high-throughput screening [4].

Amongst the ADME assays to which this approach is being increasingly applied are those which assess the potential of new candidates to be victims or perpetrators of drug–drug interactions (DDIs). Knowledge of DDIs has become a part of the process of enabling new drugs to be introduced to the market. Any DDI associated with a compound, is likely to give it a potential competitive disadvantage, leading to labelling restrictions and in extreme cases can lead to the regulatory authorities refusing drug approval or in market withdrawal, as had been the case for terfenadine and ketoconazole [5], tegafur and sorivudine [6], and mibefradil with several cardiovascular drugs [7]. The majority of DDIs result from perturbation of the Cytochrome P450 (CYP) enzyme system, with inhibition being the major reason for this type of interaction. Cytochrome P450s 1A2, 2C9, 2C19, 2D6 and 3A4 have been shown to account for

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doi:10.1016/j.jpba.2008.05.011

the majority of DDIs [8,9], however appropriate attention should be paid to the roles played by CYPs 2B6, 2C8 and 3A5 [9].

Whilst miniaturization and automation using advanced liquid handling technologies can achieve efficiency gains in conducting DDI screens using drug probes, this gain is limited by the speed of quantification. A single LC–MS/MS methodology has been established for determination of CYP inhibitory potential using human liver microsomes (HLMs) [10]. However, over recent years, the development of cocktail biology (assessment of several isozymes simultaneously) has necessitated the design of cocktail analytical assays with appropriate throughput and sensitivity to determine a test compound's CYP inhibitory potential [11–24]. A drawback with a number of these studies are their limitations in assay design, such as use of recombinant CYPs, clinically irrelevant probe substrates, high protein content potentially leading to unspecific protein binding, but also analytically with some procedures requiring sample preparation and/or longer run times.

The current study focused on the development of an automated IC_{50} cocktail assay in a miniaturized 384 well assay format. This was developed in combination with a significantly shorter HPLC separation and LC–MS/MS run-time; than those currently reported in the literature. Since its recent introduction in the scientific arena by MacNair et al. [25,26], ultra-high pressure liquid chromatography (UHPLC) is experiencing continuous growth due to the benefits in separation power and speed of analysis over the traditional HPLC and the technique combined with mass spectrometric detection has been successfully used for the bioanalysis of small molecule drug candidates in plasma [27]. This type of HPLC separation was utilized in the analysis of the DDI cocktail IC_{50} assay samples monitoring a cocktail of the 5 main CYPs probes (and their associated internal standards, IS) in acetonitrile protein crashed microsomes. To validate the usefulness of the automated and analytical methodologies, IC_{50} determinations were performed for a series of test compounds known to exhibit inhibition across the five major P450s were compared between the cocktail mix and individual substrate approaches.

2. Materials and methods

2.1. Materials

Potassium phosphate buffer, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), DL-isocitric acid trisodium salt, isocitric dehydrogenase from porcine heart, sertraline, disulfuram, ticlopidine and tacrine hydrochloride were purchased from Sigma–Aldrich Company Ltd. Magnesium chloride was purchased from BDH (Poole, UK), organic solvents were obtained from Romil Ltd. (Cambridge, UK). (S)-Mephenytoin, diclofenac, midazolam, fluconazole, fluvoxamine, ketoconazole, miconazole, paroxetine, flunitrazepam, D^3-4-hydroxymephenytoin, D^3-dextrorphan and ^{13}C6-4-hydroxydiclofenac were synthesized and purified at Pfizer Global Research and Development. Pooled human liver microsomes (HLMix-101) were obtained from BD-Gentest, Inc. (Woburn, MA). All probes and substrates were prepared in methanol (10 and 30 mM, respectively) and subsequently diluted to appropriate working solutions in 80:20 (v/v) water:methanol.

2.2. In vitro IC_{50} determinations: automated procedure

All IC_{50} determinations were performed using a MicroL-STAR Autoload with 8 channels and a 96 Head (Hamilton Robotics, UK). Test compounds were diluted to 3 mM in 80:20 (v/v) water:methanol. Compounds were manually transferred to a 384 micro-titre polypropylene plate. The automated procedure was used to dilute compounds in 80:20 (v/v) water:methanol to 600, 200, 60, 20, 6, 2, 0.6, and 0 μM.

Incubation mix (pH 7.4) was prepared using (values in parenthesis represent final concentrations); potassium phosphate buffer (50 mM), magnesium chloride (5 mM), isocitric acid (5 mM), isocitric acid dehydrogenase (1 Unit/ml), water and HLMix-101 (0.1 mg/ml). An automated procedure was used to prepare the reaction mixtures. The final incubation volume was 50 μl/well, containing incubation mix, substrate(s), inhibitor (30, 10, 3, 1, 0.3, 0.1, 0.03 and 0 μM, final organic content in the incubation was 1%) and NADPH (1 mM). Controls wells were prepared for each substrate, containing no NADPH, no substrate, and test compound with and without NADPH. Reagents removed from the reaction were replaced with assay buffer. Reducing equivalents required for P450 metabolism were provided by NADPH which was regenerated *in situ* using an isocitric acid/isocitric acid dehydrogenase system. Incubations were pre-heated for 10 min at 37 °C prior to the addition of the NADPH to initiate the reactions. Following incubations for 10 min at 37 °C, reactions were terminated using acetonitrile containing appropriate internal standards mix (0.25 μg/ml fluconazole (CYP1A2), 25 μg/ml flunitrazepam (CYP3A4), 0.25 μg/ml D^3-4-hydroxymephenytoin (CYP2C19), 0.005 μg/ml D^3-dextrorphan, 0.2 μg/ml (CYP2D6) and ^{13}C6-4-hydroxydiclofenac (CYP2C9)). The design of the automated procedure allowed simultaneous assessment of IC_{50}s for up to 16 compounds in duplicate.

2.2.1. Comparison of single substrate with substrate cocktail IC_{50}s

Incubations for single substrates and substrate cocktail mix were prepared using the same stock substrate solutions and run at the literature K_m (Table 1). The IC_{50}s for each test compounds was determined on 5 separate days against each individual P450 and the cocktail mixture.

2.3. LC–MS/MS conditions for quantification

HPLC conditions consisted of a very fast gradient over 0.60 min using 95% water 5% acetonitrile with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) as mobile phases at a flow rate of 1 ml/min. HPLC gradient program used was as follows: (1) mobile phase B was at 3% at 0 min, (2) a linear gradient was run to 90% B 0.6 min, (3) solvent composition was returned to 3% B in 0.1 min for re-equilibration for 0.2 min. A Phenomenex Synergi Fusion High pressure HPLC column, 2.0 mm × 20.0 mm, 2.5 μm particle size was used for chromatographic separation. Samples were injected using a CTC-PAL autosampler, fitted with a 6-port Rheodyne high pressure injection valve (stable to 15,000 psi, special plating of titanium, biocompatible), high pressure 10 μl sample loop, active wash station and refrigerated micro-plate stacks compatible for injection from 96 and 364 well micro-plates. The CTC autosampler was used in conjunction with a Jasco XLC 3185PU high pressure, low dead volume, binary gradient pump, Jasco XLC 3067CO column oven

Table 1
CYP DDI probe substrates used in the cocktail DDI assay for both single substrate and the cocktail assay

Enzyme	Cytochrome P450 probe substrates	Metabolite	K_{mapp} (μM), literature
CYP1A2	Tacrine	1′-Hydroxytacrine	2 [37]
CYP2C9	Diclofenac	4′-Hydroxydiclofenac	5 [10]
CYP2C19	(S)-Mephenytoin	4′-Hydroxymephenytoin	40 [10]
CYP2D6	Dextromethorphan	Dextrorphan	5 [10]
CYP3A4	Midazolam	1′-Hydroxymidazolam	2 [10]

Table 2
LC–MS/MS MRM conditions for the individual cocktail probe metabolites and associated internal standards (IS)

Isozyme	Analyte	Precursor ion > product ion mass (*m*/*z*)	Declustering potential (eV)	Collision energy (eV)	Dwell time (ms)
CYP1A2	Tacrine	152 > 110	50	30	15
	Fluconazole (IS)	307 > 220	30	25	15
CYP2C9	4OH-Diclofenac	312 > 230.1	45	45	15
	^{13}C6-4OH-Diclofenac (IS)	318.2 > 237.1	56	30	15
CYP2C19	4OH-Mephenytoin	235.1 > 150.1	45	27	15
	D3 4OH-Mephenytoin (IS)	238.2 > 150.1	50	25	15
CYP2D6	Dextrorphan	258.2 > 157.1	90	53	15
	D3-dextrophan (IS)	261.2 > 157.1	65	53	15
CYP3A4	1OH-Midazolam	342.1 > 168.1	65	53	15
	Flunitrazepam (IS)	314.2 > 268.1	90	40	15

and Jasco XLC 3080DG degasser. Sample injection volume was 5 μl using a sandwich technique. Column temperature was set at 45 °C to reduce column back pressure. Two wash solvents were used in the injection port; 100% methanol and 95% water 5% acetonitrile with 0.1% formic acid was used to wash the syringe (10 μl capacity) and injection port after each sample injection. The aqueous injection wash solvent was also used to sandwich the sample in the injection syringe prior to injection onto the HPLC system. The active wash station ensured low carry over; enabling very fast washing of high organic and then aqueous injection solvents. An Applied Biosystems/Sciex API 4000 QTRAP mass spectrometer was operated in the positive ionization mode controlled by Analyst 1.4 software using the following conditions: curtain gas, 10.00; CAD gas, 7.00; GS1, 50; GS2, 50; ion spray, 5000 eV; temperature, 450 °C; EP, 10.00 eV. The mass spectrometer was operated under unit resolution (Q1 and Q3). LC–MS/MS MRM conditions for the individual cocktail probe metabolites and associated internal standards are given in Table 2. These conditions were optimized individually by infusion. Importantly, the dwell times are reduced to 15 ms due to the very sharp eluting analyte peaks and the requisite data points required across a peak for accurate quantification. Isotopically labelled internal standards were chosen to correct (where possible) for errors in the methodology and also correct for any ion suppression from the matrix (see Section 2.2).

2.4. Method validation

Four-day validations were undertaken to assess the LC/MS/MS robustness for the simultaneous quantification of ten P450-probe metabolites and respective internal standards. Calibration standards were prepared at 8 concentrations ranging from 0.01 to 10 μM, in blank microsomal incubation matrix. Quality control samples were prepared separately at three concentrations (0.1, 0.5 and 1 μM). These samples were used to assess the accuracy and precision of the method.

2.5. Data analyses

2.5.1. IC_{50} calculations

The IC_{50} values for CYP inhibitors were estimated from the data by fitting a standard 4 parameter logistic using non-linear regression as implemented in Thermo Galileo LIMS™, an integrated informatics solution designed specifically for *in vitro* ADME/Tox profiling software. In this equation, Range is the fitted uninhibited value minus the Background, and *s* is a slope factor. The equation assumes that *y* decreases with increasing *x*.

$$y = \frac{\text{Range}}{1 + (x/\text{IC}_{50})^s} + \text{Background}$$

2.5.2. Statistical analyses

The aim of the statistical analysis is to compare the cocktail and individual methods both in terms of their average IC_{50} for each enzyme and their overall precision. In addition the effect of increasing the number of replicates on the precision of the overall IC_{50} estimate is investigated. Details of the statistics used are presented alongside the results in the Section 3.3.

3. Results

3.1. HPLC–MS/MS quantification

Low volume injection of 1–5 μl was achieved using a novel "sandwich injection" technique which allowed reproducible introduction of biofluid extracts and also band focusing of the analytes (Fig. 1). For the production assays samples were analysed using the XLC gradient with an injection volume of 5 μl. This illustrates retention CYP probes and an eloquent separation of the analytes in under 30 s; including polar and multi-metabolite products. Importantly, there is also a sensitivity gain for S-mephenytion, the most analytically challenging probe of the cocktail mixture (Fig. 1). The small sample volume together with this separation ensured that all the analytes were separated from the solvent front and any endogenous interference from the matrix.

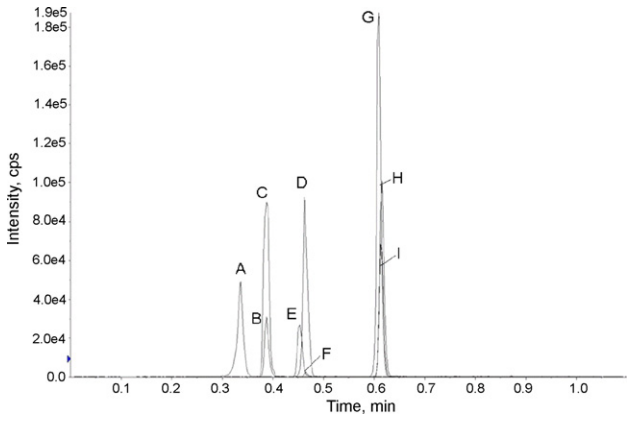

Fig. 1. Analytical trace from DDI cocktail screen analysed using XLC gradient conditions (insert shows response for S-mephenytoin in no inhibitor sample). (A) 1-OH tacrine; (B) D^3-dextrophan; (C) dextrophan; (D) 1-OH midazolam; (E) D^3-4-OH mephenytoin; (F) 4-OH mephenytoin; (G) flunitrazepam; (H) ^{13}C6-4-OH diclofenac; (I) 4-OH diclofenac.

Table 3
Performance of LC–MS/MS assay

Analyte	0.1 μM		0.5 μM		1.0 μM	
	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)
Tacrine	98.3	6.5	99.3	7.6	101.2	6.6
Fluconazole	94.5	9.7	110.0	8.1	97.6	7.5
4OH-diclofenac	104.6	4.3	89.6	5.6	293.4	7.5
^{13}C6-4OH-diclofenac	99.7	3.2	92.6	9.3	88.4	9.8
4OH-mephenytoin	89.2	8.5	112.6	10.2	91.9	10.2
D^3 4OH-mephenytoin	93.1	10.2	97.2	13.2	92.5	13.2
Dextrorphan	105.9	12.6	102.3	14.7	104.3	9.8
D^3-dextrophan	110.2	9.8	99.4	10.2	110.6	10.3
1OH-midazolam	96.3	4.5	105.4	7.6	88.4	11.2
Flunitrazepam	103.2	6.7	101.2	9.2	99.0	8.9

3.2. Analytical validation

The current method was validated, although its use is intended for screening-type analysis by solely comparing the corresponding peak areas in cocktail samples with different inhibitors. The results are shown in Table 3. Accuracies (percentage of metabolite concentrations measured relative to the known amount) for the low, middle and high QC's were within 88–113%. Precision was calculated as the relative standard deviation (R.S.D.%) and was found to be less than 15%. Sensitivity was not investigated as P450 probe turnover to their respective metabolites was sufficient to allow easy detection by LC/MS/MS.

3.3. Assay validation

3.3.1. Data transformation

A plot of the raw data is give in Fig. 2. As is generally found the IC_{50}s from the fitted 4-parameter logistic curves closely followed a log-normal distribution and so they were analysed on the logarithmic scale as this transformation stabilises the variance across the range of IC_{50}s observed.

3.3.2. Dealing with censored data

For each enzyme, compounds where the majority of IC_{50} results were recorded as greater than 30 μM or less than 0.03 μM were excluded from the analyses. This condition excluded both cocktail and individual methods for each applicable compound in almost all cases except for CYP3A4 fluconazole. Thus for the compounds excluded due to IC_{50}s being close to, or out of, the concentration range there was, in general, good agreement between the two assay methods. For the CYP3A4 fluconazole results all cocktail IC_{50}s were greater than 30 μM whilst the individual probe IC_{50}s ranged from 21.0 to 28.8 μM.

3.3.3. Comparing precision between the methods

The first step was to verify that the variation within each compound was constant for each of the methods. Levenes test [28] for

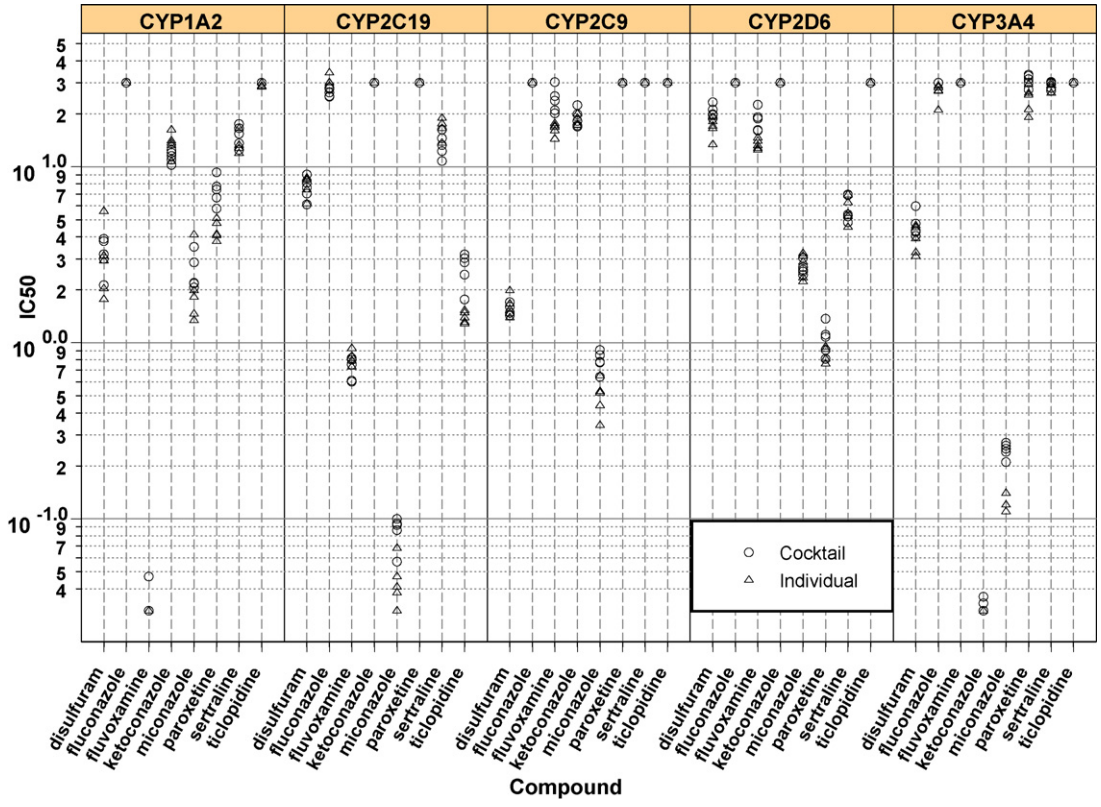

Fig. 2. Scatter plot of raw data, showing IC_{50} on the log scale against compound for each CYP enzyme with different symbols identifying assay method.

Table 4
F-tests results of comparing the variability between the cocktail and individual probe assay

Assay	Standard deviation of log IC_{50} cocktail	Standard deviation of log IC_{50} individual	*F*-test *p*-values (cocktail against individual)
CYP1A2	0.079	0.130	0.016
CYP2C9	0.054	0.065	0.25
CYP2C19	0.075	0.072	0.42
CYP2D6	0.062	0.058	0.37
CYP3A4	0.045	0.059	0.11

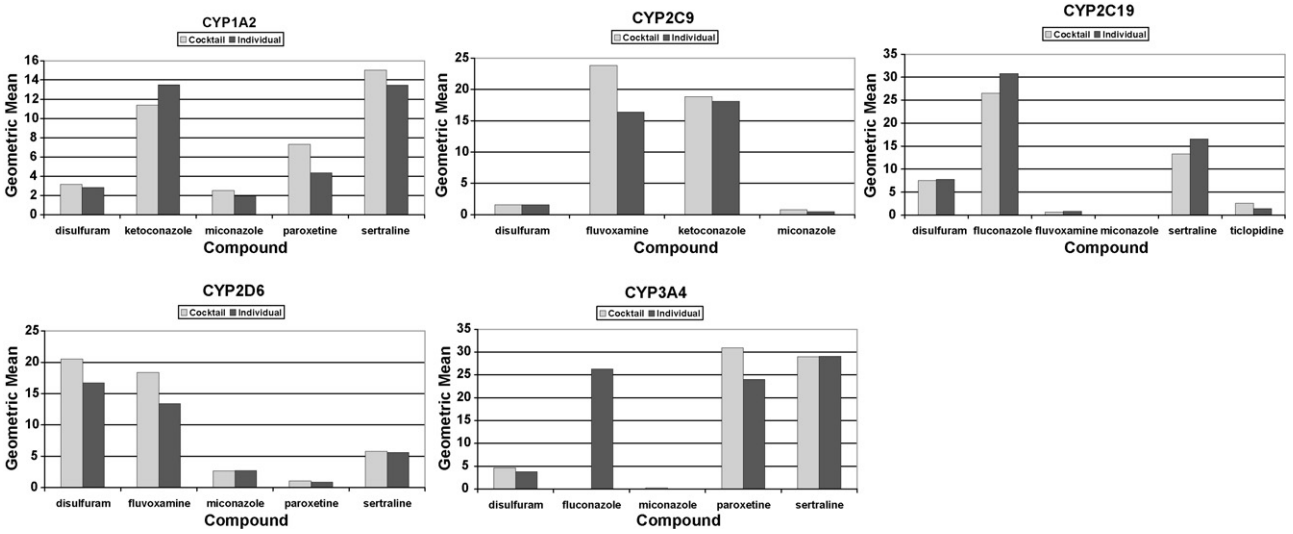

Fig. 3. Geometric mean IC_{50}s for each CYP enzyme.

differences in the variation showed no significant differences in the variability between compounds for either method for any of the enzymes. Thus it is reasonable to pool the variance across compounds to get a single estimate of variance for each method for each of the enzymes. Within each enzyme an *F*-test was used to compare the variance obtained for the cocktail method with that obtained from the individual probe method. The variance of the individual probe results for CYP1A2 was significantly higher than the variance of the cocktail results at the 5% level. For the other CYP enzymes there was no significant evidence of a difference in the assay precision for the two methods at the 5% level (Table 4).

3.3.4. *Comparing mean IC_{50} levels between the methods*

An analysis of variance (ANOVA) was used to look for differences in the mean level of IC_{50} (using a Satterthwaite adjustment [29] to allow for unequal variances in the case of CYP1A2). Note that as we are analysing the IC_{50} on the logarithmic scale then results are naturally displayed as geometric means and ratios of geometric means. Geometric means and 95% confidence intervals are given in Table 5 and displayed graphically in Fig. 3. Estimates of the true ratio of the geometric means from the two assay methods, together with 95% confidence limits, are given for each compound and for each CYP enzyme in Table 6. The 95% confidence limits may be thought of as a range in which the ratio of the true IC_{50}s from the two methods is likely to lie. Thus if the limits include a value of 1 (i.e., the true IC_{50}s from the two methods are equal) then there is no evidence to suggest the methods yield different results. This approach is equivalent to performing a significance test at the 5% level. In our case 10 of the 11 mean ratios were significantly greater than 1, though there was no evidence to suggest a trend within enzyme or compound. There is thus some evidence of an upward bias in the cocktail results for some of the compounds.

3.3.5. *Determining the variation associated with increased replication*

The observed variability between the $\log_{10}$ IC_{50}s has been used to determine the precision of estimates of the 'true' IC_{50} based on different numbers of replicates. For all but CYP1A2 the precision of the individual probe and cocktail methods was similar and had been combined. Fig. 4 shows how the precision of the estimate of IC_{50} varies when taking means across various numbers of repeat experiments. The variation in the IC_{50}s was very similar across the CYP enzymes and thus the curves in Fig. 4 are all very similar. As an example of how to use this graph consider the curve corresponding to the CYP2C19. If a single IC_{50} estimate is obtained then the fold error is approximately 1.4. This can be interpreted as follows, if the estimate of IC_{50} is 10 μM then the true IC_{50} is likely (with 95% confi-

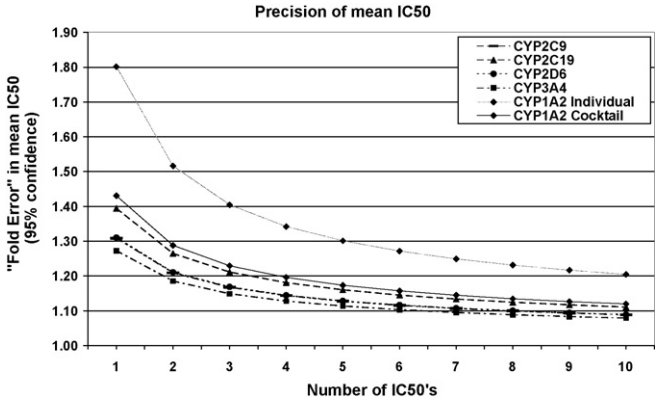

Fig. 4. Graph showing likely precision of IC_{50} estimates for the 5 Cytochrome P450s based on increasing replication.

Table 5
Geometric means from the cocktail and individual probe assay, together with 95% confidence limits

Assay	Compound	Source	*N*	GM	95% Confidence limits	
CYP1A2	Disulfuram	Cocktail	5	3.13	2.50	3.92
	Disulfuram	Individual	5	2.39	2.00	2.87
	Ketoconazole	Cocktail	5	11.40	9.11	14.27
	Ketoconazole	Individual	5	13.48	10.77	16.88
	Miconazole	Cocktail	5	2.50	2.00	3.13
	Miconazole	Individual	5	1.63	1.36	2.45
	Paroxetine	Cocktail	5	7.29	5.83	9.13
	Paroxetine	Individual	5	4.35	3.47	5.44
	Sertraline	Cocktail	5	15.01	11.99	18.79
	Sertraline	Individual	5	13.46	10.75	16.84
CYP2C9	Disulfuram	Cocktail	5	1.56	1.38	1.77
	Disulfuram	Individual	5	1.60	1.41	1.81
	Fluvoxamine	Cocktail	5	23.81	21.01	26.98
	Fluvoxamine	Individual	5	16.38	14.46	18.56
	Ketoconazole	Cocktail	5	18.82	16.61	21.32
	Ketoconazole	Individual	5	18.12	15.99	20.53
	Miconazole	Cocktail	5	0.78	0.69	0.89
	Miconazole	Individual	5	0.48	0.43	0.55
CYP2C19	Disulfuram	Cocktail	5	7.52	6.45	8.76
	Disulfuram	Individual	5	7.77	6.67	9.05
	Fluconazole	Cocktail	5	26.49	22.73	30.86
	Fluconazole	Individual	4	30.73	25.91	36.45
	Fluvoxamine	Cocktail	5	0.71	0.61	0.83
	Fluvoxamine	Individual	4	0.82	0.69	0.97
	Miconazole	Cocktail	5	0.08	0.07	0.10
	Miconazole	Individual	5	0.04	0.04	0.05
	Sertraline	Cocktail	5	13.31	11.42	15.51
	Sertraline	Individual	4	16.58	13.98	19.67
	Ticlopidine	Cocktail	5	2.60	2.23	3.02
	Ticlopidine	Individual	5	1.39	1.20	1.62
CYP2D6	Disulfuram	Cocktail	5	20.50	18.10	23.23
	Disulfuram	Individual	5	16.72	14.76	18.94
	Fluvoxamine	Cocktail	5	18.39	16.24	20.83
	Fluvoxamine	Individual	5	13.39	11.82	15.16
	Miconazole	Cocktail	5	2.65	2.34	3.00
	Miconazole	Individual	5	2.73	2.41	3.09
	Paroxetine	Cocktail	5	1.04	0.92	1.18
	Paroxetine	Individual	5	0.87	0.77	0.99
	Sertraline	Cocktail	5	5.79	5.11	6.55
	Sertraline	Individual	5	5.62	4.96	6.36
CYP3A4	Disulfuram	Cocktail	5	4.63	4.14	5.17
	Disulfuram	Individual	5	3.83	3.43	4.28
	Fluconazole	Cocktail	–	>30	–	–
	Fluconazole	Individual	5	26.27	23.50	29.37
	Miconazole	Cocktail	5	0.25	0.22	0.27
	Miconazole	Individual	5	0.12	0.11	0.13
	Paroxetine	Cocktail	5	30.96	27.70	34.61
	Paroxetine	Individual	5	24.02	21.49	26.85
	Sertraline	Cocktail	5	29.03	25.97	32.45
	Sertraline	Individual	5	29.03	25.97	32.45

Data only reported for IC_{50}s < 30 μM.

dence) to be in the range 7.1–14 μM (i.e., 10/1.4 to 10 × 1.4). Greater precision may be gained by increasing the replication, and so by using a mean of 2 IC_{50}s the fold error reduces to about 1.26. Again, if the mean estimate is 10 μM, we would be reasonably confident that the true IC_{50} was in the range 7.9– μM to 12.6 μM.

4. Discussion

One of the major challenges faced by the pharmaceutical industry is the prediction of drug–drug interactions mediated through inhibition of Cytochrome P450s, which ultimately could result in reduced metabolic clearance of itself and/or co-administered drugs potentially resulting in increased exposure that may exceed the tolerated therapeutic window. Early assessment of a, new chemical entities, propensity to elicit such interactions, is an important stage-gate during early discovery. As such the implementation of higher throughput DDI assays along side other ADME/TOX assays and biological screens can provide the foundation from which potential development candidates are identified, at the same time reducing attrition later on.

With a capability of large pharmaceutical companies to rapidly synthesise large numbers of compounds, an obvious need has risen to design innovative automated high-throughput solutions to allow rapid turn around of ADME data without compromising on quality. Whilst miniaturization using advanced liquid handling technologies can achieve efficiency gains in conducting DDI screens using drug probes, this efficiency can ultimately be limited by the speed of analysis, detection and quantification. Numerous *in vitro* assays to assess CYP inhibition have been developed and adapted for drug discovery [11–24]. The differences between these systems are CYP enzyme source and composition (i.e., recombinant (cDNA expressed) human CYP (rhCYP) isozymes, human liver microsomes

Table 6
Ratio of the geometric mean IC_{50}'s with 95% confidence intervals

Assay	Compound	Ratio	95% Confidence limits	
CYP1A2	Disulfuram	1.10	0.80	1.52
	Ketoconazole	0.85	0.61	1.16
	Miconazole	1.28	0.93	1.76
	Paroxetine	1.68	1.22	2.31[a]
	Sertraline	1.12	0.81	1.54
CYP2C9	Disulfuram	0.97	0.82	1.16
	Fluvoxamine	1.45	1.22	1.73[a]
	Ketoconazole	1.04	0.87	1.24
	Miconazole	1.62	1.36	1.93[a]
CYP2C19	Disulfuram	0.97	0.78	1.20
	Fluconazole	0.86	0.69	1.08
	Fluvoxamine	0.87	0.69	1.09
	Miconazole	1.95	1.57	2.42[a]
	Sertraline	0.80	0.64	1.01
	Ticlopidine	1.86	1.50	2.31[a]
CYP2D6	Disulfuram	1.23	1.03	1.46[a]
	Fluvoxamine	1.37	1.15	1.64[a]
	Miconazole	0.97	0.82	1.16
	Paroxetine	1.19	1.00	1.42
	Sertraline	1.03	0.86	1.23
CYP3A4	Disulfuram	1.21	1.03	1.41[a]
	Miconazole	2.05	1.75	2.40[a]
	Paroxetine	1.29	1.10	1.51[a]
	Sertraline	1.00	0.85	1.17

[a] Indicates significance at the 5% level.

(HLM)), probe substrates, and detection methods (i.e., radioactivity, fluorescence, luminescence and LC–MS) [30]. However, the two most popular approaches utilized to monitor DDIs through CYP inhibition are: (i) rhCYP isozymes with coumarin derivative probe substrates and fluorescence detection (rhCYP-fluorescent) [31] and (ii) HLM with drug probe substrates and LC–MS detection (HLM LC–MS) [10].

Since fluorescent-probe substrates lack specificity for each CYP isozyme, a single purified rhCYP enzyme is used in each assay and HLM are not used. The increase cost associated with use of rhCYPs together with issues around fluorescence quenching (interference by test compounds), is a limiting factor in the adoption of this approach. Historically, a single LC–MS/MS method has been used for determination of CYP inhibitory potential using HLMs [10]. However, over recent years, the development of cocktail biology (assessment of several isozymes simultaneously) has necessitated the design of cocktail analytical assays with appropriate throughput and sensitivity to determine a NCEs CYP inhibitory potential [11–24]. However, numerous factors must be taken into consideration when establishing a cocktail assay. Firstly, probe substrates and their metabolites should exhibit minimal interference with each other. In the current study, inclusion of isotopically labelled internal standards wherever possible helped correct for any suppression effects that may occur. Ion suppression effects are further reduced due to the separation obtained from the background matrix and solvent front. As important as this, is the specificity of probes substrates for each CYP. In this regard the probes substrates used in the current study are those recognized by the Food and Drug Administration (FDA; http://www.fda.gov/cder/drug/drugInteractions/tableSubstrates.htm#inVitro) as being clinically relevant and/or specific to the CYPs being investigated.

The current cocktail approach employed a single probe in the assessment of CYP3A4. It has been suggested that a better assessment of CYP3A4 inhibition is achieved through the assessment of more than one substrate, for example testosterone, nifedipine and/or felodipine [9,32]. However, Obach et al. [33] have recently reported that whilst many CYP3A inhibitors show different inhibitory potency for three different CYP3A markers, those differences did not generally alter the conclusion regarding whether that drug would cause a CYP3A4 DDI *in vivo*. Hence, in the discovery phase, where a cocktail assay of this type would be used, the inclusion of just a single probe for CYP3A4 seems acceptable. When moving into the drug development stage, definitive *in v*itro drug interactions are needed for clinical drug interaction study planning and for supplementing drug product labelling, which may present a more appropriate stage for investigating more than one CYP3A marker substrate. The current assay described here also used human liver microsomes as the CYP source. Whilst use of this type of matrix affords numerous advantages, human liver microsomes are a complex system, as they exhibit both CYP and non-CYP inhibition activities. Amongst non-CYP inhibition activities are protein binding and non-specific lipid binding properties which influence the free concentration of test compound [34,35]. The use of mass spectroscopy, which offers increased selectivity and sensitivity allowed a low protein concentration to be used; 0.1 mg/mL, mitigating the effects of binding and hence making the data reflective of the actual (intrinsic) IC_{50}. Moreover, the increased sensitivity offered by the XLC system allowed measurement of (*S*)-Mephenytoin 4′-hydroxylase activity (a substrate marker for CYP2C19), which is known to require higher protein concentrations and increased incubation time to generate sufficient metabolite for reliable determination of CYP2C19 inhibition [10].

Although numerous studies have been reported to measure CYP inhibition in a cocktail format [11–24], the need to support growing discovery libraries not only relies on higher throughput assays but quicker analytical run times. The use of HPLC instrumentation and column technology has shown to increase sample throughput by reducing run times, without compromising analytical sensitivity or analyte resolution and has been demonstrated to be both practical and robust [36]. The present method offers a significant advantage over some of the existing cocktail analytical methodology in that separation can be achieved with run times as short as 1 min without compromising data integrity. Moreover, the current approach boasts a superior analytical end-point compared with existing procedures, together with increased sample capacity and reproducibility, pre-requisites for an efficient and reliable automated assay. As alluded to earlier, lack in specificity of probe substrates and potential interference could significantly impact the measured intrinsic inhibition of a NCE against the CYPs under investigation. Eight compounds known to display a broad range of inhibitory potencies across the 5 major CYPs were studied as part of a cocktail assay, and against each of the CYPs individually. Estimates of the true ratio of the geometric means between the two assay methods, together with 95% confidence limits, found 10 of the 11 mean ratios were significantly greater than 1, indicating an upward bias in the cocktail results compared with the single probe approach. There is no clear reason to explain this, except a potential rate suppression of probe turnover by the myriad of reaction components within the cocktail incubation compared to the single probe incubations. However, this upward bias was not attributed to any one single P450, which may discount this theory. Nonetheless, closer examination of the data showed that whilst the IC_{50}s were higher with cocktail incubations for some compounds, that the actual geometric mean IC_{50}s were similar, and hence unlikely to significantly influence the predicted clinical DDI risk. With regard to reproducibility, F-tests at the 5% level showed that there was no clear evidence that the cocktail IC_{50}s exhibited higher variability than the individual probe IC_{50}s over the five runs. Together, these results show that the experimental component of the current assay provides reliable CYP DDI information.

In conclusion, the challenged faced by the pharmaceutical and biotech companies is beginning to shift from liquid handling aspects of automation technologies towards high-end quantification systems. To this end the current study reports a miniaturized high-throughput cocktail IC_{50} assay designed to simultaneously assess IC_{50}s for up to 16 compounds in duplicate, in conjunction with a robust, rapid resolution LC–MS/MS end-point offering increased sample throughput without compromising analytical sensitivity or analyte resolution.

Acknowledgements

The authors gratefully acknowledge the contributions made by Kiran Succaram and Mark Savage for their contribution to the generation of the IC_{50} data and mass spectrometric optimization, respectively.

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