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Analytical approaches to determine cytochrome P450 inhibitory potential of new chemical entities in drug discovery

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Abstract

The use of a cassette incubation of probe substrates with human liver microsomes (HLM) – also known as the 'cocktail' approach – is becoming a widely accepted approach to determine the interaction of new chemical entities (NCEs) with cytochrome P450 enzymes (CYP450) in early drug discovery. This article describes two LC–MS/MS-based analytical methods used at the high-throughput (HT) stage and late discovery (LD) stage for analysis of 'cocktail' incubates to analyze the probe metabolites 1′-hydroxymidazolam (CYP3A4), 4′-hydroxydiclofenac (CYP2C9), dextrorphan (CYP2D6), 1′-hydroxytacrine (CYP1A2) and 4′-hydroxymephenytoin (CYP2C19). The analytical methods are advantageous over currently reported methods due to their sensitivity, shorter analyses times (<2 min/sample for the HT method and 4 min/sample for the LD method) and their ability to monitor a unique set of clinically relevant probe metabolites from a biological incubate containing low microsomal protein (0.1 mg/mL). The analytical methods employ the same mobile phase, acetonitrile and 0.1% formic acid, under similar LC–MS/MS conditions. In the HT method, the chromatographic method consists of a short robust step-gradient where the probe metabolites are simultaneously and quickly eluted to enhance throughput. The probe metabolites are chromatographically resolved in the LD stage by utilizing a true linear gradient to obtain optimal peak separation. The IC_{50} data generated by both analytical methods using single incubations *versus* cocktail incubations for various test compounds are in good agreement (correlation coefficient (r^2) ≥ 0.98). The scientist conducting the analysis is provided with a choice of method selection depending on the stage of the test compound and on whether throughput or minimizing interference from other co-eluting metabolites is the most important criterion.

Keywords: *In vitro* cytochrome P450 inhibition assay; LC–MS/MS; DDI; Cocktail DDI analysis; Bioanalysis; CYP3A4; CYP2D6; CYP2C19; CYP2C9; CYP1A2; High-throughput analysis; Drug discovery

1. Introduction

The pharmaceutical industry continues to face an overall high attrition rate, primarily due to lack of efficacy and safety. Hence, there is a demand on pharmaceutical companies to meet their business objectives and to think about ways to achieve efficiencies [1]. Currently, a vast number of new chemical entities (NCEs) are being generated by combinatorial chemistry and tested using *in silico* and *in vitro* technologies. These tools are widely used in the industry in various disciplines to predict *in vivo* behavior in an effort to minimize resource consumption and enhance throughput. Drug–drug interaction (DDI) is one such area where *in vitro* results are used to predict a compound's inhibitory potential towards the cytochrome P450 enzymes, which serve as the major clearance pathway for hepatically cleared compounds. There are serious ramifications to the marketability when a compound's CYP inhibitory potential is identified during clinical development [2]; hence it is essential to utilize *in vitro* technologies in early discovery for DDI risk assessment.

Although more than 40 human cytochrome P450 enzymes have been identified, 5 enzymes are responsible for more than 87% of human drug metabolism: CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19 [3]. The cocktail DDI approach is an *in vitro* tool that is becoming widely accepted in the early drug discovery stages among researchers. Early on Breimer and Schellens demonstrated the feasibility and utility of the cocktail strategy [4]. It deviates from the traditional singlet incubation assay by pooling multiple CYP450 probe substrates into a

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single human microsomal incubation under the same biological conditions [5–9]. The cocktail assay has allowed researchers rapid and simple assessment of the potential inhibitory effect of test compounds on CYP450s.

Throughout drug discovery, analytical methodology is designed to provide the necessary speed and quality predicated on demand. In early discovery several hundreds to thousands of compounds are screened per week. To assess CYP inhibitory potentials, fluorescent techniques are typically used at this stage for high-throughput. For example, CYP3A4 interaction potential is assessed using 7-benzyloxy-4-trifluoromethylcoumarin (BFC)/vivid red as substrates [10]. Fluorescent assays often require the use of expressed CYP450/recombinant enzymes instead of human liver microsomes (HLM) due to poor specificity of the substrates [11]. Typically, *in vitro* DDI data (% inhibition from fluorescent methods) at this stage is used in conjunction with other *in vitro* parameters such as potency, selectivity and safety to bin and effectively screen high-risk compounds.

After this initial stage, the number of compounds is often in the few hundreds or less per week. During this phase, CYP inhibitory potential data are used to guide structural modification of compounds to mitigate risk. CYP inhibitory potentials can usually be determined using non-fluorescent techniques or clinically relevant probes in a human liver microsomal incubation by LC–MS/MS. Either % inhibition or IC_{50} can be determined using extrapolation from single concentration data in this phase. Yet, determination of IC_{50} involving incubations at various concentrations of test compound would be resource consuming due to the large number (several hundreds) of compounds in this stage gate. In the late stages of drug discovery where the number of compounds is fewer, definitive assays are conducted to determine IC_{50}. The test compound is incubated at different concentrations (determined based on projected free efficacious concentrations) with the probe substrates in human liver microsomes. The IC_{50} value in conjunction with the compounds free efficacious concentration is used for risk assessment in the clinic. At this stage, analyses of the microsomal incubations require robust, reliable analytical techniques to separate the probe metabolite from the parent compound and other metabolites at higher concentrations of the test compound to prevent ion suppression and to ensure quality. Typically, this data can be used up to IND submissions, to predict clinical risk of DDIs, to increase patient safety and to design early phase DDI studies in healthy volunteers. Additionally, throughput is not a concern, considering only a small percentage of compounds progress to this stage of testing.

Traditionally, prior to the development of cocktail biology, singlet enzyme incubation in conjunction with a single LC–MS/MS method has been used for determination of CYP inhibitory potential [12]. Upon the development of a substrate cocktail biological incubation assay there was a need for a cocktail analytical assay with appropriate throughput and sensitivity to determine a test compound's CYP450 inhibitory potential. Several LC–MS/MS-based cocktail analytical assays have been reported in the literature [13–28]. However, many of these methods suffer from limitations such as use of recombinant CYPs, clinically irrelevant probe substrates, intensive sample preparation, use of sample preparation HPLC columns, longer run times and higher protein (HLM) content in biological incubations that provides higher substrate turnover; however, could result in non-specific binding. Reported herein are two LC–MS/MS-based analytical methods that are stage gate aligned (high-throughput (HT)/late discovery (LD)) to enable appropriate throughput, have shorter run times, possess increased sensitivity and enable analysis of metabolites for a unique set of clinically relevant probe substrates (Table 1). The appropriate choice of analytical conditions and hindrances that were faced during method development are discussed.

Table 1
CYP enzymes of interest and their clinically relevant probe substrates and metabolites measured in the HT and LD assays

Enzyme	Substrate	Metabolite
CYP3A4	Midazolam	1′-Hydroxymidazolam
CYP2D6	Dextromethorphan	Dextrorphan
CYP2C9	Diclofenac	4′-Hydroxydiclofenac
CYP2C19	*S*-mephenytoin	4′-Hydroxymephenytoin
CYP1A2	Tacrine	1′-Hydroxytacrine

2. Experimental methods

2.1. Materials

Diclofenac sodium salt, sulfaphenazole, ketoconazole, testosterone, 6β-hydroxytestosterone, phenacetin, acetamidophenol, furafylline, quinidine, dextrorphan, ticlodipine, fluconazole, fluoxetine, benidipine HCl, potassium phosphate buffer (pH 7.4) and the reduced form of NADPH were purchased from Sigma–Aldrich (St. Louis, MO). Human liver microsomes (HLM), midazolam hydrochloride, 1′-hydroxymidazolam, *S*-mephenytoin, 4′-hydroxydiclofenac and 4′-hydroxymephenytoin were obtained from Gentest Corp. (Woburn, MA). Dextromethorphan and Triazolam were purchased at Sigma RBI (Natick, MA). Tacrine and 1′-hydroxytacrine were synthesized in house (Pfizer Inc., Ann Arbor, MI). Acetonitrile (ACN), methanol, HPLC grade water and formic acid were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ).

2.2. In vitro incubations

Reaction preparation was automated using a Tecan® Genesis 200 robotic system. The reaction volume for both singlet and cocktail incubation was 500 μL, which consisted of 5 μL of the test compound, 400 μL of substrate, 70 μL of HLM and 25 μL of 1 mM NADPH regeneration system. The final HLM protein concentration of the cocktail incubation is 0.1 mg/mL, with 0.1% DMSO concentration. These final conditions were derived to ensure a substrate depletion of ≤20%, linearity in rate of product formation over ≥8 min period, analytical cross-talk of <5% and no detectable biological assay cross-talk. Substrates consisted of tacrine (1A2), *S*-mephenytoin (2C19), midazolam (3A4), diclofenac (2C9) and dextromethorphan (2D6). Sub-

Table 2
LC conditions for HT analytical method; LC conditions for LD analytical method

Step	Time (min)	Flow rate (μL/min)	Gradient profile	% Aqueous	% Organic
HT assay step-gradient conditions					
0	0.0	800	1.0	50	50
1	0.9	800	1.0	50	50
2	1.0	800	1.0	0	100
3	1.7	800	1.0	0	100
4	1.8	800	1.0	50	50
5	2.0	800	1.0	50	50
LD assay gradient conditions					
0	0.0	300	1.0	100	0
1	2.0	300	1.0	20	80
2	3.0	300	1.0	20	80
3	3.5	450	1.0	100	0
4	4.0	300	1.0	100	0

strates in both the single and cocktail incubations were made from the same stock solution in 100 mM potassium phosphate buffer. Final substrate concentrations were at their respective K_ms tacrine (2.0 μM), *S*-mephenytoin (40.0 μM), midazolam (2.0 μM), diclofenac (5.0 μM) and dextromethorphan (5.0 μM).

Incubation was conducted in a water-heated block using 1.2 mL mini tubes at 37 °C. The pre-incubation reaction of 5 min consisted of test compound, substrate or cocktail of substrates, HLM and 100 mM potasssium phosphate buffer. The reaction was initiated with the addition of 25 μL NADPH (1 mM final) and quenched with 500 μL of cold ACN after an 8-min incubation. The 200 μL of sample was aliquoted to a 96-deepwell round bottom plate. Triazolam in acetonitrile (25 μL of a 250 ng/mL) was added to each sample as an internal standard (IS). Samples were vortexed and centrifuged for 10 min at 4000 rpm for LC–MS/MS analysis.

Reference inhibitors consisted of miconazole (2D6, 2C9, 2C19, 1A2, 3A4), sulfaphenazole (2C9), ketoconazole (3A4), furafylline (1A2), quinidine (2D6) and ticlodipine (2C19). Reference compounds were tested at 10 different concentrations ranging from 0.06 to 30 μM based on literature reported IC_{50}s.

2.3. Standard and QC preparation

The standard curve for the cocktail DDI assay was prepared as follows: 100 μL of 0.2 mg/mL standard stock (in water) of the metabolites (4′-hydroxymephenytoin, 4′-hydroxydiclofenac, 1′-hydroxymidazolam, dextrorphan and 1′-hydroxytacrine) was added to 500 μL of acetonitrile to produce 20 mg/mL cocktail stock solution. Final standard curve was prepared in an incubation mixture (0.5 mL) containing 0.405 mL substrate/KPO_4 mix, 0.07 mL microsomes/KPO_4 mix and 0.025 mL of diluted standard stocks resulting in standards of concentrations ranging from 500 to 0.488 ng/mL. Subsequently, the standards were treated with 0.5 mL acetonitrile to simulate a matrix similar to the samples.

Quality control (QC) samples for the individual analytes to validate the analytical assay were prepared similar to the standards, as described above, using separately weighed stock solutions. Concentrations of the low, medium and high QCs were 12.5, 150 and 375 ng/mL, respectively.

2.4. Data analysis

IC_{50} results were calculated using the internal Pfizer software (Pfizer Inc.). An one-site competition equation (Eq. (1)) was used to estimate IC_{50}, where Y is the response and X is the logarithm of the agonist concentration.

$$Y = \frac{V_{max}}{1 + 10^{\log IC_{50} - X}} \quad (1)$$

2.5. LC–MS conditions

2.5.1. HT method

LC conditions consisted of a step-gradient (Table 2) using 0.1% formic acid in water (A) and acetonitrile (B) as

Table 3
MS conditions for the probe metabolites

Isozyme	Analyte	Precursor ion mass (*m/z*)	Product ion mass (*m/z*)	Dwell time (ms)	Declustering potential (eV)	Collision energy (eV)
3A4	1′-Hydroxymidazolam	342.1	203.2	100	70	40
2C9	4′-Hydroxydiclofenac	312.0	231.1	100	25	30
1A2	1′-Hydroxytacrine	215.3	182.1	100	45	11
1A2	Phenacetin	152.1	110.2	100	25	20
2C19	4′-Hydroxymephenytoin	235.1	150.2	200	25	30
2D6	Dextrorphan	258.2	157.3	100	61	57
Internal standard	Triazolam	343.2	308.1	100	70	40

mobile phases. The specific mass spectrometric conditions are listed in Table 3. A Waters YMC Basic Guard Cartridge, 2.0 mm × 20.0 mm, 5 μm particle size was used for chromatographic separation. A Valco six-port switching valve was operated to divert flow between the waste and the mass spectrometer. The flow was diverted to waste from 0 to 0.3 min and from 1.8 to 2.0 min. A LEAP CTC-PAL autosampler was utilized in conjunction with two parallel Perkin-Elmer micro pumps. Sample injection volume was 10 μL. An Applied Biosystems/Sciex API 4000 mass spectrometer was operated in the positive ionization mode controlled by Analyst 1.2 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). A 20/80 mixture of 0.2% formic acid/acetonitrile was used to wash the syringe (100 μL capacity) (×1) and injection port (×4) after each sample injection.

2.5.2. LD method

The MS conditions for this method were the same as that used in the HT method. The LC conditions (Table 2) for this method consisted of an analytical column; Waters, Atlantis dC_{18}, 50 mm × 2.1 mm, 3 μm particle size, with acetonitrile and 0.1% formic acid in water as mobile phase delivered in gradient mode by the Shimadzu LC–10Advp pumps. A LEAP CTC-PAL autosampler was used to inject 3 μL sample volumes. Post-column flow was diverted to waste between 0–1.5 and 3–4 min using a Valco six port switching valve. A 50/50 0.1% formic acid/acetonitrile mix was used to wash the syringe (100 μL capacity) (×1) and injection port (×3) after each sample injection.

3. Results and discussions

The methodology reported here consists of a cocktail biological incubation of five probe substrates (most of which are recommended by Pharmaceutical Research and Manufactures of America (PhRMA)) for the five major CYPs in HLM with the NCE and monitors for their respective probe metabolites using LC–MS/MS as listed in Table 1 [29]. The development of cocktail biology will be reported separately. Fig. 1 depicts the appropriate technologies used to determine DDI potential of NCEs at the various stages of drug discovery. Figs. 2 and 3 show the chromatogram of the five probe metabolites from a cocktail incubation as obtained from the HT and LD methods, respectively.

Prior to the development of the cocktail approach, fluorescent technology was used at the ultra-HT and HT stages while at the LD stage inhibition potential of the NCE towards each CYP was assessed separately using five different incubation conditions and five different LC–MS/MS methods for the probe metabolites. Hence, the LC–MS/MS conditions were different for each of the analytes and the ionization mode (ESI *versus* APCI and positive *versus* negative) was chosen to provide the maximum sensitivity. Analytes such as hydroxydiclofenac and hydroxymephenytoin were monitored in the negative ion mode in the mass spectrometer. However, the goal of the cocktail approach was to improve throughput of the biology and the analysis; *i.e.* to measure all probe metabolites in a single analysis. Towards this

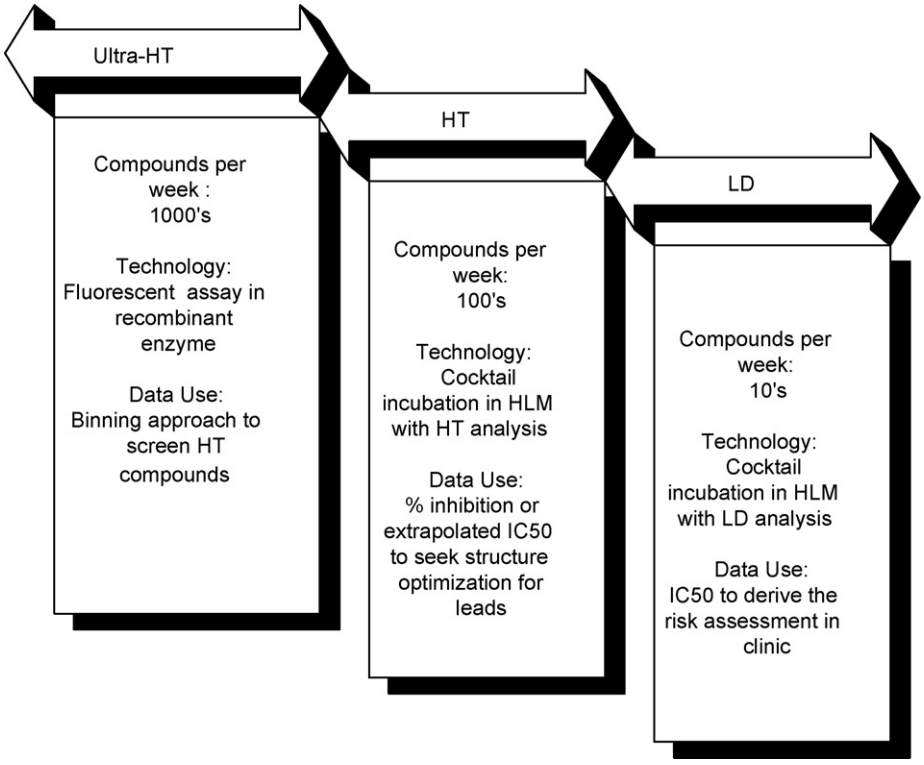

Fig. 1. Drug discovery continuum: *in vitro* CYP assays used at the different stages of drug discovery and development.

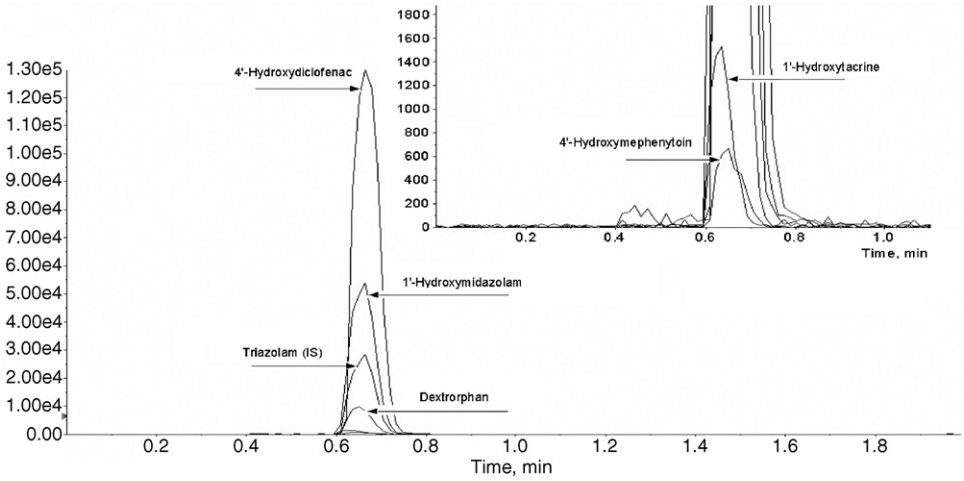

Fig. 2. LC–MS/MS total ion chromatogram of the analytes at zero inhibitor concentration in a microsomal matrix using HT analytical method.

a single ionization mode was preferred to prevent duplicate analysis. All the probe metabolites were amenable for analysis in the electrospray positive ion mode and with the availability of MS platforms with improved sensitivity such as the API 4000, this was readily feasible. Table 3 shows the probe metabolite, their respective precursor, product ion transitions and optimal mass spectrometric conditions that were analyte dependent. Since the assay involves quantitation of multiple analytes from a single LC–MS/MS analysis it was imperative to evaluate the mass spectrometric cross-talk (multiple response monitoring (MRM) channel interference) of each of the analyte against the other analytes to ensure specificity. Each analyte was injected separately while monitoring the signal in all mass spectrometric channels. Signal was observed only in the analyte channel of interest, demonstrating lack of mass spectrometric cross-talk and ensuring method specificity.

In an effort to keep the method(s) user-friendly and robust, commonly used organic mobile phases such as acetonitrile/methanol and modifiers such as formic acid/acetic acid were tested during method development. According to Zhou et al. [30], the sensitivity of a given analyte in positive or negative ionization mode has been shown to depend on mobile phase composition. We found that the combination of acetonitrile/0.1% formic acid as mobile phases provided the best chromatographic results and sensitivity in the positive ionization mode.

Of the five probe metabolites, the sensitivity requirement for 1′-hydroxymephenytoin [HMP] was most stringent due to the low level of formation in comparison with the other probe metabolites of interest. The total amount of HMP generated under the given condition of 0.1 mg/mL HLM for 8-min incubation was approximately 1–2 ng/mL. The different factors that would improve the sensitivity for HMP were evaluated. The MS conditions were optimized to yield the maximum possible sensitivity for HMP without compromising signal for other analytes. The effect of either acetonitrile or methanol as the organic mobile phase component was tested. The analyte signal intensity observed was not dependent on the choice of organic mobile phase component. Hence, there was no significant value to changing the organic make up of the mobile phase. Acetonitrile remained the organic solvent for all further studies.

The total amount of DMSO used to solvate the compound influenced the analyte's signal intensity. Fig. 4 shows the signal for HMP (area counts) in the presence of increasing % DMSO concentrations. HMP was evaluated due to its sensitivity limita-

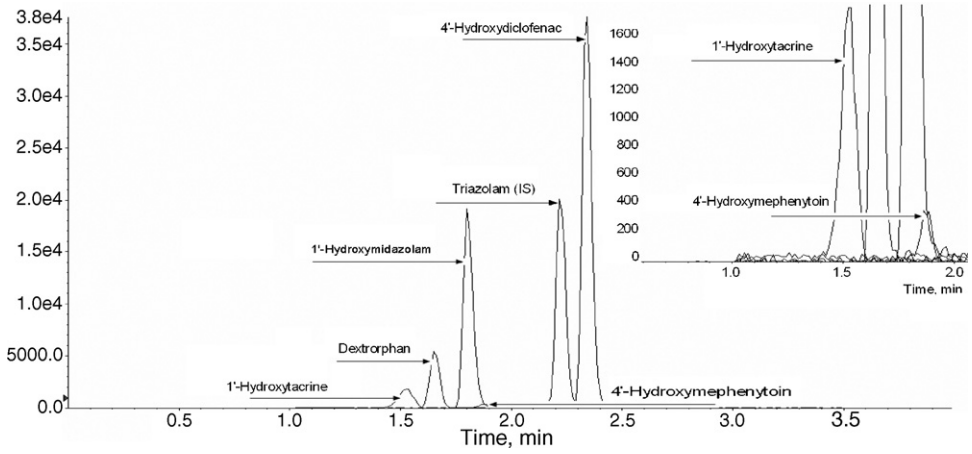

Fig. 3. LC–MS/MS total ion chromatogram of the analytes at zero inhibitor concentration in a microsomal matrix using post-LD analytical method.

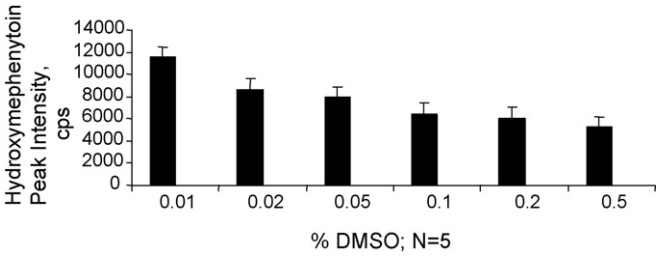

Fig. 4. Difference in turnover for hydroxymephenytoin analyte (as shown by peak area) with different % of total DMSO in biological incubations containing 0.1 mg/mL of protein and incubation period of 8 min.

tions compared to the other analytes. Lowering the total DMSO content in the cocktail incubate allows higher enzyme turnover and hence the amount of metabolite formed.

Midazolam, testosterone and felodipine are commonly used as specific substrates for the major human enzyme CYP3A4. To definitively understand total CYP3A4 inhibition at least two probe substrates are typically used because CYP3A4 possesses two active sites that accommodate substrates of different properties [31]. Early in-house investigations that compared cocktail *versus* singlet biological incubations showed that testosterone when used as the 3A4 probe substrate was inhibiting *S*-mephenytoin (2C19 substrate) turnover. For this reason, midazolam was chosen as the CYP3A4 probe substrate in the cocktail rather than testosterone. The final cocktail assay was validated using midazolam as the CYP3A4 probe substrate. Currently, midazolam is the probe of choice to test *in vivo* DDI in the clinic. Binding/inhibitory potential of NCEs to the testosterone-like or felodipine-like binding pocket of 3A4 is tested using a singlet incubation in the LD stage on an as needed basis. The LD-analytical assay has the capability to analyze the metabolites of testosterone (6β-hydroxytestosterone) and felodipine (dehydrofelodipine). In-house data (not reported) show that midazolam as a probe provides adequate evidence towards the binding affinity of an NCE toward 3A4 in most cases.

A significant decrease in signal for HMP was observed when using midazolam maleate instead of midazolam HCL as the 3A4 substrate. During assay development either salts of midazolam were used interchangeably and a significant change in HMP signal was observed in each case. The reason for difference in signal for HMP was investigated and it was found that the use of the different salt forms of midazolam was the primary factor since all other variables were constant. Use of the maleate salt form of midazolam caused more than a six-fold decrease

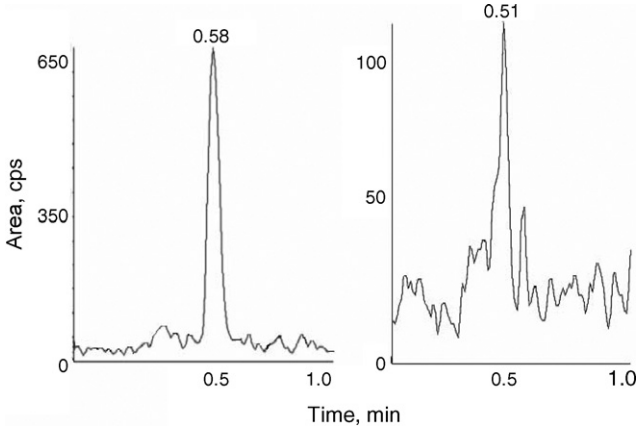

Fig. 5. Difference in LC–MS/MS sensitivity for hydroxymephenytoin analyte in incubate containing midazolam hydrochloride salt (left panel) *vs.* midazolam maleate salt (right panel) in biological incubations containing 0.1 mg/mL of protein, zero inhibitor concentration and incubation period of 8 min.

in analyte peak intensity, possibly due to ion suppression in the LC–MS/MS analysis. Fig. 5 shows the signal for HMP when using the HCl salt *versus* the maleate salt. Hence, further experiments used midazolam hydrochloride salt as the substrate in all incubations. The final biological assay conditions after optimizations allowed for linear kinetics for all the enzymes with <20% substrate turnover [32].

The HT cocktail analytical method was developed with focus on simplicity in instrumentation set-up, sensitivity and robustness. Cocktail HT analyses were conducted on the same instruments supporting other HT analytical measurements such as metabolic stability and permeability measurements of potential new chemical entities. In order to batch the HT DDI samples with other screening samples, it was critical to ensure that the assay conditions were relatively generic. This minimized the time and effort to switch ionization sources/conditions and mobile phases between diverse assays. Triazolam is used as the internal standard for both methods. As shown in Fig. 2, the HT method has a total run time of <2 min. Throughput and robustness are the major attributes of this method. The retention times of the analytes were around 0.60 min. In this step-gradient method all the analytes, along with the internal standard triazolam, co-eluted without chromatographic resolution. The analytical performance characteristics of the HT method are listed in Table 4.

Fig. 3 shows chromatographic separation, using a linear gradient in the LD method. All of the analytes in this method were separated from each other except 1′-hydroxymephenytoin

Table 4
Analytical performance of the HT and LD cocktail LC–MS/MS assay

Analyte	HT		LD	
	Coefficient of determination of the standard curve (r^2)	Limit of quantitation (ng/mL)	Coefficient of determination of the standard curve (r^2)	Limit of quantitation (ng/mL)
4′-Hydroxydiclofenac	0.9991	3.90	0.9994	0.488
4′-Hydroxymephenytoin	0.9997	0.488	0.9971	0.488
Dextrorphan	0.9998	0.976	0.9831	0.488
1′-Hydroxymidazolam	0.9990	0.488	0.9954	0.488
1′-Hydroxytacrine	0.9997	0.488	0.9895	0.488

and 1′-hydroxymidazolam. However, the precursor and product transitions of the two analytes were different and the potential interference was minimal as demonstrated by the lack of MS cross-talk. The linear gradient also allowed separation of the analytes of interest from both the test compound and its potential metabolites. In some therapeutic areas where the target concentration may be high, such as anti-bacterial agents, the test compound may be incubated at concentrations as high as 1000 μM. In these situations, the potential for analytical interference from the compound is high. The LD method can resolve the interfering peaks from the probe metabolites to a greater extent than the step-gradient utilized in the HT method and provide more confidence in the data generated.

The LD method was also able to retain more polar analytes such as acetamidophenol, a clinically relevant 1A2 probe metabolite of phenacetin, with a capacity factor ~2. The HT step-gradient method was not able to retain acetamidophenol under its chromatographic conditions. Drastically different LC and mobile phase conditions were needed for good chromatographic conditions of phenacetin in the HT step-gradient method. Thus, phenacetin was substituted with tacrine as the probe substrate for the CYP1A2 in the cocktail to minimize the complexity of the assay, because retention of 1′-hydroxytacrine by the HT method was not an issue. The LD method uses a silica-based column of difunctionally bonded reversed-phase C_{18} with balanced ligand density that allowed the retention of more polar compounds due to both silica and reverse-phase interactions. The LD method retained compounds ranging from log *P* of 0.494 to log *P* of 4.2; exhibiting acceptable peak shape. Table 4 shows the assay performance for each of the analytes from the LD method. The assay provided desired sensitivity and linear dynamic range for each analyte.

As described previously, prior to the development of the cocktail biology and analysis methods, five different singlet biology and single LC–MS/MS methods were used. To validate the LD cocktail LC–MS/MS method, samples from the singlet incubations were analyzed using either the singlet analytical LC–MS/MS method (not describe here) or the cocktail analytical LC–MS/MS method; concentrations determined from either method from the same sample showed good correlation. Thus, the cocktail analytical method was cross-validated against the standard singlet analytical method for each probe metabolite. Example data comparing 1′-hydroxymephenytoin concentrations using a singlet analytical method or the LD cocktail analytical method is shown in Fig. 6. Quality controls analyzed using the LD cocktail analytical method show % CV (intraday) of <15% and % RE of <15% at three different concentrations within the dynamic range indicative of acceptable precision and accuracy.

Fig. 7 compares the IC_{50} values obtained from the cocktail incubation conditions using either the HT analytical method or the LD analytical method for eight control inhibitors at a concentration range of 0.06–30 μM. The eight compounds were inhibitors of multiple CYPs. The IC_{50}s were in good agreement with a correlation coefficient (r^2) of 0.99. These data provide confidence in using the cocktail biological assay in conjunction with either LC–MS/MS method.

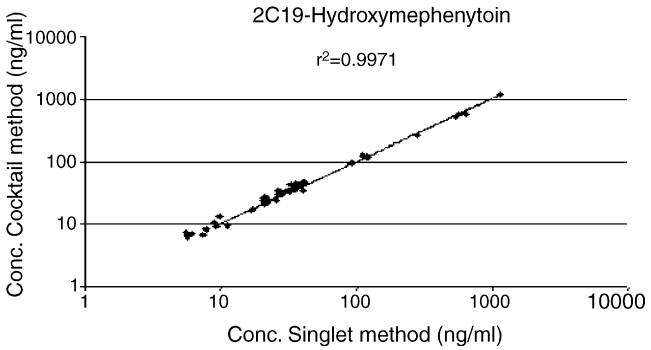

Fig. 6. Correlation of concentrations ($n = 65$) determined using the single-analysis method and the cocktail-analysis (post-LD) method for *in vitro* samples used to test 2C19 inhibitory potential of test compounds.

The HT method is currently used to support the cocktail incubation at a single (3 μM) concentration for each compound. Either % inhibition or extrapolated IC_{50} calculations are obtained based on peak area of the probe metabolite. The internal standard area counts are monitored in each run to assess instrumental drift or matrix effects. The cartridge used in the HT method allows for more than 1000 injections and has been shown to be robust. Changes in peak shape or sensitivity (peak area counts for the probe metabolites in the incubation with no control inhibitor) are routinely monitored to maintain quality of the assay.

The analytical column used in the LD method has exhibited acceptable analytical performance for >1500 injections. Both assays consist of the same mobile phases, similar LC–MS/MS conditions and a straightforward set-up. No additional sample preparation step is involved in either method; acetonitrile supernatant from the biological incubation is directly injected into the LC–MS system. Multiple scientists have used this assay on different instruments to obtain reproducible results. Both the HT and LD methods are suitable for their respective stage in the discovery process and the data from the LD stage can be used to support IND submission.

As proof of the robustness and reproducibility of results, data were compiled over a 1-month period that represents IC_{50} and % inhibition data for NCEs enabling the potential CYP450 risk assessment using the cocktail incubation and the two different analytical approaches. Fig. 8 shows data using the HT method where the number of compounds screened is approximately 1024 per month. Fig. 9 shows the data using the LD method

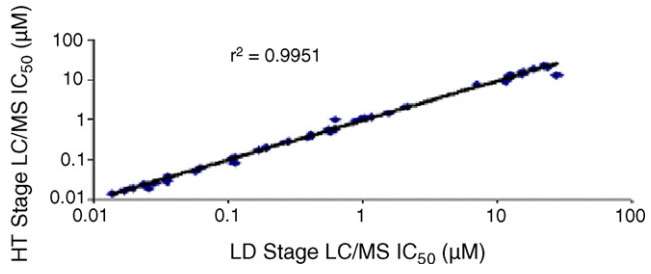

Fig. 7. Correlation of IC_{50} generated from cocktail incubate samples using the LD method *vs.* the HT method. Eight compounds are represented ($N = 2$) and each IC_{50} is shown. The eight compounds were inhibitors of multiple CYPS.

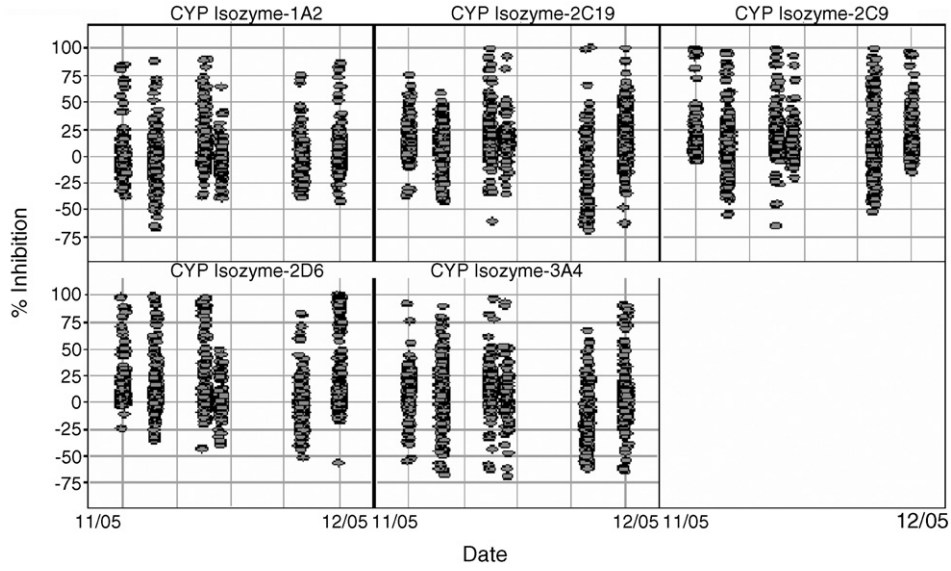

Fig. 8. Trellis plot by CYP Isozyme at the HT stage of NCEs. Data (% inhibition) is shown for a set of compounds analyzed during a 1-month time period. $N = 1024$.

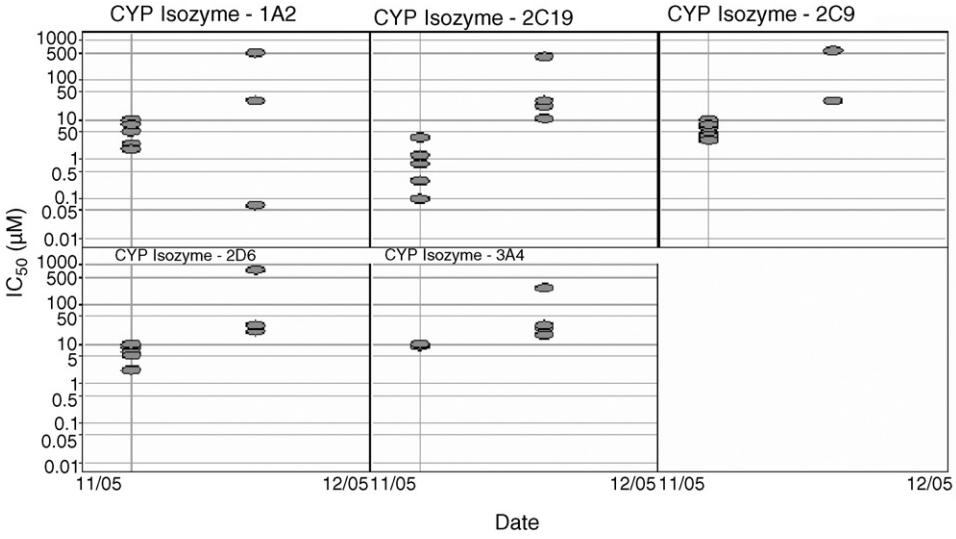

Fig. 9. Trellis plot by CYP Isozyme at the LD stage of NCEs. Data (IC_{50}) is shown for a set of compounds analyzed during a 1-month period. $N = 13$.

where the number of compounds is approximately 13 per month, significantly lower than the number at the HT stage. The data in both figures demonstrate how the two analytical methods are used at the different stages of drug discovery appropriately to provide adequate throughput and quality data to enable necessary decision-making. Thus, this new 'cocktail' (biology in conjunction with analytical methodology) concept has allowed an improved and more efficient approach in advancing NCEs in early drug discovery.

4. Conclusions

We have presented analytical approaches that can be combined with an *in vitro* cocktail incubation to assess *in vitro* CYP inhibitory potential for NCEs. The two LC–MS/MS-based methods are robust and provide reliable data fit for the stage (HT and LD) at which they are used. These methods provide exemplary sensitivity for the 4′-hydroxymephenytoin probe metabolite for the 2C19 isozyme over other existing methods. Both methods employ the same combination of acetonitrile and water (0.1% formic acid) as mobile phase and are able to monitor five different probe metabolites along with an internal standard. The HT analytical method uses a step-gradient and has a run time <2 min, allowing analysis of ∼1500 compounds/week at a single test concentration. The LD method employs a linear gradient that provides adequate resolution between the analytes of interest and potential test compound or metabolite interference. The LD method also has a <5 min/sample cycle time, shorter than most published methods of this nature. The method can be used to measure IC_{50} or K_is for definitive analysis. The IC_{50} data generated from the cocktail incubation using the HT assay or the LD method show good agreement. The scientist is provided with a choice of method selection depending on the stage at which the information is requested to ensure appropriate throughput.

References

[1] I. Kola, J. Landis, Nat. Rev. Drug Discov. 3 (2004) 711.
[2] R.H. Levy, K.E. Thummel, W.F. Trager, P.D. Hansten, M. Eichelbaum (Eds.), Metabolic Drug Interactions, Lippincott, Williams & Wilkins, Philadelphia, PA, 2000.
[3] F.P. Guengerich, Adv. Pharmacol. 43 (1997) 7.
[4] D.D. Breimer, J.H. Schellens, Trends Pharmacol. Sci. 11 (1990) 223.
[5] R.F. Frye, G.R. Matzke, A. Adedoyin, J.A. Porter, R.A. Branch, Clin. Pharmacol. Ther. 62 (1997) 365.
[6] R.J. Scott, J. Palmer, I.A.S. Lewis, S. Pleasance, Rapid Commun. Mass Spectrom. 13 (1999) 2305.
[7] D.S. Streetman, J.F. Bleakley, J.S. Kim, A.N. Nafziger, J.S. Leeder, A. Gaedigk, R. Gotschall, G.L. Kearns, J.S. Bertino Jr., Clin. Pharmacol. Ther. 68 (2000) 375.
[8] B. Zhu, D.S. Ou-Yang, X.P. Chen, S.L. Huang, Z.R. Tan, N. He, H.H. Zhou, Clin. Pharmacol. Ther. 70 (2001) 455.
[9] E.A. Dierks, K.R. Stams, H.K. Lim, G. Cornelius, H. Zhang, S.E. Ball, Drug Metab. Dispos. 29 (2001) 23.
[10] D.M. Stresser, S.D. Turner, A.P. Blanchard, V.P. Miller, C.L. Crespi, Drug Metab. Dispos. 30 (2002) 845.
[11] C.L. Crespi, V.P. Miller, D.M. Stresser, Methods Enzymol. 357 (2002) 276.
[12] R.L. Walsky, R.S. Obach, Drug Metab. Dispos. 32 (2004) 647.
[13] J. Ayrton, R. Plumb, W.J. Leavens, D. Mallett, M. Dickins, G.J. Dear, Rapid Commun. Mass Spectrom. 12 (1998) 217.
[14] I. Chu, L. Favreau, T. Soares, C. Lin, A.A. Nomeir, Rapid Commun. Mass Spectrom. 14 (2000) 207.
[15] H. Yin, J. Racha, S.Y. Li, N. Olejnik, H. Satoh, D. Moore, Xenobiotica 30 (2000) 141.
[16] H.Z. Bu, L. Magis, K. Knuth, P. Teitelbaum, Rapid Commun. Mass Spectrom. 14 (2000) 1619.
[17] H.Z. Bu, K. Knuth, L. Magis, P. Teitelbaum, Rapid Commun. Mass Spectrom. 14 (2000) 1943.
[18] H.Z. Bu, L. Magis, K. Knuth, P. Teitelbaum, J. Chromatogr. B 753 (2001) 321.
[19] H.Z. Bu, K. Knuth, L. Magis, P. Teitelbaum, J. Pharm. Biomed. Anal. 25 (2001) 437.
[20] H.Z. Bu, L. Magis, K. Knuth, P. Teitelbaum, Rapid Commun. Mass Spectrom. 15 (2001) 741.
[21] E.S.M. Lutz, M.E. Markling, C.M. Masimirembwa, J. Chromatogr. B 780 (2002) 205.
[22] T. Zhang, Y. Zhu, C. Gunaratna, J. Chromatogr. B 780 (2002) 371.
[23] C. Yu, Y.G. Shin, J.W. Kosmeder, J.M. Pezzuto, R.B. van Breeman, Rapid Commun. Mass Spectrom. 17 (2003) 307.
[24] S.A. Testino Jr., G. Patonay, J. Pharm. Biomed. Anal. 30 (2003) 1459.
[25] R. Weaver, K.S. Graham, I.G. Beattie, R.J. Riley, Drug Metab. Dispos. 31 (2003) 955.
[26] S.X. Peng, A.G. Barbone, D.M. Ritchie, Rapid Commun. Mass Spectrom. 17 (2003) 509.
[27] M.J. Kim, H. Kim, I.J. Cha, J.S. Park, J.H. Shon, K.H. Liu, J.G. Shin, Rapid Commun. Mass Spectrom. 19 (2005) 2651.
[28] T.D. Bjornsson, J.T. Callaghan, H.J. Einolf, V. Fisher, L. Gan, S. Grimm, J. Kao, S.P. King, G. Miwa, L. Ni, G. Kumar, J. McLeod, R.S. Obach, S. Roberts, A. Roe, A. Shah, F. Snikeris, J.T. Sullivan, D. Tweedie, J.M. Vega, J. Walsh, S.A. Wrighton, Drug Metab. Dispos. 31 (2003) 815.
[29] M. Turpeinen, U. Jouko, J. Jorma, P. Olavi, Eur. J. Pharm. Sci. 24 (2005) 123.
[30] S. Zhou, M. Hamburger, Rapid Commun. Mass Spectrom. 9 (1995) 1516.
[31] S. Ekins, D.M. Stresser, J.A. Williams, Trends Pharmacol. Sci. 24 (2003) 4.
[32] G.T. Tucker, J.B. Houston, S.M. Huang, Br. J. Clin. Pharmacol. 52 (2001) 107.