P122 Validation of Cocktail Enzyme Assay in 96-well Plated Human Hepatocytes for CYP1A2, CYP2B6, and CYP3A4 Induction Studies with LC-MS/MS Analysis

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Abstract

Assessment of changes in enzyme activity as an endpoint is common in the determination of induction potential of CYPs for a potential inducer. Although regulatory agencies recommend that mRNA be a primary endpoint, the measurement of enzyme activity can be very useful in interpretation of induction data. Historically, these activities for various CYP isoforms were measured in separate and single incubations with their respective probe substrates. Herein, we developed and validated a cocktail strategy that will enable a robust evaluation of CYP1A2, CYP2B6, and CYP3A4 enzyme activity in basal, as well as induced samples by concomitant incubation of selected probes recommended by the FDA and EMA[1,2]. Human hepatocytes from three individual donors were plated in 96-well Collagen I-coated microplates. The hepatocytes were treated for two days with solvent vehicle control DMSO and prototypical inducers (100 µM omeprazole for CYP1A2, 1 mM phenobarbital for CYP2B6, and 10 µM rifampicin for CYP3A4). After treatment, enzyme activity for each isoform was measured in incubations with single and cocktail probe substrates (phenacetin for CYP1A2, bupropion for CYP2B6, and midazolam for CYP3A4). In a separate experiment, the linearity of metabolite formation in both basal and induced hepatocyte cultures with incubation time for each enzyme was examined. Metabolite formation for basal and induced CYP1A2 and CYP2B6 was linear up to 120 min. Interestingly, the linearity of 1'-hydroxymidazolam formation was dependent on the enzyme level and substrate concentrations. For the induced CYP3A4 samples, the metabolite formation was not linear at 30 µM over 30 min. To ensure adequate sensitivity for detection of all metabolites measured in the cocktail assay, it was necessary to incubate for longer than 30 min. The linear formation of 1'-hydroxymidazolam up to 60 min. with negligible effect on the amount of metabolite produced was achieved by reducing the concentration of midazolam from 30 µM to 5 µM. We compared metabolite formation in single vs. cocktail incubations for basal as well as induced CYP1A2, CYP2B6, and CYP3A4 activity. Our results demonstrate that metabolite formation in the cocktail assay was overall not considerably different from single assay for all enzymes across all three hepatocyte lots tested, implying negligible interaction between the probes used in the cocktail assay. In conclusion, we have demonstrated that the cocktail assay produced the same induction response compared with single assay and is more efficient and cost-effective for measuring enzyme activity as an endpoint for induction studies.

Introduction

Cocktail approach for the analysis of enzyme activity as an endpoint for CYP induction studies has been in use in the industry for quite some time now. However, several gaps exist in the studies reported in the literature. For example, in a study by Mohutsky, et al.[3], CYP2B6, which is one of the three isoforms recommended to be studied in an induction assay by the FDA/EMA was not included in the investigation. In another study Lahoz, et al.[4] used benzoxyresorufin, which is not considered as a preferred probe substrate of CYP2B6. Midazolam and its metabolite 4'OH midazolam are time-dependent inactivators of CYP3A4[5].Therefore, higher concentration of midazolam and 4'OH midazolam may be responsible for non-linearity in 1'OH midazolam formation. In most studies, linearity of the enzyme reaction in cocktail, especially with respect to CYP3A4 (basal and induced), was not taken into account. This is critical since non-linearity with respect to incubation time can have an impact on fold induction values. Here we have demonstrated that use of lower midazolam concentration can mitigate the non-linearity and the present work described the validation of cocktail assay for CYP1A2, 2B6, and 3A4 used for CYP induction studies.

Materials and Methods

Materials: All inducers (omeprazole, phenobarbital, rifampicin) were obtained from Sigma-Aldrich. Corning® Cryopreserved human hepatocytes were obtained from Corning Life Sciences.

Hepatocyte plating and treatment: Cryopreserved human hepatocytes (Corning Cat. No. 454550 or 454551) from three different donors were thawed using Corning Gentest™ High Viability CryoHepatocyte Recovery Kit (Corning Cat. No. 454534) and plated in Corning Collagen I-coated 96-well microplates (Corning Cat. No. 356407). Cell cultures were maintained overnight in William's Medium E supplemented with Penn-Strep Glutamine, Insulin-Transferrin-Selenium, HEPES, and Dexamethasone prior to treatment in triplicates with solvent vehicle control DMSO or inducers at single concentration of 50 µM omeprazole, 1,000 µM phenobarbital, and 10 µM rifampicin for 48 hours, respectively.

CYP activity: After two-day treatments, hepatocyte cultures were washed and incubated with single or cocktail probe substrates 100 µM phenacetin, 250 µM bupropion, and 5 µM midazolam for 30 minutes and the data with single and cocktail substrate were used for comparison. In addition, a time-course (0, 15, 30, 45, and 60 min,) for the cocktail assay for both basal and induced activity was conducted. In a separate experiment, a time-course of basal and inducted CYP3A4 activity from a single donor (lot 385) was determined at 5 and 30 µM midazolam. All enzyme activities were determined by measuring the metabolite formation of the specific probe substrate for each enzyme. The standard curves with single metabolite or cocktail metabolites were prepared and analyzed by LC-MS/MS for single and cocktail assays, respectively.

Data analysis: The quantitation of metabolite (µM) formed in hepatocytes was calculated from the standard curve. The fold inductions were calculated by dividing the activity observed in treated samples by the mean activity found in solvent samples (DMSO). For inter-assay (single vs. cocktail assays) comparison, a mean of triplicate samples were initially calculated for single and cocktail samples, followed by calculation of mean and standard deviation of these two duplicate values. Coefficient of variation (CV%) was then calculated and used for inter-assay variation analysis.

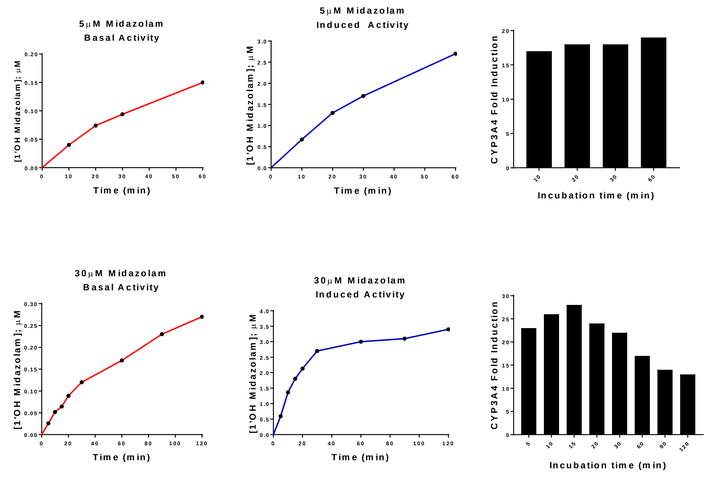
$$Inter\text{-}assay\ variation\,(CV\%) = \frac{SD\,(single\ and\ cocktail)}{Mean\,(single\ and\ cocktail)} * 100$$

Results and Discussion

The linearity of metabolite formation over reaction time was assessed in control, as well as induced hepatocytes. Probe substrates were incubated from 0-120 min. at 37ºC and respective metabolites were quantified. Formation of corresponding metabolite was linear up to 60 min. for phenacetin and bupropion assays in both control and induced hepatocytes (data not shown). At 30 µM midazolam, formation of 1'OH-midazolam was linear up to 20 min. in control hepatocytes, but non-linearity was observed from 30-120 min. in hepatocytes induced with rifampicin. As a consequence, this non-linearity resulted in a decrease in fold induction values from 30-120 min. (**Figure 1**). Decrease in midazolam concentration from 30 µM to 5 µM resulted in linear metabolite formation up to 60 min. in both control, as well as induced hepatocytes. In addition, this fold induction was consistent at 5 µM throughout the incubation period **(Figure 1).** The K_m for midazolam was approximately 2.5 µM (data not shown). Lowering probe substrate concentration did not compromise the analytical sensitivity of midazolam assay.

The inter-assay variation of single vs. cocktail substrates was evaluated with CV% for both assays in three lots of hepatocytes and two separate experiments (**Figure 2**). Overall, data generated from single and cocktail assays were equivalent for basal and induced activities, as well as fold induction across all isoforms and donors. The CV% for both assays is shown in **Table 1**. Among 36 data points, 29 data points exhibited CV% of <25% and 4 data points exhibited the CV% of >30%. Given that only 4 out of the 36 data points had higher CV% in the range of 30% to 40% that was not specific to any one isoform or donor implies that variation seen was probably due to experimental variability in either the single or cocktail experiment.

Figure 1. Effect of change in midazolam concentration on the formation of the CYP3A4 metabolite, 1'OH midazolam in control as well as induced hepatocytes

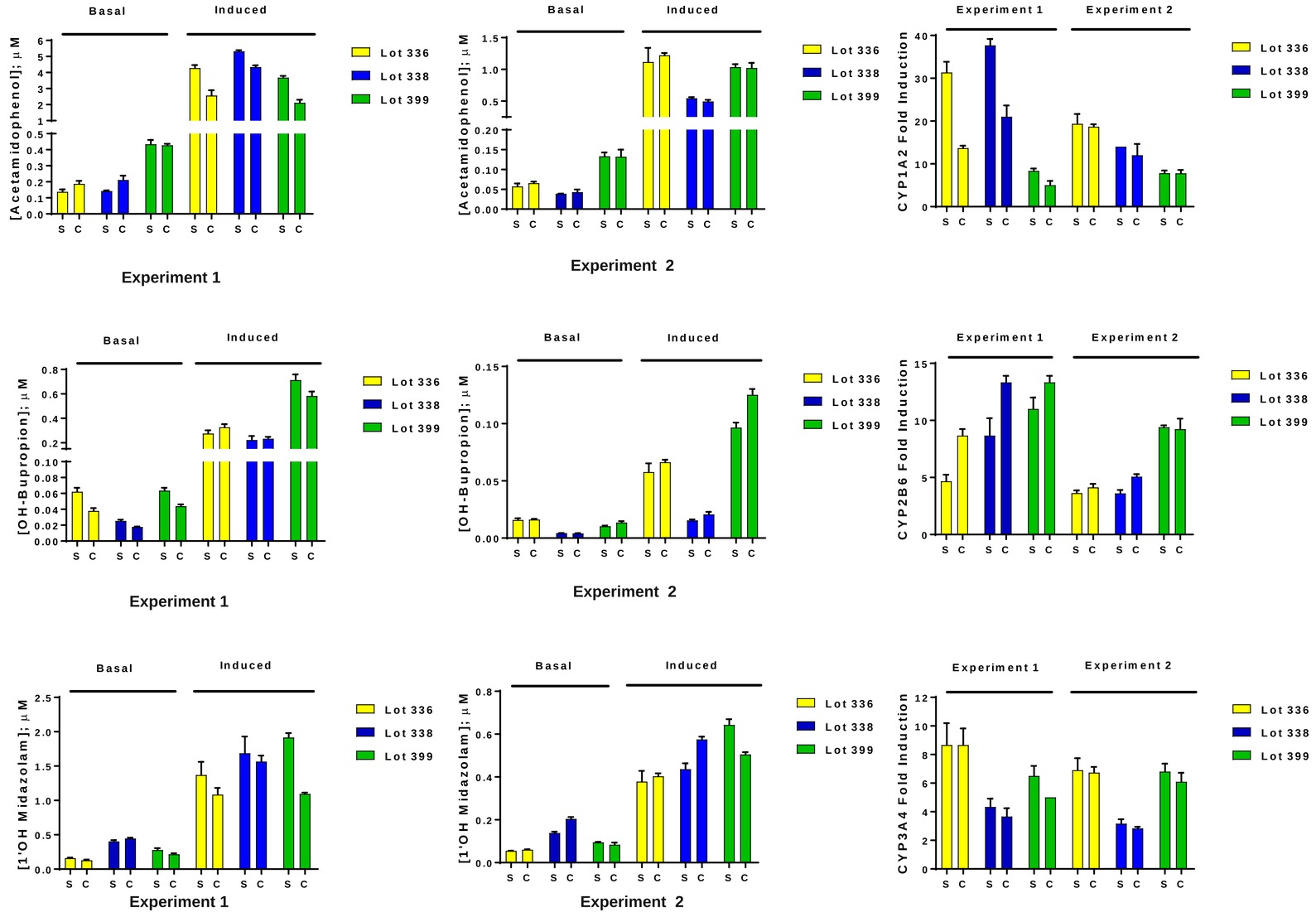


Data are expressed as mean of triplicate measurements performed using a single donor of hepatocytes.

Table 1. Inter-assay variability in basal and induced activity values between single and cocktail incubations for CYP1A2, CYP2B6, and CYP3A4.

Co-efficient of Variation (CV%)		CYP1A2		CYP2B6		CYP3A4	
		Basal	Induced	Basal	Induced	Basal	Induced
Experiment 1	Lot 336	22	35	34	12	15	16
	Lot 338	28	14	25	3	7	5
	Lot 399	1	38	26	14	18	39
Experiment 2	Lot 336	9	6	1	10	6	5
	Lot 338	7	7	3	21	27	19
	Lot 399	1	1	20	18	8	17

Figure 2. Comparison of basal activity, induced activity, and fold induction obtained using the single vs. cocktail assays for CYP1A2, CYP2B6, and CYP3A4 isoforms in cryopreserved primary hepatocytes



Data are the mean ±SD from 3 wells. S = Single incubation; C = Cocktail incubation

Conclusion

In conclusion, we have demonstrated that –

- 5 µM midazolam is optimal as a substrate for the cocktail activity assay to successfully determine CYP3A4 induction potential.
- Cocktail assay produced equivalent induction response compared with single assay for CYP1A2, CYP2B6, and CYP3A4 using FDA/EMA recommended probe substrates.
- Cocktail assay is more efficient and cost-effective for measuring enzyme activity as an endpoint for induction studies.

References

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5. Giragossian C, LaPerle J, Kosa R, Gillian S. (2009). Impact of time-dependent inactivation on the estimation of enzyme kinetic parameters for midazolam. Drug Metab Lett 3:45-53.