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# The Use of a Substrate Cassette Strategy to Improve the Capacity and Throughput of Cytochrome P450 Induction Studies in Human Hepatocytes

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# Substrate cassette strategy in human hepatocyte induction studies

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## Nonstandard abbreviations

CYPs, cytochromes P450

PXR, pregnane X receptor

AhR, aryl hydrocarbon receptor

CAR, constitutive androstane receptor

NCE, new chemical entity

HMM, hepatocyte maintenance media

CV, coefficient of variation

CI, confidence interval

**Abstract**

The gold standard for estimating the ability of a new chemical entity to induce the human cytochromes P450 (CYPs) is to determine the increase in catalytic activity of these enzymes after treatment of primary cultures of human hepatocytes with the potential inducer. The limited availability of fresh human hepatocytes makes these studies impossible to perform on demand. A substrate cassette strategy in which probe substrates for three different CYPs were simultaneously added to hepatocyte preparations was studied to determine if this would be a viable method to increase the capacity of induction studies. The biotransformations of phenacetin (CYP1A2), diclofenac (CYP2C9), and midazolam (CYP3A) were compared when administered to 4 different hepatocyte preparations (± inducer) individually or simultaneously as a cassette. The determinations of fold induction in response to known inducers were not significantly affected, although slight differences were occasionally observed between the various CYP activities, whether determined individually or in the cassette. In total, for three CYP activities in the four hepatocyte preparations with and without inducer, no trend demonstrating a drug-drug interaction between any of the three probes was detected. The lack of interactions between the probe substrates demonstrate that this cassette strategy may be used in primary human hepatocyte induction studies without concern that the interactions between the substrates may be affecting the results as have been seen in other cassette dosing experiments. Therefore, this substrate cassette is an excellent method for increasing the

capacity and throughput of human hepatocyte induction studies by combining three experiments into one.

## Introduction

Induction of hepatic drug-metabolizing enzymes, the increase in steady-state concentration of enzyme after exposure to an appropriate stimulus, has been shown to be a significant cause of drug-drug interactions in patients. A signature group of sometimes overlapping enzymes are induced by the activation of several nuclear receptors including the pregnane X receptor (PXR), the aryl hydrocarbon receptor (AhR), and the constitutive androstane receptor (CAR) (Wang and LeCluyse, 2003). Two basic assay formats have been developed to predict the ability of a new chemical entity (NCE) to induce the drug metabolizing enzymes such as the cytochromes P450 (CYP). Early in drug discovery reporter gene assays that determine the activation of a receptor, for example PXR, or receptor ligand displacement assays, are employed as a measure of induction potential by the pharmaceutical industry and academic investigators. However, such specific interactions of the NCE with a single nuclear receptor fail to account for the emerging understanding of the coordinated regulation of expression of the CYPs by multiple nuclear receptors and induction mechanisms other than transcriptional regulation (Pascussi et al., 2003; Handschin and Meyer, 2003.). Thus the industry standard for predicting clinically significant induction of the CYPs by a NCE is to determine the change in catalytic activity of the CYPs in cultured primary human hepatocytes with and without exposure to potential inducers (Bjornsson et al., 2003). These hepatocyte assays in which the ultimate endpoint of clinical interest, change in CYP catalytic activity, is determined have the advantage over other model systems in that the response is

the culmination of the interactions with all the various receptors and induction mechanisms.

Prototypical inducers of the CYPs functioning through the major receptor pathways have been identified. Rifampicin, an inducer of CYP3A4 and CYP2C9, has been shown to induce primarily through a PXR mechanism (Wang and LeCluyse, 2003). Omeprazole, an inducer of CYP1A has been shown to induce the enzyme through an AhR mechanism (Wang and LeCluyse, 2003). Induction through the CAR mechanism is difficult to distinguish from response by the PXR mechanism since the ligands of CAR are often ligands of PXR and CAR response elements are often found in tandem with PXR response elements (Maglich et al., 2002; Pascussi et al., 2003). Thus, most studies examining the potential of a NCE or other agents to induce the CYPs focus on the induction of CYP3A and CYP1A2 as sentinel responses representing the large number of genes activated by the various mechanisms (Bjornsson et al., 2003; Wang and LeCluyse, 2003).

In an effort to increase the throughput of the pharmacokinetic studies during drug discovery, a strategy has evolved utilizing the administration of several compounds simultaneously as a cassette to a single animal. However, use of a cassette strategy can lead to uncertain results due to one or more compound interfering with the clearance of a co-administered compound (White and Manitpisitkul, 2001). This is a particular issue when compounds are dosed together for which no study of the potential for metabolic interaction between the compounds has been performed. However, combinations of 3 to 6 drugs,

demonstrated not to interact with each other, have been very successfully used in clinical studies as a cocktail to simultaneously determine the in vivo activities of several CYPs (Zhou et al., 2004). A major concern with the primary human hepatocyte induction model is the limited availability of high quality cells. Thus, by determining the activities of multiple CYPs per well of hepatocytes, a substrate cassette strategy would dramatically increase the information obtained from each preparation of human hepatocytes. Therefore, in the current study the potential interaction between the different probe substrates for three CYPs: midazolam for CYP3A4, diclofenac for CYP2C9, and phenacetin for CYP1A2, has been investigated in the primary human hepatocyte induction model when administered as a substrate cassette.

## Materials and Methods

### Materials

Midazolam maleate, diclofenac sodium, phenacetin, omeprazole, rifampicin, mefenamic acid and dicumarol were obtained from Sigma (St. Louis, MO). [$^{2}H_4$]- Acetaminophen was obtained from C/D/N Isotopes (Pointe-Claire, Canada). [$^{13}C_5$]-midazolam was synthesized and [$^{13}C_5$]-1'-hydroxymidazolam generated biosynthetically at Eli Lilly and Company. Hepatocyte maintenance media (HMM) was obtained from Cambrex Bio Science (Walkersville MD) supplemented with insulin and dexamethasone to a final concentration of 100 nM, gentamicin to a final concentration of 50 µg/ml, and amphotericin B to final concentration of 50 ng/ml. Acetonitrile was obtained from Burdick and Jackson (Muskegon MI). Formic acid was obtained from Mallinckrodt Baker (Paris KY). Human hepatocytes HH1114, HH1119 and HH1148 were obtained from Dr. Stephen Strom (University of Pittsburgh) and IVT 3/19/2004 was obtained from In Vitro Technologies (Baltimore, MD). Demographic information concerning the liver donors is presented in Table 1.

### Methods

*Incubation of Hepatocytes*

Media from the shipped hepatocytes was removed, replaced with fresh supplemented HMM and allowed to incubate for 24 hours at 37°C, 98% humidity and 5% carbon dioxide before initiating the experiment. The experiment was then initiated by replacement of the media with supplemented HMM containing

10 µM rifampicin for induction through a PXR mechanism, 50 µM omeprazole for induction through an AhR mechanism, or carrier (0.1% dimethylsulfoxide). Media containing the prototypical inducers or carrier were changed at 24 and 48 hours. At 72 hours the media containing the inducer or carrier were removed, replaced with fresh supplemented media and allowed to incubate for 10 minutes. The media were removed and dicumarol (1 mM) supplemented media (Bolanowska and Gessner, 1978) containing diclofenac (50 µM), midazolam (50 µM), and phenacetin (100 µM) were placed in triplicate wells both as a single substrate and as a cassette containing all three substrate probes at the indicated concentrations. As diclofenac and midazolam were added in water, the organic solvent burden of all incubations was only 0.1% DMSO. After the substrates were incubated for 30 minutes the media were removed from the plates and frozen at -80°C until analyzed. Formations of acetaminophen, 4'-hydroxydiclofenac and 1'-hydroxymidazolam were linear to at least 30 minutes after an expected loading phase.

Cellular protein was then determined by adding 1 ml of 100 mM potassium phosphate buffer to the wells and scraping the cells using a disposable cell scraper. Cells were sonicated and the protein content was measured using the Bradford protein assay (Bradford, 1976).

*Analysis of Enzyme Activity*

Enzyme activity was analyzed by adding 100 µl of sample medium to

100 µl of methanol containing internal standards. Internal standards used for the quantification of metabolites were mefenamic acid for 4'-hydroxydiclofenac, [$^{13}C_5$]-1'-hydroxymidazolam for 1'-hydroxymidazolam, and [$^{2}H_4$]-acetaminophen for acetaminophen.

Separation of the metabolites and internal standards were performed using a Javelin Aquasil C-18 (2.1 X 20 mm) pre-column and a Keystone ODS-AQ C18 (2 X 100 mm) analytical column. Sample, 50 µL, was injected using a HTC PAL injector (CTC Analytics). The high performance liquid chromatography system consisted of a Shimadzu LC AD90 with an isocratic pump (mobile phase 0.1% formic acid), pump A (0.1 % formic acid in water with 5% acetonitrile) and pump B (0.1% formic acid in water with 95% acetonitrile). Sample was injected onto the precolumn and washed with the isocratic pump operating at 0.75 ml/min for 1.5 min. A switching valve was then activated allowing for the solvent from gradient pumps A and B to elute the analytes from the precolumn onto the analytical column. The gradient used was: t = 0 min %B = 9.5, t = 1.55 min %B = 9.5, t = 6.2 min %B = 43, t = 7 min %B = 100, t = 8.5 min %B = 100, t = 9.0 min %B = 9.5, total runtime = 10.5 min.

Mass spectrometry was performed on a Micromass Quattro LC mass spectrometer operating in electrospray positive ionization mode using the MassLynx v 4.0 software package. Mass transitions monitored were acetaminophen 151.9 > 110.0 with internal standard [$^{2}H_4$]-acetaminophen 155.9 > 114.0, 1'-hydroxymidazolam 342.1 >168.1 with internal standard [$^{13}C_5$] 1'-hydroxymidazolam 347.0 >173.0, and 4'-hydroxydiclofenac 311.9 > 230.0 with

internal standard mefenamic acid 242.0 >180.0. Cone voltages and collision energies for each of the analytes were acetaminophen 16 V and 16 eV, [$^{2}H_{4}$]-acetaminophen 16 V and 20 eV, 1'-hydroxymidazolam and [$^{13}C_{5}$] 1'-hydroxymidazolam 20 V and 40 eV, diclofenac and mefenamic acid 40 V and 30 eV respectively. Retention times were acetaminophen and [$^{2}H_{4}$]-acetaminophen 4.4 min., 1'-hydroxymidazolam and [$^{13}C_{5}$] 1'-hydroxymidazolam 6.3 min., diclofenac 8.0 min., and mefenamic acid 8.7 min. Formation of metabolite was quantitated by comparing peak area ratios of (metabolite / internal standard) to the peak area ratios generated with a standard curve using the QuanLynx v 4.0 software system. Standard curve correlation coefficients ($r^2$ values) were greater than 0.99.

Quantitation of acetaminophen was linear from 78 to 10000 nM with an inter-day variability (% coefficient of variation, CV) of 13.2% at 78 nM, 4.7% at 1250 nM, and 1.5% at 10000 nM. The lower limit of quantitation and upper limit of quantiation for 1'-hydroxymidazolam were 625 nM and 10000 nM respectively. The inter-day %CVs observed were 13.8% at 625 nM, 6.5% at 2500 nM, and 5.5% at 10000 nM. Finally, 156 nM and 20000 nM were the lower limit of quantitation and upper limit of quantitation respectively for 4'-hydroxydiclofenac. Interday %CVs were 15.7% at 156 nM, 6.2% at 2500 nM, and 1.6% at 20000 nM.

*Statistical Analysis*

The formation rate data were log transformed to allow for an evaluation of differences in fold-induction between the two dosing methods. The mean and variance was then determined for each group and fold induction calculated by

taking the difference of the mean positive control and mean vehicle control. To determine if the fold induction was different between dosing methods, a 95% confidence interval (CI) for the difference in the fold-induction was calculated. When the sample variances across each dosing method were the same, the CI was calculated by pooling the variances for all groups. In all cases where the sample variances across groups were not equal, the within group variances were determined to be equal. In these situations, the within group variances were pooled and the CI for the difference in the two dosing methods was determined using unequal variances. This gave a lower bound and upper bound for the difference in the fold-induction values. The CI was then transformed back to the original form of the data by taking the inverse log of lower bound and upper bound. When this interval contained 1.0, it was concluded that there was no difference in the fold induction between the single versus cassette substrate methods (Casella and Berger, 2002).

## Results and Discussion

The possibility of an interaction between the probe substrates, midazolam, phenacetin, and diclofenac, used in this study was evaluated. This was necessary even though an interaction would not be expected from information obtained from the literature, since the probes are either not substrates or are very poor substrates for the other enzymes being examined. The CYP3A probe, midazolam, has been shown to not to be a substrate for either CYP2C9 or CYP1A2 (Hamaoka et al., 2001). Phenacetin, the CYP1A2 probe, on the other hand is O-deethylated by CYP2C9, however this reaction occurs at a relatively high $K_m$ value of 566 µM compared to 31 µM for CYP1A2 (Venkatakrishnan et al., 1998) and thus should have little effect on CYP2C9 activity. Diclofenac has been shown to be metabolized by both CYP2C9 to the 4'-hydroxy metabolite and partially by CYP3A to the 5-hydroxy metabolite, an alternative pathway not measured in this assay. The reported intrinsic clearance for the formation of the 4'-hydroxy metabolite is much greater than that for the 5-hydroxy metabolite, 48 µL/min/mg and 0.36 µL/min/mg respectively (Bort et al., 1999). The predicted lack of interactions between the probe substrates was confirmed by preliminary experiments (not shown) using suspensions of cryopreserved human hepatocytes which demonstrated no interaction between these three probes and thus definitive studies with and without known inducers in plated cultures of primary human hepatocytes were undertaken.

The catalytic activities of CYP3A4, CYP2C9, and CYP1A2 were measured in cultured primary human hepatocytes using midazolam, diclofenac, and

phenacetin, respectively, as probe substrates. The results of the studies reported here demonstrated that the substrates may be administered simultaneously yielding similar results as compared to adding them to the culture as single agents. The short incubation time, low solvent load, and the fact that the observed induction responses with the prototypical inducers were similar to previous reports, indicate that little or no toxicity would be expected using the cassette dosing protocol. The 95% CI for the ratio of the fold-induction between the different substrate adminstration methods (single/cassette) was examined in four cultures of hepatocytes treated with omeprazole to induce CYP1A2 (Table 2). Three of the four CIs for the ratio of activity after single versus cassette administration contained 1.0, while the fourth (HH1119) had an upper bound of 0.9. For HH1119, this relatively small difference is not considered to be of biological significance. When examining the induction of CYP3A4 by midazolam 1'-hydroxylase activity (Table 3), all four CIs for activity ratio after single versus cassette administration contained 1.0, indicating no difference in the fold-induction by the different methods when using rifampicin as an inducer of CYP3A. Similarly, when examining CYP2C9 induction by diclofenac 4'-hydroxylase activity (Table 4) the 95% CI for the activity ratio contained 1.0 for all four cultures. In total, these results clearly demonstrate that phenacetin, midazolam, and diclofenac may be used as a substrate cassette for the simultaneous determination of the catalytic activities of CYP1A2, CYP3A4, and CYP2C9, respectively, in the determination of the fold-induction of these CYPs in cultures of human hepatocytes.

The activities observed for the different hepatocyte preparations exhibited an increase in activity of all three CYPs with exposure to a known potent inducer. Both CYP3A and CYP2C9 were induced by rifampicin in these studies and showed the expected induction pattern (Soars et al., 2004). Although CYP3A and CYP2C9 are induced through the PXR mechanism, there was a marked difference in the fold-induction for these two enzymes in this study. The observed, relatively large induction of CYP3A of 4.7 to 7.5–fold (Table 3) is likely due to the CYP3A promoter region containing both an everted repeat 6 (ER-6) and xenobiotic response element (XREM) (Goodwin et al., 1999). On the other hand, the CYP2C9 promoter region contains only an everted repeat-5 (ER-5) (Chen et al., 2004) and this likely relates to the observed lower induction response to rifampicin (1.5 to 3.4-fold) compared to CYP3A (Table 4). As shown in Table 2 induction of CYP1A2 by omeprazole also fell within the expected range (11- to 41-fold) (Soars et al., 2004). The CYP3A and CYP1A2 induction responses were well above the recently recommended minimum two-fold increase in activity as a positive measure of induction in studies using human hepatocytes (Bjornsson et al., 2003). Consistent with another report (Madan et al., 2003), CYP2C9 induction by rifampicin did not always exhibit this minimal inductive response. This low response of CYP2C9 to a strong inducer draws into question its usefulness in a routine screen of induction since the dynamic range of the response is very limited. Further, the induction response through the PXR nuclear receptor mechanism is more reliably accessed by the CYP3A induction response.

The results presented here demonstrate that the fold-induction responses of CYP1A2, CYP2C9, or CYP3A in human hepatocytes treated with rifampicin and omeprazole, measured with single substrate or the cassette, were not significantly different for all three CYPs (Tables 2-4). In conclusion, these results clearly demonstrate that a substrate cassette of phenacetin, midazolam, and diclofenac may be administered to primary cultures of human hepatocytes yielding similar results as single substrates, thus increasing the data obtained from each experiment with human hepatocytes by a factor of three.

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**Table 1. Demographic Information for Hepatocyte Donors**

| Donor | Age | Sex | Race | Drug Use History |
|---|---|---|---|---|
| HH1119 | 29 | F | Caucasian | Unknown |
| HH1139 | 3 | M | Caucasian | None |
| HH1148 | 60 | M | Caucasian | Unknown medications for hypertension, hyperlipidemia and a heart condition |
| IVT3/19/2004 | 29 | F | African American | Marijuana, cocaine, ethanol |

**Table 2. Phenacetin Deethylase Activities (pmol/mg/min) and Fold Induction by Omeprazole**

| | | Activities ± SEM | Fold Induction | | 95 % CI for Ratio |
|---|---|---|---|---|---|
| **HH1114** | Control - single | 26.4 ± 3.8 | Single | Cassette | [0.8, 1.7] |
| | Control - cassette | 16.1 ± 1.7 | | | |
| | Omeprazole - single | 331 ± 10.6 | 12.5 ± 0.4 | 14.4 ± 0.8 | |
| | Omeprazole - cassette | 232 ± 13.6 | | | |
| **HH1119** | Control - single | 15.4 ± 2.1 | Single | Cassette | [0.4, 0.9] |
| | Control - cassette | 16.5 ± 0.5 | | | |
| | Omeprazole - single | 279 ± 23.0 | 18.0 ± 1.5 | 10.9 ± 0.5 | |
| | Omeprazole - cassette | 180 ± 9.0 | | | |
| **HH1148** | Control - single | 4.7 ± 0.5 | Single | Cassette | [0.6, 1.4] |
| | Control - cassette | 5.2 ± 0.5 | | | |
| | Omeprazole - single | 195 ± 7.3 | 41.4 ± 5.7 | 37.2 ± 1.4 | |
| | Omeprazole - cassette | 194 ± 27.0 | | | |
| **IVT** | Control - single | 3.8 ± 0.0 | Single | Cassette | [0.4, 1.6] |
| | Control - cassette | 3.7 ± 0.1 | | | |
| | Omeprazole - single | 66.7 ± 14.1 | 17.2 ± 3.7 | 14.3 ± 3.0 | |
| | Omeprazole - cassette | 52.6 ± 11.2 | | | |

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**Table 3. Midazolam 1'-hydroxylase Activities (pmol/mg/min) and Fold-Induction by Rifampicin**

| | | Activities ± SEM | Fold Induction | | 95 % CI for Ratio |
|---|---|---|---|---|---|
| **HH1114** | Control - single | 99.7 ± 1.2 | Single | Cassette | [1.0, 1.4] |
| | Control - cassette | 111.9 ± 4.9 | | | |
| | Rifampicin - single | 399.1 ± 13.2 | 4.0 ± 0.1 | 4.6 ± 0.5 | |
| | Rifampicin - cassette | 520.7 ± 51.4 | | | |
| **HH1119** | Control - single | 100.3 ± 4.6 | Single | Cassette | [1.0, 1.3] |
| | Control - cassette | 101.0 ± 2.8 | | | |
| | Rifampicin - single | 408.8 ± 12.5 | 4.1 ± 0.1 | 4.7 ± 0.1 | |
| | Rifampicin - cassette | 479.0 ± 9.7 | | | |
| **HH1148** | Control - single | 48.2 ± 3.0 | Single | Cassette | [0.7, 1.8] |
| | Control - cassette | 47.7 ± 6.5 | | | |
| | Rifampicin - single | 323.0 ± 22.7 | 6.7 ± 0.5 | 7.5 ± 0.8 | |
| | Rifampicin - cassette | 357.4 ± 40.5 | | | |
| **IVT** | Control - single | 60.5 ± 3.1 | Single | Cassette | [0.9, 1.3] |
| | Control - cassette | 76.7 ± 4.4 | | | |
| | Rifampicin - single | 215.6 ± 5.4 | 3.6 ± 0.1 | 3.7 ± 0.1 | |
| | Rifampicin - cassette | 285.1 ± 9.6 | | | |

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**Table 4. Diclofenac 4'-hydroxylase Activities (pmol/mg/min) and Fold-Induction by Rifampicin**

| | | Activities ± SEM | Fold Induction | | 95 % CI for Ratio |
|---|---|---|---|---|---|
| **HH1114** | Control - single | 27.9 ± 3.6 | Single | Cassette | [0.5, 1.5] |
| | Control - cassette | 35.2 ± 3.7 | | | |
| | Rifampicin - single | 58.6 ± 8.6 | 2.1 ± 0.3 | 1.9 ± 0.1 | |
| | Rifampicin - cassette | 65.3 ± 4.7 | | | |
| **HH1119** | Control - single | 12.2 ± 0.7 | Single | Cassette | [0.5, 1.0] |
| | Control - cassette | 14.9 ± 0.7 | | | |
| | Rifampicin - single | 26.7 ± 3.8 | 2.2 ± 0.3 | 1.5 ± 0.0 | |
| | Rifampicin - cassette | 22.5 ± 0.3 | | | |
| **HH1148** | Control - single | 15.0 ± 0.4 | Single | Cassette | [0.6, 1.2] |
| | Control - cassette | 13.0 ± 1.1 | | | |
| | Rifampicin - single | 51.1 ± 1.6 | 3.4 ± 0.1 | 2.9 ± 0.7 | |
| | Rifampicin - cassette | 38.0 ± 8.9 | | | |
| **IVT** | Control - single | 13.6 ± 1.0 | Single | Cassette | [0.4, 1.1] |
| | Control - cassette | 14.0 ± 1.8 | | | |
| | Rifampicin - single | 39.5 ± 1.4 | 2.9 ± 0.1 | 2.0 ± 0.3 | |
| | Rifampicin - cassette | 28.3 ± 4.1 | | | |