#1864 Changes in Phase I and Phase II Enzyme Activities Over Time In Plated Human Hepatocytes

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ABSTRACT

It is well known that P450 activities in hepatocytes will diminish over time in cultured hepatocytes as documented in studies using rat cultured hepatocytes and human liver slices measuring P450 activities. Other reports have included P450 mRNA losses in human cultured hepatocytes but without comparative enzyme activities of the major drug metabolizing CYP enzymes. Here, we report measuring the P450 enzyme activities along with UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) in cultured human hepatocytes. Plateable cryopreserved human hepatocytes were used to determine the enzymatic activity of CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4, as well as UGT and SULT over a time course of 36 hours. There was an increase in activities for CYP2B6, 2C9, 2C19 and 2E1 in the first four hours after attachment, while the other CYPs decreased. All CYP activities trended down at the 12-, 18-, 24- and 36-hour time points. The raw activities did vary from donor to donor; however, the trend in the percent loss was similar in most CYPs with average T1/2 of 22.9 hours. UGT activity increased over first four hours, then remained stable out to 36 hours. SULT activity varied from donor to donor with an increase in the first four hours and then differences in maintaining the activity to 36 hours. One donor loss activity from the four hour value but maintained approximately the initial value measured. The other donors retained their initial increases in activity. In all, we confirmed the reported loss of activities in human hepatocytes and the stability of UGT activity. Retention of SULT activity was unique to the reported loss in human liver slices and rat hepatocyte cultures.

INTRODUCTION

Isolated hepatocytes, parenchymal cells of the liver, represent the gold standard for *in vitro* use since these cells retain most of the hepatocytes' *in vivo* activities, such as metabolic capacity and transporter expression (MacGregor 2001, Li 1999). For this reason, hepatocytes have been utilized by the pharmaceutical industry as a model for determining metabolic profiles and drug-drug interactions of new chemical entities (NCEs) (Chu 2009, Reddy 2005) and have been considered an acceptable model by the FDA for safety assessments (FDA 2006). As well, the use of primary hepatocytes as an *in vitro* model for determining hepatotoxicity has shown strong correlation to *in vivo* hepatotoxicity (Kienhuis 2009, Kikkawa 2006).

The hepatocytes present a complex system that may complicate data interpretation. One issue is the loss of CYP enzymes when cultured for extended periods of time. This phenomenon has been documented in different hepatocytic systems. When human liver slices were used to determine Phase I (CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4) and Phase II (UGT and SULT) enzyme activities over time in culture (Vandenbranden 1998), decreases in CYP and SULT activities were observed, while UGT activities were maintained. However, liver slices are not commonly used for drug screening due to a required high technical expertise and low through-put capacity of the assays. Dissociated hepatocytes in culture are more commonly used. Changes in both mRNA and enzymatic activity of CYP3A4 have been demonstrated in cultured human hepatocytes; however, other important CYP enzymes have not been studied (Lecluyse 2001). In addition, while an extensive survey of hepatocyte gene expression has been performed, the time frame did not capture the early alterations prior to 24 hours, and activity was not compared (Richert 2006). A gap in information still exists for early alterations in Phase I and Phase II activities that may affect assay design and data interpretation. Herein, we present a more extensive profile of major drug metabolizing enzymes in hepatocytes cultured over 36 hours as measured by enzymatic activity.

MATERIALS AND METHODS

Hepatocyte Preparation: Cryoplateable human hepatocyte lots AAS, KQG and SCT were obtained from BioreclamationIVT (Baltimore, Maryland) and prepared as described in product literature. Briefly, cryopreserved human hepatocytes were thawed at 37°C for approximately two minutes and transferred into 5 mL of *InVitro*GRO™ CP medium at 37°C. The hepatocyte suspension was counted for viability and cell concentration using Trypan blue exclusion. Cell density was diluted to a final concentration of 0.7 x 10^6 viable cells per mL. Cells were dispensed into 48-well collagen I coated culture plates at a final density of 140,000 viable hepatocytes per well. Plates were maintained for duration of experiment in a 37°C, 5% CO_2, humidified incubator. Hepatocytes were washed with *InVitro*GRO CP medium at two hours after plating to remove unattached cells. Plates were returned to 37°C, 5% CO_2, humidified incubator until specified times for enzymatic activities determination.

Enzymatic Activity Characterization: At the given time points of 2, 4, 12, 18, 24 and 36 hours after plating, media was changed to *InVitro*GRO KHB medium with specific substrates for Phase I and Phase II enzymes (Table 1). Hepatocytes were returned to 37°C, 5% CO_2, humidified incubator for 30 minutes. Reactions were terminated using equal volume of methanol. Each data point was performed in triplicate. Samples were stored at -80°C until analyzed using HPLC, LC/MS/MS or UPLC/MS/MS protocols.

Viability Assay: CellTiter Glo® (Promega, Madison Wisconsin) luminescent cell viability assay was used to assess culture viability by measuring ATP content. Assay was performed as described in product literature.

Data Analysis: Metabolite formation concentrations determined by bioanalytical methods were averaged from the triplicate samples for each substrate and time point. Specific activity (pmol/min/10^6) was derived by dividing the amount of metabolite (pmol) by incubation time (min) and scaled to million viable hepatocytes (10^6). Data was normalized to the two-hour time point as the baseline activity to achieve percent of activity. $T_{1/2}$ was derived using GraphPad Prism® least-squares linear regression. Graphs were plotted using Microsoft Excel®.

Enzyme	Substrate	Abbreviation	[uM]	Metabolites
CYP1A2	Phenacetin	Phen	15	Acetaminophen
CYP2A6	Coumarin	Coum	8	7-HC, 7-HCG, 7-HCS
CYP2B6	Bupropion	Bup	50	OH-Bupropion
CYP2C9	Tolbutamide	Tolb	15	4-OH Tolbutamide
CYP2C19	S-mephenytoin	Meph	20	4-OH Mephenytoin
CYP2D6	Dextromephorphan	Dex	5	Dextrorphan
CYP2E6	Chlorzoxazone	CZX	50	6-OH Chlorzoxazone
CYP3A4	Testosterone	Test	50	6β-OH Testosterone
CYP3A4	Midazolam	Mid	4	1-OH Midazolam
SULT	7-hydroxycoumarin	7-HCS	30	7-HC Sulfate
UGT	7-hydroxycoumarin	7-HCG	30	7-HC Glucuronide

Table 1. Substrates, associated CYP enzyme, final concentration of substrates in reaction buffer and metabolite formed from reaction are listed.

		$T_{1/2}$							
		Lot AAS		Lot KQG		Lot SCT		Summary	
Substrate	Enzyme	Hours	95% Confidence	Hours	95% Confidence	Hours	95% Confidence	Hours	95% Confidence
Phenacetin	CYP1A2	21.21	17.79 to 25.57	19.67	17.61 to 21.96	25.28	24.26 to 26.40	21.97	20.47 to 23.64
Coumarin	CYP2A6	NA	NA	20.92	19.76 to 22.17	25.14	24.32 to 26.03	22.85	21.85 to 23.92
Bupropion	CYP2B6	17.17	14.37 to 20.12	24.92	22.63 to 27.73	26.80	24.63 to 29.47	23.58	21.54 to 26.01
Tolbutamide	CYP2C9	26.76	22.40 to 33.70	40.27	33.89 to 51.39	24.02	21.50 to 27.17	29.74	26.47 to 34.27
s-Mephenytoin	CYP2C19	16.84	12.77 to 21.11	26.20	23.73 to 29.31	24.86	23.16 to 26.84	22.30	20.24 to 24.70
Dextromethorphan	CYP2D6	21.24	18.66 to 24.33	22.19	19.96 to 24.83	18.48	15.90 to 21.31	20.57	19.06 to 22.23
Chlorzoxazone	CYP2E1	19.85	17.36 to 22.69	18.76	16.41 to 21.35	23.46	21.50 to 25.76	20.60	19.26 to 22.05
Testosterone	CYP3A4	20.39	18.09 to 22.90	24.80	22.04 to 28.37	36.07	31.24 to 43.61	25.09	20.86 to 31.59
Midazolam	CYP3A4	18.36	16.18 to 20.71	21.39	16.95 to 27.63	22.56	20.57 to 24.87	20.69	19.02 to 22.55
						Average All CYPs		22.94	19.45 to 27.67

Table 2. $T_{1/2}$ values and 95% confidence range measured in hours from three individual lots of cryoplateable human hepatocytes and the summary of the combined data from the three lots.

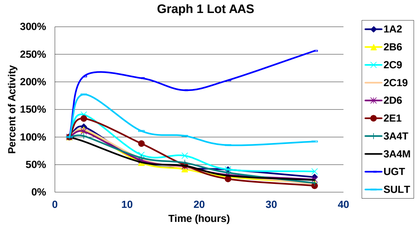
RESULTS AND DISCUSSION

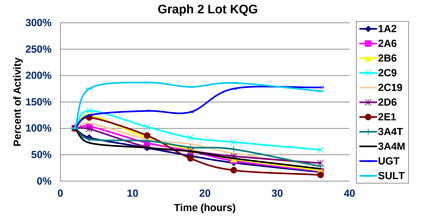
Phase I Enzymes: The hepatocytes' viability were well maintained during the 36 hours in culture as measured by CellTiterGlo assay. No significant differences were observed in the ATP levels from any of the time points (data not shown). Thus, any differences in activity would be due to enzymatic influences and not general health of the cells. All CYP enzymatic activity was reduced from the 12-hour time point to the 36-hour time point as previously observed in the literature. However, the rate of activity loss was not symmetrical across the CYPs tested (Tables 3, 4 and 5). At the 4-hour time point, CYP2B6, 2C9 and 2E1 consistently increased in activity in comparison to the 2-hour time point for the three lots, and CYP2C19 increased for two of the three lots. The reasons for this difference need to be explored. Presumably, it is not an artifact of increased co-factors since not all CYP activities were increased. In the case of CYP2E1, ligand stabilization of the enzyme may contribute to the increase in activity as found in the literature, but this increase in the first few hours after attachment has not been cited, nor has stabilization of CYP2B6, 2C9 and 2C19 been reported.

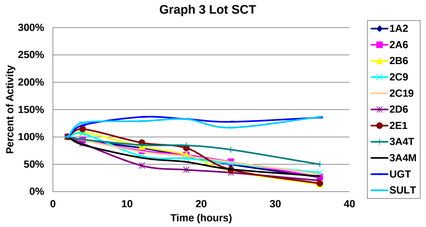
The enzyme $T_{1/2}$ was determined for each CYP (Table 2). The $T_{1/2}$ varied from lot to lot which may be due to CYP enzyme expression differences, quality of the tissue, genotype or other idiosyncratic factors. The minimum $T_{1/2}$ measured was CYP2C19 in lot AAS at 16.84 hours, though lots KQG and SCT had CYP2C19 $T_{1/2}$ of 26.20 and 24.86 hours, respectively. The highest observed $T_{1/2}$ was CYP2C9 in lot KQG at 40.27 in comparison to lot AAS at 26.76 and lot SCT at 24.02 hours. To reduce the individual variation, the data from the three lots were combined to generate an average $T_{1/2}$. In the summary data set (Table 2), the range between the lowest to highest $T_{1/2}$ was smaller with CYP2D6 as the least stable at 20.57 hours and CYP2C9 as the most stable at 29.74 hours. The latter result may be skewed due to the 40.27 hour $T_{1/2}$ observed in lot KQG. The data from all the CYPs from the three lots were combined to determine an overall $T_{1/2}$ value of 22.94 hours for CYP enzymatic activity.

Phase II Enzymes: UGT and SULT activities were found to maintain or increase for the 36 hours in culture. Again, variations were observed from lot to lot and between enzymes. UGT activities have previously been reported to be stable in liver slices (Vandenbranden 1998); however, mRNA levels in cultured hepatocytes were found to decrease in the first 24 hours (Richert 2006). We observe the same trend in cultured cryoplateable human hepatocytes as measured by enzyme activity. For lots AAS and SCT, the UGT activities increased between 2 and 4 hours, then maintained the achieved plateau for the remainder of the time course. (Tables 3 and 5). The increase in UGT activities for lot KQG was biphasic. An increase was observed between the 2- and 4-hour time points, and another between the 18- and 24-hour time points (Table 4).

SULTs have been reported to decrease in activity in human liver slices (Vandenbranden 1998). In contrast, we observed increases for all three lots between the 2- and 4-hour time points. Lots KQG and SCT maintained the plateau in activities for the duration of the experiment. Lot AAS showed an increase of UGT activities between 2 and 4 hours, but diminished to about the initial activity by the 12-hour time point. The SULT activity then stabilized and remained near initial activity for the remainder of the experiment.







Graphs 1, 2 and 3: Temporal changes of Phase I and Phase II metabolic activity measured in cryoplateable human hepatocytes using specific substrates. The activity rates were normalized to the two-hour time point.

Table 3.

Lot AAS	Phen	Coum	Bup	Tolb	Meph	Dex	CZX	Test	Mid	7-HCG	7-HCS
Time (Hr)	1A2	2A6	2B6	2C9	2C19	2D6	2E1	3A4	3A4	UGT	SULT
2	4.1	NA	1.34	11.0	2.92	4.9	39.3	31	6.25	176	39
4	4.9	NA	1.52	15.5	3.34	5.4	52.5	32	5.75	370	69
12	2.2	NA	0.72	7.5	1.77	2.8	34.7	19	3.39	364	43
18	1.8	NA	0.56	7.3	1.28	2.3	19.4	17	3.00	326	40
24	1.7	NA	0.37	4.5	0.92	1.5	9.5	11	1.87	358	33
36	1.1	NA	0.22	4.1	0.43	1.1	4.6	5	1.38	451	36

Table 4.

Lot KQG	Phen	Coum	Bup	Tolb	Meph	Dex	CZX	Test	Mid	7-HCG	7-HCS
Time (Hr)	1A2	2A6	2B6	2C9	2C19	2D6	2E1	3A4	3A4	UGT	SULT
2	11.6	90	11.9	35	0.70	51.5	25.1	183	33	458	10
4	9.6	94	14.9	47	0.77	51.0	30.2	145	24	572	18
12	7.3	65	9.7	36	0.56	33.3	21.7	140	21	610	19
18	5.6	51	7.1	29	0.49	29.4	10.8	118	19	600	18
24	4.1	35	5.0	26	0.37	24.3	5.2	111	14	803	19
36	2.0	17	2.4	21	0.20	17.6	2.9	51	8	813	17

Table 5.

Lot SCT	Phen	Coum	Bup	Tolb	Meph	Dex	CZX	Test	Mid	7-HCG	7-HCS
Time (Hr)	1A2	2A6	2B6	2C9	2C19	2D6	2E1	3A4	3A4	UGT	SULT
2	14.0	182	109	80	1.58	48.3	44.0	214	47	781	22
4	13.1	172	119	85	1.49	42.5	50.4	204	40	949	28
12	11.1	136	90	51	1.15	22.9	39.3	182	29	1066	28
18	9.1	122	74	48	1.02	19.3	35.1	181	26	1036	29
24	7.0	99	41	41	0.85	16.7	17.7	164	19	997	26
36	3.7	48	14	28	0.50	9.8	6.6	107	13	1058	30

Tables 3, 4 and 5: Phase I and Phase II metabolic activity measured in cryoplateable human hepatocytes over a 36-hour time course. The rates are expressed in pmoles of metabolite formed per minute per million viable hepatocytes (pmol/min/10^6).

CONCLUSION

The loss of CYP activity has been reported in various hepatic systems, such as liver slices and dissociated hepatocytes, and measured by enzymatic activity and mRNA levels. However, gaps in the knowledge still remain. We set out to elucidate the changes in Phase I and Phase II enzymatic activities in cryoplateable human hepatocytes within the first 36 hours after attachment. We have confirmed most of the previous observations with the overall decrease in CYP activities by the 36-hour time point. However, a subset of enzymes (CYP2B6, 2C9, 2C19 and 2E1) increased up to the 4-hour time point before decreasing. Further studies would be required to explain this phenomenon. As previously described, UGT activities were confirmed to be stable in culture as measured by activity. SULT activities, however, did not decrease as demonstrated in liver slices, but increased or at least maintained their initial activity.

The time in culture can affect the activities of Phase I and Phase II enzymes and will have to be taken into account for clearance determination. The loss of activity, however, can be advantageous when determining induction potential by lowering the basal rate of activity. Further study is needed to investigate potential influences to the enzymatic activity, such as media components, culture conditions and donor genotypes.

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