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APPLICATION PAPER

Effects of Incubation Buffer Conditions on *In Vitro* Primary Cryopreserved Hepatocyte Phase I and Phase II Metabolism

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Hepatocyte suspension metabolism assays of new chemical entities (NCE) are widely used in determining *in vitro* compound stability for use in human *in vivo* extrapolations. Preclinical species selection and incubation conditions vary across laboratories and organizations. These variations can cause significant differences in the predicted clearance values used for first in human (FIH) dosing prediction. Here we explore the impact of incubation buffer selection on the resultant Phase I and Phase II metabolism of prototypic substrates in a standard cryopreserved hepatocyte suspension assay.

Primary cryopreserved hepatocytes from multiple species including Human, Dog, and SD Rat were incubated with prototypic substrates in single time point assays in a variety of incubation buffers which included INVITROGRO™ Krebs Henseleit Buffer (KHB), standard KHB, Williams' E medium, and Phosphate Buffered Saline (PBS).[1] Moderate to significant differences were seen between species types and between the different incubation buffers, and those differences varied in both Phase I and Phase II enzymes. The metabolism of testosterone to 6β-hydroxytestosterone was on average 21% (±14%) lower in all species when compared to INVITROGRO KHB for cells incubated in standard KHB buffer. In contrast, species differences were seen between buffers in the metabolism of tolbutamide to 4-hydroxytolbutamide.

Human showed lower metabolism in standard KHB and Williams' E when compared to INVITROGRO KHB. The relationship was reversed in rat, where standard KHB and Williams' E were higher than INVITROGRO KHB by a similar magnitude. The substrate and species-specific differences between buffer systems were seen with substrates of other P450 and Phase II enzymes and are detailed in this work. These differences highlight the need for researchers to carefully examine the systematic biases in the generation of *in vitro* metabolism data with primary hepatocytes. The effects of different buffer systems can impact the estimated metabolic stability of a compound based on both which species are being tested, and which Cytochrome P450 or conjugating enzymes are involved.

Methods

Hepatocyte Metabolism

Cryopreserved hepatocytes from Sprague Dawley Rat, Beagle Dog, and human were obtained from BioIVT (Baltimore, MD). Hepatocytes were thawed according to manufacturer's protocol. Cells were thawed at 37°C for 90 seconds, transferred into INVITROGRO HT Media and spun at 50xg for 5 minutes to pellet cells. Cell pellets were resuspended in one of four different incubation buffers (INVITROGRO KHB, Sigma KHB, Williams' E, or PBS), counted and cell concentration adjusted to 2x10^6 cells/ml. Sigma KHB, Williams' E, and PBS buffers were all purchased from Sigma (St. Louis, MO). INVITROGRO KHB was obtained from BioIVT (Baltimore, MD). Metabolite formation assays were run by aliquoting 0.2x10^6 cells into 48 well plates along with CYP450 enzyme substrates including Testosterone, Midazolam, 7-ethoxycoumarin, Phenacetin, Amodiaquine, and Tolbutamide. After a 30 minute incubation, reactions were stopped by the addition of an equal volume of methanol. Substrate formation was quantitated by LC/MS/MS and calculated as mol/min/10^6 cells. Values were normalized by dividing the metabolite rate for each media within each species and substrate group by the value for the INVITROGRO sample within that group.

ATP Level Assay

A quantitative ATP assay time course was run using the CellTiter-Glo® Luminescent Cell Viability Assay from Promega (Madison, WI). One lot of cryopreserved hepatocytes from each species was tested at zero minutes, 30 minutes, and 60 minutes. Cells were thawed and counted as described previously before being diluted to a concentration of 5x10^5 cells/mL. 100µL of the diluted cells were then aliquoted into a 96-well plate.Following the CellTiter-Glo Luminescent Cell Viability Assay instructions, an equal amount of reagent was added to each well at the appropriate time points. The cells were then shaken for two minutes to induce cell lysis, left to incubate at room temperature for 10 minutes, and then the luminescent signal was read on a Wallac Victor2 plate reader.

Media Components

Formulations of tested incubation buffers expressed in g/L. Components and formulations found on vendor sites or internal documentation. INVITROGRO™ KHB contains additional proprietary components not listed.

Component	IVT KHB	Sigma KHB	Williams E	PBS
MgS04 (g/L)	0.141	0.141	0.0977	
KCl (g/L)	0.35	0.35	0.4	0.2
KH2P04 (g/L)	0.16	0.16		0.24
NaCl (g/L)	6.9	6.9	6.8	8
D-Glucose (g/L)	2	2	2	
NaHCO3 (g/L)	2.1	2.1	2.2	
Na2HPo4				1.44
CaCl (g/L)	0.147		0.2	
Cupric Sulfate (g/L)			0.0000001	
Ferric Nitrate (g/L)			0.0000001	
Magnesium Sulfate (g/L)			0.0000001	
Sodium Phosphate Monobasic (g/L)			0.122	
Zinc Sulfate (g/L)			0.0000002	
HEPES (g/L)	4.765			

Williams' E media also contains the following:
Amino Acids (g/L) *Glycine 0.05, L-Alanine 0.09, L-Arginine 0.05, L-Asparagine-H20 0.02, L-Aspartic acid 0.03, L-Cysteine 0.04, L-Cystine 2HCl 0.02607, L-Glutamic Acid 0.05, L-Histidine 0.015, L-Isoleucine 0.05, L-Leucine 0.075, L-Lysine hydrochloride 0.08746, L-Methionine 0.015, L-Phenylalanine 0.025, L-Proline 0.03, L-Serine 0.01, L-Threonine 0.04, L-Tryptophan 0.01, L-Tyrosine disodium salt dehydrate 0.05065, L-Valine 0.05.* ***Vitamins (g/L)*** *Ascorbic Acid, 0.002, Biotin 0.0005, Choline chloride 0.0015, D-Calcium pantothenate 0.001, Ergocalciferol 0.0001, Folic Acid 0.001, Menadione sodium bisulfat 0.00001, Niacinamide 0.001, Pyridoxal hydrochloride 0.001, Riboflavin 0.0001, Thiamine hydrochloride 0.001, Vitamin A (acetate) 0.0001, Vitamin B12 0.0002, alpha Tocopherol phos. Na salt 0.00001, i-Inositol 0.002.*

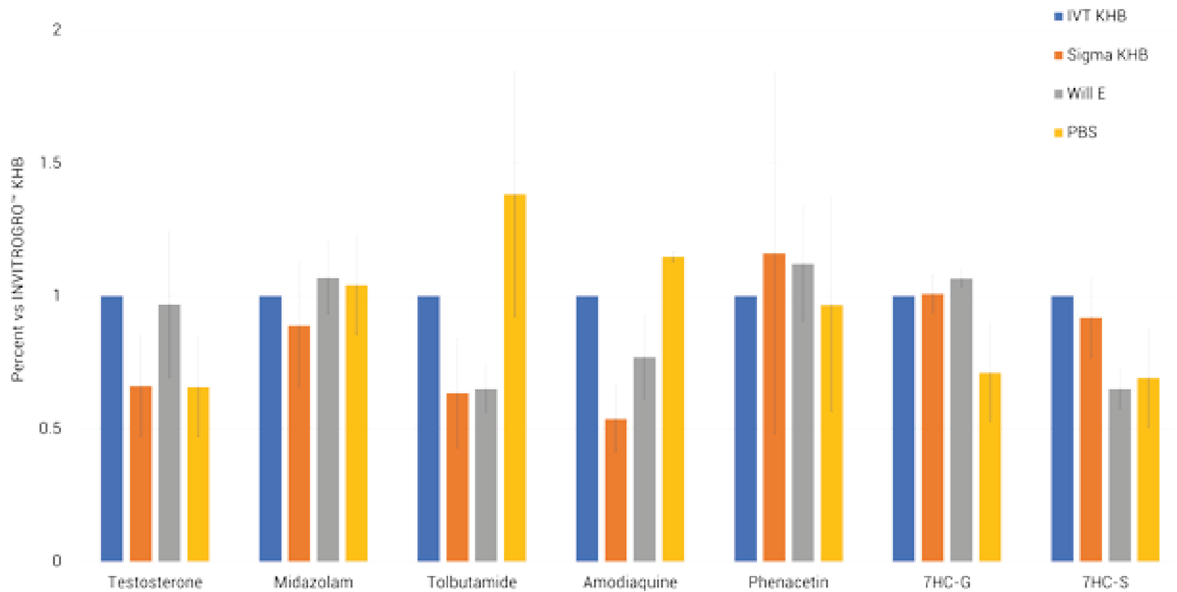
P450 Enzyme Isoforms

Prototypic substrates for human CYP450 enzymes were used for hepatocyte incubations across species. In general, genetically conserved p450 homologues were responsible for metabolite formation across species. For some substrates, in the animal species used, the assayed metabolite may be generated by more than one p450 isoform. Isoforms for beagle dog metabolism of tolbutamide and amodiaquine have not been well delineated, and in some cases, turnover is considered low. Detectable metabolites were formed for all substrate, in all species.

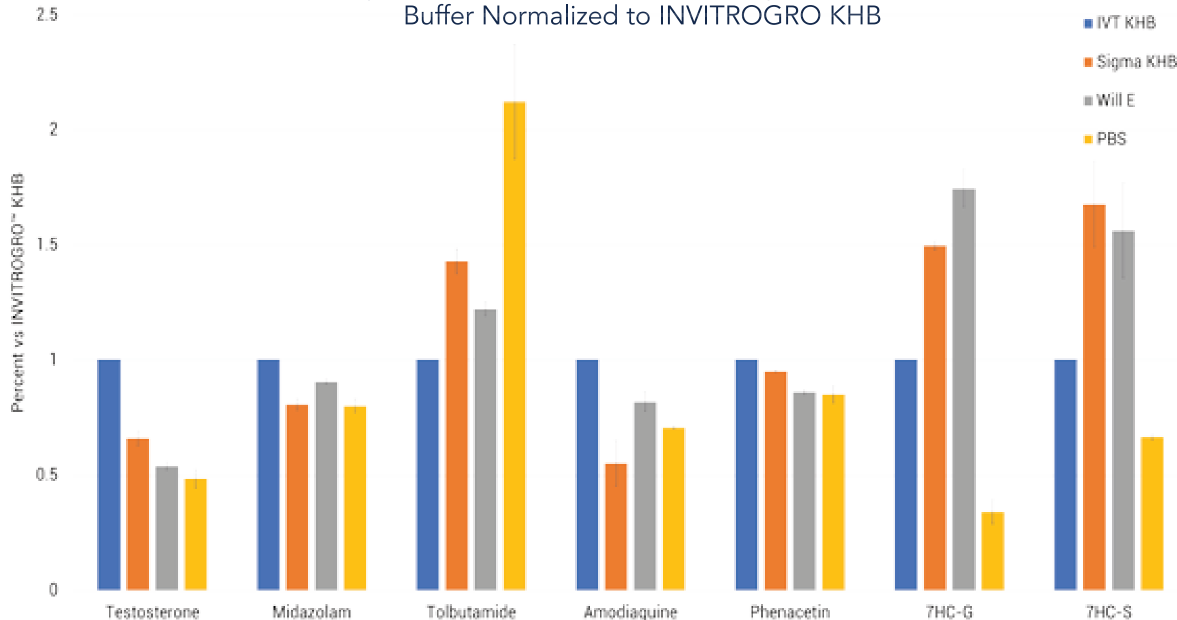
	P450 Isoform Involved in Metabolite Formation		
	Human	Rat	Dog
Phenacetin	1A2	1A,	1A1, (2A13)[2]
Testosterone	3A4	3A	3A12 [3]
Midazolam	3A4, 3A5	3A	3A, 2B11, 2C21
Tolbutamide	2C9	2C9, 38, 39	2C, ?
Amodiaquine	2C8	2C21	2C, ?

A comparison of metabolic rates by each species (human, rat, dog) of various CYP450 enzyme substrates when metabolic assays were run in different incubation buffers. Three lots each of human and dog hepatocytes were tested, along with two lots of rat hepatocytes. Each substrate set was normalized to INVITROGRO™ KHB for comparison. Different substrates showed varying levels of difference depending on the species and incubation buffer used. Midazolam metabolism rates were similar regardless of the species or incubation buffer, while Tolbutamide and Amodiaquine showed large differences in rates.

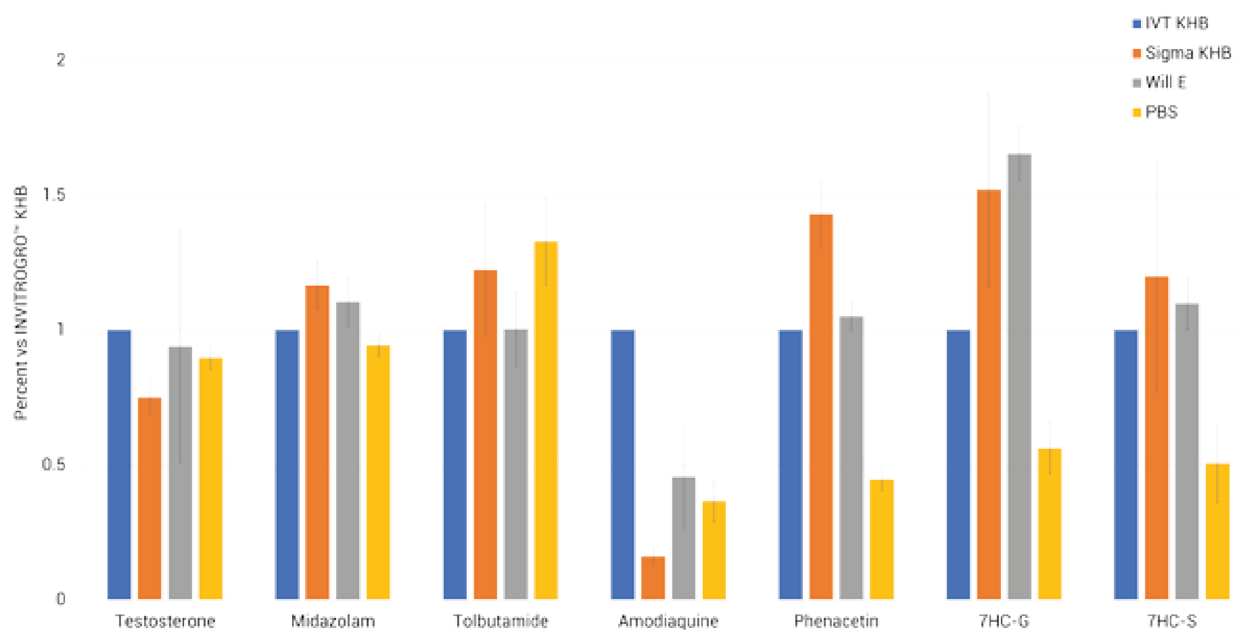
Human Hepatocyte Metabolism Rates by Cyp450 Substrate and Buffer Normalized to INVITROGRO KHB



Rat Hepatocyte Metabolism Rates by Cyp450 Substrate and Buffer Normalized to INVITROGRO KHB



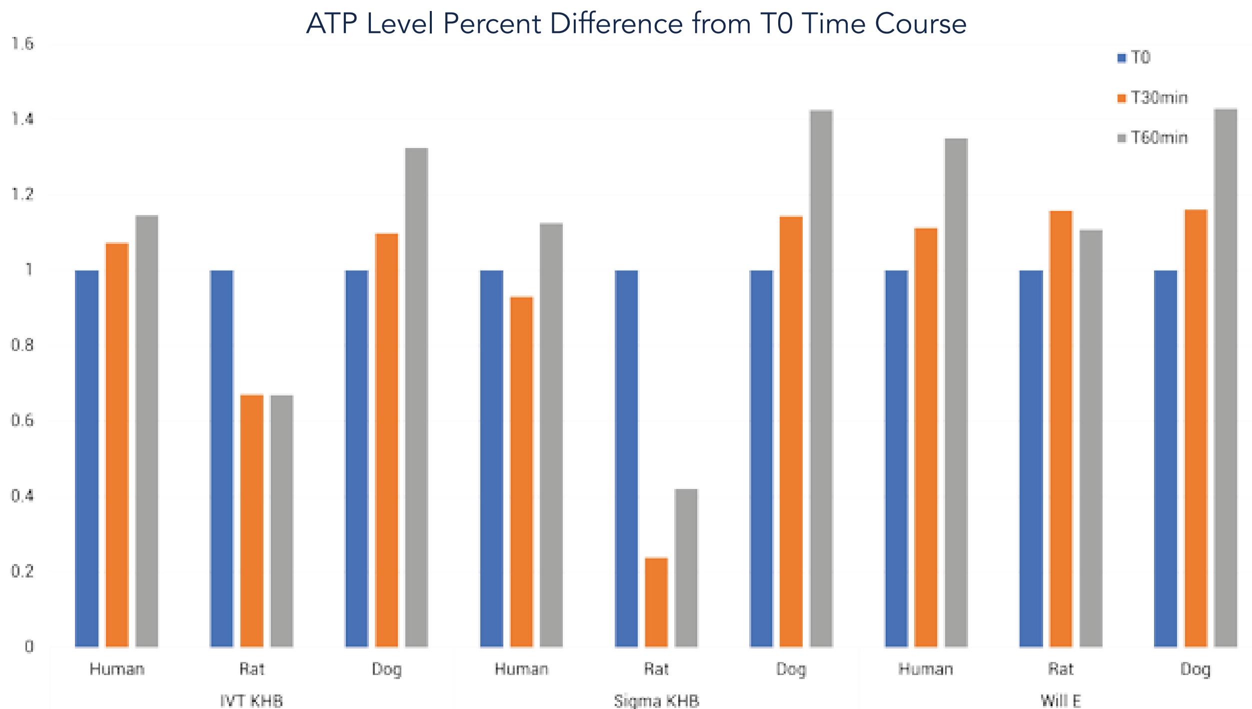
Dog Hepatocyte Metabolism Rates by Cyp450 Substrate and
Buffer Normalized to INVITROGRO KHB



CYP450 enzyme substrates and concentrations used in the hepatocyte metabolism assays.

Substrate	Conc [μM]	Metabolite
Testosterone	50	6ß-hydroxytestosterone
Midazolam	15	1-hydroxymidazolam
7-ethoxycoumarin	75	7-HC, 7-HCG, 7-HCS
Phenacetin	15	acetaminophen
Amodiaquine	20	desethylamodiaquine
Tolbutamide	150	4′-methylhydroxytolbutamide

Results shown are percentages of ATP content as compared to T0. Overall, ATP levels tended to trend upward by 30 minutes with the exception of rat. While rat did show the upward trend in Williams E, ATP levels were only 24% of their levels at T0 after 30 minutes in Sigma KHB. The reason for the decrease is unknown, as are the effects on metabolism rates.



Conclusion

The magnitude of differences seen in hepatocyte metabolism rates varied by both incubation buffer, particular species, and CYP450 enzyme substrate. No substrate was consistent across buffer and species with the exception of midazolam. An example of large variations included Amodiaquine metabolism in rat hepatocytes where the rate was 76% lower in Sigma KHB as compared to INVITROGRO™ KHB. While Williams' E and PBS had slightly higher rates, they still fell below 50% of the rate of INVITROGRO KHB. And while PBS had a lower rate for that CYP450 enzyme substrate in that species, it had higher rates than INVITROGRO KHB in other species with other CYP450 enzyme substrates. For example, in dog hepatocytes, PBS showed a Tolbutamide metabolism rate over twice what the INVITROGRO KHB rate was.

Phase II conjugation reactions with both glucuronidation and sulfation showed significant differences between buffers for all species, though the pattern of change was different between human and both the rat and dog.

The data resulting from the hepatocyte metabolism assays run in this study suggests that there is no one buffer system that is optimal in all species for all CYP450 substrates. Depending on the CYP450 enzyme or substrate being investigated, it is important to keep in mind differences between conditions when comparing or scaling clearance values from different species.

References

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