

# Comparison of the Metabolic Profile of Various Substrates: The Effect of Species and Methodology

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## Abstract

One of the primary challenges in NCE (new chemical entity) analysis is the determination of the correct animal species in which to conduct pharmacological and toxicological trials. Typically, the FDA requires one species in addition to rat for *in vivo* studies. The selection of species is critical as it may determine whether or not a drug candidate proceeds into clinical trials. A primary question is in which species is the compound metabolized most similarly to human. Prior to testing *in vivo* it is important to establish the metabolic profile of the candidate drug in humans to that of various test species. In these studies, BD Biosciences observed the metabolic profile of several compounds: testosterone, phenacetin, and phase 2 reactions, in rat, mouse, dog and human cryopreserved hepatocytes. For example, our data demonstrates a species difference in the production of 7-hydroxycoumarin sulfate such that mouse > rat > dog > human whereas the production of 7-HCG is more prevalent in human and dog. Production of 6 $\beta$ -hydroxytestosterone, however, appears to be greatest in mouse hepatocytes and least in dog hepatocytes. In addition, the viability and activity of cryopreserved hepatocytes when isolated by a non-percoll method versus a percoll gradient were compared and changes in activity between fresh and cryopreserved hepatocytes were assessed. These data demonstrate that there are inter-species differences, and that experimental factors may determine metabolic outcome.

## Introduction

Cryopreserved hepatocytes are a crucial element in drug discovery for determining toxicological and particularly, pharmacological properties of NCEs *in vitro* prior to expensive *in vivo* animal testing and human clinical trials. The data derived from these *in vitro* studies is important in eliminating unsuitable drug candidates and in determining the appropriate animal species for future animal trials. While the use of primary freshly isolated hepatocytes is preferred above that of cryopreserved cells, this is often difficult to obtain. Human donor livers are only occasionally available, and while billions of cells can be isolated in a single preparation, the freshly isolated hepatocytes rapidly lose expression of drug metabolizing enzymes and therefore must be used immediately after isolation, often making fresh cells an inconvenient research tool. Cryopreservation of hepatocytes with conservation of phase I and phase II metabolic activity allows for cell banking, maximizing the use of scarce donor tissue resources, and allows consistent testing between different lots of human and animal hepatocytes.

In this study, the metabolic activity of Phase I (P450) and Phase II (UGT, SULT) enzymes in cryopreserved human, Beagle dog, Sprague Dawley rat, and CD-1 mouse hepatocytes were analyzed. The probe substrates used in the analysis include the following: 1) Testosterone for CYP3A4 in human, CYP3A12 in dog; CYP3A in rat and mouse; 2) Phenacetin for CYP1A2 in human, CYP1A1/2 in dog, rat and mouse; 3) 7-Ethoxycoumarin which is metabolized to 7-Hydroxycoumarin (7-HC) or 7-HC directly, which is a UDP-Glucuronosyl Transferase (UGT) and a Sulfo-transferase (SULT) substrate in all species. It was also determined that the method of isolating the cryopreserved hepatocytes after thaw- by either a percoll or non-percoll method- did not impact the viability or the enzyme activity of the hepatocytes.

## Materials and Methods

### Hepatocyte Preparations

BD Gentest™ Cryopreserved Human, Male Beagle Dog, Sprague Dawley Rat, and CD-1 Mouse Hepatocytes were obtained from BD Biosciences Discovery Labware (Woburn, MA). Human hepatocytes were isolated from donor livers not suitable for transplant. Human and dog livers were obtained post-mortem within a 24-hour cold ischemic period. All hepatocytes were isolated using two modifications of the two-step collagenase perfusion method by Seglen<sup>1</sup> and cryopreserved in a controlled rate freezer using a step-down freezing program. Cryopreserved cells were stored at liquid nitrogen vapor temperatures until use. Freshly isolated hepatocytes were used at the time of isolation. Rat and mouse hepatocyte preparation represent a pool of hepatocytes from three to six animals per lot.

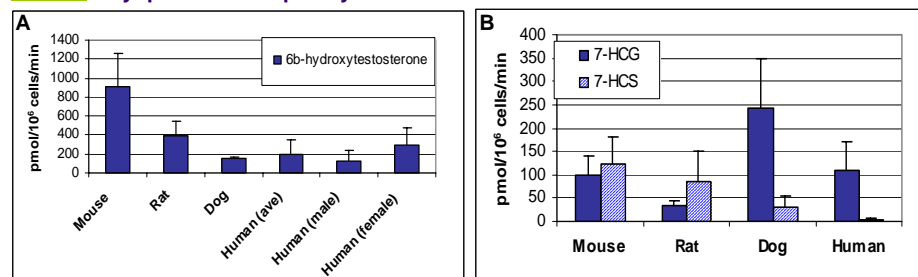
### Isolation and Metabolic Assays

Cryopreserved hepatocytes were thawed with the BD Gentest™ Purification Kit using a modified method of Kreamer.<sup>2</sup> The cells were rapidly thawed in a 37°C water bath and then added to ISOM's medium<sup>4</sup> with 24% Percoll™ (Amersham Pharmacia, Inc.) at 4°C for human, rat and mouse and at 37°C for dog. Cells were pelleted at 100 g for five minutes. The pellet was washed once with ISOM's medium, and assessed for viability by trypan blue exclusion. Alternatively, hepatocytes were thawed using the BD Gentest™ One-Step Purification Kit. Cells were rapidly thawed and added to ISOM's media<sup>3</sup> at 37°C and centrifuged at 50 g for five minutes. Hepatocytes were resuspended in either Williams' E medium at 0.5 x 10<sup>6</sup> cells/ml (human and dog) or in KHB with 3 mM Glycine and 10 mM Fructose at 0.5 x 10<sup>6</sup> cells/ml (rat) or 0.25 x 10<sup>6</sup> cells/ml (mouse). An equal volume of cell suspension was added to an equal volume of the substrate and reactions were stopped at 0, 0.5, 1, and 2 hours. Incubations were performed in either BD Falcon™ 24- or 96-well Tissue Culture Plates at 37°C, 5% CO<sub>2</sub> with 200 or 400  $\mu$ L of cell suspension/substrate mix per well, respectively. At each time point, reactions were stopped by adding the appropriate volume of stop solution from **Table 1**. All samples were analyzed by HPLC with the appropriate standards.

### T1 Assay Conditions

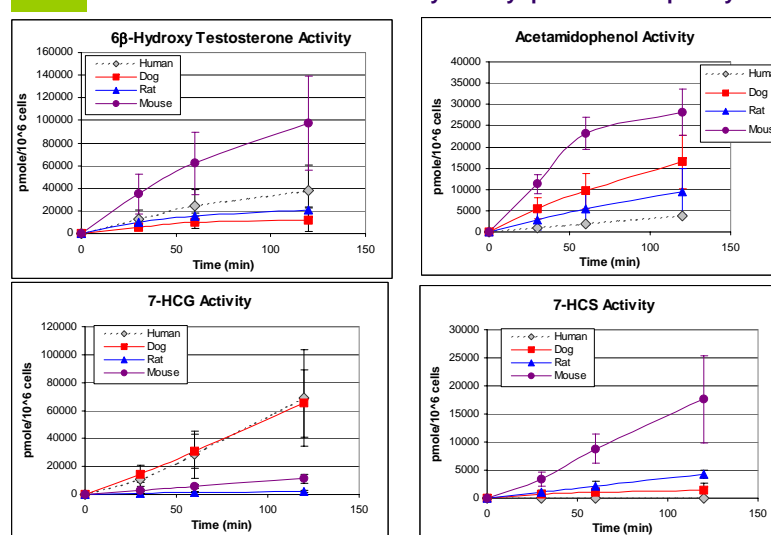
Probe Substrate (Target Enzyme)	Final Substrate Concentration	Assay Stop Solution
Testosterone CYP 3A	200 $\mu$ M	ACN
Phenacetin (CYP1A1/2)	100 $\mu$ M	70% Perchloric Acid (PCA)
7-Hydroxycoumarin (UGT, SULT)	100 $\mu$ M	PCA/ ZnSO <sub>4</sub> , BaOH

## F1 Comparison of 6 $\beta$ -Hydroxytestosterone and Phase 2 Activity in Cryopreserved Hepatocytes

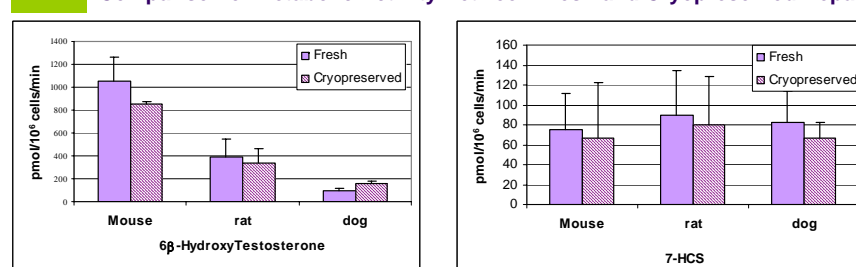


Data represents the average metabolite production and standard deviation at 30-minute incubation. All samples represent an N=3 samples.

## F2 Time Course of Metabolic Activity for Cryopreserved Hepatocytes



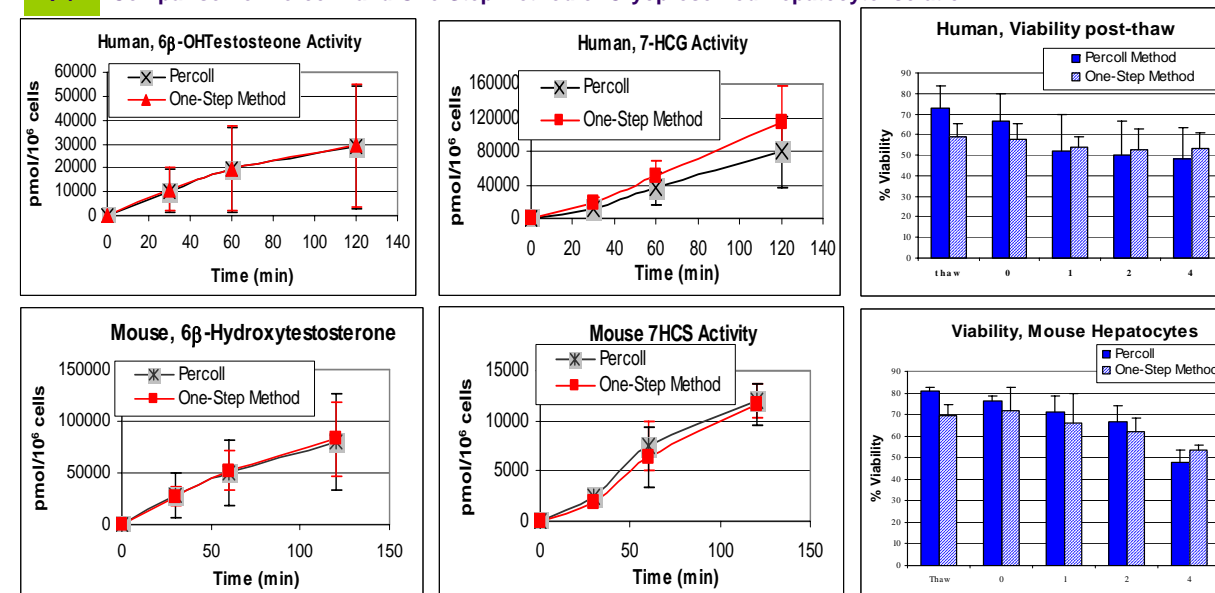
## F3 Comparison of Metabolic Activity Between Fresh and Cryopreserved Hepatocytes



## Results and Discussion

All species tested demonstrated measurable metabolic activity for both Phase 1 and Phase 2 enzymes.<sup>4</sup> CYP3A activity was measured by the metabolism of testosterone to 6 $\beta$ -hydroxytestosterone. As shown in **Figure 1A**, mouse hepatocytes were shown to have significant CYP3A activity followed by rat hepatocytes. Human female hepatocytes were shown to be more active for CYP3A4 activity than the corresponding human male hepatocytes, however, there is considerable overlap in activity due to individual variation. Sex differences were not apparent in the activities of the other enzymes tested (data not shown). Glucuronidation activity (UGT) appeared to be most similar between human and mouse hepatocytes, whereas dog hepatocytes showed more UGT activity than the other species. Both rodent species demonstrated significantly more sulfation than dog or human hepatocytes (**Figure 1B**). CYP1A2 activity as measured by metabolism of phenacetin, was found to be most similar between human and rat hepatocytes (**Figure 2**). The relative relationship of species to enzyme activity is summarized in **Table 5**.

## F4 Comparison of Percoll® and One-Step Method of Cryopreserved Hepatocyte Isolation



All samples represent an N=3 samples.

## T5 Species Differences in Phase 1 and Phase 2 Enzyme Activity

6b-OH Test	Mouse>Rat>Dog>Human
16a-OH Test	Rat>Mouse=Dog>Human
7-HCG	Dog>Mouse=Human>Rat
7-HCS	Mouse>Rat>>Dog>Human
Acetamidophenol	Mouse>Dog>Rat=Human

## Conclusions

- Phase I and 2 metabolism are conserved in all species cryopreserved hepatocytes.
- Mouse hepatocytes showed the highest CYP3A activity. Dog and rat hepatocytes showed similar activity to human hepatocytes.
- CYP1A2 activity in human hepatocytes is most similar to male rat hepatocytes.
- Phase 2 activity in rodent hepatocytes is predominately sulfation, whereas in human and dog hepatocytes it is primarily glucuronidation.
- Activity of Phase 1 and 2 enzymes is linear or curvilinear over a two-hour time course for all species of hepatocytes.
- Phase 1 and 2 enzyme activity is similar between freshly isolated and cryopreserved hepatocytes. Activity varies between 10-20%.
- Recovery of cryopreserved hepatocytes can be performed using a Percoll isolation method or the One-Step method which eliminates the Percoll step.
- Viability of hepatocytes isolated by the One-Step method is initially slightly lower than that of the Percoll method but viability is similar over a four-hour time course in culture.
- Recovery of cells by the One-Step method is generally greater than recovery after a Percoll method.
- Enzyme activity is similar between the two recovery methods.

## References

- Seglen, P.O. Preparation of rat liver cells. II. Effects of ions and chelators on tissue dispersion. *Exp. Cell Res.* 76:25 (1973).
- Kreamer, B.L. Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cell Dev. Biol.* 4:201 (1986).
- Isom, H.C. Quantitative assay for albumin-producing liver cells after simian virus 40 transformation of rat hepatocytes maintained in chemically defined medium. *Pro. Natl. Acad. Sci.* 81:6378 (1984).
- Bogaards, J.J.P. Determining the best animal model for human cytochrome p450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. *Xenobiotica* 30:1131 (2000).