In Vitro Metabolic Enzyme Activity Characterization in Cryopreserved, Freshly Isolated Hepatocytes and Liver S9 Fraction from Rainbow Trout

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Introduction

Assessment of bioaccumulation for all chemical substances with log K_{ow} ≥3 and produced in quantities of >100 tons / year will be required by regulatory agencies. We are in the process of pre-validating an *in vitro* assay utilizing rainbow trout liver S9 fraction to determine the metabolic fate for different categories of test chemicals. The *in vitro* metabolism data generated will be used in a bioconcentration factor (BCF) assessment model. Besides the trout liver S9 fraction, hepatocytes may provide additional information of the metabolic turnover of the chemicals in question. The objective of the present study was to determine and compare the metabolic enzymatic activities in cryopreserved, freshly isolated hepatocytes and liver S9 fraction from rainbow trout. The results may provide insight of any changes in enzymatic activities as a function of cryopreservation procedures. Livers were flushed with HBSS, excised and transported to another facility for further perfusion and processing. Batches of hepatocytes were separated according to sex. A portion of each lot was used to assess enzyme activities in fresh hepatocytes and the remainder was subjected to cryopreservation. In addition, livers were also collected and S9 fraction obtained for further analysis of enzyme activities. Metabolic enzymes activities (Phase I and II) were determined in fresh, cryopreserved hepatocytes and liver S9 fractions. Metabolic stability of test chemicals was determined in order to assess metabolic rates. The results indicate that there are no significant differences between fresh and cryopreserved hepatocytes when metabolic enzyme activities are compared. Liver S9 fraction enzyme activities are being further optimized to attain high enzyme activities. Rainbow trout hepatocytes and liver fractions may be considered powerful tools to determine the BCF using metabolic rates generated from *in vitro* metabolism assays.

Methods

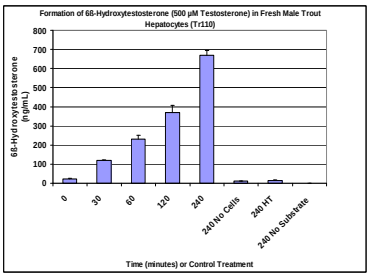
Rainbow trout were maintained under natural photoperiod (16L:8D) and temperature (about 17 °C) at the ABC Labs (Columbia, MO) (for hepatocytes and liver S9 fraction). Fish were acclimated for a week prior to sacrifice. Fish were euthanized with MS-222, livers flushed with HBSS (10 mM HEPES, 2.3 mM EDTA; pH 7.8), excised and transported to one of our facilities for further perfusion using collagenase. In addition, another set of trout were utilized for liver S9 fraction preparation. Briefly, whole livers were homogenized in 50 mM Tris-HCl pH 7.8, 150 mM KCl, 2 mM EDTA, 1 mM DTT and spun at 13,000 x g. Livers were homogenized in buffer with or without 250 mM sucrose and stability overtime was determined (presented elsewhere).

A portion of the resulting supernatant (S9) was stored at -80°C. Protein determination was performed using the Micro Lowry. Different batches of liver S9 fractions were prepared and separated according to sex. **Metabolic enzymes:** Phase I: lauric acid (data not shown) and testosterone hydroxylation; Phase II: 7OHCMN glucuronidation and sulfation (data not shown) and estradiol glucuronidation were determined in fresh and cryopreserved (one month after cryopreservation) hepatocytes as well as in trout liver S9 fractions. **Hepatocytes incubations:** Trout hepatocytes were diluted with DMEM (pH 7.8) containing 10% fetal bovine serum (FBS), centrifuged at 700 rpm for five min at approximately 4 °C and resuspended in culture media without FBS. Cells were centrifuged and decanted again and resuspended in fresh culture media without FBS. Cells were diluted to a final density of 0.5 x 10^6 cell/mL. Hepatocytes were incubated up to 240 min. **Liver S9 incubations:** All incubations were performed in 100 mM phosphate buffer pH 7.8 up to 120 min according to the specific assay. All incubations (hepatocytes and S9) were performed at T= 12 °C mimicking the trout natural environmental conditions.

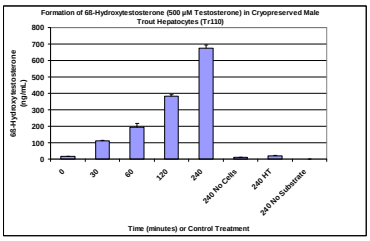
Results

Figure 1 – Testosterone hydroxylation results are comparable in both trout fresh (A) and cryopreserved hepatocytes (B)

A

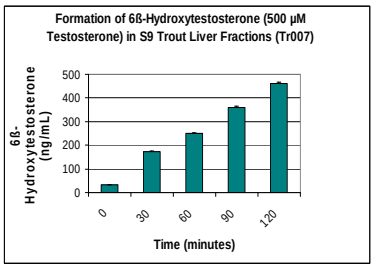


B



Incubation Conditions: 500 µM Testosterone, 0.5 x 10^6 cells/mL, pH 7.8, 12°C.

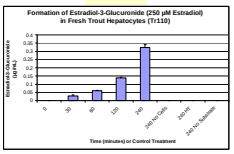
Figure 2 – Testosterone hydroxylation in liver S9 fraction



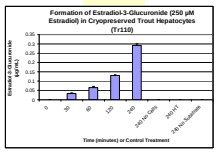
Incubation Conditions: 500 µM Testosterone, 1mg/mL, pH 7.8, 12°C.

Figure 3 – Estradiol glucuronidation in trout fresh (A) and cryopreserved hepatocytes (B) as well as in liver S9 (C) is similar

A

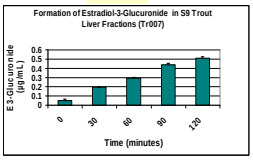


B



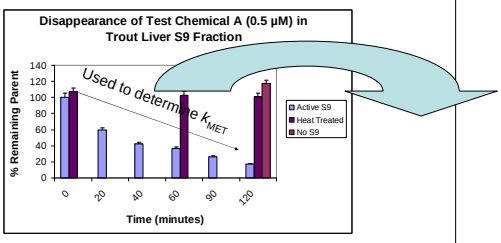
Incubation Conditions: 250 µM Estradiol, 0.5 x 10^6 cells/mL, pH 7.8, 12°C.

C



Incubation Conditions: 250 µM Estradiol, 1mg/mL, pH 7.8, 12°C.

Figure 4 – Model for whole body transformation rate and BCF determination from *in vitro* metabolism data. (Cowan-Ellsbery *et al,* 2008)



Incubation Conditions:
S9 protein: 2 mg/mL
T = 12 °C

S9 Hepatic Intrinsic Clearance Rate of Parent Chemical CL_m

↓

Hepatic Clearance CL_H

↓

Metabolic Transformation rate k_{MET}

↓

Bioconcentration Factor BCF

Conclusions

- Metabolic enzyme activities (Phase I and II): results are comparable in both fresh and cryopreserved hepatocytes.
- Likewise, trout liver S9 showed similar results in metabolic enzyme activities.
- *In vitro* metabolism utilizing trout liver S9 fraction and hepatocytes can be used to determine the BCF and therefore bioaccumulation potential of chemicals.
- The rainbow trout liver S9 fraction metabolic assay for the BCF determination is currently under further optimization.
- Fish (e.g. trout and carp) hepatocytes may represent an additional tool to determine bioaccumulation as the human hepatocytes are considered the gold standard when assessing drug metabolism.

Acknowledgments

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References

Cowan-Ellsberry, C., Dyer, S.D., Erhardt, S., Bernhard, M.J., Roe, A.L., Dowty, M.E., and Weisbrod, A.V. 2008. Approach for extrapolating *in vitro* metabolism data to refine bioconcentration factor estimates. Chemosphere 70:1804-1817.