Vitro Fish Metabolism and Metabolite Profiling of Several Fragrances using Rainbo Trout Liver S9 Fraction and Cryopreserved Hepatocytes

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Abstract

Bioaccumulation assessment of test chemicals is required by REACh. Fish bioconcentration factor (BCF) has been determined by traditional *in vivo* methodologies using large numbers of animals with costly and labor intensive procedures. *In vitro* systems have been proposed as alternative methods that may substitute for *in vivo* BCF assessment. Xenobiotic biotransformation rates can be a controlling factor for bioaccumulation estimation. The primary objective of this study was to conduct an *in vitro* metabolic stability/profiling of fragrance chemicals in fish using liver S9 fractions and hepatocytes. Four radiolabeled and non-labeled fragrances (Sanjinol, Coniferan, Precyclemone B and Vernaldehyde) were incubated in the presence of rainbow trout liver S9 fractions and cryopreserved hepatocytes. Very little difference in the extent of the metabolic turnover of each of the four compounds was found between the liver S9 fraction and hepatocyte systems. GC-MS chromatograms were next examined in an effort to identify metabolites from each of the fragrances. We identified one metabolite of Sanjinol from the S9 incubations that also appears to be present in the hepatocyte incubations. The liver S9 incubations of Coniferan showed three metabolites with two of these found in the hepatocyte incubations. Precyclemone B and Vernaldehyde both generated two metabolites in the presence of trout liver S9, but neither was observed in hepatocyte incubations. Additional trials in the presence and absence of cofactors during the S9 incubations found that the metabolites from Sanjinol, Precyclemone B and Vernaldehyde were all CYP dependent, but the three metabolites of Coniferan were not. Analysis of the mass spectra of the metabolite of Sanjinol suggests it to be an oxidation product while those from Precyclemone B and Vernaldehyde both appear to be reduction products. Two main metabolites from Coniferan appear to be deacetylation products. Trout liver S9 fractions and hepatocytes can be powerful tools to estimate BCF. In addition, metabolite profiling can further elucidate the fate of test chemicals.

Methods

The rainbow trout S9 and cryohepatocyte metabolic systems have been characterized elsewhere (Johanning *et al*., SETAC 2008 and SETAC 2009) demonstrating the activity of several important Phase I and Phase II enzymes. Test chemicals (Sanjinol, Coniferan, Precyclemone B and Vernaldehyde) were incubated with male rainbow trout (1+) cryohepatocytes or S9 fraction and cofactors (0.1 mM PAPS, 2 µM UDPGA and 1 mM NADPH) at pH =7.8 in a water bath at 12°C. Hepatocyte incubations were performed with both radioactive and non-radioactive Coniferan, Precyclemone B and Vernaldehyde. All incubations were performed in triplicate at two or three different test chemical concentrations, and with hepatocytes at 0.5 X 10^6 cells/mL or S9 protein at 0.2 and 1 mg/L concentrations. All incubations included six time points. The Sanjinol reaction was terminated by addition of ice cold methanol and extracted with ethyl acetate. Coniferan, Precyclemone B and Vernaldehyde reactions were terminated by the addition of ethyl acetate. Incubation samples were vortexed, centrifuged at 3000 rpm for 10 min at room temperature. The organic layer was transferred to a GC test vial and test chemical disappearance was measured by GC/MS. In all cases, zero-time incubations, heat-treated (inactivated, boiled at 100°C for 10 min), solvent control (SC) and no cofactors (NC) (all controls incubated for the longest time point) samples were included. Results were reported as disappearance of test chemicals over time. Metabolites were examined by GC/MS and HPLC-β-RAM. Chromatograms were compared to those from tissue extracts of rainbow trout exposed to the same chemicals. Tissue homogenates from fish fed radiolabeled Coniferan, Precyclemone B and Vernaldehyde (data not shown) over 28 days were analyzed. Homogenates were extracted with two volumes of ethyl acetate and samples of the organic phase after spinning at 3000 rpm for 5 minutes, were examined by GC-MS.

β-RAM Chromatograms of Radiolabeled Hepatocyte Incubations

Figure 12. Initial β-RAM chromatogram of Coniferan hepatocyte incubation at t= 0 min

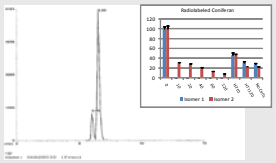


Figure 13. β-RAM chromatogram of Coniferan hepatocyte incubation after 5 min.

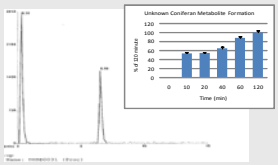


Figure 14. Initial β-RAM chromatogram of Precyclemone B hepatocyte incubation at t = 0 min

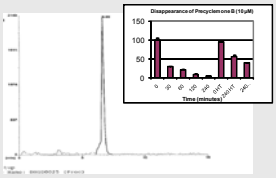
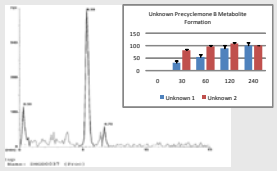


Figure 15. β-RAM chromatogram of Precyclemone B hepatocyte incubation after 240 min.



Results of the Incubations - Sanjinol

Figure 1. 120 minute incubation of Sanjinol with trout S9

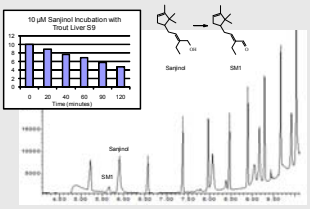
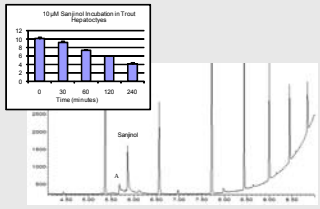


Figure 2. 240 minute incubation of Sanjinol with trout cryopreserved hepatocytes



A was found to be an impurity from the isolation and not a metabolite of Sanjinol.

Results of the Incubations - Coniferan

Figure 3. 60 minute incubation of Coniferan with trout S9

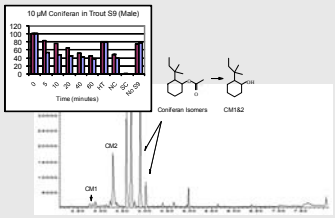
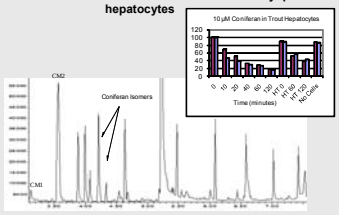


Figure 4. 120 minute incubation of Coniferan with trout cryopreserved hepatocytes



Chromatograms of Fish Tissue Extracts vs S9 Incubations

Figure 9. Chromatograms of Coniferan results from fish tissue extraction and S9 incubation

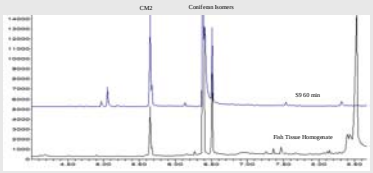


Figure 10. Chromatograms of Precyclemone B from fish tissue extraction and S9 incubation

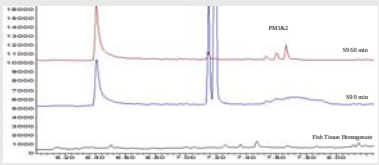
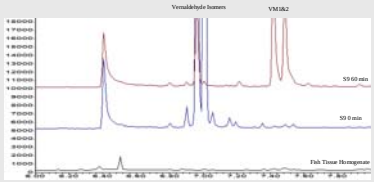


Figure 11. Chromatograms of Vernaldehyde from fish tissue extraction and S9 incubation



Results of the Incubations – Precyclemone B

Figure 5. 60 minute incubation of Precyclemone B with trout S9

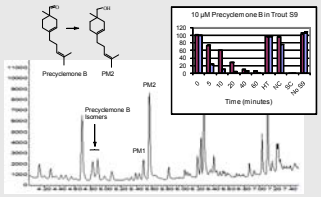
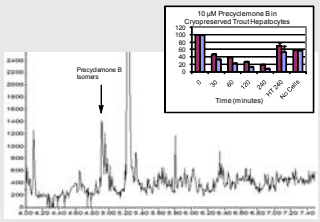


Figure 6. 240 minute incubation of Precyclemone B with trout cryopreserved hepatocytes



Results of the Incubations - Vernaldehyde

Figure 7. 60 minute incubation of Vernaldehyde with trout S9

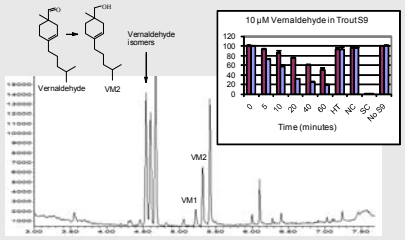
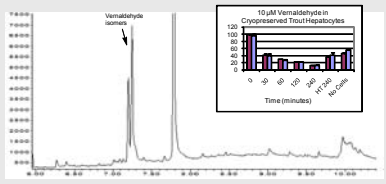


Figure 8. 240 minute incubation of Vernaldehyde with trout cryopreserved hepatocytes



Discussion / Conclusions

- A single metabolite of Sanjinol was detected and identified as the aldehyde of Sanjinol.
- The two metabolites of Coniferan were detected and identified as deacetylation products of Coniferan. A single radioactive LC component was detected from incubations of radiolabeled Coniferan with fish hepatocytes. This single radiolabeled component would be consistent with the formation of the alcohols of Coniferan with the radiolabel remaining in the acetic acid hydrolysis byproduct.
- Two metabolites of Precyclemone B and Vernaldehyde were detected and identified as the alcohols of these compounds.
- BCFs for these chemicals are all predicted to be high based on standard QSAR methods. The US EPA's PBT Profiler predicts BCF=1200 for Coniferan, and in the range of 1800 – 2300 for the others.
- Even a modest amount of metabolism would be sufficient to show that the chemicals are not bioaccumulative as predicted and according to EU and US standards.
- Metabolism has been demonstrated using S9 and trout hepatocyte *in vitro* systems and in rainbow trout *in vivo*. Although metabolic capabilities differ among the three systems, all are able to confirm significant metabolism. Rates of metabolic turnover were comparable for all four chemicals in S9 and hepatocyte systems.
- Both hepatocyte and S9 methodologies look promising as practical and efficient ways to further investigate the potential for bioaccumulation of suspect B compounds.
- Identification of metabolites was only partial, but the observed patterns of transformation and disappearance of parent chemical then major metabolites support the overall conclusions about bioconcentration/bioaccumulation.
- Methods for extrapolation of the *in vitro* results to predict whole-fish bioconcentration potential are now being tested. Extrapolation to whole organisms will be essential to be able to rely on *in vitro* tests without the guideline BCF tests that are much more time consuming, expensive, and require large numbers of animals and radiolabeled compounds. Progress has been made on extrapolation but the approach is in the process of further validation and refinement.
- Three of these chemicals (Coniferan, Precyclemone B, and Vernaldehyde) have been tested *in vivo* confirming that they are much less bioaccumulative than predicted. This work will be presented separately and compared to results of extrapolation from *in vitro* results.

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References

•Guiney, P.D., Weeks, J.A., Johanning, K.M., Hill, J.E., and Johnson, R. *In Vitro* Fish Metabolism Predicts Elimination of Suspected Bioaccumulative Chemicals. SOT 2009.
•Johanning, K., Dungan, L., Sahi, J, Embry, M., Erhardt, S., Escher, B., Halder, M., Sharpe, A., and Hill, J. Pre-Validation of the *In Vitro* Rainbow Trout Liver S9 Fraction Assay to Predict *In Vivo* Fish Metabolism of Chemicals and Other Substances. SETAC 2008.
•Johanning, K., Dungan, L., Whisnant, R., Lohnes, K., Smith, C., Amaral, K., Gauvin, R., Lehnert, K., Baucom, C., Sahi, J., and Hill, J. Comparison of Metabolic Enzyme Activities in Cryopreserved and Fresh Hepatocytes Isolated from Rainbow Trout SETAC 2009.
•Weeks, J.A., Guiney, P.D., Johanning, K.M., Gauvin, R., and Hill, J.E. Metabolic Stability of Fragrances in Rainbow Trout. SETAC 2009.