EVALUATION OF POSITIVE CONTROL SUBSTRATES FOR THE OECD319 IN VITRO RAINBOW TROUT BIOACCUMULATION ESTIMATION STUDIES

SMITHERS

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ABSTRACT

The OECD 319 guidelines for the determination of in vitro intrinsic clearance using cryopreserved rainbow trout hepatocytes (RT-HEP, OECD319A) and S9 sub-cellular fraction (RT-S9, OECD319B) were published in 2018. The studies are intended as a means of estimating the potential for bioaccumulation of chemicals in fish without the need for an in vivo fish bioconcentration factor (BCF) study (OECD305). They can be used in cases where it is not possible to run the BCF study, for example, ethical reasons such as cosmetic use, to reduce animal usage or it is not technically feasible. They may also be used to provide early screening information about bioconcentration potential or metabolism.

Since the release of the guidelines there have been discussions around the most applicable positive control substrates for the experiments. Positive controls are used to ensure the metabolic viability of the test system used (S9 or hepatocytes). A positive control usually should typically result in a minimum transformation of 20% with 10% remaining unchanged by the terminal time-point of the experiment. This can present a challenge in the case of particularly high or low turnover as the positive control could be entirely transformed or remain unchanged at the end of the experiment. To validate the OECD319 study at our facility we have tested several different substrates that have the potential to be used as a convenient positive control. These can be matched to the clearance of the chemical under investigation.

This poster presentation summarises the results from the testing of several different potential positive control substrates for both the RT-HEP and RT-S9 assays.

Introduction

- Pharmaceutical residues in surface waters can lead to bioaccumulation in fish, with the potential for chronic effect outcomes, even at trace level environmental concentrations. Sensitivity to these adverse effects is considerable due to the high evolutionary conservation of human drug targets in fish and have been associated with behavioural changes and sexual dysfunction in wild fish populations. Computational tools have been developed over the past decade to assess the BCF in fish, however, they are limited due to the lack of certainty on hepatic transformation and its subsequent impact on intrinsic clearance rate.
- OECD319A[1] and 319B[2] Test guidelines describe the *in vitro* intrinsic clearance of a test chemical in *Oncorhynchus mykiss* (Rainbow trout) cryopreserved Hepatocytes and liver S9 sub-cellular fractions, using a substrate depletion approach. Liver intrinsic clearance values can be estimated based on the $CL_{IN\ VITRO,\ INT}$ determined by these tests and can be used to inform *in silico* predictions.
- These *in vitro* assays have demonstrated good interlaboratory reproducibility and provide an ethically sound prediction of bioconcentration factors, as well as enabling mechanism-based predictions on clearing routes, such as the cytochrome P450 pathway.
- The depletion rate constants are used to calculate the bioconcentration factors (BCF) of the test substances as documented in the OECD guidance document no. 280[1].
- Positive controls are essential for ensuring the viability of the S9 fraction or hepatocytes used, usually a minimum turnover (metabolism of parent) of 20% is acceptable but neither the guidance document or guidelines specify a minimum.
- Radiolabeled ibuprofen, diclofenac and 7-ethoxycoumarin were selected because of the availability of published literature on their biotransformation in fish.

Positive control selection is critical in cases where OECD319 test data will be used to predict fish BCF values

Materials And Methods

Incubations were conducted to OECD319A/B guidelines, using radiolabelled ibuprofen, diclofenac and 7-ethoxycoumarin as reference substrates. All experiments were conducted using a single vial approach at a volume of 1000 µL, with 100 µL sub-samples taken at each interval and quenched in ice cold methanol. All incubations were carried out at 11±1°C, with 9 timepoints between 0 and 180 minutes for S9 fractions, and up to 24 hours for hepatocytes.

- Rainbow trout S9 subcellular fraction (RT-S9) and hepatocytes (RT-HEP) were purchased from Primacyt GmBH (Germany), with an S9 protein concentration of 20 mg/mL and hepatocyte cell density of $1{\times}10^7$ cells/mL.
- Hepatocytes were thawed and cell viability and yield determined with the trypan blue stain method. Cells were manually counted using a haemocytometer and microscope then diluted to give 1×10^6 cells/mL. Cells were re-counted at the end of the test to ensure cell viability was maintained. 96-97% viability was maintained after 24 hours.
- RT-S9s were thawed on ice and diluted using pH 7.8 potassium phosphate buffer, and a mix of co-factors added. NADPH was used as a phase I co-factor and GSH, UDPGA and PAPS as phase II enzymes. Alamethicin also used to reduce latency of glucuronidation activity.
- Heat-inactivated RT-S9s and RT-HEPs were utilized as negative controls to distinguish between enzymatic biotransformation and abiotic loss.
- At least two concentrations were used for each test chemical. The incubations at 10 µM were carried out in triplicate.
- The slope of the of the S9 and RT-HEP regression curves was used to calculate the in vitro intrinsic clearance (CL_{INT} *; ml / h / mg protein*).
- All analysis was carried out using a Shimadzu Nexera and Lablogic β-Ram model 5C radiodetector and LabLogic Laura v6 chromatography data system.
- Confirmation of expected metabolites was performed using a ThermoFisher Scientific Vanquish Horizon UHPLC coupled with an Orbitrap Eclipse Tribrid Mass Spectrometer and Heated Electrospray Ionisation (HESI) and Xcalibur 4 data system.

Results

- Limited metabolism was observed in rainbow trout hepatocyte incubations in early time points. This could be due to rate-limiting factors such as restricted chemical diffusion across the cell membrane.
- Ibuprofen was metabolised in both rainbow trout S9 and hepatocyte experiments. Diclofenac was metabolised at a reasonable rate in S9 experiment but was very slow in the hepatocyte experiment. No significant metabolism was observed in the 7-EC S9 experiment. In the early timepoints of the 7-EC hepatocyte experiment little metabolism was observed but greater metabolism was observed by the 24-hour time-point. Linear regression plots are shown in figure 1.

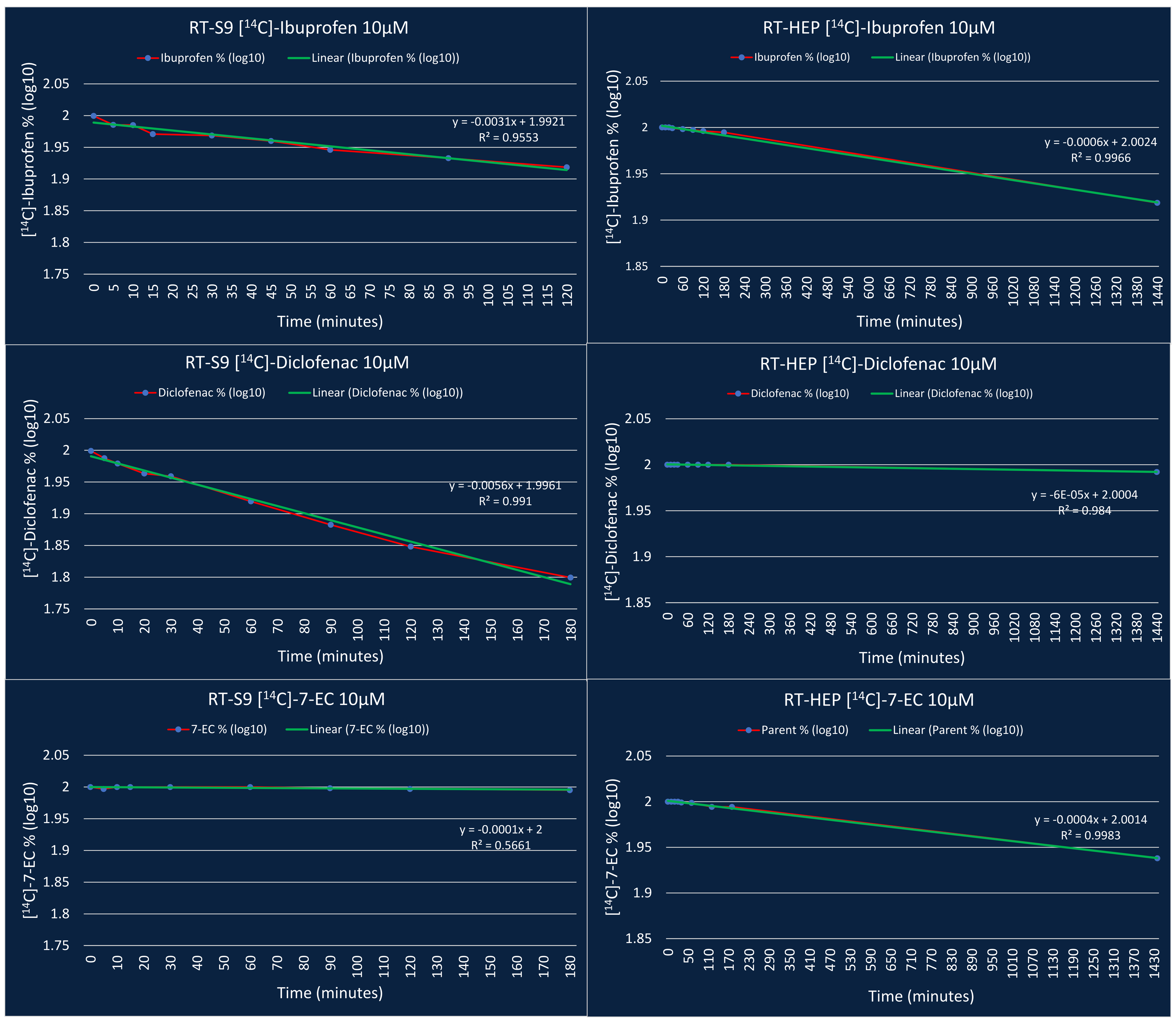

Figure 1: Ibuprofen, Diclofenac and 7-EC regression curves for Rainbow trout S9's and Hepatocytes, 10µM.

- After incubation for 3 hours with RT-S9 ibuprofen 14-28% of parent was metabolised, diclofenac had 30-37% metabolised and 7-EC had ≤2% metabolised. After 24 hours incubation with RT-HEP ibuprofen was 3-15% metabolised, diclofenac had ≤3% metabolised and 7-EC had 13-27% metabolised. Extent of metabolism was concentration dependent. Negative controls demonstrated no significant loss of test substance due to abiotic loss.
- BCF values were calculated using the spreadsheets of Nichols *et al.*[4] as published in the OECD guidance document[3] and are presented in Table 1. Ibuprofen BCF_{TOT} values for RT-S9's are comparable to findings from Pihlaja *et al.*[5] However, Diclofenac BCF_{TOT} were considerably lower.
- BCF values in fish studies vary greatly and are dose dependant. Schwaiger *et al*[6] Demonstrated Diclofenac BCF values between 12-2732 in liver, whereas other experiments have demonstrated BCF values of <10. Ibuprofen has also been given similar whole body BCF values in the <5 range.
- *In silico* values from EPISuite for ibuprofen, diclofenac and 7-EC were 436.3, 1415 and 10.06 respectively.

Test System	Test Substance	Concentration	Slope R²	In Vitro CL_{INT}	Calculated BCF_{TOT}
RT-S9	Ibuprofen	1 µM	0.76	0.01127	461.02
		10 µM	0.96	0.00713	461.29
		20 µM	0.98	0.00621	461.35
	Diclofenac	1 µM	0.96	0.01058	1556
		10 µM	0.99	0.01288	1555
	7-Ethoxycoumarin	1 µM	NC	NC	NC
		10 µM	NC	NC	NC
		100 µM	NC	NC	NC
RT-HEP	Ibuprofen	10 µM	1.00	0.00138	457.57
		100 µM	1.00	0.00138	457.57
	Diclofenac	1 µM	NC	NC	NC
		10 µM	0.98	0.000138	1556
		100 µM	0.98	0.000184	1556
	7-Ethoxycoumarin	1 µM	0.60	0.00	10.20
		10 µM	1.00	0.00	10.19
		100 µM	0.98	0.00	10.20

Table 1: Summary of data following *in vitro* incubation of various substrates with Rainbow trout S9 and Hepatocytes.

NC = Not calculated

CONCLUSIONS

- **The calculated BCF values for ibuprofen and diclofenac were consistent between RT-S9 and RT-HEP experiments.**
- **Calculated BCF values for ibuprofen by RT-S9 are comparable to literature values.**
- **7-EC is not a suitable positive control for RT-S9 or RT-HEP experiments. Diclofenac is not a suitable positive control for RT-HEP experiments**
- **The In Vitro CL_{INT} values were higher or lower than some found in the literature depending on the tissues being studied.**
- ***In silico* values were a good match to the *in vitro* experimental values but much higher than whole fish experimental values, indicating the model gives a worst-case estimation and is therefore fit for purpose.**

References

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[3] OECD (2018), Guidance Document on the Determination of in vitro intrinsic clearance using cryopreserved hepatocytes (RT-HEP) or liver S9 sub-cellular fractions (RT-S9) from rainbow trout and extrapolation to in vivo intrinsic clearance, Series on Testing and Assessment No. 280, OECD Publishing, Paris.

[4] Nichols, J.W., D.B. Huggett, J.A. Arnot, P.N. Fitzsimmons and C.E. Cowan-Ellsberry (2013). Towards improved models for predicting bioconcentration of well-metabolized compounds by rainbow trout using measured rates of in vitro intrinsic clearance. Environmental Toxicology and Chemistry 32: 1611-1622.

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