In vitro hepatic biotransformation of ^{14}C-decamethylcyclopentasiloxane (D5) and ^{14}C-decamethyltetrasiloxane (L4) in rainbow trout, carp, catfish, kestrel, quail, rat, mink, and human.

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Abstract

The ability of an organism to biotransform and eliminate chemicals plays an important role in bioconcentration (BCF), bioaccumulation (BAF) and trophic magnification (TMF) assessments. Metabolic potential must be considered when extrapolating from the laboratory to the field, with biotransformation capabilities varying widely across species. Recent efforts have been made in extrapolating *in vitro* fish biotransformation data to a whole body BCF value. By integrating biotransformation estimates into BCF models, a more realistic estimation of BCF can be calculated while providing a cost-effective assay that uses less vertebrate animals. Building on this single species extrapolation concept, biotransformation data from multiple species may be used to construct a biomagnification or trophic magnification model for a given chemical. Using ^{14}C radiolabelled compounds and high performance liquid radiochromatography, *in vitro* metabolism data utilizing liver microsomes were developed for a cyclic siloxane, D5, and a linear siloxane, L4. Of the species investigated, mink demonstrated the greatest potential to biotransform D5 and L4 siloxane. ^{14}C radiochromatograms show the metabolic loss of D5 and L4 siloxane, as well as increases in metabolite production over the 60 min incubation period. The percentage loss of D5 was similar with human and rat microsomes and greater then observed with fish. The percentage loss of D5 in birds is low compared to fish and mammals. Similar relationships for loss of parent hold true for L4 (kestrel data not available). These data suggest that D5 and L4 siloxane are biotransformed by a wide array of species, which can influence "B" assessments. In addition, these data can be used to estimate whole-body rates of metabolism for incorporation into predictive environmental assessment where data gaps exist.

Materials & Methods

Test System

Rainbow trout (*Oncorhynchus mykiss*) and common carp were obtained from Crystal Lake Fisheries (Ava, MO). Channel catfish were obtained from Pond King in Gainesville, TX. Prior to sacrifice, fish were maintained in dechlorinated tap water at the appropriate temperature in Frigid Units Living Streams. Fish were not individually labeled or tagged and were group housed. Fish were housed at the University of North Texas at least 14 d prior to use. Quail livers were obtained from Wildlife International (Easton, MD), while kestrel livers were obtained from the USGS Patuxent Wildlife Research Center (Beltsville, MD). Rat and human microsomes were obtained from Xenotech (Lenexa, KS).

Microsomal Preparations and Assay

Microsomal fractions were prepared from trout S9 fractions. S9 was placed in chilled ultracentrifuge tubes and centrifuged at 100,000g for 1 h at 4°C. The supernatant was removed from the tubes and 500 µL resuspension buffer (0.02 M KH2PO4; 0.08 M K2HPO4; 0.1 M KCl; 1 mM EDTA; 1mM DTT; 20% glycerol) was added to remove the bottom pellet. Using a thin brush, the microsomes were gently removed from the bottom of the tube and mixed in with the resuspension buffer. The resulting solution was then placed in a -80°C freezer for storage.

Biotransformation reactions (n=3) were conducted in triplicate. Microsomes were diluted to 1 mg/mL protein content, respectively in PBS (pH7.4) containing 50mM potassium phosphate and 0.15 M potassium chloride. A NADPH regeneration system was used and composed of isocitric (7 mM), isocitric dehydrogenase (0.5 units of activity ml^{-1}) and β-NADPH (600 mM) (Sigma-Aldrich, St. Louis, MO). All reagents were made in PBS (pH7.4) on the day of the assay. The regeneration system was aliquoted into 10 mL vials that contained the diluted S9 or microsomal fractions, and the reaction mix was placed in a shaking water bath (quail, kestrel, rat and human = 37ºC; catfish and carp = 25 ºC; trout = 16 ºC) and allowed to equilibrate for several minutes before adding the test material. To initiate biotransformation, an ethanol (<1% solvent) stock containing the compound (10 µM) was added to each of the reaction mixes. Over a period of 1 h, individual reaction vials were stopped with an equal amount of 80:20 acetonitrile:THF solution. Time points to assess biotransformation were 0 min, 15 min, 30 min, and 60 min. The vial was then vortexed and centrifuged to remove the supernatant for analysis. No-NADPH, heat deactivated and Fluroxypyr positive controls were also conducted to ensure quality.

Materials & Methods Continued

HPLC/β-RAM CONDITIONS

Instrument	Waters e2695 HPLC separations module
Detector	INUS systems radio HPLC detector (β-RAM, Model 3)
Column	Phenomenex Phenosphere (SCX 80A, 5 µm, 4.6 x 150 mm)
Column temperature	25°C ± 1°C
Injection volume	100 µL
Radiometric detection	Carbon-14 (C-14) detection at 98% counting efficiency calibrated using sealed C-14 reference standard (LabLogic, D1-BXX-18)
Mobile phase (and gradient)	1:1 ACN:THF

Table 1. 25 min HPLC Gradient Conditions

Time	%A (MQ Water)	%B (1:1 ACN:THF)	Flow	Scintillation
0 min	100 %	0 %	1 ml/min	3 ml/min
10 min	40 %	60 %	1 ml/min	3 ml/min
15 min	0 %	100 %	1 ml/min	3 ml/min
20 min	40 %	60 %	1 ml/min	3 ml/min
25 min	100 %	0 %	1 ml/min	3 ml/min

Results

Table 2. Percent loss of ^{14}C-D5 siloxane n microsomes from multiple species

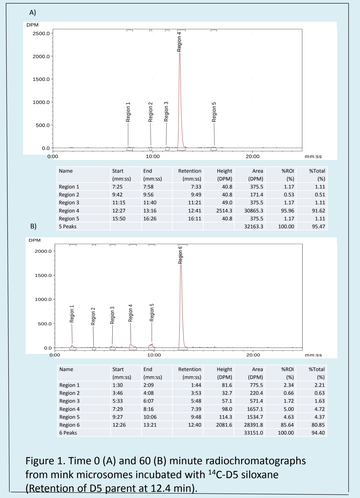
	Time Points		
		% Recovery	% Loss
Mink	0 min	92.29	
	15 min	89.10	3.46
	30 min	86.62	6.14
	60 min	82.24	10.89
Quail	0 min	93.36	
	15 min	93.07	0.31
	30 min	93.49	-0.14
	60 min	92.86	0.54
Carp	0 min	92.66	
	15 min	92.22	0.47
	30 min	92.10	0.60
	60 min	92.04	0.67
Trout	0 min	92.32	
	15 min	92.33	-0.01
	30 min	91.67	0.70
	60 min	91.29	1.12
Kestrel	0 min	92.28	
	15 min	91.75	.057
	30 min	91.13	1.25
	60 min	91.60	0.74
Rat	0 min	92.48	
	15 min	91.16	1.43
	30 min	88.98	3.78
	60 min	88.16	4.67
Human	0 min	93.12	
	15 min	90.27	3.06
	30 min	89.15	4.26
	60 min	87.58	5.95
Catfish	0 min	92.34	
	15 min	92.31	0.03
	30 min	91.12	1.32
	60 min	90.91	1.55

Table 3. Percent loss of ^{14}C-L4 siloxane in microsomes from multiple species

	Time Points		
		% Recovery	% Loss
Mink	0 min	96.45	
	15 min	89.64	7.06
	30 min	82.56	14.40
	60 min	78.64	18.47
Quail	0 min	93.46	
	15 min	92.65	1.08
	30 min	91.97	1.59
	60 min	91.79	1.79
Carp	0 min	95.46	
	15 min	94.18	1.34
	30 min	94.29	1.23
	60 min	93.97	1.56
Trout	0 min	96.31	
	15 min	95.79	0.54
	30 min	91.46	5.04
	60 min	89.73	6.83
Rat	0 min	94.46	
	15 min	92.73	1.83
	30 min	92.47	2.11
	60 min	92.01	2.59
Human	0 min	95.19	
	15 min	94.71	0.50
	30 min	91.59	3.78
	60 min	90.08	5.37
Catfish	0 min	92.48	
	15 min	92.27	0.23
	30 min	92.31	0.18
	60 min	91.94	0.58

- Total P450 was measured in each of the microsomal fractions according to Guengerich et al (2009). Measurements were taken on freshly prepared fractions. Data suggest a range of Total P450 concentrations, with rat and human being the highest. Total P450 concentrations for species of ecological interest were 5 – 50x different from rat and human preparations
- Preliminary work focused on optimizing the assay conditions , as well as initiating some "cold" experiments using the GC-MS. In these preliminary studies, it was determined that the appropriate control for these studies was the No NADPH controls. In buffer only controls, the analytes were rapidly removed from solution. The analytes remained in solution over the test period when the matrix (e.g. S9) was introduced.

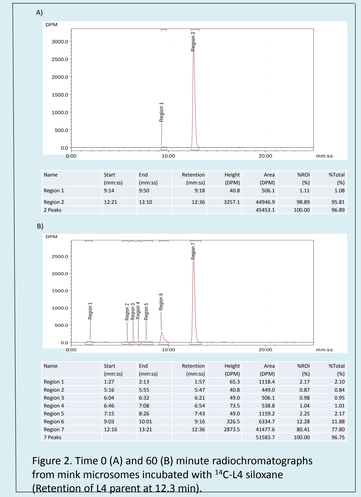
Results



Name	Start (mm:ss)	End (mm:ss)	Retention (mm:ss)	Height (DPM)	Area (DPM)	%ROI (%)	%Total (%)
Region 1	7:25	7:58	7:33	40.8	375.5	1.17	1.11
Region 2	9:42	9:56	9:49	40.8	171.4	0.53	0.51
Region 3	11:15	11:40	11:21	49.0	375.5	1.17	1.11
Region 4	12:27	13:16	12:41	2514.3	30865.3	95.96	91.62
Region 5	15:50	16:26	16:11	40.8	375.5	1.17	1.11
5 Peaks					32163.3	100.00	95.47

Name	Start (mm:ss)	End (mm:ss)	Retention (mm:ss)	Height (DPM)	Area (DPM)	%ROI (%)	%Total (%)
Region 1	1:30	2:09	1:44	81.6	775.5	2.34	2.21
Region 2	3:46	4:08	3:53	32.7	220.4	0.66	0.63
Region 3	5:33	6:07	5:48	57.1	571.4	1.72	1.63
Region 4	7:29	8:16	7:39	98.0	1657.1	5.00	4.72
Region 5	9:27	10:06	9:48	114.3	1534.7	4.63	4.37
Region 6	12:26	13:21	12:40	2081.6	28391.8	85.64	80.85
6 Peaks					33151.0	100.00	94.40

Figure 1. Time 0 (A) and 60 (B) minute radiochromatographs from mink microsomes incubated with ^{14}C-D5 siloxane (Retention of D5 parent at 12.4 min).



Name	Start (mm:ss)	End (mm:ss)	Retention (mm:ss)	Height (DPM)	Area (DPM)	%ROI (%)	%Total (%)
Region 1	9:14	9:50	9:18	40.8	506.1	1.11	1.08
Region 2	12:21	13:10	12:36	3257.1	44946.9	98.89	95.81
2 Peaks					45453.1	100.00	96.89

Name	Start (mm:ss)	End (mm:ss)	Retention (mm:ss)	Height (DPM)	Area (DPM)	%ROI (%)	%Total (%)
Region 1	1:27	2:13	1:57	65.3	1118.4	2.17	2.10
Region 2	5:16	5:55	5:47	40.8	449.0	0.87	0.84
Region 3	6:04	6:32	6:21	49.0	506.1	0.98	0.95
Region 4	6:46	7:08	6:54	73.5	538.8	1.04	1.01
Region 5	7:15	8:26	7:43	49.0	1159.2	2.25	2.17
Region 6	9:03	10:01	9:16	326.5	6334.7	12.28	11.88
Region 7	12:16	13:21	12:36	2873.5	41477.6	80.41	77.80
7 Peaks					51583.7	100.00	96.75

Figure 2. Time 0 (A) and 60 (B) minute radiochromatographs from mink microsomes incubated with ^{14}C-L4 siloxane (Retention of L4 parent at 12.3 min).

- GC-MS experiments with cold D5 were also conducted early in this study in hopes of optimizing test conditions without use of costly radiolabelled material. It was determined that GC-MS was not as sensitive as HPLC/β-RAM.
- A limited number of S9 studies with D5 siloxane were conducted. It was observed that the results of the S9 studies were similar to the microsome studies, suggesting biotransformation was Phase I dependent. Therefore, the project focus shifted to the use of liver microsomes.
- Typical liquid:liquid solvent extraction methods using hexane, hexane:ethyl acetate, etc were not effective. A 80:20 mix of acetonitrile:THF provided recoveries that were >90%.

Conclusions

- Microsomal biotransformation ^{14}C-D5 and ^{14}C-L4 is observed in a variety of species within 60 min of incubation.
  - For both D5 and L4, mink demonstrated the greatest potential for siloxane biotransformation (≥ 12% loss relative to Time 0).
  - Quail, kestrel, carp and catfish demonstrated negligible ability to biotransform D5 and L4 *in vitro*.
  - Trout, rat and human demonstrated some ability to biotransform D5 and L4.
  - Investigation of the radiochromatograms revealed the presence of biotransformation products that were more polar than parent D5 and L4.
  - Fluroxypyr positive controls revealed that the individual subcellular fractions were enzymatically active (e.g. esterase activity). Within 60 mins, the concentrations in the sub-cellular fractions were below levels of analytical detection.