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Hexadecylamine biotransformation rates in
carp and rainbow trout liver subcellular fractions

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Abstract

The aim of the present work was to investigate, using *in vitro* approaches, the fish capabilities to metabolize the cationic surfactant hexadecyl Primary Amine (C16PA) which may be present in surrounding water. The study of C16PA biotransformation rates has been undertaken using carp liver subcellular systems incubated with radio-labeled surfactant to investigate the disappearance of the parent compound and to quantify the amount of metabolites formed. For comparison, the biotransformation rates of this surfactant was also studied in rainbow trout microsomes.

The activity of the subcellular systems (homogenates and microsomes) from common carp (*Cyprinus carpio*) liver and of microsomes from rainbow trout was controlled measuring Ethoxycoumarine O-deethylase activity (ECOD).

The metabolism of the cationic surfactant hexadecylamine was investigated using radio-labeled molecule incubated with the subcellular liver fractions. The remaining parent compound as well as the resulting metabolite were analyzed by radio-TLC. The incidence of substrate concentrations and of incubation times on metabolism was tested.

Carp liver homogenates as well as microsomes were found to be able to metabolize hexadecyl Primary Amine, however, significant differences were found between fish species. The biotransformation rates of C16PA using carp liver microsomes were 5 fold higher than using trout microsomes.

Introduction

The rate of metabolic transformation is a dominant factor in determining the extent to which xenobiotics concentrate in fish. Most of the xenobiotic metabolizing enzymes are located primarily in the liver and the metabolites formed from the chemicals are largely the consequence of their hepatic biotransformation. Owing to the increasing pressure to minimize the use of whole-animal experimentations and to the central role played by hepatic xenobiotic metabolizing enzymes to biotransform chemicals, much effort has been devoted to the development of hepatic *in vitro* systems for assessing the fate of contaminants in fish. However, little information is available on the capabilities of fish to metabolize surfactants and on the usefulness of *in vitro* systems for measuring the biotransformation rates of these compounds.

In the precedent work, we investigated using *in vitro* approaches, the fish capabilities to metabolize two **anionic** surfactants (C12-LAS and C14-E2S) and two **non ionic** surfactants, (C13EO8 and C16EO8), using radiolabeled molecules incubated with carp liver subcellular fractions (Homogenates, S9 fractions and microsomes).

This work completes the data obtained on the fate of **anionic** and **non ionic** surfactants and concerns the biotransformation rates of the **cationic** surfactant hexadecyl Primary Amine (C16PA). After activity control of the subcellular fractions, radiolabeled hexadecylamine was incubated with carp liver homogenates and microsomes, then the remaining surfactant and the resulting metabolites were analysed by radio-TLC. The effects of incubation times and of substrate concentration on the biotransformation rates were investigated and a comparative study between carp and rainbow trout liver microsomes was undertaken.

Materials and Methods

1 Chemicals

7-Ethoxy[3-¹⁴C]coumarin was from Amersham (Les Ulis, France). Unlabeled ethoxycoumarin was from Sigma (Saint Quentin Fallavier, France). 1-[¹⁴C]- Hexadecylamine (specific activity 25.2 mCi/mmol) was provided by Procter and Gamble. All solvents were of analytical grade.

2 Activity control of the subcellular fractions

Carp liver microsomes and S9, and trout liver microsomes were stored at -80°C. Their conservation was controlled measuring Ethoxycoumarin *O*-Deethylase activity (ECOD):

¹⁴C-Labeled 7-ethoxycoumarin was used as substrate (10 nmol, 550,000 dpm) and incubated in 1 mL buffer PO₄ Na/K 0.1 M, 5 mM Mg Cl₂ pH 7.4 containing the subcellular fraction (10 mg protein S9 fraction or 2 mg protein microsomes) and a NADPH-generating system (NADP 1.27 mM, glucose 6-phosphate 5 mM and glucose 6-phosphate dehydrogenase 2 IU). After 2 hr incubation at 25°C, 3 ml methanol was added to stop the reaction process and samples were stored at 4°C until HPLC analysis.

After centrifugation to precipitate the proteins, samples were evaporated and analyzed by High Performance Liquid Chromatography (HPLC) as described by Zalko et al. (1998). The separations were performed on a C-18 reversed-phase column (ODS2 Spherisorb, 4.6x250 mm; 5µm; Interchrom, Montluçon, France) using a Spectra Physics P1000 system (Les Ulis, France) equipped with an on-line radioactivity detector (Radiomatic A 500, Packard Instruments Co.). Mobile phases consisted of ammonium acetate buffer (50mM, pH 5) and

acetonitrile 85 :15 (v/v) for A and ammonium acetate buffer (50mM, pH 5) and acetonitrile 20:80 (v/v) for B. The flow rate was 1ml/min and a two-step gradient was developed as follows : 0-5min, 100%A ; 5-25min, linear gradient from 100% A to 50 :50 (v/v)A/B ; linear gradient leading to 100% B. The chromatographic conditions permitted the separation and the quantification of 7-ethoxycoumarin and its major metabolite, 7-hydroxycoumarin. ECOD activity was expressed as pmol hydroxycoumarin formed/hr/mg protein.

3 Purity testing of C16 PA labeled surfactant :

The purity of the labeled surfactant (diluted solutions stored at 4°C in 100% ethanol) was controlled by thin layer chromatography (TLC) using silica gel plate 60 (without fluorescent indicator, pre-coated plates 20x20 cm, layer thickness 0.25 mm, E. Merck, Darmstadt, Germany). Chromatograms were analysed by an automatic TLC-Linear Analyzer LB 284/285 (Berthold, Wilbad, Germany) equipped with a Chroma 1D software.

The solvent mixture used was : dichloromethane/methanol/triethylamine 75:25:1 v/v in saturated cuve.

The radio-purity for ¹⁴C16PA was ca 98% and the compound was used without purification.

4 *In vitro* incubations of labeled C16PA with subcellular liver fractions (homogenates and microsomes) :

Frozen aliquots of three pools of carp liver homogenates or microsomes were thawed and kept on ice. ¹⁴C-Labeled surfactant (500 000 dpm in 2µl ethanol) was added in 1 mL buffer PO₄ Na/K 0.1M, 5 mM MgCl₂ pH 7.4 containing the subcellular fraction (homogenates 10 mg protein and microsomes 2 mg protein) and a NADPH-generating system (NADP 1.27 mM, Glucose 6-phosphate 5mM and Glucose 6-phosphate dehydrogenase 2 IU). Incubations were performed in silanized glass tubes plunged in a water bath at 25°C for 2h while shaking. Various concentrations were tested. Blanks (inactivated subcellular fraction by immersion in boiling water for about 3 min.) were systematically conducted in parallel. The reaction process was stopped by adding 3 ml methanol. Samples were kept on ice for about 10 min to precipitate the proteins and then were centrifuged at 8000 rpm for 10 min. Radioactivity was counted on an aliquot before incubation and after methanol precipitation using a Liquid Scintillation Analyzer (Tricarb 2200CA, Packard, A Canberra Company, USA) for measuring extractable radioactivity. An aliquot of the supernatant (400 µL, corresponding to ca 50 000 dpm) was evaporated in a low-binding microtube to dryness with a vacuum concentrator Speed Vac SC 110A (Thermo Savant, USA). Evaporated samples were resuspended in 20µL methanol before TLC analysis. Chromatography was carried out on silica gel plate 60 (without fluorescent indicator, pre-coated plates 20x20 cm, layer thickness 0.25 mm, E. Merck, Darmstadt, Germany). Plates were not preconditioned. The elution solvent consisted in dichloromethane/methanol/triethylamine 75:25:1 v/v in saturated cuve. TLC plates were analyzed with an automatic TLC-Linear Analyzer LB 284/285 equipped with Chroma 1D software, then the remaining surfactant and the resulting metabolites were quantified. Experiments were all performed in triplicate.

Results

1 ECOD activity control

	ECOD activity pmol/hr/mg
Carp liver homogenates	29.32 ± 6.89
Carp liver microsomes	199.66 ± 48.01
Trout liver microsomes	312.12 ± 138.79

Metabolization of 7-ethoxycoumarin obtained showed a higher activity in the microsomes of both species as compared with homogenates due to the higher P450 concentration in microsomes, which is responsible for the ethoxycoumarin deethylation.

2 C16PA biotransformation by carp liver homogenates

2.1. Typical radio TLC profile

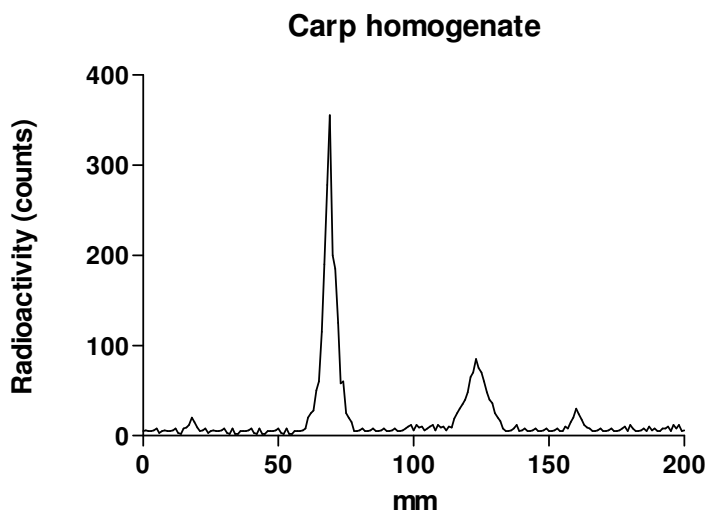


Figure 1 : Typical radio-TLC profile obtained from C16PA incubations (100 μ M, 2hr) with carp liver homogenates

The metabolic profile of C16PA incubates with carp liver homogenates exhibited the parent compound (Rf 0.34) and one peak (reaching 23% of the C16PA incubated) which corresponds to C16PA metabolite (Rf 0.60).

2.2. Kinetic study

In order to perform a time-course study, 10 μ M C16PA was incubated during 30, 60, 90, 120 and 150 minutes and the metabolite production was measured. The production of metabolite in homogenate increases with incubation time, reaching a plateau after 60 min.

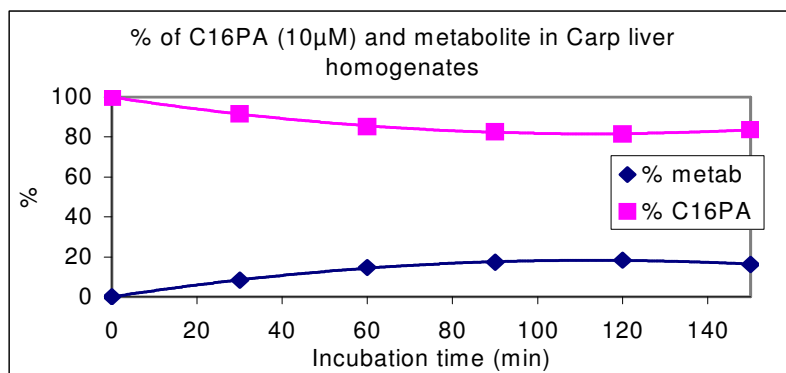


Figure 2 : Metabolite production (blue) and disappearance of C16PA (pink) in carp liver homogenate incubations (n=3).

The metabolic activity calculated in nmol metabolite formed/mg protein appears to increase linearly with incubation time.

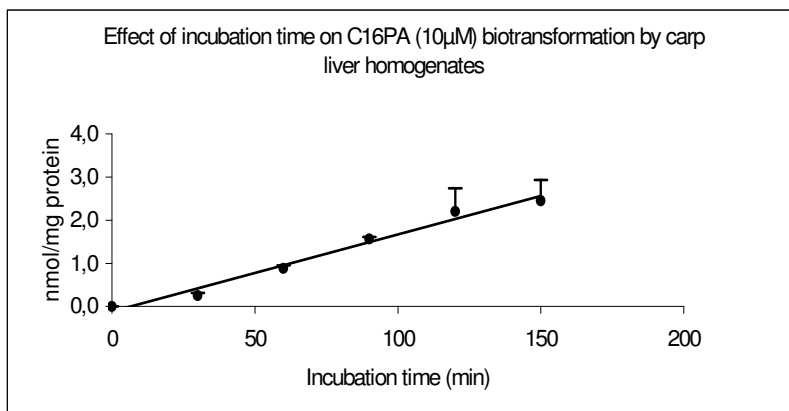


Figure 3 : Kinetic study of C16PA (10µM) metabolism by carp liver homogenates.

Data are expressed as mean \pm SD (n=3)

2.3.Effect of C16PA concentration on biotransformation rate

Six different C16PA concentrations were incubated 2 hours with carp liver homogenates. For each C16PA concentration, only one metabolite was produced and migrated at Rf 0.60. The production of this metabolite was calculated in picomoles per minute and per milligram of protein and presented versus C16PA concentration.

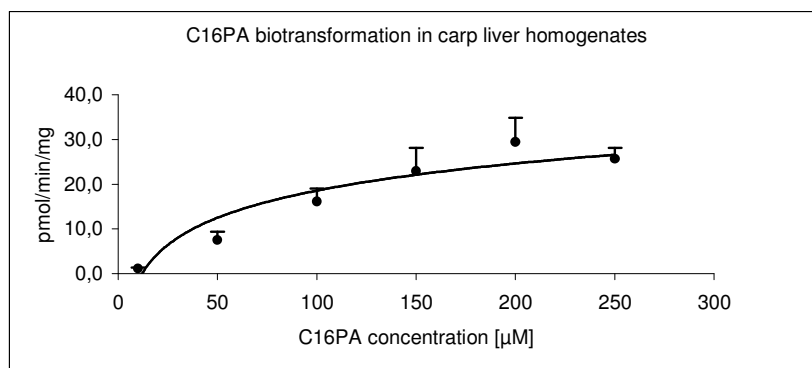


Figure 4 : C16PA metabolite production in carp liver homogenates.
Data are expressed as mean \pm SD (n=3)

The production of metabolite followed Michaelis-Menten kinetics increasing with the concentration of C16PA from 50 to 150 μ M and reaching saturation at 200 and 250 μ M.

3 C16PA biotransformation by carp liver microsomes

3.1. Typical radio TLC profile

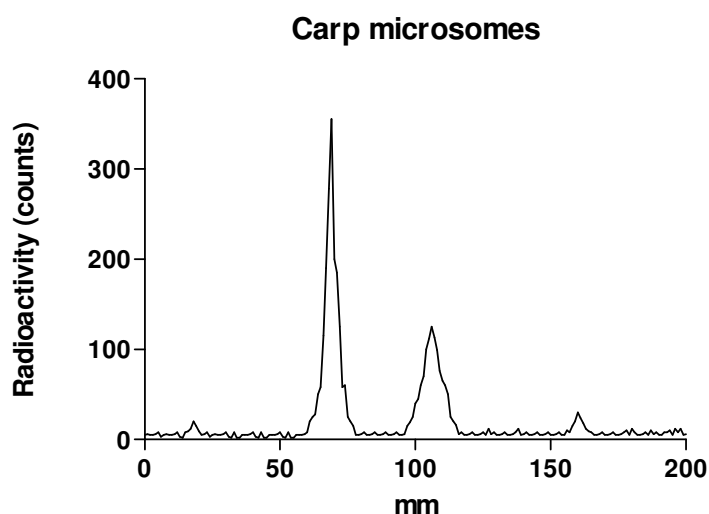


Figure 5 : Typical radio-TLC profile obtained from C16PA incubations (10 μ M, 2hr) with carp liver microsomes

As shown with carp liver homogenates, C16PA is extensively metabolized in carp liver microsomes. The metabolic profile of C16PA incubates with microsomes exhibited the parent compound (Rf 0.34) and one peak which corresponds to C16PA metabolite (Rf 0.60), this peak reaching 40% of C16PA incubated.

3.2. Kinetic study

10 μ M C16PA was incubated during 30, 60, 90, 120 and 150 minutes. The metabolite production was measured and reached 20% of C16PA incubated.

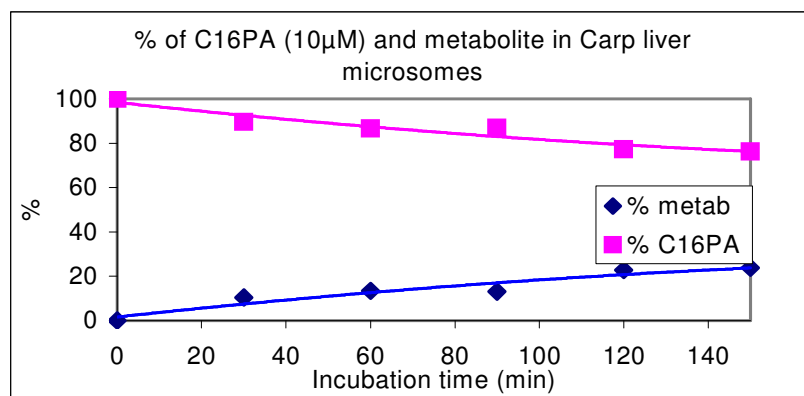


Figure 6 : Metabolite production (blue) and disappearance of C16PA (pink) in carp liver microsomes incubations (n=3).

The metabolic activity expressed in nmol metabolite produced/mg protein increases linearly with incubation time.

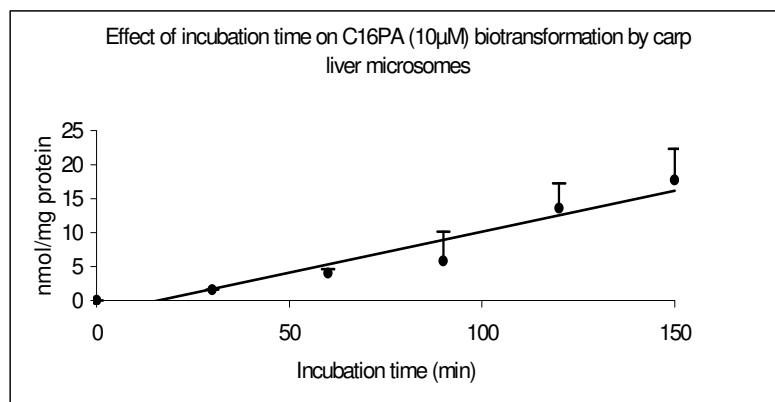


Figure 7 : Kinetic study of C16PA (10 μ M) metabolism by carp liver microsomes. Data are expressed as mean \pm SD (n=3)

3.3. Effect of C16PA concentration

C16PA at six different concentrations was incubated 2 hours with carp liver microsomes. For each C16PA concentration, only one metabolite was produced reaching 20-30% of C16PA incubated and migrated at Rf 0.60.

The production of this metabolite expressed in pmoles of C16PA metabolized per minute and per milligram of protein incubated, followed Michaelis-Menten kinetics increasing with the concentration of C16PA from 50 to 150 μ M and reaching saturation at 200 and 250 μ M.

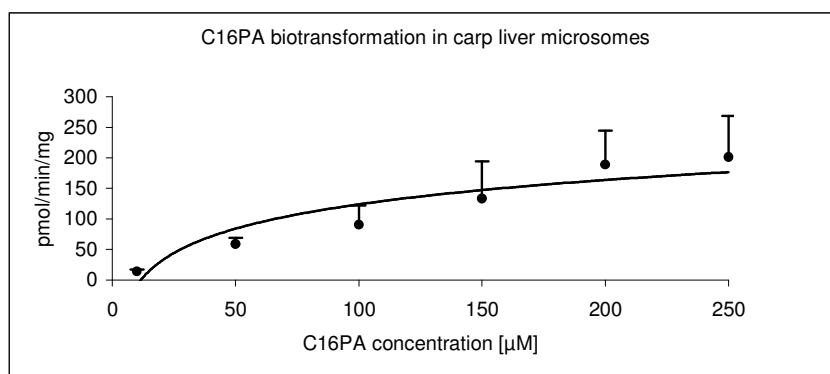


Figure 8 : C16PA metabolite production in carp liver microsomes.
Data are expressed as mean \pm SD (n=3)

4 C16PA biotransformation by rainbow trout liver microsomes

4.1. Typical radio TLC profile

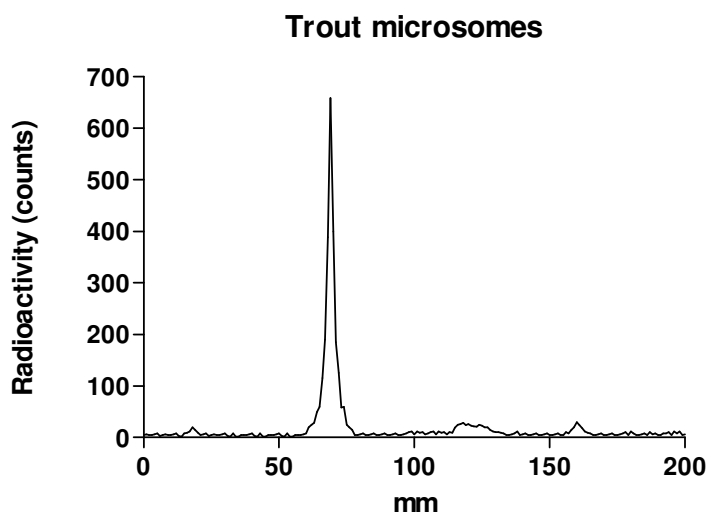


Figure 9 : Typical radio-TLC profile obtained from C16PA incubations (10 μM , 2hr) with trout liver microsomes

The metabolic profile of C16PA incubates with trout liver microsomes exhibited the parent compound (Rf 0.34) and one radioactive trace which could correspond to C16PA metabolite (Rf 0.60) but very difficult to quantify.

4.2. Effect of C16PA concentration

The six different C16PA concentrations were incubated with trout liver microsomes. TLCs were difficult to analyze, due to the low amount of metabolite produced (less than 5% total radioactivity for the peak Rf 0.60).

The results were however calculated and expressed in picomoles of metabolite produced per minute and per mg of microsomal protein. The rate does not follow Michaelis-Menten kinetics. It increases linearly with the C16PA concentration but remains very low, 45 pmoles/min/mg protein compared with the carp microsomes rates.

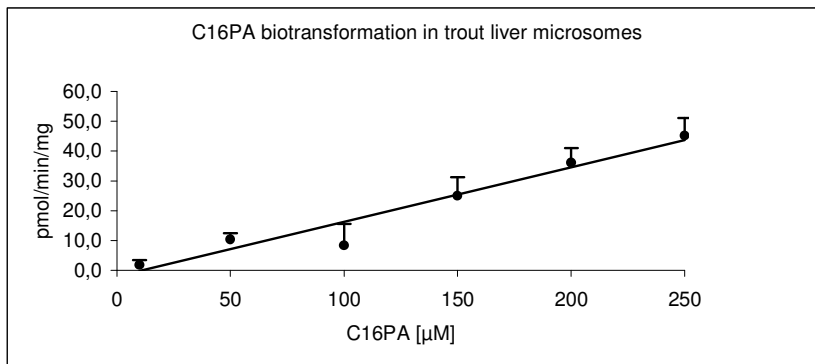


Figure 10 : C16PA metabolite production in trout liver microsomes.
Data are expressed as mean \pm SD (n=3)

5 Conclusions for C16PA biotransformation in vitro

As shown in the time-course study as well as in the concentration study, hexadecylamine is extensively metabolized in carp liver microsomes as well as in homogenates. The TLC metabolic profiles are similar in the both systems and the metabolization percent too (20% level, depending on the time or the C16PA concentration).

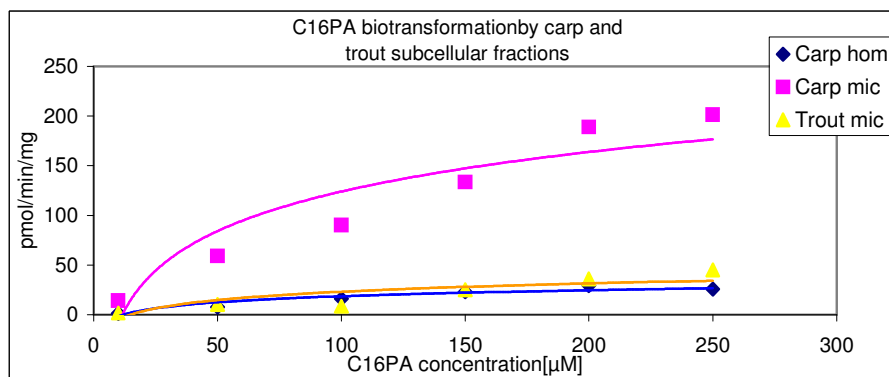


Figure 11 : C16PA metabolite production in carp liver homogenates and microsomes and in trout liver microsomes.
Data are expressed as mean \pm SD (n=3)

The biotransformation rate calculated in pmoles C16PA metabolized per minute and per milligram of protein, is higher in microsomes (200 pmol/min/mg protein) than in homogenates (20 pmol/min/mg protein) due to the high amount of proteins used in homogenates incubations.

Hexadecylamine is poorly metabolized in trout liver microsomes and lower metabolic rates were observed for trout microsomes (45 pmol/min/mg) than for carp microsomes (200 pmol/min/mg).

ANNEXES: Table of results in % values and in calculated values (nmol/mg and pmol/min/mg)

C16PA biotransformation by carp liver homogenates

Kinetic study

	Hom Carp 2	Hom Carp 3	Hom Carp 4		
Time (min)	% metab	% metab	% metab	Mean (%)	
0	0	0	0	0,00	
30	10	9	6	8,33	
60	14	16	14	14,67	
90	17	17	18	17,33	
120	18	23	14	18,33	
150	15	20	14	16,33	
Time (min)	nmol/mg	nmol/mg	nmol/mg	Mean (3)	SD (3)
0	0,00	0,00	0,00	0,00	0,00
30	0,30	0,27	0,18	0,25	0,06
60	0,84	0,96	0,84	0,88	0,07
90	1,53	1,53	1,62	1,56	0,05
120	2,16	2,76	1,68	2,20	0,54
150	2,25	3,00	2,10	2,45	0,48

Effect of C16PA concentration on biotransformation rate

	Hom Carp 2	Hom Carp 3	Hom Carp 4		
C16PA [µM]	% metab	% metab	% metab	Mean (%)	
10	12	16	13	13,67	
50	13	19	22	18,00	
100	16	19	23	19,33	
150	15	17	23	18,33	
200	14	19	20	17,67	
250	13	11	13	12,33	
C16PA [µM]	pmol/min/ mg	pmol/min/ mg	pmol/min/ mg	Mean (3)	SD (3)
10	1,00	1,33	1,08	1,14	0,17
50	5,42	7,92	9,17	7,50	1,91
100	13,33	15,83	19,17	16,11	2,93
150	18,75	21,25	28,75	22,92	5,20
200	23,33	31,67	33,33	29,44	5,36
250	27,08	22,92	27,08	25,69	2,41

C16PA biotransformation by carp liver microsomes

Kinetic study	Mic	Mic	Mic		
	Carpe 2	Carpe 3	Carpe 4		
Time(min)	metab %	metab %	metab %	Mean (%)	
0	0	0	0	0,00	
30	10	10	11	10,33	
60	14	15	11	13,33	
90	7	24	8	13,00	
120	16	28	24	22,67	
150	17	29	25	23,67	
Time(min)	nmol/mg	nmol/mg	nmol/mg	Mean (3)	SD (3)
0	0,00	0,00	0,00	0,00	0,00
30	1,50	1,50	1,65	1,55	0,09
60	4,20	4,50	3,30	4,00	0,62
90	3,15	10,80	3,60	5,85	4,29
120	9,60	16,80	14,40	13,60	3,67
150	12,75	21,75	18,75	17,75	4,58

Effect of C16PA concentration on biotransformation rate

C16PA [µM]	Mic	Mic	Mic		
	Carpe 2	Carpe 3	Carpe 4		
C16PA [µM]	% metab	% metab	% metab	Mean (%)	
10	26	40	36	34,00	
50	26	34	25	28,33	
100	20	30	15	21,67	
150	13	32	19	21,33	
200	17	30	21	22,67	
250	22	24	12	19,33	
C16PA [µM]	pmol/min/mg	pmol/min/mg	pmol/min/mg	Mean (3)	SD (3)
10	10,83	16,67	15,00	14,17	3,01
50	54,17	70,83	52,08	59,03	10,28
100	83,33	125,00	62,50	90,28	31,82
150	81,25	200,00	118,75	133,33	60,70
200	141,67	250,00	175,00	188,89	55,48
250	229,17	250,00	125,00	201,39	66,97

C16PA biotransformation by trout liver microsomes

Effect of C16PA concentration on biotransformation rate

	Mic Trout2	Mic Trout3	Mic Trout4		
C16PA [µM]	% metab	% metab	% metab	Mean %	
10	7	6	0	4,33	
50	6	4	5	5,00	
100	3	3	0	2,00	
150	5	4	3	4,00	
200	5	4	4	4,33	
250	5	4	4	4,33	
C16PA [µM]	pmol/min/ mg	pmol/min/ mg	pmol/min/ mg	Mean (3)	SD (3)
10	2,92	2,50	0,00	1,81	1,58
50	12,50	8,33	10,42	10,42	2,08
100	12,50	12,50	0,00	8,33	7,22
150	31,25	25,00	18,75	25,00	6,25
200	41,67	33,33	33,33	36,11	4,81
250	52,08	41,67	41,67	45,14	6,01