Biotransformation of an environmental azaarene pollutant and vitamin A by human, rat and fish aldehyde oxidase

Omaro A. Aburas[1], Emhamed Boras[2], Juma Ahmad Ali[3].

[1]Higher Institute of Science and Medical Technology, Tripoli/Abusalim

[2]Libyan Academy of postgraduate Studies, Life Science Department, Tripoli/ Janzor

[3]Faculty of Science, Department of Zoology, University, of Gharyan/Libya

Abstract

Aldehyde oxidase (AOX) is molybdo-flavoenzyme involved in the oxidation of hundreds of endogenous and exogenous and Nheterocyclic compounds and environmental pollutants. Uncharged Nheterocyclic aromatic compounds such phenanthridine are commonly distributed pollutants in soil, air, sediments, surface water and groundwater, and in animal and plant tissues. Retinal (vitamin A) and its metabolite retinoic acid are important in the visual system and for cell differentiation in vertebrates, so processes involved in their interconversion are important to study. Phenanthridine as uncharged N-heterocyclic aromatic compound and retinal (vitamin A) was incubated with partially purified aldehyde oxidase from fish rainbow trout (*Oncorhynchus mykiss*) liver, human liver and Wister rat liver. Reversed-phase HLPC method was used to separate the oxidation products from phenanthridine and the metabolite and retinal and the metabolite was identified. The 6(5H)-phenanthridinone was identified the major metabolite and retinoic acid the metabolite of retinal by partially purified aldehyde oxidase from fish liver, rat liver and human liver. The comparison obtained in the present study shows that phenanthridine were good substrates of human and rat aldehyde oxidase (AOX). The enzyme kinetic parameters for aldehyde oxidase (AOX) for human , rat and trout fish. The Km value of exogenous N-heterocyclic phenanthridine with trout it was 55.0 ± 1.63µM. On other hand, the Km value of mammalian enzyme Wistar rat and human with exogenous N-heterocyclic phenanthridine were 5.60 ± 0.65 µM, 3.20 ± 0.23 µM respectively. The Km of the trout fish AOX with phenanthridine was approximately 10 times greater than that with mammalian AOX. The Vmax value of Wistar rat and human with phenanthridine was approximately 2 and 4 times respectively more than that with trout fish AOX . The Km value of trout AOX with all-trans retinal was 124.7 ± 2.27 µM was approximately 4-fold greater than that measured with Wistar rat with all-trans retinal and ~ 10 fold more than that of human AOX. The Vmax value of trout AOX with all-trans retinal was 2.22 ± 0.62 nmol/min/mg protein and was approximately 3 and 4-fold lower than that Wistar rat and human respectively.

Keywords—Aldehyde oxidase (AOX) , Wister Rat, Human, Fish Rainbow trout , Phenanthridine, All trans retinal, Specificity.

Introduction

Aldehyde oxidase (AOX) is an enzyme that is involved in the metabolism of a diverse array of endogenous and exogenous aldehydes and N-heterocyclic compounds. Currently there are very few studies on aldehyde oxidase in vertebrate species such as humans that have only one functional AOX gene compared with the more extensively studied laboratory rodents that have a four functional AOX genes [1], [2], [3]. Most notably there has been no report of the biotransformation of toxic azaarenes or endogenous vitamin aldehydes by AOX in the most evolved vertebrate humans or the most primitive vertebrate class fish that have only one functional AOX gene [4], [5]. In this study two such compounds, phenanthridine and retinal, were targeted as AOX substrates for the following reasons. Phenanthridine is an azaarene pollutant found in terrestrial and aquatic environments from industrial activities associated with fossil fuels [6], [7], [8]. Azaarenes are therefore a cause for concern both to humans and aquatic wildlife [9], [10], [11]. At present it is not known whether humans or aquatic species such as fish can metabolise phenanthridine to a more polar metabolite (phenanthridone) aiding its excretion [12], [13], [14]. Retinal (vitamin A) and its metabolite retinoic acid are important in the visual system and for cell differentiation in vertebrates [15], [16], [17], so processes involved in their interconversion are important to study. In this study evaluated if AOX in humans and a representative fish and rainbow trout could biotransform phenanthridine and retinal comparing with the activity found in laboratory rat.

Materials and Methods

-*Reagents and chemicals*

All reagents and chemicals were obtained from Fisher Scientific and Sigma / Aldrich Chemical Company Ltd, Poole, UK. Solvents and mobile phase reagents were obtained from different companies but were all for HPLC grade purity. The control of substances hazardous to health (COSHH) risk assessment for all reagents and chemicals were prepared before experiments were initiated.

-*Preparation of cytosol*

The preparation of cytosol all steps were carried out at 0 – 4°C. Approximately 10 g of liver, weighed out and homogenised in 40 ml of cold buffer (0.25 M sucrose, 10 mM Tris HCL pH 7.4) with a motor-driven tissue mortar fitted with a Teflon pestle(clearance 0.15-0.23mm, speed 10000 rpm and 5 minute duration) to produce a 25% w/v homogenate. The homogenate was then centrifuged at 4°C for 15 minutes at 10,000 xg, to pellet out the nuclear/mitochondrial fractions of the homogenate. The supernatant was then removed and centrifuged for a further

60 minutes at 4°C at 105,000 xg to obtain the cytosolic fraction. This fraction was then collected and separated into 0.5 ml aliquots and stored at -80°C.

-Gel filtration of cytosol

Gel filtration of cytosol was carried out in order to remove endogenous small molecules, such as endogenous substrates and inhibitors, which may interfere with enzyme assays. A PD-10 gel filtration column was used (GE Healthcare Bio-Science, UK). Prior to use the column was allowed to drain before being equilibrated with 25 ml (5 column volumes) 50 mM Tris HCL pH 7.4. 2.5 ml of cytosol was then loaded onto the column and eluted with 3.5 ml of 50 mM Tris HCL pH 7.4, the protein containing fraction was then collected, pooled and aliquoted into 0.5 ml fractions in 1.5 ml polypropylene Eppendorf tubes to avoid repeated freeze/thawing and stored at -80°C.

-Protein determination

The amount of protein in each sample was calculated using a modification of the method described by Smith *et al*. using bovine serum albumin (BSA) as standard (Smith *et al.*, 1985). The bicinchoninic acid (BCA) based assay is available as a kit from Sigma-Aldrich Co.

-HPLC analysis of phenanthridine and all trans retinal

The analysis of phenanthridine and all trans retinal compounds and their corresponding metabolites were carried out by reverse phase HPLC. The system used comprised of a Beckman Coulter System Gold^{TM} 127 Solvent HPLC Module (dual pump) and a programmable UV detector (module 166) or a programmable diode array detector (module 168) along with injector designed with a 20 µl sample loop and auto sampler.

-Spectophotometric assay of phenanthridine and all trans retinal

AO assays were performed with 40µl of gel filtered cytosol, 10µl of 1mM of substrate (phenanthridine and all trans retinal) and 50µl Tris HCL pH 7.4 at 37°C. Spectrophotometric molybdo-flavoenzyme assays were conducted by using a microplate spectrophotometer (BioTek) at 37ºC

-Aldehyde oxidase assay

AO assay were performed using 50uL of liver cytosol and 100uM of substrate with molecular oxygen as electron acceptor. Following incubation substrates and products were resolved using an gradient HPLC system fitted with a Kromasil 5µm; C18 column (25cm x 4.6mm,) using ammonium acetate and acetonitrile as mobile phase [18], [19] and [20]. Protein concentration was determined using BCA reagent.

Results and Discussion

Phenanthridine is an pollutant found in terrestrial and aquatic environments from industrial activities associated with fossil fuels [27]. phenanthridine are therefore a cause for concern both to humans and aquatic wildlife. Several papers reported that the phenanthridine is a good substrate and has a very high affinity towards AOX [28],[29] and [21]. The Km and Vmax were calculated from linear oxidation rates using Eadie-Hofstee plot (Fig. 5,6 and 7) for N heterocyclic aromatic compound phenanthridine as aldehyde compounds by Aldehyde oxidase (AOX). The results obtained in the present study shows that phenanthridine were good substrates of human and rat aldehyde oxidase (AOX). The enzyme kinetic parameters for aldehyde oxidase (AOX) for human , rat and trout fish are listed in (table 1). The Km value of exogenous N-heterocyclic phenanthridine with trout it was 55.0 ± 1.63µM. On other hand, the Km value of mammalian enzyme Wistar rat and human with exogenous N-heterocyclic phenanthridine were 5.60 ± 0.65 µM, 3.20 ± 0.23 µM respectively (Table 1). The Km of the trout fish AOX with phenanthridine was approximately 10 times greater than that with mammalian AOX. The Vmax value of Wistar rat and human with phenanthridine was approximately 2 and 4 times respectively more than that with trout fish AOX (Table 1). It was a good substrate with both rat and human in my study and with a Km value of 5.6 ± 0.65 µM with rat and Km value of 3.2 ± 1.23µM with human. This agreement with phenanthridine as a specific substrate of AOX has been used in several papers and Km previously has been estimated were < 1 µM with rabbit and guinea pig liver AOX enzyme and 6 µM and 14 µM with rat and human liver AOX respectively [28].[29] and [22].

Table 1. Kinetic constants for phenanthridine oxidase activity in fish (Rain bow trout), human and rat liver cytosol in spectrophotometric assays

Species	K_m (µM)	V_{max} (nm/min/mg protein)
Human	3.2 ± 0.23	4.16 ± 0.14
Rat	5.60 ± 0.65	2.43 ± 0.14
Fish (rain bow trout)	55.0 ± 1.63	1.78 ± 0.55

Values shown are the mean ± S.D. The typical results are from three independent experiments. (*P< 0.05) n = 3.

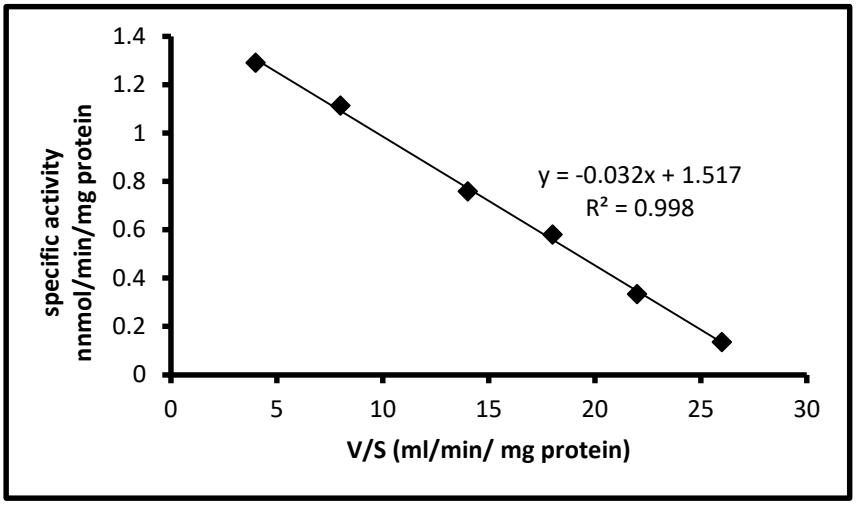


Figure 5. Eadie-Hofstee plot of phenanthridine oxidase activity in human liver cytosol

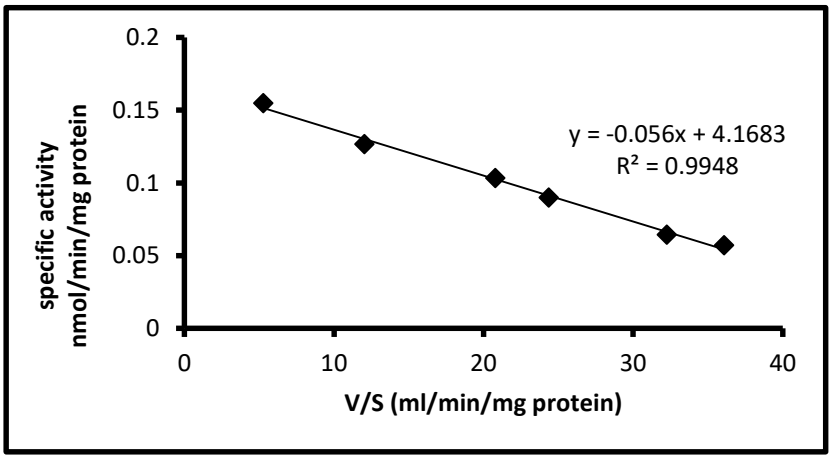


Figure 6. Eadie-Hofstee plot of phenanthridine oxidase activity in rat liver cytosol

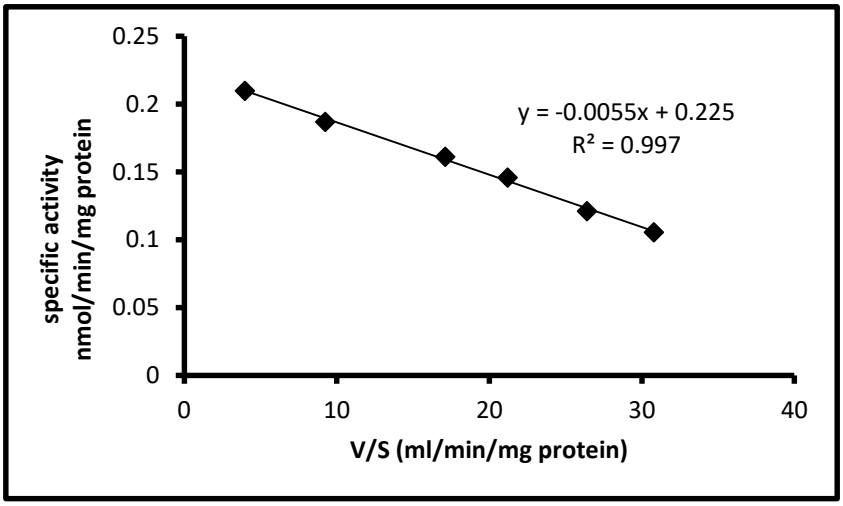


Figure 7. Eadie-Hofstee plot of phenanthridine oxidase activity in fish rainbow trout liver cytosol

Another potential substrate of AOX of physiological importance is all-trans- retinal aldehyde which it oxidised to retinoic acid [30], [23] and [24]. Retinoic acid is a key regulator of the homoeostasis of keratinized epithelia and a recognized morphogen of the vertebrate organisms [31], [25] and [26]. The Km and Vmax were calculated from linear oxidation rates using Eadie-Hofstee plot (Fig. 8,9 and 10) for endogenous vitamin(A) aldehydes. The Km value of trout AOX with all-trans retinal was 124.7 ± 2.27 µM was approximately 4-fold greater than that measured with Wistar rat with all-trans retinal and ~ 10 fold more than that of human AOX (Table 2). The Vmax value of trout AOX with all-trans retinal was 2.22 ± 0.62 nmol/min/mg protein and was approximately 3 and 4-fold lower than that Wistar rat and human respectively. The Km values found in this study were of a similar magnitude to those found by Schumann *et al*., 2009. it found the Km value of all-trans retinal aldehyde (vitamin A) with mouse AOX1 of 55.8 ± 8.8 µM [32],[29].

Table 2. Kinetic constants for All trans retinal oxidase activity in fish (rain bow trout), human and rat liver cytosol in spectrophotometric assays

Species	K_m (µM)	V_{max} (nm/min/mg protein)
Human	12.6 ± 0.58	8.32 ± 0.06
Rat	30.9 ± 1.23	5.16 ± 0.56
Fish (rain bow trout)	124.7 ± 2.27	2.22 ± 0.62

Values shown are the mean ± S.D. The typical results are from three independent experiments. (*P< 0.05) n = 3.

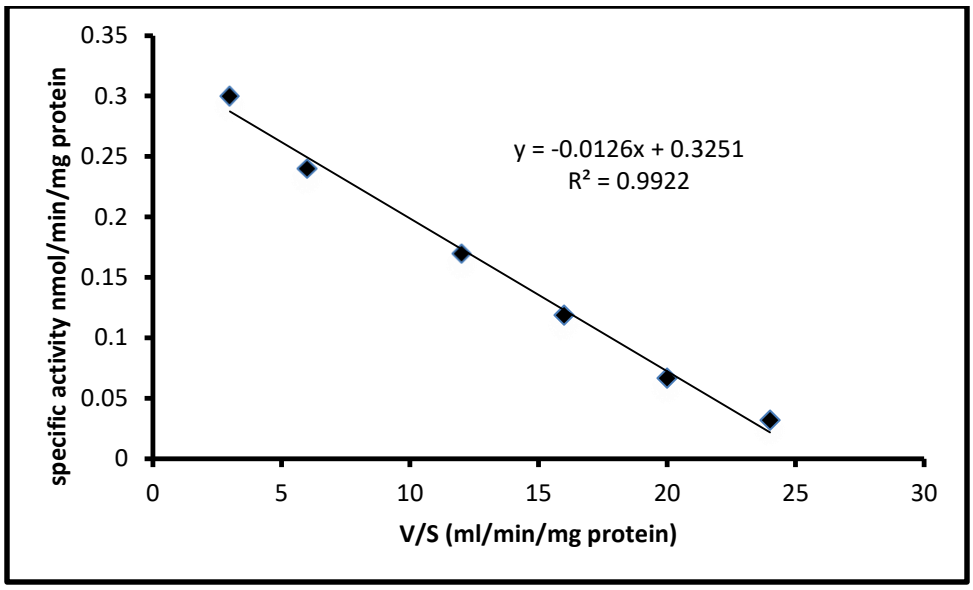


Figure 8. Eadie-Hofstee plot of all trans retinal oxidase activity in human liver cytosol

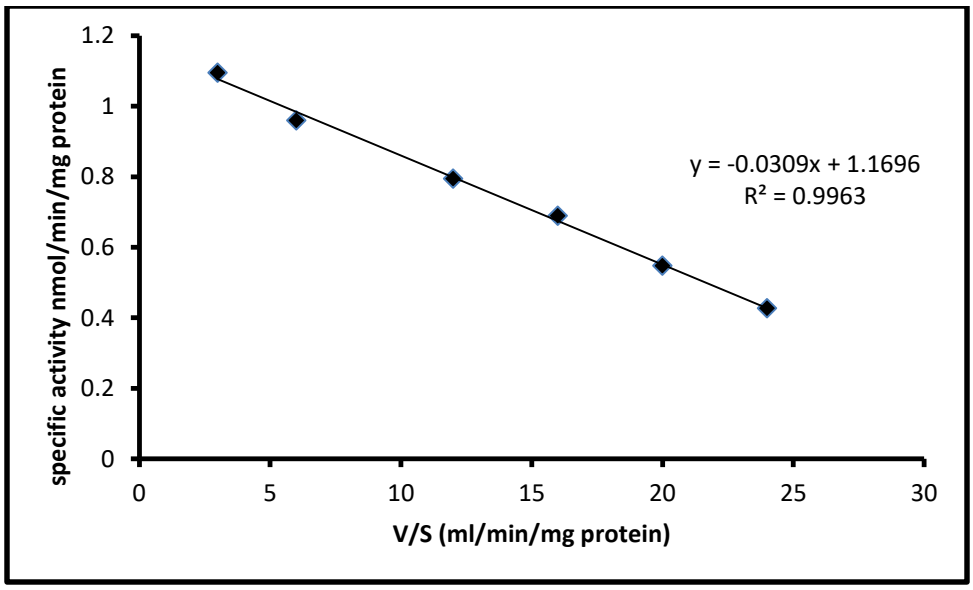


Figure 9. Eadie-Hofstee plot of all trans retinal oxidase activity in rat liver cytosol

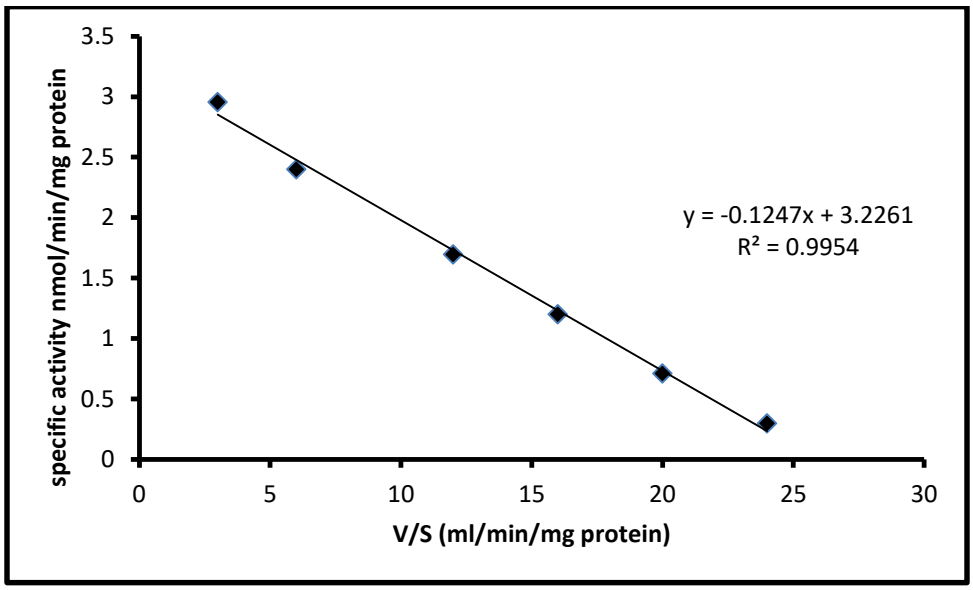
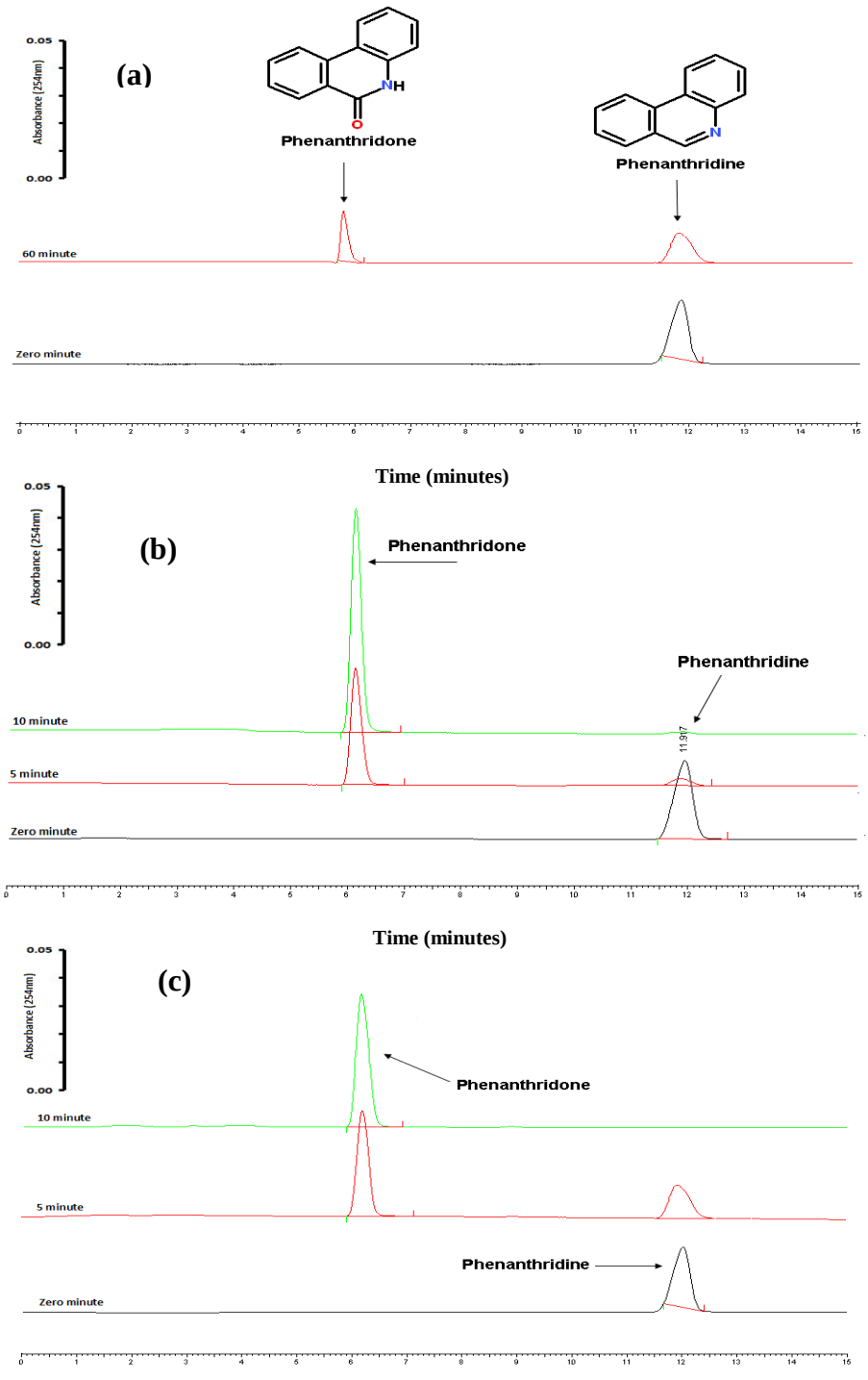


Figure 10. Eadie-Hofstee plot of all trans retinal oxidase activity in fish rainbow trout liver cytosol

HPLC analysis of the *in vitro* biotransformation of phenanthridine by Fish rainbow trout, Wistar rat, and human liver cytosol.(Figure 11).(a) rainbow trout, (b) Wistar rat, (c) human liver cytosol. HPLC chromatogram of the *in vitro* oxidation of phenanthridine to phenanthridone .Times at the left hand side of the chromatograms indicate incubation times. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 µm) and eluted with water: acetonitrile gradient system as described in materials and methods. The wavelength of the detection was 254 nm . When the prototypical N-heterocyclic AOX substrate phenanthridine was used as a substrate in HPLC assay (Figure 11) the Wistar rat and human liver cytosol had more activity than that trout liver cytosol. As there is evidence that the trout fish generates phenanthridone from phenanthridine *in vivo*. This demonstrated that rainbow trout liver cytosol AOX was able to generate measurable phenanthridone (Figure 11, a). In contrast there was more detectable phenanthridone formed with extended incubation with human and rat cytosol (Figure 11).



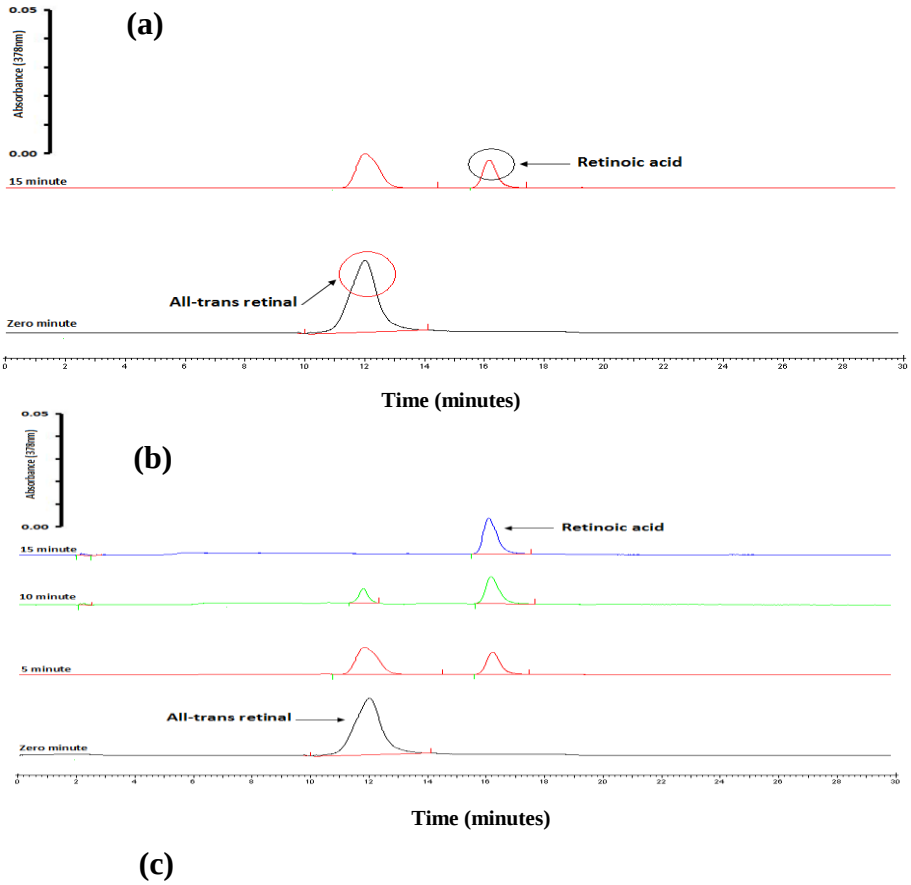
Rainbow trout
Liver
Phenanthridine
AOX

Rat
Liver
Phenanthridine
AOX

Human
Liver
Phenanthridine
AOX

Figure 11. HPLC analysis of the ınsformation of phenanthridine by rainbow trout, Wistar rat, and human liver cytosol.(a) rainbow trout, (b) Wistar rat, (c) human liver cytosol.

HPLC analysis of the *in vitro* biotransformation of endogenous compound all-trans retinal by liver cytosol from different species. HPLC chromatogram of the *in vitro* oxidation of all-trans retinal to retinoic acid by (a) Fish rainbow Trout, (b) Wistar rat, (c) human liver cytosol. Times at the left hand side of the chromatograms indicate incubation times. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 µm) and eluted with a 30 mM CH_3COONH_4: acetonitrile (30: 70) as mobile phase. The wavelength of the detection was 378 nm. When all-trans retinal (vitamin A) was incubated with rainbow trout, Wistar rat and human liver cytosol, the AOX metabolite retinoic acid was generated with cytosols from all three species (Figure 12).



Rainbow trout
Liver
Retinal
AOX

Rat
Liver
Retinal
AOX

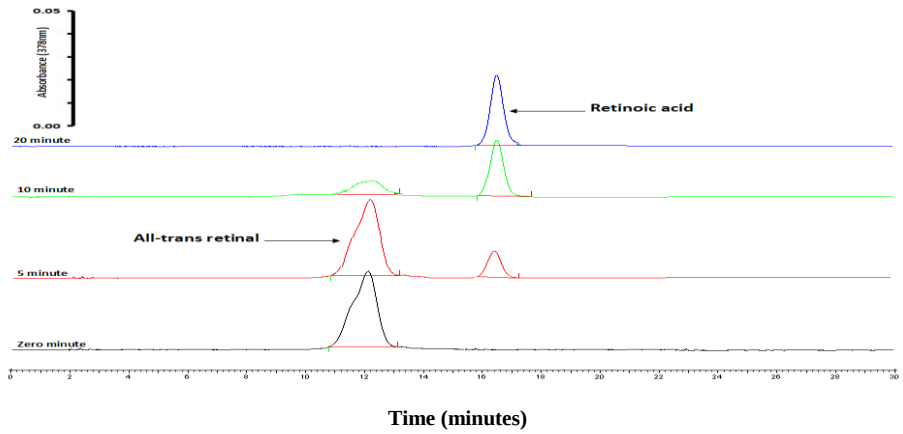


Figure 12. HPLC analysis of the *in vitro* biotransformation of endogenous compound all-trans retinal by liver cytosol from different species.HPLC chromatogram of the *in vitro* oxidation of all-trans retinal to retinoic acid by (a) rainbow Trout, (b) Wistar rat, (c) human liver cytosol.

Conclusions

This study aimed to determine whether a fish rainbow trout (*Oncorhynchus mykiss*) can biotransform AOX substrates phenanthridine and vitamin A all trans retinal aldehyde. Rainbow trout AOX were tested in the liver cytosol using HPLC and spectrophotometric assays. Hepatic cytosols of rainbow trout, human and rat were able to catalyse the oxidation of phenanthridine and vitamin A all trans retinal aldehyde to varying degrees. When Eadie-Hofstee plots were used to determine kinetic values of rainbow trout activities with phenanthridine and all trans retinal and compared with human and rat and it was found that the Km was higher and the Vmax lower in rainbow trout. This study therefore demonstrated that a trout fish AOX has the ability to metabolise phenanthridine as environmental pollutants and all trans retinal. The biotransformation by AOX this is an important detoxication mechanism as several studies *in vivo* and *ex vivo*. In conclusion this study yields new insight into groups of anthropogenic environmental pollutants and vitamins that are substrates of trout fish AOX that is an ancestral vertebrate AOX.

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