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Biological effects of contaminants: Quantification of δ-aminolevulinic acid dehydratase (ALA-D) activity in fish blood

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This document describes a colorimetric method to quantify the enzyme δ-aminolevulinic acid dehydratase (ALA-D) in fish blood. ALA-D is an enzyme in the heme synthesis pathway. The activity of the enzyme is inhibited by lead (Pb) and it has, therefore, been used as a biomarker for lead exposure and effects in mammals, birds, and fish. This paper describes optimal conditions for the kinetic determination of ALA-D activity in fish blood, standardized to protein content.

Key words: δ-aminolevulinic acid dehydratase, ALA-D, lead, biological effects, ecotoxicological effects, Atlantic cod, flounder, dab, red blood cells, heme synthesis.

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1 INTRODUCTION

Many metals are essential to all organisms, e.g., Cu, Zn, Fe, Mn, and Mo, but there are also metals for which no biological function is known, e.g., Hg, Cd, Au, Ag, and Pb. Such non-essential metals generally have much higher toxicity than the essential metals. Uptake, storage, and excretion are also less well controlled for the non-essential metals than for essential metals.

One of the most important toxic mechanisms of non-essential metals is interaction with and inhibition of enzymes, especially enzymes with metal co-factors. One such enzyme is δ-aminolevulinic acid dehydratase (ALA-D, E.C. 4.2.1.24) with Zn as a co-factor (Granick *et al.*, 1972). This enzyme is one step in the synthesis pathway for heme and is found in bacteria, plants, and invertebrates, as well as in vertebrates. Heme is incorporated in macromolecules such as hemoglobin and cytochromes. In mammals and birds, inhibition of ALA-D may lead to anemia since it is one of the rate-limiting enzymes in heme (and hence hemoglobin) synthesis. This does not appear to be the case for fish (Larsson *et al.*, 1985). The reason for ecotoxicological interest in ALA-D is its inhibition by Pb, even at very low exposure levels (Hodson *et al.*, 1984; Haux and Förlin, 1989).

ALA-D has been used to investigate Pb effects in, e.g., rainbow trout (Hodson, 1976; Hodson *et al.*, 1977, 1978; Addison *et al.*, 1990; Burden *et al.*, 1998; Sordyl and Osterland. 1990), flounder (Bogovski *et al.*, 1998), longear sunfish (Dwyer *et al.*, 1988), perch (Haux, *et al.*, 1985), crucian carp (Nakagawa *et al.*, 1997b), various catostomid species (Schmitt *et al.*, 1984), and grey mullet (Krajnovic-Ozretic and Ozretic, 1980).

The sensitivity, timing, and specificity of ALA-D inhibition in response to lead exposure were reviewed by Hodson *et al.* (1984). For rainbow trout, red blood cell ALA-D activity may be inhibited following exposure to less than 5 $\mu g\ l^{-1}$ of lead through water (Haux *et al.*, 1986) and blood lead concentration of 300 $\mu g\ l^{-1}$ (Hodson *et al.*, 1982), although other studies indicate a lower sensitivity (Burden *et al.*, 1998). In rainbow trout, ALA-D inhibition is evident 1–2 days following exposure to lead and there is a clear dose-response relationship (Hodson *et al.*, 1982). The half-life of the response will depend on the amount of lead accumulated in other tissues (in equilibrium with blood), but will generally be of the order of months. Although other metals affect ALA-D activity *in vitro*, such effects are weak or absent *in vivo* (see Section 4).

ALA-D activity in red blood cells of Atlantic cod (*Gadus morhua*), flounder (*Platichthys flesus*), and dab (*Limanda limanda*) has been used in the Norwegian JAMP[1] for five years (1997–2001; Hylland, pers. obs). In that programme, ALA-D was generally found to be inhibited in the blood of fish from areas with known urban impact and in one area with a known metal impact (see Section 3).

This paper describes the method of analysis for ALA-D in fish blood, including sampling considerations, sources of error, and quality assurance considerations.

2 TEST METHOD

2.1 Design of Study

The response in any biological effects method will be modulated by various endogenous and exogenous factors. It is crucial that relevant factors are controlled, either through sampling design or through additional analyses or observations. The same fish species must be used

[1] JAMP: Joint Assessment and Monitoring Programme; a national programme to implement the OSPAR Commission JAMP.

throughout. Although maturation and spawning are not known to affect ALA-D, there are major physiological and biochemical changes during that period. It is also known whether there are seasonal differences in basic ALA-D activity. All samples should therefore be collected within a period of one month outside the spawning season for the relevant species. For most temperate marine species, early autumn is the most appropriate period. Length, weight, liver weight, and gross external disease or parasites should be recorded for each individual. If there is a substantial heterogeneity in any of the above factors (e.g., size differences), the sample size should be increased. The required number of samples will depend on factors such as the objective of the study, local conditions, and analytical procedures. For Atlantic cod, 15–20 individual fish would be required to reject H_0: no difference between sites (α=0.05) if the true mean differed by a factor of two. The variability obviously differs between species and populations.

2.2 Chemicals

Perchloric acid, acetic acid, trichloroacetic acid (TCA), potassium hydrogen phosphate, potassium di-hydrogen phosphate, all analytical grade, can be purchased from any major supplier of chemicals. Porphobilinogen (PBG), *p*-amino benzoic acid (PAB, Ehrlich's reagent), δ-aminolevulinic acid, and Triton X-100 are available from Sigma-Aldrich.

Other chemicals such as buffer constituents should be of analytical grade and are available from most major suppliers of laboratory chemicals. PBG and δ-aminolevulinic acid should be stored dry at or below −20 °C; the remainder can be stored at room temperature (acids in appropriately ventilated cupboards or under fume hoods).

2.3 Preparation of Reagents and Buffers

Modified Ehrlich's reagent: dissolve 0.35 g of mercury chloride in 6 ml of distilled water and 20 ml of 70 % perchloric acid. Add acetic acid to a total volume of 110 ml. Dissolve 2.0 g PAB in the final volume. This solution needs to be freshly made each day and should be prepared in a fume hood.

Precipitation solution: dissolve 4.0 g trichloroacetic acid and 2.7 g mercury chloride in distilled water to a volume of 100 ml. Trichloroacetic acid must be added in a fume hood.

Dilution buffer: 0.1 M potassium phosphate buffer with 0.2 % Triton X-100. The pH of the phosphate buffer needs to be optimized for new fish species. For rainbow trout and various other freshwater fish, the optimum is pH 6.2 (Hodson *et al.*, 1984), whereas it is pH 7.0 for Atlantic cod, flounder, and dab.

ALA-reagent: dilute 33.5 mg δ-aminolevulinic acid in 50 ml dilution buffer; prepare fresh every day.

Standard solution: dissolve PBG in dilution buffer to a final concentration of 40 $\mu g\ ml^{-1}$. Prepare fresh every day.

2.4 Equipment

A refrigerated centrifuge with a fixed-angle rotor (capable of at least 10,000 × **g**) and a motorized homogenizer are needed for homogenization and separation. A plate-reader or spectrophotometer fitted with a filter of wavelength 553 (550) nm is also needed. In addition, equipment to refrigerate and store samples is required (a liquid nitrogen thermos or carrier and a –80 °C freezer).

2.5 Selection of Tissue and Treatment of Samples

ALA-D activity can be determined in liver, kidney, spleen, and red blood cells in teleosts (see Hodson *et al.*, 1984). In general, red blood cells or whole blood is the matrix of choice, both owing to the ease of sampling and the high activity of the enzyme. One ml of blood should be sampled from the caudal vein using a heparinized syringe. Whole blood and red blood cells can be used for the analyses, although the latter is preferable. Red blood cells should be separated from plasma by centrifugation at 5,000 × **g** for 5 mins (at 4 °C). Prior to centrifugation, the blood should be kept on ice. Whole blood or red blood cells should immediately be transferred to appropriate vials and frozen in liquid nitrogen. Samples may be shipped on dry-ice and stored at −80 °C prior to analysis.

2.6 Preparation of Sample Supernatant

The procedures should be performed at 0–4 °C. Frozen samples should be thawed on ice and homogenized in three volumes (v/v) of dilution buffer (if blood cells) and in an equal volume of dilution buffer (if whole blood), using a Potter-Elvehjem glass-Teflon motorized homogenizer. For small samples, the homogenization may be done directly in the sample vial using a modified Teflon pestle. The homogenization should be done on ice and the sample kept cold at all times. Samples for Pb analyses may be taken from the homogenate[2]. Following homogenization, samples should be centrifuged for 30 min at 10,000 × **g** (at 4 °C). The resulting supernatant is used for measurement of ALA-D activity and total protein.

2.7 Protein Determination

ALA-D activity can be normalized to red blood cell volume (hematocrit), whole blood volume or protein in the red blood cells. The latter is preferred to avoid problems associated with changes in blood cell volume. Protein in the red blood cell supernatant prepared in Section 2.6 may be determined by any protein assay, e.g., Bradford (Bradford, 1976; Read and Northcote, 1981) or Lowry (Lowry *et al.*, 1951). Both assays can easily be adapted to measurement by plate reader. Care should be taken to select the correct protein standard. Although used by many workers, bovine serum albumin (BSA) should be avoided in the Bradford assay because it gives around twice the absorbance of most other proteins at any given concentration. Bovine gamma globulin is recommended as a general protein standard.

2.8 Measurement of ALA-D Activity

The analytical method for ALA-D described herein includes the use of mercury salts. Alternative methods have been proposed that exclude this toxic metal (Nakagawa *et al.*, 1997b). However, the advantage of the method described here in relation to others is the stability of intermediate solutions. Some other methods appear to be sensitive to the length of time between the steps in the assay, thus increasing variability and decreasing reproducibility. Please refer to Section 2.12, below, for a description of safety procedures when handling materials with mercury.

The analysis involves three discrete steps: (i) sample dilution and initiation of reaction, (ii) stopping the reaction and separation of product from reagents, and (iii) quantifying the product.

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[2] The homogenizing buffer needs to be checked for metal content.

Step (i): dilute the supernatant (prepared in Section 2.6) 1:2 in dilution buffer. Add 50 µl of this solution into six Eppendorf tubes. Then add 200 µl of dilution buffer into three tubes and 200 µl of ALA-reagent into the remaining three tubes. Mix well and incubate for 2 h at 25 °C (room temperature, RT).

Step (ii): prepare standards from the standard solution in dilution buffer to a total volume of 400 µl; 20 µl, 40 µl, 80 µl, and 160 µl of the standard solution are generally appropriate. Then add 300 µl of precipitation reagent to all tubes (samples and standards); mix immediately and leave for 5 min, then centrifuge at 2,500 × **g** for 5 min (RT). Transfer the supernatants to clean, labelled tubes.

Step (iii): transfer 150 µl of each supernatant to wells in a 96-well plate (in triplicate) or into clean tubes, add 150 µl of modified Ehrlich's reagent to all the wells or tubes and mix well. Incubate for 15 min (RT), then read the absorbance at 553 nm using either a plate reader or a spectrophotometer. As described, the assay is linear for values exceeding those found in the temperate fish species studied.

Sample calculation: ALA-D (ng PBG min^{-1} mg^{-1} protein) = [(absorbance)/120]/protein (assuming the standard curve is prepared as ng PBG).

2.9 Additional Analyses

Inhibition of ALA-D activity indicates the presence of Pb in blood. Optimally, Pb should be analysed in blood homogenate (see Section 2.6, above). If blood is not available, data for Pb in other tissues of the same fish can be used. Care needs to be taken to achieve sufficiently low detection limits for the Pb analyses.

2.10 Expression of Results

As mentioned above, ALA-D activity can be standardized to cell volume, blood volume, or protein. The convention has been to express ALA-D activity in relation to blood cell volume or blood volume. As mentioned above, the use of hematocrit (red blood cell volume) or whole blood volume may be problematic.

On a protein basis, results should be expressed in ng (or µg) PBG min^{-1} mg^{-1} protein. Protein concentration is very often the most variable component of biochemical measurements and should also be reported (as mg ml^{-1} supernatant).

2.11 Quality Control

At present, there exists no commercially available standard reference material for fish ALA-D. Bovine ALA-D is available commercially and may be used to test the assay. The pH of the assay needs to be established for each fish species (and will differ from the pH optimum of bovine ALA-D). Suitable internal reference materials (blood supernatants from relevant fish species) should be prepared by each laboratory undertaking ALA-D analyses. Aliquots of reference material should be prepared and stored at −80 °C, for use with each batch of analyses. Control charts should be prepared during the analyses.

ALA-D was one of the methods included in the EU-funded project BEQUALM (Biological Effects Quality Assurance in Marine Monitoring Programmes; project PL97-3587). The small number of laboratories that participated in the exercise (five) precludes general conclusions on the use of the method. The exercise did show that homogeneous test samples for intercalibration purposes can be prepared from red blood cells diluted in buffer.

2.12 Laboratory Safety and Waste Treatment

The method involves the use of mercury salts and aggressive acids. All work with the reagent and solutions containing mercury should be performed in a fume hood. Rest materials with mercury need to be handled according to local guidelines, but should not be emptied into municipal sewer systems.

3 USE OF ALA-D IN MONITORING

ALA-D was used in the Norwegian JAMP in the period 1997–2001. The results for two years (2000 and 2001) clearly show that Atlantic cod is affected by lead in two areas with known inputs. The urban area is in the inner Oslofjord near Oslo, while the industrial source is located in Sørfjord, an area with a known lead input (Figure 1).

From the analyses conducted under the Norwegian JAMP, typical values from reference areas are: 15–18 ng PBG min^{-1} mg^{-1} protein for plaice and 20–25 ng PBG min^{-1} mg^{-1} protein for Atlantic cod, flounder, and dab.

4 SOURCES OF ERROR

4.1 Sample Treatment

It is recommended that all procedures be conducted on ice or at 4 °C. The enzyme appears to be stable for hours in whole heparinized blood kept on ice (Hodson *et al.*, 1984; Nakagawa *et al.*, 1997a). Protease inhibitors do not appear to be required to retain the activity of ALA-D in fish blood. As for any other enzyme, ALA-D is sensitive to warming and freezing-thawing cycles, which need to be avoided.

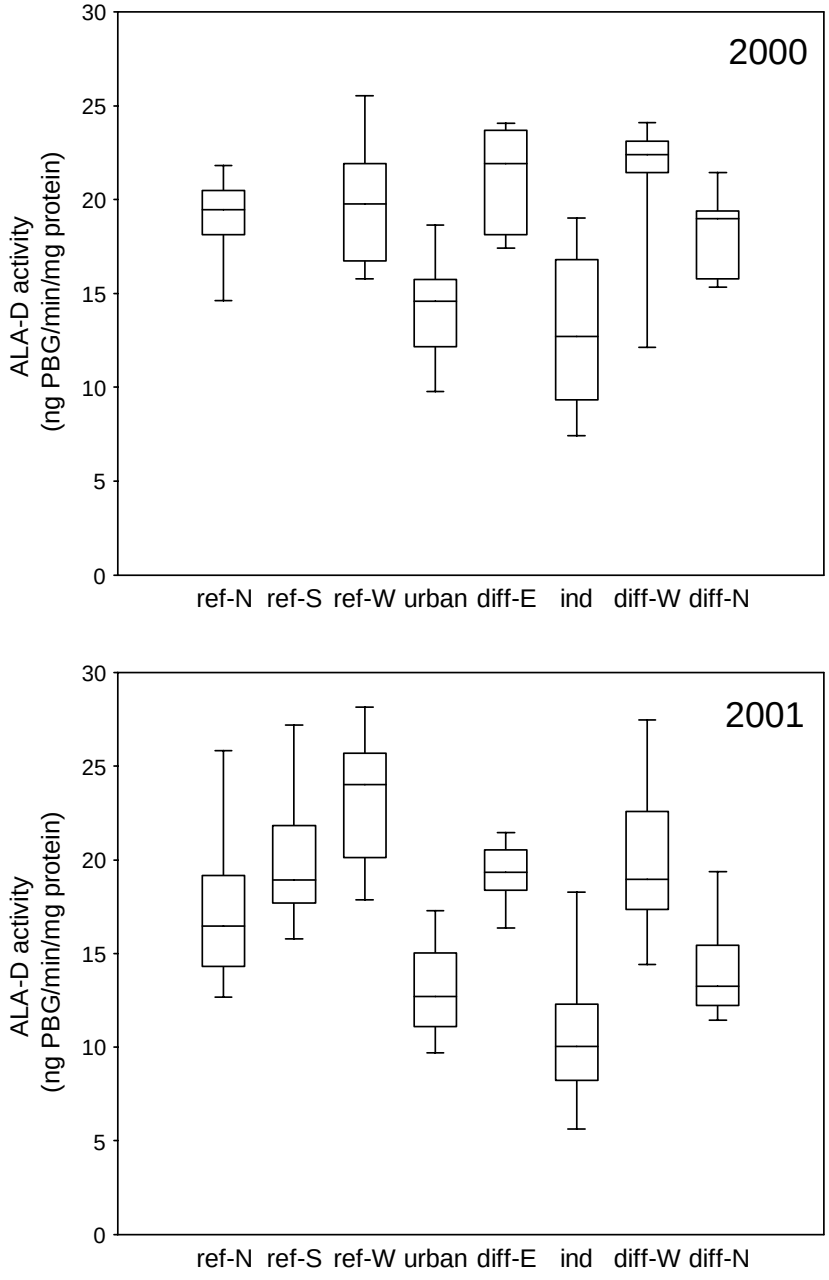
4.2 Analytical Procedures

As for all other biochemical techniques, solutions must be kept at appropriate temperatures. Samples must be kept cold at all times prior to the enzymatic reaction, which is carried out at room temperature. The method described does not appear to be sensitive to delays in reading the results after the reaction has been stopped, but intervals should be standardized as much as possible.

5 INTERPRETATION OF RESULTS

Differences between sites should be evaluated using a t-test (if there are only two locations) or analysis of variance (ANOVA; cf Underwood, 1981). If there are significant differences between sites in ANOVA, the analysis should be followed by Dunnett's test (with reference site) or either Tukey's test or the SNK test (with no reference site) (Day and Quinn, 1989).

Figure 1. ALA-D activity (ng PBG min^{-1} mg^{-1} protein) in Atlantic cod collected at locations along the Norwegian coast in 2000 and 2001. Figures show median, quartiles (boxes), and 10 and 90 percentiles (whiskers). "ref" = reference locations with no known inputs; "diff" = locations with no known point sources, but diffuse exposure; "urban" = near Oslo; "ind" = industrial area with a known lead input.



The relationship between red blood cell ALA-D activity and Pb in blood or other tissues may be investigated using simple correlation analysis (Draper and Smith, 1981; Sokal and Rohlf, 1981). A negative correlation is expected between red blood cell ALA-D activity and Pb (see, e.g., (Haux *et al.*, 1986; Hodson, 1976; Sordyl and Osterland, 1990). If the sampled fish or sample sites are heterogeneous with regard to the factors indicated in Section 2.1 (Design of study), an analysis of covariance may be appropriate to identify whether the factors influence the response.

Red blood cell ALA-D activity in fish does not, however, appear to be strongly influenced by non-metal factors.

Lead is generally found in the environment as the inorganic form, but the alkylated form may be present. Alkyllead does not affect ALA-D strongly and there may thus be a situation where there is little inhibition, but high blood lead levels (Hodson *et al.*, 1984).

Data on the influence of metals other than Pb are contradictory (Dwyer *et al.*, 1988; Jackim, 1973; Rehman, 1984; Sordyl and Osterland, 1990). The only metal that appears to affect fish red blood cell ALA-D *in vivo* is Zn (Schmitt *et al.*, 1993), although the influence was found to be weak and variable in rainbow trout (Hodson *et al.*, 1984).

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