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Metallothionein induction by Cu, Cd and Hg in *Dicentrarchus labrax* liver: assessment by RP-HPLC with fluorescent detection and spectrophotometry

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Abstract:

Metallothionein was quantified in sea bass *Dicentrarchus labrax* intraperitoneally (i.p.) injected with different Cu, Cd and Hg doses (50-250 $\mu\text{g kg}^{-1}$ wet wt) after 48 h exposure. A distinct peak with 16.8 min retention time was obtained by reversed-phase high performance liquid chromatography coupled to fluorescence detection (RP-HPLC-FD) with the three metals. Total metallothionein levels assayed in unheated liver extracts by RP-HPLC-FD were significantly higher (1.3 to 1.95-fold) than those obtained by the well-established spectrophotometric method. In the RP-HPLC-FD method, metallothionein increased linearly with Cu and Hg doses, being saturated beyond 100 $\mu\text{g kg}^{-1}$ Cd. Maximum induction was obtained at 100 $\mu\text{g kg}^{-1}$ Cd (5.3-fold), and 250 $\mu\text{g kg}^{-1}$ Cu or Hg (8- and 5.1-fold, respectively). At low doses no metallothionein induction was shown by the less sensitive spectrophotometric assay.

Keywords: Metal pollution biomarker; Fish; Reversed-Phase High Performance Liquid Chromatography; Fluorescence Detection; Spectrophotometry.

1. Introduction

Metallothioneins (MTs) are low Mr (~7 kDa), cysteine-rich, metal-binding proteins of widespread expression throughout all eukaryotes and some prokaryotes. They play crucial

roles in homeostasis of essential trace metals (Cu, Zn, Co, Ni) (Bremner and Beattie, 1990), in detoxification of excess intracellular levels of these and of non-essential metals (Cd, Hg, Ag, Pb), directly or indirectly by displacing MT synthesis inducers (Cu, Zn) from other metalloproteins, and in protection against various forms of oxidative injury (Kagi, 1991; Klaassen et al., 1999; Miles et al., 2000; Viarengo et al., 2000; Amiard et al., 2006).

Even though Cu is essential for metabolic processes, being a cofactor of ~30 enzymes, it can be very toxic to fish at low concentrations (Klaassen et al., 1999). High Cu levels in aquatic environments derive from geochemical and anthropogenic processes (mining and smelting, agriculture, water treatment, industrial wastes). Copper is used in aquaculture for treatment of algae and ectoparasite infestations, although tolerance levels of fish are not well-known (Schlenk et al., 1999). Cd and Hg are ubiquitous aquatic pollutants of important ecological and human health concern (Klaassen et al., 1999). Cd is dramatically increasing due to its industrial uses (batteries, electroplating, pigments, plastic stabilizers, alloys, phosphate fertilizers). Cd produces various adverse alterations in different fish organs (Castaño et al., 1998; Klaassen et al., 1999; De Smet and Blust, 2001; Giari et al., 2007). Inorganic Hg is converted by microorganisms into methyl-Hg, a potent neurotoxin readily accumulated by aquatic biota and strongly biomagnified along the food chain, being finally ingested by humans (Aschner, 2002; Bebianno et al., 2007; Ullrich et al., 2007).

Induction of MT synthesis by metals (Cd, Cu, Hg, Ag, Zn...), widely established, has led to its use as specific biomarker of metal exposure in aquatic invertebrates (Roesijadi, 1992; Cosson, 2000; Amiard et al., 2006) and fish (Kille et al., 1992; Roesijadi, 1992; Chan, 1995; Aschner, 2002). Accurate MT assessment is crucial for its use as a biomarker and to identify new biological roles (Dabrio et al., 2002). Many techniques and methodologies have been developed for purification and quantification of total MTs, although MT concentrations reported widely differ among laboratories (Cosson, 2000; Dabrio et al., 2002; Amiard et al.,

2006). We have described a method for total MT quantification by reversed-phase high performance liquid chromatography coupled to fluorescence detection (RP-HPLC-FD) in digestive gland of clams, successfully applied to assess metal pollution status of South Spanish littoral (Alhama et al., 2006) and Guadalquivir Estuary (SW Spain) (Romero-Ruiz et al., submitted). Now, the utility of the new chromatographic method is studied in sea bass specimens intraperitoneally injected with Cu, Cd and Hg by comparing the results obtained with the RP-HPLC-FD and the spectrophotometric method reported by Viarengo et al. (1997).

2. Materials and Methods

2.1. Materials. GSH, 5,5'-dithio-bis-2-nitrobenzoic acid, DL-dithiothreitol (DTT), leupeptin, phenylmethylsulfonyl fluoride (PMSF) and rabbit liver MT-I, were from Sigma (Spain). HPLC-grade acetonitrile was from Panreac (Spain). Monobromobimane (mBBBr), CuCl₂, CdCl₂ and HgCl₂ were from Fluka (Spain). Trifluoroacetic acid (TFA), Tris base and sodium dodecyl sulfate (SDS) were from Merck (Germany), and protein dye binding reagent from Bio-Rad (USA). Other chemicals were reagent quality.

2.2. Animals and experimental exposure. Specimens of sea bass (*Dicentrarchus labrax*) were purchased from the aquaculture farm of the National Institute of Marine Sciences and Technology (Monastir, Tunisia). Fish (80-150 g, 18-25 cm length) were maintained in 280-L tanks at 16 °C with aerated fresh seawater and fed *ad libitum* for 15 days. After the acclimation period, CuCl₂, CdCl₂ and HgCl₂ were dissolved in 9‰ NaCl, and the fish (n=6) were injected (i.p.) with various volumes to obtain final doses of 50, 100 and 250 µg kg⁻¹ for each metal, according to fish wet weight. Control fish were injected with the vehicle. After 48 h, 6 fish were sacrificed per condition; their livers were removed, pooled, washed briefly in ice-cold homogenizing buffer and frozen at -80°C until further analysis.

2.3. Metallothionein determination by spectrophotometric analysis. MT content was analyzed in frozen/ground livers by the assay of Viarengo et al. (1997). Tissue (1 g) was homogenized in 3 ml of 20 mM Tris-HCl buffer (pH 8.6) containing 0.5 M sucrose, 0.006 mM leupeptin, 0.5 mM PMSF and 0.01% β -mercaptoethanol, and spun 20 min at 30 000 x g. To 1 ml supernatant, 1.05 ml of cold (-20°C) absolute methanol and 80 μ l chloroform were added and the sample was spun at 6 000 x g for 10 min at 4°C. To the supernatant, 1 mg RNA, 40 μ l 37% HCl and 3 volumes of cold ethanol were added, and after 1 hour at -20°C the mixture was spun as indicated above. The MT-containing pellet was washed with 2 ml 87% ethanol, 1% chloroform and 12% homogenizing buffer, spun and dried under a N₂ stream. The pellet was resuspended in 150 μ l 0.25 M NaCl, and the metals released from MT with 150 μ l of 1 N HCl plus 4 mM EDTA. To assess MT content of a sample, 4.2 ml of 2 M NaCl with 0.43 mM 5,5'-dithio-bis-2-nitrobenzoic acid adjusted to pH 8 with 0.2 M Na-phosphate were added at room temperature. After centrifugation at 3 000 x g for 5 min, supernatant absorbance was measured at 412 nm and the MT concentration was estimated using GSH as a reference standard (Viarengo et al., 1997).

2.4. Metallothionein determination by RP-HPLC fluorescence detection. Total MT levels were quantified by RP-HPLC-FD in unheated liver extracts (Alhama et al., 2006; Romero-Ruiz et al., submitted). Pooled frozen/ground livers (0.3 g) were homogenised in 0.9 ml of 0.1 M Tris-HCl buffer (pH 9.5) including 1 mM DTT, 50 μ M PMSF and 6 μ M leupeptin, and spun 20 min at 35 000 x g. MTs thiols were labelled with the fluorogenic reagent mBBBr (12 mM final concentration) by heating (70°C, 20 min) in the presence of EDTA, DTT and SDS (2 mM, 12 mM and 3% final concentrations, respectively). Derivatized proteins were separated in a Supelcosil LC-18 column (0.46 x 35 cm, 5 μ m) by a gradient of acetonitrile containing 0.1% TFA (Alhama et al., 2006). Fluorescence of mBBBr-labelled proteins was measured with excitation at 382 nm and emission at 470 nm, using rabbit liver MT-I as a

reference standard. For the calibration line, different amounts of MT-I were labelled with mBBBr (12 mM final concentration) by heating (70°C, 20 min) in the presence of optimal EDTA, DTT and SDS concentrations, 2 mM, 2 mM and 3% final concentrations, respectively (Romero-Ruiz et al., submitted).

2.5. Protein determination. Protein concentration was determined by the dye-binding method (Bradford, 1976), using bovine serum albumin as standard.

2.6. Statistical analysis. All analyses were carried out in five replicates. Results are expressed as the means $\pm$ SD. The data were analyzed by one-way analysis of variance (ANOVA) using the SPSS software. The means obtained from each set were compared using the Duncan's Multiple Range test at 0.05 confidence level. Statistical significance of the results is indicated as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

To test in fish the utility and sensitivity of the new RP-HPLC-FD method for MT quantification, it was first adapted to fish liver extracts. Different DTT, SDS and mBBBr concentrations were assayed to optimize derivatization conditions. As previously shown for *Scrobicularia plana*, optimal labelling of the extracts required 12 mM DTT, 3% SDS and 12 mM mBBBr (Romero-Ruiz et al., submitted). Figure 1 shows the chromatographic patterns of rabbit liver MT-I and of *D. labrax* extracts from unexposed fish or exposed to metals, after being labelled with mBBBr. Comparing controls and fish injected with Cu, Cd, and Hg a peak eluting at 16.8 min, present in metal-exposed animals and absent in controls, was the main difference, thus being considered to correspond to *D. labrax* MT. This retention time was slightly higher than that of purified rabbit liver MT-I, 16.3 min, probably due to the higher

protein content of fish extracts, as confirmed by the more complex patterns observed at the initial part of the elution profile in all fish extracts, particularly between 3 and 10 minutes.

Figure 2 shows a plot of the peak areas obtained by RP-HPLC-FD *versus* rabbit liver MT-I content following separation of increasing amounts of this purified protein. An excellent linearity ($r = 0.9997$) was observed from 0.6 to 4.8 μg (0.098-0.786 nmol) of rabbit liver MT-I. From these results the following equation was calculated for the calibration line: MT (nmol) = $1.34 \cdot 10^{-7} \times \text{peak area} - 0.038$.

Once the new assay was optimized and its sensitivity documented, inducibility of MT synthesis was studied in sea bass exposed for 48 h to increasing doses of three model metal inducers, Cu, Cd and Hg. Figure 3 summarizes the results obtained by comparing the MT levels determined by RP-HPLC-FD with those obtained by the spectrophotometric method that titrates the -SH groups released from metal-stripped MT with Ellman's reagent (Viarengo et al., 1997). Both MT methods showed a clear and similar dose-response relationship after exposure to all metals studied. However, although no significant differences were found in controls, the MTs levels quantified by RP-HPLC-FD in all metal-exposed fish were significantly higher (1.31-1.95-fold) than those determined by the spectrophotometric assay, illustrating the higher sensitivity of the new chromatographic method.

The MT content determined by RP-HPLC-FD was directly proportional to Cu ($y = 0.42 x + 15.93$, $r^2=0.99$) and Hg ($y = 0.24 x + 20.70$, $r^2=0.98$) doses. With Cd, this straight relationship was visible only up to 100 $\mu\text{g kg}^{-1}$ ($y = 0.66 x + 10.63$, $r^2=0.94$), since MT content decreased somewhat after exposure to higher Cd doses. Fish exposed to all doses and metals showed significant differences in MT levels assayed by RP-HPLC-FD compared to unexposed fish. The MT content assayed by RP-HPLC-FD showed a 5.3-fold maximum induction at 100 $\mu\text{g kg}^{-1}$ Cd and of 8.0- and 5.1-fold at 250 $\mu\text{g kg}^{-1}$ Cu and Hg, respectively.

In contrast to the sensitive response of the RP-HPLC-FD method, fish injected with 50 $\mu\text{g kg}^{-1}$ Cu, Cd or Hg failed to show significant MT induction if assayed by the conventional spectrophotometric method. In fact, MT induction was detected only above 100 $\mu\text{g kg}^{-1}$ of the three model metals, and the straight lines showed clearly smaller slopes (Cu, $y = 0.17 x + 19.50$, $r^2 = 0.99$; Hg, $y = 0.13 x + 20.70$, $r^2 = 0.98$; Cd, $y = 0.37 x + 13.84$, $r^2 = 0.73$). Lower maximum inductions were also obtained with this spectrophotometric method, 2.8-fold for Cd (at 100 $\mu\text{g kg}^{-1}$), 3.1- and 2.6-fold for Cu and Hg, respectively (at 250 $\mu\text{g kg}^{-1}$).

4. Discussion

This work intended to confirm in fish the utility of the RP-HPLC-FD MT assay, first described in *Chamaelea gallina* clams (Alhama et al., 2006), and improved by using unheated digestive gland extracts of *Scrobicularia plana* clams (Romero-Ruiz et al., submitted). It should be noticed that the elution profiles obtained in *D. labrax* liver were somewhat more complex than those reported in digestive gland of clams (Alhama et al., 2006; Romero-Ruiz et al., submitted), probably reflecting the higher physiological and biochemical complexity of fish liver compared to bivalve digestive gland.

The chromatographic profiles obtained in animals injected with any of the three model metals confirmed that the peak of mBBBr-fluorescent protein eluting at 16.8 min corresponded to *D. labrax* MT, since it was found in metal-exposed fish but was totally absent in controls. Although the retention time of rabbit liver MT-I was slightly smaller, 16.3 min, this difference could be due either to species differences or to the higher protein content of cell-free extracts compared to purified rabbit MT-I. The linearity range (0.098-0.786 nmol) of the new RP-HPLC-FD assay developed for *D. labrax* MT renders it suitable for its direct use with cell-free extracts, according to the MT concentrations previously reported in other organisms (Dabrio et al., 2002; Amiard et al., 2006).

To test the utility of the chromatographic-fluorimetric assay and to compare its sensitivity to that of spectrophotometric method (Viarengo et al., 1997), sea bass fish were exposed for 48 h to Cu, Cd and Hg doses (50-250 $\mu\text{g kg}^{-1}$), well below the LD_{50} defined in *D. labrax* for Cu and Cd, 3000 and 2500 $\mu\text{g kg}^{-1}$, respectively (Roméo et al., 2000). Compared to the spectrophotometric assay (Viarengo et al., 1997), the RP-HPLC-FD method detected significantly higher MT content in all metal-exposed animals, confirming the results obtained in clams, where much higher MT levels were detected by RP-HPLC-FD (Alhama et al., 2006). The discrepancy between both methods could be attributed to under-estimation of the MT content due to the use of GSH as standard instead of MTs, and/or to partial co-precipitation of MT with hydrophobic proteins during the solvent extraction required before the spectrophotometric assay (Cosson, 2000; Erk et al., 2002; Alhama et al., 2006;). The lack of MT induction by low Cu, Cd and Hg doses, and the lesser slope of the dose-response relationships obtained with the spectrophotometric method for all model metals confirms the higher sensitivity of the RP-HPLC-FD assay (Alhama et al., 2006; Romero-Ruiz et al., submitted).

Excellent linear correlations were obtained between Cu, Hg and MT levels up to the higher metal doses tested. In contrast, this relationship deviated from linearity beyond 100 $\mu\text{g kg}^{-1}$ Cd. These results agree with those reported in the turbot (*Scophthalmus maximus*) (George et al., 1996) and the greater amberjack (*Seriola dumerilli*) (Jebali et al., 2006). At acute Cd doses (over 200 $\mu\text{g kg}^{-1}$), synthesis of hepatic MT is clearly reduced, becoming limiting due to a progressive inhibition of critical metabolic processes (cytotoxicity) (Bremner and Beattie, 1990; Roesijadi, 1992; George et al., 1996; Jebali et al., 2006) by one of the most deleterious heavy metals in fish (Sörensen, 1991). European sea bass (*D. labrax*) exposed to different Cd concentrations showed several toxic effects, including cellular alterations and anomalies in the liver and other organs (Klaassen et al., 1999; Roméo et al., 2000; Giari et al.,

2007). Severe ultrastructural changes in liver cells affected endoplasmic reticulum (proliferation, degranulation, dilatation and vesiculation), mitochondria (swelling, cristae disappearance), and nuclei, including formation of myelinoid bodies, steatosis, cellular oedema and cytoplasm rarefaction (Berntssen et al., 2001; Giari et al., 2007). Alterations in Cd-treated fish were also apparent at biochemical and physiological levels, by inducing stress responses and affecting carbohydrate, protein and energy metabolism (Pratap and Wendelaar-Bonga, 1990; Cattani et al., 1996; Roméo et al., 2000; Wu et al., 2007).

With Cu, a 30% decrease of hepatic MT was reported in *D. labrax* injected with 500 $\mu\text{g kg}^{-1}$ for 48 h, an effect attributed to metal overload and hepatotoxicity at such a high dose (Roméo et al., 1997). Since we used 2-fold smaller Cu dose, its toxic effect in sea bass probably would appear at doses beyond those tested here, that is between 250-500 $\mu\text{g kg}^{-1}$. The dissimilar toxic effects of Cd and Cu in sea bass could be due to their differential uptake and accumulation: compared to controls, Cd accumulated (205-fold) well in excess to Cu (2.6-fold) after 48 hours injection with 1000 $\mu\text{g kg}^{-1}$ (Roméo et al., 2000). Metal bioaccumulation and toxicity and MT induction varies in metal-, organ- and species-specific ways (George et al., 1996; Roméo et al., 2000; De Smet and Blust, 2001; De Boeck et al., 2003; Amiard et al., 2006;). In *D. labrax* kidney, Cu is more toxic than Cd, due to increased lipid peroxidation and lower lysosomal membrane stability (Roméo et al., 2000), at difference with the higher toxicity shown by Cd in liver.

Mercury is an extremely toxic element, largely distributed in aquatic environments, and of great concern for human health since fish diet is the most critical source of Hg (Aschner, 2002; Bebianno et al., 2007; Ullrich et al., 2007). MT induction has a significant role in Hg detoxification in fish liver (Bebianno et al., 2007), probably affording protection against its cytotoxic effects (Aschner, 2002). A 5.1-fold MT induction was obtained in the present work at the higher Hg dose tested, 250 $\mu\text{g kg}^{-1}$, in agreement with the 3-fold induction

obtained after 4 days exposure to $100 \mu\text{g l}^{-1}$ in the rainbow trout (*Oncorhynchus mykiss*) (Angelow and Nicholls, 1991) and the 5- and 8-fold increase of MT-I and MT-II gene expression, measured by QRT-PCR in the barbell (*Barbus graellsii*) (Quirós et al., 2007).

In conclusion, quantification of total MTs by RP-HPLC-FD in unheated fish extracts allows the evaluation of metal effects with higher sensitivity and specificity than the spectrophotometric assay, and could be proposed as a fish biomarker in biomonitoring programmes.

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Figure legends

Figure 1: RP-HPLC-FD profile of mBBBr derivatives corresponding to 0.4 nmol of purified rabbit liver MT-I, to unheated sea bass liver extracts of control fish, and of fish injected with $250 \mu\text{g kg}^{-1}$ Cu, $100 \mu\text{g kg}^{-1}$ Cd, and $250 \mu\text{g kg}^{-1}$ Hg after 48 h exposure. The arrows indicate the peak of rabbit liver MT-I and that of MT induced after Cu, Cd and Hg exposure, respectively.

Figure 2: Calibration line for rabbit liver MT-I obtained by RP-HPLC-FD. The indicated amounts of purified rabbit liver MT-I were processed as described in Materials and Methods. Peak areas, expressed in arbitrary units, are plotted versus the corresponding MT contents.

Figure 3: Metallothionein content in the liver of the sea bass *D. labrax* injected with different Cu (A), Cd (B) and Hg (C) concentrations after 48 h exposure. Significant differences

between exposed and control groups are shown by (*) for the results obtained with the RP-HPLC-FD method, and by (#) for those obtained with the spectrophotometric method. Significant differences between the results obtained with both methods in the same experimental condition are indicated by (\$). Statistical significance of the differences is shown as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Figure 1

Figure 2

Figure 3