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EFFECTS OF CHLORAMINE-T AND $CuSO_4$ ON ENZYME ACTIVITY OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM RAINBOW TROUT *(ONCORHYNCHUS MYKISS)* ERYTHROCYTES *IN VITRO* AND *IN VIVO*

Abdulkadir Çiltas[1]*, Orhan Erdogan[1], Olcay Hisar[1] and Mehmet Çiftçi[2]

[1] *Department of Aquaculture, Agriculture Faculty, Atatürk University, Erzurum 25240, Turkey*

[2] *Biotechnology Application and Research Center, Atatürk University, Erzurum 25240, Turkey*

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Abstract

Traditional treatments of parasitic and bacterial diseases are based on chemotherapeutic compounds such as chloramine-T and $CuSO_4$. Although many compounds are used in fish treatments, their undesirable effects are not known. In this study, glucose 6-phosphate dehydrogenase (G6PD) was purified from rainbow trout (*Oncorhynchus mykiss*) erythrocytes by hemolysate preparation, ammonium sulfate precipitation and 2', 5'-ADP Sepharose 4B affinity gel chromatography in a single day. The enzyme, with a specific activity of 14.51 EU/mg protein, was purified 906.8-fold with a yield of 70.38%. To check the purity of the enzyme, SDS polyacrylamide gel electrophoresis was performed, which showed a single band. The effects of chloramine-T and copper sulfate ($CuSO_4$) on the G6PD were investigated *in vitro*. Chloramine-T and $CuSO_4$ had inhibitory effects on the enzyme. I_{50} values of the chemotherapeutic compounds were determined by plotting percent activity and K_i values, and types of inhibition were determined for each compound by means of Lineweaver-Burk graphs. *In vivo* studies showed that G6PD in rainbow trout erythrocytes was significantly inhibited by $CuSO_4$ in one hour but not inhibited by chloramine-T.

Introduction

Chloramine-T (n-sodium-n-chloro-q-toluenesulfonamide) is a widely used disinfectant or prophylactic in freshwater aquaculture for the treatment of bacterial and parasitic diseases in gills (Bullock et al., 1991; Thorburn and Moccia, 1993) and skin (Cross and Hursey, 1973). Such diseases are considered serious limiting factors to freshwater aquaculture production (Speare and Ferguson, 1989). Bills et al. (1988) established the 3-hour LC_{50} value of

* Corresponding author. Tel.: +90-442-2313482, fax: +90-442-2360948, e-mail: akciltas@atauni.edu.tr

chloramine-T for rainbow trout in soft water as 43.0 mg/l (36.9-50.1). Toxicity was greater in soft, acidic waters than in hard, alkaline waters. Bullock et al. (1991) recommended a bath treatment of 10 mg chloramine-T per liter for one hour as an effective treatment for bacterial gill disease in cultured salmonids. Chloramine-T degrades in solution due to nucleophilic substitution, releasing a hypochlorite ion and paratoluenesulfonamide (pTSA). It is believed that the release of hypochlorite is the primary mechanism of both therapeutic action (antibacterial disinfection) and toxicity (ultra-structural injury to epithelia; increased mucus secretion; Powell et al., 1995; Powell and Perry, 1996). Hypochlorite is acutely toxic to fish (Brooks and Bartos, 1984).

Copper sulfate ($CuSO_4$) is indicated for the treatment of algae and various ectoparasitic infestations; it is widely used as a therapeutic for some waterborne fish diseases (Straus and Tucker, 1993; Schlenk et al., 1999). Exposure to $CuSO_4$ causes physiological changes in fish that are similar to changes induced by other physical or chemical stressors (Barton and Iwama, 1991). Griffin et al. (1999) reported that therapeutic doses of $CuSO_4$ (1.7 mg $CuSO_4$ /l for 24 h), although acutely stressful, cause a mild degree of stress in channel catfish compared to other agents of stress.

It is generally recognized that the cell has four major NADPH-production systems that correspond to the activities of four cytoplasmatic enzymes: glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) belonging to the pentose phosphate pathway, malic enzyme (ME) and NADP-dependent isocitrate dehydrogenase (NADP-IDH). G6PD (D-glucose 6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49) is the first enzyme in the pentose phosphate pathway. The main physiological function of G6PD is to produce NADPH and ribose 5-phosphate, which are essential for reductive biosynthesis and nucleic acid synthesis (Kuo et al., 2000; Bianchi et al., 2001). The major role of NADPH in erythrocytes is the regeneration of reduced glutathione (GSH), which preserves the integrity of red blood cell membrane sulfhydryl groups and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells (Deutsch, 1983; Weksler et al., 1990).

Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme (Hoechster et al., 1972). Despite widespread use of chemotherapeutic compounds, relatively little is known about the physiological consequences of these treatments in fish. Therefore, in the present study, we investigated the effects of chloramine-T and $CuSO_4$ on fish erythrocyte G6PD *in vitro* and *in vivo*. By determining the K_i and I_{50} values, undesirable side effects on G6PD activity and body metabolism can be diminished.

Materials and Methods

Chemicals. 2', 5'-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, glucose 6-phosphate, protein assay reagent, and chemicals for electrophoresis were purchased from Sigma Chemical Co. All other chemicals used for analytical grade were purchased from either Sigma or Merck.

Fish husbandry and maintenance. Twenty one-year-old rainbow trout (mean wt 200±20 g) were used for the purification of G6PD enzyme from erythrocytes. The average water temperature was 9±2°C, dissolved oxygen was 8-9 ppm, pH was 7.8 and total hardness was 102 mg as $CaCO_3$ during the tests. At the time of sampling, fish were fed a commercial pelleted trout feed twice a day at 1% of their body weight per day.

Preparation of the hemolysate. Blood was sampled from the caudal vein using a 10-ml heparinized (5 IU/ml) plastic syringe. The blood samples were transferred in tubes and centrifuged at 2,500 x g for 15 min. The plasma was removed by drip. The packed red blood cells were washed with KCl solution (0.16 M) three times, centrifuged at 2,500 x g each time and the supernatants were removed. The erythrocytes were hemolyzed with 5 volumes of ice-cold water and centrifuged (4°C, 10,000 x g) for 30 min to remove the ghosts and intact cells (Ninfali et al., 1990).

Ammonium sulfate fractionation and dialysis. The hemolysate was precipitated with increasing amounts of ammonium sulfate (10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70% and 70-80%), centrifuged at 5,000 x g (5 min) and dissolved in 50mM of phosphate buffer (pH 7.0). For each respective precipitation, the enzyme activity was determined both in the supernatant and in the precipitate. The enzyme was observed to precipitate at 40-65%. The enzyme solution was then dialyzed at 4°C in 50 mM K-acetate per 5 mM K-phosphate buffer (pH 7.0) for two hours with two changes of buffer (Ninfali et al., 1990).

2', 5'-ADP Sepharose 4B affinity chromatography. For 10 ml of bed volume, 2 g of dry 2', 5'-ADP Sepharose 4B was washed several times in 400 ml of distilled water. With the washings, the impurities were removed and the gel conditioned. After removal of the air, the gel was resuspended in the buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) at a ratio of 25% buffer and 75% gel and packed in a column (1 x 10 cm). After precipitation of the gel, it was equilibrated with the same buffer by means of a peristaltic pump (flow rate 50 ml/h). The dialyzed enzyme solution obtained previously was loaded on the column, and the flow rate was adjusted to 20 ml/h. The column was sequentially washed with 25 ml 0.1 M K-acetate + 0.1 M K-phosphate (pH 6.0) and 25 ml 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). The second washing (pH 7.85) continued until the final absorbance difference became 0.05. Finally, the enzyme was eluted with a solution of 80 mM K-phosphate + 80 mM KCl + 0.5mM $NADP^+$ + 10 mM EDTA (pH 7.85). Enzyme activity was measured in the final fractions and the activity-containing tubes were collected together. Protein was determined in the resultant solution. During all procedures, the temperature was kept at 4°C (Morelli et al., 1978; Ninfali et al., 1990).

Activity determination. The enzymatic activity was measured by Beutler's method (1971). One enzyme unit was defined as the amount of enzyme that reduced 1 µmol $NADP^+$ in 1 min.

Protein determination. Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (1976), with bovine serum albumin used as a standard.

SDS polyacrylamide gel electrophoresis (SDS-PAGE). To determine the enzyme purity, Laemmli's procedure (1970) was carried out in 3% and 10% acrylamide concentrations containing 10% SDS for running and stacking gel, respectively. The gel was stabilized in the solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. The staining was made for about two hours in a solution of 0.1% Coommassie Brillant Blue R-250 + 50% methanol + 10% acetic acid + 40% distilled water. Finally, the washing was carried out in the same solution without the dye until protein bands were cleared.

In vitro studies for chloramine-T and $CuSO_4$. Chloramine-T and $CuSO_4$ were used as inhibitors. Five substrate concentrations (0.15, 0.30, 0.45, 0.60 and 0.90 mM) were used. Inhibitor solutions were added to the substrate media, resulting in three fixed concentrations for each inhibitor: 0.283, 1.135 and 2.13 mM for chloramine-T and 0.312, 1.250 and 2.343 mM for $CuSO_4$ per 1 ml total reaction volume. To draw Lineweaver-Burk graphs by using 1/V and 1/[S] values, regression analysis was carried out and the equations obtained from the analysis were used to draw graphs for each inhibitor concentration. K_i values were calculated from the Lineweaver-Burk graphs. To determinate I_{50} values, inhibition percent values were obtained from different inhibitor concentrations (0.284, 0.568, 0.852, 1.136, 2.130 and 3.550 mM for chloramine-T; 0.312, 0.624, 0.937, 1.249, 1.562 and 2.343 mM for $CuSO_4$) with 0.6 mM constant substrate concentration (G6-P). Regression analysis graphs were drawn using percent inhibition values by a statistical packing computer program. The inhibitor concentrations causing up to 50% inhibition (I_{50}) were determined from these graphs.

In vivo studies for chloramine-T and $CuSO_4$. Ten rainbow trout (250±24g) were selected for each chemotherapeutic compound. Blood samples of 0.5 ml were taken and placed into a heparinized vacutainer. The

first group was bathed in 2 mg chloramine-T per liter water and the second in 0.2 mg $CuSO_4$ per liter water (Scott, 1993). Blood samples were taken from each trout two, four and six hours after the baths. The blood samples were centrifuged at 2500 g, then the erythrocyte pellet was washed with 0.16 M KCl three times and the supernatant was discarded. One volume from the resultant erythrocyte pellet was hemolyzed in five volumes of ice water to prepare the hemolysate. Studies were carried out at 4°C. G6PD activity was assayed by the method of Beutler (1994). Obtained data were subjected to statistical analysis by *t* test, followed by Duncan's multiple range test to determine significant differences among means at the α = 0.05 level (Duncan, 1971).

Results

G6PD was purified 906.8-fold with a yield of 70.386% by using ammonium sulfate precipitation and 2', 5'-ADP Sepharose 4B affinity gel (Table 1). Only a single band was observed on the gel electrophoresis (Fig. 1). Both chloramine-T and $CuSO_4$ inhibited the G6PD activity in rainbow trout erythrocytes *in vitro.* Percent activity values of G6PD for six concentrations of chloramine-T and $CuSO_4$ are shown in Figs. 2 and 3. K_i values were calculated from Lineweaver-Burk graphs (Figs. 4 and 5). I_{50} and K_i values obtained from *in vitro* studies are shown in Table 2. The type of inhibition of the chloramine-T and the $CuSO_4$ was noncompetitive. K_i and I_{50} values were 0.52±0.17 mM and 0.927 mM for chloramine-T and 3.967±0.82 mM and 2.156 mM for $CuSO_4$, respectively. G6PD was inhibited by $CuSO_4$ in the *in vivo* studies, but chloramine-T had no effect on this enzyme (Table 3).

Discussion

G6PD is the key and first enzyme on the pentose phosphate metabolic pathway. It catalyzes the conversion of glucose 6-phosphate to 6-phosphogluconate in the presence of $NADP^+$. The major role of NADPH in the erythrocyte is regeneration of reduced glutathione (GSH). GSH is used by antioxidant defense mechanisms and produced by

Table 1. Purification of glucose-6-phosphate dehydrogenase from rainbow trout erythrocytes.

Purification step	*Activity (U/ml)*	*Total volume (ml)*	*Protein (mg/ml)*	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Yield (%)*	*Purification factor*
1. Hemolysate	0.371	80	32.5	975	29.68	0.011	100	1
2. Ammonium sulfate precipitation (40-65%)	0.864	25	19.3	289.5	21.60	0.044	72.77	4
3. Affinity chromatography	2.612	8	0.18	1.8	20.89	14.51	70.38	906.8

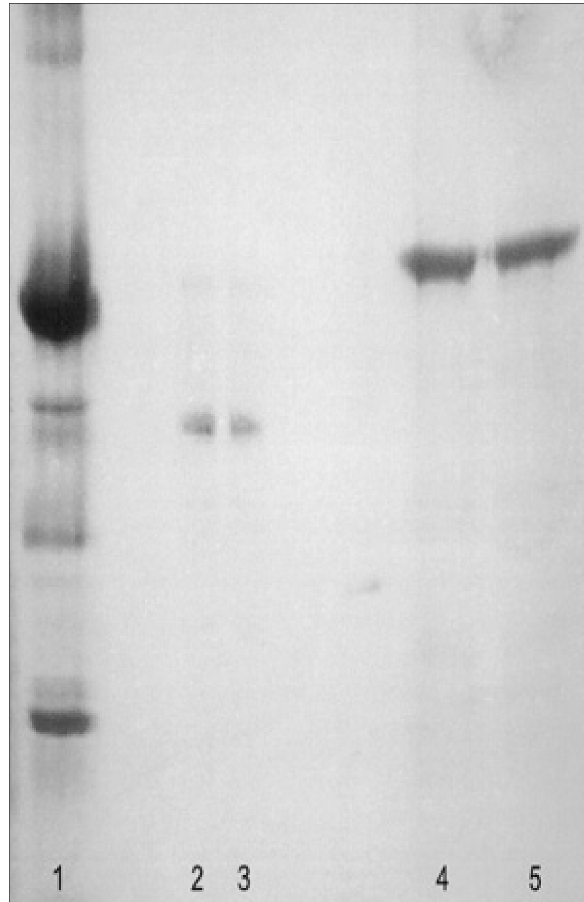

Fig. 1. SDS-PAGE bands of G6PD. Lane 1 - standard proteins; yeast hexokinase (100 kDa), rabbit heart creatine phosphokinase (81 kDa), bovine serum albumin (66 kDa), bovine liver glutamic dhydrogenase (55 kDa), bovine spleen deoksiribonuclease (38 kDa); lanes 2-3 - rainbow trout G6PD; lanes 4-5 - bovine lactoperoxidase (80 kDa).

NADPH synthesized in the pentose phosphate metabolic pathway thanks to G6PD and 6PGD (Beutler, 1994; Lehninger, 2000). For this reason, G6PD can be considered an antioxidant enzyme and very important for living cells.

The toxicity of chloramine-T decreases as the stocking density increases and increases as pH decreases, temperature rises and water hardness drops (Bills et al., 1988). Chloramine-T and its degradation product paratoluenesulphonamide (pTSA) are taken up across the gill, but both are rapidly eliminated (Powell and Perry, 1996).

High doses of $CuSO_4$ may be acutely toxic to fish but copper compounds quickly precipitate from water as copper oxide and toxicity can be avoided if the dose does not exceed one hundredth of the total alkalinity concentration of the water to be treated. Some copper may be absorbed by organisms, but concentrations in tissues are no greater than

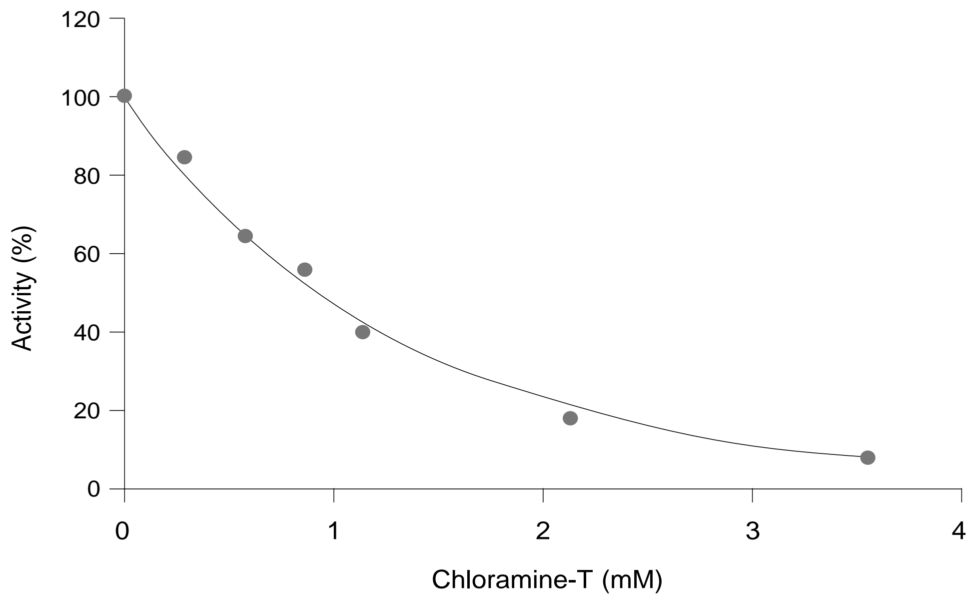

Fig. 2. Percent activity vs chloramine-T regression analysis graphs for G6PD from rainbow trout erythrocytes in the presence of six different chloramine-T concentrations.

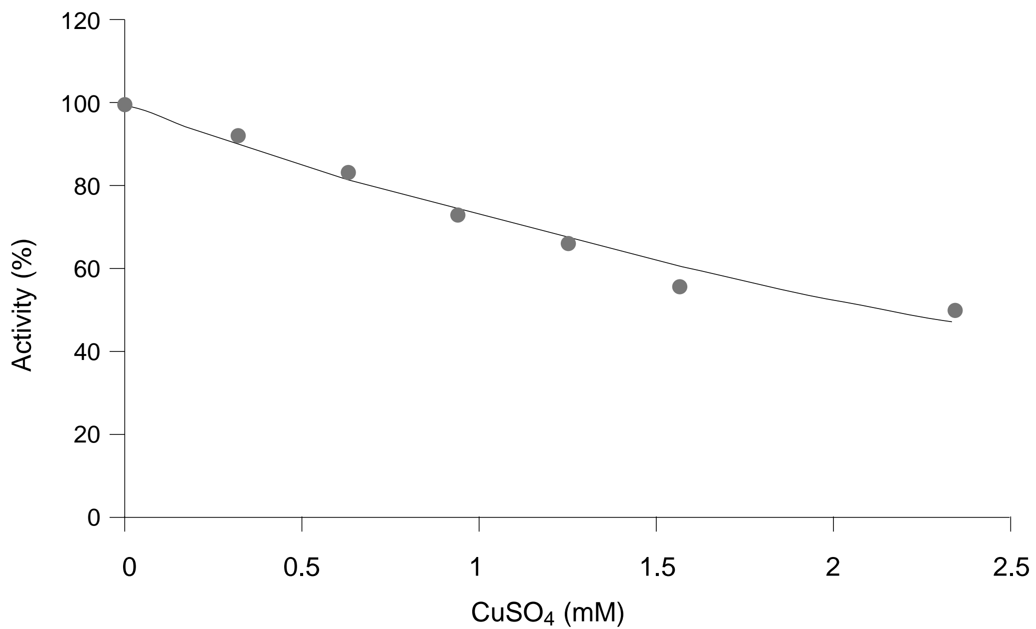

Fig. 3. Percent activity vs $CuSO_4$ regression analysis graphs for G6PD from rainbow trout erythrocytes in the presence of six different $CuSO_4$ concentrations.

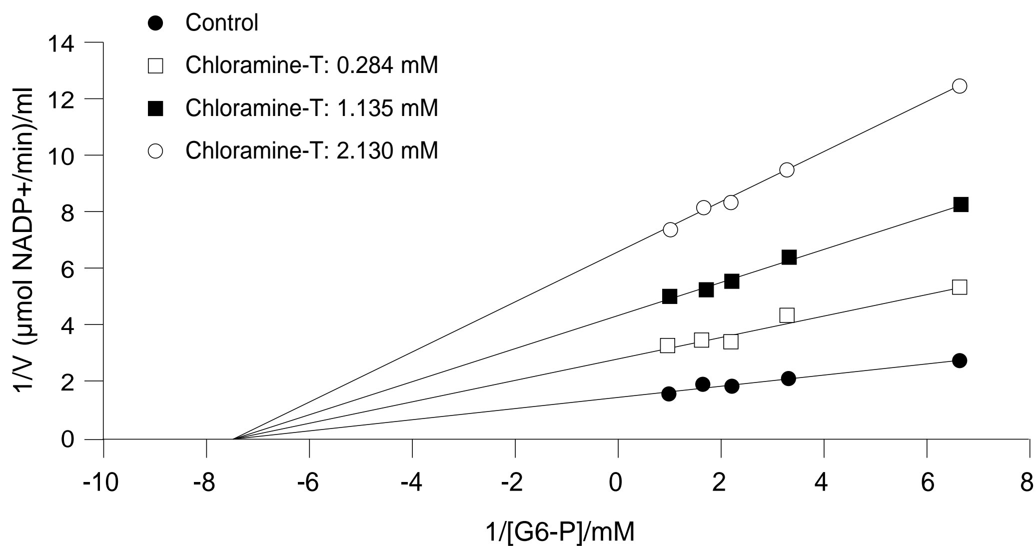

Fig. 4. Lineweaver-Burk graph for five substrate (G6-P) concentrations and three chloramine-T concentrations, used to determine K_i for chloramine-T.

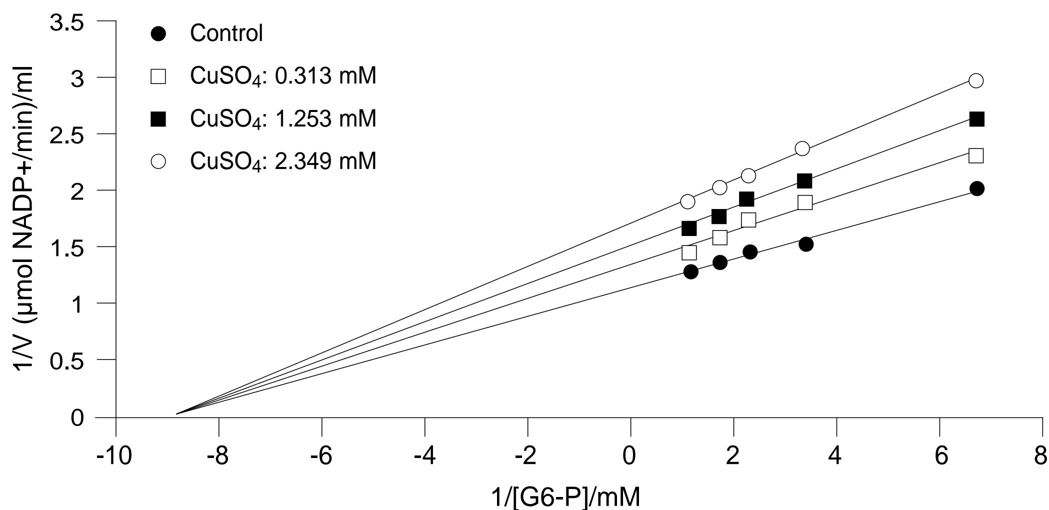

Fig. 5. Lineweaver-Burk graph for five substrate (G6-P) concentrations and three $CuSO_4$ concentrations, used to determine K_i for $CuSO_4$.

Table 2. K_i values obtained from Lineweaver-Burk graph for G6PD in the presence of three fixed inhibitors and four substrate concentrations for chloramine-T and $CuSO_4$.

Inhibitor	*[I] (mM)*	*K_i (mM)*	*K_i (mean mM ±SD)*	*Inhibition*	*I_{50} (mM)*
Chloramine-T	0.284	0.324			
	1.136	0.594	0.52±0.17	Noncompetitive	0.927
	2.130	0.644			
$CuSO_4$	0.513	3.187			
	1.253	3.891	3.967±0.82	Noncompetitive	2.156
	2.349	4.823			

Table 3. Statistical values obtained from *in vivo* studies for chloramine-T and $CuSO_4$.

Chemotherapeutic compound	*Hour*	*EU (g/Hb; mean±SD)*
Chloramine-T	Control	24.16±6.39[a]
	1 h	21.96±7.09[a]
	2 h	21.88±7.03[a]
	3 h	22.36±4.41[a]
$CuSO_4$	Control	35.62±7.29[a]
	1 h	19.22±4.41[b]
	2 h	25.81±2.00[a]
	3 h	25.72±2.14[a]

Means with different superscripts differ significantly ($p<0.05$).

those normally found in native plants and fish (Boyd and Massaut, 1999).

Many drugs have adverse effects on an organism when used for therapeutic or other purposes (Hochster et al., 1972). The effects can be dramatic and systemic (Christensen et al., 1982). Similarly, acetazolamide inhibits carbonic anhydrase, giving rise to severe diuresis (Warnock, 1989). On the other hand, human G6PD is affected by sodium ampicillin, netilmicin sulfate and metamizol while carbonic anhydrase isozymes are affected by sodium ampicillin and metamizol (Çiftçi et al., 2000, 2001).

While the most suitable parameter is the K_i constant, some researchers use the I_{50} value

to indicate inhibition effects. In this study, we determined both K_i and I_{50} for the studied chemotherapeutic compounds. In the *in vitro* studies, both chloramine-T and $CuSO_4$ inhibited the enzyme to different degrees. The I_{50} values of both compounds almost fit their K_i values. The I_{50} and K_i for chloramine-T are lower than for $CuSO_4$, suggesting greater inhibitory effects of chloramine-T than $CuSO_4$ when compared *in vitro*.

In the *in vivo* studies, the G6PD enzyme was inhibited by $CuSO_4$ but not by chloramine-T, indicating that $CuSO_4$ entered the erythrocytes but chloramine-T did not. Chloramine-T inhibits the activity of the G6PD enzyme *in vitro*, but has no effect on the enzyme *in vivo*. Apparently, chloramine-T metabolizes in the liver or is rapidly eliminated. Therefore, chloramine-T appears to be a good candidate for use as a therapeutic in salmonid hatcheries and the use of $CuSO_4$, recommended as a bath treatment of 0.2 mg/l dosage (Scott, 1993), cannot be recommended for this enzyme.

References

Barton B.A. and G.K. Iwama, 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish Dis.*, 1:3-26.

Beutler E., 1971. *Red Cell Metabolism Manual of Biochemical Methods*. Academic Press, London. 188 pp.

Beutler E., 1994. Glucose-6-phosphate dehydrogenase deficiency. *Blood,* 84(11):3613-3636.

Bianchi D., Bertrant O., Haupt K. and N. Coello, 2001. Effect of gluconic acid as a secondary carbon source on non-growing L-lysine producers cells of *Corynebacterium glutamicum*. Purification and properties of 6-phosphogluconate dehydrogenase. *Enzyme Microbial Technol.,* 28:754-759.

Bills T.D., Marking L.L., Dawson V.K. and J.J. Rach, 1988. Effects of environmental factors on the toxicity of chloramine-T to fish. *US Dept. Int. Fish Wildlf. Serv. Invest. Fish Cont.*, 96:1-6.

Boyd C.E. and L. Massaut, 1999. Risks associated with the use of chemicals in pond aquaculture. *Aquacult. Eng.*, 20:113-132.

Bradford M.M., 1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* 72:248-251.

Brooks A.S. and J.M. Bartos, 1984. Effects of free and combined chlorine and duration on rainbow trout, channel catfish and emerald shiners. *Trans. Am. Fish. Soc.*, 113:786-793.

Bullock G.L., Herman R.L. and C. Waggy, 1991. Hatchery trials with chloramine T for control of bacterial gill disease. *J. Aquat. Anim. Health*, 3:48-50.

Christensen G.M., Olson D. and B. Riedel, 1982. Chemical effects on the activity of eight enzymes: A review and discussion relevant to environmental monitoring. *Environ. Res.*, 29:247-255.

Çiftçi M., Küfrevioglu Ö.I., Gündogdu M. and I. Özmen, 2000. Effects of some antibiotics on enzyme activity of glucose-6-phosphate dehydrogenase from human erythrocytes. *Pharmacol Res.*, 41:109-113.

Çiftçi M., Özmen I., Büyükokuroglu M.E., Pençe S. and Ö.I. Küfrevioglu, 2001. Effects of metamizol and magnesium sulfate on enzyme activity of glucose-6-phosphate dehydrogenase from human erythrocytes *in vitro* and rat erythrocyte *in vivo*. *Clinical Biochem.*, 34: 297-302.

Cross D.G. and P.A. Hursey, 1973. Chloramine-T for the control of *Ichthyophthirius multifilis* (Fouquet). *J. Fish Biol.,* 5:789-798.

Deutsch J., 1983. Glucose-6-phosphate dehydrogenase. pp. 190-196. In: H.U. Bergmeyer, J. Bergmeyer (eds.). *Methods of Enzymatic Analysis,* Vol. 3. Verlagsgerellschaff, VCH.

Duncan D.B., 1971. Multiple range and multiple F-tests. *Biometrics*, 11:313-323.

Griffin B.R., Davis K.B. and D. Schlenke, 1999. Effect of simulated copper sulfate therapy on stress indicators in channel catfish. *J. Aquat. Anim. Health*, 11:231-236.

Hochster R.M., Kates M. and J.H. Quastel, 1972. pp. 71-89. In: *Metabolic Inhibitors*. Vols. 3 and 4. Academic Press, New York.

Kuo W., Lin J. and T.K. Tang, 2000. Human

glucose-6-phosphate dehydrogenase (G6PD) gene transforms nih 3t3 cells and induces tumors in nude mice. *Int. J. Cancer*, 85:857-864.

Laemmli D.K., 1970. Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227:680-683.

Lehninger A.L., Nelson D.L. and M.M. Cox, 2000. pp. 558-560. In: *Principles of Biochemistry*, 2nd ed. Worth Publ. Inc., New York.

Morelli A., Benatti U., Gaetani G.F. and A. De Flora, 1978. Biochemical mechanisms of glucose-6-phosphate dehydrogenase deficiency. *Proc. Natl. Acad. Sci.*, 75:1979-1983.

Ninfali P., Orsenigo T., Barociani L. and S. Rapa, 1990. Rapid purification of glucose-6-phosphate dehydrogenase from mammal's erythrocyte. *Prep. Biochem.,* 20:297-309.

Powell M.D. and S.F. Perry, 1996. Respiratory and acid-base disturbances in rainbow trout *(Oncorhynchus mykiss)* blood during exposure to chloramine-T, paratoluenesulphonamide and hypochlorite. *Can. J. Fish. Aquat. Sci.*, 53:701-708.

Powell M.D., Wright G.M. and D.J. Speare, 1995. Morphological changes in rainbow trout (*Oncorhynchus mykiss*) gill epithelia following repeated intermittent exposure to chloramine-T. *Can. J. Zool.*, 73:154-165.

Schlenk D., Davis K.B. and B.R. Griffin, 1999. Relationship between expression of hepatic metallothionein, and sublethal stress in channel catfish following acute exposure to copper sulfate. *Aquaculture*, 117:367-379.

Scott P., 1993. Therapy in aquaculture. pp. 135-136. In: L. Brown (ed.). *Aquaculture for Veterinarians Fish Husbandry and Medicine*. Pergamon Press Inc., New York.

Speare D.J. and H.W. Ferguson, 1989. Clinical and pathological features of common gill diseases of cultured salmonids. *Can. Vet. J.,* 30:882-887.

Straus D.L. and C.S. Tucker, 1993. Acute toxicity of copper sulfate and chelated copper to channel catfish, *Ictalurus punctatus*. *J. World Aquacult. Soc.,* 24:390-395.

Thorburn M.A. and R.D. Moccia, 1993. Use of chemotherapeutics on trout farms in Ontario. *J. Aquat. Anim. Health*, 5:85-91.

Warnock D.G., 1989. Diuretic agent. pp. 183-197. In: B.G. Katzung (ed.). *Basic and Clinical Pharmacology,* 4th ed. Appleton and Lange, USA.

Weksler B.B., Moore A. and J. Tepler, 1990. Hematology. pp. 341-363. In: T.E. Andreoli, C.C.J. Carpenter, F. Plum, L.H. Smith (eds.). *Cecil Essentials of Medicine,* 2nd ed. WB Saunders Co., Philadelphia.