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Research Article

Inhibition Effects of Some Antibiotics on the Activity of Glucose 6-Phosphate Dehydrogenase Enzyme from Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1792) Erythrocytes

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Abstract: Inhibitory effects of some antibiotics on glucose 6-phosphate dehydrogenase from the erythrocytes of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) were investigated. For this purpose, initially erythrocyte glucose 6-phosphate dehydrogenase was purified 1271.19-fold in a yield of 70.40% by using ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity gel. A constant temperature (+4 ºC) was maintained during the purification process. Enzyme activity was determined with the Beutler method by using a spectrophotometer at 340 nm. This method was utilized for all kinetic studies. Thiamphenicol, amikacin, gentamicin, and netilmicin were used as antibiotics. All the antibiotics exhibited inhibitory effects on the enzyme. K_i constants for glucose 6-phosphate dehydrogenase were found by means of Lineweaver-Burk graphs. While thiamphenicol and amikacin showed competitive inhibition, gentamicin and netilmicin displayed noncompetitive inhibition. In addition, I_{50} values of the antibiotics were determined by plotting activity % vs. [I].

Key Words: G6PD enzyme, *Oncorhynchus mykiss*, inhibition, antibiotic

Gökkuşağı Alabalığı (*Oncorhynchus mykiss* Walbaum, 1792) Eritrositlerindeki Glikoz 6-Fosfat Dehidrogenaz Enzimi Aktivitesi Üzerine Bazı Antibiyotiklerin Inhibisyon Etkileri

Özet: Bazı antibiyotiklerin, gökkuşağı alabalığı (*Oncorhynchus mykiss* Walbaum, 1792) eritrositlerindeki glikoz 6-fosfat dehidrogenaz enzimi üzerine inhibisyon etkileri araştırılmıştır. Bu amaçla başlangıçta, eritrositlerdeki glukoz 6-fosfat dehidrogenaz enzimi, amonyum sülfat çöktürmesi ve 2',5' Sefaroz 4B afinite jeli kullanılarak, %70,40 oranında 1271.19 kat saflaştırılmıştır. Saflaştırma esnasında sıcaklık +4 ºC' de sabit tutulmuş, enzim aktivitesi spektofotometre ile 340 nm'de Beutler metoduna göre ölçülmüştür. Bütün kinetic çalışmalarında bu metot kullanılmıştır. Antibiyotiklerden; tiamfenikol, amikasin, gentamisin ve netilmisin kullanılmıştır. Bu antibiyotiklerin hepsi enzim üzerinde inhibitor etki göstermiştir. Glukoz 6-fosfat dehidrogenaz enzimi için K_i değeri Lineweaver-Burk grafiklerinden tespit edilmiştir. Tiamfenikol ve amikasin yarışmalı bir inhibisyon gösterirken, gentamisin ve netilmisin yarışmasız bir inhibisyon göstermiştir. Buna ilaveten, antibiyotiklerin I_{50} değerleri, % vs [I] aktivitesinden tayin edilmiştir.

Anahtar Sözcükler: G6PD enzimi, *Oncorhynchus mykiss*, inhibisyon, antibiyotik

Introduction

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49; G6PD) 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, nucleic acid and membrane lipids synthesis (1-3). NADPH in erythrocytes is a regeneration of reduced glutathione, which prevents hemoglobin denaturation, preserves the integrity of red blood cell membrane sulfhydryl groups, and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells (4,5). When one molecule palmitate is synthesized in the biosynthesis of fatty acids, 14 NADPH molecules are used. The 6 molecules of NADPH are synthesized in the pentose phosphate pathway. Essentially, the pentose phosphate pathway is more active adipose tissue than muscle. This shows that the G6PD enzyme is very

important in the biosynthesis of fatty acids (3,6). Growth rate in fish is influenced by various factors such as dietary regime, and feeding competition and frequency (7). NADPH plays an important role in initiating protein synthesis (8). Fish in general require more dietary protein than do other vertebrates. NADPH is essential to growth and proliferation processes, serving as they do as hydrogen and electron sources for a variety of reductive biosynthetic reactions, including the synthesis of fatty acids and cholesterol (9,10).

Although the effects of many drugs on human and rat G6PD enzyme activity have been investigated (11,12) there have been no studies on rainbow trout erythrocyte G6PD enzyme activity.

Therefore, in vitro effects of some antibiotics, used against fish diseases, on rainbow trout red blood cell G6PD activity were investigated in this study.

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. All other chemicals used were of analytical grade and were purchased from either Sigma or Merck.

Fish husbandry and maintenance

Fish samples were obtained from the rainbow trout farm of the Department of Aquaculture, Faculty of Agriculture, Atatürk University. The rainbow trout (n: 50) used in this study were mature, healthy, 4-5 years old and with an average weight of 1.6-2.2 kg. The average water temperature was 9 ± 2 °C during the tests. At the time of sample collection fish were fed a commercial trout feed at 2% body weight twice per day. Prior to the experiment, fish in each group were kept in 1 x 1.2 m (wide-deep) fiber-glass tanks for one month. Tanks were supplied with fresh water at a flow rate of 0.01 l/min per kg of body weight. The water quality parameters were measured as O_2 = 8.8 ppm, pH = 8.1, SO_4 = 0.33 mg/l, PO_4 = trace, NO_3 = 3.45 mg/l, NO_2 = trace, and conductivity = 240 µs/cm.

Preparation of the Hemolysate

Blood was sampled from the caudal vein using a 10 ml plastic heparinized syringe (5 IU/ml). It was then transferred to tubes and centrifuged (Hettich® Micro 22 R, refrigerated centrifuge) at 2,500 x g for 15 min. The plasma was removed by drip. After the package of red cells was washed with KCl solution (0.16 M) 3 times, the samples were centrifuged at 2,500 x g each time and 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 (13).

Ammonium Sulphate Fractionation and Dialysis

The hemolysate was subjected to progressive precipitation with ammonium sulfate (10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70% and 70-80%). Ammonium sulfate was slowly added to the hemolysate for complete dissolution. This mixture was centrifuged at 5,000 x g for 15 min and the precipitate was dissolved in 50 mM phosphate buffer (pH 7.0). For each respective precipitation, the enzyme activity was determined in both the supernatant and precipitate. The enzyme was observed to precipitate at 40-65% precipitation. It was then dialyzed at 4 °C in 50 mM K-acetate/5 mM K-phosphate buffer (pH 7.0) for 2 h with 2 changes of buffer (13).

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. During several washings the impurities were removed and the gel was conditioned. After the removal of the air in the gel, it was resuspended in the buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) at a ratio of 25% buffer to 75% gel and was 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 of 0.1 M K-acetate + 0.1 M K-phosphate (pH 6.0) and 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). The washing with 0.1 M KCl + 0.1 M K-phosphate (pH 7.85) was 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 + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). The enzyme activity was measured in final fractions, and the activity-containing tubes were collected together. The protein was determined in the resultant solution. During all procedures, the temperature was kept at +4 °C (13-15).

Optimal pH Determination

For the optimal pH determination, the enzyme activity was measured in 1 M Tris-HCl and phosphate buffers within the pH ranges of 7.2 to 8.9 and of 4.9 to 8.0, respectively.

Activity Determination

The enzymatic activity was measured by Beutler's method (16). One enzyme unit was defined as the enzyme amount reducing 1 µmol of $NADP^+$ per min at optimum pH.

Protein Determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (17), with bovine serum albumin being used as a standard.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity, using Laemmli's procedure (18), was carried out in 3% and 8% acrylamide concentrations for running and stacking gel, respectively. To the gel solution was added 10% SDS. The gel was stabilized in a solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. Staining was performed for about 2 h in a solution of 0.1% Coommassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid. Finally, washing was carried out in a solution of 50% methanol + 10% acetic acid + 40% distilled water until the protein bands were cleared.

Inhibitor Studies

Thiamphenicol, amikacin, gentamicin, and netilmicin were used as inhibitors. In the media with or without inhibitor, the substrate (G6-P) concentrations were 0.15 mM, 0.30 mM, 0.45 mM, 0.60 mM, and 0.90 mM. Inhibitor (antibiotics) solutions were added to the reaction medium, resulting in 3 different fixed concentrations of inhibitors in 1 ml of total reaction volume. For the 3 fixed inhibitor concentration values mentioned, the values were obtained by using 5 substrate concentrations. To draw Lineweaver-Burk graphs (19) by using 1/V vs. 1/[S] values, regression analysis (n = 5) was carried out and equations obtained from regression analysis were used to draw graphs for each fixed inhibitor concentration. K_i values were calculated from these Lineweaver-Burk graphs.

In order to determine I_{50} values, activities were calculated with different inhibitor concentrations, and a 0.60 mM constant substrate (G6-P) concentration (thiamphenicol: 21, 42, 84, 168, and 210; amikacin: 21.25, 42.50, 85, 106.25, and 170; gentamicin: 12.52, 16.70, 25.05, 33.40, and 41,75; netilmicin: 6.92, 13.85, 20.77, 27.70, 34.62, 55.40, and 62.32 mM). Drugless cuvette activity was taken as 100%. Regression analysis graphs were drawn using inhibition % values by a statistical package on a computer. The inhibitor concentrations causing up to 50% inhibition (I_{50}) were determined from the graphs.

Results

G6PD was purified 1271.19-fold in a yield of 70.40% by using ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity gel. SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme, and the electrophoretic pattern was photographed (Figure 1). Optimal pH of G6PD was determined as 8.0 using 1 M Tris-HCl.

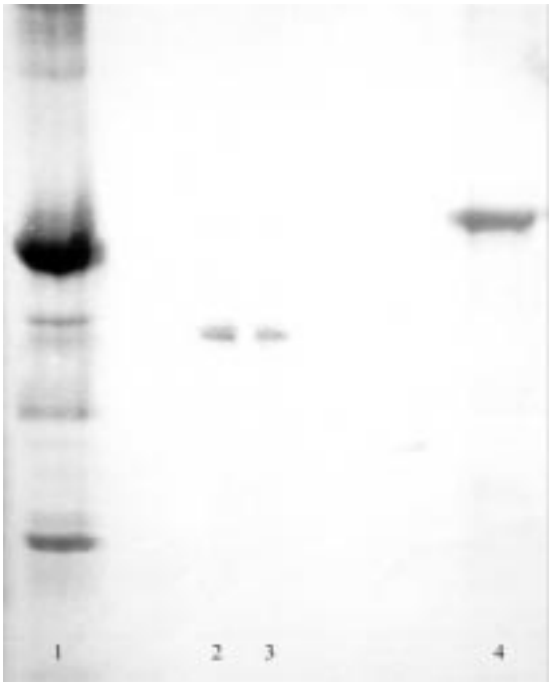

Figure 1. SDS-PAGE bands of G6PD (Lane 1: Standard proteins; yeast hexokinase (100 kDa), rabbit heart creatine phosphokinase (81 kDa), bovine serum albumin (66 kDa), bovine liver glutamic dehydrogenase (55 kDa), bovine spleen deoxyribonuclease (38 kDa), Lane 2-3: rainbow trout G6PD and Lane 4: bovine lactoperoxidase (80 kDa).

For each antibiotic the Lineweaver-Burk graphs were drawn and are shown in Figures 2-5.

K_i constant and inhibition type were determined as 33.35 ± 11.42, 14.13 ± 2.20, 3.34 ± 1.60, and 2.65 ± 0.31 mM from the graphs for thiamphenicol (competitive), amikacin (competitive), gentamicin (noncompetitive), and netilmicin (noncompetitive), respectively.

In addition, [Antibiotic] vs. activity % graphs were drawn for the antibiotics and are shown in Figures 6-9.

I_{50} values were calculated as 212.99, 56.18, 22.22, and 16.28 mM from the graphs for thiamphenicol, amikacin, gentamicin, and netilmicin, respectively. I_{50} values, K_i constants and inhibition type are presented in the Table.

Discussion

The importance of G6PD in metabolism has been well known for many years. GSH is used by antioxidant defense mechanisms and its production requires NADPH to be synthesized in the pentose phosphate metabolic pathway in which G6PD and 6PGD participate. For this reason, G6PD and 6PGD were considered as antioxidant enzymes (20). In the present study, the in vitro effects of some antibiotics, used in aquaculture, on G6PD have been investigated.

We think that the easier purification methods for the enzyme make it possible that investigations on the subject can be easily performed. A high purity for the enzyme was obtained (Figure 1). The pH determined was similar to those in previous studies (16,21,22).

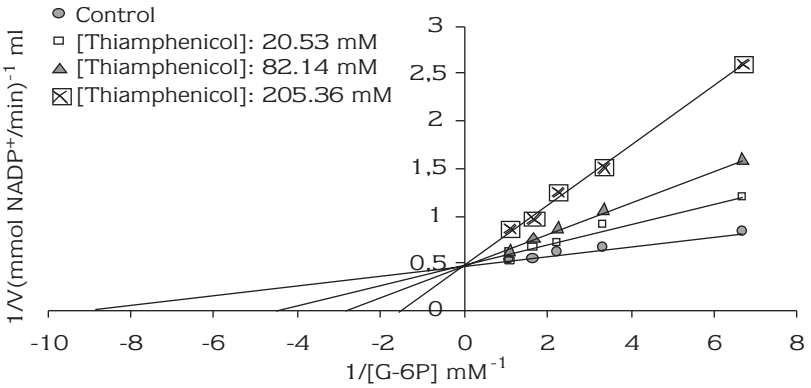

Figure 2. Lineweaver-Burk graph in 5 different substrate (G6-P) concentrations and in 3 different thiamphenicol concentrations for determination of K_i for thiamphenicol.

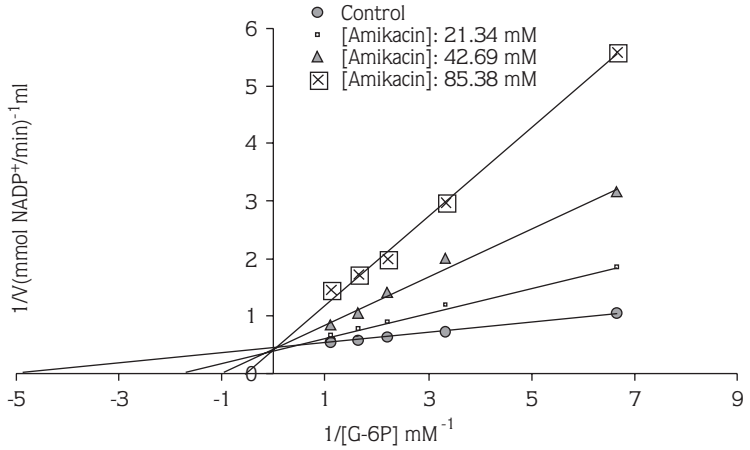

Figure 3. Lineweaver-Burk graph in 5 different substrate (G6-P) concentrations and in 3 different amikacin concentrations for determination of K_i for amikacin.

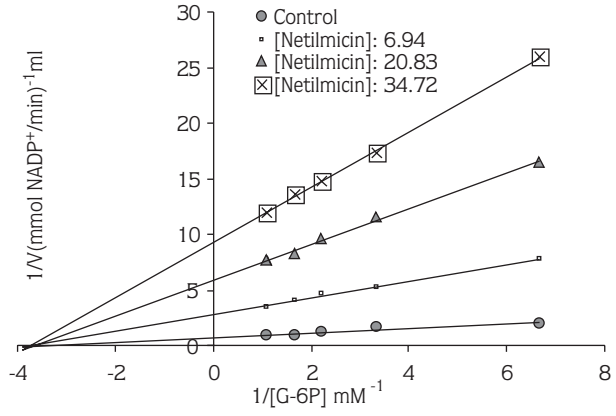

Figure 4. Lineweaver-Burk graph in 5 different substrate (G6-P) concentrations and in 3 different netilmicin concentrations for determination of K_i for netilmicin.

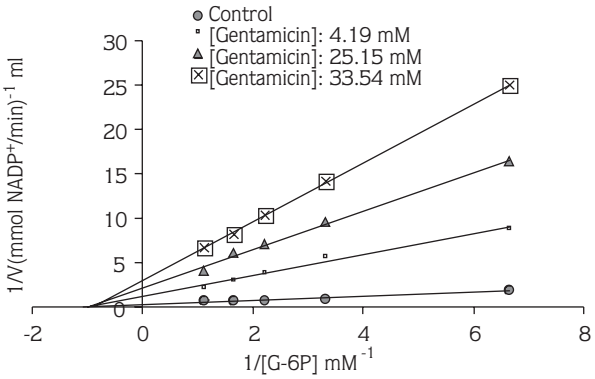

Figure 5. Lineweaver-Burk graph in 5 different substrate (G6-P) concentrations and in 3 different gentamicin concentrations for determination of K_i for gentamicin.

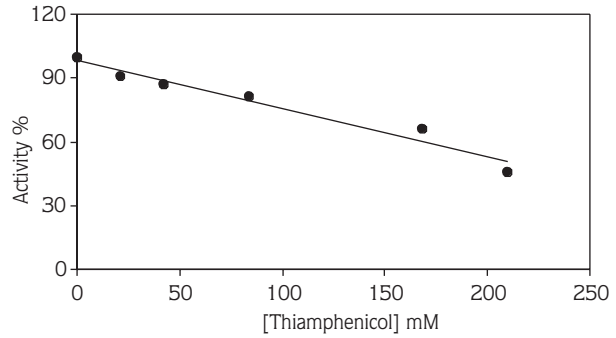

Figure 6. Activity % vs. [thiamphenicol] regression analysis graphs for rainbow trout erythrocytes G6PD in the presence of 5 different thiamphenicol concentrations.

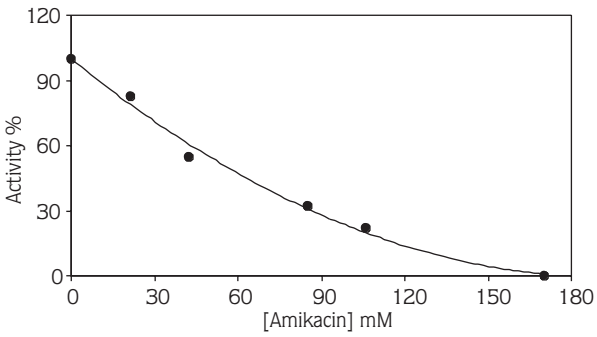

Figure 7. Activity % vs. [amikacin] regression analysis graphs for rainbow trout erythrocytes G6PD in the presence of 5 different amikacin concentrations.

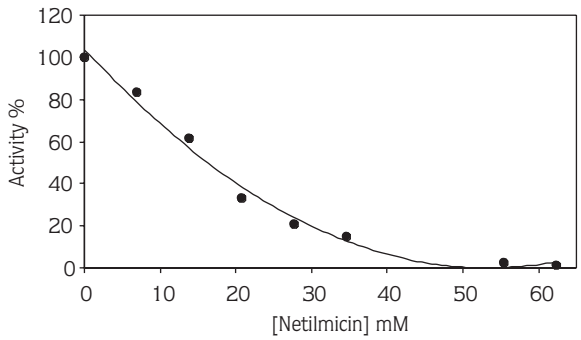

Figure 8. Activity % vs. [netilmicin] regression analysis graphs for rainbow trout erythrocytes G6PD in the presence of 7 different netilmicin concentrations.

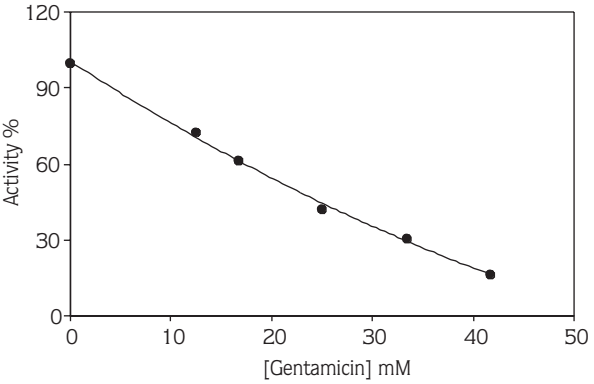

Figure 9. Activity % vs. [gentamicin] regression analysis graphs for rainbow trout erythrocytes G6PD in the presence of 5 different gentamicin concentrations.

Table. K_i values obtained from Lineweaver-Burk graphs for G6PD in the presence of 3 fixed inhibitors and 4 substrate (G6-P) concentrations for different antibiotics.

Antibiotics	I_{50} (mM)	K_i (mM)	Mean ± SD	Inhibition type
		20.29		
Thiamphenicol	212.99	38.27	33.35 ± 11.42	Competitive
		41.50		
		16.66		
Amikacin	56.18	12.96	14.13 ± 2.20	Competitive
		12.76		
		1.50		
Gentamicin	22.22	4.48	3.34 ± 1.60	Noncompetitive
		4.03		
		2.29		
Netilmicin	16.28	2.82	2.65 ± 0.31	Noncompetitive
		2.83		

It is known that many drugs have adverse effects on the organism when used for therapeutic or other purposes (23). These effects may be dramatic and systematic (11). A good example of this is that in 1926 pamaquine used for malaria treatment caused severe adverse effects in patients within a few days, resulting in black urination, hyperbilirubinemia, a dramatic decrease in blood Hb levels, and finally death, which occurred in cases of severe G6PD deficiency (6). Similarly, acetazolamide inhibits carbonic anhydrase (CA), giving rise to severe diuresis (24).

The antibacterial efficiency of amikacin, gentamicin, netilmicin and thiamphenicol against bacterial fish pathogens has been investigated by many authors (25-28). However, the inhibitory effects of these antibiotics on G6PD in fish have not been studied. Inhibitory effects of some antibiotics on enzymatic activities in other animal species and human beings have been reported in many investigations. For example, ampicillin inhibits human red cells' G6PD and CA, netilmicin inhibits human red cells' G6PD, and metamizol inhibits human red cells' G6PD and activates human red cells' CA. In addition, it has been reported that ampicillin and metamizol inhibit rat CA in vivo (11,12,29,30).

In order to show inhibitory effects, while the most suitable parameter is the K_i constant, some researchers use the I_{50} value. Therefore, in this study, both the K_i and I_{50} parameters of these antibiotics for G6PD were determined.

As shown in the Table, K_i values are 33.35 ± 11.42, 14.13 ± 2.20, 3.34 ± 1.60 mM and 2.65 ± 0.31 mM and I_{50} values are 212.99, 56.18, 22.22 and 16.28 mM for thiamphenicol, amikacin, gentamicin and netilmicin, respectively. These values show that netilmicin had the highest inhibitor effect, followed by gentamicin, amikacin and thiamphenicol, respectively. I_{50} values showed the same trend. K_i and I_{50} values for gentamicin and netilmicin were similar to the values obtained from gentamicin and netilmicin for the G6PD enzyme of human erythrocytes (11).

In another study, it was shown that amikacin inhibited G6PD enzyme activity (in vivo and in vitro) in rat erythrocytes (31). We also found similar results for amikacin.

No references in the literatures have been found about thiamphenicol inhibiting G6PD.

In this investigation, these drugs showed highly inhibitory effects on the G6PD enzyme activity of trout erythrocytes. By using the obtained K_i and I_{50} values, undesirable side effects on G6PD activity and body metabolism and fatty acid synthesis can be reduced.

If it is necessary to give these antibiotics to fish, their dosage should be carefully determined to reduce their hemolytic side effects, since these drugs may damage the health of fish and lead to fatal results.

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