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Research Article

In Vitro Effects of Some Antibiotics on Glutathione Reductase Obtained from Chicken Liver

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Abstract: Glutathione reductase (GR) is important in maintaining high levels of reduced glutathione in cells. The enzyme is inhibited by some drugs used in antimicrobial chemotherapy. The effects of some antibiotics on GR activity were investigated in this study.

At first, the enzyme, having a specific activity of 88.28 EU/mg proteins, was purified 1636-fold with a yield of 35%. Purity of the enzyme was checked by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Activity of the enzyme was determined spectrophotometrically at 340 nm and in vitro effects of ofloxacin, levofloxacin, cefazolin, and cefepime were determined.

The antibiotics used in this study showed inhibitory effects on the enzyme. Concentrations of inhibitors producing 50% inhibition (I_{50}) and their inhibition constants (K_i) were estimated to be 0.159 mM and 0.5840 ± 0.3121 mM for ofloxacin, 0.090 mM and 0.3833 ± 0.2725 mM for levofloxacin, 14.871 mM and 34.5002 ± 4.2628 mM for cefazolin, and 4.589 mM and 4.9504 ± 3.8928 mM for cefepime, by using activity-[Drug] and Lineweaver-Burk graphs. Of these 4 antibiotics, ofloxacin, levofloxacin, and cefazolin were non-competitive inhibitors, whereas, cefepime was a competitive inhibitor for the enzyme. Therefore, when these drugs are given to chickens, their dosages should be carefully controlled in order to prevent their adverse effects.

Key Words: Glutathione reductase, chicken, liver, ofloxacin, levofloxacin, cefazolin, cefepime

Tavuk Karaciğerinden Elde Edilen Glutatyon Redüktaz Enzimi Üzerine Bazı Antibiyotiklerin In Vitro Etkileri

Özet: Glutatyon redüktaz (GR) hücrede indirgenmiş glutatyonun yüksek seviyelerde tutulması için önemlidir. Bu enzim antimikrobiyal kemoterapide kullanılan bazı ilaçlar tarafından inhibe olur. Bu çalışmada bazı antibiyotiklerin glutatyon redüktaz enzim aktivitesi üzerindeki etkileri araştırıldı.

Öncelikle enzim, 88,28 spesifik aktiviteye sahip, %35 verim ile 1.636 kat saflaştırıldı. Enzimin saflığı sodyum dodesil sülfat poliakrilamid jel elektroforezi (SDS-PAGE) ile kontrol edildi. Enzim aktivitesi 340 nm'de spektrofotometrik olarak belirlendi ve ofloksasin, levofloksasin, sefazolin, and sefepim'in enzim üzerine in vitro etkileri tespit edildi.

Araştırmada kullanılan bütün antibiyotikler enzim üzerine inhibitör etkisi gösterdiler. %50 inhibisyon oluşturan inhibitor konsantrasyonları (I_{50}) ve inhibisyon sabitleri (K_i), Aktivite-[İlaç] ve Lineweaver-Burk grafikleri kullanılarak, sırayla ofloksasin için 0,159 mM ve 0,5840 ± 0,3121 mM, levofloksasin için 0,090 mM ve 0,3833 ± 0,2725 mM, sefsazolin için 14,871 mM ve 34,5002 ± 4,2628 mM ve sefepim için 4,589 mM ve 4,9504 ± 3,8928 mM oldukları hesaplandı. Enzim için bu dört antibiyotikten ofloksasin, levofloksasin ve sefazolin yarışmasız, sefepim ise yarışmalı inhibitörler idi. Bu yüzden, bu ilaçlar tavuklara verildiği zaman olumsuz etkilerini önlemek için dozlarının iyi ayarlanması gerekir.

Anahtar Sözcükler: Glutatyon redüktaz, tavuk, karaciğer, ofloksasin, levofloksasin, sefazolin, sefepim

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Introduction

Glutathione reductase (glutathione: $NADP^+$ oxidoreductase, E.C.1.8.1.7; GR) is a crucial flavoprotein responsible for maintaining a high ratio of reduced to oxidized glutathione in the cells of most organisms. Reduced glutathione (GSH) is a reaction partner for the detoxification of endobiotics and xenobiotics, and a storage and transport form of cysteine. Its function is important for protection against oxidative stress, for maintaining the thiol redox potential in cells that keep sulfhydryl groups of intracellular proteins in the reduced form, and in the production of deoxyribonucleotides (1). In these reactions, 2 GSH molecules are oxidized to the disulfide form (GSSG). GSH is regenerated by means of reduction with NADPH produced by the enzymes of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Decreased GSH levels have been reported in several diseases, such as diabetes (2), acquired immune deficiency syndrome (AIDS) (3), adult respiratory distress syndrome (4), and Parkinson's disease (5). On the other hand, high GSSG concentrations inhibit a number of important enzyme systems, including protein synthesis (6). Increasing the intracellular concentration of GSSG by oxidative stress is positively correlated with an increase in protein-GSH mixed disulfides (7).

Ofloxacin and levofloxacin, 2 of the more recent introduced fluorinated 4-quinolones, represent an important therapeutic advance. Cefazolin and cefepime, first-generation and fourth-generation cephalosporins, respectively, are stable to hydrolysis by many of the previously identified plasmid-encoded β-lactamases (8). These antibiotics used in therapies have been determined to have inhibitory effects for GR obtained from sheep liver (9), but no studies were encountered related to changes in this enzyme's activity in chickens. For this reason, in the present study, the in vitro effects of the 4 antibiotics on GR purified from chicken liver were investigated.

Materials and Methods

Materials

2', 5'-ADP Sepharose 4B affinity column material was obtained from Pharmacia (Sweden). Sephadex G-200, GSSG, NADPH, and protein assay reagents, and chemicals for electrophoresis were obtained from Sigma Chemical Co. (USA). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich (USA) or Merck (Germany).

Purification procedure

Fresh chicken liver (40 g) was washed in isotonic saline solution containing 1 mM EDTA, cut into small pieces, and homogenized in a Waring blender with 80 ml of 0.1 M Tris-HCl buffer (pH 7.4), which contained 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 15,000 rpm for 60 min, and the precipitate was removed. This process was repeated twice and the supernatant was used as a crude extract. The sample was brought to a 30%-70% ammonium sulfate saturation with solid $(NH_4)_2SO_4$. The precipitate was dissolved in a small amount of 0.1 M Tris-HCl buffer, which contained 1 mM EDTA (pH 7.4), and then it was dialyzed against the same buffer (10).

The dialyzed sample was loaded onto the washed and equilibrated 2', 5'-ADP Sepharose 4B affinity column (1 x 10 cm) and then washed successively 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 latter washing continued with 50 mM K-phosphate buffer, which contained 1 mM EDTA (pH 7.4) and 0.4 mM $NADP^+$, until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted successively with a gradient of 0-1 mM NADPH in a solution of 80 mM K-phosphate + 80 mM KCl + 10 mM EDTA (pH 7.4) (10). Active fractions were collected and dialyzed with equilibration buffer.

The sample obtained from the affinity column was loaded onto an equilibrated Sephadex G-200 gel filtration column (1.5 x 70). Flow rate was adjusted to 15 ml/h by means of a peristaltic pump and elutions were collected in 1.8-ml Eppendorf tubes containing equilibration buffer (50 mM Tris-HCl, 50 mM KCl buffer (pH 7.4), which contained 50 mM glycerol). Active fractions were lyophilized and stored at –85 °C for checking the enzyme purity by electrophoresis and for determination of the effects of the antibiotics. During all purification procedures, the temperature was kept at 4-6 °C.

Activity Determination

Enzymatic activity was measured spectrophotometrically at 25 °C, according to the modified method of Carlberg and Mannervik (11). The

assay system contained 0.75 mM Tris-HCl buffer (pH 7.0), which contained 1 mM EDTA, 1 mM GSSG, and 0.1 mM NADPH, in a total volume of 1 ml. The decrease in absorbance at 340 nm was followed with a Shimadzu spectrophotometer (UV-1208, Japan). The reaction was initiated by the addition of the enzyme solution. One enzyme unit was defined as the oxidation of 1 µmol NADPH/min under the assay conditions (ε_{340} = 6.2 mM^{-1} cm^{-1} for NADPH).

Protein Determination

Protein concentrations were estimated from measurements of absorbance at 595 nm, according to Bradford's method (12), with bovine serum albumin as a standard.

SDS-PAGE

In order to check enzyme purity, SDS-PAGE was performed with Laemmli's method (13), with bovine carbonic anhydrase (29 kDa), chicken ovalbumin (45 kDa), and bovine albumin (66 kDa) used as standard proteins.

In vitro effects of antibiotics

In order to determine the effects of the antibiotics on GR, some concentrations of ofloxacin (0.055-0.275 mM), levofloxacin (0.011-0.353 mM), cefazolin (5.247-14.69 mM), and cefepime (0.65-10.40 mM) were added to separate cuvettes containing purified enzyme. Selected values were in a range of concentrations producing about 20%-80% inhibition for each drug. Control cuvette activities in the absence of the drugs were taken as 100%. For each antibiotic having inhibitory effects, an activity-[drug] graph was drawn and drug concentrations producing 50% inhibition (I_{50}) were calculated from these graphs.

For determining K_i constants (equilibrium constant of enzyme-inhibitor complex), 3 fixed inhibitor concentrations (0.275, 0.330, and 0.413 mM for ofloxacin; 0.177, 0.266, and 0.354 mM for levofloxacin; 10.50, 15.75, and 21.00 mM for cefazolin; and 5.2, 7.8, and 10.4 mM for cefepime) were tested. In these studies, GSSG was used as a substrate with 5 different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 mM). The Lineweaver-Burk graphs were obtained for each inhibitor by using 1/V and 1/[S] values (14). K_i constants and inhibition types were estimated from these graphs.

Statistical analysis

The data obtained were analyzed by t-test and are given as $\overline{X}$ + SD.

Results

Chicken liver GR was purified 1636-fold with a specific activity of 88.28 EU/mg proteins and a yield of 35% in this study. Figure 1 shows the SDS-PAGE made for the purity of the enzyme. Effects of ofloxacin, levofloxacin, cefazolin, and cefepime were examined, and all 4 antibiotics were determined to be inhibitors of the enzyme. I_{50} values were estimated as 0.159 mM for ofloxacin, 0.090 mM for levofloxacin, 14.871 mM for cefazolin, and 4.589 mM for cefepime (Figure 2). K_i constants were calculated as 0.5840 ± 0.3121 mM for ofloxacin, 0.3833 ± 0.2725 mM for levofloxacin, 34.5002 ± 4.2628 mM for cefazolin, and 4.9504 ± 3.8928 mM for cefepime (Figure 3, Table). Ofloxacin, levofloxacin, and cefazolin were determined to be non-competitive inhibitors, whereas cefepime was determined to be a competitive inhibitor of the enzyme.

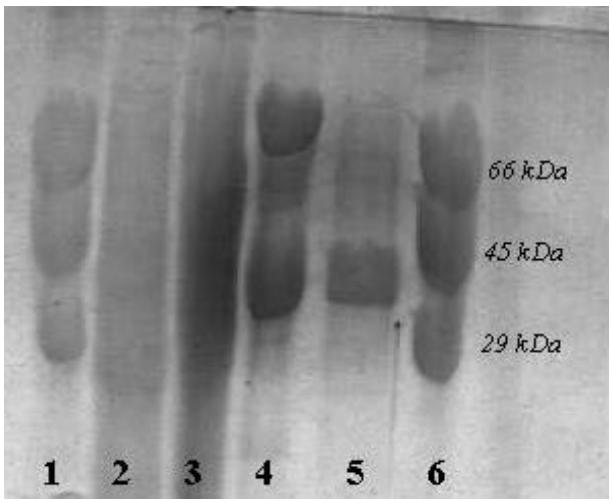

Figure 1. SDS-PAGE. Lanes 1 and 6: standard proteins [bovine carbonic anhydrase (29 kDa), chicken ovalbumin (45 kDa), and bovine albumin (66 kDa) (Sigma: MW-SDS-200)]; Lane 2: homogenate; Lane 3: ammonium sulfate precipitate; Lane 4: sample from 2', 5'-ADP Sepharose 4B affinity column; Lane 5: purified GR from Sephadex G-200 gel filtration column.

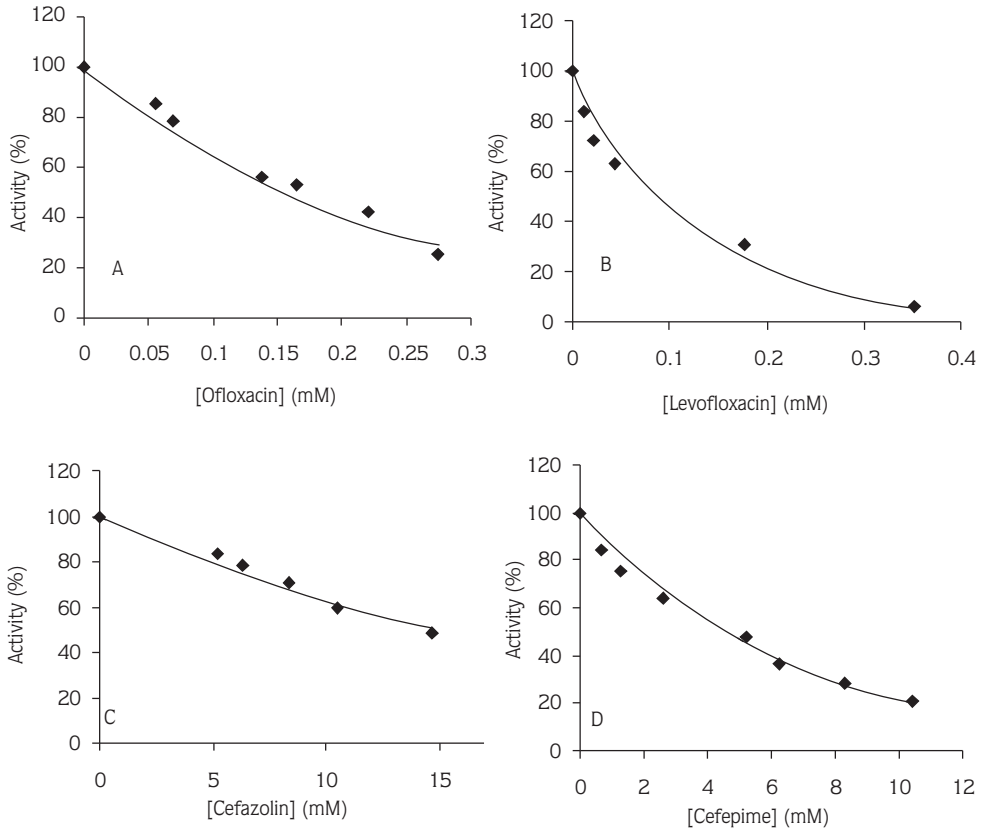

Figure 2. Activity (%)-[drug] graphs for GR in the presence of different antibiotics concentrations; (A) ofloxacin, (B) levofloxacin, (C) cefazolin, and (D) cefepime.

Discussion

Oxidative stress, which refers to the unusually high presence of molecules with a high potency to abstract electrons from biomolecules, plays an important role in the pathogenesis of various disorders (15). The most important oxidative stress agents are free radicals and reactive oxygen species (ROS). Undesirable biological effects of these highly reactive molecules disappear due to enzymatic and non-enzymatic antioxidant defense systems. GSH and GSH-related enzymes are one of the most important protective systems in cells. GSH can be involved either as a substrate in the cytosolic GSH redox cycle, or is able to directly inactivate free radicals and ROS (16). For this reason, GSH and GR are considered non-enzymatic and enzymatic antioxidants, respectively.

Some chemicals and drugs at relatively low dosages affect the metabolism of biota by altering their normal enzyme activity, particularly inhibition of a specific enzyme (17). For example, GR has been inhibited by nitrofurazone, nitrofurantoin, 5-nitroindol, 5-nitro-2-furoic acid, 2,4,6-trinitrobenzene sulfonate (18), and some hydroxylamine derivatives (19). Benzoazepin, crystal violet, a large group of tricyclics based on acridine, polyamine derivatives, phenothiazine, isoalloxazine, and pyridoquinoline ring structures, which are inhibitors of the other enzymes of thiol metabolism, have competitively inhibited GR activity (20). Arsenical compounds have also been found to be competitive inhibitors of the enzyme. Trivalent arsenicals were more potent inhibitors than their pentavalent analogs, and methylated trivalent arsenicals were more potent inhibitors than inorganic trivalent arsenicals (21).

Ofloxacin and levofloxacin, both fluoroquinolones, are potent bactericidal agents against *E. coli* and various species of *Salmonella, Shegella, Enterobacter, Campilobacter,* and *Neisseria* (22). These antibiotics target bacterial DNA gyrase and topoisomerase IV (8).

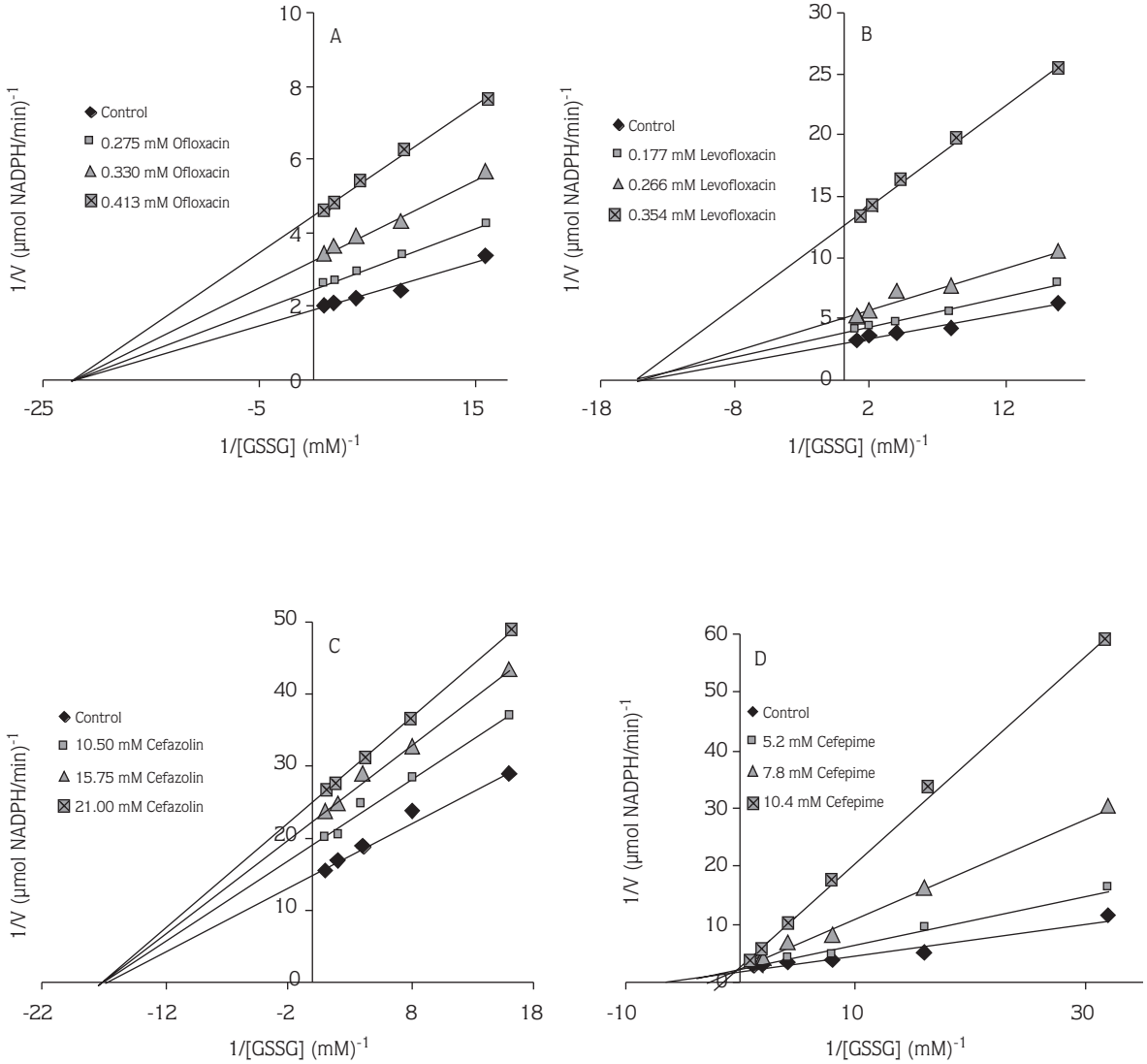

Figure 3. Lineweaver-Burk graph in different substrate (GSSG) concentrations and in 3 fixed drug concentrations for the determination of K_i values for ofloxacin (A), levofloxacin (B), cefazolin (C), and cefepime (D).

For many gram-positive bacteria (such as *S. aureus*), topoisomerase IV is the primary activity inhibited by the quinolones (23). In contrast, for many gram-negative bacteria, such as *E. coli*, DNA gyrase is the primary quinolone target (24).

Cephalosporins, like other β-lactam antibiotics, have certain general toxicities; however, they are usually very well tolerated. Cefazolin, a first-generation cephalosporin, is more active against *E. coli* and Klebsiella species (25). Cefepime, a fourth-generation cephalosporin, is a poor inducer of type I β-lactamases. It is thus active against many *Enterobacteriaceae* that are resistant to other cephalosporins, via induction of type I β-lactamases, but remains susceptible to many bacteria expressing extended spectrum plasmid-mediated β-lactamases (8).

In this study, GR was purified from chicken liver by ammonium sulfate precipitation, 2', 5'-ADP Sepharose 4B affinity chromatography, and Sephadex G-200 gel filtration chromatography. Thanks to the 3 consecutive

Table. I_{50} values, K_i constants, and inhibition types of ofloxacin, levofloxacin, cefazolin, and cefepime on chicken liver GR.

Inhibitors	I_{50} values (mM)	K_i constants (mM)	Mean K_i constants (mM)	Inhibition types
Ofloxacin	0.159	0.9209 0.5256 0.3047	0.5840 ± 0.3121	Non-competitive
Levofloxacin	0.090	0.6569 0.3813 0.1121	0.3833 ± 0.2725	Non-competitive
Cefazolin	14.871	39.4131 32.3058 31.7816	34.5002 ± 4.2628	Non-competitive
Cefepime	4.589	9.3286 3.6428 1.8797	4.9504 ± 3.8928	Competitive

procedures, the enzyme, having the specific activity of 88.28 EU/mg proteins, was purified with a yield of 35% and a 1636-fold purity. Purity of the enzyme was checked by SDS-PAGE and a single band was cleared on the gel after the final chromatographic step (Figure 1). Ofloxacin, levofloxacin, cefazolin, and cefepime were chosen for investigation of their inhibition effects. All of these antibiotics showed an inhibitory effect on the enzyme activity (Figure 2); however, ofloxacin and levofloxacin were rather more active inhibitors than cefazolin and cefepime. In order to show inhibition 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 were determined for GR and these values are shown in the Table.

The inhibitor concentrations that caused up to 50% inhibition were determined from activity-[drug] graphs. The I_{50} values of each antibiotic shown in Figure 2 almost match the obtained K_i values of the same antibiotic (Figure 3). Based on our results, it is understood that the enzyme was inhibited by the 4 drugs. Ofloxacin, levofloxacin, and cefazolin were non-competitive inhibitors, whereas cefepime was a competitive inhibitor. In general, the inhibition ratio may be decreased by increasing the concentration of the substrate in the competitive inhibitors, but it is impossible in the non-competitive inhibitors. Therefore, these drugs should not be used for therapy. If these drugs are given to chickens, in particular ofloxacin, levofloxacin, and cefazolin, (non-competitive inhibitors), their dosages should be carefully controlled in order to prevent their side effects.

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