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In Vitro Effects of Some Antibiotics on Glutathione Reductase from Sheep Liver

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The effects of gentamicin sulphate, thiamphenicol, ofloxacin, levofloxacin, cefepime, and cefazolin were investigated on the *in vitro* enzyme activity of glutathione reductase. The enzyme was purified 1,850-fold with a yield 18.76% from sheep liver using ammonium sulphate precipitation, 2′, 5′-ADP Sepharose 4B affinity chromatography, and Sephadex G-200 gel filtration chromatography. The purified enzyme showed a single band on sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE). The enzyme activity was measured spectrophotometrically at 340 nm, according to the method of Carlberg and Mannervik. From these six antibiotics, Ofloxacin, levofloxacin, cefepime, and cefazolin inhibited the activity of the purified enzyme; gentamicin sulphate and thiamphenicol showed little effect on the enzyme activity. The I_{50} values for these four antibiotics were 0.150 mM, 0.154 mM, 3.395 mM, and 18.629 mM, respectively. The K_i constants were 0.047 ± 0.034 mM, 0.066 ± 0.038 mM, 4.885 ± 3.624 mM, and 6.511 ± 1.894 mM, respectively and they were competitive inhibitors.

Keywords: Glutathione reductase; Sheep Liver; Thiamphenicol; Antibiotics; Gentamycin; Ofloxacin; Levofloxacin; Cefepime; Cefazolin

INTRODUCTION

Glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7; GR) is the key enzyme in glutathione metabolism. This flavin enzyme is essential for reduction of glutathione disulfide (GSSG) to the reduced form (GSH), necessary for protection of the cells against oxidative stress as an antioxidant. GSH is also a reaction partner for the detoxification of xenobiotics, is a cofactor in isomerization reactions, and is a storage and transport form of cysteine[1,2] and maintains the thiol redox potential in cells keeping sulfhydryl groups of intracellular proteins in the reduced form.[3] Decreased GSH levels have been reported in several diseases, such as acquired immune deficiency syndrome (AIDS),[4] adult respiratory distress syndrome,[5] Parkinson's disease,[6] and diabetes.[7] In addition, a recent result suggests that GSH is essential for cell proliferation[8] and it plays a role in the regulation of apoptosis.[9] Alternatively, high GSSG concentrations inhibit a number of important enzyme systems including protein synthesis.[10]

From a pharmacological point of view, GR is an attractive target for antimalarial and antitumor drugs.[11] Thiol metabolism in parasitic protozoa is crucial for protozoal infections. The enzymes of thiol metabolism, and in some cases the thiols themselves of parasitic protozoa differ in many interesting ways from those of mammals. Trypanosomes and *Leishmania* are most remarkable in that they have trypanothione reductase (TryR) instead of GR.[12] The crucial role of TryR for thiol homeostasis and its absence from mammalian cells suggest that it might be well suited as a target molecule for rational drug development. Different classes of compounds have been revealed as selective inhibitors of TryR; they have much weaker interactions with the closest related mammalian enzyme GR. Many of the compounds act as competitive inhibitors including polyamine derivatives,[13] crystal violet,[14] phenothiazine,[15] and benzoazepine.[16] A second class of compounds

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attracting interest are the so-called turncoat inhibitors, such as nitrofuran and naphthoquinone derivatives.[11] Covalent inhibitors are another type of drug candidate that has promise, although a selective compound for TryR has not yet been found. Cytostatic nitrosourea drugs like carmustine (BCNU) covalently inhibit TryR, but also GR.[11] Ajoene, a garlic-derived natural compound, is also a covalent inhibitor of TryR and GR.[17]

Antibiotics are used to deal with various disorders but there are few reports of their effects on enzyme activities. Some studies found either increases or decreases in mammalian enzyme activities and the inhibitor or activator effects of some antibiotics have been investigated.[18–21] The present study therefore investigated the *in vitro* effects of gentamicin sulphate, thiamphenicol, ofloxacin, levofloxacin, cefepime, and cefazolin on GR purified from sheep liver.

MATERIALS AND METHODS

Materials

Sephadex G-200, NADPH, GSSG, and protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. 2′, 5′-ADP Sepharose 4B was obtained from Pharmacia. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of the Homogenate

Livers from adult sheeps were washed in isotonic saline containing 1 mM EDTA, and stored at −85°C before use. 20 g of liver was first cut into small pieces and homogenized in a Waring blender with 50 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 15.000 rpm for 60 min and the precipitate removed. This process was repeated twice and the supernatant was used as a crude extract. The crude extract was brought to 30–70% ammonium sulphate precipitation with solid $(NH_4)_2SO_4$ and the precipitate gathered. The precipitate was dissolved in a small amount of 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.0 and then dialyzed at 4°C in the same buffer.[22]

2′, 5′-ADP Sepharose 4B Affinity Chromatography

2 g dried 2′, 5′-ADP Sepharose 4B gel was used for a 10 mL column size. The gel was washed with 300 mL distilled water, to remove foreign bodies and air, and suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0) and packed in a column. After precipitation of the gel, it was equilibrated with 50 mM K-phosphate buffer containing 1 mM EDTA, pH 6.0. The flow rates for washing and equilibration were adjusted to 20 mL/h. The dialysed sample obtained previously was loaded onto the 2′, 5′-ADP Sepharose 4B affinity column and 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 was continued with 50 mM K-phosphate buffer containing 1 mM EDTA, pH 7.0 until the final absorbance difference was 0.05 at 280 nm. The enzyme was eluted successively with a gradient of 0–0.5 mM GSH and 0–1 mM NADPH in 50 mM K-phosphate buffer, containing 1 mM EDTA (pH 7.0). Active fractions were collected and dialyzed with equilibration buffer. All procedures were performed at 4°C.[23–25]

Sephadex G-200 Gel Filtration Chromatography

Dried Sephadex G-200 (2 g) was used for a 165 mL column size and the gel was incubated in distilled water at 90°C for 5 h. After removal of the air in the gel, it was loaded onto the column (2 × 50 cm). The flow rate was adjusted to 15 mL/h. The column was equilibrated with 50 mM Tris-HCl, 50 mM KCl buffer, pH 7.0 until the final absorbance difference became zero at 280 nm and the pH was that of the equilibration buffer. The dialysate from the affinity chromatography column was mixed with 55 mM glycerol. The mixture was loaded onto the column and eluted with equilibration buffer containing 55 mM glycerol. Eluates were collected as 2 mL fractions and their activity values determined at 340 nm. The active fractions were lyophilized and stored at −85°C until required.

Activity Determination

Enzyme activity was assayed spectrophotometrically at 25°C according to the method of Carlberg and Mannervik.[26] The assay system contained 435 mM K-phosphate buffer pH 7.0, containing 1 mM EDTA, 1 mM GSSG and 0.1 mM NADPH. The decrease in absorbance at 340 nm was followed with a Shimadzu Spectrophotometer UV-(1208). The reaction was initiated by the addition of the enzyme solution. One enzyme unit is defined as the oxidation of 1 μmol NADPH per min under the assay conditions used.

Protein Determination

Quantitative protein determination was measured spectrophotometrically at 595 nm according to the method of Bradford, with bovine serum albumin as a standard.[27]

SDS-page

SDS-PAGE was performed by Laemmli's method to control enzyme purity.[28] The acrylamide concentration of the stacking and the separating gels was 4% and 10%, respectively, and 1% SDS was also added to the gel solution. The gel was stabilized in a solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. Staining was conducted for about 2 h in a solution of 0.1% Coomassie Brillant Blue R-250 + 50% methanol + 10% acetic acid + 39.9% distilled water. The gel was washed with several changes of the same solvent without the dye until the protein bands were clear.

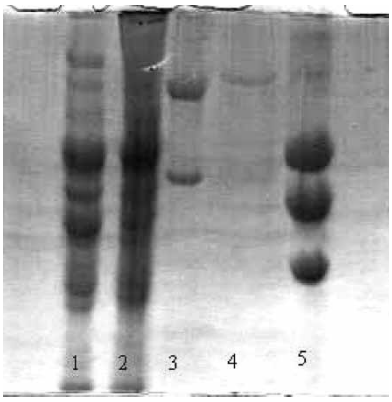

FIGURE 1 SDS-polyacrylamide gel electrophoresis of purified GR. Lane 1: homogenate; Lane 2: ammonium sulphate precipitation; Lane 3: affinity chromatography; Lane 4: gel filtration chromatography; Lane 5: standard proteins (rabbit phosphorylase b, 97,400 Da; bovine serum albumin, 66,000 Da; chicken ovalbumin, 45,000 Da; and bovine carbonic anhydrase, 29,000 Da).

In Vitro Drug Studies

To determine the effects of the antibiotics on GR, enzyme activities were measured for gentamicin sulphate (0.84–5.04 mM), thiamphenicol (8.42–50.52 mM), ofloxacin (0.055–0.44 mM), levofloxacin (0.14–0.42 mM), cefepime (2.08–12.48 mM), and cefazolin (10.29–77.75 mM) at these cuvette concentrations. Control cuvette activity in the absence of drug was taken as 100%. For each antibiotic an Activity-[Drug] graph was drawn. For four of these antibiotics (ofloxacin, levofloxacin, cefepime, and cefazolin) which had an inhibitory effect on the enzyme, drug concentrations that produced 50% inhibition (I_{50}) were calculated from these graphs (Figure 3).

For determining K_i constants, three different inhibitor concentrations (0.11–0.22 mM for ofloxacin, 0.14–0.28 mM for levofloxacin, 6.24–10.40 mM for cefepime, and 21.05–42.10 mM for cefazolin) were used. In these studies, GSSG was used as substrate at five different concentrations (0.031–0.500 mM). The Lineweaver-Burk graphs (1/V *vs* 1/[S]) were obtained for each inhibitor. K_i constants and inhibition types were estimated from the graphs (Figure 4). Analysis of data obtained was made by at test and they are given as $\bar{X} \pm SD$

RESULTS

In this study, glutathione reductase was purified 1,850-fold with a yield of 18.76% and a specific activity of 62.22 EU/mg protein. Figure 1 shows the SDS-PAGE obtained for determining the purity of the enzyme. Effects of gentamicin sulphate, thiamphenicol, ofloxacin, levofloxacin, cefepime, and cefazolin were investigated on the purified enzyme. Two antibiotics (gentamicin sulphate and thiamphenicol) have insignificant effects on the enzyme's activity (Figure 2). Four antibiotics (ofloxacin, levofloxacin, cefepime, and cefazolin) inhibited the enzyme with increased concentration of the drugs. I_{50} values were estimated as 0.150 mM, 0.154 mM, 3.395 mM, and 18.629 mM (Figure 3) respectively; K_i constants were 0.047 ± 0.034 mM, 0.066 ± 0.038 mM, 4.885 ± 3.624 mM, and 6.511 ± 1.894 mM, respectively (Figure 4, Table I). The inhibition type for these four antibiotics was competitive.

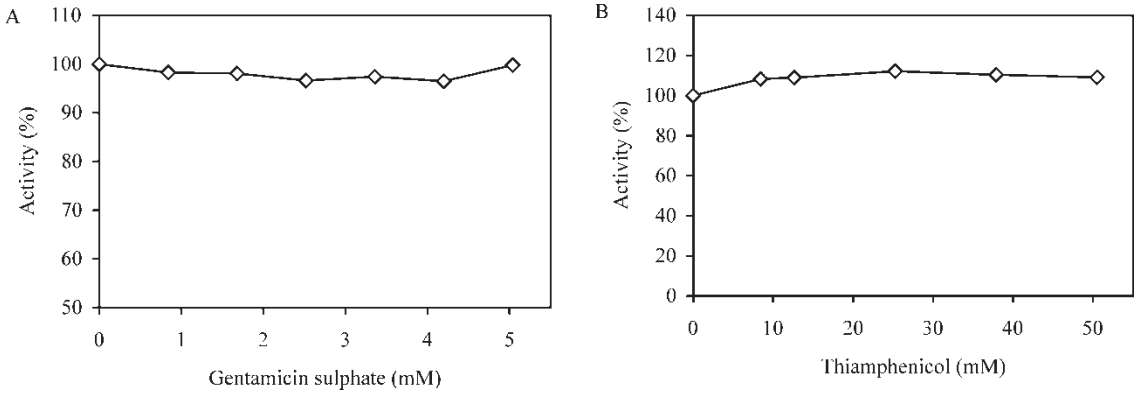

FIGURE 2 % Activity-[drug] graphs for GR in presence of gentamicin sulphate (A) and thiamphenicol (B).

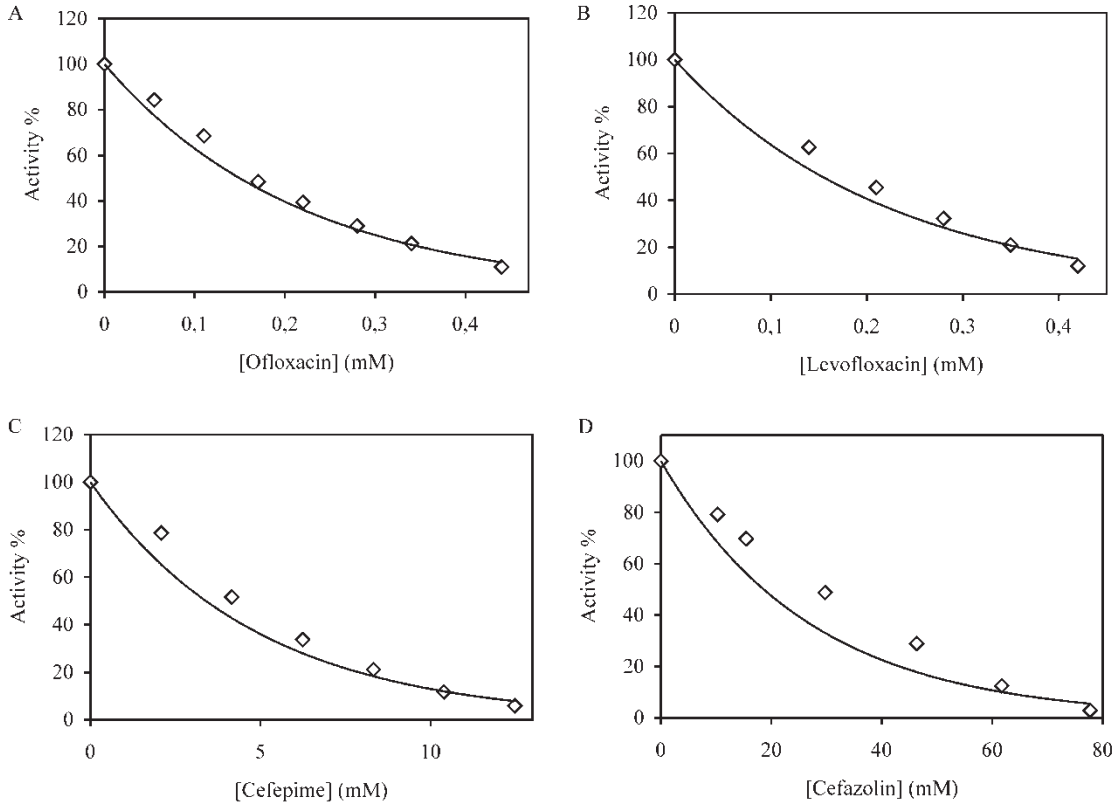

FIGURE 3 % Activity-[drug] graphs for GR in presence of four antibiotics; (A) ofloxacin; (B) levofloxacin; (C) cefepim; and (D) cefazolin.

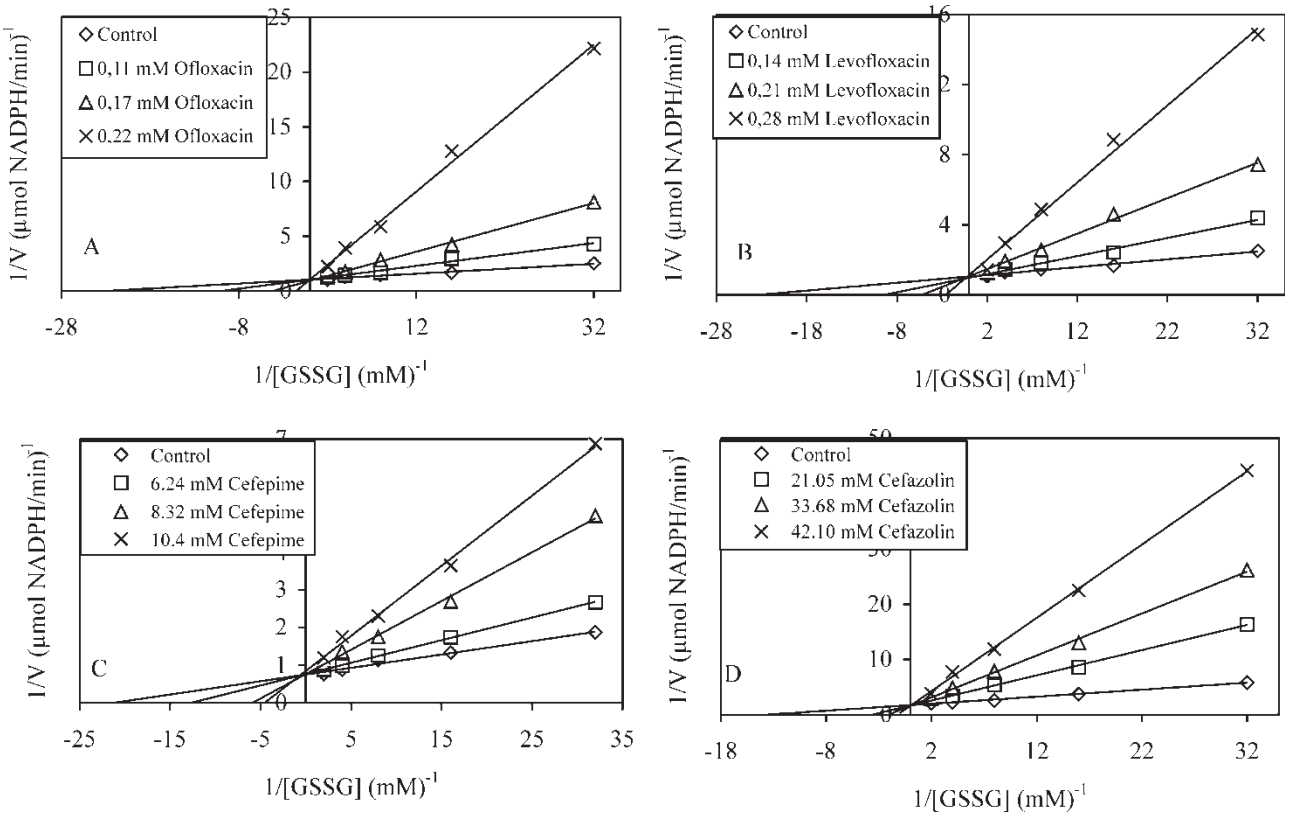

FIGURE 4 Lineweaver-Burk graphs for 5 different substrate (GSSG) concentrations and 3 different antibiotic concentrations for determination of K_i constants; (A) ofloxacin; (B) levofloxacin; (C) cefepime; and (D) cefazolin.

TABLE I I_{50} values, K_i constants, and inhibition types of ofloxacin, levofloxacin, cefepime, and cefazolin for sheep liver GR

Inhibitors	I_{50} values (mM)	[I] (mM)	K_i constants (mM)	Mean K_i constants (mM)	Inhibition type
		0.11	0.083		
Ofloxacin	0.150	0.17	0.043	0.047 ± 0.034	Competitive
		0.22	0.016		
		0.14	0.107		
Levofloxacin	0.154	0.21	0.059	0.066 ± 0.038	Competitive
		0.28	0.032		
		6.24	9.051		
Cefepime	3.395	8.32	3.150	4.885 ± 3.624	Competitive
		10.40	2.454		
		21.05	8.230		
Cefazolin	18.629	33.68	6.822	6.511 ± 1.894	Competitive
		42.10	4.481		

DISCUSSION

Oxidative stress has a crucial role in a number of pathophysiological processes including DNA damage and lipid peroxidation. The most important oxidative stress agents are free radicals and reactive oxygen species (ROS). ROS includes non-organic molecules i.e. superoxide radical anion ($O_2^{\cdot -}$), hydroxyl radicals ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2), as well as organic molecules such as alkoxyl and peroxyl radicals. ROS are continuously generated during oxidative metabolizm. In order to avoid damage caused by ROS, such as lipid peroxidation, protein modification, and DNA strand breaks, mechanisms exist which remove ROS or prevent the generation of ROS.[29,30] For example, the removal of superoxide and H_2O_2 prevents the generation of hydroxyl radicals, which are formed by the iron-catalyzed Fenton Reaction or by the Haber-Weiss-Reaction[31,32] and are the most reactive species within the ROS family. Undesirable biological effects of these highly reactive molecules are dispersed by enzymatic and non-enzymatic antioxidant defense systems. Enzymatic defense is provided by many enzyme systems such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, glutathione S-transferase (GST), aldoketoreductase and DNA repair enzymes. Non-enzymatic antioxidant defense systems include many different agents such as vitamins (e.g. A, E and C), transferrin, ceruloplasmin, lactoferrin, uric acid, taurine, GSH, cysteamine, cysteine and thioredoxin. The 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 reactive oxygen species.[33] The metabolism of exogenous compounds such as carcinogens, toxins and drugs, known as xenobiotics, usually involves phase I and phase II reactions. Phase I metabolism includes oxidation-reduction or dealkylation of the xenobiotics by cytochrome P-450 mono oxygenases.[34] Xenobiotics are generally converted to more polar hydroxylated derivatives in these reactions. In phase II reactions, these derivatives are conjugated with molecules such as glutathione, sulfate or glucuronic acid. These reactions are catalyzed by GST, sulfo transferases and glucuronyl transferases.[35–37] Conjugates produced as a result of these reactions are more soluble in water and are excreted readily in the urine or the bile.

Many chemicals and drugs at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme.[38] For example, GR enzyme have been inhibited by nitrosourea drugs,[39] nitro-compounds such as nitrofurantoin, nitrofurazone, and 5-nitroindol,[40] (2,2′:6′,2″-Terpyridine)platinium(II) complexes,[41] some sulphydryl agents such as arsenicals and 2,4,6,-trinitrobenzene sulphonate (TNBS),[42] polyamine derivatives, phenothiazine and ajone, a garlic-derived natural compound.[17]

In this study, GR has been purified from sheep liver by 2′, 5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel filtration chromatography. Before affinity chromatography so as to remove the impurities and to obtain a concentrated enzyme, ammonium sulphate precipitation[43] was conducted. Using the procedure described in this study, an enzyme with a specific activity of 62.22 EU/mL protein was to purified from 20 g sheep liver homogenate within a day with a yield of 18.76% and 1,850-fold concentration. The purified enzyme showed a single band on the SDS-PAGE (Figure 1).

Gentamicin sulphate, an aminoglycoside, is used for treatment of many aerobic gram-negative infections such as by *Escherichia coli, Klebsiella, Enterobacter, Proteus, and Serratia* and infection by methicillin-resistant *Staphylococci*. Thiamphenicol, an antibiotic that resembles chloramphenicol, inhibits the synthesis of proteins of the inner mitochondria membrane that are synthesized within mitochondria, probably by inhibition of ribosomal peptidyltransferase.[44] These group antibiotics inhibit hepatic microsomal cytochrome P-450 enzymes, and thus may prolong the half-lives of drugs that are metabolized by this system.[45] In this study,

we found that gentamicin sulphate and thiamphenicol had an insignificant effect on GR an activity. Nevertheless, sheep red blood cell glucose 6-phosphate dehydrogenase (G6PD) and human erythrocyte G6PD have been inhibited by gentamicin sulphate.[19,21] Ofloxacin and levofloxacin, fluoroquinolones, are potent bactericidal agents against *E coli* and various species of *Salmonella*, *Shegella*, *Enterobacter, Campilobacter, and Neisseria*.[46] Quinolones and fluoroquinolones are generally well tolerated,[47] the most common adverse reactions involve the gastrointestinal tract, and central nervous system side effects,[44] of Cefepime, a fourth-generation cephalosporin, is stable to hydrolysis by many of the previously identified plasmid encoded β-lactamases. Against the fastidious gram-negative bacteria (*H.influenzae, Neisseria gohorrhoeae and Neisseria meningitides*), cefepime has comparable or greater *in vitro* activity than other cephalosporins and has excellent penetration into the CSF in animal models of meningitis. Cefazolin, a first generation cephalosporin, is more active against *E. Coli* and *Klebsiella* species, but it is somewhat more sensitive to staphylococcal betalactamase than cephalothin.[48] Çiftçi *et al.* reported that sheep liver G6PD have also been inhibited by cefazolin.[18]

This study showed that ofloxacin, levofloxacin, cefepime, and cefazolin have strong inhibitory effects on GR activity. Since effects of these antibiotics on enzyme activity have not been previously reported, these results are of interest for further researches. Both the K_i constant and I_{50} values were determined for four of the studied antibiotics that inhibited the activity of sheep liver GR. K_i constants and I_{50} values in Table I show that ofloxacin is the most potent inhibitory. Therefore, if these antibiotics are given to sheep, their dosages should be very well controlled to prevent adverse effects on the GR enzyme.

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