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Effects of some antibiotics on glutathione reductase activities from human erythrocytes *in vitro* and from rat erythrocytes *in vivo*

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Abstract

The effects of streptomycin sulfate, gentamicin sulfate, thiamphenicol, penicillin G, teicoplanin, ampicillin, cefotaxime, and cefodizime on the enzyme activity of glutathione reductase (GR) were studied using human and rat erythrocyte GR enzymes in *in vitro* and *in vivo* studies, respectively. The enzyme was purified 5,342-fold from human erythrocytes in a yield of 29% with 50.75 U/mg. The purification procedure involved the preparation of hemolysate, ammonium sulfate precipitation, 2′,5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel filtration chromatography. Purified enzyme was used in the *in vitro* studies, and rat erythrocyte hemolysate was used in the *in vivo* studies. In the *in vitro* studies, I_{50} and K_i values were 12.179 mM and 6.5123 ± 4.1139 mM for cefotaxime, and 1.682 mM and 0.7446 ± 0.2216 mM for cefodizime, respectively, showing the inhibition effects on the purified enzyme. Inhibition types were noncompetitive for cefotaxime and competitive for cefodizime. In the *in vivo* studies, 300 mg/kg cefotaxime and 1000 mg/kg cefodizime when administered to rats inhibited enzyme activity during the first 2 h ($p < 0.01$). Cefotaxime led to increased enzyme activity at 4 h ($p < 0.05$), but neither cefotaxime nor cefodizime had any significant inhibition or activation effects over 6 h ($p > 0.05$).

Keywords: *Glutathione reductase, glutathione, drug, erythrocyte, inhibition*

Introduction

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 diseases [1,2]. The undesirable biological effects of these highly reactive molecules are eliminated by enzymatic and non-enzymatic antioxidant defence systems. Enzymatic defence is provided by many enzyme systems such as superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, glutathione reductase, aldoketoreductases and DNA repair enzymes. Non-enzymatic antioxidant defence systems include many different agents, such as transferrin, ceruloplasmin, lactoferrin, vitamins (e.g., A, E and C), uric acid, taurine, glutathione (GSH), cysteamine and cysteine. The GSH and GSH-related enzymes responsible for glutathione metabolism make up one of the most important protective systems in cells [3].

Glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7; GR) plays a central role in glutathione metabolism. This flavin enzyme is essential for reducing glutathione disulfide (GSSG) to the tripeptide form. GSH not only counteracts oxidative agents but also protects the sulfhydryl groups of intracellular proteins in the erythrocytes. The GSH concentration needs to be about 2 mM to keep red blood cells alive and not being hemolysed [4]. On the other hand, a high GSSG concentration inhibits a number of important enzyme systems including protein synthesis [5]. Decreased GSH levels have also been reported in several diseases, such as acquired immune deficiency syndrome (AIDS) [6], adult respiratory distress syndrome [7], Parkinson's disease [8], and diabetes [9].

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Many drugs have activation or inhibition effects on the body's enzyme systems [10,11,12]. Many drugs may show the same effects under both *in vivo* and *in vitro* conditions; however, some of them may not show the same effects on enzymes [13,14].

We have not encountered any studies on the effects of streptomycin sulfate, gentamicin sulfate, thiamphenicol, penicillin G, teicoplanin, ampicillin, cefotaxime, or cefodizime on human erythrocyte GR activity. The objective of this study was to investigate any possible *in vitro* effects of these antibiotics on purified human erythrocyte GR, and the *in vivo* effects of cefotaxime and cefodizime, which behave as inhibitors, on rat erythrocyte GR.

Materials and methods

Materials

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

Preparation of the hemolysate

Fresh human blood from one single subject was collected into a tube containing 0.1 M Na-citrate, 0.16 M glucose, 0.016 M Na-phosphate, and 2.59 mM adenine for anticoagulation. The sample was centrifuged at 3000 g for 15 min and the plasma and leukocyte coat were removed. The erythrocytes were washed three times with isotonic NaCl solution including 1 mM EDTA, the samples being centrifuged at 3000 g each time and the supernatants removed. The washed erythrocytes were hemolyzed with 5 volumes of ice-cold distilled water containing 2.7 mM EDTA and 0.7 mM β-mercaptoethanol, and then centrifuged at 4°C, 20,000 g for 30 min to remove residual intact cells and membranes [15].

Ammonium sulfate fractionation and dialysis

In order to determine the optimal precipitation of the enzyme, the hemolysate was brought to 0–10%, 10–20%, 20–30%, 30–40%, 40–50%, 50–60% and 60–70% with solid $(NH_4)_2SO_4$. Ammonium sulfate was added slowly to the hemolysate, followed by stirring until it dissolved completely. The mixture was centrifuged at 5000 g for 15 min. The precipitate was dissolved in 50 mM of phosphate buffer including 1 mM EDTA (pH 7.0). For each precipitation step, the enzyme activity was assayed in both supernatant and precipitate. The enzyme was observed to precipitate at 30–70% saturation. Precipitates in the range (30–70%) were collected and dialyzed at 4°C in the same buffer for 2 h with two changes of buffer [12].

2′,5′-ADP sepharose 4B affinity chromatography

Dry 2′,5′-ADP Sepharose 4B (2 g) was washed and swelled in distilled and deionized water. After removal of the air in the gel, it was resuspended in 0.1 M K-acetate/0.1 M K-phosphate buffer, pH 6.0, and packed into a small column (1 × 10 cm). After precipitation of the gel, it was equilibrated with the same buffer by means of a peristaltic pump with the flow rate adjusted to 20 ml/h. The dialyzed sample obtained from ammonium sulfate precipitation was loaded onto the column, and washed with 25 ml of 0.1 M K-acetate +0.1 M K-phosphate, pH 6 and 25 ml of 0.1 M K-acetate +0.1 M K-phosphate, pH 7.85. Washing continued with 50 mM of K-phosphate including 1 mM EDTA (pH 7.0) until the final absorbance difference was 0.05 at 280 nm. Proteins bound on the gel were eluted with a gradient of 0–0.5 mM GSH and 0–1 mM NADPH in 50 mM K-phosphate, containing 1 mM EDTA (pH 7.0). Active fractions were collected and dialyzed with 50 mM of K-phosphate including 1 mM EDTA (pH 7.0) at 4°C [16,17,18,19].

Sephadex G-200 gel filtration chromatography

Dry Sephadex G-200 (5 g) was incubated in distilled water at 90°C for 5 h. After removal of the air in the gel, it was loaded onto a column (2 × 50 cm). The flow rate was adjusted to 15 ml/h by means of a peristaltic pump. The column was equilibrated with 50 mM Tris-HCl, and 50 mM KCl buffer (pH 7.0) until the final absorbance difference became zero at 280 nm and the pH was the same as that of the equilibration buffer. The dialyzate from the affinity chromatography column was mixed with glycerol in a proportion of 5%. The final sample was loaded onto the column and 2 ml of each elution was collected. The absorbance values were determined at 280 nm and 340 nm in each fraction. Active fractions were lyophilized and stored at − 85°C for use in the *in vitro* studies.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the enzyme's purity, SDS-PAGE was performed according to Laemmli's method [20]. The acrylamide concentration of the stacking and separating gels was 3% and 8%, respectively, and 1% SDS was also added to the gel solution. The gel was stained for 2 h in 0.1% Coomassie Brilliant Blue R-250 containing 50% methanol, 10% acetic acid and 40% distilled water. Then the gel was washed with many changes of the same solvent without dye. Cleared protein bands were photographed (Figure 1).

Activity assay

Glutathione reductase activity was determined by the method of Carlberg and Mannervik [21] with a

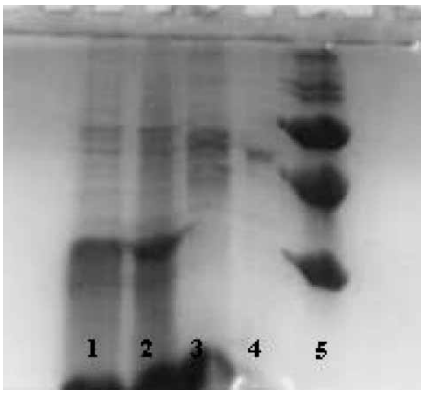

Figure 1. SDS-PAGE photograph: Lane 1, hemolysate. Lane 2, ammonium sulfate precipitate. Lane 3, sample obtained from 2′,5′-ADP Sepharose 4B affinity column. Lane 4, purified enzyme from Sephadex G-200 gel filtration. Lane 5, standard proteins: rabbit myosin (205 kDa), *E.coli* β-galactosidase (116 kDa), rabbit phosphorylase B (97.4 kDa), bovine albumin (66 kDa), chicken ovalbumin (45 kDa), and bovine carbonic anhydrase (29 kDa).

Shimadzu Spectrophotometer UV-(1208) at 25°C. The assay system contained 40 mM Tris-HCl buffer, pH 8.0, including 0.8 mM EDTA, 1 mM GSSG and 0.1 mM NADPH in 1 ml total reaction volume. The activity was measured by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH at 25°C. One enzyme unit is defined as the oxidation of 1 μmol NADPH per min under the assay conditions.

Protein determination

Quantitative protein determination was performed spectrophotometrically at 595 nm according to Bradford's method [22], using bovine serum albumin as a standard. Qualitative protein determination was also performed spectrophotometrically at 280 nm according to Segel's method [23].

In vitro *drug studies*

Streptomycin sulfate, gentamicin sulfate, thiamphenicol, penicillin G, teicoplanin, ampicillin, cefotaxime, and cefodizime were tested to determine their effects on human erythrocyte GR activity. Assays were performed in cuvette concentrations of 0.69–20.5 mM streptomycin sulfate; 0.42–4.20 mM gentamicin sulfate; 2.1–21.05 mM thiamphenicol; 1.03–16.45 mM penicillin G; 7.22–43.32 mM teicoplanin; 1.43–85.84 mM ampicillin; 0.8–26.2 mM cefotaxime; and 1.07–2.68 mM cefodizime. The cuvette GR activity in the absence of drug was taken as 100%. For each drug, an Activity%-[Drug] graph was drawn (Figures 2 and 3) and drug concentrations producing 50% inhibition (I_{50}) were graphically calculated for cefotaxime and cefodizime. In the media with or without inhibitor, five substrate (GSSG) concentrations (6.25–50 mM for cefotaxime, and 1.075–18 mM for cefodizime) were used. Three different fixed concentrations of inhibitor solution selected from Activity%-[Drug] graphs were added to the reaction medium in a total reaction volume of 1 ml for each fixed inhibitor concentration. Regression analysis provided the equations to be used for drawing the Lineweaver-Burk graphs (Figures 4 and 5 and Table II) using 1/V and 1/[S] values. The K_i values were graphically calculated for these drugs.

In vivo *drug studies*

Twelve male Sprague-Dawley rats (200 ± 20 g) were used in the *in vivo* studies for cefotaxime and cefodizime. The animals were housed individually and fed with standard laboratory chow and water before the experiment. The animal rooms were windowless with automatic temperature (22 ± 1°C) and lighting controls (14 h light/10 h dark cycles). For the control cuvettes, 0.5 ml blood samples were taken from a tail vein before the drug was administered and put into tubes containing anticoagulant. Then 300 mg/kg cefotaxime was i.m. administered to the first group, and 0.5 ml blood samples were taken again from a tail vein 2, 4 and 6 h after the drug was administered. In the second group, 1000 mg/kg cefodizime was i.m. injected; 2, 4, and 6 h after drug administration, 0.5 ml blood samples were taken again from a tail vein. All blood samples were put into tubes with anticoagulant. Hemolysate was prepared as described in *in vitro* studies. Activity measurement and other *in vivo* studies were also carried out at 25°C. The data obtained were analyzed by Student's *t*-test, and the results are given as means ± standard deviation.

Results

Human erythrocyte GR was purified 5,342-fold with a yield of 29% using 2′,5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel filtration chromatography after ammonium sulfate precipitation of the hemolysate (Table I). The purity of the enzyme was determined by SDS-PAGE and a single band was observed on the gel after the final chromatographic step (Figure 1). Our *in vitro* studies showed that streptomycin sulfate, gentamicin sulfate, thiamphenicol, and penicillin G activated human erythrocyte GR activity, whereas cefotaxime and cefodizime inhibited enzyme activity. I_{50} and K_i values were 12.179 mM and 6.5123 ± 4.1139 mM for cefotaxime, and 1.682 mM and 0.7446 ± 0.2216 mM for cefodizime, respectively. Inhibition types were determined to be non-competitive for cefotaxime and competitive for cefodizime (Table II).

We used only cefotaxime and cefodizime in our *in vivo* studies. The results showed that cefotaxime and cefodizime significantly inhibited GR activity to the extent of 46% and 21%, respectively, during the

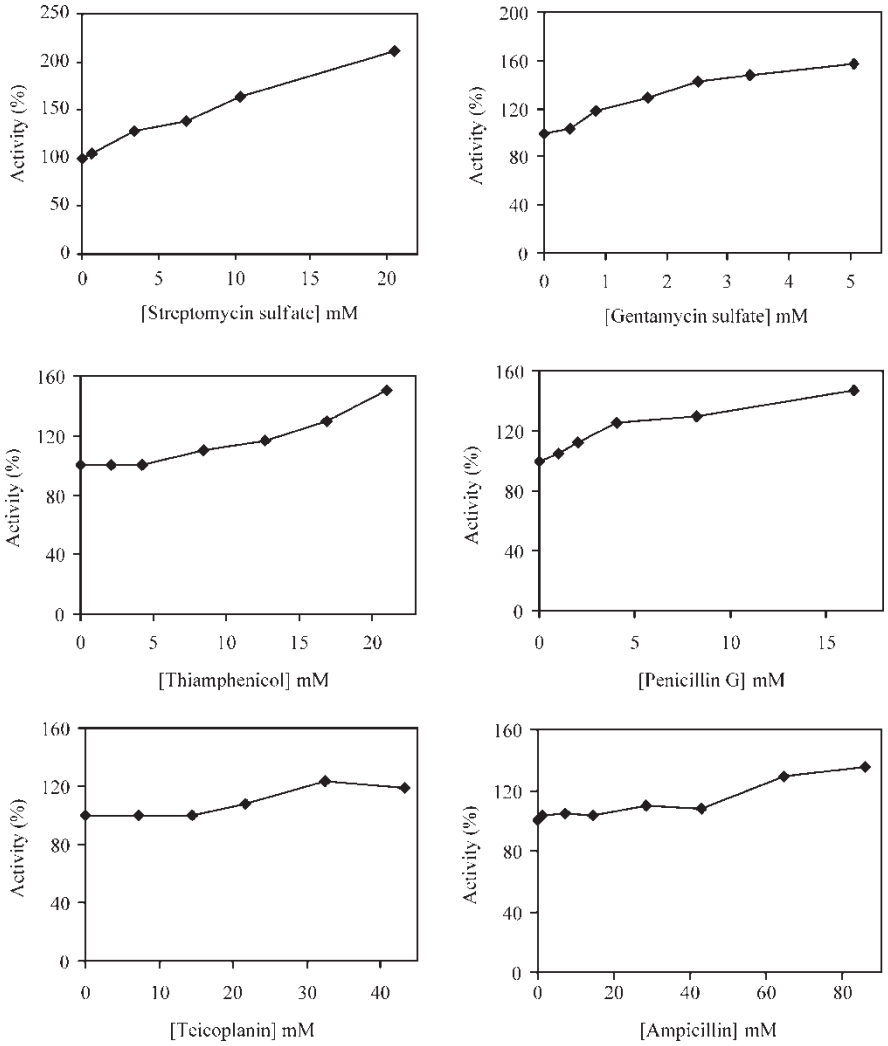

Figure 2. Dose dependency effect of streptomycin sulfate, gentamycin sulfate, thiamphenicol, penicillin G, teicoplanin, and ampicillin on the activity of GR.

first 2 h following administration ($p < 0.01$; Tables III and IV). After 4 h and 6 h, the inhibition effect of cefotaxime not only declined to zero, but also activity increased according to the control to the extent of 1.1% after 4 h ($p < 0.05$) and 0.3% after 6 h ($p > 0.05$) (Table III). The inhibition effect of cefodizime decreased as well, to the extent of 0.9% after 4 h ($p < 0.05$) and 0.3% after 6 h ($p < 0.05$), in comparison with the control (Table IV).

Discussion

GR is essential for the maintenance of cellular glutathione in its reduced form, which is highly

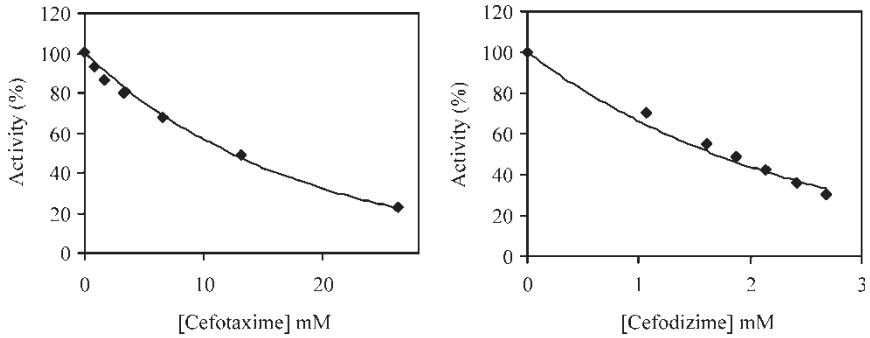

Figure 3. Dose dependency effect of cefotaxime and cefodizime on the activity of GR.

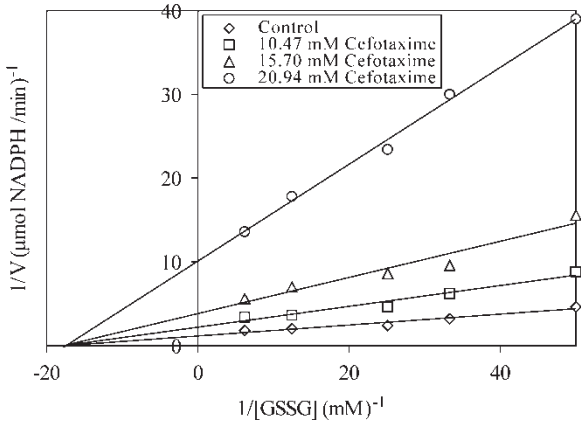

Figure 4. Lineweaver-Burk plot for 5 different substrate (GSSG) concentrations and 3 different cefotaxime concentrations for the determination of K_i.

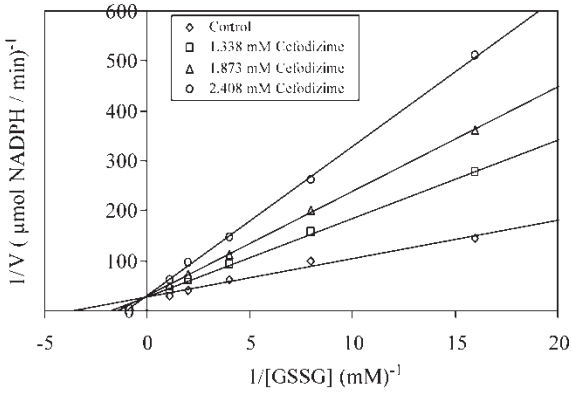

Figure 5. Lineweaver-Burk plot for 5 different substrate (GSSG) concentrations and 3 different cefodizime concentrations for the determination of K_i.

nucleophilic for many reactive electrophils [24]. GSH is involved either as a substrate in the cytosolic GSH redox cycle, or is able to directly inactivate free radicals and reactive oxygen species (ROS), which are known to be effective stress agents [25].

Many chemicals and drugs are known to have adverse or beneficial effects on human enzyme and metabolic events and the effects can be dramatic and systemic [26]. The inhibition of some important enzymes plays a key role in a metabolic pathway. e.g. some metabolic diseases such as diabetes mellitus are affected by enzyme activity [27]. Similarly, acetazolamide has an inhibitory effect on the carbonic anhydrase (CA) enzyme leading to diuresis [28]. Additionally, epiandrosterone was found to inhibit red blood cell glucose 6-phosphate dehydrogenase (G6PD) uncompetitively and suppress hexose monophosphate shunt activity by more than 95% [29]. Some chemicals and drugs, such as nitrofurazone, nitrofurantoin, 5-nitroindol, 5-nitro-2-furoic acid, 2,4,6-trinitrobenzene sulfonate (TNBS) [30] and polyphenolic compounds, also inhibit GR enzyme activity [31]. Furthermore, it is reported that human erythrocyte CA-I and CA-II are inhibited by ampicillin; [13] human erythrocyte G6PD by netilmicin, gentamicin, streptomycin, ampicillin [32], metamizol and magnesium sulfate; [33] and bovine erythrocyte GR by cefotaxime and cefodizime [12].

Many drugs are commonly used in the therapy of human diseases but we have not encountered any investigations of their inhibition or activation effects on GR enzyme in the literature. In the present paper, the effects of some commonly used antibiotics on human erythrocyte GR enzyme were investigated, and I_{50} and K_i values were estimated for the drugs showing inhibition effects.

Table I. Purification scheme for glutathione reductase from human erythrocytes.

Purification step	Total volume (ml)	Activity (EU/ml)	Protein (mg/ml)	Total activity (mg)	Total protein (EU)	Specific activity (EU/mg)	Yield (%)	Purification (fold)
Hemolysate	50	0.208	22.008	10.4	1100.4	0.0095	100.00	1.00
Ammonium sulfate precipitation (30–70%)	15	0.545	25.975	8.175	389.63	0.0210	78.61	2.21
2′,5′-ADP Sepharose 4B affinity chromatography	8.5	0.505	0.050	4.293	0.43	10.1	41.28	1,063
Sephadex G-200 gel filtration chromatography	15	0.203	0.004	3.045	0.06	50.75	29.28	5,342

Table II. I_{50}, K_i values and inhibition types for cefotaxime and cefodizime towards human erythrocyte GR.

Inhibitors	I_{50} values (mM)	[I] (mM)	K_i values (mM)	Mean K_i values (mM)	Inhibition types
Cefotaxime	12.179	10.470	10.7607	6.5123 ± 4.1139	Noncompetitive
		15.700	6.2287		
		20.940	2.5476		
Cefodizime	1.682	1.338	0.5401	0.7446 ± 0.2216	Competitive
		1.873	0.7137		
		2.408	0.9801		

Table III. Effect of cefotaxime on rat erythrocyte GR activity *in vivo*.

Time (h)	Activity (EU/gHb) (Mean ± Standard Deviation, n=6)	*t*	*p*
Control	4.686 ± 0.477	–	–
2	2.522 ± 0.601	6.524	<0.01
4	5.198 ± 0.228	−2.976	<0.05
6	4.819 ± 0.678	−0.393	>0.05

Table IV. Effect of cefodizime on rat erythrocyte GR activity *in vivo*.

Time (h)	Activity (EU/gHb) (Mean ± Standard Deviation, n=6)	*t*	*p*
Control	7.650 ± 0.251	–	–
2	6.028 ± 0.223	14.776	<0.01
4	6.966 ± 0.587	2.409	>0.05
6	7.424 ± 0.317	1.679	>0.05

Streptomycin sulfate, gentamicin sulfate, and other aminoglycoside drugs contain one or more sugar molecules and the sugar moieties have one or more amino groups. The side effects of these antibiotics are ototoxicity and nephrotoxicity. The high concentration of drug that accumulates in the fluids of these tissues probably plays a role [34]. Here, it was observed that streptomycin sulfate and gentamicin sulfate increased human erythrocyte GR enzyme activity by therapentically relative drug concentrations. In the same way, thiamphenicol and penicillin G also caused increases in activity, but teicoplanin and ampicillin did not exhibit effective inhibition or activation effects on the enzyme (Figure 2).

Cefotaxime and cefodizime are third generation cephalosporins, and are commonly used in the treatment of many infection diseases. In the present study, it was seen that human erythrocyte GR was efficiently inhibited by both cefotaxime and cefodizime (Figure 3). For these drugs, the determined I_{50} and K_i values showed that cefodizime was a more effective inhibitor than cefotaxime. In addition, inhibition types were determined to be noncompetitive for cefotaxime and competitive for cefodizime (Figures 4 and 5, Table II).

These two drugs showed an inhibitory effect in human purified enzyme and when injected in rats intramuscularly the enzyme was effectively inhibited within the first 2 h by both cefotaxime and cefodizime. Over 4 h, the inhibitory effects of cefotaxime declined completely, and its significant activating effect appeared, whereas the inhibitory effect of cefodizime was only decreased completely at both 4 h and 6 h (Tables III and IV).

Hence, the use of cefotaxime and cefodizime is undesirable for the enzyme. Therefore, we think that if these drugs are given to humans, their dosages should be very carefully controlled in order to prevent side effects on red blood cell GR activity.

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