Effects of some antibiotics on glutathione reductase from bovine erythrocytes

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ABSTRACT: This paper describes effects of some antibiotics on glutathione reductase *in vitro*. Glutathione reductase was purified from bovine erythrocytes. Purification procedure consisted of four steps; preparation of hemolysate, ammonium sulphate precipitation, 2′,5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel filtration chromatography. As a result of these four consecutive procedures, the enzyme having the specific activity of 62.5 EU/mg proteins was purified 31 250-fold with a yield of 11.39%. The purified enzyme showed a single band on sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE). The effects of eight different antibiotics-streptomycin sulphate, gentamicin sulphate, netilmicin, teicoplanine, thiamphenicol, ampicillin, cefotaxime, and cefodizime- were investigated on the purified enzyme. Six of these antibiotics (streptomycin sulphate, gentamicin sulphate, netilmicin, teicoplanine, thiamphenicol, and ampicillin) increased the activity of glutathione reductase with increase in their concentrations while the two (cefotaxime and cefodizime) inhibited the enzyme activity. I_{50} values were 7.713 mM and 1.954 mM, and K_i constants were 11.011 mM and 8.956 mM for cefotaxime and cefodizime, respectively. Their inhibition types were competitive.

Keywords: glutathione reductase; bovine erythrocyte; antibiotic

Glutathione reductase (Glutathione:$NADP^+$ oxidoreductase, EC.1.8.1.7; GR) is the key enzyme of glutathione metabolism and is widespread in all tissues and blood cells. This enzyme catalyses reduction of oxidized glutathione (GSSG) to glutathione (GSH) in the presence of NADPH and maintains a high intracellular GSH/GSSG ratio of about 500 in red blood cells (Kondo *et al*., 1980). GSH is the most abundant low molecular weight thiol in almost all cells and is involved in a wide range of enzymatic reactions (Meister, 1981). Functions of GSH in reductive processes are essential for protein synthesis, regulation of enzyme, and protection of the cells against reactive oxygen species and free radicals generated in cellular metabolism (Meister, 1988). On the other hand, GSSG inhibits a number of important enzyme systems including protein synthesis (Deneke and Fanburg, 1989). Since GSH has an important role in detoxification of xenobiotics, such as carcinogens, toxins and drugs, inhibition of GR may lead to low concentration of GSH and concomitantly a high concentration of exogenous compounds in the tissues, resulting in severe pathological conditions, such as hemolytic anemia, diabetes and neurologic disorders (Beutler, 1984; Jacobasch and Rappoport, 1996; Knapen *et al*., 1999; Gul *et al*., 2000).

Most of the drugs affect the enzyme systems as an activator or inhibitor (Edward and Morse, 1988; Jacobasch and Rappoport, 1996; Çiftçi *et al*., 2002). Many drugs exhibit the same effects both *in vivo* and *in vitro*, but some of them may not show the same effects on enzymes (Beydemir *et al*., 2000).

We are not aware of any reports on the effects of streptomycin sulphate, gentamicin sulphate, ampicillin, netilmicin, cefotaxime sodium, cefodizime sodium, teicoplanine, and thiamphenicol on the activity of GR from bovine erythrocyte. Therefore, objective of this study was to investigate any possible *in vitro* effects of some commonly used antibiotics on the bovine erythrocyte GR activity.

MATERIAL AND METHODS

Material

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

Preparation of the hemolysate

Bovine blood souples were obtained from local slaughter house and collected in tube with anticoagulant (EDTA). The blood samples were centrifuged at 3 000×g for 15 min and the plasma was removed. The erythrocytes were washed three times with isotonic NaCl solution containing 1 mM EDTA. The washed erythrocytes were hemolysed with 5 volumes of ice-cold distilled water containing 2.7 mM EDTA and 0.7 mM β-mercaptoethanol. The hemolysate was centrifuged at 20 000×g for 30 min and the ghosts and intact cells were removed (Beutler, 1984). The hemolysate was subjected to ammonium sulphate precipitation about 30–70% saturation with solid $(NH_4)_2SO_4$. The precipitate was dissolved in a small amount of 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.3 and then dialyzed at 4°C in the same buffer (Acan and Tezcan, 1989).

Affinity chromatography on 2′,5′-ADP Sepharose 4B

2 g dried 2′,5′-ADP Sepharose 4B was used for a small column (10 × 1 cm). 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 column. After precipitation of the gel, it was equilibrated with 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.3 by means of a peristaltic pump. The flow rate was adjusted to 20 ml/h. The dialyzed sample obtained from ammonium sulphate precipitation was loaded onto the column, and washed with 25 ml 0.1 M K-acetate + 0.1 M K-phosphate, pH 6 and 25 ml 0.1 M K-acetate + 0.1 M K-phosphate, pH 7.85. Then the washing was continued with 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.3 until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted with a gradient of 0 to 0.5 mM GSH and 0 to 1 mM NADPH in 50 mM K-phosphate, containing 1 mM EDTA (pH 7.3). Active fractions were collected and dialyzed with equilibration buffer at 4°C (Boggaram *et al.*, 1979; Carlberg and Mannervik, 1981; Acan and Tezcan, 1989).

Gel filtration chromatography on Sephadex G-200

5 g dried Sephadex G-200 was used for a 165 lm bed volume. The gel was incubated in the distilled water at 90°C for 5 hours. After removal of the air in the gel, it was loaded onto a column (2 × 50 cm). Flow rate was adjusted to 15 ml/h by means of peristaltic pump. The column was equilibrated with 50 mM Tris-HCl, 50 mM KCl buffer, pH 7.3 until the final absorbance difference became zero at 280 nm and pH value became same with that of equilibration buffer. The dialyzed from affinity chromatography column was mixed with glycerol at the rate of 5%. The final sample was loaded onto the column and elutions were collected as 2.5 ml in tubes. In each fraction activity values were determined at 340 nm. Active fractions were lyophilized and stored at –85°C for measuring the enzyme purity by the electrophoresis and the antibiotic effects on its activity.

Activity determination

Glutathione reductase activity was determined spectrophotometrically with a Shimadzu Spectrophotometer UV-(1208), at 25°C, according to the method of Carlberg and Mannervik (Carlberg and Mannervik, 1985). The assay system contained 435 mM K-phosphate buffer pH 7.3, including 1 mM EDTA, 1 mM GSSG and 0.1 mM NADPH. One enzyme unit is defined as the oxidation of 1 μmol NADPH per min under the assay condition.

Protein determination

Protein concentration was determined at 595 nm according to the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard.

Sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE)

To check the enzyme purity, SDS-PAGE was performed by Laemmli's method (Laemmli, 1970). The acrylamide concentration of the stacking and the separating gels were 3% and 8%, respectively, and 1% SDS was also added to the gel solution. Gel was stained for two hours in 0.1% Coomassie Brillant Blue R-250 containing 50% methanol, 10% acetic acid and 40% distilled water. The gel was washed with several changes of the same solvent without dye. Cleared protein bands were photographed (Figure 1).

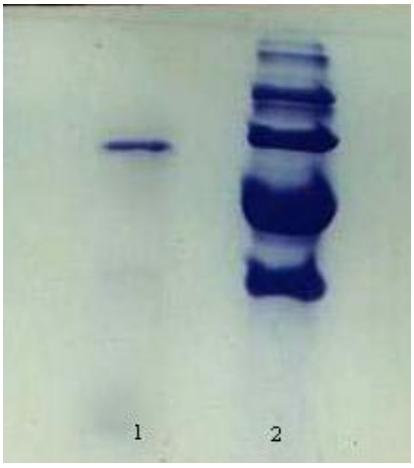

Figure 1. SDS-PAGE photograph of purified bovine erythrocyte GR. Lane 1: Bovine erythrocyte GR (118 000); Lane 2: Standard proteins: rabbit myosin (205 000 Da), *E. coli* β-galactosidase (116 000 Da), rabbit phosporilase B (97 400 Da), bovine albumin (66 000 Da), chicken ovalbumin (45 000 Da), and bovine carbonic anhydrase (29 000 Da)

In vitro drug studies

Different concentration of various antibiotics were used to determine their effects on GR activity. The final concentrations are given in Table 1. Control cuvette activity in the absence of drug was taken as 100%. For each antibiotic an Activity-[Drug] graph was drawn. For cefotaxime and cefodizime showing inhibition effect on the enzyme the drug concentrations that produce 50% inhibition (I_{50}) were calculated from these graphs (Figure 2, Table 2).

In order to determine K_i constants, inhibitor solutions were added to the reaction medium, resulting in three different concentrations of inhibitors in 1 ml total reaction volume. For these three inhibitor concentrations, the values were obtained by using five different substrate concentrations. To draw Lineweaver-Burk graphs by using 1/V and 1/[S] values were used to draw graphs for each fixed inhibitor concentration. K_i values and inhibition types were estimated from these graphs (Figures 3 and 4, Table 2). Analysis of data was by *t*-test and the results are given as mean $\bar{x}$± SD.

RESULTS AND DISCUSSION

Living cells are subjected to exposure to various foreign chemicals such as drugs, food additives and pollutants. These chemicals are called xenobiotics and are usually electrophilic compounds (Murray *et al.*, 1996). The metabolism of xenobiotics usually involves two distinct stages, commonly referred to phases I and II. Phase I metabolism involves an initial oxidation, reduction or dealkylation of the xenobiotic

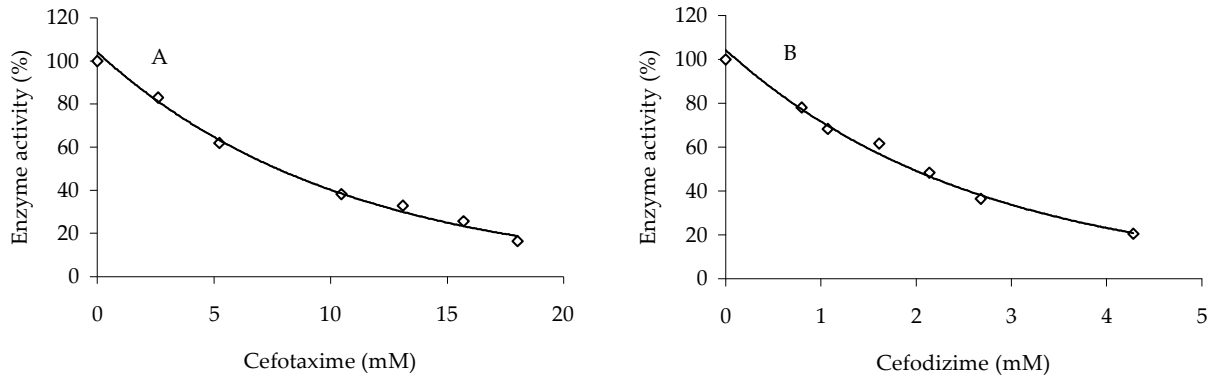

Figure 2. Effects of different concentrations of cefotaxime and cefodizime on GR activity

Table 1. GR activity at different drug concentrations. Drugless activity was accepted as 100%

Drug	Concentration (mM)	Activity (mean ± SD) in %	Drug	Concentration (mM)	Activity (mean ± SD) in %
Streptomycin sulphate	0.00	100 ± 0.00	Teicoplanin	0.00	100 ± 0.00
	0.69	103 ± 1.53^{-}		7.22	100 ± 1.07–
	3.43	119 ± 1.53*		14.4	102 ± 0.52+
	6.86	141 ± 2.08*		21.6	108 ± 1.82+
	10.4	154 ± 0.58*		32.5	122 ± 1.35*
	20.5	162 ± 1.00*		43.3	123 ± 0.50*
	30.8	171 ± 1.16*			
Gentamicin sulphate	0.00	100 ± 0.00	Ampicillin	0.00	100 ± 0.00
	0.42	103 ± 1.92^{-}		1.43	104± 1.74^{+}
	0.84	116 ± 1.95^{+}		7.16	105 ± 1.32^{+}
	1.68	127 ± 1.87*		14.3	107 ± 0.88*
	2.52	142 ± 1.89*		28.6	115 ± 1.77*
	3.36	147 ± 1.70*		42.9	118 ± 0.76*
	5.04	156 ± 3.46*		64.9	131 ± 2.81*
	6.72	172 ± 2.33*			
Netilmicin	0.00	100 ± 0.00	Cefotaxime	0.00	100 ± 0.00
	0.17	115 ± 1.62*		2.62	83.1 ± 0.98*
	0.34	126 ± 1.70*		5.24	61.9 ± 1.13*
	0.51	135 ± 2.38*		10.5	38.3 ± 0.87*
	0.68.	154 ± 2.92*		13.1	33.1 ± 0.92*
	1.02	157 ± 1.04*		15.7	25.5 ± 1.76*
	1.36	168 ± 1.35*		18.0	16.4 ± 1.22*
Thiamphenicol	0.00	100 ± 0.00	Cefodizime	0.00	100 ± 0.00
	2.10	103 ± 0.67^{+}		0.80	77.9 ± 1.87*
	4.21	109 ± 1.20*		1.07	68.4 ± 2.53*
	8.42	113 ± 1.97*		1.61	64.6 ± 1.16*
	12.6	125 ± 1.26*		2.14	48.3 ± 1.27*
	16.8	131 ± 1.64*		2.68	36.3 ± 1.20*
	25.3	149 ± 1.28*		4.28	20.51 ± 1.85*
	33.7	161 ± 1.96*			

$n = 3$, $^{*}P < 0.01$, $^{+}P < 0.05$, $^{-}P > 0.05$

Table 2. I_{50} values, K_i constants, and inhibition types of cefotaxime and cefodizime for bovine erythrocyte GR

Inhibitors	I_{50} values (mM)	I (mM)	K_i constants (mM)	Mean K_i constants (mM)	Inhibition types
Cefotaxime	7.713	0.79	15.9	11.0 ± 6.05	competitive
		1.05	12.9		
		1.31	4.24		
Cefodizime	1.954	0.80	11.3	8.96 ± 2.34	competitive
		1.07	8.95		
		3.21	6.62		

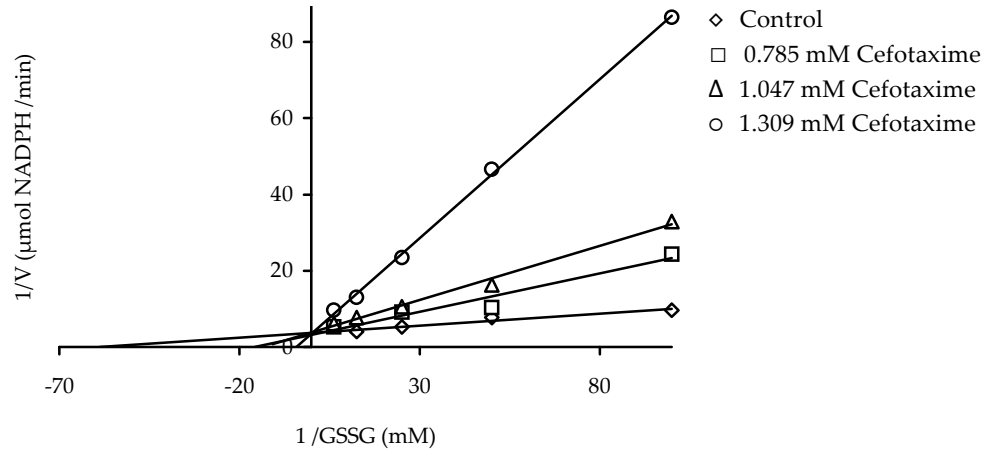

Figure 3. Lineweaver-Burk graph in 5 different substrate (GSSG) concentrations and in 3 different cefotaxime concentrations for determination of K_i constant for cefotaxime

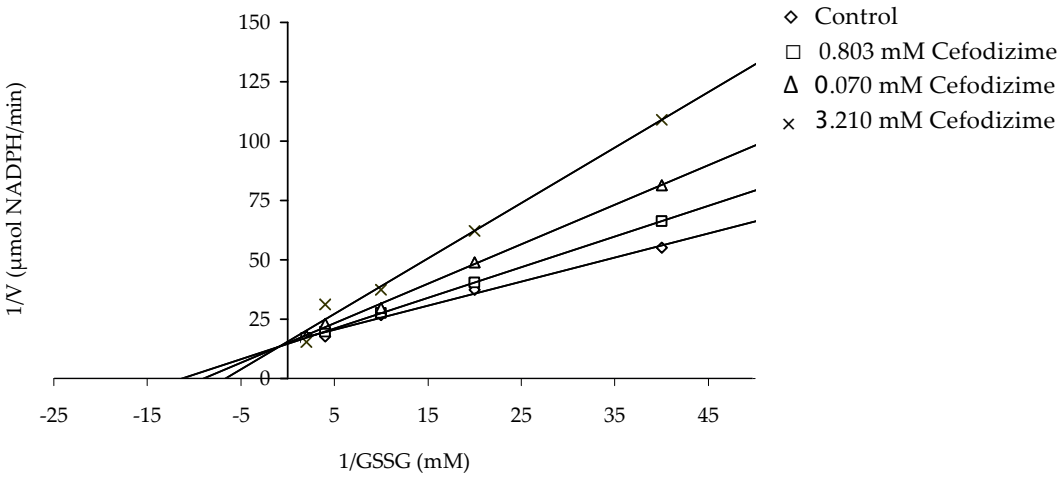

Figure 4. Lineweaver-Burk graph in 5 different substrate (GSSG) concentrations and in 3 different cefodizime concentrations for determination of K_i constant for cefodizime

by cytochrome P-450 monooxigenases (Guengerich, 1991). This step is often needed to provide a molecule with hydroxyl- or amino- groups, which are essential for phase II reactions. Phase II metabolism generally adds hydrophilic moieties, thereby making a toxin more water soluble and less biologically active. Frequently involved phase II conjugation reactions are catalyzed by glutathione S-transferases (GSTs) (Beckett and Hayes, 1993), sulfotransferases (Falany, 1991) and UDP-glucuronyl- transferases (Bock, 1991). The GSTs catalyze the addition of GSH to a wide variety of exogenous compounds. GR, a flavo enzyme, is essential for the maintenance of cellular glutathione in its reduced form, which is high nucleophilic potent for many reactive electrophils (Carlberg and Mannervik, 1975).

Many chemicals and drugs affect the metabolism of living organisms by altering normal enzyme activity, particularly inhibition of a specific enzyme (Hochster *et al.*, 1973). The effects can be dramatic and systemic (Christensen *et al.*, 1982). Some chemicals and drugs inhibit GR enzyme activity, such as nitrofurazone, nitrofurantione, 5-nitroindol, 5-nitro-2-furoic acid, 2,4,6-trinitrobenzene sulphonate (TNBS) (McCallum and Barrett, 1995) and some polyphenolic compounds (Zihang *et al.*, 1997). Similarly, it is reported that human erythrocyte carbonic anhydrase I and II are inhibited by ampicillin (Beydemir *et al.*, 2000), human erythrocyte G6PD by netilmicin, gentamicin, streptomycin and ampicillin (Çiftçi *et al.*, 2000), and sheep liver erythrocyte G6PD by penicillin, sulbactam,

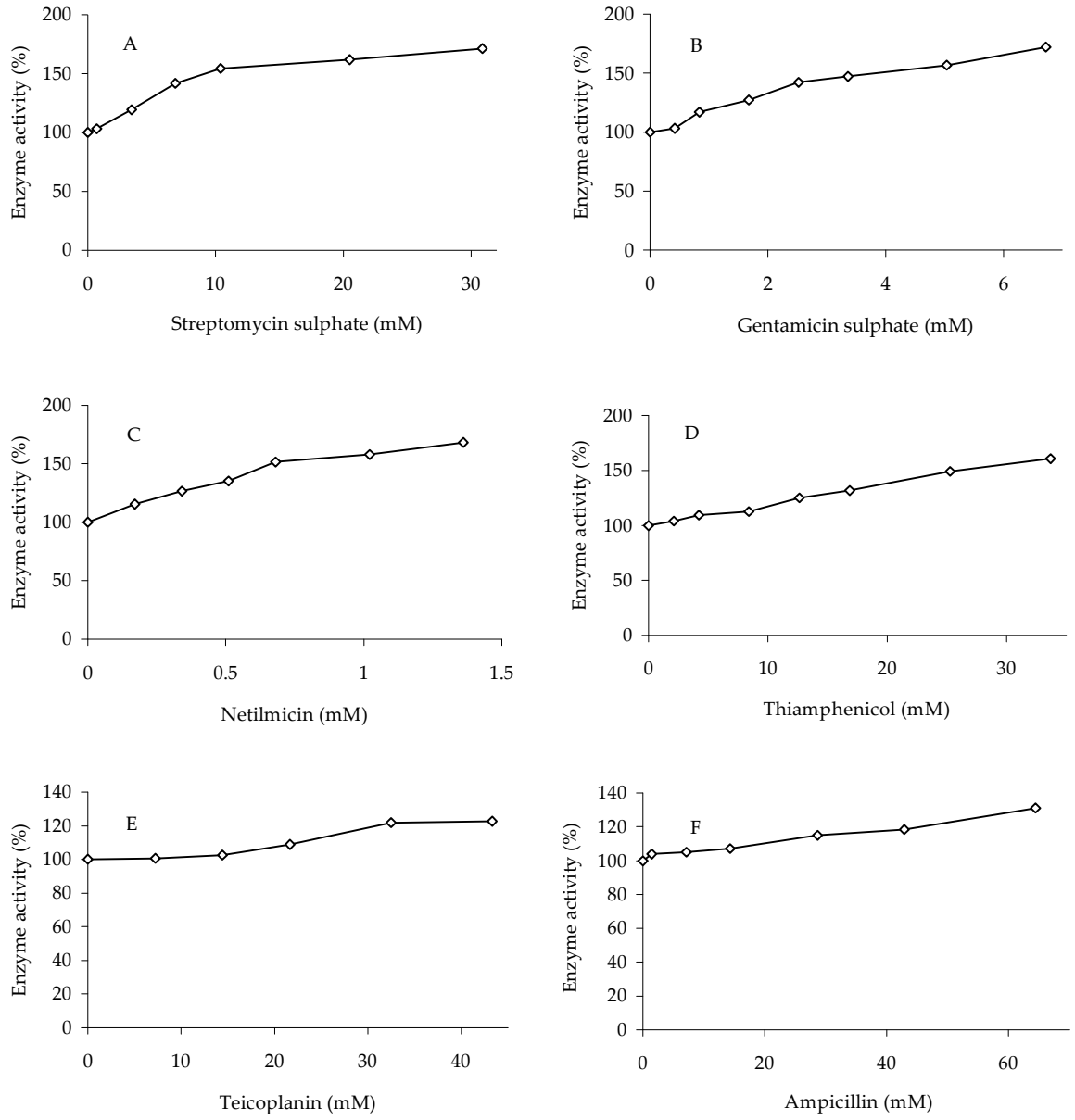

Figure 5. Effects of different concentrations of six antibiotics on GR activity

cefazolin and amikacin (Çiftçi *et al*., 2002). A lot of drugs are commonly used for the therapy of animal diseases. To our knowledge, inhibition or activation GR enzyme has not been investigated. Hence the effects of some commonly used antibiotics on bovine erythrocyte GR enzyme were investigated and I_{50} and K_i values estimated for the drugs that showed inhibition effect.

Bovine erythrocyte GR was purified 31 250-fold with a yield of 11.39% by using 2′,5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel filtration chromatography after ammonium sulfate precipitation of the hemolysate. Purity of the enzyme was tested by SDS-PAGE and single band was observed on the gel after final chromatographic step (Figure 1). Then effects of eight antibiotics (streptomycin sulphate, gentamicin sulphate, netilmicin, thiamphenicol, teicoplanin and ampicillin cefotaxime, and cefodizime) were investigated on the activity of purified enzyme.

Gentamicin sulphate, streptomycin sulphate and netilmicin, amino glycoside drugs, contain one or more sugar molecule and the sugar moieties have one or more amino group. The sugar moieties are joined to each other or to other moieties by glycosidic links. The amino glycosides are very soluble

in water and they have activity against both gram-positive and gram-negative organisms. The side effects of these antibiotics are ototoxicity and nephrotoxicity (Smith and Reynard, 1992). As shown in Figure 5, enzyme activity was increased with increased concentration of six antibiotics, streptomycin sulphate, gentamicin sulphate, netilmicin, teicoplanin, and ampicillin. Therefore, we think that these antibiotics may be used in an animal disease with GR deficiency.

Cephalosporins are generally toxic but not nephrotoxic. However, this toxicity may be tolerable. Hepatic enzymes may be reversibly and transiently elevated during therapy, but true hepatoxicity is rare (Kayaalp, 1998). Cefotaxime and cefodizime are third generation cephalosporins, and they are commonly used for the treatment of a lot of infection diseases. In this study, it was seen that bovine erythrocyte GR was inhibited by cefotaxime and cefodizime. In addition, estimated I_{50} and K_i values show that cefodizime is the more effective inhibitor than cefotaxime for the enzyme. Besides, inhibition types of two antibiotics were determined to be competitive (Figures 3 and 4, Table 2). Hence, the use of cafotaxime and cefodizime should be minimum and if given to bovine, their dosages should be very well controlled in order to prevent their adverse effects on GR activity in red blood cells.

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