**In Vitro Effects of Some Drugs on Glutathione Reductase Enzyme Purified from Sheep Spleen Tissue**

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| **Abstract**  In this study, the effects of dexketoprofen, meloxicam, phenyramidol HCl and hyoscine-N-butylbromide drugs on the enzyme Glutathione reductase (EC 1.8.1.7; GR, Glutathione: NADP+ oxidoreductase), which is purified from sheep spleen tissue and which is the most important enzyme of antioxidant metabolism, was investigated. Firstly, GR enzyme was purified from sheep spleen tissue by ammonium sulfate precipitation between 20-70% and 2', 5' ADP-Sepharose 4B gel affinity chromatography with 44.61% yield and 1476,6 fold. The purity of the enzyme was checked by the SDS-PAGE method. In the next step, the effects of dexketoprofen, meloxicam, phenyramidol HCl and hyoscine-N-butylbromide on the enzyme activity were investigated. Activity%-[I] and Lineweaver-Burk graphs were drawn to find the IC50 value and Ki constant for hyoscine-N-butylbromide, which showed an inhibitory effect. The IC50 value of the drug hyoscine-N-butylbromide was calculated as 31,36 mM. In addition, the Ki constant of this drug was 21.21±4.2 mM and the inhibition type was determined non-competitive. It was determined that dexketoprofen, meloxicam, and phenyramidol HCl drugs did not have a significant activation or inhibition effect on the enzyme. |
| Keywords: Glutathione reductase, buscopan, inhibition |

1. **Introduction**

Glutathione reductase (E.C. 1.8.1.7; GR), called NADP+ oxidoreductase, is a very important enzyme that functions in the intracellular defense system by catalyzing the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) [1]. The GR enzyme keeps the GSH/GSSG ratio, which is vital for the cell, at a certain level. In studies, this ratio is approximately 500/1 for erythrocyte cells, and a decrease in this ratio causes hemolysis [2] Since the GSH molecule contains a large amount of -SH group in its structure, it has a protective antioxidant effect especially against free radicals. Free radicals that arise due to some factors such as harmful habits such as smoking, alcohol, and adequate and unbalanced nutrition that disrupt the biochemical order in the cell, especially the superoxide radical, are broken down into water and oxygen by the -SH group of the GSH molecule. Thus, metabolic order is provided in the cell [3, 4].

In order for the GR enzyme to react, it needs nicotinamide adenine dinucleotide phosphate (NADPH), which is supplied from the pentose phosphate pathway and functions in reducing biosynthesis events. The production of NADPH is produced by the enzyme glucose 6-phosphate dehydrogenase (G6PD), the first enzyme of the pentose phosphate pathway, in the presence of nicotinamide adenine dinucleotide (NAD+). The G6PD enzyme is an enzyme that synthesizes ribose phosphates, which are components of vital biomolecules such as DNA, FAD, RNA, ATP, CoA and NAD, along with the production of NADPH. The need for NADPH in the reaction catalyzed by the GR enzyme also reveals the relationship between G6PD and GR. Therefore, the inactivity of the G6PD enzyme causes a decrease in NADPH production. This situation also negatively affects the GR enzyme, and as a result, the formation of GSH may also be negatively affected [5, 6]. As a result, the reaction in which GSSG is converted to GSH with the use of NADPH to protect the cell against various types of oxidative stress is catalyzed by GR [7].

Numerous inhibition studies have been carried out on enzymes that function at key points in cell metabolism, such as the GR enzyme. Inhibitors are defined as substances that reduce or completely eliminate the activity of the enzyme. Many drugs and chemicals used for human, animal and plant health perform their functions by affecting the activities of enzymes and proteins in metabolism [5, 6, 8]. The inhibition effect of these chemicals on regulatory enzymes at important points of metabolism may allow the elimination of a disorder in that region. From this point of view, enzymes constitute an important field in pharmacological studies [9]. It is known that the GR enzyme has been purified from many living tissues, its kinetic properties have been determined, and the effects of many drugs and chemical substances on enzyme activity have been investigated so far [10-12]. However, in the literature review, studies investigating the effects of **deksketoprofen,** meloxicam, phenyramidol HCl and hyoscine-N-butylbromide drugs related to the sheep spleen GR enzyme were not found.

Among these drugs, dexketoprofen is a drug with analgesic, anti-inflammatory and antipyretic effects, included in the anti-inflammatory drug group. It is widely used in the symptomatic treatment of mild to moderate pain such as musculoskeletal pain, toothache, postoperative pain. In addition, it binds to the plasma membrane at high rates (95% 98%) and has analgesic, antipyretic and anti-inflammatory effects. Meloxicam is a member of the enolic group of non-steroidal anti-inflammatory drugs. It has strong analgesic, antipyretic and ant rheumatic activity. Phenyramidol HCl is used as a pain reliever and muscle relax. Hyoscine-N-butylbromide is an anticholinergic drug that has both spasm-relieving and pain-relieving effects, and also helps to relax the digestive system. It is effective in relieving sudden and severe spasms in the muscles of the stomach, intestines, urinary bladder, urinary tract and bile ducts [13]. In this study, it was aimed to examine the effects of these drugs on GR activity.

1. **Materials and Methods**

**2.1. Materials**

The drugs used in the study were obtained from the pharmacy. 2′, 5′-ADP-Sepharose 4B, NADPH, electrophoresis chemicals, GSSG, hydrochloric acid, sodium chloride, glycine, Tris, electrophoresis chemicals, protein standards, ammonium sulfate and other chemicals were obtained from Sigma-Aldrich Com. (St. Louis, MO) and Merck (Darmstadt, Germany).

**2.2. Preparation of Homogenate**

The sheep spleen tissue used in this study was obtained from the Combined Meat and Milk Institution of Bingöl according to the cold chain rules. 15 grams of tissue brought to the laboratory was taken and divided into small pieces and suspended in 45 mL of 50 mM KH2PO4 (pH= 8.0) buffer. After the obtained suspension was centrifuged for 1 hour at 10,000xg and the precipitate was discarded and homogenate was formed [8, 10].

**2.3. Ammonium Sulphate Precipitation and Dialysis**

For the homogenate obtained from sheep spleen tissue, partial precipitation was performed with ammonium sulfate salt, which has a highly soluble feature in water, at 20-70% saturation concentrations. The obtained salty homogenates were centrifuged at 10,000xg for 15 minutes each time and the supernatant part was removed. The remaining precipitate was treated until dissolved with 50 mM KH2PO4 (pH= 8.0) buffer and activity control for the enzyme was performed. Then, the enzyme solution was placed in dialysis bags and dialyzed with dialysis buffer (10 mM K-phosphate, 1 mM EDTA) adjusted to pH= 7.5 for 2 hours [8, 10, 14].

**2.4. Enzyme Purification by 2', 5' ADP Sepharose-4B Affinity Chromatography**

2', 5' ADP Sepharose-4B affinity chromatography was used to purify the enzyme sample, which was partially purified by ammonium sulfate precipitation. First, an affinity column was prepared. For a bed volume of 10 mL; The 2', 5'-ADP sepharose 4B gel was weighed 2 g dry. Afterwards, this gel was washed several times with 400 mL of distilled water to remove solids from the gel, while the gel was swollen. The air caused by swelling was removed by vacuuming using a water trumpet and the gel was suspended by adding equilibration buffer (50 mM KH2PO4/1 mM EDTA, 1mM DTT, pH: 6.0). The prepared gel was packed into a cooled column and waited for the gel to precipitate. The equilibration of the column was understood from the approximate equalization of the absorbance and pH of the eluate and buffer at 280 nm. 16 mL enzyme sample was applied to the prepared column. Then the column was washed with three separate wash solutions. 25 mL of each of the prepared washing solutions was used in turn. The column was first washed with 0.1 M K-acetate/0.1 M K-phosphate (pH=6) buffer. Then 0.1 M K-phosphate/0.1 M KCl (pH=7.85) buffer was used for washing and finally washed with 50 mM KH2PO4/1 mM EDTA (pH= 7) buffer. The washing process was followed in the spectrophotometer and the absorbance values ​​were determined to be approximately equal to the blank. After washing, the enzyme was eluted with elution buffer (50 mM KH2PO4/1 mM EDTA, 1mM GSH and 0.5 mM NADPH (pH= 7.3) [8, 10, 15-17]. Elutions were taken into 1.5 mL Eppendorf tubes and their activity values ​​were checked. The enzyme samples we obtained were checked for purity by SDS-PAGE method.

**2.5. Enzyme Activity Measurement**

The activity of glutathione reductase enzyme was measured spectrophotometrically at 340 nm. This situation was determined by the decreasing amount of NADPH due to the oxidation of NADPH in the presence of GSSG [17].

**2.6. Protein Determination**

Quantitative protein amount was determined spectrophotometrically at 595 nm according to the Bradford method. Bovine serum albumin protein was used to draw the standard graph [18].

**2.7. Enzyme Purity Control with SDS-PAGE**

Purity control of the enzyme was carried out using 3-8% batch sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method [19].

**2.7. Kinetic Studies**

The effects of deksketoprofen, meloxicam, phenyramidol HCl and hyoscine-N-butylbromide on enzyme activity were investigated in kinetic studies. Different concentrations of these drugs were taken and added to the cuvette medium. As a result of the activity measurements, experiments were made up to the highest possible inhibitor concentration, and the IC50 value was calculated by plotting the % Activity-[I] graph for hyoscine-N-butylbromide, which showed an inhibitory effect. In addition, in order to determine the Ki constant and inhibition type of this drug, preliminary experiments were carried out and five different substrate concentrations were determined, and measurements were made for each substrate concentration with three different inhibitor concentrations and Lineweaver-Burk plots were drawn [20].

**3. Results and Discussion**

**3.1. Enzyme Purification**

In this study, homogenate, ammonium sulfate precipitate and pure enzyme were purified 1476.6 times with a protein specific activity of 15.80 EU/mg and a yield of 44.61% from the standard graph used for the quantitative determination of proteins by Bradford method, and the results are shown in Table 1.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Purification steps | Total volume  (mL) | Activity  (EU/mL) | Protein  (mg/mL) | Total  protein  (mg) | Total  activity | Specific  activity  (EU/mg protein) | Yield% | Purification fold |
| Homogenate | 25 | 0,581 | 52,59 | 1315 | 14,525 | 0,0110 | 100 | 1 |
| Ammonium sulfate precipitation  (20-70%) | 16 | 0,697 | 64,57 | 1033 | 11,152 | 0,0107 | 76,78 | 0,972 |
| 2',5'-ADP Sepharose-4B affinity chromatography | 10 | 0,648 | 0,041 | 0,41 | 6,48 | 15,80 | 44,61 | 1476,6 |

**Table 1.** Sheep spleen tissue GR enzyme purification steps

**3.2. SDS-PAGE**

SDS-PAGE was performed to determine the enzyme purity. The single band obtained on the gel is shown in Figure 1.

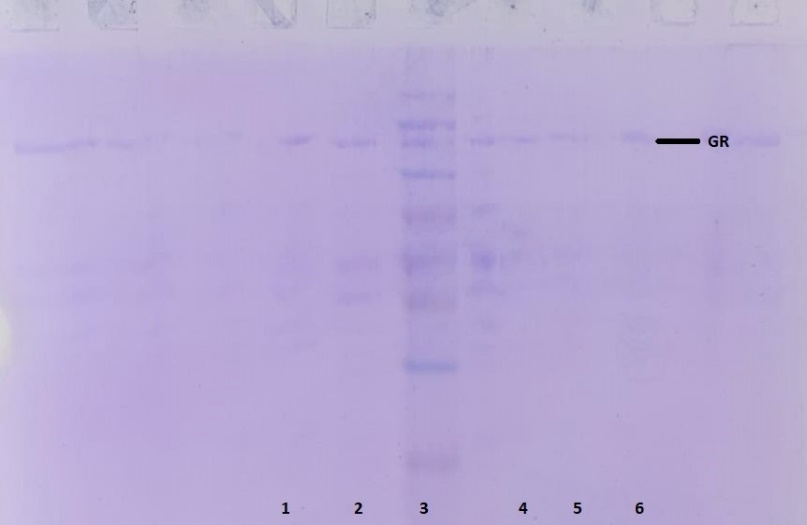


Figure 1. Lanes 4,5 and 6: Pure GR enzyme eluted from the affinity column. Lane 3: standard proteins (14kDa - 175 kDa).

**3.3. Kinetic Studies**

In kinetic studies, the effects of deksketoprofen, meloxicam, phenyramidol HCl and hyoscine-N-butylbromide drugs on GR enzyme activity at different concentrations were investigated. Among these drugs, deksketoprofen at 0.33, 0.66, 1.32, 1.98 and 2.64 mM concentrations, meloxicam at 0.028, 0.056 and 0.084 mM concentrations, phenyramidol HCl at 0.93, 1.86 and 2.79 mM concentrations and hyoscine-N-butylbromide concentrations of 0.227, 1.362, 2.27, 4.54, 9.08, 18.16, 24.97, 26.33, and 27.24 mM were used. The % Activity-[I] graph was drawn for hyoscine-N-butylbromide, which showed an inhibitory effect, and the IC50 value was calculated as 23.79 mM using the graph equation (Figure 2). In addition, Linevawer-Burk plot was drawn for the GR enzyme at 3 different inhibitors and 5 different substrate concentrations, and the Ki constant was determined as 21.21±4.2 mM and the inhibition type was non-competitive (Figure 3). It was determined that deksketoprofen, meloxicam and phenyramidol HCl drugs did not have a significant activation or inhibition effect on the enzyme.

**Figure 2.** Effect of hyoscine-N-butylbromide on sheep spleen GR enzyme

**Figure 3.** Lineweaver-Burk plot plotted for sheep spleen GR enzyme at 5 different substrates and 3 different hyoscine-N-butylbromide concentrations.

**3.4. Discussion**

The GR enzyme, which is involved in the intracellular defense system, belongs to the oxidoreductase enzyme group and catalyzes the conversion of GSSG to GSH. It is very important to keep the GSH/GSSG ratio at a certain level in metabolism. For example, this ratio is 500/1 in erythrocytes, and a decrease in this value leads to hemolysis [2]. In a study on antioxidants, the enzymes of antioxidant systems such as GR and G6PD decreased as a result of exposure of erythrocytes to oxidative stress for various reasons. The increase in malondialdehyde (MDA), an indicator of lipid peroxidation, determined this situation [21]. Radicals formed as a result of oxidative stress in the cell are eliminated by the regeneration and increase of the GSH molecule, which is the most important antioxidant system, and intracellular stress tolerance is provided [22].

In this study, the effects of deksketoprofen, meloxicam, phenyramidol HCl and hyoscine-N-butylbromide drugs on the enzyme were investigated by purifying the GR enzyme from sheep spleen tissue. In the purification of the enzyme, 20-70% ammonium sulfate precipitation was made in the homogenate prepared first [10]. Ammonium sulfate precipitation is important in terms of removing impurities from the enzyme sample and thus making the proteins more concentrated [23]. The enzyme sample obtained from the ammonium sulfate precipitation was applied to the 2', 5'-ADP Sepharose 4B affinity column and the enzyme with a specific activity of 15.80 EU/mg.protein was purified 1476.6-fold with a yield of 44.61%. In another purification performed on the sheep spleen GR enzyme, the enzyme was purified with a yield of 40.61% and 1564.8 times [10]. In addition, in another study, GR enzyme was purified 1654 times from rainbow trout liver with 2', 5'-ADP Sepharose-4B affinity chromatography with 41% yield [24]. In another study, GR enzyme was purified 1028 times from quail erythrocytes with 46.2% yield [12].

In the second phase of our study, the effects of deksketoprofen, meloxicam, phenyramidol HCl and hiyoscine-N-butylbromide drugs, which are widely used in human and animal health, on the enzyme were investigated. When the study results were analyzed, it was observed that deksketoprofen, meloxicam and phenyramidol HCl did not have any activation or inhibition effects on enzyme activity. Hyoscine-N-butylbromide on the other hand, was found to have an inhibitory effect on the enzyme with an IC50 value of 23.79 mM. In addition, the Ki constant and inhibition type were determined in the 5 most suitable substrates, which were determined by the preliminary experiments, of this drug, which showed an inhibitory effect, first in the medium without inhibitor, and then at 3 different fixed drug concentrations. According to this; Ki constant was 21.21±4.2 mM, inhibition type was determined without non-competition.

In the literature research, the GR enzyme was characterized by being purified from different tissues with the help of different chromatographic techniques, and it was determined that the effects of some drugs and organic compounds on the enzyme activity were investigated. In an inhibition study conducted by purifying the GR enzyme from human erythrocyte cells, the drugs diclofenac sodium, tenoxicam, etomidate, ketoprofen, lornoxicam, morphine and propofol were investigated on the enzyme. As a result, it was determined that propofol showed non-competitive inhibition, while the others showed competitive inhibition [25] (Şentürk et al., 2009).

In another study, it was determined that the GR enzyme purified from rat heart and lung tissues was inhibited by cefazolin, ceftazidime, cefuroxime furosemide, gentamicin, levofloxacin, methylprednisol, and teicoplanin [26]. In another study on sheep spleen tissue, the effects of some antibiotics and anti-inflammatory drugs on GR enzyme activity were investigated. It has been observed that ampicillin and gentamicin are non-competitive, while streptomycin sulfate, cefoperazone sodium and precort-lyo drugs inhibit the enzyme competitively [10]. In addition, the inhibitory effects of gentamicin sulfate, thiamphenicol, oflaxacin, levoflaxacin, cefepime and cefazolin on sheep liver GR enzyme activity were investigated. It has been determined that oflaxacin, levoflaxacin, cefepime and cefazolin antibiotics inhibit the enzyme semi-competitively [27].

In conclusion, inhibitors are of great importance for enzyme-treated approaches. In this study, it was determined that hyoscine-N-butylbromide inhibited the GR enzyme without competition. For this reason, it will be beneficial to be more careful with dose adjustments in the use of hyoscine-N-butylbromide.

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