Araştırma Makalesi / *Research Article* **Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 9(4): 2140-2147, 2019**
Kimya / Chemistry ***Journal of the Institute of Science and Technology,* 9(4): 2140-2147, 2019**

DOI: 10.21597/jist.525154 **ISSN: 2146-0574, eISSN: 2536-4618**

Purification of Glutathione Reductase from Human Erythrocytes: Inhibition Profile of Some Anti-Epileptic Drugs

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ABSTRACT: Glutathione reductase (GR) is found in the NADPH-dependent oxidoreductase family. GR has various important functions in the cell, such as protein and DNA biosynthesis, the detoxification of reactive oxygen species and free radicals. The purpose of this research was to perform the *in vitro* inhibition effects of anti-epileptic drugs (phenytoin, gabapentin, and primidone) on GR enzyme. In the current study, the GR enzyme was purified from human erythrocytes with a specific activity of 20.08 EU/mg protein and 2135.97-purification fold. To determine the inhibition effects of anti-epileptic drugs on GR enzyme, Lineweaver-Burk graphs were drawn for each inhibitor. K_i values and inhibition types were determined from these plotted graphs. The K_i values of drugs were found in ranging from 0.15± 0.03-5.74±1.14 mM. Phenytoin was shown the most effective inhibitor feature with a competitive inhibition type.

Keywords: anti-epileptic drugs, enzyme inhibition, enzyme purification, glutathione reductase

Glutatyon Redüktaz Enziminin İnsan Eritrositlerinden Saflaştırılması: Bazı Anti-epileptik ilaçların İnhibisyon Profili

ÖZET: Glutatyon redüktaz (GR), NADPH'ye bağlı oksidoredüktaz ailesinde bulunur. GR hücrede protein ve DNA biyosentezi, reaktif oksijen türlerinin ve serbest radikallerin detoksifikasyonu gibi çeşitli önemli fonksiyonlara sahiptir. Bu çalışmanın amacı, GR enzimi üzerine antiepileptik ilaçların (fenintoin, gabapentin ve pirimidon) *in vitro* etkilerini belirlemektir. Bu çalışmada, GR enzimi insan eritrositlerinden 20.08 EU/mg protein spesifik aktivite ve 2135.97 kat saflaştırıldı. Anti-epileptik ilaçların GR enzimi üzerindeki inhibisyon etkisini belirlemek için Lineweaver-Burk grafikleri çizildi; K_i sabiti ve inhibisyon tipleri bu çizilen grafiklerden hesaplandı. K_i değerleri 0.15± 0.03-5.74±1.14 mM aralığında bulundu. Fenintoin en etkili inhibitör özelliğini yarışmalı inhibisyon tipi ile göstermiştir.

Anahtar Kelimeler: Anti-epileptik ilaçlar, enzim inhibisyonu, enzim saflaştırılması, glutatyon redüktaz

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Geliş tarihi / *Received:* 10.02.2019
Kabul tarihi / *Accepted:* 15.06.2019

INTRODUCTION

Free radicals are carried many electrons at molecular orbitals. Free radicals such as alkoxyl, hydroxyl, superoxide, peroxyl, nitrogen dioxide, and nitric oxide are derived from oxygen (Ozaslan et al., 2017) These compounds are formed in organisms by the external effects or via metabolic ways and bind irreversibly to DNA and proteins (Ozaslan et al., 2018). They also cause degradation of structural coenzymes, nucleotide, and DNA in tissues and cells (Ceylan et al., 2019; Demir and Köksal, 2019). Also, they can bind covalently to enzymes, proteins, and lipids; break down cell membranes; damage transport systems; change enzyme activities; and cause metabolic diseases (Demir et al., 2017a; 2018a).

Epilepsy is a serious, chronic, and prevalent neurologic disease. Epilepsy treatment requires antiepileptic drugs (Beydemir and Demir 2016). Phenytoin is employed as an anti-arrhythmic and anti-convulsant drug. It is effective for the prevalent forms of epileptic seizures (Farber et al. 2002). Primidone is an anticonvulsant drug. It is used generally in the treatment of psychiatric problem and bipolar disorder (Yamatogi et al., 2004). Gabapentin is a useful to prevent repeated migraine headaches, neuropathic pain, treatment of neuropathic pain in diabetic neuropathy, and nystagmus (Heli et al., 2012).

It is well known that enzymes are crucial bio-catalyzers in the metabolism. Therefore, all substances taken in the body may interact with various enzymes. Especially some enzymes are called drug-target and chemical target (Caglayan et al., 2018; Parham et al., 2018a). Glutathione reductase (GR) is found in the NADPH-dependent oxidoreductase family. The enzyme catalyzes the reduction of glutathione disulfide to the sulfhydryl form glutathione. GR has various important functions of the cell, such as protein and DNA biosynthesis, the detoxification of reactive oxygen species and free radicals (Budak et al., 2014).

In the present study, it was evaluated the impacts of antiepileptic drugs such as phenytoin, gabapentin, and primidone (Figure 1) on human GR enzyme activity.

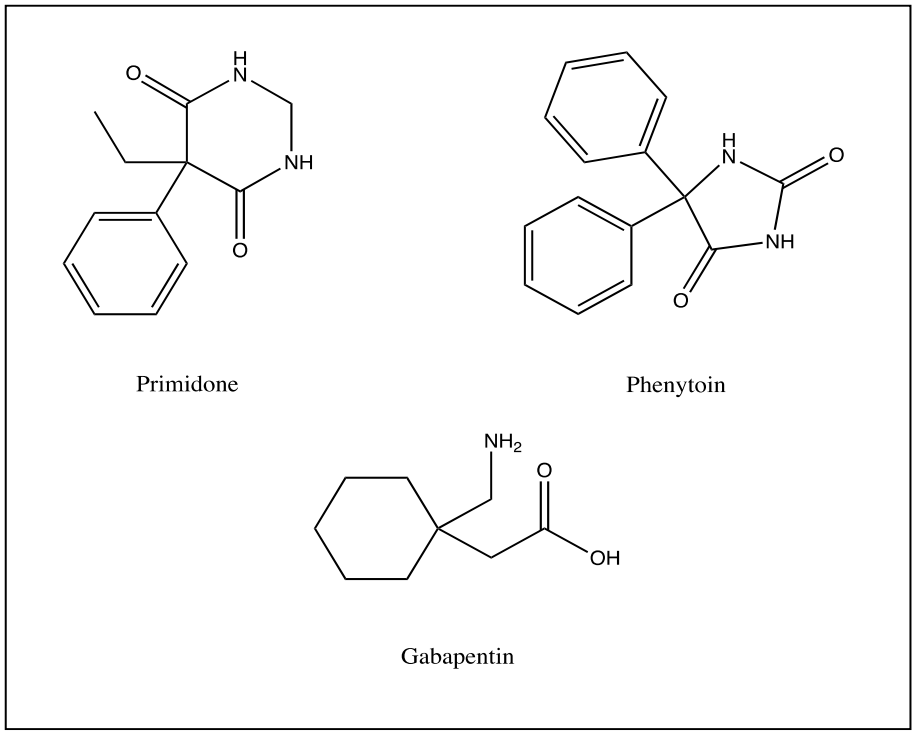

Figure 1. The molecular structure of anti-epileptic drugs used in this study.

MATERIAL AND METHOD

Chemicals

All chemicals were obtained from Sigma Chemical Co. Standard protein markers for electrophoresis were obtained from Thermo Fisher Scientific Company.

Human Erythrocytes Hemolysate Preparation

Fresh blood samples taken into the tubes including EDTA were centrifugated at 1500 *xg* for 15 min. Thus, three phases were obtained as leukocyte, plasma, and erythrocytes. Plasma and leukocyte phases were removed. The erythrocytes were taken, and they were washed with 0.9% NaCl solution three times. Subsequently, pure erythrocytes were shaken by adding 5x ice water throughout 30 minutes. To remove the impurities, the hemolysate was centrifuged at 15.000 *xg* for 30 min, and the supernatant was used for the purification process and activity of the enzyme (Aslan et al., 2018; Turkan et al., 2019).

Activity assay

GR activity was performed by the method of Carlberg and Mannervik (1985).

Purification of GR

Human erythrocytes hemolysate applied to 2'5'-ADP Sepharose-4B affinity column equilibrated with 0.1 M K-phosphate buffer + 0.1 M K-acetate (pH 6.0). The column was washed with 30 ml of 0.1 M K-phosphate buffer + 0.1 M K-acetate (pH 6.0), and 30 ml of 0.1 M K-phosphate buffer+ 0.1 M K-acetate (pH 7.8). Additional washing was carried out 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.0. Elutions were collected in 1 mL aliquots, and enzyme activity was performed at 340 nm (Şentürk et al., 2008).

Protein quantity assay

Quantitative amounts of GR enzyme were determined according to the Bradford (1976) procedure at 595 nm, spectrophotometrically.

SDS-polyacrylamide gel electrophoresis

According to Laemmli's procedure (1970), the presence and purity of GR were observed by the SDS-PAGE technique. The method was performed according to our previous studies (Demir and Beydemir 2015; Demir et al., 2017b; 2018b).

In vitro inhibition studies

The inhibition effects of epilepsy drugs were investigated at least five different inhibitor concentrations on GR enzyme. The K_i values and inhibition types were found by Lineweaver and Burk's curves (1934).

RESULTS AND DISCUSSION

Oxidative stress occurs overproduction of free radicals and oxidants cause the homeostasis in the body and afterward leading to serious imbalance. It arises from different sources of lifestyle or disease state such as chronic hyperglycemia or episodes of ketosis, excessive nutrient intake, and sleep restriction (Türkeş et al., 2019). The imbalance between antioxidants and oxidants impair the lipids, DNA, and proteins which in turn bring about cell death and physiological dysfunction of the cells. Oxidative stress is thought as the most important contributor in the cardiovascular disease, a neurodegenerative disease like Alzheimer and epilepsy (Isık et al., 2015; Demir et al., 2016; Taslimi et al., 2018b).

Enzymatic defense is supplied by a number of enzyme systems such as DNA repair enzymes glutathione reductase, glutathione S-transferase, aldo-ketoreductase, catalase, glutathione peroxidase, and superoxide dismutase(Alım et al., 2019). In the presence of NADPH, GR catalyzes the reduction of oxidized glutathione. Reduced glutathione is used in the protection of the thiol groups of intracellular proteins, detoxification of xenobiotics, counteraction of oxidative events, and scavenging of H_2O_2 and other organic peroxides (Erat et al., 2005).

Metabolic processes in living organisms are closely related to the catalytic activity of enzymes. Changes in enzyme activity can cause various diseases. Thus, compounds which inhibit enzyme activity must be beneficial therapeutic agents (Kırıcı et al., 2016). Hence, it was focused on the *in vitro* inhibition role of some epilepsy drugs (phenytoin, gabapentin, and primidone) on GR enzyme activity in the present study. For this purpose, GR was purified from human erythrocytes using the simple chromatic method. The GR enzyme was purified with a specific activity of 20.08 EU/mg protein and 2135.97-purification fold. (Table 1). SDS-PAGE is generally used determination of molecular weight of enzyme. After purification by affinity chromatography, the enzyme eluate was electrophoretically analyzed by SDS PAGE. According to PAGE results, the molecular weight of GR from human erythrocytes was near approximately 51 kDa (Figure 2).

IC_{50} values of phenytoin, gabapentin, and primidone were found 1.72, 2.06 and 3.09 mM respectively (Figure 3).

Table 1. Summary of the GR purification procedure from human erythrocytes

Purification Steps	Total volume (mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Purification fold	Yield (%)
Hemolysate	20	605.00	5.70	$9.4x10^{-3}$	1	100
2'-5' ADP- Sepharose 4B Affinity Chromatography	4	0.128	2.57	20.08	2135.97	45.09

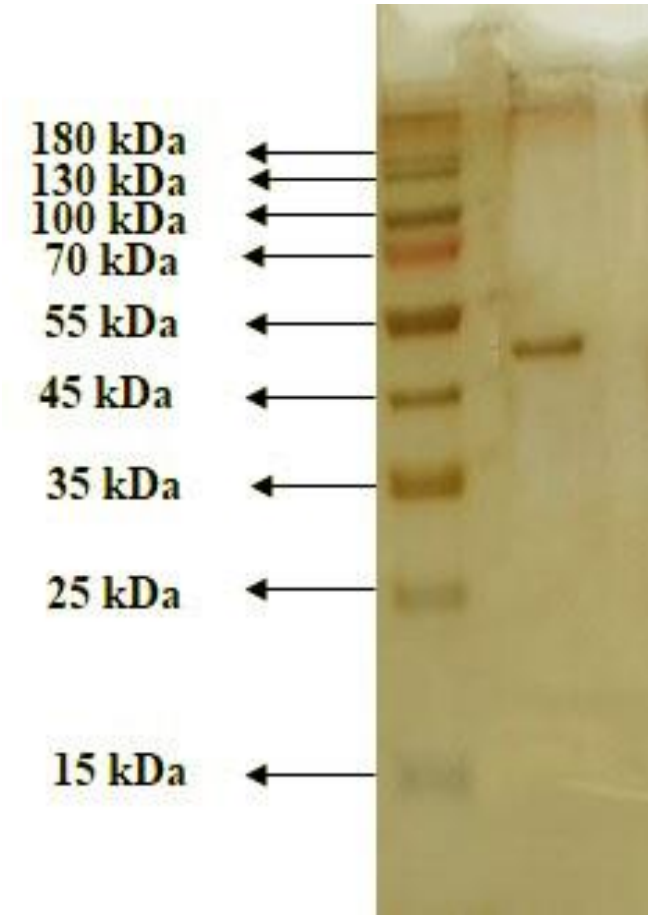

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis for GR enzyme

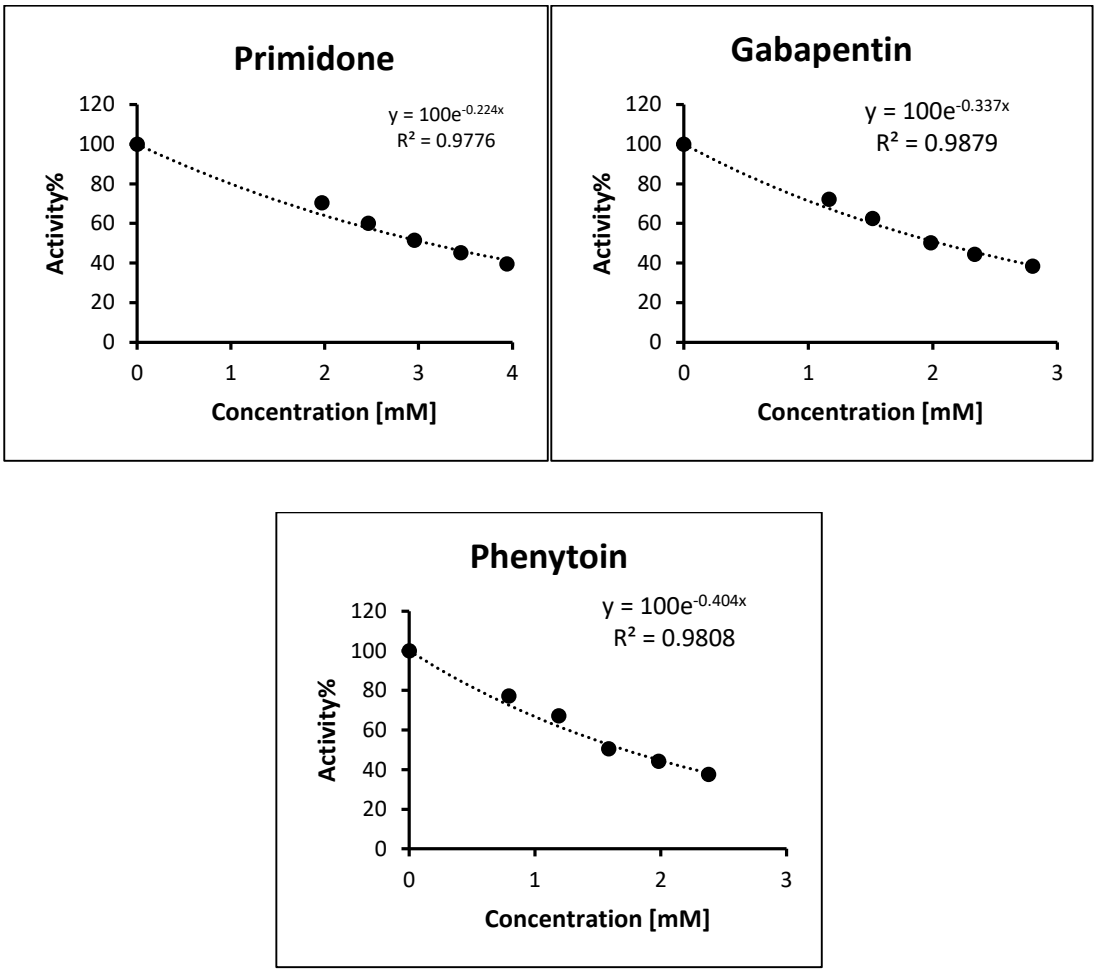

Figure 3. The half maximal inhibitory concentration values (IC_{50}) of drugs for GR

This study showed that K_i values order of compounds showing inhibitory potency was phenytoin (0.15± 0.03 mM) > gabapentin (0.19±0.05 mM) > primidone (5.74±1.14 mM) against purified GR. It is reported that these drugs, which show potential inhibitor properties for GR, reduced the enzyme activity at low concentrations (Table 2).

Table 2. IC_{50}, K_i values and inhibition types of epilepsy drugs on GR

Epilepsy Drugs	IC_{50} (mM)	K_i (mM)	Inhibition type
Phenytoin	1.72	0.15± 0.03	Competitive
Gabapentin	2.06	0.19±0.05	Competitive
Primidone	3.09	5.74±1.14	Noncompetitive

According to our results, phenytoin showed a stronger inhibitory effect against GR while primidone showed the lowest inhibitory effect. It was found that phenytoin and gabapentin showed competitive inhibition effect, primidone showed non-competitive inhibition. It may have an interaction with the amino acids of the active site (Figure 4).

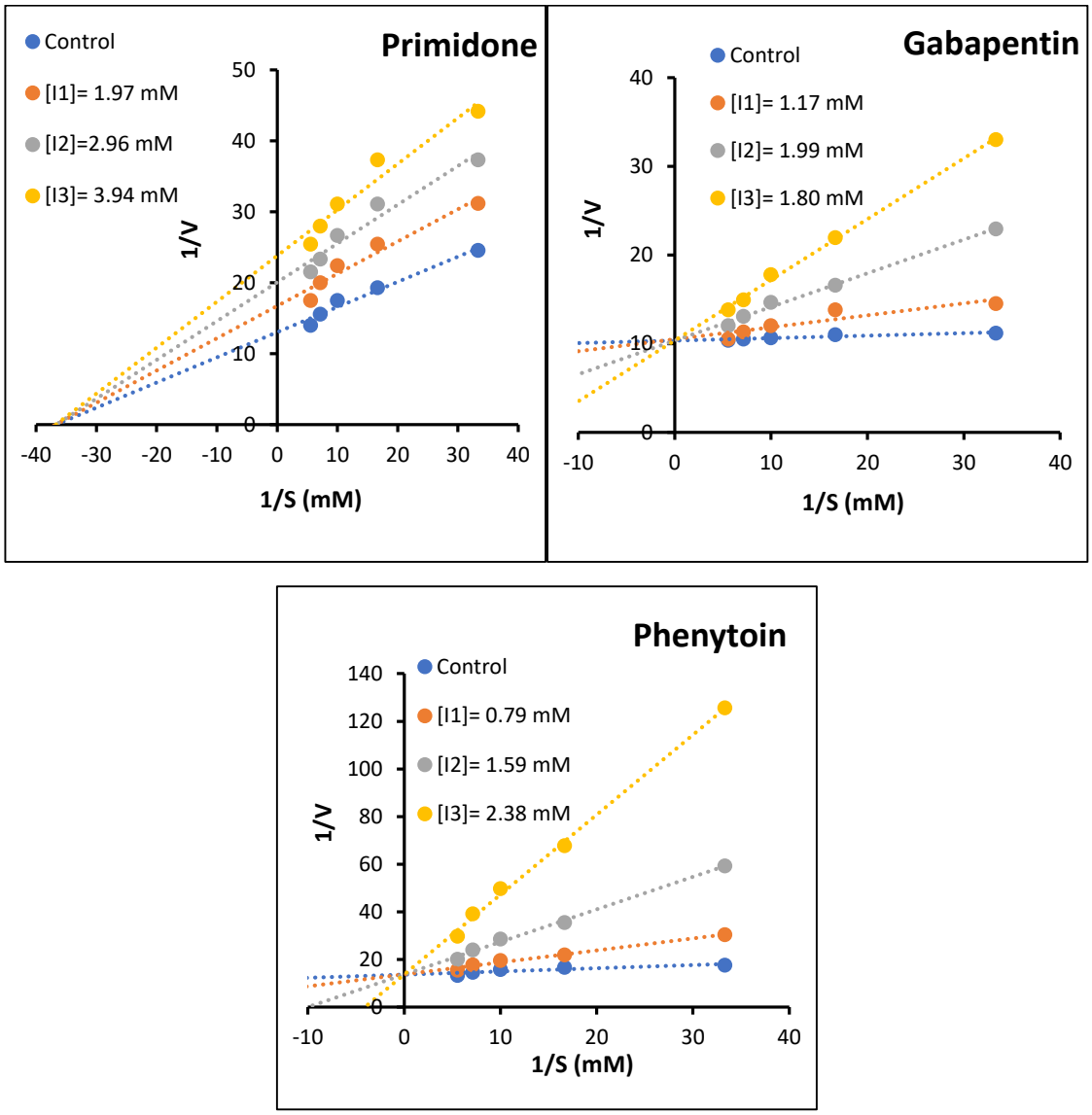

Figure 4. Determination of Lineweaver-Burk graphs for drugs on GR

Some reports are found on GR inhibition by drugs, and various chemicals. For instance, Şentürk *et al.*, (2008) investigated the inhibition of some antibiotics on purified human erythrocyte GR. They found that sulfanylacetamide, rifamycin imipenem, ceftazidime, ceftriaxone, chloramphenicol, vancomycin, ornidazole, and cefuroxime showed inhibitory effects, but lincomycin, amikacin clindamycin, and amoxicillin showed activatory effects on the enzyme. According to this study, imipenem was showed the best inhibition effect. In another study, the inhibition of methotrexate, dacarbazine, pantoprazole sodium, 5-fluorouracil thiocolchicoside, and olanzapine was found on GR. The enzyme is mostly inhibited by dacarbazine (Akkemik et al., 2011). Researchers studied that the impacts of Schiff base derivatives on baker's yeast GR. These compounds were displayed as effective inhibition profiles (Balaydın et al., 2018).

CONCLUSION

In conclusion that GR enzyme was purified from human erythrocytes. After then, *in vitro* inhibition studies of anti-epileptic drugs were performed on this enzyme activity. It can be considered that epilepsy drugs may be effective on GR. The findings from this study make several contributions to the literature. However, further biological studies such as gene expression and *in vivo* experiment should be done for these drugs.

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