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The Effects of Amoxicillin and Vancomycin Hydrochloride Hydrate on Glutathione S-Transferase Enzyme Activity: *An in vitro study*

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ABSTRACT: Glutathione S-transferase (GST) enzymes detoxify a broad spectrum of xenobiotics, including chemotherapeutic drugs, environmental carcinogens, and endogenous molecules. Glutathione transferases catalyze the conversion of some metabolites into less toxic substances. Phase II reactions, which often result in detoxification, are conjugation reactions of glutathione through GST enzymes that protect the organism from the attacks of highly reactive electrophilic substances. Since the GST enzyme can metabolize toxic exogenous compounds, it has been extensively investigated in mammals. In this study, the GST enzyme was purified in human erythrocytes with a specific activity of 5.56 EU mg^{-1} protein and a yield of 2316.0 fold with 65% efficienc. SDS-polyacrylamide gel electrophoresis was performed to check the purity of the purified enzyme and the molecular mass was determined to be about 26 kDa. The IC_{50} and Ki values for amoxycillin and vancomycin hydrochloride drugs on the purified GST enzyme were calculated and the type of inhibition was determined. As a result of the inhibition studies, we observed that these antibiotics inhibited the GST enzyme at low doses.

Keywords: Amoxicillin, vancomycin hydrochloride hydrate, glutathione s-transferase

Glutatyon S-Transferaz Enzim Aktivitesi Üzerine Amoksilin ve Vankomisin Hidroklorid Hidratın Etkisi: Bir in vitro çalışma

ÖZET: Glutatyon S-transferaz enzimleri kemoterapik ilaçlar, çevresel karsinojenler, endojen moleküller olmak üzere ksenobiyotiklerin geniş bir spektrumunu detoksifiye ederler. Glutatyon transferazlar, bazı metabolitleri daha az toksik olan maddelere dönüşmesini katalize eder. Çoğunlukla detoksifikasyonla sonuçlanan faz II reaksiyonları arasında organizmayı son derece reaktif elektrofilik maddelerin ataklarından koruyan GST enzimleri aracılığıyla gerçekleşen glutatyonun konjugasyon reaksiyonlarıdır. GST enzimi toksik ekzojen bileşikleri metabolize edebildiğinden dolayı ayrıntılı biçimde memelilerde araştırılmıştır. Bu çalışmada GST enzimi insan eritrositlerinde 5.56 EU mg^{-1} protein spesifik aktiviteyle ve 65 %verimle 2316.0 kat saflaştırıldı. Saflaştırılan enzimin saflığını kontrol etmek için SDS-poliakrilamid jel elektroforezi yapıldı ve molekül kütlesi yaklaşık 26 kDa olarak belirlendi. Saflaştırılan GST enzimi üzerine inhibisyon etkisi gösteren amoksisilin ve vankomisin hidroklorür için IC_{50} değerleri ve Ki sabitleri hesaplanarak inhibisyon tipleri belirlendi. İnhibisyon çalışmalarının sonucu olarak, bu antibiyotiklerin düşük dozlarda GST enzimini inhibe ettiği gözlendi.

Anahtar Kelimeler: Amoksisilin, vankomisin hidroklorür, glutatyon s- transferaz

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INTRODUCTION

Glutathione S-transferase (EC 2.5.1.18) plays an important role in the elimination of endogenous and exogenous xenobiotics. Since most xenobiotics are lipophilic, they can easily be absorbed. Enzymatic detoxification of xenobiotics occurs in three different phases. Phase I and II involve lipophilic exchange. In phase III, xenobiotics are formed which are more water-soluble and non-polar and more easily removed by cells. The glutathione transferases which are generally easier to remove and catalyze the conversion to less toxic metabolites by providing conjugation of endogenous and exogenous sources of electrophilic and hydrophobic compounds with glutathione are a member of the family of phase II detoxification enzymes (Hayes et al., 2005; Halliwell, 2009; Mazzetti et al., 2015). Glutathione S-transferases that catalyzes the first step in the formation of the water soluble end product mercapturic acid in the detoxification metabolic pathway is a multifunctional enzyme.

Mercapturic acids are latest products into water-soluble. The acids formed in the liver are transferred to the bile from the canaliculus, and thus can be excreted by the bile into the urine. (Stevens and Jones, 1989; Hinchman et al., 1998).

Detoxification mechanisms are as divided into phase 1 and phase 2 reactions. Phase I; It contains the cytochrome P450 enzyme system, which in general acts as the first defense system in the body against foreign components. Phosphorus substrates prepare substrates for phase II conjugation by adding substrate active groups with reactions such as oxidation, reduction, hydrolysis. This system assumes a group of reactions where oxidation is at an important level. (Guengerich, 1990).

In Phase II, conjugation reactions of polar compounds formed as a result of Phase I reactions occur. It is a complex and multifunctional enzyme family that plays a role in Phase II reactions and catalyzes the conjugation of a large part of the electrophiles with glutathione (GSH). At this phase occurs conjugation reactions of polar compounds formed as a result of phase I reactions (Mann, 1996; Hatey et al., 1998; Sheehan et al., 2001). Conjugation reactions are steps that chemical substances combine with endogenous substances in the organism to facilitate their elimination from the body. GSTs is a complex and multifunctional enzyme family which role in phase II reaction and catalyzes the conjugation of a large part of the electrophiles with glutathione (Sheehan et al., 2001).

Antibiotics are naturally occurring that they often act by inhibiting or killing the synthesis of a bacterial cell. Antibiotics have an important role to stimulate digestion in animals, banning bacterial infection and in the beneficial use of nutrients (Zaman et al., 2017).

Amoxicillin is frequently used antibiotic agents with a wide antibacterial spectrum and semisynthetic β -lactam active against a wide range of gram-positive and a limited range of gram-negative organisms (Sutherland et al., 1972; Hardman et al., 2001).

Vancomycin hydrochloride hydrate, is an amphoteric glycopeptide antibiotic and is a water-soluble. It has a strong bactericidal activity, inhibiting the cell wall synthesis.

Due to its toxic effect, methicillin and penicillin resistant vancomycin were used as an alternative in the treatment. (Liu et al., 2015; Serrı et al., 2017). So that in this study we investigated the effects of amoxicillin and vancomycin hydrochloride hydrate antibiotics on total GST activity in human erythrocytes.

MATERIALS AND METHODS

Materials and Chemicals

Standard protein markers for electrophoresis were obtained from Thermo and 1-chloro-2,4-dinitrobenzene (CDNB) were provided by Sigma-Aldrich (USA). All other chemicals were obtained from Merck (Germany).

Preparation of Hemolysate From Blood Samples

Fresh blood was taken in volunteer people at the blood centre of Atatürk University Research Hospital. The samples taken to the hemogram tubes having EDTA anticoagulant and were centrifuged at 1 500xg for 15 min. Then, the supernatant leukocyte and plasma were carefully removed using the dropper. The volume of erythrocytes which collapsed in the similar to volume was added the isotonic 0.9% NaCl solution in tube. The sample is similarly centrifuged again and the supernatant was removed. For the thorough purification of erythrocytes from other substances, washing operation was performed three times. After erythrocytes was hemolyzed with ice water. hemolysate was centrifuged at 15 000xg and at +4 °C for 30 min.

The Supernatant at the upper was carefully removed by using dropper (Alim et al., 2015).

Purification of GST Enzyme

After pH adjusted to 8.7 for the hemolysate with solid Tris, It was applied to Glutathione-Agarose affinity column. The column was previously washed with with buffer (10 mM phosphate buffer, pH 7.4, 150 mM NaCl) and quilibrated the like buffer. In this GST the bound is washed with buffer (50 mM Tris-HCI buffer pH 9.0 and up to 10 mM GSH) and eluted fractions in 2 mL volume were collected. The active fractions were separated and this fractions stored at +4 °C for the protein and enzyme activity measured (Demirdağ et al., 2012).

GST Assay

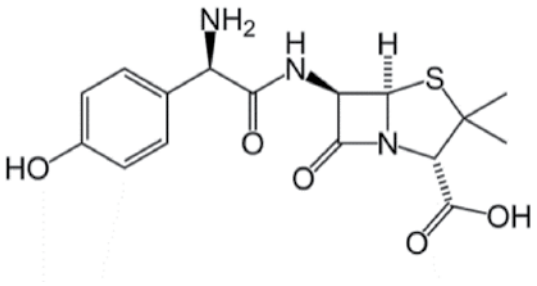
GST activity was measured at 25°C using CDNB as a model substrate. The assay system included a phosphate buffer (pH 6.5), GSH (20 mM) and CDNB (25 mM). A spectrophotometer was used to estimate the changes in absorbance at 340 nm for 3 min (Çomaklı et al., 2011). GST activity was assayed spectro photometrically at 25°C with reduced glutathione (GSH) and CDNB as substrates.

Protein Quantity Assay

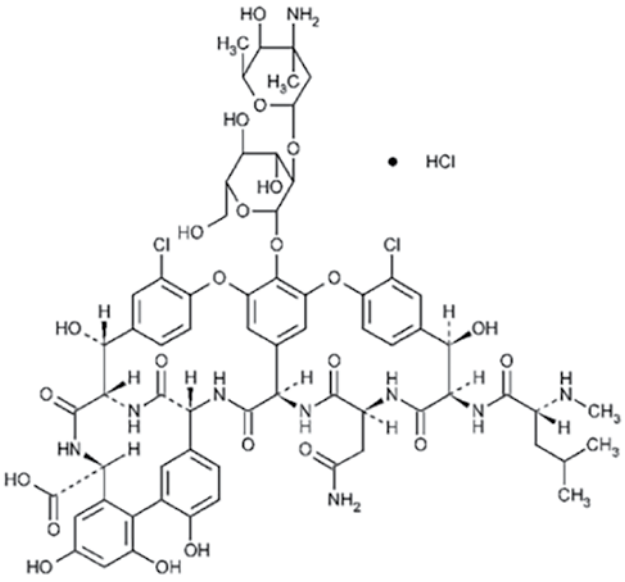
Bradford method was used for determination of colorimetric protein assay. The Bradford assay requires the reagent Coomassie Brilliant Blue G-250 dissolved in an acidic solution (Bradford, 1976).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The purity and presence of GST was performed by the SDS-PAGE technique according to Laemmli's procedure and our previous practice (Laemmli, 1970). After this procedure, a single band was seen for GST.



Amoxicillin
$C_{16}H_{19}N_3O_5S$



Vancomycin Hydrochloride
$C_{66}H_{75}Cl_2N_9O_{24}\cdot HCl$

Figure 1. Chemical structures of antibiotics used for in vivo studies

RESULTS AND DISCUSSION

It shows that the GST enzyme was purified from human erythrocytes (Table 1). In this, a specific activity of 5.56 EU mg^{-1} proteins expressed about 2 316.47 fold and yield of 65.86 % by using GSH-affinity chromatographic method. A single band protein was appeared on SDS-PAGE after purification process which was applied GSH-affinity chromatography. The molecular weight of GST enzyme was estimated to be 26 kDa (Figure 2).

Table 1. Summary of the GST purification procedure

Purification steps	Activity (EU/mL)	Protein (mg/mL)	Total volume (mL)	Total activity (EU)	Total Protein (mg)	Specific activity (EU/mg)	Yield (%)	Purification fold
Hemolysate	20	0.084	36.22	598.2	1.418	0.0024	1	100
affinity chromatography	4	0.264	0.078	0.168	0.934	5.56	2316.47	65.86

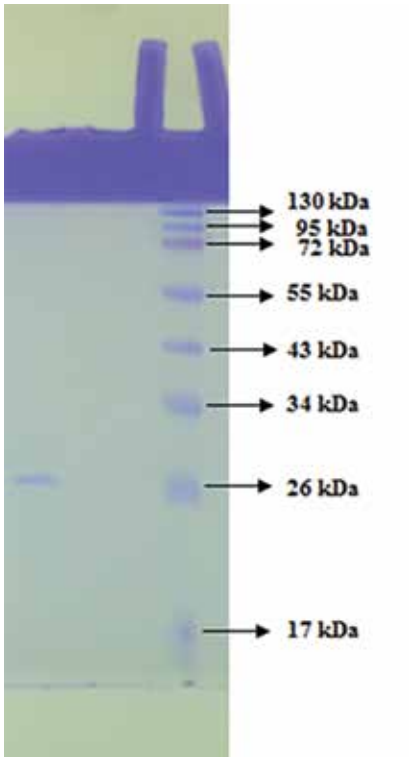


Figure 2. Molecular weight of proteins (kDa) in SDS-PAGE.

GST is one of the enzymes of xenobiotic metabolism and has gold specs in broad spectrum. Because of this feature, the GST enzyme acts as a defense in the organism exposed to endogenous and exogenous chemicals. GST is required for the activation and inactivation of many electrophilic materials. This enzyme inactivates the electrophilic sites of toxic compounds with reduced -SH group of glutathione. As a product, water-soluble mercapturic acid is formed and excreted in the urine from the body (Hee-Joong et al., 2013).

Because of their ability to metabolize GST enzyme toxic exogenous compounds has been explored in detail in mammals. The GST enzyme has been extensively investigated in mammals because of their ability to metabolize toxic exogenous compounds. This enzyme has both been purified from many sources such as insects, bacteria, plants and studied in many areas. Since the GST enzyme is a very important enzyme in the defense system, its characterization and characterization has become important today (Erat and Sakiroglu, 2013).

Thus, the purification of the GST enzyme was performed in two steps. First the homogenate is prepared and then GST enzyme purified by means

of glutathione-agarose affinity gel chromatography. The GST enzyme in human erythrocytes has been purified 1143 times and specific activity of 16 EU mg^{-1} protein previously by Erat and Sakiroglu (2013) with a with glutathione-agarose affinity column (Türkan et al., 2014).

In our work, when the values are checked after purification, GST enzyme specific activity reached 5.56 EU mg^{-1} protein, 65% yield and 2316.0 fold purification was achieved. Furthermore, Gülçin et al. had realized purification process according to the procedure of Toribo et al. (1996) and Güvercin et al. (2008) that they performed in two steps, such as preparation of the hemolysate and glutathione-agarose affinity gel chromatography. So, erythrocyte GST enzyme with 12.2 EU mg^{-1} protein specific activity was purified 8.714-fold with a yield of 90% (Riol et al., 2001; Gülçin et al., 2015).

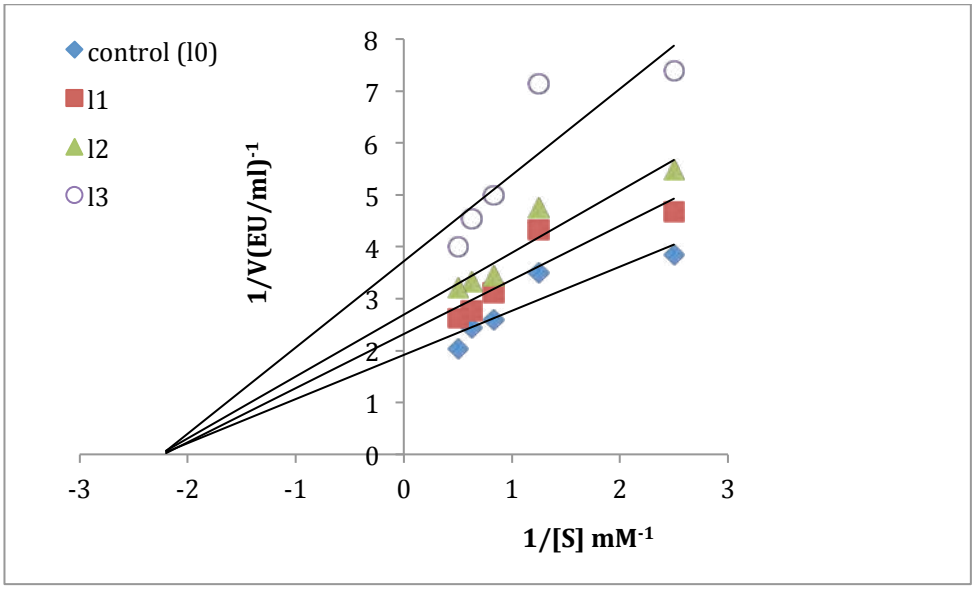
There are many sources in the literature that the molecular mass of GST enzyme is different. such as the molecular mass of GST enzyme purified from the rainbow trout liver was determined to be 23 kDa, molecular mass of GST enzyme purified from rat liver 26 kDa, and molecular mass of the GST enzyme isolated from *E.coli* is approximately 22.5 kDa. Similarly, in other studies has been found near molecular weight for GST. In another study, after purification by enzyme affinity column, the purity was checked by SDS-PAGE. As a result, a single band of the homodimer structure was obtained. The molecular mass of GST enzyme purified from bovine erythrocytes was determined to be about 27 kDa (Güvercin et al., 2008). In our work, the molecular mass of GST enzyme purified from human erythrocytes determined about 26 kDa (Lizuka et al.,1989; Young and Brıedis, 1989; Novoa–Valinas et al., 2004).

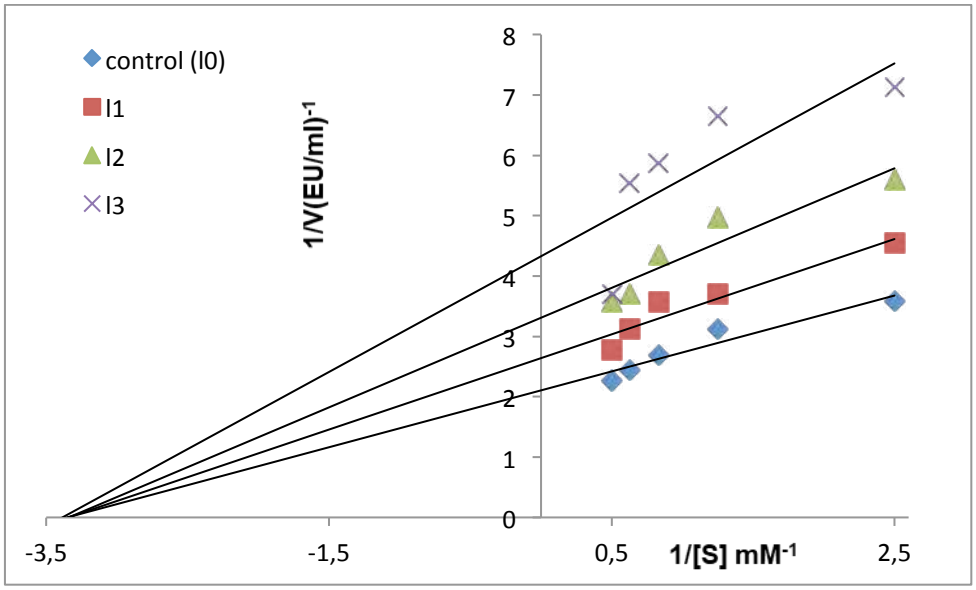
Antibiotics are known to have negative effects on the organism (Çiftçi et al., 2007) and sometimes, the use of antibiotics in patients may also generate adverse effects. But, GST inhibitors may contribute to promising therapeautic agents for the development of new drugs. Thus, in this work inhibitor effects of amoxicillin and vancomycin hydrochloride antibiotics examined on enzyme activity of glutathione S-transferase purified from human erythrocytes.

Inhibition effect by performed kinetic study was determined for amoxicillin and vancomycin hydrochloride antibiotics. Which IC_{50} değerleri for amoxicillin was determined 12.96 mM, IC_{50} value for vancomycin hydrochloride was calculated as 22.48 mM. Further, K_i constants were calculated for these two antibiotics. As a result of K_i constant for amoxicillin and vancomycin hydrochloride were determined 48.744± 18.088 and 0.453±0.255 mM respectively. When looking at the type of inhibition for these two antibiotics, both amoxicillin and vancomycin hydrochloride showed non-competitive inhibition type (Table 2 and Figure 3).

Table 2. The calculated IC_{50}, K_i values and inhibition type for amoxicillin and vancomycin hydrochloride antibiotics on GST enzyme activity

GST			
İnhibitor	**IC_{50} (mM)**	**K_i (mM)**	**Inhibition type**
Amoxicillin	12.96	48.744±18.088	Non-competitive
Vancomycin Hydrochloride	22.48	0.453±0.255	Non-competitive





Figures 3. Lineweaver–Burk graph of Amoxicillin and Vancomycin Hydrochloride for determination of K_i and inhibition type for GST.

Many similar studies are available in the literature. In a study, the in vitro interaction of some antibiotics on the activity of GST enzyme has been investigated and it has been reported that ampicillin which used as one of these antibiotics had reduced GST enzyme activity by 45% and another is novalgin which reduced GST enzyme activity by 70%. Moreover another study was demonstrated that gentamicin inhibition is caused by GST enzyme (Riol et al., 2001).

The most effective parameter showing the inhibitory effect is the Ki constant and IC_{50} values for any inhibitör (Gülçin et al, 2016). In this study, inhibitory effects of amoxicillin and vancomycin hydrochloride antibiotics on GST enzyme activities were reported. According to K_i constant and IC_{50} values, we observed that both amoxicillin and vancomycin hydrochloride had inhibitory effect on GST enzyme. These antibiotics inhibit GST enzyme and seriously leading to insufficiency in detoxification reactions.

Both in the results of our work and in other studies it is seen that GST enzyme, which has a very important role in the detoxification system, causes a decrease in the enzyme activity at certain doses of antibiotics used in treatment.

CONCLUSION

The in vitro inhibition study of these drugs against GST enzyme activity has been studied for the first time. Significant decrease in activation was observed in enzyme activation in both drugs. However, the use of antibiotics in patients may also generate adverse effects. To minimise of the side effects of antibiotics should be used carefully and their doses should be adjusted correctly. Studies in the literature on this enzyme involved in detoxification are quite extensive. Because inhibition of this enzyme causes important problems in metabolism. It is advisable to avoid using inhibitors that lower enzyme activity or to keep the doses low. The enzyme has been obtained pure by the proposed method and the results of the purification have been found to be in accordance with the literature.

As a result of the study, purified GST enzyme in erythrocytes of humans was purified. Inhibitory effects of amoxicillin and vancomycin hydrochloride on the activity of this enzyme have been investigated. IC_{50} and K_i constants of these antibiotics were calculated and inhibition types were determined. As a result of the inhibition studies, we observed that these antibiotics inhibited the GST enzyme at low doses. Vancomycin hydrochloride also showed the best inhibition effect.

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