# *In Vitro* Inhibition of Human Plasma and Erythrocyte AChE by Various Drugs

# İnsan Plazma ve Eritrosit AChE'nin Çeşitli İlaçlarla *in Vitro* İnhibisyonu

Research Article

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## ABSTRACT

In the present study, the in vitro effects of eight structurally different antibiotics (cefazolin, kanamycin, gentamicin, clindamycin, ivermectin and ampicillin) and painkiller (metamizole and diclofenac) on human erythrocyte and plasma acetylcholinesterase activity (AChE; EC 3.1.1.7) were investigated. For this, $IC_{50}$ and $K_I$ values of the drugs were calculated for determining the inhibition caused by these drugs. Acetylcholinesterase activity was determined spectrophotometrically, according to Ellman's method. Then the enzyme activity exposed to the drugs with different concentrations was measured. AChE activity vs. drug concentration and Mihealis-Menten graphs were obtained. Lastly, $IC_{50}$ and $K_I$ values were estimated from these graphs. $IC_{50}$ values were estimated to be 1.78 x$10^{-8}$ M of diclofenac for plasma AChE and 0.85x$10^{-8}$ M of gentamicin for erythrocyte AChE. $K_I$ values were 1.36 x$10^{-8}$ M for diclofenac and 1.16 x$10^{-8}$ M for gentamicin. Also, $IC_{50}$ and $K_I$ values for ivermectin, clindamycin, kanamycin, ampicillin were obtained. Diclofenac exhibited the highest inhibitory effect on plasma AChE activity. On the other hand, gentamicin showed the highest inhibitory effect on erythrocyte AChE activity, which seems to be better for inhibition of AChE than those reported previously.

**Key Words**
Acetylcholinesterase, enzyme inhibition, enzyme assay, medicinal drug, human erythrocyte and plasma, *in vitro* kinetics.

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## ÖZET

Bu çalışmada; yapısal olarak farklı olan sekiz antibiyotik (sefazolin, kanamisin, gentamisin, klindamisin, ivermektin ve ampisilin) ve ağrı kesicinin (metamizol ve diklofenak) insan eritrosit ve plazma asetilkolinesteraz (AChE; EC 3.1.1.7) enzimi üzerine in vitro etkileri araştırılmıştır. Bu amaçla; farklı derişimlere sahip ilaçların neden olduğu inhibisyonu belirlemek için ilaçların $IC_{50}$ ve $K_I$ değerleri hesaplandı. Asetilkolinesteraz aktivitesi spektrofotometrik olarak Ellman yöntemiyle belirlendi. Daha sonra, ilaçlara maruz kalan enzim aktivitesi ölçüldü. AChE aktivitesine karşı ilaç derişimi ve Michealis-Menten grafikleri çizildi. Son olarak, bu grafikler yardımıyla $IC_{50}$ ve $K_I$ değerleri hesaplandı. $IC_{50}$ değerleri, plazma AChE'sine diklofenak için 1.78 x$10^{-8}$ M ve eritrosit AChE'sine gentamisin için 0.85 x$10^{-8}$ M olduğu belirlendi. $K_I$ değerleri ise diklofenak için 1.36 x$10^{-8}$ M ve gentamisin için 1.16 x$10^{-8}$ M olarak hesaplandı. Ayrıca, ivermektin, klindamisin, kanamisin, ampisilin için $IC_{50}$ ve $K_I$ değerleri elde edildi. Diklofenak, plazma AChE aktivitesi üzerine gentamisin de eritrosit AChE aktivitesi üzerine en yüksek inhibitör etkisi göstermiştir. Bulunan bu değerlerin daha önce yapılan benzer çalışmalardaki inhibisyon değerlerinden daha yüksek olduğu görülmektedir [1,2].

**Anahtar Kelimeler**
Asetilkolinesteraz, enzim inhibisyonu, enzim analizi, tıbbi ilaçlar, insan eritrositi ve plazması, in vitro kinetikleri.

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## INTRODUCTION

Acetylcholinesterase (AChE; E.C.3.1.1.7) hydrolyses the neurotransmitter acetylcholine to choline and acetate. Furthermore, AChE balances the impulse transmission at cholorgenic synapses [1-3]. There are two type cholinesterases according to the reaction catalyzed by them. The first one is true AChE and the second one is butyrylcholinesterase [4]. AChE exists in liver, muscle, kidney, spleen, brain, erythrocyte, and chorgenic neurons [5-7]. AChE belonging to its two forms is found either asymmetric or globular. They change from tissue to tissue [8]. Thus, the previous kinetic studies showed that the active site of AChE consist of two subsites, "esteric" and "anionic" (Scheme 1) [9]. The enzyme generally exists as hydrophilic modes in the brain, muscle and the other tissues, which contains disulfide-linked oligomers with collagenous or lipid-containing structural subunits. Amphipathic AChE dimmers with globular structures are attached to the erythrocyte membrane [10,11].

Pseudocholinesterase (PChE) and acetyl cholinesterase (AChE) are isoforms of cholinesterase (ChE) [12]. AChE and PChE are determined by their substrates and some specific inhibitors can affect their activity [13]. The basic role of AChE is the termination of neurotransmission by hydrolyzing of the neurotransmitter acetylcholine at intern euronal synapses in the brain and neuromuscular junctions [14]. As well-known, plasma cholinesterase is generally found in tissues and plasma. Moreover, it works as assistance of AChE because it can catalyze the hydrolysis of various acetylcholines like AChE [15].

Inhibition of both cholinesterase activities are higher in humans affected by neuro degeneration. Subversive processes that can stimulate the death of neurons are connected with cholinesterase activities. In spite of the fact that AChE and PChE have many inhibitors, most of them are very toxic, so they cannot be considered as drugs [16].

Antibiotics are among the most widely used drugs inpatient and outpatient. As these antimicrobial agents are commonly well-tolerated, these drugs have some adverse effects, both dose dependent and idiosyncratic in nature. While diarrhea is generally related reverse effect of them, toxic effects on the center of nervous system may be slightly distinguished [17]. A few factors of drug metabolism can enhance inclination to neurotoxicity like an individual's nutritional condition, partial blood flow and tissue ingression, rate of absorption of the medication, line of drug delivery to target tissue, activation and elimination of the drug along with preservative replies the individual [18,19]. There are some other factors that can be involved genetic factors, switched in pharmacokinetics of drug in the event of nephritic deficiency and center of nervous system penetration can be connected with leading neurotoxicity [20]. Drugs have generally shown activator or inhibitor on enzyme systems of the body [21,22].

The objective of this in vitro study was to investigate the effect of eight structurally different drugs i.e., ivermectin, diclofenac, clindamycin, gentamicin, kanamycin, ampicillin, cefazolin, and metamizole on both human plasma and erythrocyte AChE.

## MATERIALS AND METHODS

### Chemicals and Reagents

All drugs i.e., ivermectin, diclofenac, clindamycin, gentamicin, kanamycin, ampicillin, cefazolin and metamizole were purchased from a local pharmacia in Van, Turkey. The structures of these drugs were listed in Table 1. The other chemicals, acetylthiocholineiodide, 5,5'-dithio-2-bis-nitrobenzoate (DTNB) and sodium phosphate dibasic ($NaH_2PO_4.2H_2O$) were obtained from Sigma Aldrich, Germany. For the experimental studies, a unit of blood with antiquagulant was obtained from the Red Crescent, Turkey.

### Preparation of Eryhtrocyte, Plasma and Drugs

Fresh human bloods were firstly centrifuged at 3.000 rpm for 15 min. Plasma and erythrocyte were appropriately collected. Plasma and erythrocyte were stored in the deep freeze for further kinetic studies. Erythrocytes were washed three times with isotonic NaCl solution and centrifuged at 3.000 rpm then the supernatants
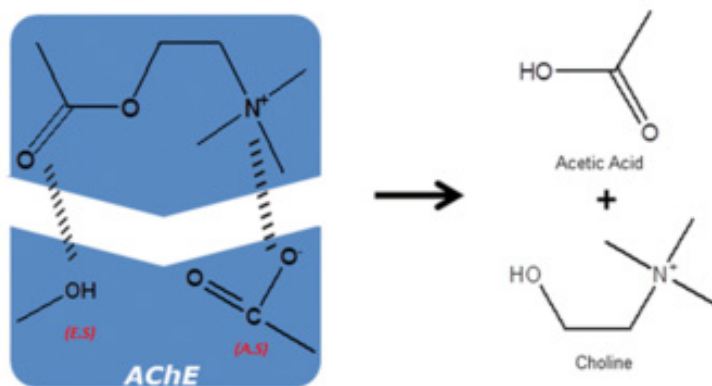
**Scheme 1.** Representative illustration of AChE.
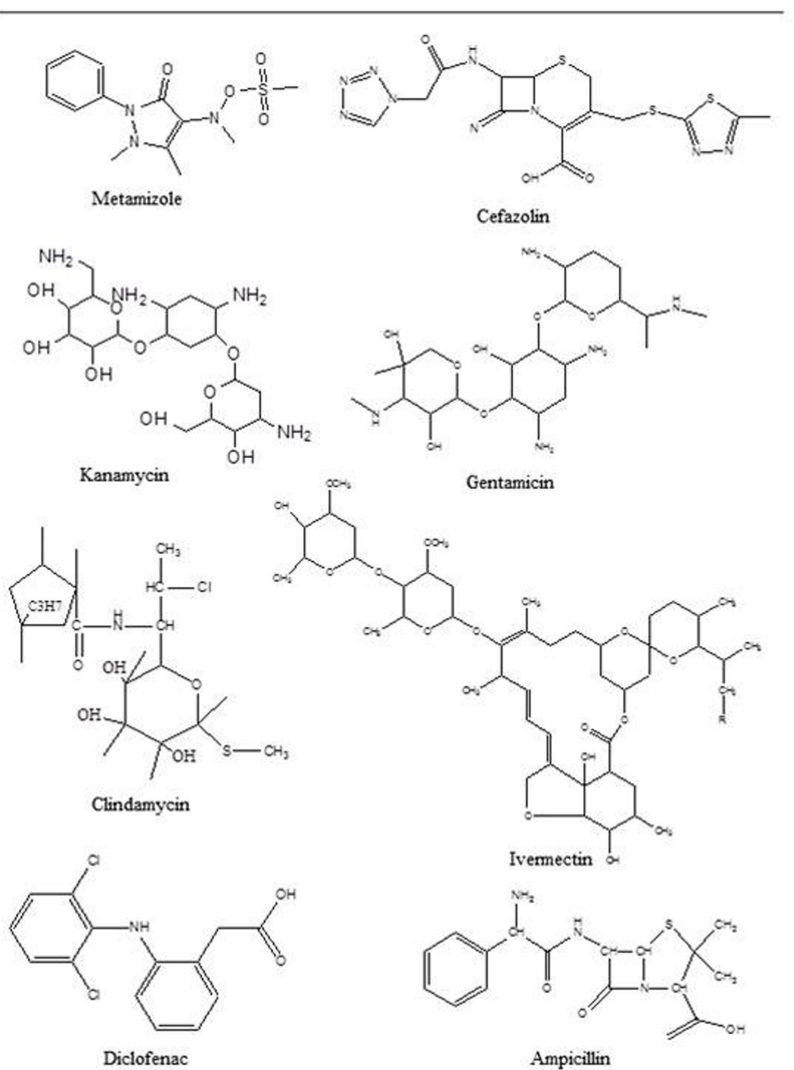
**Table 1.** Chemical structures of drugs.

**Table 2.** Drug solutions.

| | Concentrations, ($10^{-8}$M) | |
|---|---|---|
| | **Drug Plasma** | **Erythrocyte** |
| **İvermectin** | 0.354, 1.41, 3.18 | 0.354, 1.41, 3.18 |
| **Diclofenac** | 0.236, 0.943, 2.12 | 0.236, 0.943, 2.12 |
| **Clindamycin** | 8.91, 35.64, 80.19 | 0.594, 2.376, 5.346 |
| **Gentamicin** | 0.375, 1.5, 3.375 | 0.375, 0.666, 1.041 |
| **Ampicillin** | 20.2, 80.8, 181.8<br>323.8, 504.8 | 20.2, 80.8, 181.8 |
| **Kanamycin** | 0.172, 1.076, 4.303<br>6.198, 13.94 | 1.55, 2.11, 2.74 |
| **Cefazolin** | 1.746, 3.935, 6.993<br>10.93, 15.74 | 1.746, 6.993, 15.74<br>27.97, 43.72 |
| **Metamizole** | 0.05, 0.19, 0.448<br>0.798, 1.248 | 0.05, 0.19, 0.448<br>0.798, 1.248 |

were removed. The washed erythrocytes were hemolyzed with 5 volumes of ice-cold bi-distilled water and then centrifuged at 4°C (20.000 rpm for 30 min to remove residual intact cells and membranes) [23].

Drug solutions i.e., ivermectin, diclofenac, clindamycin, gentamicin, kanamycin, ampicillin, cefazolin and metamizole were freshly prepared in 56 mM phosphate buffer solution (pH 8.0) at different concentrations that were listed in Table 2.

### Activity Assay

Acetylcholinesterase activity was measured spectrophotometrically with a Shimadzu spectrophotometry UV-(1208), Japan at 25°C, 412 nm, according to Ellman's method [24]. The assay mixture (3 ml) contained 3 mM of acetylthiocholine iodide, 10 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid) and 100 µL of plasma or erythrocyte (2.37 or 1.28 Eu/ml) in 56 mM-Na-buffer ($NaHPO_4.2H_2O$, pH 8) and different concentrations of the drug active compounds were diluted with bi-distilled water or Na-buffer. $K_m$ and $V_{max}$ values were determined by Lineweaver-Burk plots using acetylthiocholineiodide concentrations from 0.05 mM to 0.15 mM. All enzyme activity assays were performed at least four different experiments, each one performed in triplicate. AChE activity

values of both erythrocyte and plasma were calculated with the Equation (1):

$$A = \frac{OD}{13.6} \times \frac{V_C}{V_E} \times f \qquad (1)$$

A: Activity
OD: Optic density's diversity at 412 nm, per minute
$V_C$: Volume of cuvette
$V_E$: Volume of enzyme solution (homogenated plasma or erythrocyte)
f: Dilution factor
13.6: Extinction coefficient for AChE

### Detection of $IC_{50}$ and $K_I$ Values

$IC_{50}$ and $K_I$ values of each drug were calculated from the activity % vs. inhibitor concentration and the Lineweaver-Burk graph was used for determination of inhibition type and $K_I$ constants. The $K_I$ value was determined according to the Equation (2):

$$V_{max} = V_{max} / (1 + ([I]/K_I)) \qquad (2)$$

### RESULTS

Herein, to evaluate the effect of ivermectin, diclofenac, clindamycin, gentamicin, kanamycin, ampicillin, cefazolin, and metamizole on AChE activity, $K_I$ and $IC_{50}$ values were calculated
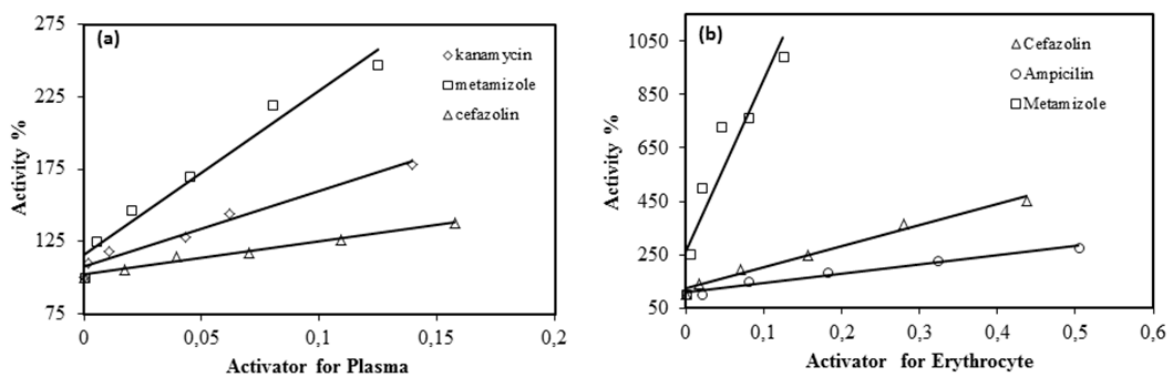
**Figure 1.** Activator for plasma (a) and erythrocyte (b).

**Table 3.** The $IC_{50}$ and $K_I$ values for drug.

| Sample type | Name of Drug | $IC_{50}(10^{-8})M$ | $K_I(10^{-8})M$ |
|---|---|---|---|
| **Plasma** | Ivermectin | 1.89 | 1.48 |
| | Diclofenac | 1.78 | 1.36 |
| | Clindamycin | 50 | 804 |
| | Gentamicin | 3.6 | 2.16 |
| | Ampicillin | 140 | 1.56 |
| | Cefazolin | - | - |
| | Kanamycin | - | - |
| | Metamizole | - | - |
| **Erythrocyte** | Ivermectin | 1.78 | 1.61 |
| | Diclofenac | 1.39 | 0.99 |
| | Clindamycin | 3.31 | 3.74 |
| | Gentamicin | 0.85 | 1.16 |
| | Kanamycin | 1.65 | 0.87 |
| | Ampicillin | - | - |
| | Cefazolin | - | - |
| | Metamizole | - | - |

from Lineweaver-Burk and drug concentration vs. activity %, respectively. They were tested on human plasma and erythrocyte acetylcholinesterase activity. $IC_{50}$ for plasma AChE was 1.78 $x10^{-8}$ M of diclofenac, $1.89x10^{-8}$ M of ivermectin, $3.6x10^{-8}$ M of gentamicin, $50x10^{-8}$ M of clindamycin and $140x10^{-8}$ M of ampicillin. $IC_{50}$ for erythrocyte AChE was $0.85x10^{-8}$ M of gentamicin, $1.39x10^{-8}$ M of diclofenac, $1.65x10^{-8}$ M of kanamycin, $1.78x10^{-8}$ M of ivermectin, $3.31x10^{-8}$

M of clindamycin (Table 3). In all cases, the drugs possessing inhibition effect on both plasma and erythrocyte showed non-competitive inhibition. The drugs showing activator effect on both plasma and erythrocyte AChE are shown in Figure 1.

The type of inhibition caused by the drugs was assessed by Lineweaver-Burk plots which were drawn by using 1/V vs 1/S values. $K_I$ constants were calculated from these graphs and shown in
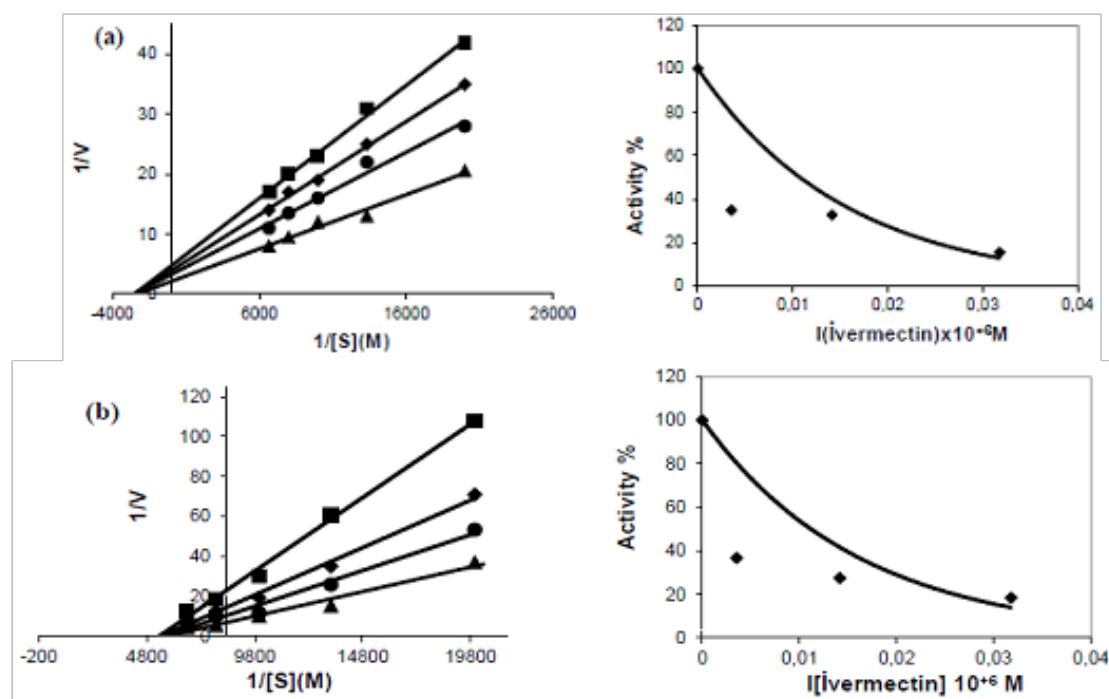
highest inhibitory effect on erythrocyte followed by diclofenac, kanamycin, ivermectin and clindamycin, respectively. The values obtained show that these drugs exhibit desirable side effects on AChE activity. It is reported that while gentamicin and kanamycin, aminoglycosides, inhibited AChE; cefazolin, cephalosporins, ampicillin and penicillin caused the inhibition of GABA-A receptors [19]. Stoytcheva and Zlatev found that eserine, nicotinic acid, atropine and caffeine show competitive inhibition and thiamine and quinine noncompetitive show inhibition on AChE activity [28]. To analyze the structure of these active substances, it is seen that active groups of them didn't fit clearly compared to AChE active site. Generally, effect of hydroxyl, amino and oxygen of carbonyl groups are probably connected with the weak bonds to amino acid residues and with outside the active center of the enzyme. It means that these drugs attach to differrent sites of AChE out of the active center of the enzyme, which causes steric effect due to their structures.

As known, enzymes are macromolecules and have isoenzymes in varied tissues and body fluids. Compounds that interact with them cause to change activation of enzyme. The data obtained here show that as the effects of drugs ampicillin, metamizole and kanamycin that link to outside of the enzyme's active site causes the change in the 3-D structure of enzyme. Among these drugs, while ampicillin inhibited plasma AChE, it activated erythrocyte AChE. Like this, although kanamycin inhibited erythrocyte AChE, it activated plasma AChE. The different effect of these drugs on plasma and erythrocyte AChE demonstrates that these isoenzymes can respond to the drugs differentially because of structural difference of the isoenzymes. Thus, these induce alterations in enzyme activity.

## CONCLUCIONS

Here, we investigated in vitro studies of some drugs as inhibitor or activator of the acetylcholinesterase on human erythrocyte and plasma. $IC_{50}$ and $K_I$ values of drugs were calculated. As $IC_{50}$ and $K_I$ values were $1.78 \times 10^{-8}$ M and $1.36 \times 10^{-8}$ M for diclofenac which has the best inhibitory

effect on plasma, $0.85 \times 10^{-8}$ M and $1.16 \times 10^{-8}$ M for gentamicin which has the best inhibitory effect on erythrocyte. As a result, a lot of drugs inhibition types were reversible and noncompetitive.

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