In vitro Inhibition of Human Plasma and Erythrocyte Cholinesterases by a Selected Drugs (Enzymes Inhibitors)

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Abstract

The inhibitory effect for human plasma and erythrocyte cholinesterase activity by six drugs already known as an enzyme inhibitors (Cimetidine, Ciprofloxacin, Donepezil, Sildenafil, Erythromycin, and Chlorpheniramine) was tested *in vitro*. The affinity toward the cholinesterase system was evaluated by using different concentrations of the investigated drugs. The cholinesterase activities were determined by using the modified electrometric method. Each drug showed different ability to inhibit cholinesterase activity depending on its concentration and its selectivity to the either plasma or erythrocyte cholinesterase; only Donepezil and Chlorpheniramine showed higher inhibitory effect. Ciprofloxacin showed moderate inhibition of cholinesterase but still statistically insignificant. Cimetidine, Sildenafil and Erythromycin showed little inhibitory activity although they have a good inhibitory effect on the liver enzyme system Cytochrome P450 (CYP450).

تنشيط إنزيم الكولين استيريز في كريات الدم الحمر وبلازما الدم في الانسان بواسطة عدد من الادوية

الملخص

تمت دراسة التأثير التثبيطي لخميرة الكولين استراز في كل من البلازما وكريات الدم الحمراء في الإنسان بواسطة عدد من الأدوية المعروفة بقابليتها التثبيطية للإنزيمات داخل الجسم وهي كل من (الساميتيدين و السيبروفلوكساسين و الدونبيزيل و سلدينافيل و الاريثروماسين و كذلك الكلورامفينيرامين) وذلك باستخدام الأنابيب المختبرية. وكذلك تم تقيم قوة قابلية كل من هذه الادوية على الارتباط مع أنواع خميرة الكولين استراز باستخدام عدة تراكيز من ه ذه الأدوية وذلك بإتباع الطريقة الكهرومترية المحورة لقياس نشاط خميرة الكولين استراز. اظهر كل دواء قابليات مختلفة لتثبيط خميرة الإنزيم اعتمادا على تركيز الدواء المستخدم و على قوة قابليته على الارتباط مع خميرة البلازما أو كريات الدم الحمراء . أظهرت النتائج أن كل من الدونبيزيل والكلورامفينيرامين حصلوا على أعلى قوة للارتباط بخميرة الإنزيم ، بينما السيبروفلوكساسين اظهر فعالية متوسطة للارتباط ومن ثم إحداث تثبيط للخميرة ولكن ليس إلى حد كافي لاعتباره مثبط حقيقي بصورة عملية . أما كل من السايمتدين والارثرومايسين والسيلدينافيل اظهروا قوة قليلة جدا للارتباط مع خميرة الإنزيم على الرغم من قوة قابليتهم المعروفة لتثبيط إنزيمات الكبد الأخرى (ساتوكروم P450) .

Introduction

Cholinesterases (ChE) are a family of enzymes that catalyze the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation (1).

There are two types (2):

- Acetylcholinesterase (EC 3.1.1.7) (AChE), also known as true or RBC cholinesterase, erythrocyte cholinesterase, found primarily in the erythrocytes and neural synapses. (2)
- Pseudocholinesterase (EC 3.1.1.8) (BChE), also known as plasma cholinesterase, butyrylcholinesterase, found primarily in the plasma and the liver. (2)

The physiological functions of erythrocyte and plasma ChE are not known, but one idea is that they protect the body from natural anti-ChE agents (e.g. physostigmine, a plant alkaloid) encountered during the evolution of species (3).

A cholinesterase inhibitor (or "anticholinesterase") suppresses the action of the enzyme. Because of its essential function, chemicals that interfere with the action of cholinesterase are potent neurotoxins, causing excessive salivation and eye-watering in low doses, followed by muscle spasms and ultimately death (examples are nerve gases sarin and VX). One counteracting medication is pralidoxime (4). Anticholinesterases are also used for reversing medication induced paralysis during anesthesia; as well as in the treatment of myasthenia gravis, glaucoma, and Alzheimer's disease (3,5). Such compounds are used as insecticides in a range of products including sheep dip, organophosphate pesticides, and carbamate pesticides. In addition to acute poisoning as described above, a semi-acute poisoning characterized by strong mental disturbances can occur. (3,6). Cimetidine : H2- receptor antagonist mainly is used in gastric and duodenal ulceration and other conditions where gastric acid reduction is beneficial, it is well known that cimetidine retards oxidative hepatic drug metabolism (enzyme inhibitor), so that care should be taken when cimetidine used with drugs having low therapeutic index and metabolized by the liver (7,8). Ciprofloxacin: It is a quinolone antibacterial drug particularly active against gram-negative bacteria and has moderate activity against gram-positive bacteria, so that it used mainly in the urinary tract infections (typhoid fever) (7). Coadministration of Ciprofloxacin with other drugs primarily metabolized by liver results in increased plasma concentrations of these drugs (8,9). Donepezil is an acetylcholinesterase inhibitor, mainly used in Alzheimer's disease, it reversibly inhibits acetylcholinesterase (9,10).In the current study we try to evaluate its cholinesterase's inhibitory action by using different concentrations. Sildenafil : is a phosphodiesterase inhibitor used for the treatment of erectile dysfunction and pulmonary arterial hypertension (10). It is a weak inhibitor of cytochrome P.450 (11,12). Erythromycin : it is a macrolide antibacterial with spectrum that is similar but not identical to that of penicillin, it is thus an alternative in penicillin-allergic patients (8). It inhibits the cytochrome P.450 system affecting the metabolism of many drugs (13). Chlorpheniramine : is an ethanolamine derivative, nonselective histamine H1-receptor antagonist, mainly used for allergy symptoms caused by histamine release (anaphylaxis, rhinitis and dermatoses), also can be used for nausea and vertigo and motion sickness (14).Chlorpheniramine is previously known to inhibit both erythrocyte and plasma cholinesterase *in vitro* and *in vivo* in experimental animals (mice and horses) (15,16). All these drugs are well known as enzyme inhibitors as they decrease the activity of many enzyme systems in the body depending on its different mechanisms of action.(8,9,10). The aims of this study was to evaluate the cholinesterase inhibitory effect of 6 drugs already known as enzymes inhibitors from different groups (Cimetidine, Ciprofloxacin, Donepezil, Sildenafil, Erythromycin, and Chlorpheniramine), by using healthy human plasma and erythrocyte (*in vitro*).

Materials and methods

The subjects included in this study (10 males and 13 females, age 30+/- 10 years) were

apparently healthy with no history of exposure to anti-ChE insecticides or drugs. Blood samples were collected in 5 ml EDTA-treated test tubes then centrifuged (Centurion, UK) at 3000 rpm for 15 min. The erythrocytes and plasma were separately pooled and kept on ice for ChE assay.

Electrometric assay of ChE activity

In current study the modified electrometric method was used, which was validated in human (17,18). The reaction mixture in a 10 ml beaker contained 3 ml distilled water, 0.2 ml plasma or erythrocytes and 3 ml pH 8.1 barbital-phosphate buffer (19). The pH of the mixture (pH1) was measured with glass electrode using pH meter (Hanna Instruments, Romania), then 0.1 ml of aqueous solution of acetylthicholine (7.5%) was added to the reaction mixture which was incubated at 37°C in a water bath (Shaker bath 5BS30, UK) for 20 min. At the end of the incubation period, the pH of the reaction mixture (pH2) was measured. The enzyme activity was calculated as follows:

ChE activity (ΔpH/20 min.) = (pH1-pH2)-ΔpH of blank

The blank was without the blood aliquot. The barbital-phosphate buffer solution consist of 1.24g sodium barbital (BDH), 0.163g potassium dihydrogen phosphate (Merck, Germany), and 35.07g sodium chloride (BDH) dissolved in one liter distilled water (17,19). The pH of the buffer was adjusted to 8.1 with 1N HCL.

In vitro ChE inhibition by the drugs

Plasma and erythrocyte samples were collected from healthy volunteers. Different drug concentrations were prepared then they were individually added in a volume of 0.1 ml to the reaction mixture to obtain final concentrations as follows:

Cimetidine: 25, 50, 100, 200 and 400 μM

Ciprofloxacin: 6.25, 12.5, 25, 50 and 100 μM

Donepezil: 12.5, 50, 75, 150 and 300 μM

Sildenafil: 12.5, 25, 50, 100, 200 and 400 μM

Erythromycin: 25, 50, 100, 200 and 400 μM

Chlorpheniramine: 25, 50, 100 and 200 μM

The concentrations of the drugs used in the present study were obtained from preliminary experiments to validate the experimental concentrations. The inhibitors were prepared in distilled water and individually added in a 0.1ml to the reaction mixture of the plasma and erythrocytes. The reaction mixtures containing inhibitors were incubated at 37ºC for 10 min. Thereafter, the residual ChE activity in mixture was measured (20). The % of enzyme inhibition was calculated as follows:

% ChE inhibition= [ChE activity (without inhibitor)-ChE activity (with inhibitor)/ChE activity (without inhibitor)]X100 (20)

Determination of IC50 values

The % inhibition of control activity was plotted against logarithm of inhibitor concentration. The IC50 values were determined by linear regression of the inhibition curve from 20-80% inhibition. When inhibition values was less than 50% the IC50 value was determined by extrapolation (21).

Statistical Analysis

When applicable, the data were subjected to analysis of variance followed by the least of significant difference test (22). Paired Student's t-test was used for the means of 2 groups (23). The level of significance was $P<0.05$.

Results

Tables 1-6 show in vitro inhibition of plasma and eryrthocyte ChEs by Cimetidine, Ciprofloxacin, Donepezil, Sildenafil, Erythromycin, and Chlorpheniramine. These drugs in a concentration- dependent manner variably inhibited plasma and erythrocyte ChE activities *in vitro* (Tables 1-6).

Table (1):- *In vitro* inhibition of plasma and erythrocyte cholinesterase activities by Cimetidine in normal subjects

Cimetidine (μM)	Plasma ChE		Erythrocyte ChE	
	Δ pH/20 min	% Inhibition	Δ pH/20 min	% Inhibition
0 μM	1.22 ± 0.004	0.00	0.97 ± 0.010	0.00
25 μM^{+}	1.04 ± 0.010	0.00	1.03 ± 0.022	0.00
50 μM^{+}	1.15 ± 0.002	0.00	0.95 ± 0.013	5.5
100 μM^{+}	1.04 ± 0.030	5.48	0.91 ± 0.00	8.08
200 μM^{+}	1.07 ± 0.002	12.65	1.20 ± 0.005	0.00
400 μM^{+}	0.92 ± 0.01*	25.31	0.88± 0.020	10.66

N=2-3 / concentration groups.
ChE values are means ± SE
$^{+}$ Cholinesterase inhibition was detected after 10 min incubation of the sample with Cimetidine.
* Significantly different from the respective control (0) group, P<0.05

Table (2):- In vitro inhibition of plasma and erythrocyte cholinesterase activities by Ciprofloxacin in normal subjects

Ciprofloxacin (μM)	Plasma ChE		Erythrocyte ChE	
	Δ pH/20 min	% Inhibition	Δ pH/20 min	% Inhibition
0 μM	1.23 ± 0.005	0.00	0.99 ± 0.011	0.00
6.25 μM^{+}	1.27 ± 0.001	0.00	1.01 ± 0.011	0.00
12.5 μM^{+}	1.19 ± 0.002	0.00	0.98 ± 0.003	0.50
25 μM^{+}	1.22 ± 0.004	0.82	0.81 ± 0.012*	18.27
50 μM^{+}	1.10 ± 0.00	11.65	0.85 ± 0.056*	16.25
100 μM^{+}	0.77 ± 0.001*	30.14	0.16± 0.052*	86.69

N=2-3 / concentration groups.
ChE values are means ± SE
$^{+}$ Cholinesterase inhibition was detected after 10 min incubation of the sample with Ciprofloxacin.
* Significantly different from the respective control (0) group, P<0.05

Table (3):- *In vitro* inhibition of plasma and erythrocyte cholinesterase activities by Donepezil in normal subjects

Donepezil (μM)	Plasma ChE		Erythrocyte ChE	
	Δ pH/20 min	% Inhibition	Δ pH/20 min	% Inhibition
0 μM	1.20 ± 0.003	0.00	0.77 ± 0.003	0.00
12.5 μM^{+}	0.77 ± 0.001*	30.14	0.11 ± 0.004*	86.7
50 μM^{+}	0.66 ± 0.002*	40.81	0.033 ± 0.032*	95.74
75 μM^{+}	0.49 ± 0.023*	62.69	0.015± 0.009*	98.05
150 μM^{+}	0.26 ± 0.004*	79.85	0.03 ± 0.017*	96.11
300 μM^{+}	0.24 ± 0.002*	81.54	0.045± 0.012*	94.16

N=2-3 / concentration groups.
ChE values are means ± SE
$^{+}$ Cholinesterase inhibition was detected after 10 min incubation of the sample with Donepezil.
* Significantly different from the respective control (0) group, P<0.05

Table (4):- In vitro inhibition of plasma and erythrocyte cholinesterase activities by Sildenafil in normal subjects

Sildenafil (μM)	Plasma ChE		Erythrocyte ChE	
	Δ pH/20 min	% Inhibition	Δ pH/20 min	% Inhibition
0 μM	1.23 ± 0.013	0.00	0.99 ± 0.010	0.00
12.5 μM^{+}	1.05 ± 0.084	4.11	0.84 ± 0.019*	14.18
25 μM^{+}	1.05 ± 0.027	4.57	0.83 ± 0.015*	16.5
50 μM^{+}	0.99 ± 0.003	9.13	0.835 ± 0.006*	15.6
100 μM^{+}	1.09 ± 0.015*	13.88	0.85 ± 0.000*	13.71
200 μM^{+}	1.07 ± 0.006*	13.06	0.89 ± 0.005*	10.15
400 μM^{+}	0.86 ± 0.015*	29.79	0.80± 0.053*	18.79

N=2-3 / concentration groups.
ChE values are means ± SE
$^{+}$ Cholinesterase inhibition was detected after 10 min incubation of the sample with Sildenafil.
* Significantly different from the respective control (0) group, P<0.05

Table (5):- *In vitro* inhibition of plasma and erythrocyte cholinesterase activities by Erythromycin in normal subjects

Erythromycin (μM)	Plasma ChE		Erythrocyte ChE	
	Δ pH/20 min	% Inhibition	Δ pH/20 min	% Inhibition
0 μM	1.13 ± 0.01	0.00	0.94± 0.03	0.00
25 μM^{+}	1.15 ± 0.05	0.00	1.00 ± 0.002	0.00
50 μM^{+}	1.35 ± 0.01	0.00	1.22 ± 0.021	0.00
100 μM^{+}	1.10 ± 0.075	8.84	1.18 ± 0.082	0.00
200 μM^{+}	1.13 ± 0.012	4.41	0.91 ± 0.003	4.74
400 μM^{+}	1.05± 0.04*	25.31	1.23± 0.062	0.00

N=2-3 / concentration groups.
ChE values are means ± SE
$^{+}$ Cholinesterase inhibition was detected after 10 min incubation of the sample with Erythromycin.
* Significantly different from the respective control (0) group, P<0.05

Table (6):- *In vitro* inhibition of plasma and erythrocyte cholinesterase activities by Chlorpheniramine in normal subjects

Chlorpheniramine (μM)	Plasma ChE		Erythrocyte ChE	
	Δ pH/20 min	% Inhibition	Δ pH/20 min	% Inhibition
0 μM	1.14 ± 0.016	0.00	0.95 ± 0.012	0.00
25 μM^{+}	0.86 ± 0.02*	24.67	0.96 ± 0.002	0.00
50 μM^{+}	0.73 ± 0.041*	35.68	1.00 ± 0.028	0.00
100 μM^{+}	0.59 ± 0.016*	47.58	0.94 ± 0.003	1.053
200 μM^{+}	0.46 ± 0.00*	59.47	0.81± 0.002*	14.74

N=2-3 / concentration groups.
ChE values are means ± SE
$^{+}$ Cholinesterase inhibition was detected after 10 min incubation of the sample with Chlorpheniramine.
* Significantly different from the respective control (0) group, P<0.05

Table 7 shows pIC50 values for the investigated drugs. Where the pIC50 values ranged from -4.344 to -1.649 and from -177.75 to 6.004 for plasma and erythrocyte, respectively.

Table (7):- The pIC50 values of the investigated drugs

Drugs	pIC50	
	Plasma	RBC
Cimetidine	-3.962	-10.565
Ciprofloxacin	-3.133	-1.806
Donepezil	-1.649	6.004
Sildenafil	-4.344	-177.75
Erythromycin	-4.313	-33.248
Chlorpheniramine	-2.060	-4.912

Discussion

In vitro ChE inhibition is a useful technique for detecting the potential anti-ChE activity of chemicals and drugs, providing the basis for development of useful and reliable methods to assess possible anticholinesterase activities. It was found that there is a good correlation between ChE inhibitions in vitro and in vivo (24). In this study in vitro inhibition of human plasma and erythrocyte ChE by Cimetidine, Ciprofloxacin, Donepezil, Sildenafil, Erythromycin, and Chlorpheniramine, the choice of these drugs is based on their ability to inhibit many enzyme system in the body (8), suggesting that they may also inhibit the cholinesterase system either as (strong – weak or very weak) inhibitors. This may help in any further study concern these drugs regarding increased toxicity or protection against the insecticides when used together. We found that Cimetidine, Sildenafil and Erythromycin have a similar activity against cholinesterase and they can be considered as very weak inhibitors, as they posses very low % enzyme inhibition even at high concentrations (Tables 1,4,5). Cimetidine and Erythromycin showed a slightly higher activity on plasma than on erythrocyte cholinesterase (Table 1,5) . Whereas Sildenafil showed similar activity on plasma than on erythrocyte cholinesterase (table 4), but this activity started weakly in low concentration and stayed in low % enzyme inhibition even in very high Sildenafil concentrations (Table 4), in addition the results showed that the affinity of Sildenafil toward cholinesterase was higher than those of both Cimetidine and Erythromycin at statistical level (Tables 1,4,5). Erythromycin and Sildenafil used here as etheylsuccinate and acetate salts respectively (the same salt used in the oral form of the drugs). Both salts are known as weak water soluble so that they show low affinity and % enzyme inhibition as the reaction mixture is an aqueous one, suggesting that these two drugs may show different ways and degrees of cholinesterase inhibition if they are used as more water soluble salts. Ciprofloxacin showed higher activity even at low concentration and had higher activity for the erythrocyte than plasma cholinesterase reaching to 86.69 % of erythrocyte for 100 μM concentration. Suggesting that Ciprofloxacin having higher affinity for cholinesterase than Cimetidine, Sildenafil and Erythromycin, but still considered a very weak inhibitor at statistical level (Table 2). Donepezil which is a known cholinesterase inhibitor (in this study we tested its ability to inhibit the cholinesterases in different concentration), we found that it is a

potent inhibitor, having a very high affinity toward erythrocyte than plasma cholinesterase (Table 3). This is in agreement with previous studies (25,26). Chlorpheniramine also can be considered as a weak inhibitor having a very higher affinity to plasma than erythrocyte cholinesterase , shows statistical significant result even at low concentration (table 6) , this is in agreement with the in vivo study (27). *In vitro* inhibition (expressed as pIC50) is considered as a simple, economic, rapid, relatively non-invasive and sufficiently correlated to in vivo studies (LD50) for toxicity risk assessment (28, 29). The pIC50 is of greatest value for prediction of toxicity when measured for cholinesterase because that is the enzyme related to toxicity (28, 30). Drugs used in this study were arranged in an ascending manner from the lower to the higher toxic one in the following order (Sildenafil < Erythromycin < Cimetidine < Ciprofloxacin < Chlorpheniramine < Donepezil) according to their plasma pIC50 and (Sildenafil < Erythromycin < Cimetidine < Chlorpheniramine < Ciprofloxacin < Donepezil) according to their erythrocyte pIC50.

Conclusion

The term (enzyme inhibitor) does not definitely involve all the enzyme systems in the body (i.e. an enzyme inhibitor may inhibit human enzymes in different ways and by different concentrations for each system). All drugs used in this study are well known as enzyme inhibitors, but only Donepezil and Chlorpheniramine showed higher affinity toward cholinesterase system , and Ciprofloxacin showed moderate activity to inhibit cholinesterase system but still not statistically significant. On the other hand Cimetidine, Sildenafil and Erythromycin showed little activity although they possess a good inhibitory effect on the liver enzyme system Cytochrome P.450 (CYP.450).

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