Chapter 8

Effect of antimalarials on human plasma butyrylcholinesterase

Introduction

The vertebrates contain two types of cholinesterases. acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1 1.8). The two enzymes differ with respect to their substrate and inhibitor specificity (1,2). Although the two enzymes show considerable sequence homology they differ with respect to the make-up of active site and differences in peripheral anionic site (3,4). Thus, both enzymes use serine-histidine-glutamate relay system for substrate hydrolysis. However, AChE has an acyl pocket and a cluster of aromatic amino acid residues near the lip of the active site gorge (5). In BuChE the F288 is replaced by valine, which results in enhanced butyrylcholine hydrolysis and also increases the sensitivity to bulky organophosphorous inhibitors like iso-OMPA (6). Besides, compared to 14 aromatic amino acids residue present at the lip of AChE , in BuChE 6 of them are replaced by aliphatic amino acids. The volume of the AChE active site gorge is 302.21 A^{o3} which is less than the van der-Walls volume of 317.6 A^{o3} for ethopropazine (7). Hence, ethopropazine is a poor inhibitor. The volume of BuChE active site gorge is ~ 200 A^{o3} larger than that of AChE gorge (7). This accounts for the differences in the binding of the bulky substrates and inhibitors to the two cholinesterases (3,7).

Earlier studies (Chapter 7) showed that human erythrocyte membrane AChE is inhibited by the antimalarials quinine, chloroquine and primaquine. The inhibition was of mixed type. Nevertheless, the antimalarials showed differential effect with respect to IC33 and IC67 and the Ki values; especially the values were very high for quinine. This was attributed to inaccessibility of quinine to the active site gorge.

Since as mentioned above the active site gorge has considerably larger volume in BuChE, this offers an opportunity to evaluate if three antimalarials have accessibility to the active site or still show differential inhibitory action on human BuChE, similar to that noted earlier for AChE (Chapter 7). The results of these experiments are described below.

Materials and Methods

Chemicals

Chloroquine phosphate, primaquine phosphate, quinine hydrochloride, and butyrylthiocholine iodide (BCTI) were purchased from Sigma Chemical Company, U.S.A. DTNB was purchased from SRL, India. All other chemicals were of analytical reagent grade and were purchased locally.

Assay of BuChE activity

Blood was collected from normal healthy volunteers (both male and female, age 22-45 Yr) in heparinized vials The plasma was separated by centrifugation at 2000 rpm for 10 min. The BuChE activity was assayed by the method of Ellman et al. with some modifications as described previously (8,9). The assay medium consisted of 100 mM Tris.HCl buffer (pH 7 4), 5 mM BCTI, 320μM DTNB, and about 10-20 μl of 1:20 diluted plasma as the source of the enzyme After incubating for 2 min at 37 °C, the reaction was started by the addition of the substrate and the increase in absorbance at 412 nm was recorded spectrophotometrically at intervals of 5 seconds. The activity is expressed as μmoles of BCTI hydrolyzed / min / ml of plasma

For the substrate kinetics studies the concentration of BCTI was in the range of 25 μM to 5 mM.

Studies with inhibitor

For inhibition studies the antimalarial were used in the range of 0.01 μM to 10 mM as indicated in Fig. 1.

Inhibition kinetics analysis were carried out using the equation (10):

$$Ki = \frac{i}{\frac{Kp(1+i/Ki)}{Ks} - 1} \qquad \text{and } K'i = \frac{i}{\frac{V}{Vp} - 1}$$

where Kp and Ks are Michaelis constants in the presence and the absence of inhibitor respectively, Vp and V are the velocities in the presence and the absence of inhibitor(10), and the Ki values were calculated.

Hill plot analysis for substrate-dependent increase in the velocity was by using the equation:

$$\log \left[\frac{v}{V-v}\right] = n \log [S] - \log k'$$

where V is the maximum velocity, v is the velocity in the presence of substrate concentration [S], k' is the constant and the Hill's coefficient (n) reflects the number of substrate molecules bound. The plot of log [v/(V-v)] vs log [S] is expected to be a straight line, the slope of which gives the Hill coefficient, n (10).

Hill plot analysis for antimalarials concentration-dependent inhibition of enzyme activity was by using the equation:

$$\log \left[\frac{V-v}{v} \right] = n \log [I] - \log k'$$

where V is the maximum velocity, v is the velocity in the presence of inhibitor concentration [I], k' is the inhibitor constant and the Hill's coefficient (n) reflects the number of inhibotor molecules bound. The plots of log [(V-v)/v] vs log [I] is expected to be a straight line, the slope of which gives the Hill coefficient, n (10).

The data for substrate kinetics were analyzed by the Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods for the determination of Km and Vmax (10). The values of Km and Vmax obtained by the three methods were in close agreement and were averaged

All the kinetics data were computer analyzed employing Sigma plot version 5.0 (11,12).

Results

As was noted for AChE (Chapter 7) incubation with the antimalarials also resulted in the inhibition of BuChE. The typical inhibition plots are shown in Fig I. As can be seen the three inhibition plots seemed to follow an identical pattern. This contrasts with the effect noted for AChE earlier (Chapter 7). From the individual plots the concentrations which bring about 33% (IC33) and 67% inhibition (IC67) were calculated. These values are given in Table I Thus 33% inhibition of the enzyme activity was obtained with a quinine concentration of 60 μM. for chloroquine and primaquine also these concentration were

Fig 1 Inhibition of BuChE by (A) Quinine, (B) Chloroquine and (C) Primaquine. The enzyme activity was determined in the presence of varying concentrations of the antimalarials The % of remaining activity (compared to the activity of uninhibited enzyme under standard assay conditions) is plotted on ordinate against log of antimalarial concentration (I) used on abscissa. The results are typical of 8 independent observations.

Fig. 1

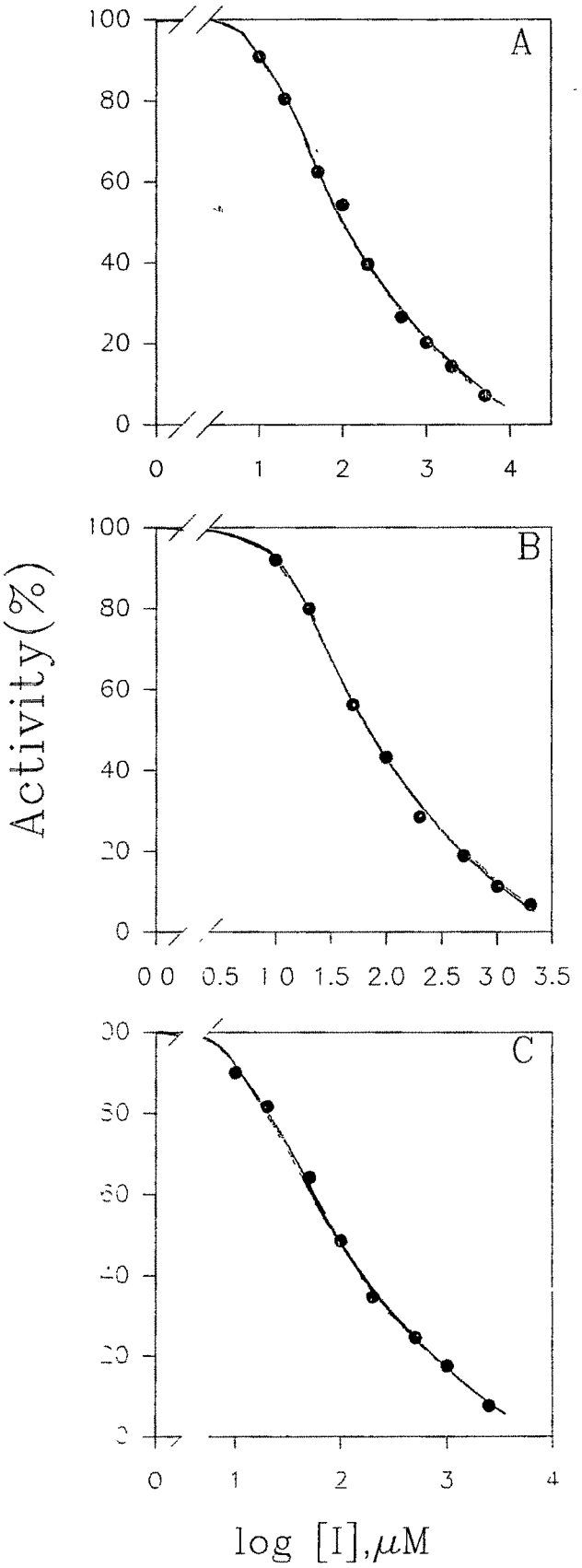

A
B
C
100
80
60
40
20
0
0 1 2 3 4
0 0 0.5 1 0 1.5 2.0 2.5 3 0 3.5
Activity(%)
log [I],μM

Table 1 IC33 and IC67 Concentration of Antimalarials for Human Plasma Butyrylcholinesterase

Antimalarial	IC33 (μM)	IC67 (μM)
Quinine	59.62±3.71	493.67±25.08
Chloroquine	37.31±3.19	230.30±7.41
Primaquine	50.62±1.24	427.01±22.96

IC33 and IC67 represents the concentration of antimalarial (μM) at which 33% and 67% inhibition was obtained. The results are given as mean ± SEM of eight independent observations.

comparable i.e. 40 and 50 μM respectively. Similarly, 67% inhibition of the enzyme activity was obtained at 500 μM concentration of quinine, for chloroquine and primaquine the corrosponding concentrations were 230 and 425 μM respectively It is obvious that the amount of antimalarials required to obtain 67% inhibition was about 6-8 times higher than that required for the 33% inhibition and not double. The results are consistent with the non-linear pattern of inhibition (Fig. 1).

It has been reported previously that chloroquine binds with BuChE (13). Since the antimalarials have the same basic quinoline ring structure but differ with respect to side chain substitutions, it was of interest to check the binding properties of the three antimalarials with the enzyme. This was evaluated by Hill plot analysis of the data obtained on the inhibition studies (Fig 1). Fig 2 shows the typical Hill plots for the three antimalarials. Quinine and primaquine showed biphasic plots and the value of Hill coefficient (n) decreased with increase in the concentration of the inhibitor. This indicated de-binding of the two antimalarials at higher concentrations. The transition in the binding pattern was seen at 84 90±5 95 and 78.11± 7.45 μM concentrations respectively of quinine and primaquine. By contrast, the Hill plots for chloroquine was a monophasic straight line with value of n equal to 1 over the entire concentration range. The value of Hill coefficient (n) for the three antimalarials are given in Table 2.

The typical substrate saturation curves and the corresponding Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden plots for uninhibited enzyme are shown in Fig. 3 from which it can be noted that BuChE resolved in three components differing in their kinetic properties i e. Km and Vmax values. These observations are consistent with the previously reported findings from our laboratory. (9)

Fig 2 Typical Hill plots for BuChE inhibition in the presence of (A) Quinine, (B) Chloroquine and (C) Primaquine. The experimental details and the concentration of antimalarials are as given in the text and in Fig 1. The transition point at which chloroquine shows de-binding is shown by vertical line.

Fig. 2

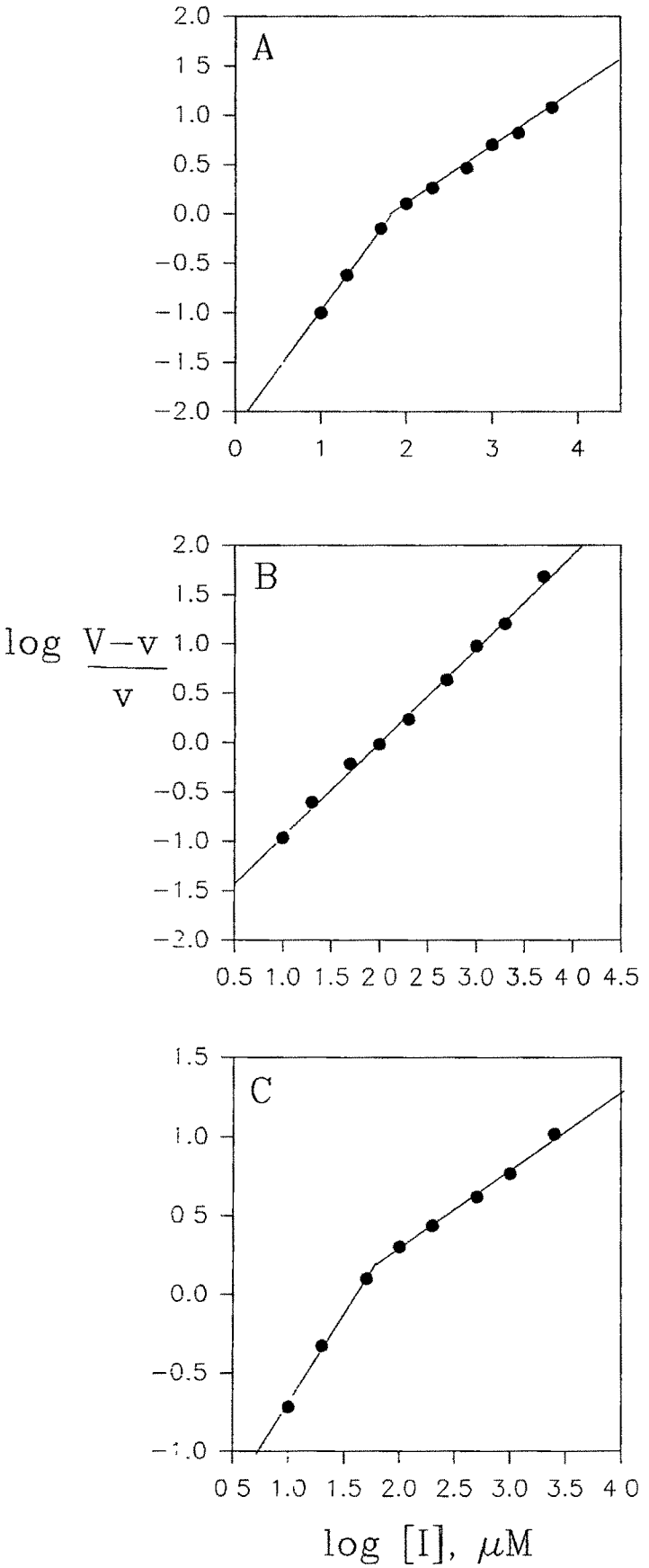

A
B
C
log V−v / v
log [I], μM
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
0
1
2
3
4
0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

Table 2 Binding of antimalarials to plasma BuChE.

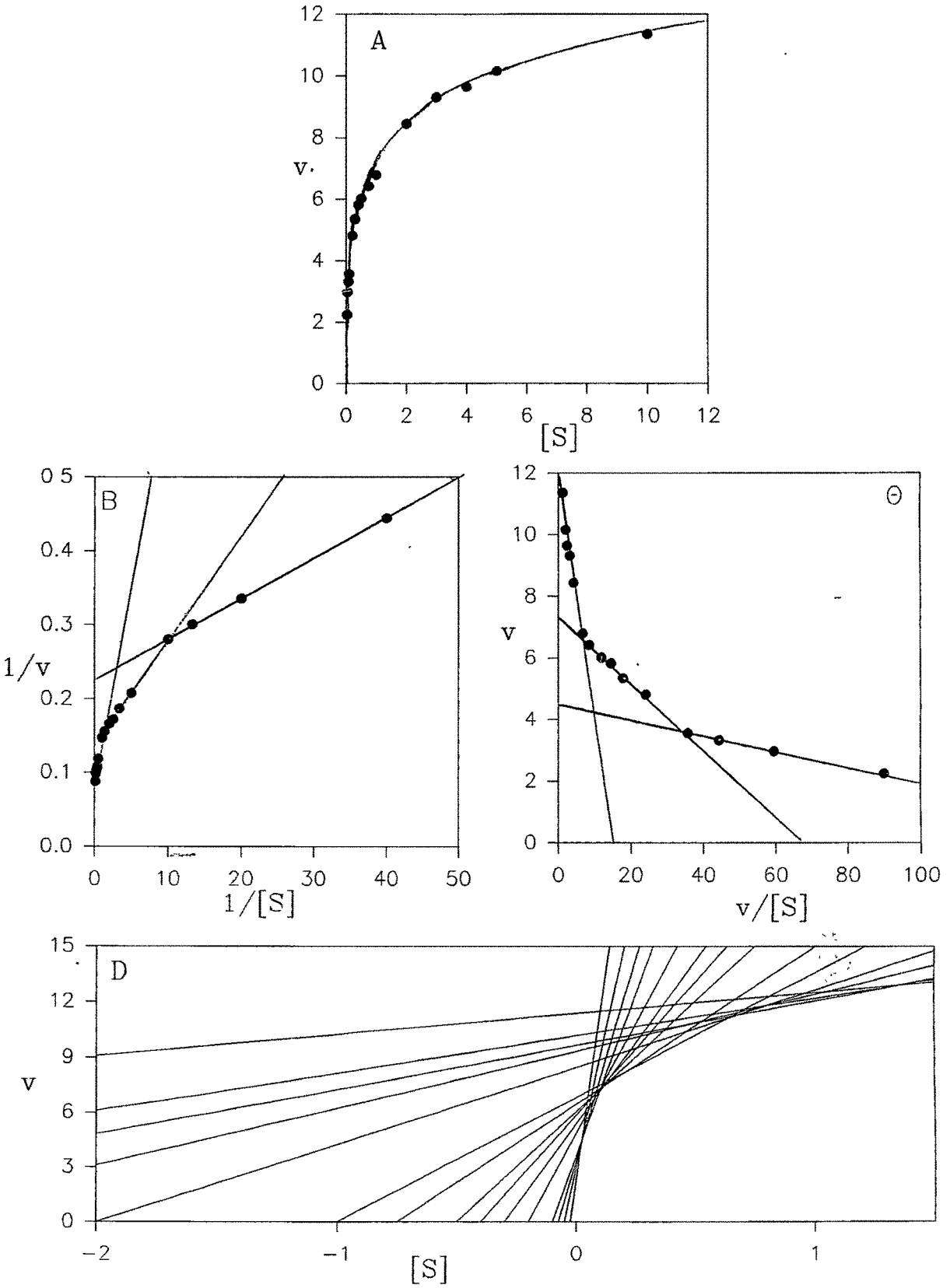
Antimalarial	Hill coefficient		Transitition point conc.(μM)
	n1	n2	
Quinine	1.08±0.10	0.62±0.04	84.90±5.95
Chloroquine	1.22±0.05	--	--
Primaquine	1.18±0.11	0.59±0.03	78.11±7.45

The Hill coefficients were calculated from the corresponding Hill plots.

The results are given as mean ± SEM of eight independent observations.

Fig. 3 Substrate kinetic analysis of uninhibited BuChE (A) substrate saturation, (B) lineweaver-Burk plot, (C) Eadie-Hofstee plot and (D) Eisenthal Cornish-Bowden plot. The results are typical of 8 independent observations for each group.

Fig. 3



To get an insight into the mechanism of inhibition by the antimalarials the substrate kinetic studies were carried out in the presence of the two inhibitory concentration i.e. IC33 and IC67 of quinine, chloroquine and primaquine. The typical substrate saturation plots in comparison with uninhibited enzyme are shown in Fig. 4. As is expected the decrease in activity was observed with increasing inhibitor concentration in the case of all the three antimalarials. The typical Lineweaver- Burk plots of the six different groups (referred to above) are shown in Fig 5. Under all the experimental conditions once again three components of BuChE were noted in all the cases except for IC67 of primaquine where only two components were evident. The computed values of Km and Vmax are given in Table 3. Thus the increase in quinine concentration caused proportionate increase in Km of all the components; simultaneously, the Vmax also decreased A similar trend was seen for chloroquine (Table 4). For primaquine a similar trend was noted for components I and III. Interestingly, component II seems to be completely inhibited (Table 5).

In order to get better insight into the nature of inhibition, separate Lineweaver- Burk plots for component I . II and III were plotted taking into considerations the componentwise data points(e g see Fig. 4) These plots are shown in Fig. 6 from which it can be noted that the inhibition in all the cases was of mixed type. This type of mixed inhibition is characterized by decrease in Vmax, increase in Km and intersection above the plane. For calculating the inhibitor rate constant (K_I) of this type of inhibition i.e. the mixed type, the equation used was the same as described in chapter 7 and in 'Materials and Methods' section. The Ki values of the antimalarials for the three components are given in Table 6. It was interesting to note that the Ki values for all the antimalarials were in the μM range. Thus for component I the K_I was lowest with chloroquine (~5 μM) followed by quinine (13 μM)

Fig. 4 Substrate saturation plots for BuChE in the presence of (A) Quinine, (B) Chloroquine and (C) Primaquine. The experimental details are as given in the text. The results are typical of 8 independent observations for each group

Fig. 4

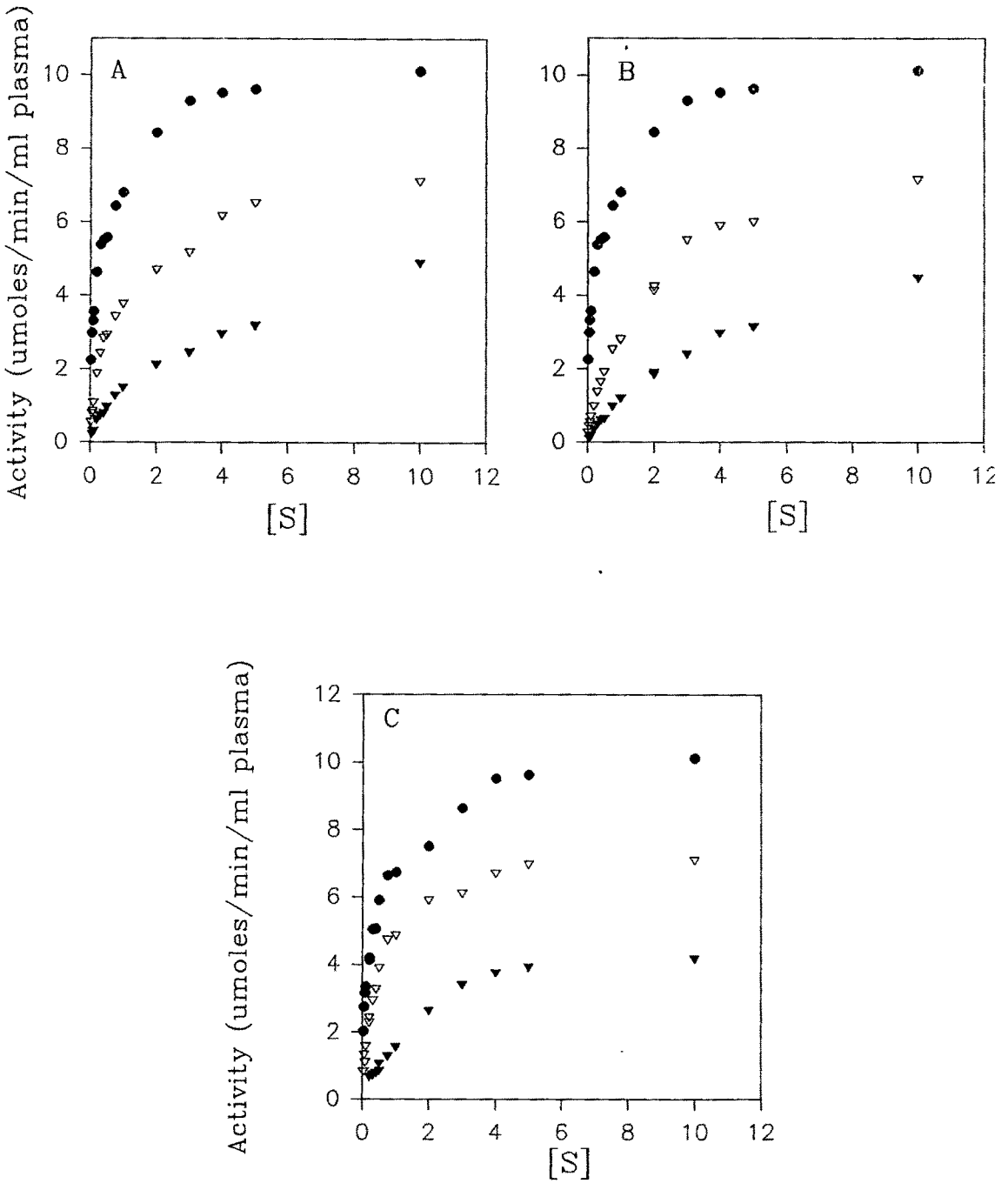


Fig 5 Lineweaver-Burk plots of BuChE in the presence IC33 and IC67 concentration of Quinine(A,B), Chloroquine (C,D) and Primaquine (E,F). The results are typical of 8 independent observations for each group

Fig. 5

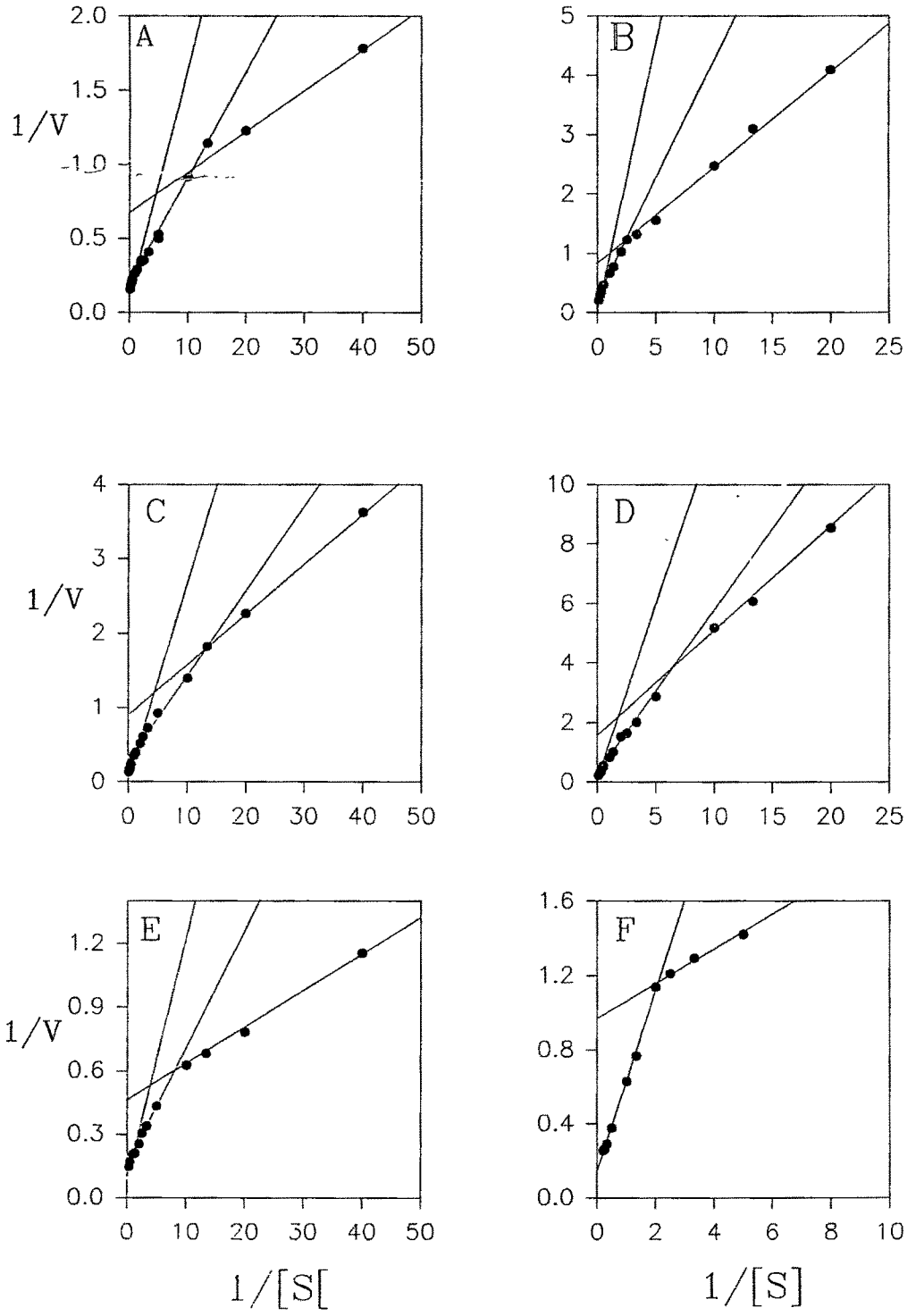


Table 3 Effect of quinine on substrate kinetics of BuChE.

	Component I		Component II		Component III	
Enzyme	Km	Vmax	Km	Vmax	Km	Vmax
Uninhibited enzyme	0.02±0.00	3.41±0.34	0.16±0.01	6.8±0.39	0.65±0.05	9.17±0.61
+Quinine IC33	0.08±0.01	1.64±0.26	0.44±0.08	4.84±0.24	1.27±0.07	7.61±0.16
+Quinine IC67	0.40±0.08	1.35±0.07	1.35±0.32	2.79±0.25	3.97±0.21	5.39±0.15

Experimental details are as given in the text. When quinine effect was studied the plasma was pre-incubated with two different concentrations of quinine for 2 min.
Km =μM;
Vmax is given as μmoles/min/ ml of plasma.
The Km and Vmax values were calculated by three different methods and averaged as described in text.
The results are given as mean ± SEM of eight independent observations.

Table 4 Effect of chloroquine on substrate kinetics of BuChE.

Enzyme	Component I		Component II		Component III	
	Km	Vmax	Km	Vmax	Km	Vmax
Uninhibited enzyme	0.02±0.00	3.41±0.34	0.16±0.01	6.78±0.39	0.65±0.05	9.17±0.60
+Chloroquine IC33	0.13±0.02	1.43±0.22	0.89±0.07	4.12±0.41	2.31±0.34	7.22±0.62
+Chloroquine IC67	0.39±0.11	1.04±0.25	1.78±0.31	3.10±0.10	4.93±0.22	6.27±0.49

Experimental details are as given in the text. When chloroquine effect was studied the plasma was pre-incubated with two different concentrations of chloroquine for 2 min.
Km =μM;
Vmax is given as μmoles/min/ ml of plasma.
The Km and Vmax values were calculated by three different methods and averaged as described in text.
The results are given as mean ± SEM of eight independent observations.

Table 5 Effect of primaquine on substrate kinetics of BuChE.

Enzyme	Component I		Component II		Component III	
	Km	Vmax	Km	Vmax	Km	Vmax
Uninhibited enzyme	0.02±0.00	3.41±0.34	0.16±0.01	6.80±0.30	0.65±0.05	9.17±0.60
+Primaquine IC33	0.06±0.01	2.50±0.31	0.28±0.03	5.66±0.39	1.09±0.07	8.97±0.65
+Primaquine IC67	0.32±0.06	1.58±0.24	---	---	3.82±0.52	7.03±0.23

Experimental details are as given in the text. When primaquine effect was studied the plasma was pre-incubated with two different concentrations of primaquine for 2 min.
Km =μM;
Vmax is given as μmoles/min/ ml of plasma.
The Km and Vmax values were calculated by three different methods and averaged as described in text.
The results are given as mean ± SEM of eight independent observations.

Fig 6 Lineweaver-Burk plots for component I (A,C,E) and component II (B,D,F) of BuChE in the presence of IC33 and IC67 concentration of Quinine(A,B), Chloroquine (C,D) and Primaquine (E,F). --■-- represents the uninhibited enzyme, --Δ-- represents the IC33 and --▲-- represents the IC67 group. The results are typical of 8 independent observations for each group.

Fig. 6

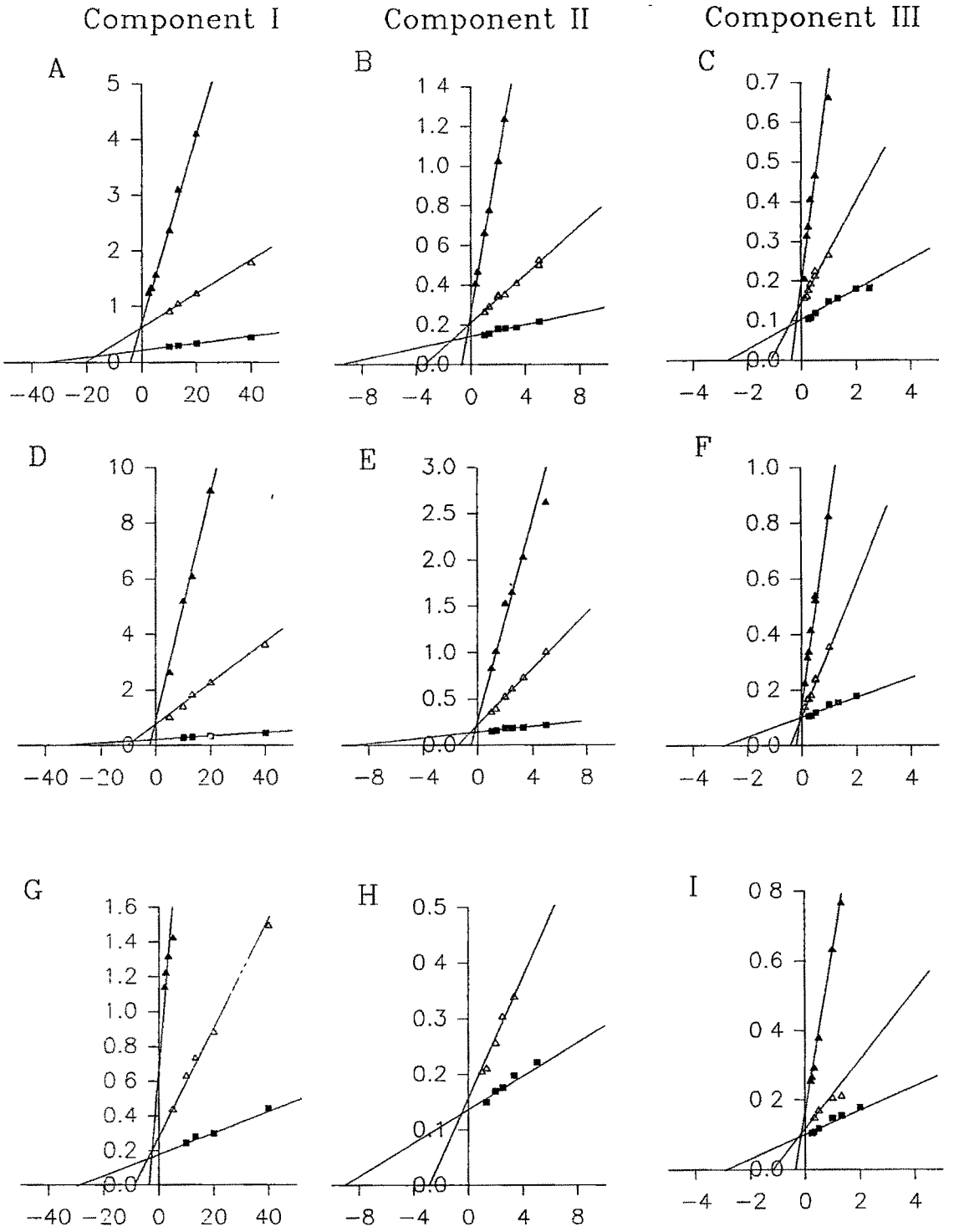


Table 6 The Ki values of antimalarials for BuChE.

Antimalarial	Ki, (μM)		
	Component I	Component II	Component III
Quinine	13.43±0.90	27.09±1.72	53.10±6.81
Chloroquine	4.56±0.49	8.75±1.58	19.67±2.42
Primaquine	18.63±1.79	54.89±5.17	89.64±14.74

The Ki values were calculated by using the equation given in the text.

The results are given as mean ±SEM of eight independent observations.

and primaquine (19 μM). For component II these values were doubled while for component III the values were 4 times higher (Table 6)

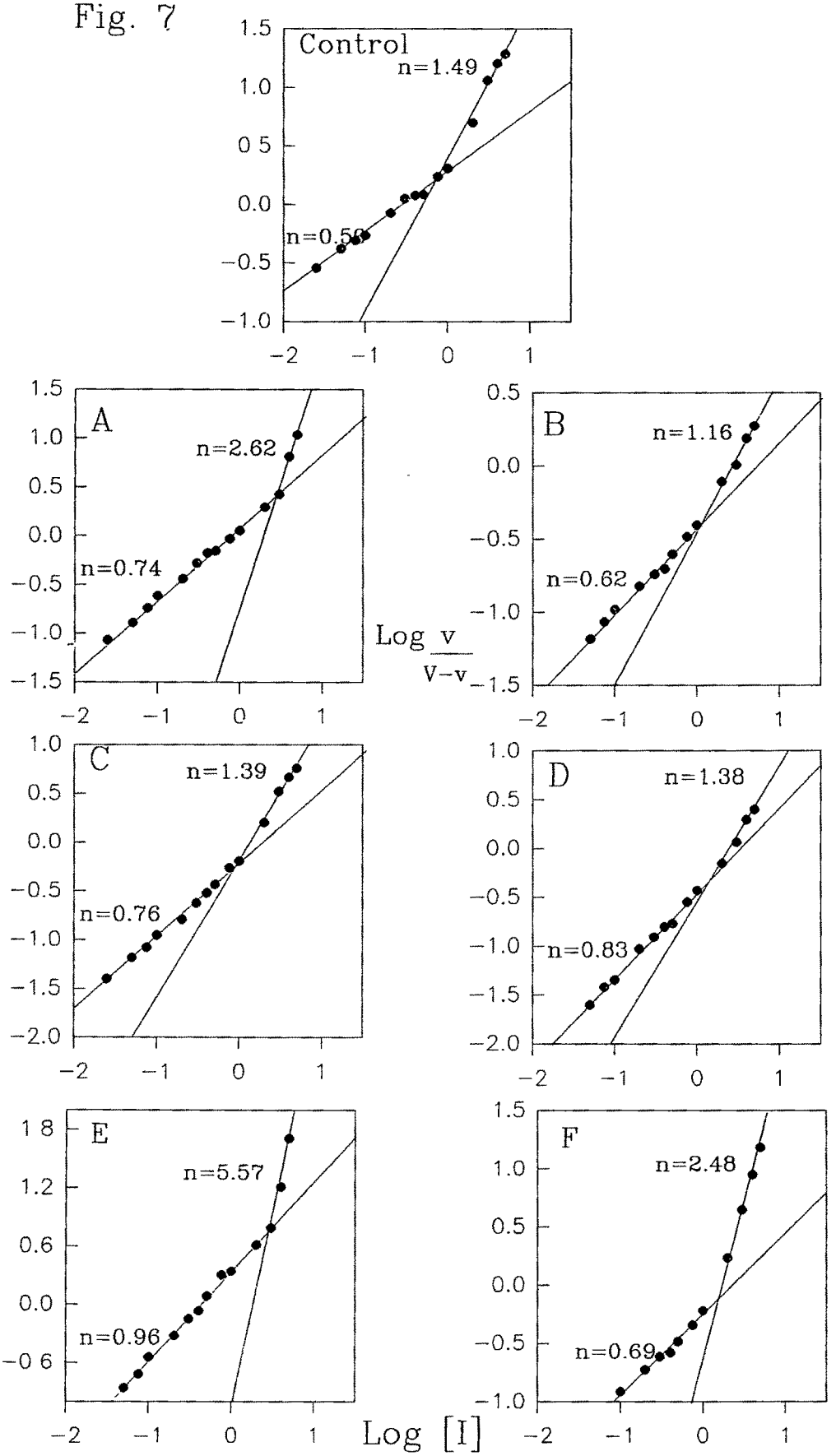
To ascertain the effect of antimalarials on binding of substrate molecules to BuChE in the presence of the antimalarials, Hill plot analysis was performed on the data given in Fig.4. The typical Hill plots are shown in Fig. 7. As can be noted, in the uninhibited enzyme one molecule of substrate bound to BuChE in low substrate concentration range whereas beyond 1 mM BCTI concentration, 2 molecules were bound. Both quinine and primaquine, in high substrate concentrations increased the number of substrate molecules bound in the presence of IC33 concentration; this number decreased in the presence of high (IC67) concentrations of antimalarials. These observations correlate well with the observed de-binding of these antimalarials at higher concentrations (Fig.2, Table 2) Chloroquine on the other hand did not show de-binding and hence the value of n remained unchanged throughout the entire substrate concentration range and the values were comparable to the control group

Discussion

From the data presented it is clear that the three antimalarials were almost equally effective in inhibiting the BuChE activity. Thus the IC33 and IC67 values were almost comparable for the three antimalarials although minor differences existed (Fig.1, Table1). These observations are in contrast to those noted earlier for the inhibition of AChE (Chapter 7 of the thesis); especially quinine was the least inhibitory for AChE. The three antimalarials also resulted in progressive inhibition of Vmax; at the same time increase in the Km values was noteworthy especially in the presence of IC67 concentrations. Thus the Km of

Fig 7 Hill plot analysis of the data presented in Fig 4. Quinine (A) IC33, (B) IC67, Chloroquine (C) IC33, (D) IC67 and Primaquine (E) IC33 and (F) IC67. n is the Hill's coefficient. The experimental details are as given in the text and in Fig. 4.

Fig. 7


Control
n=1.49
n=0.50
A
n=2.62
n=0.74
B
n=1.16
n=0.62
C
n=1.39
n=0.76
D
n=1.38
n=0.83
E
n=5.57
n=0.96
F
n=2.48
n=0.69
Log v/(V-v)
Log [I]

component I increased by 16-20 folds in the presence of IC67 concentrations of all the antimalarials (Table 3-5) The Km of component II increased by 8-11 fold in the presence of quinine and chloroquine respectively while this component was completely inhibited by primaquine The effect on Km of component III was the least in that the increase amounted to 4-6 fold Thus on a comparative scale it may be inferred that at the higher substrate concentrations inhibitory effects of antimalarials were partially overcome. The higher efficacy of antimalarials with respect to inhibition of BuChE activity is in contrast to that noted earlier (Chapter 7 of the thesis) with respect to AChE where the Km had increased for component I by 5-8 folds and for component II only by 2-3 fold.

The common denominator was that the inhibition of the three components of BuChE was of mixed type as in the case of AChE (Fig.6). Interestingly, however, the Ki values were comparatively very low. Also the Ki value for component II were about doubled compared to component I and increased by a factor of four for component III (Table 6). This is in contrast to the earlier observation that Ki values of the three antimalarials for the two components of AChE were almost the same (Chapter 7 of the thesis). It has been reported that the human plasma contains three isoforms of BuChE designated C1, C3 and C4 which represent respectively the monomeric, dimeric and tetrameric forms (1,14). The proportionate increase in Ki which is observed here (Table 6) is consistent with the reported polymorphism of plasma BuChE.

The peripheral anionic site of BuChE has D70 residue which is 1.1963 nm from the hydroxyl group of the active serine (S198) (3). Also W82 at the bottom of the active site gorge serves as a site for binding choline during catalysis (3). It is now generally agreed that D70 is a component of peripheral anionic site which functions to transmit the signal

from the top of the gorge to the bottom of the gorge resulting in the conformational change (3)

Viewed in this context, results of the present study suggest several things. Firstly, it would appear that the antimalarials which are quinoline-derived compounds, interact with the carboxyl group of D70 thereby interfering with the transport of the substrate to the active site gorge. It is possible that the interaction of the antimalarials with the D70 could induce conformational changes of different nature than those induced by substrate binding (3). However, this possibility needs to be verified experimentally. Nevertheless, assuming that the conformational changes occur (which is a distinct possibility), these would lead to alteration in substrate transport and hence the activity.

It has been suggested for cholinesterases that the entry of the inhibitor into the active site gorge can interfere with the binding of the substrate at the active site thereby resulting in decreased reaction velocity and apparent inhibition (9). The active site gorge of BuChE is larger in volume than that of AChE (7). The difference in the inhibitor specificity has been explained on the basis of accessibility of the inhibitor at the active site gorge (1,7). The results on inhibition with IC67 concentration directly relate to the aforementioned facts.

Thus as has been pointed above, the Km values increased in the presence of the three antimalarials and the effect was more dramatic when IC67 concentrations were used (Table 3-5) The effects of antimalarials in the lower concentration range i.e. IC33 may relate to the interference with D70 residue involved in the initial substrate binding and transport (3) On the other hand when IC67 concentrations were used (which are about 8 times higher e g. see Table 1), under these conditions the antimalarials will not only

interact with D70 but may also reach the active site gorge and interfere with the binding of the substrate at the active site. The substantial increase in the Km values noted under these conditions (Tables 3-5) is consistent with such an assumption.

BuChE is known to be synthesized in liver and is secreted in the plasma (15). The exact functions of BuChE are not known. However, it is known to hydrolyze drugs such as aspirin, succinylcholine, heroin and cocaine (16-18). In addition BuChE has a positive effect against organophosphate poison when administered as a prophylactic treatment in mice, rats and non human primates (19-22). This suggests that it may serve as a scavenging enzyme in the detoxification of natural compounds. Recently a mutant BuChE the G117H mutant has been produced which can hydrolyze organophosphates (23,24). Thus the results of the present study suggest that prolong treatment with antimalarials can compromise the scavenging and detoxifying functions of BuChE.

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Summary

The antimalarials quinine, chloroquine and primaquine were found to be potent inhibitors of human plasma butyrylcholinesterase (BuChE). Chloroquine was the most effective inhibitor, followed by quinine and primaquine. The IC33 and IC67 concentrations were in the micromolar range and about 7-8 times higher concentration of antimalarials were required to obtain 67% inhibition than those required for 33% inhibition Hill plot analysis revealed that primaquine and quinine showed de-binding above 80-85μM concentration. The three antimalarials inhibited all the three kinetic components of BuChE. The three antimalarials affected the kinetic properties in almost a similar manner; Vmax values decreased while Km values increased significantly with the effect being greater at IC67 concentrations The inhibition was of mixed type The Ki values for component I were in μM range with the value being the lowest for chloroquine. Quinine and primaquine showed 3 and and 5 times higher values compared to chloroquine. Compared to component I the Ki values were 2 and 4 times higher for component II and component III respectively