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Graphical Kinetic Approach for Estimation of Various New Constants for Inhibition of Acetylcholinesterase by Cisplatin

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Abstract: This study is designed to determine new, rapid and efficient kinetic parameters of the inhibition of human erythrocyte membrane-bound acetylcholinesterase (AChE) by an expensive metallic chemical, "cisplatin". These kinetic parameters in the presence of inhibitor are as follows: Percent maximal activity of the enzyme (V_{mc}), inhibition rate of the reaction at the first phase of the reaction system (IR_{r1}), inhibition rate of the reaction at the second phase of the reaction system (IR_{r2}), time constant (k_t), velocity constant (k_v), fifty percent inhibition time (t_{50}), inhibition rate constant (K_{ir}), 50 % inhibition constant (K_{I50}), 99% inhibition constant (K_{I99}) and overall inhibition constant with respect to time factor (K_{is}). The current study is a short approach to the investigation of the new kinetic parameters of inhibition of AChE by cisplatin. It is useful approach for the estimation of several inhibition constants of enzymes by variety of chemicals and drugs.

Key words: Acetylcholinesterase, cisplatin, erythrocyte, enzyme, inhibition, kinetics

Introduction

Cis-Platinum (II) diammine dichloride (cisplatin) [$PtCl_2(NH_3)_2$] is one of the most efficient drugs currently available for chemotherapy against various human tumors. Its clinical usefulness is compromised due to its side effects which include, the inhibition of various enzymes [catalase, glutathione peroxidase and glutathione S-transferase] (Sadzuka *et al.*, 1992; Dedon and Borch, 1987), the frequent development of cisplatin resistance, nephrotoxicity (its effects on the proximal tubular apparatus, which can be detected by increased urinary excretion of brush-border enzymes, such as l-alanine-aminopeptidase and magnesium (Bokemeyer *et al.*, 1996). Cisplatin has also various side effects, such as nausea, vomiting, and loss of appetite. As a precautionary measure, this medication should not be taken by women who are pregnant or breast feeding. Since this medication potentiates the production of sperm in men, a reliable form of birth control is recommended while on medication (web-site). Cisplatin has been often used in combination with other anti-cancer drugs to achieve better results and minimize side effects. A combination chemotherapy consisting of cisplatin such as M-VAC (methotrexate, vinblastine, adriamycin and cisplatin) has been affective against invasive or metastatic transitional cell carcinoma (Noguchi *et al.*, 1994).

Cholinesterases (butyrylcholinesterase, EC 3.1.1.8 and acetylcholinesterase, EC. 3.1.1.7, AChE) are serine hydrolytic enzymes, classified according to their catalytic and inhibition specificity characteristics. Generally, they are measured to assess exposures to various toxic compounds such as war gases, nerve agents and insecticides intoxication but now-a-days, these are the major target enzymes for testing of the new anti-Alzheimer's drugs because those agents that have the greatest potency in the therapy of Alzheimer's disease are cholinesterase inhibitors (Kamal and Al-Jafari, 1999; Kamal *et al.*, 2000). Two forms of these cholinesterases coexist ubiquitously throughout the body and although highly homologous as being product of different genes on chromosomes 7 and 3 in humans, respectively (Yu *et al.*, 1999). AChE play a key role in hydrolysis of the neurotransmitter, acetylcholine at cholinergic synapses in the central and peripheral nervous systems at rates near that of a diffusion-controlled process, and thus terminate impulse of neurotransmission. In our previous reports, it was demonstrated that cisplatin has the ability to inhibit AChE activity *in vitro* but its mode of inhibition was very unusual, therefore, in the present study, we adopted new graphical approach to investigate several new inhibition constants (IR_{r1}, IR_{r2}, k_t, K_{I50}, K_{I99}, k_{stma}, C_{pkst5}, K_i, K_r, k_{tmin}, k_{tmax}, $Ci50$, K_h, t_{50max} and k_v) for exploring its mechanism of inhibition (Al-Jafari *et al.*, 1995; Kamal *et al.*, 1996a).

Materials and Methods

Materials: Acetylthiocholine iodide (ASCh; used as substrate), adenine, citrate-phosphate-dextrose solution, cisplatin and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (U.S.A.). Triton X-100 was purchased from Merck and bovine serum albumin was obtained from Fluka Chemika-Biochemika (Switzerland). Other chemicals of highest quality were purchased from various commercial sources.

Enzyme Preparation and Enzyme Assay: Human blood was collected from healthy males. Colorless ghosts were prepared essentially as reported previously (Kamal *et al.*, 1996b). Human erythrocyte membranes were homogenized in 1% Triton X-100. After centrifugation at 100, 000 g for 60 min at 4°C (Beckman ultracentrifuge L8-80), AChE activity was recovered in the supernatant. The enzyme assays were performed according to the spectrophotometric method of Ellman *et al.* (1961). The assay mixture contained 0.25 mM ASCh and 0.25 mM DTNB. Other assay conditions have been reported previously (Kamal and Al-Jafari, 1999).

Estimation of Protein: The protein content of the enzyme preparation was estimated according to the method of Lowry *et al*. (1951), using bovine serum albumin as standard. The interference by Triton X-100 was corrected as described previously (Al-Jafari *et al*., 1998).

Graphics: The plots were prepared by using GraFit program (Leatherbarrow, 1992). The correlation coefficient was computed by the linear regression analysis using the same program.

Results and Discussion

The log percentage activity of AChE inhibition by cisplatin (0.50-14.0 mM) at various incubation periods was analyzed by two types of analysis, first by polynomial regression analysis while secondly by linear regression analysis as shown in Fig. 1A and 1B

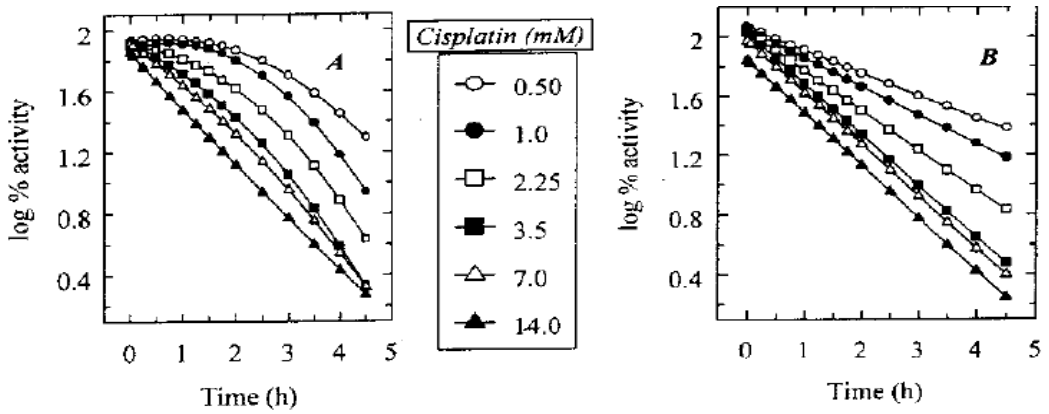

Fig. 1: A: Polynomial regression analysis of the inhibition of human erythrocyte AChE at different concentrations of cisplatin. The concentrations of cisplatin are presented in the box. B: Presentation of the same data with linear regression analysis, correlation coefficient was found to be 0.92, 0.91, 0.96, 0.96, 0.96 and 0.95 for 0.5, 1.0, 2.25, 3.5, 7.0 and 14.0 mM cisplatin respectively. Each point represents the mean of three trials

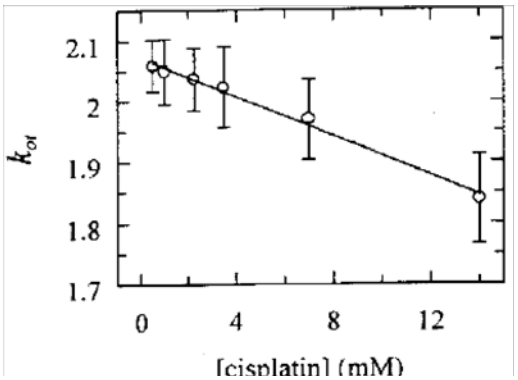

Fig. 2: Secondary replot of primary plots from Fig. 1. In this plot, k_{ot} (ordinate of each line of the primary plot) was plotted against concentration of cisplatin. Bars indicates standard error obtained by same linear regression analysis. The correlation coefficient was found to be 1.0

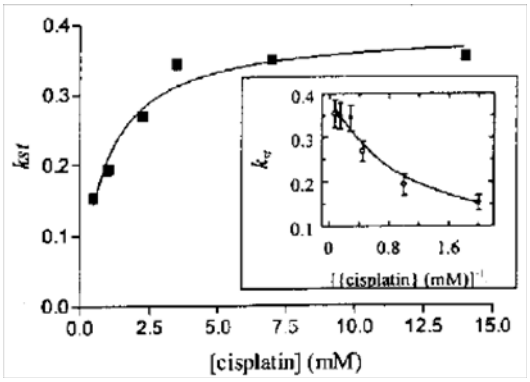

Fig. 3: Secondary replot of primary plots from Fig. 1. In this plot, k_{st} (slope of each line of the primary plot) was plotted against concentration of cisplatin. Inset represents plot of k_{st} against reciprocal concentration of cisplatin. Bars indicates standard error obtained by the linear regression analysis

respectively. The pattern of calculated regression lines for various concentrations of cisplatin versus the reaction time demonstrated that the reaction rate was variable. The V_{mc}; maximal activity of the enzyme in the presence of the cisplatin, IR_{r1} ; inhibition rate of the reaction in the presence of inhibitor at first phase of the reaction system and IR_{r2} ; inhibition rate of the reaction in the presence of inhibitor at second phase of the reaction system were estimated by polynomial regression analysis of the data and presented in Table 1. The time required for 50 % inhibition of AChE by cisplatin (t_{50}) was calculated from the following equation:

$$t_{50} = (\text{FP}-k_{ot})/k_{st}$$

where k_{ot} is the intercept and k_{st} is the slope. The intercept and slope were obtained after linear regression analysis of the data (Fig. 1B). The time constant (k_t) was also determined for each concentration of cisplatin from Fig. 1B, i.e., $k_t = k_{ot}/k_{st}$ (k_{ot} = intercept, k_{st} = slope).

A secondary replot of the primary plot from Fig. 1B is represented in Fig. 2. In this plot, the ordinate of each line of the primary plot was plotted against cisplatin concentration and the theoretical line of linear regression analysis was drawn through the experimental data point of the k_{ot}. This new plot demonstrated that the ordinate values were inversely proportional to the concentrations of cisplatin. This plot was useful for determining two inhibition parameters, K_{I50} and K_{I99} which indicated the sensitivity of enzyme for drug. The K_{I50} and K_{I99} is the concentration of inhibitor which blocks 50% and 99% of enzyme activity at overall incubation time respectively. The K_{I50} was calculated as follows:

$$K_{I50} = (\text{FP}-k_{oto})/k_{ots}$$

where k_{oto} is the intercept, k_{ots} is the slope and FP is for fifty percent. The intercept and slope were obtained from linear regression analysis. The K_{I99} was calculated as follows:

$$K_{I99} = (\log 1-k_{oto})/k_{ots}$$

By using these two equations, the K_{I50} and the K_{I99} were estimated to be 23 mM and 128 mM respectively.

The secondary replot of primary plot (i.e., log percentage activity of AChE as a function of time; Fig. 1B) is represented in Fig. 3. Where the slope (k_{st}) of each line of the primary plot was plotted against the concentration of cisplatin. The plot was found hyperbolic, therefore data was fitted in the nonlinear regression with the one-site binding curve model using the following

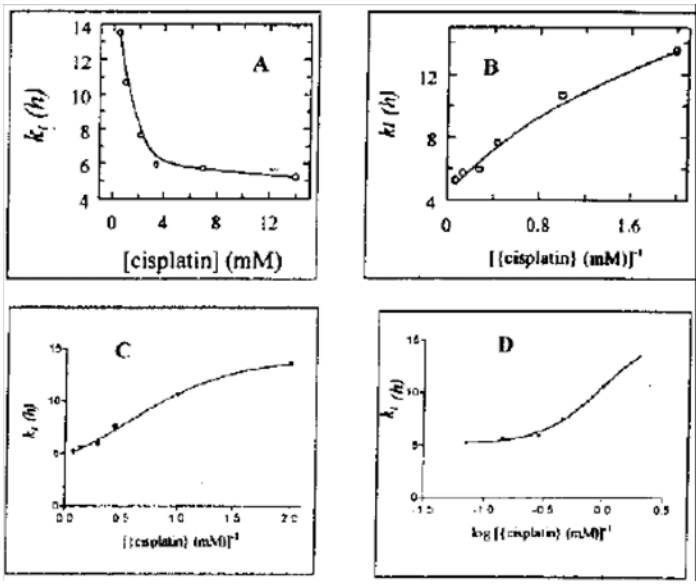

Fig. 4: Dependence of k_t on concentration of cisplatin for estimation of various parameters. A: Original data of k_t versus concentration of cisplatin. B: k_t versus reciprocal concentration of cisplatin. C: A plot of k_t against reciprocal concentration of cisplatin, data fitted in the equation for sigmoidal model. D: Replotted the same data in transform way i.e. transformation of cisplatin concentration into logarithm form for a standard sigmoidal model

Table 1: Maximal activity (%) of the AChE and its inhibition rate of the reactions in the presence of the cisplatin obtained by polynomial regression analysis.

[Cisplatin] (mM)	$^{!}V_{mc}$ (%)	$^{@}IR_{r1}$ $(h)^{-1}$	$\#IR_{r2}$ $(h)^{-1}$	$^{\$}$R. Ch. S.
0.5	83.64 ± 1.3	0.064 ± 0.036	-0.046 ± 0.0	0.0000801
1	76.47 ± 1.3	0.097 ± 0.036	-0.068 ± 0.0	0.000132
2.3	79.73 ± 0.0	-0.032 ± 0.0	-0.056 ± 0.0	0.0049
3.5	83.12 ± 0.0	-0.162 ± 0.0	-0.043 ± 0.0	0.021
7	82.55 ± 0.0	-0.257 ± 0.0	-0.022 ± 0.0	0.026
14	71.85 ± 0.0	-0.3854 ± 0.0	0.0075 ± 0.0	0.0324

$^{!}V_{mc}$, presents maximal activity of the enzyme in the presence of the cisplatin; $^{@}IR_{r1}$, inhibition rate of the reaction in the presence of inhibitor at first phase of the reaction system; $\#IR_{r2}$, inhibition rate of the reaction in the presence of inhibitor at second phase of the reaction system; $^{\$}$Reduced Chi squared

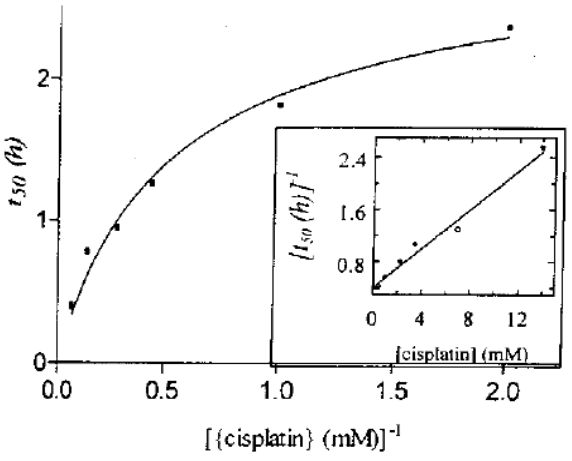

Fig. 5: Dependence of t_{50} with reciprocal concentration of cisplatin for the demonstration of inversely proportional behavior to cisplatin concentration. The inset represents the reciprocal of t_{50} against concentration of the cisplatin with linear regression analysis

equation:

$$k_{st} = k_{stmax} . [C_p] / C_{pkst50} + [C_p]$$

where C_p is for cisplatin. The value of k_{stmax} and C_{pkst50} were found as 0.39 ± 0.018 $(h)^{-1}$ and 0.86 ± 0.17 mM respectively while the value of R^2 was 0.956. When the k_{st} values were re-plotted against reciprocal of cisplatin concentrations, a single exponential decay type graph was obtained, therefore, polynomial regression analysis was applied and presented in the inset of Fig. 3, the value of reduced Chi^2 was found as 0.00038. This plot is valuable, because it was used to calculate K_{is}:

$$K_{is} = k_{sis}/k_{ois}$$

where k_{ois} is the intercept and k_{sis} is the slope. The intercept and slope were obtained from the regression analysis. From this analysis, the value of K_{is} was found to be 0.681 mM.

The plot for k_t against concentrations of cisplatin are shown in Fig. 4A. The shape of the plot was like decaying curve i.e. hypobolic. For this reason the k_t values were replotted against the reciprocal of cisplatin concentration, the plot was became approximate linear, therefore, its polynomial regression analysis was carried out and presented in the Fig. 4B. This plot

is useful for estimation of another new kinetic parameter known as inhibition rate constant (K_{ir}). This can be calculated by using the following equation:

$$K_{ir} = k_{oik}/k_{sik}$$

where k_{oik} is the intercept and k_{sik} is the slope. The intercept and the slope were obtained from the polynomial regression analysis of the k_t curve from Fig. 4B versus 1/[cisplatin]. The K_{ir} was calculated to be 0.596 mM, while the value of the reduced Chi^2 was found as 0.148. For a precautionary point of view, the data of k_t values were replotted against the reciprocal of cisplatin concentration by fitting in the following equation for testing its sigmoidicity:

$$k_t = k_{tmin} + [\ (k_{tmax} - k_{tmin})/1 + 10^{Ci50 - Ci).\ Hs}\ ]$$

where k_{tmin} and k_{tmax} is the minimum and maximum k_t respectively. While Ci, Ci50 and Hs are for cisplatin, that concentration of the cisplatin which gives a response halfway between k_{tmin} and k_{tmax} and Hill slope respectively. This testing plot (Fig. 4C), confirmed sigmoidal behavior due to the value of R^2 which was 0.995, therefore, finally, replotted the data in formal way by applying same equation after little modification due to transformation of cisplatin concentration into logarithm form (Fig. 4D) as follows:

$$k_t = k_{tmin} + [(k_{tmax} - k_{tmin})/1 + 10^{(LogCi50 - Ci).\ Hs}\ ]$$

The curve in Fig. 4D was found steeper having a value of 1.774 ± 0.454 for its Hs. The value of k_{tmin} , k_{tmax} and Ci50 were found 5.11 ± 0.37 h, 15.66 ± 1.82 h and 0.94 mM respectively (the value of R^2 was 0.996).

The plot for t_{50} against reciprocal form of the concentration of cisplatin is shown in Fig. 5, which is a rectangular hyperbolic, therefore data was fitted in the following equation:

$$t_{50} = t_{50max} \cdot [1/C_p]/1/K_h + [1/C_p]$$

where K_h is the equilibrium dissociation constant. The value of K_h and t_{50max} was estimated as 1.85 ± 0.34 mM and 2.91 ± 0.21 h respectively (the value of R^2 was 0.981). However, its reciprocal form was found to be linear so linear regression analysis applied and got a straight plot with correlation coefficient of 0.99 (Fig. 5 inset). Another new kinetic parameter (velocity constant, k_v) for this inhibition reaction was calculated from the slope of this plot. The value of k_v was obtained 0.149 ± 0.009 (mM h^{-1}).

The use of this unique approach may open up new avenues for the estimation of several such kinetic parameters of enzyme inhibition by various chemicals and drugs. The importance of these characteristic constants will be further revealed in the future after investigation of various inhibitory studies of different enzymes and receptors by low and high affinity ligands according to current analysis. The main beauty of this investigation is that so many parameters can be estimated by only one type of experiment, such as presented in Fig. 1. A computerized program package is also under consideration, which will facilitate the automatic drawing and calculations of these kinetic constants from secondary plots (Fig. 2-5) of Fig. 1. Moreover, these new kinetic constants will be fruitful in understanding the binding isotherm of various inhibitors with different enzymes *ex vivo*.

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