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Electrochemical and Spectroscopic Studies of Interaction of Clopidogrel Bisulphate with Calf Thymus DNA

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The DNA binding properties of clopidogrel bisulphate, was examined by cyclic voltammetry, fluorescence and UV spectroscopy. Shift in peak potential, decrease in peak current in cyclic voltammetry, hypochromic effect in UV titration and quenched emission intensity of ethidium bromide bound to calf thymus DNA signal indicated clopidogrel bisulphate has displaced ethidium bromide from its binding site in DNA. The binding constant is calculated using absorbance and is found to be closer to that estimated in voltammetric measurements. The results also corroborated well with fluorescence measurements and represent that the binding proceeds by intercalation

Keywords: Clopidogrel bisulphate, DNA binding, Cyclic voltammetry, Spectrophotometry.

INTRODUCTION

DNA strands are vital for life processes and their interplay of gene expression forms the basis for many myocardial diseases. The interaction of cardiovascular and myocardial drugs with nucleic acids is a subject of extensive research that provides an insight of the functioning of these drugs at a molecular level which may help in minimizing the side effects [1]. DNA also have a significant biological role as receptor molecules with which various drugs interact [2,3]. Electrochemical methods have since decades successfully proved to be simple and sensitive to investigate the binding between some ligands and DNA [4-12].

Clopidogrel bisulphate, an antiplatelet, blood clot inhibiting drug, preferred over its predecessor ticlopidine, is the world's second best selling drug which is now available to patients as generic drug. Clopidogrel bisulphate is routinely used in secondary prevention of ischemic stroke and myocardial infarction. Clopidogrel bisulphate [(methyl (2S)-2- (2-chlorophenyl)-2-(6,7-dihydro-4*H*-thieno[3,2-*c*]pyridin-5-yl)acetate] is an antiplatelet prodrug which generates a carboxylic acid metabolite *in vitro* [13-15] on hepatic metabolism [16]. The other degradation products are studied by reversed-phase HPLC [17] characterized by solid state stress conditions [18] and the structures are elucidated using LC-MS/TOF and LC-MS. Bulk clopidogrel and the tablet form are assayed by RP-HPLC [19], chemometry [20], spectrophotometry [21], TLC [22], HPTLC [23], normal phase LC [24] and squre wave voltammetry [25]. The redox behaviour was studied using cyclic voltammetry and differential pulse voltammetry techniques [26]. *In vivo* binding of active and inactive metabolites of clopidogrel with plasma protein [27], it's mechanism of action on P2Y12 receptors [28] and the influence exerted by genetic polymorphism [29] suggests that such interactions may suppress or enhance the availability and the metabolic pathway of the drug.

However, the interaction of clopidogrel bisulphate with ctDNA in solution has not been investigated by electrochemical methods. In the present work, the interaction of clopidogrel bisulphate with ctDNA was investigated by cyclic voltammetry, UV absorption and fluorescence spectroscopic experiments under neutral pH conditions.

EXPERIMENTAL

Calf thymus DNA and *tris*(hydroxymethyl)aminomethane-ticlopidine hydrochloride were purchased from Sigma Aldrich India. Clopidogrel bisulphate was of acceptable grade of purity received by Apotex India Ltd as a gift sample and used without further purification.

Spectroscopic measurements were made on Shimadzu double beam UV spectrophotometer UV-1800 (JAPAN) using 1 × 1 cm quartz cuvette. Cyclic voltammetry was performed in a potentiostat CH instruments USA model CHI660E. All the experiments were carried out in a conventional electrochemical cell. The electrode system contained a glassy carbon electrode as working electrode, a platinum wire as counter electrode and potassium chloride saturated calomel reference electrode. The

pH measurements were carried out with a Systronics335 Elico IL-120pH meter with a glass electrode. All the experiments were carried out at room temperature (26 ± 1 ºC). All fluorescence measurements were carried out on a F-4500 spectrometer (Hitachi, Tokyo, Japan) equipped with a 150W Xenon lamp source and 1cm quartz cells.

General procedure: 50 mM Tris-HCl was dissolved in aqueous buffer at pH = 7.1, filtered with a 0.8 µM Millipore filter before using. Calf thymus DNA was dissolved in Tris-HCl by incubation at 4 ºC for 24 h with occasional stirring to ensure homogeneity of solution. The DNA concentration was determined using ε259 nm = 6600 M^{-1} cm^{-1}. All other chemicals used were of analytical purity and all the solutions were prepared in double distilled deionized water.

RESULTS AND DISCUSSION

Voltammetric studies of interaction of clopidogrel with DNA: To investigate the scan rate effect, a cyclic voltamogram of 1 × 10^{-3} M clopidogrel in presence of in 0.1 M KCl as a supporting electrolyte was obtained showing a stable anodic peak at 1.18 V without ctDNA.

Voltamograms at different scan rates over a range of 0.05 to 0.3 V s^{-1} were obtained and overlaid in Fig. 1. It can be seen that peak current is proportional to the root of the scan rate while a minute potential shift is observed. The plots of anodic peak current *versus* square root of the scan rate (inset in Fig. 1) depicts an increasing trend suggesting that the electrochemical process is diffusion controlled with the regression equation and I_{pa} = 1.534 $\nu^{1/2}$($V^{1/2}$ $s^{-1/2}$) + 0.81

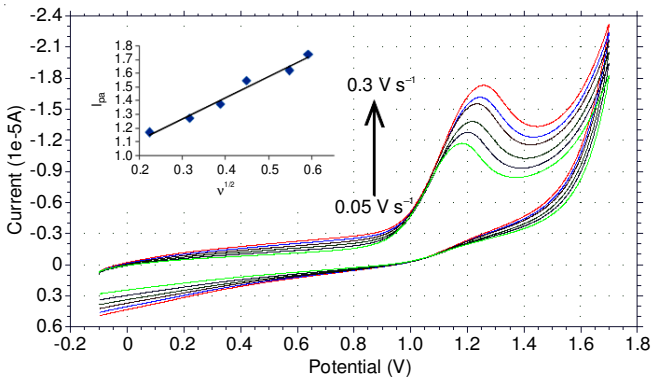

Fig. 1. Cyclic voltammogram for the oxidation of 1 × 10^{-3} M clopidogrel at different scan rates (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 V s^{-1})

Cyclic voltammograms of 1 × 10^{-3} M clopidogrel in the absence and presence of various concentrations of DNA in 50 mM *tris*-HCl/NaCl buffer pH 7.1 were obtained (Fig. 2). The peak current decreases upon the addition of the increasing concentrations of DNA (1.25 × 10^{-5} to 5 × 10^{-5} M), owing to the formation of electroinactive clopidogrel-ctDNA complex. This leads to a decrease in the diffusion co-efficient and thereby a decrease in electroactive species [5,10].

The peak potential shifted to a more positive value in the presence of ctDNA. This is a characteristic behaviour of the intercalation of clopidogrel into ctDNA double helix [30]. A plot of 1/1-(I_o/I) *versus* 1/[DNA] was constructed in Fig. 3 and from the ratio of intercept to slope, the value of K is calculated to be 2.3 × 10^2 indicating a weak interaction.

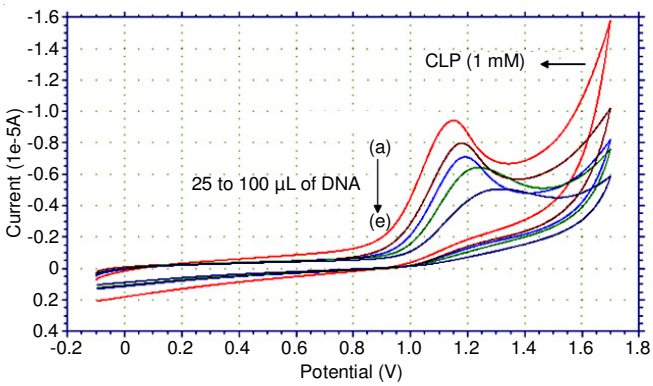

Fig. 2. Cyclic voltammograms of 1 × 10^{-3} M clopidogrel in 50 mM *tris*-HCl buffer of pH 7.1 in presence of (a) 0 µL DNA (b) 25 µL DNA (c) 50 µL DNA (d) 75 µL DNA (e) 100 µL DNA

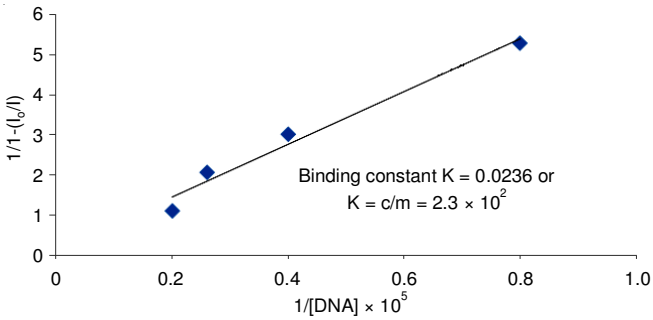

Fig. 3. Plot of 1/[1-(I_o/I)] *versus* 1/[DNA]

The binding site size can be obtained by the plot of C_b/C_f *versus* [DNA] (Fig. 4). The C_b/C_f ratio was determined by the equation $C_b/C_f = (I_o/I)/I$ [31]. The small value of the slope 0.1818 obtained from the plot confirms the electrostatic interaction of clopidogrel with ctDNA.

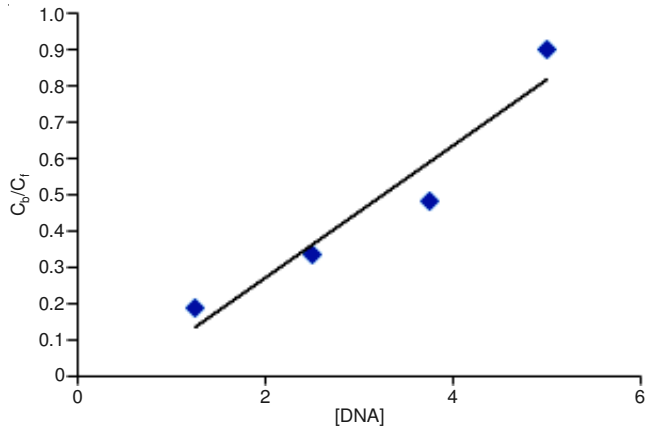

Fig. 4. Plot of C_b/C_f *versus* [DNA]

Absorption studies of interaction of clopidogrel with DNA: An absorption titration was carried out with fixed concentration of clopidogrel and varying concentrations of ctDNA recorded. The scanning range was set between 200 and 400 nm. The final volume was maintained at 3 mL by using 50 mM of *tris*-HCl buffer (pH 7.1). The addition of increasing concentrations of ctDNA (25 to 300 µL or 1.25 × 10^{-5} to 5 × 10^{-5} M) reduces the intensity in absorbance of clopidogrel and the hypochromism was found to be 98%. The electronic stacking interaction (Fig. 5) of clopidogrel with the base pairs of the helix indicates an intercalation of clopidogrel with ctDNA.

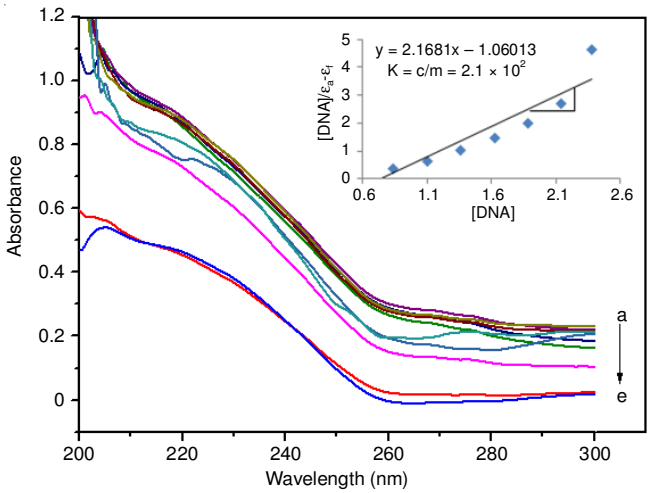

Fig. 5. UV-visible spectra of 1×10^{-3} M clopidogrel in presence of (a) 0 µL DNA (b) 25 µL DNA (c) 50 µL DNA (d) 75 µL DNA (e) 100 µL DNA

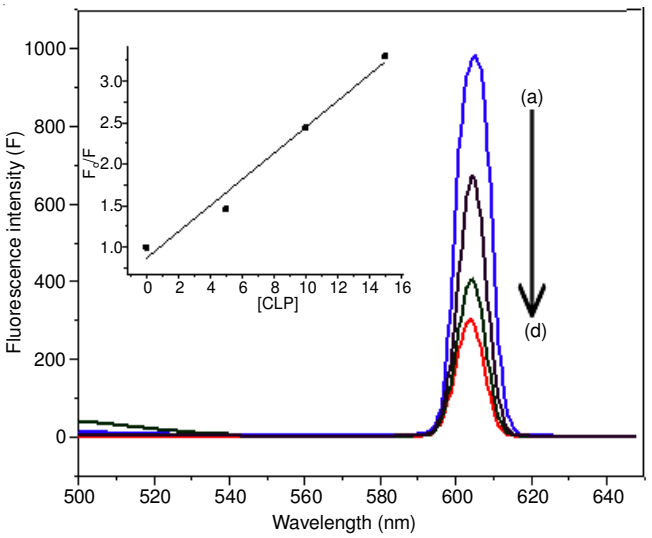

Fig. 6. Fluorescence spectra of 1×10^{-3} M ethidium bromide bound to ctDNA in the presence of (a) 0 µL clopidogrel (b) 25 µL clopidogrel (c) 50 µL clopidogrel (d) 75 µL clopidogrel (e) 100 µL clopidogrel

Binding constant K, was determined using the equation [32]:

$$\frac{[DNA]}{\varepsilon_a - \varepsilon_f} = \frac{[DNA]}{\varepsilon_b - \varepsilon_f} + \frac{1}{K_b(\varepsilon_b - \varepsilon_f)} \quad (1)$$

where [DNA] is the concentration of ctDNA in base pairs, ε_f, ε_a and ε_b correspond to extinction coefficient of free clopidogrel and bound to ctDNA, K_b is the binding constant obtained by the ratio of slope to the intercept and found to be 2.1×10^2 which is very close to the binding constant obtained cyclic voltammetric techniques. The Gibbs free energy at room temperature was found to be $\Delta G = -0.082 \times 293 \times 2.3 \times 100 = -5.1896$ KJ/mol.

Fluorescence studies of interaction of clopidogrel with DNA: In competition binding experiments, DNA and ethidium bromide concentrations were 1×10^{-3} M, while that of clopidogrel varied from 1.25×10^{-5} to 5×10^{-5} M. The excitation wavelength 440 nm was chosen and the emission spectra were recorded from 500 to 800 nm. In the reverse titrations, DNA samples with different concentration of complex was titrated with ethidium bromide and the fluorescence intensity recorded at 604.73 nm. The emission spectra of ethidium bromide bound to ctDNA in the absence and presence of increasing concentrations of ethidium bromide is shown in Fig. 6.

Fluorescence intensity of the signal decreased progressively due to quenching with an increase in concentration of clopidogrel indicating an intercalative mode of interaction [33]. The hypochromic effect in this experiment suggests a strong interaction between electronic states of the intercalating chromophore and that of DNA bases where the magnitude of this electronic interaction is expected to decrease as the cube of distance of separation between the chromophore and DNA bases [34]. The Stern-Volmer quenching constant K_{sv} was found to be 12.7×10^2 from the slope of plot F_o/F *versus* [CLP] (inset in Fig. 6) which is according to Stern-Volmer equation [35] $F_o/F = 1 + K_{sv}[CLP]$. The calculated binding constant in fluorescence competition binding study is larger than the value that was achieved from absorbance data. This is because the Stern-Volmer equation (eqn. 1) is based on the assumption that each base is a binding site. However, the bound complex actually covers three base pairs of DNA. Thus, for a double helix ctDNA, dividing $[DNA]_{total}$ by 6, K_{sv} obtained by absorbance studies would be six times smaller than K_b from the competitive binding experiment [36].

Conclusion

In the present work, the interaction of clopidogrel with ctDNA was studied using cyclic voltammetry, UV-visible spectroscopic and fluorescence spectroscopy. Experiments revealed the formation of complex between clopidogrel and ctDNA through intercalation. Cyclic voltammetry revealed the formation of electro-inactive species, UV-visible studies represented the decreasing of clopidogrel transitions due to the coordination with base pairs of ctDNA and fluorescence studies showed ethidium bromide in the presence of DNA was quenched by adding clopidogrel. Further research at the molecular level may provide a better understanding of exact mechanism behind the quenching mechanism of clopidogrel and ethidium bromide-ctDNA system.

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