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CHARACTERIZATION OF BINDING SITES OF CLOPIDOGREL AND INTERFERENCE OF LINOLEIC ACID AT THE BINDING SITE ON BOVINE SERUM ALBUMIN

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Abstract: Binding of clopidogrel to serum albumin has been characterized in the presence and absence of linoleic acid by equilibrium dialysis method where ranitidine and diazepam were used as specific probes. Our findings suggested two binding sites for clopidogrel: a high affinity site ($k_1 = 11.5 \times 10^5$ M^{-1}) with low capacity ($n_1 = 1.2$) and low affinity site ($k_2 = 2.1 \times 10^5$ M^{-1}) with high capacity ($n_2 = 9.3$). Interaction of linoleic acid with clopidogrel in the presence of ranitidine shows an increment of clopidogrel from 71 to 85.5% at concentration of (1×10^{-5} M) to (6×10^{-5} M). However, interaction of linoleic acid with clopidogrel in the presence of diazepam exhibits significant rise in free fraction of clopidogrel from 93 to 116% at concentration of (0×10^{-5} M) to (4×10^{-5} M). At higher concentrations, linoleic acid displaced clopidogrel from its binding sites on serum albumin. This may cause escalation of free drug in the blood, which alters pharmacokinetic properties of clopidogrel taken with high fat diet.

Keywords: clopidogrel, linoleic acid, bovine serum albumin, equilibrium dialysis, protein binding

Multiple factors impact the distribution of drugs in blood and tissues among which protein binding plays critical role (1). The binding nature of drug to plasma proteins is reversible, which allows slow transfer of the drug to the site of action (2). Drug-protein binding is non-selective and is not determined by chemical structure (3). Pharmacokinetics and pharmacodynamics properties of drugs are, therefore, influenced by protein binding. The most important drug-binding protein is albumin, a 65 kDa protein that is present in the blood, normally at a concentration of approximately 4.5%. Investigations based on probe displacement method revealed that human serum albumin (HSA) has three relatively high affinity binding sites commonly known as the warfarin or ranitidine site (site I), the benzodiazepine site (site II) and the digoxin binding site (site III) (2, 4-6). Drug binding to albumin may display low capacity or high capacity with varying affinities (3). Binding of other ligands including fatty acids, amino acids, steroids, metals, etc. is a remarkable feature of albumin (7-10). Besides albumin, α_1-acid glycoprotein binds several cationic drugs and macromolecules such as lipoprotein (for some lipophilic drugs) and globulin (for corticosteroids) may play a role in binding of drugs (11).

Dietary fatty acids are stored as fat in our body for two reasons: to build up lipid membrane around the cells and to release ATP (adenosine triphosphate) (12). Under circumstances, the stored fat releases fatty acids into the blood stream and bound to serum albumin which are eventually carried to different parts of the body. Each serum albumin has the capacity to carry seven fatty acid molecules and they are transported in such a way that their carbon-rich tail remains covered inside the deep crevices of protein (13).

Clopidogrel, an antiplatelet drug, is used to prevent myocardial infarction and stroke and to inhibit blood clots in coronary artery disease, cerebrovascular disease and peripheral vascular disease (14, 15). In clinical practice, prescribing clopidogrel with other drugs is very common. Co-administration of clopidogrel with some drugs including proton pump inhibitors, calcium channel blockers, etc., was reported to reduce the efficacy, whereas drugs

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including aspirin, angiotensin-converting enzyme inhibitors, etc., were reported to increase the efficacy of clopidogrel (16). Dietary factors such as curcumin, grapefruit juice have also been noted to interfere with clopidogel efficacy. Addition of ethyl esters of ω-3 fatty acids to the combination of aspirin and clopidogrel has been claimed to potentiate significantly platelet response to clopidogrel after percutaneous coronary intervention (17). However, till date, there are no such studies undertaken to demonstrate the interactions of dietary fatty acids with clopidogrel on the binding sites of serum albumin.

The concentration of free fatty acids in blood is increased by the consumption of fatty diet. Since free fatty acids are highly protein bound (18, 19), therefore, we assumed that dietary fat derived free fatty acids may displace or relocate the bound clopidogrel from its binding sites on albumin. This, in turn, may affect the pharmacokinetic property of clopidogrel. Therefore, the current *in vitro* study has been carried out to characterize the binding profiles of clopidogrel on serum albumin and to observe the interference of dietary linoleic acid with the binding of clopidogrel (2, 20). In this study, ranitidine was used as site I specific probe and diazepam was used as site II specific probe, because ranitidine binds specially on site I and diazepam binds specially on site II of albumin. Present study, carried out using bovine serum albumin (BSA) instead of HSA because of its structural similarity (21, 22), for the first time characterized the binding of clopidogerel with albumin in the presence of dietary fatty acids.

MATERIALS AND METHODS

Materials and instruments

Dialysis membrane (molecular weight cut off at 3500 Da) was purchased from Medicell International Ltd., UK. Bovine serum albumin (molecular weight approximately 66 kDa, fatty acid free, fraction V, 96-98%) and linoleic acid were obtained from Sigma-Aldrich, Germany. Ranitidine hydrochloride, diazepam, clopidogrel were gifted by Renata Ltd. (Bangladesh). Disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck, Germany. All other chemicals used were of commercial grade and purchased from local sources. UV-VIS spectrophotomer (UV-1800, Shimadzu Corporation, Japan) and water bath (Model: WS17-2; temperature range: 25-80°C, Sheldon Manufacturing Inc., USA) were used to carry out the experiment.

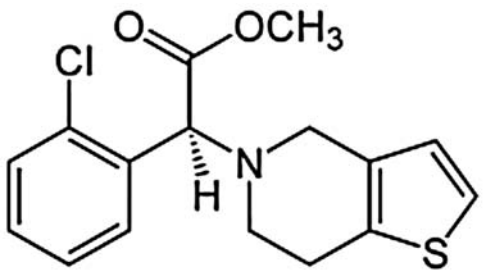

Figure 1. Chemical structure of clopidogrel (molecular weight = 321.82)

Preparation of dialysis membrane

To remove sulfur, dialysis membrane was cut into small pieces and then boiled for 8 h at 65-70°C in deionized water.

Preparation of standard calibration curve

Standard curves were prepared by plotting the absorbance against concentration taking different concentrations (0×10^{-5} M, 0.5×10^{-5} M, 1×10^{-5} M, 2×10^{-5} M, 4×10^{-5} M, 8×10^{-5} M, 12×10^{-5} M and 16×10^{-5} M of ranitidine hydrochloride, diazepam and clopidogrel in phosphate buffer (pH 7.4). Absorbance values were recorded at λ_{max} 318, 235 and 240 nm for ranitidine, diazepam and clopidogrel, respectively. At experimental conditions clopidogrel showed maximum absorbance of UV light at 240 nm, hence it was used to record the absorbance.

Determination of association constant

The association constant was determined using the previously established method (2). Briefly, for the determination of association constant, different concentration of clopidogrel solution (0.5×10^{-5} M, 1×10^{-5} M, 2×10^{-5} M, 4×10^{-5} M, 8×10^{-5} M, 12×10^{-5} M and 16×10^{-5} M) were prepared by mixing with freshly prepared BSA solution (2×10^{-5} M) in phosphate buffer at pH 7.4. After 12 h dialysis at 37°C in 20 rpm, samples were withdrawn and absorbances were recorded using UV-spectrophotometer.

Determination of binding site of clopidogrel

For the determination of the binding sites of clopidogrel two probes: ranitidine and diazepam were used as site I specific probe and site II specific probe, respectively. Both the probe concentrations were kept in 1 : 1 ratio with clopidogrel while varying the ratio between clopidrogrel and protein at a ratio of 1 : 1, 2 : 1, 4 : 1, 6 : 1, 8 : 1 and 10 : 1. After dialysis, free concentration of ranitidine hydrochlo-

ride and diazepam were calculated from standard calibration curve taking absorbances at λ_{max} 318 nm and 235 nm, respectively (2).

Effect of linoleic acid on clopidogrel binding to BSA

Interference of linoleic acid with clopidogrel binding on BSA was studied using the established methods (2). Briefly, clopidogrel (2×10^{-5} M), BSA (2×10^{-5} M) and linoleic acid (0 to 20×10^{-5} M) were mixed at a ratio of 1 : 1 : 0, 1 : 1 :1, 1 : 1 : 2, 1 : 1 : 4, 1 : 1 : 6, 1 : 1 : 8, 1 : 1 : 10 in the absence and presence of probes, respectively. Probes were added at two folds higher concentration than BSA and clopidogrel. After dialysis, absorbances were recorded and free fraction of clopidogrel was measured in each experiment.

RESULTS AND DISCUSSION

Estimation of binding parameters

The binding parameters of clopidogrel at pH 7.4 and at 37°C with albumin were evaluated by Scatchard plot (Fig. 2). Scatchard analysis showed a non-liner curve and extrapolation of the curve suggested that clopidogrel may bind with BSA at least on two sites: a high affinity binding site (n_1 = 1.2 approximately) with low capacity and a low affinity binding site (n_2 = 9.3 approximately) with high capacity. The estimation of association constant (Table 1) of clopidogrel to BSA at pH 7.4 showed that primary association constant termed as high affinity association constant ($k_1 = 11.5 \times 10^5$ M^{-1}) was reasonably higher. On the other hand, secondary association constant termed as low affinity association constant ($k_2 = 2.1 \times 10^5$ M^{-1}) was found to be about 6 fold lower in comparison to it high affinity association constant, which indicates that clopidogrel might bind to serum albumin with higher affinity.

Table 1. Estimated association constant (K_a) for clopidogrel at pH 7.4 and 37°C.

Association constant	
K_1 ($\times 10^{-5}$ M)	K_2 ($\times 10^{-5}$ M)
11.5 ± 0.76	2.1 ± 0.43

Values expressed as the mean ± standard error of the mean (SEM).

Identification and characterization of binding sites

Some drug molecules bind with specific site on serum albumin and can be used as probes for identification of binding site of other drugs. Ranitidine hydrochloride binds particularly on site I, whereas diazepam binds with site II of serum albumin (2). Hence, to identify the characteristic of binding of clopidogrel with albumin, it was induced for a reaction with BSA in the presence of ranitidine (as site I probe) and diazepam (as site II probe) and the results are presented in Figure 3. It is evident from this Figure that clopidogrel was able to displace both

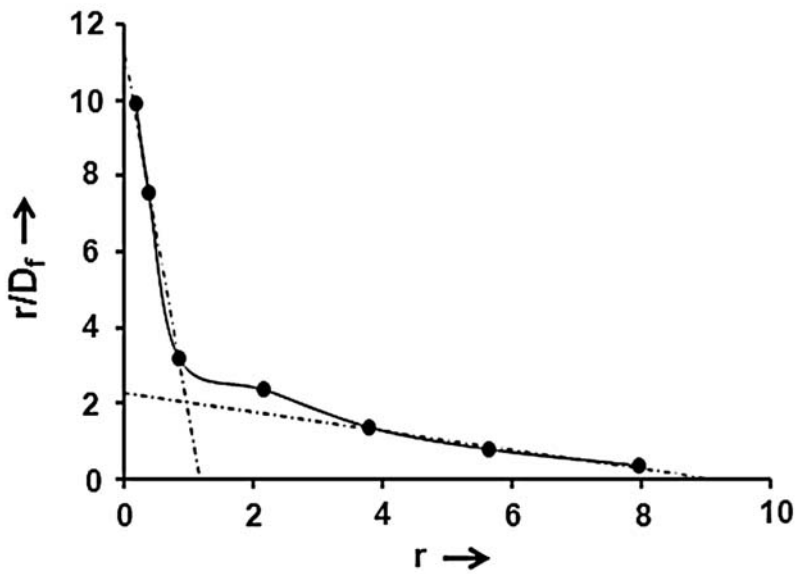

Figure 2. Scatchard plot. Clopidogrel at increasing concentration [0 to 20×10^{-5} M] was mixed with BSA [2×10^{-5} M] at pH 7.4 and 37°C. After dialysis, free fraction of clopidogrel was measured as described in the text. The ratio (r) of the molar concentration of bound drug to the molar concentration of protein was plotted against r/D_f where D_f is molar concentration of free drug

ranitidine and diazepam from its binding sites on BSA. However, the free fraction (%) of ranitidine hydrochloride was found to be increased from 0.835 to 5.3% (as percent of initial), from concentration 0 to 1×10^{-5} M. But, when increasing concentration of clopidogrel was added to BSA and ranitidine mixture, it was found that free fraction (%) of ranitidine decreases from 5.3 to 3.61% as percent of initial. This indicates that clopidogrel at higher concentration may enhance the binding of ranitidine with albumin.

On the other side, free fraction (%) of diazepam by clopidogrel was greatly increased from 8.6 to 27.3% (as percent of initial) from concentration 0 to 4×10^{-5} M and decreases to 23.35% at further higher concentration of clopidogrel (Fig. 3). These data provide the evidence that clopidogrel binds preferentially on site II because it displaces diazepam at higher level than ranitidine. This finding indicates that clopidogrel binds at site II on serum albumin with high affinity. This means that site II on albumin is the high affinity binding site for

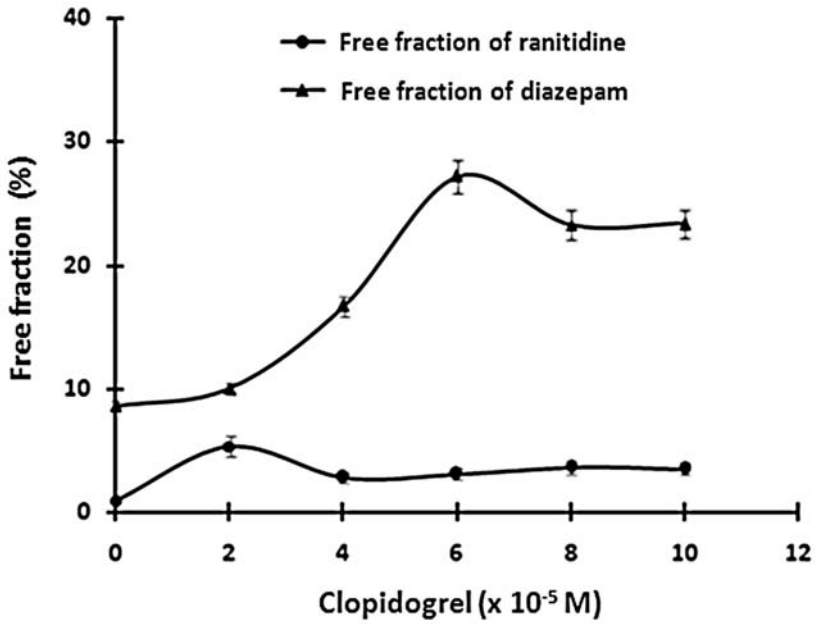

Figure 3. Release (%) of ranitidine and diazepam by clopidogrel from the binding sites on BSA. Clopidogrel was added to BSA-ranitidine (1 : 1) or BSA-diazepam (1 : 1) mixture at pH 7.4 and 28°C as described in the text. Free fraction of ranitidine or diazepam was estimated using standard calibration curve

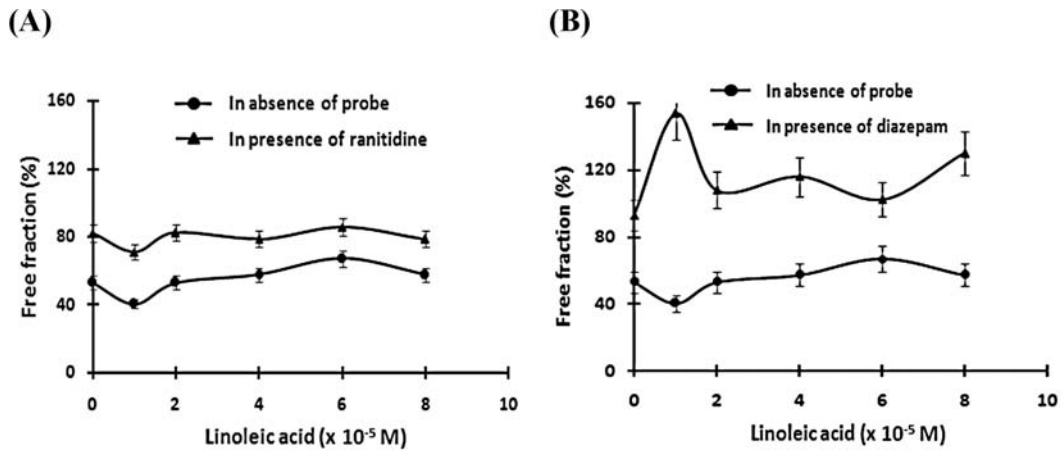

Figure 4. Displacement (%) of clopidogrel by linoleic aicd from the binding sites on BSA (A) in the absence and presence of ranitidine, a site I specific probe; and (B) in the absence and presence of diazepam, a site II specific probe

Figure 5. Interaction between clopidogrel and linoleic acid on binding sites of BSA (a proposed model)

clopidogrel. However, clopidogrel also displaces ranitidine to some extent from its binding site on BSA, and from this finding it could be assumed that at higher dose clopidogrel may also bind with site I on serum albumin as its low affinity binding site.

Interference between clopidogrel and linoleic acid for binding sites on BSA

To explore the possible interference of linoleic acid, a most common essential fatty acid in diet, with clopidogrel at the binding sites on albumin, binding studies were carried out in the presence and absence of probes as described under experimental section and the data are summarized in Figure 4.

In Figure 4, it is evident that with the increased concentrations of linoleic acid, the free drug percentage of clopidogrel increases, as drug displacement occurs from the binding sites on albumin. In the absence of ranitidine, a site I specific probe, linoleic acid was found to displace clopidogrel from the binding sites from 40.5 to 67% (as percent of initial). But in the presence of ranitidine, it was increased from 71 to 85.5% and then decreased to 78.5% (as percent of initial) at concentration of 8×10^{-5} M or higher. This fluctuation in free fraction may be due to the fatty acid binding to a complimentary site other than the drug binding site. Due to this, there might be a change in the shape of the drug binding site on BSA, which may lead to an increase in the half-life of the drug.

On the other hand, binding studies taking diazepam as site II specific probe showed (Fig. 4) that diazepam and linoleic acid bound at the same site and the free drug percentage of clopidogrel increased from 93 to 116% (as percent of initial) at concentrations from 0 to 4×10^{-5} M. Both diazepam and linoleic acid competitively bind to site II displacing clopidogrel, hence increasing the free drug concentration (Fig. 5). But at concentration of 6×10^{-5} M to 8×10^{-5} M, it is seen that in the absence of probe (diazepam) the free drug percentage of clopidogrel decreases compared to the presence of probe. This signifies that the fatty acid is binding to a different site other than site II in BSA. Moreover, comparing the displacement of clopidogrel by linoleic in the presence of ranitidine as site I and diazepam as site II specific probes, it was observed that in the presence of diazepam displacement of clopidogrel by linoleic acid was very high in comparison to ranitidine. It implies that ranitidine does insignificantly interference with clopidogrel since it binds with different site on albumin. This finding is in line with a recent study that oral ranitidine therapy with clopidogrel does not affect the efficacy of clopidorel significantly (23).

The protein binding characteristic of a drug is estimated by reviewing its capacity to displace specific probes from the binding sites on albumin. The binding of drugs to serum album strongly influences their pharmacokinetic behavior and is associated with drug safety issues, low clearance, low tissue penetration as well as drug-drug interactions (2, 5). In this study, it was found that clopidogrel has higher affinity for site II or the benzodiazepine site than site I or the ranitidine site.

Previous studies revealed that food derived fatty acids are highly protein bound and has the ability to displace other drugs, endogenous molecules from their binding sites, thus resulting in a pharmacological or physiological imbalance (5, 18). *In vitro* interaction of a polyunsaturated fatty acid, linoleic acid with clopidogrel has been studied here, on binding sites of BSA molecule where linoleic acid was found to displace clopidogrel from its binding sites on BSA.

Pharmacokinetic properties of a drug are influenced by various compounds and molecules. At times when the pharmacological properties of drugs

are related to its protein binding characteristic, especially in case of protein bound drugs, the therapeutic effect is related to the free drug plasma concentration (24). Any factor causing an alteration in the affinity of the drug towards albumin will directly cause the pharmacological property of the drug to be changed but this is not always indicated by its tissue distribution, elimination and activity (25, 26).

There are other drugs from different classes which also binds to site II or the benzodiazepine site on the BSA molecule like valsartan (angiotensin-II receptor antagonist), metoprolol succinate (β-blocker) and losartan potassium (angiotensin-II receptor antagonist) (2, 5, 26). Combination therapy, including any of these drugs if given along with clopidogrel, might be a chance of competitive displacement among drugs from the binding site II on the BSA molecule, whereby increasing the plasma concentration of the drug. Besides drugs, the same effect may be given by intake of foods containing linoleic acid (ω-3 fatty acid) like walnuts, fishes like shrimp, salmon, sardines, vegetables like Brussels sprouts, cauliflower, winter squash, chicken fat (27), certain vegetable oils like olive oil (28), sesame oil, sunflower oil, peanut oil. If any of these dietary sources are consumed in excess along with clopidogrel, then it may interfere with the plasma concentration of the drug. However, consequences in the changes of drug protein binding are complex and still debatable (29-31).

CONCLUSIONS

The objective of this particular investigation was to understand the interaction of dietary fatty acids with clopidogrel, an anti-platelet agent. It was observed that linoleic acid, a polyunsaturated fatty acid (PUFA), causes the release of clopidogrel from its binding site on albumin molecule and increases free fraction of clopidogrel. This finding indicates that consumption of PUFA may increase free fraction of clopidogrel in blood and enhances pharmacological activity of clopidogrel. Since clopidogrel is a narrow therapeutic drug, precautions must be taken and care should be exercised when taking this drug with PUFA-rich diet. But these data are at primary level and necessitate further intensive studies using *in vivo* experimental model. Only then we could be able to make conclusion about the pharmacokinetic behavior and pharmacological responses of the drug taking with fatty diet. However, findings of the present study with the present advancement on pharmacokinetic profiles of clopidogrel might be useful to understand the binding manners of the clopidogrel with HSA.

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Conflict of interest

The authors declare no conflict of interest.

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