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Research Article

Interaction of Palmitic Acid with Metoprolol Succinate at the Binding Sites of Bovine Serum Albumin

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Abstract
Purpose: The aim of this study was to characterize the binding profile as well as to notify the interaction of palmitic acid with metoprolol succinate at its binding site on albumin.
Methods: The binding of metoprolol succinate to bovine serum albumin (BSA) was studied by equilibrium dialysis method (ED) at 27°C and pH 7.4, in order to have an insight in the binding chemistry of the drug to BSA in presence and absence of palmitic acid. The study was carried out using ranitidine as site-1 and diazepam as site-2 specific probe.
Results: Different analysis of binding of metoprolol succinate to bovine serum albumin suggested two sets of association constants: high affinity association constant (k_1 = 11.0 x 10^5 M^{-1}) with low capacity (n_1 = 2) and low affinity association (k_2 = 4.0×10^5 M^{-1}) constant with high capacity (n_2 = 8) at pH 7.4 and 27°C. During concurrent administration of palmitic acid and metoprolol succinate in presence or absence of ranitidine or diazepam, it was found that palmitic acid displaced metoprolol succinate from its binding site on BSA resulting reduced binding of metoprolol succinate to BSA. The increment in free fraction of metoprolol succinate was from 26.27% to 55.08% upon the addition of increased concentration of palmitic acid at a concentration of 0×10^{-5} M to 16×10^{-5} M. In presence of ranitidine and diazepam, palmitic acid further increases the free fraction of metoprolol succinate from 33.05% to 66.95% and 40.68% to 72.88%, respectively.
Conclusion: This data provided the evidence of interaction at higher concentration of palmitic acid at the binding sites on BSA, which might change the pharmacokinetic properties of metoprolol succinate.

Introduction

Most of the drugs form a drug-protein complex with plasma proteins like albumin, alpha-1-acid glycoproteins, lipoproteins. Plasma protein binding of a drug is one of the determinants of drug's pharmacokinetic parameters. Serum albumin is produced by the lliver, with a molecular weight of 65000 – 69000 Da. This protein serves as a depot and transport protein for numerous endogenous and exogenous compounds, which plays an important role in binding phenomenon.[1] Albumin has the ability to bind reversibly with different kind of ligands. These ligands represent a spectrum of chemically diverse molecules, including fatty acids, amino acids (tryptophan and cysteine), steroids, metals such as calcium, copper and zinc, and numerous pharmaceuticals.[2,3]
Human serum albumin (HAS) has three highly specific binding sites. These sites are called the warfarin/ranitidine, the benzodiazepine and the digoxin binding sites, and are also denoted as site-1, site-2 and site-3 respectively.[4,5] Free fatty acids are highly protein bound and replace many drugs and other ligands from its binding site on albumin.[6,7]
Metoprolol succinate is a cardioselective β-blocker used in the management of hypertension, angina pectoris, cardiac arrhythmias, myocardial infarction and heart failure.[8] Due to its selective ß$_1$ receptor blocking property it is also prescribed in performance anxiety, social anxiety disorder and other anxiety disorders. A study has shown that metoprolol succinate is highly protein bound and suprapharmacologic concentration of NSAIDs increases the free fraction of this drug.[9] However until now, no report has been published about the interference of free plasma fatty acid in binding profile of metoprolol succinate. The concentration of free fatty acid in blood is increased by the intake of fatty diet. Since free fatty acid is highly protein bound, therefore, we hypothesized that dietary fat originates free fatty acid may displace the bound metoprolol succinate from its albumin binding site and also may interfere with the pharmacokinetic property of metoprolol succinate. Therefore present study was undertaken to characterize the binding profile as well as to notify the interaction of palmitic acid with metoprolol succinate at its binding site on albumin using bovine serum albumin employing equilibrium dialysis method.

Materials and Methods

Materials and instruments

Dialysis membrane (molecular weight cut off at 3500 Daltons) was purchased from Medicell International Ltd., UK and bovine serum albumin (molecular weight

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approximately 66210 Dalton, fatty acid free, fraction V, 96-98%) from Sigma Chemical Co., USA. Ranitidine hydrochloride, diazepam, metoprolol succinate were gifted by Novo Healthcare and Pharma Ltd. (Bangladesh). High-resolution UV-VIS spectrophotomer (SP8-400 UV/VIS Spectrophotometer, Thermospectronic, England) and Metabolic Shaking Incubator (Clifton Shaking Bath, Nickel Electro Ltd., England) were used in the experiment. All other chemicals used in the experiment were of commercial grade. Dialysis membrane used in the experiment was cut into small pieces and was boiled for 8 hours at 65-70°C in de-ionized water to remove sulfur.

Preparation of standard curve

For the preparation of standard curves of ranitidine hydrochloride, diazepam and metoprolol succinate solutions of different concentrations (0×10^{-5} M to16×10^{-5} M) of these drugs were prepared in phosphate buffer of pH 7.4 and absorbance values were taken at λ_{max} 318 nm, 235 nm and 222 nm respectively. Standard curves were obtained by plotting the absorbance values against the corresponding concentrations.

Estimation of association constant

To determine the association constant of metoprolol succinate, different concentrations (2×10^{-5} M to 20×10^{-5} M) of metoprolol succinate solutions were mixed with prepared BSA solution (2×10^{-5} M in phosphate buffered saline, pH 7.4) to get a final volume of 5 ml each. These solutions were allowed to stand for 10 minutes for the maximum binding of metoprolol succinate to BSA. From each mixture, 3.5 ml of solution was withdrawn and poured into previously prepared semi-permeable membrane tubes and both sides of the membranes were sealed properly. The membrane tubes containing drug-protein mixture were immersed in conical flasks containing 30 ml of 0.067 M phosphate buffer solution(pH 7.4). All the conical flasks were placed in a metabolic shaker for dialysis for 12 hours at 27°C in 20 rpm. This temperature was selected due to get higher binding affinity with BSA of drug.[10] Buffer samples were collected from each conical flask after dialysis and free fraction of metoprolol succinate was measured by UV spectrophotometer (λ_{max} 222 nm).[10]

Determination of binding site of metoprolol using ranitidine hydrochloride as site-1 specific probe

To determine the binding sites of metoprolol succinate at BSA, the concentrations of BSA and probe (ranitidine hydrochloride as site-1 specific probe) were remained fixed in 1:1 ratio (2×10^{-5} M: 2×10^{-5} M) and the concentration of metoprolol succinate was added in increased concentration (0 to 20×10^{-5} M). So, the final ratio of BSA: Probe: Metoprolol succinate were 1:1:0, 1:1:2, 1:1:4, 1:1: 6, 1:1:8, 1:1:10. The dialysis was carried out as described above and free concentration of ranitidine hydrochloride was measured at λ_{max} 318 nm. Only BSA solution and mixture of BSA and metoprolol succinate were used as positive and negative control during the measurement.[10]

Determination of binding site of metoprolol succinate using diazepam as site-2 specific probe

To determine the binding sites of metoprolol succinate at BSA, the concentrations of BSA and probe (diazepam as site-2 specific probe) were remained fixed in 1:1 ratio (2×10^{-5} M: 2×10^{-5} M) and the concentration of metoprolol succinate was added in increased concentration (0 to 20×10^{-5} M). So, the final ratio of BSA: Probe: Metoprolol succinate were 1:1:0, 1:1:2, 1:1:4, 1:1:6, 1:1:8, 1:1:10. The dialysis was carried out as described above and free concentration of diazepam was measured at λ_{max} 235 nm. Only BSA solution and mixture of BSA and metoprolol succinate were used as positive and negative control during the measurement.[10]

Effect of palmitic acid on metoprolol succinate binding to BSA

The effect of palmitic acid on metoprolol succinate, when bound to BSA was estimated in absence and in presence of site specific probes (using ranitidine hydrochloride as site-1 specific and diazepam as site-2 specific probes). In absence of site specific probes, the BSA and metoprolol succinate was mixed at 1:1 ratio (2×10^{-5} M: 2×10^{-5} M) and then palmitic acid was added in increasing concentration (0 to 20×10^{-5} M) to make final ratio of BSA, metoprolol succinate and palmitic acid in each experiment as 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:6, 1:1:8, 1:1:10. While in presence of probes; BSA, probe and metoprolol succinate were mixed at ratio of 1:2:1 and palmitic acid was added in increasing concentration to make the final ratio of BSA, probe, metoprolol succinate and palmitic acid as 1:2:1:0, 1:2:1:1, 1:2:1:2, 1:2:1:4, 1:2:1:6, 1:2:1:8, 1:2:1:10 in each analysis. Dialysis was carried out and the amount of free metoprolol succinate was measured in absence and in presence of probes as described above. Only BSA was used as positive control, mixture of BSA and palmitic acid, and mixture of BSA, palmitic acid and probe were used as negative control.[10]

Results and Discussion

Estimation of binding parameters

The binding parameters of metoprolol succinate have been characterized using Scatchard type of analyses of the binding of drug to albumin (Figure 1). Scatchard analysis of the binding of the drug at pH 7.4 and at 27°C provided a non-liner curve, suggesting the presence of at least two classes of binding sites for the binding of metoprolol succinate to BSA. As shown in Table 1, the number of high affinity binding site (n_1) for metoprolol succinate was approximately 2.1 (low capacity) and the number of low affinity binding site (n_2) was approximately 8 (high capacity). The high affinity association constant (k_1) for the metoprolol succinate binding to BSA at pH 7.4 is quite high (11.2×10^{5} M^{-1}), while the low affinity association constant (k_2) for this drug to BSA is about 3 fold lower (4×10^{5} M^{-1}) than that of primary association constant.

These findings indicate that metoprolol succinate is significantly bound to BSA.

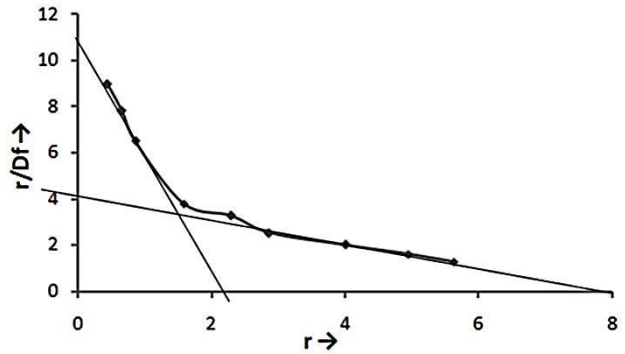

Figure 1. Scatchard plot for the binding of metoprolol succinate to BSA at pH 7.4 and 27°C. Metoprolol Succinate [0×10^{-5} M to 20×10^{-5} M] was added to BSA [2×10^{-5} M] and dialyzed as described under materials and methods. Free fraction of metoprolol was measured by UV-spectroscopic method and analyzed. r, the ratio of the molar concentration of bound drug to the molar concentration of protein; D_f, molar concentration of free drug.

Table 1. Association constant of metoprolol succinate bound to BSA at pH 7.4 (27°C)

pH	Association constant	
	$k_1(n_1=2.1\pm0.1)$(high affinity)	$k_2(n_2=8.0\pm0.1)$(low affinity)
7.4	11.0 ± 0.07	4.0 ± 0.03

Value represents the mean ± SEM of three independent experiments

Interaction of metoprolol succinate with site specific probes

The effects of metoprolol succinate on the binding of site specific probes were examined to determine whether metoprolol succinate binds preferentially with site-1 or site-2 on BSA. As mentioned under materials and methods, site specific probe and BSA were mixed at 1:1 molar ratio and metoprolol succinate was added in an increased concentration from 0×10^{-5} M to 16×10^{-5} M. The result showed that metoprolol succinate increased the free fraction of ranitidine hydrochloride and diazepam from 18.13% to 22.5% and 19.73% to 44.58%, respectively (Figure 2).

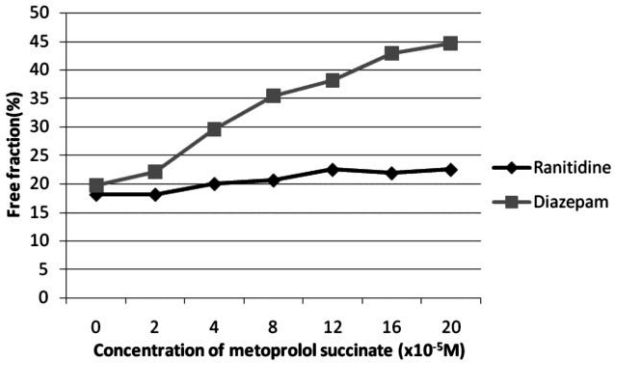

Figure 2. Free fraction of ranitidine or diazepam bound to BSA (1:1) upon the addition of metoprolol succinate. The concentrations used in the binding study were: [BSA] = [ranitidine] = 2×10^{-5} M; [BSA] = [diazepam] = 2×10^{-5} M; and [metoprolol succinate] = 2-20×10^{-5} M.

Interaction of palmitic acid with site specific probes

The effect of palmitic acid was measured to know whether it can release the ranitidine and diazepam from their binding sites or not. It was found that palmitic acid at a concentration of 0×10^{-5} M to 16×10^{-5} M increased the free fraction ranitidine from 16.0 to 39.8% and of diazepam from 11.5% to 58.7% (Figure 3).

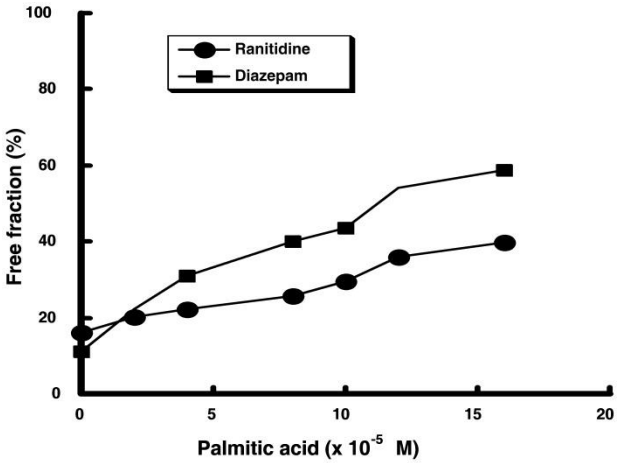

Figure 3. Free fraction of ranitidine (●) or diazepam (■) bound to BSA (1:1) upon the addition of palmitic acid at 27°C and pH 7.4. The concentrations used in the binding study were: [BSA] = [ranitidine] = 2×10^{-5} M; [BSA] = [diazepam] = 2×10^{-5} M; and [Metoprolol] = 0-16×10^{-5} M.

Interaction of palmitic acid with metoprolol succinate at the binding sites on BSA

The interactions at bindings sites on BSA were measured between metoprolol succinate and palmtic acid in the absence or in presence of site specific probes ranitidine and diazepam, respectively. In absence of both ranitidine and diazepam, palmitic acid increased the free fraction of metoprolol succinate from 26.27% to 55.08% with the addition of palmitic acid from 0×10^{-5} M to 16×10^{-5} M (Figure 4). Whereas, in presence of ranitidine and diazepam, palmitic acid at the same concentration, increased the free fraction of metoprolol succinate from 33.05% to 66.95% and 40.68% to 72.88%, respectively.

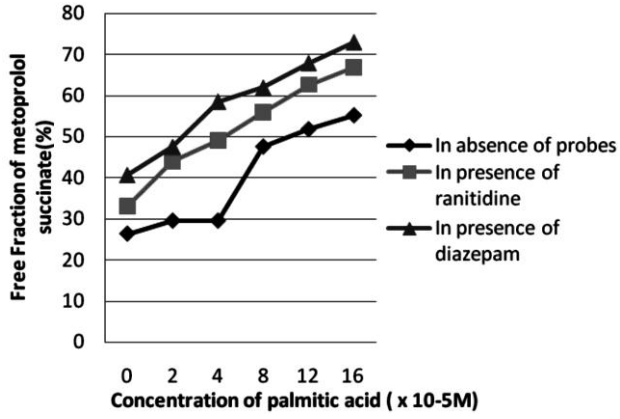

Figure 4. Free fraction of metoprolol succinate in the absence or presence of probes to BSA at 2:1 molar ratio upon the addition of palmitic acid at 27°C and pH 7.4. The molar concentrations used in the binding study were: [BSA]: [probe]: [metoprolol succinate] = 1:2:1; and [palmitic acid] = 0-16×10^{-5} M.

Binding of drugs are determined by studying its ability to displace the site specific probes. The association constants as shown in Table 1 indicate that metoprolol succinate is highly bound to BSA. Figure 2 shows the change in free concentration of ranitidine and diazepam by metoprolol succinate. It is seen that the free concentration of ranitidine hydrochloride increased from 18.13% to 22.50%, whereas, the free concentration of diazepam was increased from 19.73% to 44.58% by the same drug. From this observation it can be said that metoprolol succinate at higher concentration displaced diazepam to a greater extent as compared to ranitidine, so metoprolol succinate has greater affinity for site-2 than for site-1 on the BSA molecule. This implies that at a lower drug to BSA ratio, metoprolol succinate binds to its high affinity site i.e., site-2 or the benzodiazepine site, whereas at higher ratio it not only binds to its high affinity site but also to its low affinity site i.e., site-1 or the warfarin/ranitidine site on the BSA molecule.

The pharmacokinetic properties of drugs are influenced by exogenous as well as endogenous compounds by binding to serum albumin in a reversible manner. Regular diet contains various fats and fatty acids. These fatty acids are highly protein bound and displaces drugs and other endogenous molecules from their binding sites on albumin.[7-9] Plasma protein binding properties are considered to be the primary determinants of the pharmacokinetic properties of many drugs. Therefore any alteration or change in the serum albumin binding of these drugs might lead to a change in the pharmacokinetic properties of these drugs. Figure 4 showed the change in the free concentration of metoprolol succinate bound to BSA in the presence of palmitic acid at pH 7.4 and at 27ºC. As observed in Figure 4, the free fraction of metoprolol succinate was increased from 26.27% to 55.08% by palmitic acid in the absence of probes. Whereas in the presence of ranitidine, this increment was from 33.05% to 66.95%. While site-2 was blocked by sufficient amount of diazepam, palmitic acid increased the free fraction of metoprolol succinate from 40.68% to 72.88% as shown in Figure 4. These findings do not contradict with the number of high and low affinity binding sites of metoprolol succinate on BSA. This suggests that metoprolol succinate is displaced to a greater extent by palmitic acid in the presence of diazepam compared to ranitidine. We found that palmitic acid, a saturated fatty acid, displaces metoprolol from its binding sites on BSA. As there is strong analogy between BSA and HSA, it is assumed that similar types of binding characteristics will be exhibited by metoprolol succinate when bound to HSA. Sometimes the pharmacologic activity of a drug is related to its protein binding. If a drug shows less affinity for albumin due to any alteration in protein binding, the pharmacologic effect of the drug may be significantly altered.[11-13] Because, therapeutic effect of a protein bound drug is related to its free drug concentration in plasma and at the target site.[14] But this is not always indicative of its tissue distribution, its elimination or its activity.

Conclusion

In our study, palmitic acid increases the free fraction of metoprolol succinate. This probably enhance the pharmacological activity of metoprolol succinate. Therefore, precaution should be taken while using therapeutically. However, the results of the present study in combination with the current advances in the binding of metoprolol succinate and interaction with fatty acid might be helpful in realizing the overall binding behavior of the drug with human serum albumin. Nevertheless, it is too early to draw such conclusion about the pharmacokinetic/pharmacological properties of the drug from our limited data. It deserves a more detailed study using *in vivo* experimental model.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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