Identification and Characterization of the Binding Sites of Amitriptyline on Bovine Serum Albumin

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Abstract: Background: Depression is a mental disorder marked by alteration mood. An estimated 3 to 5% of the world's population experience depression on any given date. Amitriptyline is an antidepressant drug. Combination therapy is common practice now a day. During the concurrent use of drugs all the drugs may exert their effects independently or may interfere or interact with each other in biopharmaceutical, biochemical or in pharmacological point of view. No previous report is available concerning binding affinities and specificity of binding of amitriptyline. **Results:** The binding of amitriptyline on Bovine Serum Albumin (BSA) was studied by Equilibrium Dialysis (ED) method using warfarin (as site-I specific probe) and diazepam (as site-II specific probe) to determine the binding sites of amitriptyline to BSA. The data obtained showed that the free concentration of warfarin increased very rapidly by the addition of amitriptyline while the free concentration of diazepam increased slowly by the addition of amitriptyline. Again in the reverse experiment, it is shown that the free concentration of amitriptyline increases very rapidly by the addition of warfarin, while the free concentration of amitriptyline increased slowly by the addition of diazepam. **Conclusion:** This site-specific probe displacement data implied that site-I (warfarin site) is high affinity site while site-II (diazepam site) is low affinity site of amitriptyline to the BSA.

Key words: Amitriptyline, binding site, BSA, drug interaction, equilibrium dialysis

INTRODUCTION

The formation of drug plasma protein complex is often termed as drug plasma protein binding. Serum albumins, the most abundant protein in blood, play a very important role in the binding phenomenon and serves as a depot protein and transport protein for numerous endogenous and exogenous compounds (Kragh-Hansen, 1981). The reduction in the extent of binding of a drug to protein occurred by the presence of other drugs is termed as drug-drug interaction or drug displacement. Since the number of protein binding sites is limited, competition will exist between two drugs and the drugs with higher affinity will displace the other causing increased free drug concentration leading to increased toxicity (Rahman, 1994).

It has found that sequences of BSA and HSA have striking homology (Brown, 1977). On the basis of probe displacement method there are at least three relatively high specific drug-binding sites on the HSA molecule (Tian *et al.*, 2005). These sites are generally called the

warfarin-binding site (site-I), the benzodiazepine-binding site (site-II) and digoxin-binding site (site-III) (Sudlow *et al.*, 1975). The ability of one drug to inhibit the other is a function of their relative concentration, binding affinities and specificity of binding (Norouzzian *et al.*, 2007; Kouch-Weser and Sellers, 1976).

Keeping this consideration in mind, the tricyclic antidepressant drug amitriptyline has been used in the study to determine its binding site. As the cardiovascular events related to depression in older women (Wassertheil-Smoller *et al.*, 2004) and does depression specifically increase cardiovascular mortality (Vinkers *et al.*, 2005), depression as a risk factor for the incidence of first-ever stroke in 85-year-olds (Liebtrau *et al.*, 2008), stress and heart disease is related (Warren-Findlow, 2006).

Thus when studying with amitriptyline-drug interaction, more specifically the drug displacement, the possibility of the occurrence of site-to-site displacement should also be considered, as there will be a difference between the free concentration of a displaced drug with

or without site-to-site displacement. Moreover, protein-binding property of a drug is not a phenomenon particular to the plasma (Mahbubul Alam *et al.*, 2004a). Plasma protein binding properties are related to plasma clearance, elimination half-life, apparent volume of the distribution and area under the curve. BSA and HSA have structural similarity (Brown, 1977). In this study BSA, in lieu of Human Serum Albumin (HSA), was used because of its low cost and easy availability.

MATERIALS AND METHODS

Determination of binding site of amitriptyline using warfarin as a site-I specific probe: Three milliliter BSA solution of 2×10^{-5} M concentration was taken in each of the eight cleaned and dried test tubes. 1×10^{-3} M warfarin solution was added by using micropipette to the seven out of 8 test tubes so that the final ratio of protein and Warfarin was 1:1 (2×10^{-5} M: 2×10^{-5} M) in each of these seven test tubes. The eighth test tube containing only BSA solution was marked as "blank" or "Control". These mixtures were allowed to stand for 10 min for allowing binding of the warfarin. Amitriptyline solutions were added with increasing concentrations into six out of seven test tubes containing 1:1 mixture of protein-warfarin. The final ratios of protein: warfarin: amitriptyline were 1:1:0, 1:1:1, 1:1:2, 1:1:3, 1:1:4, 1:1:5 and 1:1:6. Then stand for 10 min to ensure maximum binding of amitriptyline to site-I and thereby displacing the probe from site-I on BSA. From each test tube 2 mL of solution was taken into seven different semi-permeable membrane tubes. Two end of the membrane a tube were clipped and was ensured that there was no leakage. The membrane tubes were then immersed in seven separates 50 mL conical flasks containing 30 mL of phosphate buffer solution of pH 7.4. The conical flasks were then placed in a metabolic shaker for dialysis at 25°C and 20 rpm and shaking was continued for 10 h. The free concentration of warfarin was measured by a UV spectrophotometer at 308 nm (BP 2000).

Determination of binding site of amitriptyline using diazepam as a site-II specific probe: A similar protocol as for binding site determination using warfarin was followed except here diazepam was used instead of warfarin. When dialysis was completed, buffer solutions were collected from each conical flask and the free concentration of diazepam was measured by a UV spectrophotometer at a wavelength of 235 nm (BP 2000).

Determination of binding site of amitriptyline using warfarin as a site-I specific probe (reverse experiment): Three milliliter BSA solution of 2×10^{-5} M concentration was taken in each of the eight cleaned and dried test

tubes. 1×10^{-3} M amitriptyline added to the seven out of 8 test tubes so that the final ratio of protein and amitriptyline was 1:1 (2×10^{-5} M: 2×10^{-5} M) in each of these seven test tubes. The eighth test tube containing only BSA solution was marked as "blank" or "Control". These mixtures were allowed to stand for 10 min for allowing binding of the amitriptyline. Warfarin solutions were added with increasing concentrations into six out of seven test tubes containing 1:1 mixture of protein-amitriptyline. The final ratios of protein: amitriptyline: warfarin were 1:1:0, 1:1:1, 1:1:2, 1:1:3, 1:1:4, 1:1:5 and 1:1:6. Then stand for 10 minutes to ensure maximum binding of warfarin to site-I and thereby displacing the probe from site-I on BSA. From each test tube 2 mL of solution was taken into seven different semi-permeable membrane tubes. Two end of the membrane a tube were clipped and was ensured that there was no leakage. The membrane tubes were then immersed in seven separates 50 mL conical flasks containing 30 mL of phosphate buffer solution of pH 7.4. The conical flasks were then placed in a metabolic shaker for dialysis at 25°C and 20 rpm and shaking was continued for 10 h. The free concentration of amitriptyline was measured by a UV spectrophotometer at 239 (BP 2000).

Determination of binding site of amitriptyline using diazepam as a site-II specific probe (Reverse Experiment): A similar protocol as for binding site determination using warfarin was followed except here diazepam was used instead of warfarin. When dialysis was completed, buffer solutions were collected from each conical flask and the free concentration of amitriptyline was measured by a UV spectrophotometer at a wavelength of 239 nm (BP 2000).

Drugs used in the experiment: Diazepam (Navana Pharmaceuticals Ltd. Bangladesh), Amitriptyline HCl (Opsonin Pharma Ltd. Bangladesh), Warfarin-Na (Incepta Pharmaceuticals Ltd. Bangladesh).

Reagents used in the experiment: Disodium hydrogen phosphate (Na_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), (Analytical grade, Glaxo, UK), Cellulose Membrane (Medical International Ltd., Liverpool Road, London; mol. wt. 1200 Daltons) Bovine Serum Albumin (BSA) (fatty acid free, fraction V, 96-98%, Mol. Wt 66500 and purchased from the Sigma Chemical Co., USA).

Instruments used in the experiment: pH Meter (HANNA Microprocessor pH Meter, Portugal), SP8-400 UV/VIS Spectrophotometer (Thermospectronic, England), Metabolic Shaking Incubator (Clifton Shaking Bath, Nickel Electro Ltd., England.), Micro syringe (Well. Liang. Jin. Yang. q. I., China). Equilibrium Dialysis method was employed in this study (Singlas, 1987a, b).

RESULTS

To characterized the binding site of amitriptyline, the free concentration of Warfarin (site-I specific probe) bound to BSA was measured upon the addition of amitriptyline. It was found that the free concentration of warfarin was increased from 100% (as % of initial) to 277.78% when the ratio Amitriptyline to BSA was increased 1 to 6. In contrast, under the same experimental conditions, when in diazepam was used as site-II specific probe, the increment of the free concentration of diazepam by amitriptyline was from 100% (as % of initial) to 152.09%. From these data this is evident that the increment of free concentration of warfarin is obviously greater than that of diazepam by amitriptyline (Fig. 1). So it can be concluded that Amitriptyline preferentially binds

Fig. 1: Free concentration of diazepam (■) and warfarin Na (●) bound to BSA (1:1) upon the addition of amitriptyline at pH 7.4 and 25°C, concentration used: [BSA] = 2×10^{-5} M, [warfarin] = 2×10^{-5} M, [diazepam] = 2×10^{-5} M, [amitriptyline] = 0.12×10^{-5} M

Fig. 2: Proposed models of the amitriptyline binding site to BSA, when; (A) = using warfarin binding to BSA, when [BSA] = 2×10^{-5} M = [warfarin] and [amitriptyline] = 0.12×10^{-5} M; (B) = using diazepam binding to BSA, when [BSA] = 2×10^{-5} M = [diazepam] and [amitriptyline] = 0.12×10^{-5} M

to site-I. Again as the displacement of diazepam is quite enough it can be also suggested that amitriptyline in addition to site-I also binds to site-II on the BSA molecule but to a lower extent (Fig. 2).

Again in the reverse experiment, the free concentration of amitriptyline was increase from 100% (as % of initial) to 248.38% when warfarin to BSA ratio was 1 to 6. On the other hand the free concentration of amitriptyline was increase from 100% (as % initial) to 240% when the ratio of Diazepam to BSA was also 1 to 6 (Fig. 3). From the data it is clear that the increment of amitriptyline due to displacement by warfarin (Site-I probe) is higher than that of amitriptyline when displaced by diazepam (site-II probe). Thus this reverse experiment also agrees with that of the previous experiment (Fig. 4).

Fig. 3: Free concentration of amitriptyline when used with diazepam (■) and warfarin Na (●) bound to [BSA] (1:1) (reverse experiment), concentration used: [BSA] = 2×10^{-5} M, [warfarin] = 0.12×10^{-5} M, [diazepam] = 0.12×10^{-5} M, [amitriptyline] = 2×10^{-5} M

Fig. 4: Proposed models of the amitriptyline binding site to BSA, when; (a) = using warfarin binding to BSA, when [BSA] = 2×10^{-5} M = [Amitriptyline] and [warfarin] = 0.12×10^{-5} M; (b) = using diazepam binding to BSA, when [BSA] = 2×10^{-5} M = [amitriptyline] and [diazepam] = 0.12×10^{-5} M (reverse experiment)

DISCUSSION

Plasma protein binding properties are primary determinants of the pharmacokinetic properties of most of the drugs such as plasma clearance, elimination half-life, apparent volume of the distribution and area under the curve (Jiunn *et al.*, 1987). During concurrent administration of drugs site-to-site displacement will take place and may change pharmacodynamic properties of the drugs (Mahbubul Alam *et al.*, 2004b). One study on tetracycline revealed that warfarin site (site-I) is the high affinity binding site and benzodiazepine site (site-II) is the low affinity binding site on BSA (Mahbubul Alam *et al.*, 2004c). Another study on arsenic showed that it changes the pharmacokinetics of drugs, during concurrent administration of arsenic and such drugs, so care should be taken for prescribing of those drugs to the arsenic affected people (Uddin *et al.*, 2004).

The initiation and intensity of pharmacologic response of a drug is related to its free concentration. So, from the pharmacologic and pharmacodynamic view point, the concept site-to-site displacement should be brought into consideration when calculating dose of a drug, during concurrent administration of two drugs (Rahman *et al.*, 2001).

So in suffering to depression and if prescribe drugs having high affinity for site-I may result in rapid action or rapid excretion from the body or even may cause toxicity at the normal doses. This is due to the fact that amitriptyline has high affinity for site-I and concurrent presence of amitriptyline and drug with affinity for site-I. So care should be taken if a drug is prescribed with amitriptyline with site-I binding drug.

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