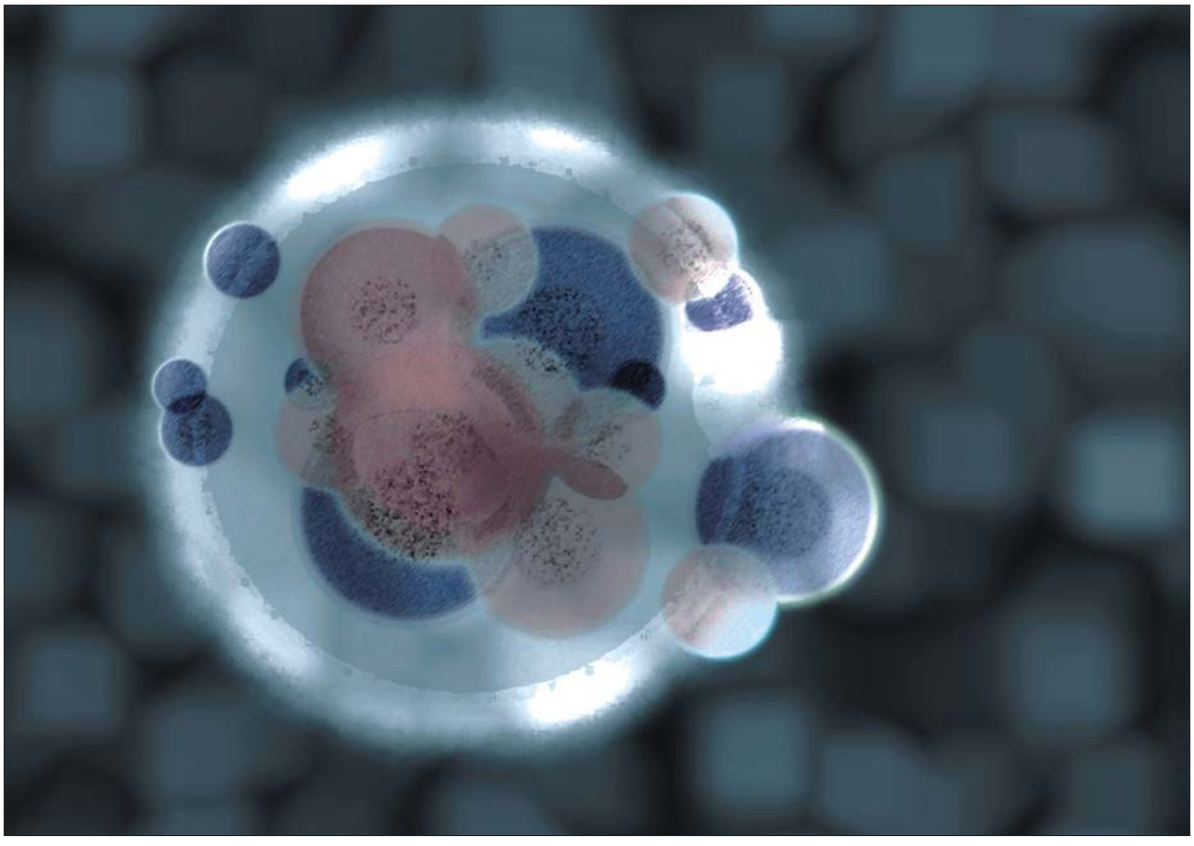

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Determination of the Binding Sites of Arsenic on Bovine Serum Albumin Using Warfarin (Site-I Specific Probe) and Diazepam (Site-II Specific Probe)

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Abstract: The binding of arsenic (As) on bovine serum albumin (BSA) was studied by equilibrium dialysis (ED) method using warfarin sodium (as site-I specific probe) and diazepam (as site-II specific probe) to determine the binding sites of arsenic to BSA. The data obtained showed that the free concentration of warfarin sodium increased very slowly by the addition of arsenic, while the free concentration of diazepam increased rapidly by the addition of arsenic. The free concentration of warfarin was 10.5% in absence of arsenic whereas this release was up to 20% when arsenic was added to BSA with an increasing concentration from $0.5x10^{-5}$ to $10x10^{-5}$M. The free concentration of diazepam was 11% in absence of arsenic whereas this release was up to 50% when arsenic was added to BSA with an increasing concentration from $0.5x10^{-5}$ to $10x10^{-5}$M. This site-specific probe displacement data implied that site-I (warfarin site) is low affinity site while site-II (diazepam site) is high affinity site of arsenic to the BSA.

Key words: Equilibrium dialysis, arsenic, diazepam, warfarin sodium, binding site

INTRODUCTION

The formation of a drug-protein complex is often termed drug-protein binding. Plasma protein binding is one of the pharmacokinetic parameters of a drug or of any compound and takes its place along those parameters relating to absorption, distribution, biotransformation and excretion. Albumin is the major component of plasma proteins responsible for reversible drug or compound binding. Albumin is distributed in the plasma and in the extra cellular fluids of skin, muscle and various other tissues in the body[1]. According to Krag-Hansen[1], BSA is composed of three homologous domains. The primary structure of HSA was elucidated by Meloun *et al.*[2] and Brown[3]. It is a large multi-domain protein folded into three domains, each of which is built of three loops. On the basis of probe displacement method, there lie at least three relatively high specific binding sites on the BSA molecule. These sites are generally called the warfarin-binding site, the benzodiazepine-binding site and digoxin-binding site and are denoted as site-I, II and III, respectively[4,5]. Site-II is more specific than site-I whereas site-III is an independent binding site. Serum albumin, the most abundant protein in the blood, plays a very important role in the binding phenomenon and serves as a depot protein and transport protein for numerous endogenous compounds[1]. Displacement of drug is defined as reduction in the extent of binding of a drug to protein caused by competition of another drug or any other agent, the displacer. This type of interaction may occur when two drugs or agents, capable of binding to proteins, are administered concurrently. Competitive displacement is more significant, when two drugs or agents are capable of binding to the same sites on the protein. From different investigations, it has been suggested that human serum albumin (HSA) has limited number of binding sites[6-8]. Since the number of protein binding sites is limited, competition will exist between drugs or drugs with metals or other agents and the agent with higher affinity will displace the other causing increased free drug concentration leading to higher toxicity or short duration of action[9].

The ability of one drug to inhibit the other is a function of their relative concentration, binding affinities and specificity of binding[10].

Now there is a high risk of arsenicosis from arsenic ingestion than previously thought. People living in Bangladesh have become terror-stricken when it revealed that underground water in parts of the country is tainted by deadly arsenic. The three major bio-chemical actions of arsenic is coagulation of proteins, complexation with co-enzymes, uncoupling of phosphorylation. Arsenic also has high tendency to deposit in the body for a long time in the tissues, nail, hair and to some proteins[11].

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Thus when studying with arsenic-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. 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[3]. In this study BSA, in lieu of human serum albumin (HSA), was used because of its low cost and easy availability.

MATERIALS AND METHODS

Drugs and reagents used in the experiment: The two probes used in the experiment, warfarin sodium and diazepam were supplied by Gaco Pharmaceuticals Ltd. Bangladesh. Disodium hydrogen phosphate (Na_2HPO_4), potassium di-hydrogen phosphate (KH_2PO_4), borax ($NaB_4O_7H_2O$), cellulose membrane (Medicel International Ltd. Liverpool Road, London; mol. Wt. 1200 Daltons), bovine serum albumin (BSA) (fatty acid free, fraction V, Mol. Wt. 66500 from Sigma Chemical Ltd.), arsenic oxide(As_2O_3) and Na-arsenate are also used.

Instruments used: pH Meter (HANNA Microprocessor pH Meter, Portugal), SP8-400 UV/VIS Spectrophotometer (Thermospectronic, England), Metabolic Shaking Incubator (Clifton Shaking Bath, Nical electro Ltd., England.) Micro Syringe (well. Liang. Jin. Yang. q.I., China) are used.

Method used: Equilibrium dialysis method was employed in the study[12,13].

Determination of binding site of arsenic to BSA at pH 7.4 and 37°C

By using warfarin sodium as the site-I specific probe: Five milliliter of 2×10^{-5} MBSA solution was taken in each of the 8 test tubes. Ten μL of 1×10^{-3} M warfarin sodium solution was added to the seven test tubes out of eight. The final ratio between protein and warfarin was 1:1 (2×10^{-5} M: 2×10^{-5} M) in each of the test tube. The eighth test tube containing only BSA (2×10^{-5} M) solution was marked as 'control' or 'blank'. Arsenic solution was added in increasing concentrations into six out of seven test tubes containing 1:1 mixture of protein and warfarin. The final ratios between arsenic and protein were 0.5:1, 2:1, 4:1, 6:1, 8:1 and 10:1. Into the seventh test tube no arsenic solution was added and it contained only protein-warfarin solution of 1:1 mixture. These solutions were then mixed and allowed to stand for 15 min so that maximum binding of arsenic to BSA was ensured. Two milliliter of solution was then pipette out and poured into eight membrane tubes. The tubes containing the drug-protein mixture were then immersed in separate 50 mL conical flasks containing 20 mL of phosphate buffer solution of pH 7.4. The conical flasks were placed in a metabolic shaker for dialysis at 37°C and 20 rpm. Dialysis was continued for about 10 h with continuous shaking so that equilibrium state inside and outside the membrane is reached. When dialysis was completed, buffer solutions were collected from each conical flask. The free concentration of warfarin sodium was measured by UV spectrophotometer at a wavelength of 308 nm (BP 2000).

By using diazepam as the 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).

RESULTS AND DISCUSSION

The complex of the drug, which forms with the protein, governs protein binding of a drug. There are two main types of protein binding, strong affinity binding to a small number of sites and weak affinity binding to a large number of sites. Since binding is almost exclusively to albumin and the number of sites available is limited, the protein-binding of some drugs depends on the plasma albumin concentration.

Adequate knowledge about composition, size and location of binding as well as the probable interactions at binding sites of HSA along with all the binding of plasma protein is required for proper explanation of pharmacokinetic aspect of drugs. It is important, for the rational understanding of drug-serum albumin binding during concurrent administration and its consequences in drug actions.

Arsenic-BSA interactions and characterization of binding sites of arsenic on BSA was studied by equilibrium dialysis method. It provided the possibility of *in vitro* estimates of protein binding of arsenic. The relative strength and specificity of binding of arsenic to BSA was determined by its ability to displace the probes (warfarin as site-I specific probe and diazepam as site-II specific probe) specific for particular sites (site-I or site-II) on the BSA molecule (Fig. 2).

By measuring the free concentration of the site-specific probe it is inferred about the binding of arsenic to BSA. In the study it was observed that the free fraction of

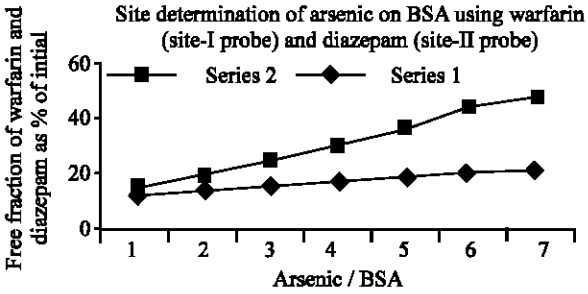

Fig. 1: Free fraction of warfarin and diazepam bound to BSA in presence of arsenic. Series 1 = increment of warfarin and Series 2 = increment of diazepam, when [BSA] = $2x10^{-5}$, [arsenic] = $0\text{-}10x10^{-5}$M and [Warfarin] = $2x10^{-5}$M = [Diazepam]

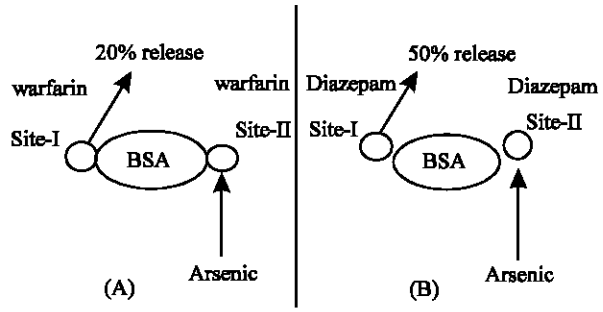

Fig. 2: Proposed models of the arsenic binding site to BSA, when; (A) = Using warfarin binding to BSA, when [BSA] = $2x10^{-5}$M = [Warfarin] and [arsenic] = $10x10^{-5}$M; (B) = Using Diazepam binding to BSA, when [BSA] = $2x10^{-5}$M= [Diazepam] and [arsenic] = $10x10^{-5}$M

warfarin (site-I specific probe) increased form 10.5% (as % of initial) to 20% with an increasing concentration from $0.5x10^{-5}$ to $10x10^{-5}$M of arsenic. On the other hand the free fraction of diazepam (site-II specific probe) increased from 11% (as % of initial) to 50% with the same increment of arsenic (Fig. 1). That is, the % of increment of free fraction of diazepam was more than that of warfarin. So this data suggested that arsenic, the metalloid under study, binds strongly to site-II (the diazepam site) than that of site-I (the warfarin site) on the BSA molecules. It revealed the fact that at a lower arsenic to BSA ratio, arsenic binds to site-II (high affinity binding site), whereas at a high arsenic to BSA ratio, arsenic binds not only at site-II (high affinity binding site) but also at site-I (low affinity binding site) on BSA molecule (Fig. 2).

Feshke *et al.*[6,14] suggested that the tyrosine residues are involved in the diazepam binding site (site-II) and the tryptophan residue is a part of the warfarin binding site (site-I). According to He and Carter[15], site-I is located in sub-domain IIA and that of site-II (diazepam site) in sub-domain IIIA. So it is quite reasonable to believe that the high affinity and low affinity binding sites for arsenic is located in the sub-domain I and sub-domain IIIA.

Drugs are bound to plasma proteins at sites located on the surface of the protein. The idea of binding sites is suggested by the relative size of the drugs and proteins. Protein binding of a drug is governed by the complex of the drug, which forms with the protein. There are two main types of protein binding, strong affinity binding to a small number of sites and weak affinity binding to a large number of sites. Since binding is almost exclusively to albumin and the number of sites available is limited, the protein of some drugs depends on the plasma albumin concentration.

Here arsenic increases the free concentration of diazepam to a greater extent than that of warfarin, so arsenic may probably bind in the high affinity binding to site-II and low affinity binding to site-I in the BSA. In the highly arsenic affected area, people suffering from any disease along with arsenicosis if takes drug having high affinity for site-II may result in rapid action or rapid excretion from the body or even may causes toxicity at the normal doses. This is due to the fact that arsenic has high affinity for site-II and concurrent presence of arsenic and drug with affinity for site-II. Since arsenic changes the pharmacokinetics of these drugs during concurrent administration of arsenic and such drugs, care should be taken for prescribing of those drug to the arsenic affected people.

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