

# Surface Plasmon Resonance Binding Study on the Interaction of Acetazolamide and Bovine Serum Albumin

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## ABSTRACT

**Objective:** Serum albumins are major plasma proteins in systemic blood circulation and act as transport proteins for endogenous and exogenous compounds such as drugs. In pharmaceutical applications, it is essential to characterize how drugs bind to serum albumin in the evaluation of drug candidates. Surface plasmon resonance (SPR) is fast, real-time, label-free optical based detection technique that offers the monitoring of molecular interactions, analyzing binding reactions and determining the affinity constants with real-time and high sensitivity. Acetazolamide (AZA) is used in the treatment of epilepsy and glaucoma.

**Methods:** To determine the binding kinetics of AZA-Bovine serum albumin (BSA) interaction, (i) SPR gold sensor surface was functionalized, (ii) amine coupling procedure was applied to activate the surface group and BSA was immobilized on functionalized sensor surface, (iii) the concentration series of AZA (10, 25, 50, 75, 100, 150, 200 and 250  $\mu$ M) was injected to SPR system and (iv) kinetic values were measured using the software of SPR system.

**Results:** 5 mM MUA was coated for surface functionalization. 250  $\mu$ g/mL BSA as ligand, 30  $\mu$ L/min flow rate, 1X PBS buffer (pH 7.4) and 10 mM acetate buffer (pH 5.2) as running and coupling buffers, respectively, were performed for SPR binding study. According to result, equilibrium constant ( $K_d$ ) of AZA-BSA was determined as 67.72  $\mu$ M.

**Conclusion:** In this study, we investigated the AZA-BSA binding interaction using SPR system based on Kretschmann configuration. The study designed with fast, label-free and real-time approach will provide valuable knowledge for pharmaceutical and clinical applications.

**Keywords:** Acetazolamide, bovine serum albumin, protein-drug interaction, surface plasmon resonance.

## 1. INTRODUCTION

Acetazolamide with molecular weight of 222.24 g/mol possesses physicochemical properties such as (i) white–yellowish crystalline powder, (ii) poorly soluble and (iii) soluble in alkaline conditions (1,2). AZA is mainly used in the treatment of epilepsy (3,4) and glaucoma (5,6) and some studies have focused on its antibacterial (7), antitumor (8), teratogenic (9) features, esterase (10-12) and intraocular pressure-lowering activities (13). After oral use, absorption of AZA is entirely actualized in gastrointestinal tract (14). AZA is nearly 95% bound to plasma proteins (15). The drug does not undergo metabolic changes in the body and is excreted by kidney in the urine within 24 hours (16).

Blood consists of great number of blood cells and plasma. The plasma also contains many plasma proteins including human serum albumin (HSA),  $\alpha$ 1-acid glycoprotein, lipoproteins (17) and globulins (18). HSA is well characterized plasma protein in intravascular compartment. Due to extraordinary

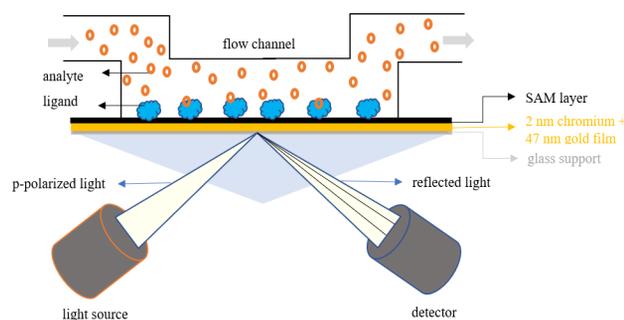
ligand-binding capacity, the transport of many endogenous and exogenous compounds such as fatty acids, steroids, thyroid hormones and drugs is one of the main functions of albumin (19). It also has essential functions such as free radical scavenging, regulation of capillary membrane permeability and colloid osmotic pressure. It constitutes approximately 55% of the total protein content in the plasma of a healthy individual, which makes it the most abundant protein of the plasma (20). HSA is an important biomarker of various diseases including post-menopausal obesity, ischemia, rheumatoid arthritis, acute graft-versus-host disease and cancer. In addition to being used as a biomarker, HSA is also used in the treatment of various diseases such as hypoalbuminemia, burns, shock, hypovolemia, trauma, hemorrhage, acute respiratory distress syndrome, chronic liver disease, acute liver failure and hemodialysis. In addition to that, biotechnological applications of HSA, including surgical sealants and adhesives, biochromatography, fusion

proteins, ligand trapping and implantable biomaterials, have been reported (21).

Serum albumins can be obtained from different mammalian species and these proteins show various similarities in physico-chemical characteristics (22). HSA and BSA are often used in biochemical and biophysical studies due to their similar folding, well known primary structure and possible binding interactions with various small molecules (23). BSA and HSA display around 76% sequence homology and a repeating pattern of disulfides. Although sequence homology does not seem too high, physico-chemical properties do not vary significantly. Due to binding properties, medical care availability and low cost, BSA is usually used in binding affinity experiments over HSA (24).

Plasma protein-drug interactions have been investigated for many years since plasma proteins establish an important part of the human proteome (25). Drugs are transported in the circulation either free or bound to plasma proteins. There are many drugs that bind reversibly to plasma proteins, making plasma proteins a crucial factor in determining the pharmacokinetics and pharmacological effects of drugs (26). Only free drugs have the capacity to interact effectively with the target. Therefore, it is important for there to be a balance between the drug and the carrier so that it is strong enough to aid in the transport but also weak enough to release the drug to the target (27). Serum albumins include several binding regions which have different affinities for analytes and bindings of these analytes with albumin occur at the side I and II on the protein (28). Several established techniques can be used to evaluate BSA-compound interactions including isothermal titration calorimetry, quartz crystal microbalance, spectroscopic (UV/VIS, fluorescence) techniques, FT-IR and SPR (29,30).

SPR is an optical based detection and/or sensing tool which offers real-time and label-free immunoassays having high specificity and sensitivity with short response time (31). Due to these unique characteristics, SPR has emerged a powerful technology in diagnosis (32), environmental monitoring (33), cellular imaging (34), genotyping (35), analysis of biomolecular bindings/interactions (36) between ligands and analytes generating kinetic and affinity data (37). SPR is classified as (i) prism coupling, (ii) integrated optical waveguide coupling, (iii) fiber coupling and (iv) grating coupling according to sensing structure (38) and prism-based or Kretschmann's configuration types are the most widely used SPR sensors (39). Aluminum, copper, gold and silver have been used as metal surface layer to generate surface plasmons for SPR sensing platforms (40). However, gold is the most commonly used surface layer for SPR instruments because of its outstanding durability, chemical stability and low oxidizing power (Figure 1) (41). Several functional chip surface modifications have been designed to enhance ligand and/or receptor immobilization in protein-drug binding investigations such as carboxymethylated dextran (42), 11-mercaptoundecanoic acid (MUA) (43) and 3-mercaptopropionic acid (44) on gold surface.



**Figure 1.** Schematic representation of SPR sensor based on Kretschmann configuration and sensor chip configuration.

Understanding the binding affinity and pharmacological activity of drugs in both normal and disease states is useful in terms of drug design and the use of effective medication and treatment for patients (45,46). To the best of our knowledge, the interaction of AZA and BSA has not been evaluated using the SPR technique. Due to the broad use of AZA, its interaction with albumin needs to be addressed. Therefore, we investigated the binding properties of AZA with BSA using SPR method in this study.

## 2. METHODS

### 2.1. Chemical and Materials

Acetazolamide, bovine serum albumin, 11-mercaptoundecanoic acid, *N*-ethyl-*N'*-(3-(dimethylamino)propyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), ethanolamine hydrochloride (EA-HCl),  $\text{KH}_2\text{PO}_4$ ,  $\text{Na}_2\text{HPO}_4$ , sodium acetate, NaOH and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) were purchased Sigma-Aldrich (Saint Louis, MO, USA). KCl and NaCl were commercially obtained from Carlo Erba. Sulfuric acid ( $\text{H}_2\text{SO}_4$ ), acetic acid and ethanol were purchased from Isolab (Turkey). The bare gold SPR sensor chip and matching fluid were obtained from Biosensing Instrument Inc. (Tempe, AZ, USA). BSA, stock solutions of chemicals and  $\text{H}_2\text{O}_2$  were stored at 4°C in refrigerator, EDC was kept at -20°C in a freezer.

### 2.2. Instrumentation

All water used in the preparation of samples and buffers was obtained from a water purification system (Direct Q<sup>®</sup>3 UV, Millipore Corp., France). Single channel pipettes were used for liquid transfer (Eppendorf Research Plus, Eppendorf AG, Germany). Routine weighing measurements in the study were performed by an analytical balance (Ohaus PA224C, Ohaus Corp., USA). The pH values of the solutions were determined with a pH meter (Seven Compact, Mettler Toledo, Switzerland). A vortex mixer (ZX3 Advanced Vortex Mixer, Velp Scientifica, Italy) was used to mix small volume of liquids and an ultrasonic bath (Elmasonic S 60 H, Elma Schmidbauer GmbH, Germany) was used to degas of the solutions.

Binding study of AZA-BSA was analyzed using BI-4500A SPR instrument (Biosensing Instrument Inc., Tempe, AZ, USA) with p-polarized laser light ( $\lambda = 670$  nm). SPR instrument was combined with autosampler, syringe pump as a flow injection system, computer and software as control system.

### 2.3. Preparation of Self-Assemble Monolayer (SAM) on Gold Chip

All chip surfaces were cleaned with piranha solution [ $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$  (7:1 v/v)] before the functionalization of gold surfaces. Then, the clean gold chip was immersed in the 5 mM MUA solution for 24 hours at 22°C for functionalization of the chip surface. After 24 hours, the chip was rinsed 3 times with ethanol to remove unbound MUA.

### 2.4. BSA Immobilization on Gold Chip

Before the experiment, the device was rinsed with phosphate-buffered saline (PBS) (pH 7.4) buffer and the functionalized chip was mounted onto prism of the SPR device. Sensor temperature was set to 25°C. Syringes were filled with degassed PBS buffer. A stable baseline was achieved before the experiment. During the initial phase, only the PBS buffer was run on the functionalized gold chip. For the activation of MUA modified gold surface, EDC-NHS mixture 1:1 (v/v) (NHS 0.05 M + EDC 0.2 M) was injected for 600 sec with the flow rate of 30  $\mu\text{L}/\text{min}$ . BSA (250  $\mu\text{g}/\text{mL}$ , prepared in 10 mM acetate buffer, pH 5.2) was injected with the flow rate of 30  $\mu\text{L}/\text{min}$  for 200 sec immediately after activation. BSA was not injected to the reference channel. After BSA injection, 1.0 M EA-HCl solution was injected onto the surface to block non-specific binding. Before kinetic analysis, the sensor surface was cleaned with 50 mM NaOH for 5 minutes. PBS was used as running buffer during the entire procedure.

### 2.5. Kinetic Analysis of AZA Interaction with BSA

All binding interactions between AZA-BSA were performed using double referencing and DMSO calibration protocols. Different concentrations of AZA solution (10, 25, 50, 75, 100, 150, 200 and 250  $\mu\text{M}$ ) were injected to the channels for 60 sec with the flow rate of 30  $\mu\text{L}/\text{min}$  to calculate the kinetic parameters. After each injection 50 mM NaOH solution was used for regeneration process for 60 sec. Since BSA was not immobilized to second channel, this channel was used as reference. PBS (pH 7.4) was used as running buffer during the entire procedure. All kinetics of AZA-BSA binding were used to calculate the association rate constant ( $k_a$ ), the dissociation rate constant ( $k_d$ ) as well as the equilibrium constant ( $K_D$ ) by SPR Data Analysis Software of Biosensing Instrument (version 3.10.5).

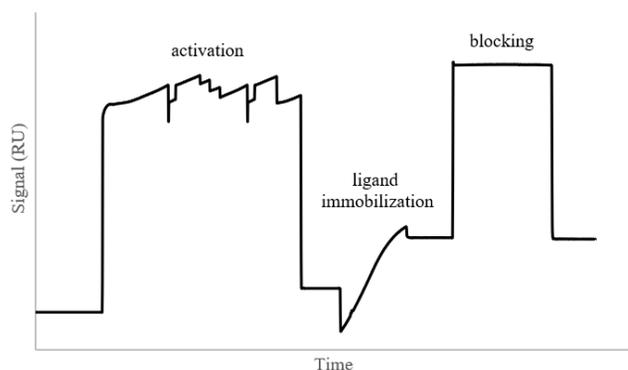
## 3. RESULTS

### 3.1. Construction of SAM and BSA Immobilization

MUA is one of the commonly used long-chain alkanethiol in the preparation of SAMs for SPR binding studies. MUA is negatively charged chemical at neutral pH (47). Surface functionalization should be designed to avoid insufficient analyte binding signal and/or some secondary effects like including steric hindrance and mass limited transport. 1 mM and 5 mM concentration of MUA were preferred in the immobilization of albumin onto the SAMs (36,48). In the study, 5 mM MUA demonstrated better surface coverage density for surface demand of BSA than 1 mM MUA. This functionalized sensor surface is one of the widely used sensor surface for amine coupling applications (43,49). As seen in Figure 2, freshly prepared EDC-NHS solution was applied to MUA functionalized SAM on the gold sensor surface for activating channels. After activation with EDC-NHS, BSA was immobilized on MUA based SAM. In this process, firstly the signal increased sharply as soon as the activation mixture contacted on SAM surface. The signal continued high level during the exposure of activation mixture. This mixture activates the MUA which increases surface quality. After exposure of activation mixture, signal turned to baseline. Then BSA solution was injected to SPR system. Signal increased relatively high levels which showed successful BSA immobilization on activated sensor surface (50). And EA-HCl solution was injected to block remaining active binding sites. This step is also crucial for removing loosely bound proteins from activated sensor surface (51). All steps including activation, ligand immobilization and blocking have been illustrated in Figure 3. In this study, the ligand has been injected into a channel for binding analysis and another channel was applied as reference channel. In binding experiments using SPR, the bulk index of refraction shifts and non-specific signals and also temperature drift are compensated by the reference channel (52).



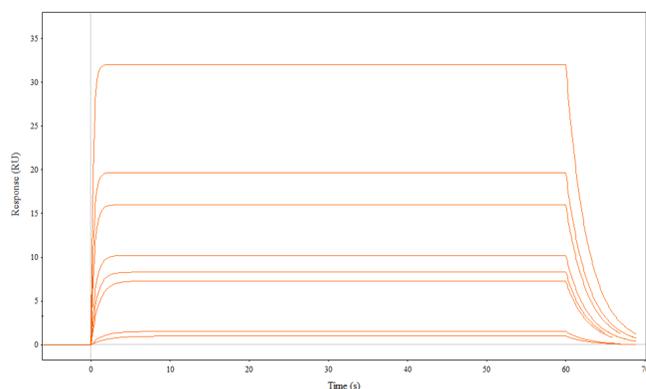
**Figure 2.** BSA as a ligand immobilization process on MUA based SAM: (a) MUA coated gold chip surface, (b) activation with NHS-EDC, (c) immobilization of BSA, (d) blocking of remaining activating groups (50).



**Figure 3.** SPR sensorgram illustrations of BSA immobilization step on 11-MUA based SAM.

### 3.2. SPR Data Analysis

Association rate constant ( $k_a$ ) demonstrates the number of complex formations formed per second. Dissociation rate constant ( $k_d$ ) shows fraction of complexes decayed per second.  $k_a$  and  $k_d$  were carried out by SPR studies. Finally, equilibrium constant ( $K_D$ ) which illustrates the ligand affinity to any molecule was calculated (53).  $k_a$ ,  $k_d$  and  $K_D$  values were calculated by BI-Data Analysis Program. As seen in Figure 4, dose response sensorgrams of AZA and BSA were investigated after reference subtraction. According to result, association rate constant ( $k_a$ ), dissociation rate constant ( $k_d$ ), and equilibrium constants ( $K_D$ ) values of AZA interaction with BSA are  $12.84 \times 10^3$  (1/M  $\times$  s),  $8.69 \times 10^{-1}$ (1/s) and 67.72  $\mu$ M, respectively.



**Figure 4.** Simulated SPR binding curves of BSA with AZA at different concentrations (10-250  $\mu$ M).

## 4. DISCUSSION

Most drugs have the ability of binding albumins in systemic blood circulation (54,55). Moderate binding to proteins is ideal in most cases. In therapeutic applications, when affinity binding of receptor and analyte is too high, effective drug action cannot occur. If the molecular binding is too weak, the

drug can undergo some possible fates, it can be metabolized and eliminated by kidney filtrations (56). HSA typically exhibits an ability to bind endogenous and exogenous compound/ligands with affinity constants in the range of  $10^{-4}$  to  $10^{-6}$  M (57). AZA possesses moderate affinity for albumin under *in vitro* conditions (58). The decrease in the binding of AZA with albumin will cause changes in the distribution and elimination of the drug. The increased concentration of free drug will cause both active tubular secretion from the kidneys and increased uptake by red blood cells (59).

There have been many investigations in the drug-serum albumin binding in recent years (60). The interactions of levothyroxine (61), resveratrol (62), diazepam, warfarin, phenylbutazone, iodipamide, azapropazone, tolbutamide, iophenoxic acid, indomethacin, furosemide, bucolome, sulfisoxazole, diclofenac and more drugs with albumin have been analyzed by several methods (45). Kurkov et al. (58) showed the *in vitro* binding behavior of AZA on HSA. According to result, AZA showed medium binding affinity to HSA. Another useful study conducted by Rich et al. (63) contains equilibrium constants values obtained by SPR. In this study, ten drug compounds including naproxen, digitoxin, sulfadimethoxine, ketanserin, pyrimethamine, rifampicin, salicylic acid, coumarin, salbutamol and warfarin which used as control group were injected to SPR system to analyze the binding interactions with HSA.

SPR based binding studies of HSA with cyclolinopeptides (64), rosuvastatin (36) and BSA with piperacillin (65), sunitinib malate (53), rifampicin (24) and neomycin (66) were performed to determine the drug affinities to serum albumins at temperatures of about 298 K. Table 1 illustrates that neomycin, piperacillin, rifampicin and sunitinib malate bind to the BSA molecule with equilibrium constants of  $1.50$ - $2.22 \times 10^{-5}$  M. Rosuvastatin shows high affinity to HSA with a low  $K_D$  value ( $1.55 \times 10^{-8}$  M). And other peptide-based biomolecules have the binding affinity for HSA in the range of  $8.27 \times 10^{-5}$ - $1.43 \times 10^{-2}$  M.

**Table 1.** Equilibrium constant values of some drug interactions with albumins at nearly 298 K.

Drug	Ligand	Equilibrium constant ( $K_D$ )	T (K)	Ref
Cyclolinopeptides	HSA	$8.27 \times 10^{-5} - 1.43 \times 10^{-2}$ M	~298	64
Rosuvastatin	HSA	$1.55 \times 10^{-8}$ M	298	36
Piperacillin	BSA	$2.22 \times 10^{-5}$ M	298	65
Sunitinib malate	BSA	$1.91 \times 10^{-5}$ M	296	53
Rifampicin	BSA	$1.50 \times 10^{-5}$ M	298	24
Neomycin	BSA	$2.21 \times 10^{-5}$ M	298	66
Acetazolamide	BSA	$6.77 \times 10^{-5}$ M	298	This study

## 5. CONCLUSION

SPR system based on Kretschmann configuration was performed to monitor binding of AZA to immobilized BSA on MUA based SAM. SPR is a fast, sensitive, label-free and non-invasive optical based detection techniques and

also allows real-time monitoring in drug-protein binding studies requiring small sample volumes. There is growing interest in pharmaceutical industry about drug-serum albumin interactions to determine accurate and reliable pharmacokinetics of the therapeutic agents. The study designed with fast, label-free and real-time approach will provide valuable knowledge about the affinity between AZA and BSA for pharmaceutical and clinical applications.

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**Author Contribution:**

Research idea: EAT

Design of the study: EAT, FGA

Acquisition of data for the study: EAT, FGA

Analysis of data for the study: EAT

Interpretation of data for the study: EAT

Drafting the manuscript: EAT, FGA

Revising it critically for important intellectual content: EAT, FGA

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