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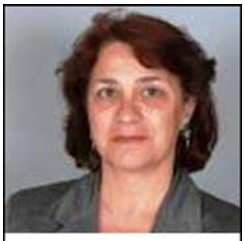
Studies on Drug – Human Serum Albumin Binding: The Current State of the Matter

Zvetanka D. Zhivkova*

Faculty of Pharmacy, Medical University of Sofia, 2 Dunav Street, Sofia 1000, Bulgaria



Zvetanka D. Zhivkova

Abstract: Human serum albumin (HSA) is the major plasma protein with vital functions acting as depot and career for many endogenous (fatty acids, bilirubin, *etc.*) and exogenous substances (drugs, nutrients, *etc.*) in the blood. Binding to HSA controls the free, active concentration of the drug and may affect considerably the overall pharmacodynamic and pharmacokinetic profile. Studies on drug – protein binding are important from both theoretical and practical point of view as they allow better understanding of the processes underlying drug disposition and elimination and the effect of several pathological states or co-administered drugs on drug delivery and efficacy.

The present review focuses on the current state of drug – HSA binding studies. The major functions and consequences of drug – protein binding are described. The X-ray structure of HSA is discussed focusing on the location and the architecture of the primary drug and fatty acids binding sites. Some of the most commonly used methods for drug – HSA binding assay are presented together with examples for their application. The most extensive studied topics in the area are discussed including quantitative characterization of drug – HSA complexation, identification of the binding sites, stereoselectivity of drug – HSA interactions, and thermodynamic characterization of the binding process. A short section is devoted to *in silico* prediction of drug – HSA binding as an important step in drug design and development.

Keywords: Human serum albumin (HSA), binding sites, stereoselective binding, thermodynamics, *in silico* prediction.

BINDING OF DRUGS TO PLASMA PROTEINS – FUNCTIONS AND CONSEQUENCES

The majority of drugs bind reversibly to various plasma proteins: serum albumin, alpha-1-acid glycoprotein (AAG), lipoproteins, etc. As a result, the drug is present in the plasma in two forms (free and bound), which are in equilibrium with each other. Plasma protein binding (PPB) exerts two vital functions – transport and depot. The binding with proteins facilitates the transport of drugs (especially highly lipo-solible molecules) to various tissues and organs in the body. The complex drug – protein serves as a depot from which the drug is gradually released thus providing a relatively constant plasma concentration.

PPB has significant pharmacodynamic (PD) and pharmacokinetic (PK) consequences. It is generally accepted that only the free fraction of the drug is pharmacologically active [1]. Highly bound drugs may require higher doses to achieve the effective concentration *in vivo*, and usually have restricted distribution to the sites of action and elimination from the body.

PPB may be crucial for the overall PK behavior affecting important PK parameters such as the apparent volume of distribution $\mathbf{V_d}$, the clearance **CL**, the bioavailability **F** and the half-life $\mathbf{t_{1/2}}$. $\mathbf{V_d}$ is related to the free (unbound) fraction of the drug in plasma $f_{u,\,p}$ as follows:

$$\mathbf{V_d} = \mathbf{V_p} + \mathbf{V_t}\frac{\mathbf{f_{u,p}}}{\mathbf{f_{u,t}}} \quad (1)$$

where $\mathbf{V_p}$ and $\mathbf{V_t}$ are the volumes of the plasma and the tissues in which the drug is distributed, and $\mathbf{f_{u,\,t}}$ is the unbound fraction of the drug in the tissues [2]. The effect of PPB on $\mathbf{V_d}$ depends on the distribution patterns. For drugs which are poorly distributed in the tissues $\mathbf{V_d}$ is close to $\mathbf{V_p}$ (~ 3L) and is independent of PPB. For extensively distributed drugs $\mathbf{V_d}$ increases almost linearly with the increase in $\mathbf{f_{u,\,p}}$.

Similarly, PPB may have either restrictive or permissive effect on drug **CL**. According to the "well-stirred model", the hepatic clearance $\mathbf{CL_H}$ is given by the equation:

$$\mathbf{CL_H} = \frac{\mathbf{Q_H f_{u,p} CL_{int}}}{\mathbf{Q_H} + \mathbf{f_{u,p} CL_{int}}} \quad (2)$$

where $\mathbf{Q_H}$ is the liver blood flow, and $\mathbf{CL_{int}}$ – the intrinsic clearance of the free drug [2]. The effect of PPB depends on the drugs extraction ratio **E** which accounts for the fraction of the drug, eliminated by the liver. Drugs with high **E** (> 0.7) are eliminated with high $\mathbf{CL_H}$, close to $\mathbf{Q_H}$ and independent of PPB. In contrast, drugs with low **E** (< 0.3) have low $\mathbf{CL_H}$, proportional to $\mathbf{f_{u,\,p}}$, restricted by PPB. The same applies to the renal excretion. Drugs that are excreted solely by glomerular filtration have low **CL**, restricted by PPB, while drugs, substrates of active secretion transporters, are eliminated with high **CL,** independent of PPB.

Ultimately, PPB may have a profound effect on the bioavailability. The fraction escaped elimination by the liver $\mathbf{F_H}$ is the upper limit of the oral bioavailability [2] and is given by the equation:

$$\mathbf{F_H} = \frac{\mathbf{Q_H}}{\mathbf{Q_H} + \mathbf{f_{u,p} CL_{int}}} \quad (3)$$

For drugs with negligible *first-pass metabolism* $\mathbf{F_H}$ is close to 1 and is independent of PPB. However, for highly extracted drugs $\mathbf{F_H}$ is inversely proportional to $\mathbf{f_{u,\,p}}$; the decrease of PPB decreases bioavailability by facilitating the *first pass metabolism*.

The effect of changes in $\mathbf{f_{u,\,p}}$ on $\mathbf{t_{1/2}}$ reflects the changes in $\mathbf{V_d}$ and **CL** as the three parameters are related according the equation:

$$\mathbf{t_{1/2}} = \frac{\mathbf{0.693 V_d}}{\mathbf{CL}} \quad (4)$$

The clinical significance of the change in PPB depends on the magnitude of the respective pharmacokinetic parameters and the

*Address correspondence to this author at the Faculty of Pharmacy, Medical University of Sofia, 2 Dunav Street, Sofia 1000, Bulgaria;
Tel: +35929236514; Fax: +35929879874;
Emails: zdzhivkova@pharmfac.acad,bg; zdzhivkova@yahoo.com

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route of administration. Most of the drugs are given by multiply dose regimen, and relay on the establishment of a *steady state* when the rate of drug input is equal to the rate of elimination. The average steady state concentration $\mathbf{C}_{\mathbf{av}}^{\mathbf{ss}}$ is given by the equation:

$$\mathbf{C}_{\mathbf{av}}^{\mathbf{ss}} = \frac{\mathbf{FD}}{\mathbf{CL}\tau} \quad (5)$$

where **F** is the bioavailability, **D** – the dose, and τ - the dosing interval [2]. Consequently, the steady state concentration of the free drug $\mathbf{C}_{\mathbf{av,u}}^{\mathbf{ss}}$ is equal to:

$$\mathbf{C}_{\mathbf{av,u}}^{\mathbf{ss}} = \frac{\mathbf{f_{u,p}FD}}{\mathbf{CL}\tau} \quad (6)$$

For low-clearance drugs the change in $\mathbf{f}_{\mathbf{u,\,p}}$ results in a proportional change in the **CL,** and $\mathbf{C}_{\mathbf{av,u}}^{\mathbf{ss}}$ remains unaffected. However for high clearance drugs the effect of changes in PPB depends on the route of administration. Following *iv* administration (**F** = 1) the change in $\mathbf{f}_{\mathbf{u,\,p}}$ will alter $\mathbf{C}_{\mathbf{av,u}}^{\mathbf{ss}}$ because the **CL** is constant, close to the blood flow. For *po* administered drugs the change in $\mathbf{f}_{\mathbf{u,\,p}}$ results in a inversely proportional change in **F,** so that $\mathbf{C}_{\mathbf{av,u}}^{\mathbf{ss}}$ remains almost unchanged. Therefore, the conclusions about the clinical significance of the change in PPB must be made after a careful analysis of the multiple effects of PPB on the various pharmacokinetic parameters. The complex effects of PPB on PK are discussed in several reviews and books [3-7].

The extent of PPB depends on the concentration of the binding proteins and their affinity for the drugs. Any change in the concentration, or modification of protein structure may have considerable effect on PPB. Protein concentration varies with the age and at several physiological and disease states [5, 7 – 10]. The binding affinity can be changed due to competition with endogenous ligands such as fatty acids (FAs), or with co-administered drugs [11, 12]. A drug can be displaced by the co-binding agent resulting in a higher free fraction in plasma. The clinical significance of displacement phenomena is a source of a never – ending debate. For years they have been considered as the main reason for the observed side and toxic effects by drugs co-administration. Classical examples are the competition between warfarin (WAR) and phenylbutazone (PBZ) leading to a marked increase in the anticoagulant action of WAR [13], and the displacement of tolbutamide by sulphonamides, resulting in a hypoglycaemia [14]. Consequently it was suggested, that other mechanisms are responsible for the increase of the free drug concentration, rather than displacement, *e. g.* inhibition of the metabolism of WAR by PBZ and reduction of tolbutamide **CL** by sulfonamides [15, 16]. Nowadays there is an agreement that displacement phenomena are overestimated and are of low clinical relevance for the majority of the drugs [17 – 19]. However, displacement interactions may affect the patient exposure to the drug in several cases: following *iv* administration of highly bound drugs with a narrow therapeutic index and high extraction ratio, or after *po* administration of drugs with a fast onset of action [19]. An algorithm for evaluation of the clinical significance of potential protein binding displacement interactions was proposed [18].

HSA is the major protein in plasma accounting for about 50-60% of all plasma proteins. Its high concentration (~ 40 g/L or 0.6 mM), notable affinity to a diversity of chemical structures, and relatively wide substrate specificity determine its extraordinary binding capacity. HSA is the principle carrier of a number of endogenous lipophilic ligands as FAs, bilirubin, hematin, bile acids, thyroxin, etc. [20]. In addition, HSA binds with high affinity a huge number of drugs and metabolites. It is generally accepted, that HSA is primarily responsible for the binding of acidic and neutral drugs, however many reports suggest considerable affinity for basic structures [21 – 24]**.** With its amazing properties and functions HSA is the most extensively explored protein, frequently used as a model for studying ligand – biomacromolecule interactions.

CRYSTALLOGRAPHIC STRUCTURE OF HSA AND ARCHIRTECTURE OF THE SPECIFIC BINDING SITES

Structure of HSA

The complete amino acid sequence of HSA was cleared by Meloun *et al.* on the basis of molecule cleavage and chemical analysis [25]. It was suggested that the protein consists of a single polypeptide chain containing 585 amino acid residuals, roughly equal number of non polar and polar, most of them with acidic or basic function. Characteristic feature was the presence of 35 cysteine residues, forming 17 disulphide bridges, with Cys34 being the only one with a free sulfhydril group. A high level of homology was observed in the distribution of several amino acids leading to the proposal, that the HSA chain is built up of subunits with almost equal distances between the disulphide bonds. This sequence was largely confirmed by the gene sequence of HSA [26].

The first crystallographic structure of HSA was reported in 1989 by Carter *et al.* [27], and further refined at 2.8 Å resolution [28, 29]. HSA molecule resembles a heart shape with approximate dimensions of 80 x 80 x30 Å. The polypeptide chain is organized in homologous domains (I, II and III), each in turn comprising two sub-domains (A and B). HSA has a high α-helical content (~ 67%). Each domain consists of 10 helical segments, and the sub-domains are linked by non-helical extended and flexible regions. The disulphide bridges are located between the helical segments, so they are well protected and inaccessible to the solvent. This explains the relative stability of HSA under a variety of harsh experimental conditions [29].

Subsequently, the X-ray structure of another crystal form of defatted HSA at 2.5 Å resolution was reported which confirmed entirely the findings of Carter's group and described in more detail the location of the sub-domains and mutual interactions between them [30].

Specific Binding Sites

Determination of the 3D structure of HSA inspired numerous X-ray crystallographic studies on complexes of HSA with various ligands. The results provided valuable structural information on the architecture of the binding sites and the modes of the complexation. Two primary binding sites for drugs and seven FA binding sites on HSA were identified [28, 29, 31 – 33]. The FA binding sites are distributed asymmetrically throughout the whole molecule encompassing all sub-domains, while the binding of drugs is restricted to two major binding sites in sub-domains IIA and IIIA.

According to the earliest crystallographic studies the principal regions of drug binding are located in the hydrophobic cavities in sub-domains IIA and IIIA [28]. The binding pockets are lined with hydrophobic side chains, forming a local points around few polar residues (Lys199, His242, Tyr411, Arg410). Both hydrophobic and electrostatic interactions (hydrogen bonding and salt bridges) are supposed to be involved in the ligand – protein complexation. The binding cavity in sub-domain IIIA is considered as the most active and accommodating in HSA, with affinity to diazepam (DAZ), digoxin, clofibrate, ibuprofen, and 3'-azido-3'-deoxythymidine. Aspirin, iodinited salicylic acid and triiodobenzoic acid show nearly equal distribution between both binding sites. WAR occupies a single site in sub-domain IIA. It was suggested, that sub-domains IIA and IIIA shared a common interface, which was considered as the reason for the conformational changes in sub-domain IIA caused by ligand binding in sub-domain IIIA. These X-ray results

are in a good agreement with the predicted locations of the binding sites, based on competitive inhibition and spectroscopic studies [34 – 36]. Residues Trp214, Lys199, Tyr411 have been implicated in the binding process by several studies, and they all are located either in sub-domain IIA, or IIIA. Therefore, it was suggested, that the binding pocket in the sub-domain IIA corresponds to Sudlow's Site I, while the binding cavity in sub-domain IIIA – to Sudlow's Site II [29].

So far the most detailed and precise information regarding the two primary drug binding sites on HSA was gained from the X-ray analysis of complexes of HSA with 12 drugs and toxins – in the absence, and presence of FAs [37]. This study describes the geometry of drug Site 1 and Site 2, identifies the key determinants of binding specificity, reveals the capacity of both pockets for flexible accommodation, identifies numerous secondary binding sites, and explains the effect of FAs on the binding affinity for Site 1 drugs.

Despite the topological similarity, Site 1 and Site 2 differ considerable in their shape, size and polarity which determine the binding specificities of the two pockets. Drug Site 1 (sub-domain IIA) consists of a central zone and three extended sub-chambers. The interior of the pocket is predominantly non-polar. Two clusters of polar residues are identified: one of them – towards the bottom of the pocket (Tyr150, His 242, Arg 257) and the other – at the pocket entrance (Lys195, Lys199, Arg218, Arg222). WAR, PBZ, oxyphenbutazone and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid are found to cluster in the centre of the pocket. Hydrophobic contacts are essential for complexation, besides there are a number of specific interactions with the polar residues. The central role in drug binding to Site 1 seems to have Tyr150 as it is involved in hydrogen bonds with all of the studied drugs. His242, Lys199, Arg222 and Arg257 are also supposed to participate in salt bridges or hydrogen bonding. The crystallographic structure of HSA complexed with myristate and phenylbutazone is shown in Fig. 1. The structure of the pocket and the mode of complexation of various Site 1 drugs imply that this site has preference for molecules with two anionic or electronegative features on the opposite sides of the ligand molecule that can simultaneously interact with the two polar patches [37]. Quite different binding behavior was observed for indomethacin. This drug was bound in the front sub-chamber only after ligand-induced conformational changes in the pocket; it did not displace FAs from their low-affinity site in the centre of pocket, and it was able to bind simultaneously with PBZ or azapropazone whereby it was neither displaced nor was displaced by any of them.

Drug Site 2 (sub-domain IIIA) is smaller than Site 1 and consists of one sub-chamber corresponding to the right sub-chamber of Site 1. It was observed, that this site was accessed only following ligand-induced side chain movement [37]. The entrance of the pocket is open, well exposed to the solvent. There is a single polar cluster, located close to one side of the entrance of the pocket including Arg410, Tyr411, Lys414 and Ser489 (Fig. **1**). The presence of only one basic polar patch, located unilaterally in the hydrophobic pocket, determines the specificity of Site 2 for drugs with peripherally located electronegative group. All of the studied Site 2 drugs (DAZ, diflunisal, ibuprofen and indoxyl sulphate) are located in the centre of the pocket and interact with Tyr411. It is assumed that Arg410 and Ser489 are also involved in salt bridges and hydrogen bonding.

The X-ray studies suggested that the binding of drugs to both Site 1 and Site 2 may induce conformational changes in the HSA molecule, affecting the binding affinity. It was found that Site 1 extends significantly beyond the core of sub-domain IIA as a result of ligand induced side-chain movement. In turn, although relatively small, Site 2 is able to accommodate large molecules like indomethacin, or two molecules of long-chain FA, or 1 molecule thyroxine. These local ligand-induced conformational changes determine the high adaptability of both the drug binding sites [37].

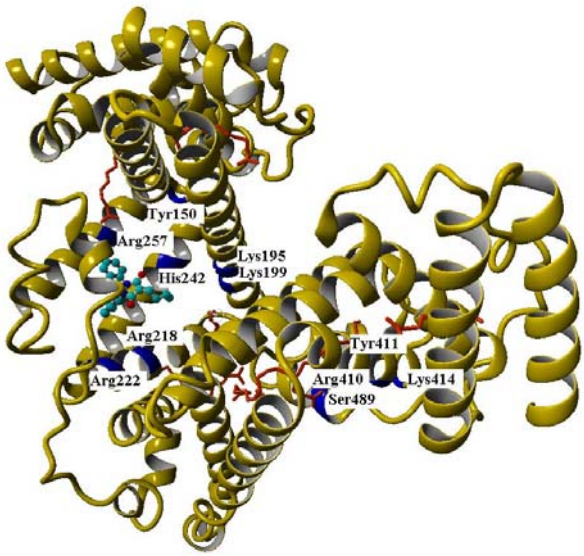

Fig. (1). Crystallographic structure of HSA complexed with myristate and phenylbutazone (taken from PDB entry 2BHP:
www.rcsb.org/pdb). The polar residues lining drug Site 1 and Site 2 are shown. Myristate molecules are represented in red.

Seven distinct binding sites have been identified for long and middle chain FAs – the natural substrates of HSA [31 – 33]. One of them is located in the centre of Site 1 [33] and is likely to be a low-affinity site, as it is not displaced by any of the studied Site 1 drugs [37]. Two other sites, considered as high-affinity FA binding sites, coincide with drug Site 2 [33]. The distribution of FAs and drug binding sites on HSA molecule is shown in Fig. **2**. Binding of FAs to HSA is accompanied by dramatic conformational changes in protein molecule, involving rotation of domains I and III relative to domain II. The changes significantly affect Site 1 through a rearrangement of the polar residues, opening a solvent channel between Tyr150 and Gln196, increase in the volume of the pocket and modification of the polarity. As a result remarkable differences in the binding mode have been observed for several Site 1 drugs as compared with defatted HSA [37]. Crystallographic analysis of the complex of WAR with HSA in the presence of myristic acid revealed a rearrangement of amino acid side chains in Site 1 providing a more hydrophobic environment for the coumarin moiety and

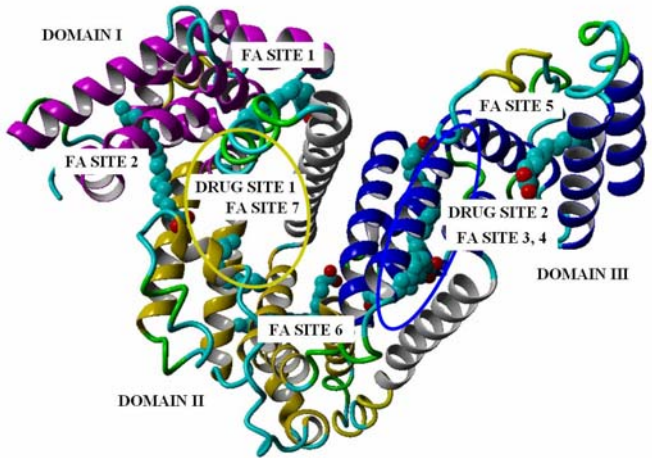

Fig. (2). Disrtibution of FAs and drug binding sites on HSA (taken from PDB entry 2BXM:
www.rcsb.org/pdb).

thus increasing the binding affinity for WAR [38]. Recently Zhu *et al.* suggested the appearance of a new sub-site in Site 1 as a result of FA induced conformational changes [39]. Thereby the X-ray structure analysis of drug – HSA – FA complexes reveal the complex nature of the interactions between drugs and FAs on their binding to HSA and provided a reasonable explanation for the observed cooperative and competitive effects [40 – 43].

METHODS FOR STUDYING DRUG INTERACTIONS WITH HUMAN SERUM ALBUMIN

Numerous analytical methods have been used in drug – protein binding studies, and they are continuously improved and added according to the extended knowledge on the complex mechanisms involved in the binding process. The most frequent methods can be classified in to three groups: conventional, spectroscopic and chromatographic methods. The current state of the methodology is critically reviewed in several publications [44 – 46]. The principles and the applications of the most commonly used techniques are described briefly.

Conventional Methods

These methods are based on a separation of the free and the bound drug (ligand). The most popular techniques are equilibrium dialysis and ultrafiltration preferred for their simplicity and general applicability for many different systems *in vitro* and *ex vivo*.

Equilibrium Dialysis (ED)

ED consists of partitioning of the ligand between two compartments (buffer and protein solutions) separated by a semi – permeable membrane impenetrable for high-molecular weight substances. At equilibrium the concentration of the free ligand on both sides of the membrane is equal. Data for the free and the total ligand concentration enable quantitative characterization of the binding process. ED is considered as the "gold standard" in studying drug – protein binding, and is frequently used as a reference technique against other methods. However, ED suffers from several shortcomings: a relatively long time for reaching equilibrium, dilution of the protein compartment, Donnan effects, etc. The methodology is instantly improved, and several high-throughput ED methods have been developed recently considerably shortening the dialysis time while increasing the reproducibility and accuracy [47, 48].

Ultrafiltration (UF)

In the UF technique the separation is achieved by applying a pressure gradient to the protein – ligand mixture. The ultrafiltrate containing only free ligand is filtered through a membrane with a proper molecular size cut-off. UF is superior with respect to time and lack of dilution effects during separation; however it also shows drawbacks such as non specific adsorption of the ligand on the membrane or leakage of drug or protein which decrease the accuracy and reproducibility of the assay [44, 46]. Nevertheless, UF remains widely applied technique which, in combination with other methods, allows rapid assay of drug – protein binding [49 - 51].

Spectroscopic Methods

They are based on the spectroscopic properties of the protein and their change in the course of ligand – protein binding. Spectroscopic techniques allow estimation of the free and/or bound ligand concentration without separation and provide valuable information on the changes in the protein structure on complexation.

Fluorescence Spectroscopy

Fluorescence spectroscopy is the most widely used spectroscopic methods suitable for studying various aspects of drug – protein interactions. HSA exhibits intrinsic fluorescence mainly due to the single Trp residue while the contribution of the other fluorophores, Tyr and Phe, is considered as negligible [20, 52, 53]. The fluorescence spectrum of HSA is sensitive to any change in the microenvironment of the fluorophore. The decrease in the fluorescence intensity (fluorescence quenching) in the presence of a ligand is an indication for ligand – HSA complexation. Therefore, measurement of fluorescence quenching is a convenient approach for studying the binding process. Two quenching mechanisms are distinguished. Dynamic quenching is observed when the ligand complexes with the fluorophore in its excited state. In the case of static quenching complexation occurs in the ground state, and the complex is non-fluorescent [54]. Fluorescence quenching studies are based on the assessment of the effect of the increasing ligand concentration on the fluorescence intensity of the protein. The type of quenching can be identified on the basis of different dependence of the quenching constant on temperature. The dynamic quenching constant increases with the temperature, while the static quenching constant decreases, suggesting the presence of a strong complex between the ligand and the protein in its ground state [53 – 55]. The quantitative parameters of binding (the number of binding sites and the association constant) can be calculated by using the modified Stern-Volmer or Scatchard equation [52 – 54, 56, 57]. Quenching studies can also provide a deeper insight into the binding mode. The change in the polarity of the microenvironment surrounding the fluorophores (Trp, Tyr and Phe) can be evaluated by synchronous spectra. The synchronous spectra are obtained by scanning the excitation and emission monochromators with a constant difference between excitation and emission wavelengths. The change of the position of the synchronous fluorescence maximum reflects the change in the polarity of the microenvironment, surrounding the respective amino-acid. A blue shift of the fluorescent maximum suggests that the fluorophore is buried in a more hydrophobic environment, and is less exposed to the solvent, while a red shift implies, that it is located in a polar environment [56 – 58]. The distance between the bound ligand and the fluorophore can be estimated using the Foster free energy transfer theory [24, 57]. Detailed studies of the use of fluorescence quenching for quantitative and qualitative characterization of drug – HSA binding were published recently, concerning phenothiazine drugs [24], coumarin derivatives [52], hippuric acid [53], ketoprofen [54], osthole [56], carboxiphenoxathiin [57], WAR – ferulic acid interactions [58], *etc.*

Another application of the fluorescent spectroscopy to drug – HSA binding studies is based on fluorescent probes – small molecules, whose fluorescent properties are changed by covalent or non-covalent interactions with other molecules. Fluorescent probes have been widely used for characterization of drug binding sites on HSA. The specific binding of dansylamide and dansylsacrosine at two distinct binding sites on HSA led to the identification of Sudlow's Site 1 and Site 2 [34]. Another 12 dansylaminoacids were classified as Site 1 probes, and 8 dansylaminoacids – as Site 2 probes [59]. It was suggested that dansylglycine could be used as a specific marker for Site 2 [60]. The wide applicability of dansylated aminoacids arises from the fact that their fluorescence significantly increases by binding to HSA. A blue shift in the maximum of the emission light is typically observed suggesting a movement of the probes from the aqueous medium to the hydrophobic cavity of the protein. Therefore, the change of the fluorescence of HSA – dansyl amino acid complex in the presence of competing drug is informative for the binding site of the drug [61].

Circular Dichroism (CD) Spectroscopy

CD spectroscopy is an optical technique based on the difference in the absorption of the left and the right circularly polarized light which arise due to structural asymmetry. HSA shows a characteristic CD spectrum in the near and the far UV region with two negative bands at 208 and 222 nm, attributed to the α-helical structure [53, 57]. The binding of ligands to HSA may induce a change in the normal CD spectrum known as extrinsic Cotton effect. This effect is quantified as molar ellipticity and serves as a measure for the concentration of the ligand – protein complex. The method is very sensitive as small changes in the asymmetrical environment may result in considerable changes in the CD spectra. Therefore, CD

spectroscopy is a convenient approach for studying different aspects of drug – HSA binding. The main principles and applications of CD are subject of several reviews [62, 63].

The earliest CD studies provided valuable information about the chemical nature of the HSA binding sites for a number of classical highly bound drugs – PBZ [64], flufenamic acid and its derivatives [65], WAR [36, 66], dicoumarol [66], indomethacin [67], *etc*. The applicability of the CD for quantifying protein binding was pioneered by Rosen who proposed the first mathematical approach for data analysis [68]. Although CD spectroscopy was considered as a less accurate method for quantitative characterization of the binding process [44], many reports have shown that the binding characteristics of drugs obtained by CD are comparable with those acquired by other methods [69, 70].

CD spectroscopy was widely used for studying drug – drug interactions at HSA – binding level. By means of CD, Sebille *et al.* first suggested the cooperative effects of FAs on WAR binding and explained them with conformational changes of the drug binding site caused by FAs [41]. A CD study on atovaquone – HSA binding revealed the presence of two high affinity binding sites with contribution of the both principal drug binding sites [71]. Lipocrine – a new candidate for acetylcholynestherase inhibitor, demonstrated direct competition with Site 2 drugs and bilirubin, and non-cooperative effects on Site 1 [72]. Ethacrinic acid also competed directly with Site 2 ligands and FAs, while influenced indirectly Site 1 binding [73]. A site – to – site displacement of carprofen and diclofenac by ibuprofen was also suggested – from the high affinity binding Site 2 to the low affinity Site 1 [74, 75]. Based on CD studies, a two step mechanism was proposed for ketoprofen complexation: initial binding to a high affinity site followed by considerable increase of the number of binding sites and 100-fold decrease of the binding affinity due to conformational changes in the protein [76]. Similarly, an opening of a number of additional binding sites in the course of sulindac complexation at its primary binding site on HSA was observed reflecting alteration of protein structure [77]. Analysis of the CD spectra allowed hypothesis to be made concerning the nature of the binding sites and the binding mode.

The utility of CD spectroscopy for studying enantioselectivity in drug – HSA binding was critically reviewed by Ascoli *et al.* [78, 79]**.** Recently CD spectroscopy is used mainly in combination with other spectroscopic techniques for monitoring the conformational changes of HSA accompanying ligand binding. The decrease in the negative bands at 208 and 222 nm is indicative of a disruption of the α-helical network, and the degree of disruption can be calculated from the mean residue ellipticity [80 – 84].

Chromatographic Methods

The progress in the chromatographic technology led to the development of a variety of chromatographic methods for studying drug – protein binding. They overcome the disadvantages of the conventional methods (ED and UF) and are beneficial in terms of precision, speed, reproducibility and capability for automation. The most frequently used chromatographic methods are the high performance frontal analysis, the Hummel-Dreyer method and the high performance liquid affinity chromatography with protein immobilized stationary phase.

High Performance Frontal Analysis (HPFA)

HPFA uses a restricted-access type HPLC (high performance liquid chromatography) column which combines the principles of the size exclusion and the partitioning separation [85]. The method allows a simple binding assay following direct injection of drug – protein mixture onto the column. Both unbound and total drug concentrations can be measured simultaneously and the binding parameters can be estimated using traditional mathematical approaches. By coupling with a chiral stationary phase, the enantioselectivity of drug – protein binding can also be studied. The principles of HPFA are described in detail in several papers [86 – 88].

Hummel – Dreyer Method

In the Hummel – Dreyer method a known quantity of the protein is injected onto a size exclusion chromatography column and is eluted with mobile phases containing the ligand at known, variable concentrations. A negative peak appears in the chromatogram corresponding to the bound ligand concentration. The main advantage of the method is that the binding equilibrium is not disturbed during the separation, and that the concentration of the bound drug is measured, which increases the accuracy of the analysis [89]. A comprehensive review on the advantages and applications of the Hummel and Dreyer method was published by Soltes [90].

High Performance Liquid Affinity Chromatography (HPLAC) with Protein Immobilized Stationary Phase

In the last three decades HPLAC was outlined as a powerful tool for studying biomolecular interactions [91]. The method is based on monitoring the behavior of a ligand (injected as an analyte) in a chromatographic system, consisting of a protein immobilized column and predominantly aqueous mobile phase. The mobile phase contains another ligand (competitor or marker) at variable concentrations. The retention of the analyte in the column serves as a quantitative measure for its binding affinity. It is dependent on the concentration of the competitor in the mobile phase. Using the same drug as analyte and competitor allows estimation of the binding characteristics of the drug. Using different ligands as analyte and competitor the mutual interactions between both substances can be investigated [92].

HPLAC provides a good simulation of the *in vivo* conditions since the passage of the ligand through the column occurs *via* a series of drug – protein binding equilibria. It is believed that the immobilized HSA retains the binding properties and conformational mobility of the native protein. This was supported by the good agreement of the results obtained by HPLAC and these acquired by other conventional methods [93 – 95].

The existence of a commercially available HSA immobilized column (purchased by *Hypersil*, Runcorn, UK) gave rise to a large number of studies on different aspects of ligand – HSA binding: estimation of the affinity constants of drugs, characterization of binding regions using markers for principle binding sites, examination of stereoselective binding, *etc.* Several mathematical approaches have been proposed for quantifying the binding process based on data for the chromatographic capacity factor and competitor concentration in the mobile phase. Most of the studies assumed competition between competitor and analyte for only one type of binding sites [96]. Consequently, a modified mathematical approach was proposed, based on a model for two-site competition [92]. The new mathematical equation was able to fit the observed biphasic relationship between the capacity factor and the marker concentration – an indication for the existence of two different types of binding site on the HSA immobilized column.

The utility of this approach was proved in a series of studies on different aspects of drug – HSA binding [69, 100 – 103). In a study on characterization of the main drug binding sites the classical markers PBZ and DAZ were tested. Each drug was used once as an analyte, and once as a marker. It was suggested that both drugs bind to two types of binding sites defined as a high- and a low-affinity site. Competition between PBZ and DAZ was observed for the high-affinity PBZ-site as well for the low-affinity DAZ-site, which led to the hypothesis that PBZ and DAZ binding sites are not independent, but overlap partially [97]. The reliability of the two-site model for quantitative characterization of the binding was tested with a few non-steroidal anti-inflammatory agents (indomethacin, sulindac, diclofenac and piroxicam). All of them were found to bind with comparable affinity to the high-affinity DAZ and PBZ binding sites and to the low-affinity DAZ sites. The estimated values for the

binding constant were in a complete agreement with those obtained by other traditional methods [69, 101].

HPLAC with HSA immobilized stationary phase is used most frequently for the characterization of the specific binding sites on HSA, in competitive studies. It was suggested, that verapamil enantiomers bind with high and comparable affinity at Sudlow's Site 1, and affect allosterically Site 2 [104]. Using the same approach, it was found that imipramine binds at one high-affinity site, identified as Site 2, and at few low-affinity, non-specific regions [105]. The study on HSA-binding of the sulphonylurea antidiabetic drugs tolbutamide and acetihexamide demonstrated that both drugs bind with high and comparable affinity at Site 1 and Site 2 [106]. The protein binding of lidocaine was studied using HSA and AAG immobilized columns. The principal binding protein for this basic drug was found to be AAG, however weak to moderate binding at Site 2 on HSA was also suggested [107].

A new trend in HPLAC is to study the changes in drug – protein binding in the case of glycation of HSA – a problem of considerable importance for diabetic patients. To this end chromatographic columns containing HSA at various stages of glycation are used. A study on the binding of sulphonylurea drug tolbutamide revealed that the drug binds with high and comparable affinity to both Site 1 and Site 2, and glycation results in an increased affinity of both of them [108]. Similar results were reported for the binding behavior of glibenclamide. It was found that the binding affinity of both Site 1 and Site 2 increased considerably by glycation. [109]. Such studies demonstrate the applicability of HPLAC as a tool for exploring the interactions of drugs with modified proteins and enable more complete understanding of the effect of glycation on drug binding in the blood. The principles and the application of HPLAC with protein immobilized stationary phase for studying drug – protein interactions have been discussed in several comprehensive reviews [110 – 113].

In addition to the traditional binding studies, HPLAC can assist in the screening of new drug candidates with appropriate binding characteristics in drug discovery and development. As the chromatographic capacity factor reflects the strength of the binding, it is frequently used as a quantitative measure for the binding affinity. Several reports demonstrate a good correlation between the retention on HSA immobilized column and the percentage of binding to HSA for structurally unrelated drugs and drug candidates [114 – 116]. Data for the capacity factors of 94 diverse drugs on HSA – immobilized column acquired by Colmenarejo *et al.* [117] have been used for the development of several *in silico* models for prediction of HSA binding affinity (considered in detail in the Section 5 *IN SILICO* PREDICTION OF DRUG – HSA BINDING AFFINITY).

Molecular Docking and Molecular Dynamics

Molecular docking and molecular dynamics are key structure – based computational (*in silico*) techniques. The aim of both methods is to predict the binding mode of a ligand to a protein with known 3D structure. Based on statistical criteria, the best fit orientation of the ligand at the binding site is calculated. The methods enable quantitative characterization of the strength of binding (free energy and association constant) and give insight into the chemical nature of the binding sites and the intermolecular forces governing complexation. Molecular dynamic simulations allow deeper understanding of the binding process. They monitor the time-dependent behavior of the complex and provide information about the complex effect of the solvent on the protein structure and the stability of the complex. The underplaying principles, software and utility of molecular docking and molecular mechanics are subject of several reviews [118 – 122].

Integration of the traditional analytical methods with molecular docking/dynamics turns to be a beneficial strategy for studying drug binding phenomena. A good number of reports were published recently revealing the good agreement and complementarity between the results obtained by experimental and by *in silico* approaches [52, 53, 81, 123 – 125]. The dynamic behavior of HSA binding sites was studied by explicit water, unrestrained molecular dynamics. It was suggested that the relative motion of the three domains caused enlargement of the entrance of drug binding Site 1 considerably increasing its volume and allowing accommodation of larger ligands than expected [126].

THE MOST WIDELY STUDIED ASPECTS OF DRUG – HSA BINDING

The recognized importance of plasma protein binding for PK and PD stimulated a great deal of research on different aspect of drug – HSA complexation. A few of the most important problems are discussed below.

Quantitative Characterization of Drug – HSA Binding

A quantitative measure of the affinity of HSA for a particular ligand is the value of the equilibrium association constant. Considering the equilibrium

$$\mathbf{D + P \rightleftharpoons DP}$$

where ***D***, ***P*** and ***DP*** represent the drug, the protein and the drug – protein complex, respectively, the equilibrium association constant $\mathbf{K_a}$ is expressed as follows:

$$\mathbf{K_a = \frac{[D][P]}{[DP]}} \quad (7)$$

The most popular method for estimation of $\mathbf{K_a}$ is based on the Scatchard model according to which a number of pre-formed and independent binding sites reside on the protein molecule [127]. Several classes of binding sites with different affinity for the drug may exist. For one class of binding sites the following equation was derived:

$$\mathbf{\bar{\nu} = \frac{nK_a[D]}{1 + K_a[D]}} \quad (8)$$

where $\bar{\nu}$ is the number of moles of bound drug per mol protein, and **n** – the number of binding sites. Transformation to linear form (known as a Scatchard equation) allows estimation of **n** and $\mathbf{K_a}$:

$$\mathbf{\frac{\bar{\nu}}{[D]} = nK_a - \bar{\nu}K_A} \quad (9)$$

The Scatchard method requires data for the free (or the bound) ligand concentration at constant protein and different total ligand concentration. Therefore the Scatchard equation is directly applicable for data acquired by separation techniques like ED and UF, as well as by HPFA and Hummel – Dreyer method. The data from spectroscopic methods may be processed by means of modified forms of the Scatchard equation.

A large number of values for **n** and $\mathbf{K_a}$ are available in the literature for a variety of drugs and endogenous ligands, and extensive databases have been published [8, 29, 128]. Binding affinity data vary significantly from report to report as a result of differences in the methodology, the experimental conditions, and the mathematical approaches.

Identification of the Binding Sites

Long before the X-ray determination of the HSA structure it was recognized that the binding of drugs to HSA occurred at a small number of distinct binding sites with different specificity [20,

29]. A large number of reports have been published targeted to identification of the chemical nature of drug binding sites. In general, the studies were based on displacement experiments. By means of fluorescent probes it was suggested that WAR and PBZ bound in hydrophobic regions [64, 66]. It was proposed that the aromatic moieties of flufenamic acid and its derivatives occupied a hydrophobic crevice in HSA while the carboxylic groups interacted with cationic site at the protein surface. It was stated that the binding of anionic drugs to HSA was governed by hydrophobic and electrostatic interactions [65]. The first attempt for mapping a HSA binding site was made by Swaney and Klotz [35]. They isolated a peptide segment around the single Trp residue and suggested the presence of a non polar cavity with one Trp residue at the bottom, surrounded by positively charged groups (Arg and Lys). A combination of a hydrophobic region and a negatively charged surface was considered as a prerequisite for strong binding of anionic drugs. Sudlow *et al.* defined two distinct binding sites on HSA for anionic drugs and proposed some structural requirements for specific binding [34]. Large heterocyclic molecules with a negative charge localized at the centre of a largely non polar region were classified as Site 1 drugs. Site 2 drugs showed preference toward aromatic carboxylic acids with a generally extended shape and a negative charge at one end of the molecule away from the hydrophobic center. Fehske *et al.* suggested that the lone Trp residue was involved in the high affinity WAR binding site*, i. e.* the main part of Site1 was located in domain II of HSA [36]. A distinct binding site, situated in domain III, was assumed for benzodiazepines. A third binding site was also supposed, and WAR, DAZ and digoxin were proposed as specific markers for the three primary binding sites on HSA [129]. Based on the large amount of binding reports it was argued that WAR, PBZ, phencoumon, acenocoumarin, oxyphenbutazone, azapropazone, sulphinpyrazone, iophenoxic acid, indomethacin, *etc.* bound specifically to Site 1 while arylpropionic non-steroidal anti inflammatory drugs (flurbiprofen, ibuprofen, naproxen, flufenamic acid), a few antidiabetic agents and benzodiazepines bound to Site 2 [59, 130].

More detailed studies have shown that the classical binding sites may comprise of more than one sub-sites. Site 1 was described as capacious and flexible region containing a large number of distinct ligand binding sites, sometimes independent, but often mutually affected [131]. Two partially overlapping binding sites were suggested for WAR and PBZ [98] as well as for WAR and azapropazone [132]. Site 2, initially described as a small, receptor like site, turned out to consist of more than two subsites [96, 133].

Competition studies have also revealed that binding of a ligand to its specific binding site may cause conformational changes in the protein and may affect the binding of second compound to a distinct binding site. Co-binding of two drugs to a protein may be classified as one of four types: independent (two drugs bind to completely independent sites and do not influence each other); cooperative (the binding of one drug to its specific site induces higher affinity of a second site for another drug); anticooperative (allosteric interactions between two sites decrease the binding affinity) and competitive (two drugs share the same site and the less tightly bound drug is displaced by simultaneous binding) [78]. In fact, the broad selectivity of HSA, the possibility to complex with a variety of structures with high affinity is considered to derive from the significant allosteric effects [134]. The presence of cooperative and allosteric modulation between the binding sites is discussed in detail by Ascenzi *et al.* [12]**.**

There is much evidence that the major drug binding sites are not completely independent. Site 2 drugs were classified into two groups depending on whether or not the drug influenced Site 1 [59, 135]. The Site 2 drugs flurbiprofen and ibuprofen were found to cause allosteric change on Site 1 [59]. Indirect competition was observed during the co-binding of Site 1 and Site 2 drugs: salicylates and DAZ, PBZ and ibuprofen [78], DAZ and PBZ [100]. This could be attributed either to overlapping of the binding sites, or to allosteric interactions between them [78].

There are numerous reports that the binding of FAs to their primary binding sites causes allosteric modification of the drug binding sites. Conformational change in both Site 1 and 2 was observed in the presence of stearic acid [59]. Long chain FAs at low molar ratio (up to 4 moles acid per mol HSA) were found to enhance the binding of WAR to its primary Site 1 [43]. The allosteric effects of FAs on the binding of WAR were demonstrated recently by means of molecular dynamics approach [136]. Allosteric modification of the secondary binding sites of ketoprofen was also supposed [137]. The occurrence of dramatic conformational changes in HSA structure in the presence of FAs was confirmed by numerous X-ray studies [31 – 33, 37].

Taking into account the high flexibility of the protein molecule, the possibility of allosteric interactions during co-binding and the evidences of numerous sub-sites within the classical binding Sites 1 and 2, the presence of a small number of pre-formed binding sites on HSA seems questionable. It is more probable that the binding sites are formed by extensive conformational changes during the binding process [138]. The possibility of binding of drugs at larger, less localized regions on the protein rather than at particular small receptor like sites was also hypothesized [139, 140]**.** Therefore, it is reasonable to replace the term "binding sites" with "binding regions" of less specific nature capable to rearrange properly for the best fit of the ligand.

Almost all hypothesis regarding the chemical nature of the specific binding sites and their location on HSA were confirmed in X-ray studies of drug – HSA complexes revealing that Site 1 corresponds to the hydrophobic pocket in sub-domain IIA, and Site 2 – to the pocket within sub-domain IIA [38]. This confirms the reliability of the conventional displacement techniques for studying the nature of binding sites.

Stereoselectivity of Drug – HSA Interactions

Many drugs are chiral molecules used as racemates in medicinal practice. The binding of racemic drugs to plasma proteins is potentially stereoselective as a consequence of the chiral discriminative properties of the binding sites [44]. The different affinity of the proteins for enantiomers may result in a different PK and PD profile. Therefore identification of the molecular mechanisms underlying stereodiscrimination by plasma proteins is important field in drug binding research. It is believed that HSA possesses the highest potential stereoselectivity among all plasma proteins [79]. The binding of acidic drugs is in general enantioselective, while the process is relatively non-selective for basic drugs which bind primarily to AAG. Reverse enantioselectivity towards HSA and AAG was reported for several drugs. S-amlodipine binds to a higher extent to HSA compared with its R-isomer, while the opposite affinity is observed for AAG [141]. Calcium antagonist semotiadil (R-isomer) and its S-isomer (levosemotiadil) bind strongly and enantioselectively to both HSA and AAG, manifesting reverse selectivity of the two plasma proteins [142].

Stereoselectivity is generally assigned to the high-affinity binding site. However, the secondary site may also be selective. For example, ketoprofen and suprofen enantiomers were selectively displaced from the WAR site while their primary site (DAZ site) was found to be non-enantioselective [99]. Another study on ketoprofen binding revealed a stereoselectivity of the high-affinity PBZ and low-affinity DAZ-sites with preference for the R-enantiomer [99].

Enantioselectivity may result either from the binding of the two chiral forms with different affinity to a common binding site, or by complexation at different binding regions. For example, WAR enantiomers bind to a common binding site on HSA, Site 1, but S-WAR is more strongly bound that its R-isomer. Evidence for differ-

ent binding mechanisms for WAR antipodes to Site 1 was obtained in reciprocal competition experiments. (S)-WAR was shown to be a direct competitor for its R-isomer, while R-WAR was an indirect competitor for its antipode [143]. Thermodynamic study revealed that the binding of S-WAR was dominated by electrostatic interactions, while less specific hydrophobic interactions were involved in the complexation of R-WAR at the same binding site [144]. In contrast, for many Site 2 drugs enantioselective binding to different regions was observed. It was found that ibuprofen enantiomers shared one common binding site with a preference for R-ibuprofen, while the S-isomer bound to at least one distinct high-affinity site [97]. Two independent binding sites for oxazepam hemisuxinate (OXH) enantiomers were suggested. In a displacement study with ibuprofen (marker for Site 2) it was observed that S-OXH was more tightly bound to HSA than its R-antipode, and was competitively displaced by ibuprofen. In contrast, R-OXH was not displaced [93]. At least five different stereoselective binding sites were proposed for benzodiazepines each of which have different structural requirements [96]. On the basis of quantitative structure – HSA binding study the enantioselective binding sites were classified into two classes – for bensodiazepines in P and M conformation, respectively [145].

Enantioselectivity is commonly studied by using displacers or markers for the specific binding sites. When two enantiomers share a common binding region, and displacement is simply competitive, the less-tightly bound enantiomer is displaced to a lesser extent. Competitive enantioselective inhibition of HSA binding by lithocholate was suggested for racemic ketoprofen, naproxen and suprofen with greater displacement of the S-enantiomers [146]. However, displacers frequently cause allosteric modification of the binding sites and may alter their chiral recognition ability. It was found that octanoic acid induced conformational changes in Site 1, resulting in allosterically mediated enantioselective displacement of suprofen and ketoprofen antipodes from their secondary binding site. In contrast, WAR enantiomers were competitively displaced from the same site [99]. Allosteric effect of octanoic acid on enantioselective binding of ketorolac was demonstrated resulting in a change of the unbound R/S ratio with an increasing concentration of the displacer [95]. The presence of WAR and ketoprofen used as markers for the major binding sites was shown to reduce and even to eliminate the enantioselectivity in the binding of the antiparkinson drug rotigotine (S-isomer) and its R-antipode [147]. It was found that acetylsalicylic acid and its metabolite salicylic acid displace stereoselectively propranolol enantiomers from their binding sites and could change the unbound R/S ratio which may affect significantly the PK profile of the drug [148]. Therefore, the possibility of allosterically mediated or inverted enantioselectivity must be taken into account by interpretation of the results from displacement protein binding studies. The methodology for evaluating stereoselectivity of PPB is subject of several reviews [44, 78, 79, 149].

Thermodynamic Characterization of Drug – HSA Binding

Thermodynamic characterization involves estimation of the change in the main thermodynamic parameters accompanying the complex formation: free energy (**ΔG**), enthalpy (**ΔH**) and entropy (**ΔS**), related by the equation:

$$\Delta G = \Delta H - T\Delta S \quad (10)$$

There are two commonly used methodologies for thermodynamic studies. The first one is based on a measurement of the equilibrium association constant K_a at several temperatures. The following relation exists between K_a and **ΔG**:

$$\Delta G = -RT \ln K_a \quad (11)$$

where **R** is the universal gas constant and **T** is the absolute temperature. In a narrow temperature interval (commonly used in protein binding experiments) **ΔG** are independent of temperature and can be calculated by means of the van't Hoff's equation:

$$\ln K_a = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R} \quad (12)$$

The other approach is based on the isothermal titration calorimetry (ITC). ITC is a widespread simple method which allows simultaneous determination of K_a and **ΔH** in one single experiment and further calculation of **ΔG** and **ΔS**. The principles and application of ITC for protein – ligand interactions are subject of numerous papers and reviews [150, 151].

A large negative value of **ΔG** is a prerequisite for spontaneous binding. For highly bound drugs with K_a of the order of $10^{-6} - 10^{-7}$ the value of **ΔG** is in the range (– 31) ÷ (– 37) kJ/mol. The binding process is either enthalpy driven (with $\Delta H << 0$) or entropy driven (with $\Delta H \geq 0$ and $\Delta S >> 0$) [152]. However, the two energy terms are not completely independent but are related through the so called "enthalpy – entropy compensation". It is characterized by a linear relationship between **ΔH** and **ΔS** for a series of related ligands, as well as for a given temperature interval [153]. Any increase in the binding enthalpy is compensated by a parallel increase of the binding entropy, and *vice versa*, so that the binding affinity remains relatively constant. According to the most recent hypotheses concerning drug – receptor interaction mechanisms, these thermodynamic phenomena appear to be a consequence of the rearrangement of solvent molecules during the binding [152].

The two components of **ΔG** represent two classes of processes responsible for binding: intermolecular interactions like hydrogen bonding, electrostatic and van der Waals interactions related mainly to the enthalpy term, and solvent reorganization associated with the entropy term [152]. The values of **ΔH** and **ΔS** are informative for the nature of the forces governing the binding process. A high positive **ΔS** is considered as an evidence for hydrophobic interactions; $\Delta H < 0$ and $\Delta S < 0$ suggests hydrogen bonding or Van der Waals interactions, while $\Delta H \leq 0$ and $\Delta S > 0$ imply electrostatatic interactions [54]. Detailed thermodynamic analysis of the binding process provides a more comprehensive view of the various types of intermolecular forces involved in the complexation.

Thermodynamic study on the binding of sulindac to HSA by means of HPLAC suggested spontaneous binding with a high negative **ΔG**. Both **ΔH** and **ΔS** were negative, and the absolute value of **ΔH** highly exceeded the entropy term **TΔS**. Therefore it was supposed that the binding was dominated by hydrogen bonds and salt linkages while hydrophobic interactions were of less importance. These data allowed hypothesis to be made about the nature of the binding site and the binding mechanism [103, 144]. Thermodynamic study also revealed different binding mechanisms for R- and S-WAR, responsible for the observed stereodiscrimination by the common binding site on HSA [144]. The binding of R-WAR was accompanied by a high positive **ΔS** and a negligible $\Delta H \approx 0$ suggesting the contribution of hydrophobic interactions. In contrast, a large negative **ΔH** and a negative **ΔS** were found for S-WAR, indicative for hydrogen bonding. The higher specificity of the hydrogen bonding compared with the non-specific hydrophobic interactions explain the stronger binding of the S-isomer. However, one must be very careful while interpreting the results from thermodynamic studies. The binding process is a very complicated phenomenon where the various types of interaction can hardly be clearly differentiated. During the binding one or other type of interaction may prevail giving the predominant role of the related parameter (**ΔH** or **ΔS**) and thus assigning them higher contribution to **ΔG**. The "enthalpy – entropy compensation" effect should also be considered.

IN SILICO PREDICTION OF DRUG – HSA BINDING AFFINITY

The application of combinatorial chemistry methods in drug development has led to extensively growing number of structures with drug-like activities. Unfortunately, the majority of drug candidates fail to become marketable products due to poor PK [155]. It is recognized that the success of a new drug candidate is determined not only by its high efficacy and safety, but also by proper PK behavior. The understanding of the importance of the PK inspired an intense research focused on the prediction of the PK properties of drug-candidates as early as possible. One of the most reliable and widely used approaches for prediction of PK properties is the computational (*in silico*) modeling. It enables construction of quantitative structure – property relationships (QSPR) based on molecular descriptors. QSPR models allow prediction of PK properties at early stages of drug discovery, even on virtual compounds.

A good number of studies on the prediction of PPB of drugs have been published in the last two decades which is not surprising given the impact of PPB on the overall PK behavior. Such models are valuable for both theory and practice. They give insights into the chemical nature of drug – protein interaction and assist in the design of new compounds with desired PPB. Recently, this strategy was successfully applied for prediction of diflunisal analogues with reduced affinity to human serum albumin [156].

A few studies concerning congeneric series of drugs – β-lactam antibiotics [157, 158], COX-2 inhibitors [159], *etc.* Lipophilicity [157] and several topological indices [158] (electron accessibility of the aromatic ring, presence of Cl or F atoms, methylene groups, *etc.*) were found to contribute positively to PPB, while amine groups or carbonyl O-atom disfavor HSA binding. In general, QSPRs derived on congeneric series have higher predictive power since a binding to common binding sites on HSA is expected. However, these models are local models, only valid within the studied series. The construction of a global model requires a large dataset, encompassing diverse chemical space – as wide, as possible.

A number of QSPR studies were performed on one and the same dataset comprising data for 94 diverse drugs and drug like compounds, compiled by Colmenarejo [117]. The chromatographic capacity factor, determined by HPLAC with HSA immobilized column was used as a quantitative measure for the HSA binding affinity. The studies differed in the type and the number of descriptor used (constitutional, topological, geometrical, electrostatic, quantum chemical, molecular docking descriptors, or combination of them). A wide array of statistical methods were used to derive the model: multiple linear regression (MLR) [117, 160 – 163], artificial neural networks (ANN) [164, 165], support vector machine (CVM) [162, 163], *etc*. Variable selection was performed by stepwise regression, genetic algorithm, ant colony optimization, cluster analysis, *etc*. Analysis of the generated QSPRs identified the hydrophobicity (measured by logP [106, 150, 152] or molecular surface area [151]) as the most significant property determining the binding affinity of HSA. In addition, several structural features appear to be important: type, number and substituents in the rings [117, 160, 162], presence of aliphatic CH groups [160, 161], halogens (F and Cl) [160], hydrogen bonding ability [162], *etc*. Most of the models have high predictive power as assessed by the values of the coefficient of determination for the external test set ranging between 0.64 and 0.89. The best statistics showed the non-linear SVM approach however on account of the lack of interpretability.

A few QSPR models were proposed based on topological substructural molecular descriptors [166, 167]. This approach is beneficial as it provides information about the contribution of different groups and fragments to the drug binding to HSA. A group contribution model for ligand binding to domain III (Site 2) of HSA was developed using data for the experimentally obtained dissociation constants for 889 chemically diverse compounds [166]. It was suggested that the presence of cyclic or acyclic amines (positively charged at physiological pH) contributes negatively to Site 2 binding, while anionic groups (carboxylic or sulphonic acids) have a positive impact. With some exceptions, the hydrophobicity of the fragments was also favorable for PPB. The integration of fragment – based QSPRs and molecular modeling confirmed the predominant contribution of hydrophobic moieties, but also the significance of polar groups for specific binding of drugs to Site 1 and 2 on HSA [167].

Several QSPR models are based on pharmacophoric similarity principle. It is expected that molecules with similar distribution of the pharmacophoric units (hydrogen bond donors/acceptors or hydrophobic regions) will interact in similar manner with HSA and will manifest comparable affinity [128, 168, 169]. Similarity concept coupled with partial linear regression (PLS) was used to generate a model for prediction of the primary association constant of the drugs to HSA. The presence of cationic nitrogen was considered as one of the criteria for low binding, while a typical pharmacophoric combination in the high binders was the presence of two hydrogen bond acceptors, separated by a hydrophobic fragment of 5 to 16 bond units. A pharmacophore for specific binding of acidic drugs was also proposed, consisting of two of the three units: hydrophobic center, aromatic center and hydrogen bond donor, located within a distance of 4-5 Å [168]**.** A combination between cluster analysis and 4D fingerprint molecular similarity analysis was used for prediction of the primary association constant of 115 drugs. The key role of the spatial relation between a non polar region and hydrogen bond donor was suggested showing that both hydrophobic interactions and hydrogen bonding are essential for drug – HSA complexation [169].

In several QSPR studies the extent of PPB is quantified by the percentage of drug bound to plasma proteins – a parameter accounting for the binding to all plasma proteins (HSA, AAG, lipoproteins, etc.) The most comprehensive QSPR analysis on PPB was published by Votano *et al.* [170]. Four modeling techniques including MLR, ANN, k nearest neighbors and SVM were applied on a dataset for 1008 drugs. The best model was ANN. In another report a stepwise MLR and a few non-linear regression tree techniques were used to generate QSPR model based on data for 794 compounds [171]. Analysis showed that lipophilicity was the major determinant of PPB for highly lipophilic drugs; however hydrogen bonding interactions contributed greatly to the binding affinity. In general, drugs with higher negative charge surfaces demonstrated higher PPB.

It is generally accepted that the acidic drugs bind preferably to HSA, while bases manifest greater affinity to AAG. Therefore different structural requirements for binding to various plasma proteins could be expected, and separate QSPR analysis is reasonable. A QSPR model for prediction of PPB of acidic drugs was published recently revealing that the lipophilicity, the presence of aromatic rings, cyano groups and H-bond donor-acceptor pairs increase the PPB, whereas the presence of a tertiary C-atoms, four-member ring or iodine atoms are unfavorable for complexation [172].

Most of the studies discussed use ligand – based methodologies. The determination of the X-ray structure of HSA and the identification of the topology of the major drug binding sites inspired a lot of structure – based *in silico* studies on drug – HSA interactions. These studies have theoretical advantages because they reveal the actual structural determinants of the affinity. A QSPR model based on molecular docking descriptors was proposed and the notable importance of considering protein flexibility was discussed [163]. Recently, an SVM based model and molecular docking algorithm were integrated into a free, web based platform [173]. This platform enables prediction of HSA complexation of a query drug, identification of the most probable ligand binding site on HSA, calculation of the complex geometry using molecular docking calculations, *etc.*

Docking simulations using template structures of HSA from X-structures of complexes have demonstrated that most of the currently available profen drugs should bind preferably to Site 2 [174]. Molecular docking studies are increasingly widely applied, alone or in combination with other methods for studying the molecular mechanisms of drug – HSA binding.

Evidently, numerous successful QSPR models have been developed for prediction of HSA binding affinity with comparable predictive power. There is no superior *in silico* methodology – each one has its advantages and shortcomings. Application of two or more methods to one dataset increases the confidence in the results. Most models identify lipophilicity as the major factor determining HSA binding affinity. However, structural features like size, shape, and presence of particular atoms, groups or fragments may be crucial for HSA binding affinity.

In summary, although the binding of drugs and other exogenous and endogenous ligands to HSA has been studied for more than 50 years, despite thousands of published papers, reviews and books, the knowledge on the binding phenomena is still incomplete and remains an area of intense research.

ABBREVIATIONS AND SYMBOLS

AAG	=	alpha – 1 – acid glycoprotein
ANN	=	artificial neural networks
C^{ss}_{av}	=	average steady state concentration of the drug
$C^{ss}_{av, u}$	=	average steady state concentration of the free drug
CD	=	circular dichroism
CL	=	clearance
CL_{int}	=	intrinsic clearance
D	=	dose
DAZ	=	diazepam
E	=	drug extraction ratio
ED	=	equilibrium dyalisis
F	=	bioavailability
F_H	=	the fraction of the drug, escaped first pass metabolism
FA	=	fatty acid
$f_{u, p}$	=	free fraction of the drug in the plasma
$f_{u, t}$	=	free fraction of the drug in the tissues
HPFA	=	high performance frontal analysis
HPLAC	=	high performance liquid affinity chromatography
HPLC	=	high performance liquid chromatography
HSA	=	human serum albumin
ITC	=	isothermal titration calorimetry
K_a	=	equilibrium (association) constant
MLR	=	multiple linear regression
PBZ	=	phenylbutazone
PD	=	pharmacodynamics
PK	=	pharmacokinetics
PLS	=	partial least square
PPB	=	plasma protein binding
Q_H	=	liver blood flow
QSPR	=	quantitative structure – property relationship
SVM	=	support vector machine
$t_{1/2}$	=	half-life
UF	=	ultrafiltration
V_d	=	apparent volume of distribution
V_p	=	volume of the plasma
V_t	=	volume of the tissues, in which the drug is distributed
WAR	=	warfarin
ΔG	=	change in the free Gibbs energy
ΔH	=	change in the enthalpy
ΔS	=	change in the entropy
$\bar{\nu}$	=	number of moles bound ligand per 1 mol protein
τ	=	dosing interval

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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