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Review

Enantioresolution and Binding Affinity Studies on Human Serum Albumin: Recent Applications and Trends

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Abstract: The interaction between proteins and drugs or other bioactive compounds has been widely explored over the past years. Several methods for analysis of this phenomenon have been developed and improved. Nowadays, increasing attention is paid to innovative methods, such as high performance affinity liquid chromatography (HPALC) and affinity capillary electrophoresis (ACE), taking into account various advantages. Moreover, the development of separation methods for the analysis and resolution of chiral drugs has been an area of ongoing interest in analytical and medicinal chemistry research. In addition to bioaffinity binding studies, both HPALC and ACE al-low one to perform other type of analyses, namely, displacement studies and enantioseparation of racemic or enantiomeric mixtures. Actually, proteins used as chiral selectors in chromatographic and electrophoretic methods have unique enantioselective properties demonstrating suitability for the enantioseparation of a large variety of chiral drugs or other bioactive compounds. This review is mainly focused in chromatographic and electrophoretic methods using human serum albumin (HSA), the most abundant plasma protein, as chiral selector for binding affinity analysis and enantioresolution of drugs. For both analytical purposes, updated examples are presented to highlight recent applications and current trends.

Keywords: affinity capillary electrophoresis; binding affinity; docking; enantiomeric separation; enantioselectivity; high performance affinity liquid chromatography; human serum albumin; plasma proteins

Citation: Cardoso, T.; Almeida, A.S.; Remião, F.; Fernandes, C. Enantioresolution and Binding Affinity Studies on Human Serum Albumin: Recent Applications and Trends. *Chemosensors* **2021**, *9*, 304. https://doi.org/10.3390/chemosensors9110304

Academic Editor: Jin-Ming Lin

Received: 14 September 2021
Accepted: 22 October 2021
Published: 25 October 2021

1. Introduction

Plasma proteins are capable of interacting with about 43% of the 1500 drugs most commonly used in clinical practice [1,2]. Moreover, the drugs frequently show a high affinity for these proteins (about 90% or more of the administered dose binds to plasma proteins) [3]. Consequently, the interaction between drugs and plasma proteins is quite significant, which is reflected in the biological action of drugs [1,2]. It should be noted that the interactions established between proteins and drugs are reversible, since the purpose is to transport the compounds to the different tissues in order to achieve their biotargets. The binding affinity study of the drugs and other bioactive molecules to plasma proteins is crucial for understanding how they are transported in the body, as well as to determine the unbound portion, which is responsible for the pharmacological/biological effect. The binding that drugs establish with plasma proteins also interferes in the control of various processes related to their pharmacokinetics and pharmacodynamics, such as

volume of distribution, excretion, toxicity, among others [1,2,4,5]. However, in the body, these interactions may not always occur in the same way, since competition between two drugs or between a drug and an endogenous molecule or a dietary compound, for the same plasma proteins, can occur, with consequences on their pharmacological activity or toxicity [6–8]. Saturation of plasma proteins may also occur in cases where the blood concentrations of administered drugs are high, resulting in an increase in the free fraction of these drugs. This phenomenon of plasma protein saturation is important especially for drugs that have a great capacity to bind to plasma proteins, where small variations in the amount of free fraction may have a consequent impact on the therapeutic effects of the drugs [9,10]. The binding of drugs with proteins and other agents in serum is also of interest in personalized medicine because this process can affect the dosage and action of drugs. The extent of this binding may also vary with a given disease state [11]. Moreover, in the early stages of drug discovery, beyond the biological activity evaluation, each compound should also be characterized by its physico-chemical properties, such as lipophilicity, ionization, solubility, as well as metabolic profile and protein-binding affinity [12,13]. These are some of the reasons that justify the importance of plasma protein binding affinity studies.

Curiously, nowadays, plasma protein binding remains a controversial topic in drug discovery and development [14]. There are researchers that question whether changes in plasma protein binding actually have a clinical relevance [15,16]. Some arguments put forward are that the number of drugs for which changes in plasma protein binding may be clinically relevant is small and that the toxicity of these drugs can be explained by other pharmacokinetic and/or pharmacodynamic phenomena [17]. Furthermore, the increase in free drug concentration is typically transient, since redistribution in the body and increased clearance will occur, i.e., the free fraction will return to its original concentration and, therefore, the effect will remain the same [18]. Instead, we and many other research groups find that the drug–drug interactions related to plasma protein binding are very relevant, which can lead to an increase in the free fraction of drugs in the blood. This concern is based on the fact that when a compound is displaced from plasma protein binding, its concentration in free form increases. As is known, the free drug concentration corresponds to the fraction responsible for the therapeutic effect; if it increases, there will consequently be an upsurge in the effect of the drug and it may become potentially toxic [15,19]. Warfarin (anticoagulant) is a classic example to explain this phenomenon. In fact, it has been observed that when taken simultaneously with phenylbutazone (anti-inflammatory), for example, an increase in warfarin's anticoagulant effect occurred, leading to serious bleeding. One of the reasons for this effect is that phenylbutazone has a higher affinity than warfarin for plasma proteins, thus leading to an increase in the free fraction of the anticoagulant. In addition, phenylbutazone also causes a decrease in the clearance of warfarin [20]. Another example is phenytoin (antiepileptic) that displays changes in binding to plasma proteins when administered together with sodium valproate (anticonvulsant). In fact, it was found that the sodium valproate caused a significant increase in the free fraction of phenytoin, as it showed a higher affinity for plasma proteins. This increase in the concentration of free phenytoin in the blood can cause nausea, drowsiness and even convulsions. In addition, sodium valproate also causes an inhibition of phenytoin metabolism [21].

Considering the interaction with chiral molecules, plasma proteins, as well as other biomolecules comprising intrinsic chirality, such as enzymes, transporters or receptors, generally have a higher affinity for one of the enantiomers (enantioselectivity) [22]. As a consequence, the enantiomers may exhibit different pharmacodynamic, pharmacokinetic and/or toxicological properties [23–26]. Typically, when one enantiomer is responsible for the biological activity of interest, the other could be inactive, possess lower activity, be an antagonist of the active enantiomer or have a different activity that could be desirable or undesirable [27,28]. There are many studies concerning enantioselective binding to plasma proteins [29,30]. A recent example described that for some profens, such as etodolac, indoprofen, ibuprofen, and ketoprofen, the plasma protein bond was strongest for the

(*R*)-enantiomer. Additionally, it was found that the different interaction that occurs between plasma proteins with the profen enantiomers influenced the metabolic behavior and drug interactions [31]. Other recent examples concern the stereoselective binding of cytosine nucleoside enantiomers [32], lactic acid [33] and ginsenoside Rh2 [34] to human serum albumin (HSA).

Many chiral drugs used in clinical practice were administered as racemates. However, this trend was changed mainly because of the regulatory requirements [35,36]. In fact, the investigation and production of chiral drugs began to have real significance from new guidelines adopted since 1992, by the Food and Drug Administration (FDA), in U.S.A., present in a document entitled Policy Statement for the Development of New Stereoisomeric Drugs [37]. In 1994, the European Committee for Proprietary Medical Products (CPMP) also issued formal guidelines called Investigation of Chiral Active Substances [38]. In addition to the regulatory requirements, also the advances in enantioselective synthesis [39–42] and enantioresolution techniques [43–45], as well as the "chiral switch" phenomenon [46,47], contributed to the increasing development of enantiomerically pure drugs rather than racemates [36,48]. In fact, it is known that enantiomerically pure drugs can have advantages, such as more effective therapy, reduction of the dose to be administered, less variability of the metabolic profile and therapeutic response, reduction of side effects and interactions, among others [22,49].

Studies of the interaction of chiral drugs with plasma proteins can provide valuable information for understanding the differences that are observed between a pair of enantiomers. In addition, they can help to predict the behavior of a chiral compound in the human body, to understand whether there are interactions between drugs and between the enantiomers of a compound and, above all, to help in determining the appropriate therapeutic doses [1,2,50]. These are some of the reasons that justify why a drug is only approved for marketing after various studies have been carried out, which include plasma protein binding affinity. If it is a chiral drug, both enantiomers must be tested. This pre-assessment of drugs also helps to understand whether the drug is more effective in its enantiomerically pure or racemic form [2].

The plasma proteins that frequently interact with drugs are HSA and α-acid glycoprotein (AGP). Lipoproteins, erythrocytes or even platelets can also interact with diverse compounds [51]. Although there are other proteins in human blood plasma capable of binding drugs, HSA is the most abundant protein [52] and, consequently, the most studied in protein binding affinity and enantioseparation studies [53,54].

This review is focused on HSA protein applied for binding analysis and enantioresolution of drugs. An overview of the different methods for determination of protein binding affinity is described with special focus to those that have aroused more significance over the last years. The current trends and perspectives in the field of HSA binding affinity are evidenced by considering updated examples of both chromatographic and electrophoretic methods.

2. Human Serum Albumin

As stated before, HSA is the most expressed plasma protein in human blood, accounting for about 50–60% of plasma proteins. It has the particularity of being synthesized only in the liver (about 12–25 g/day) and not being glycosylated like other plasma proteins. The normal range of HSA concentration in blood is 30–50 g/L. Furthermore, it has a half-life of about 20 days. Its degradation (about 14 g/day) is mainly carried out in muscle tissue, skin, kidney and gastrointestinal tract as well as to a limited extent in the liver. HSA has a pK of 4.8 which means that at physiological pH (~7.4) it ionizes, which explains its high solubility in water [55,56].

Structurally, HSA consists of a single polypeptide chain composed of 585 amino acids, with a molecular weight of about 66 kDa, and comprising six helix-shaped domains (Figure 1), with domains I–III being the most important for binding endogenous and exogenous compounds; each of these is subdivided into A and B subdomains. Domains

IIA and IIIA are also known to be the preferential binding sites for exogenous compounds, and are referred to as Drug Site I or Sudlow's site I and Drug Site II or Sudlow's site II, respectively [57–59]. HSA has 35 thiol groups, of which 34 form disulfide bonds between them, resulting in the helical shape of the protein and being responsible for its structural and thermostability [52,60]. The remaining thiol group is responsible for about 80% of the antioxidant capacity of albumin, as it is able to neutralize reactive oxygen species (ROS) and nitrogen species (RNS), as well as free radicals. In addition, it has a wide variety of other functions in the body (Figure 1), which explains its designation as a "multifunctional" protein [56].

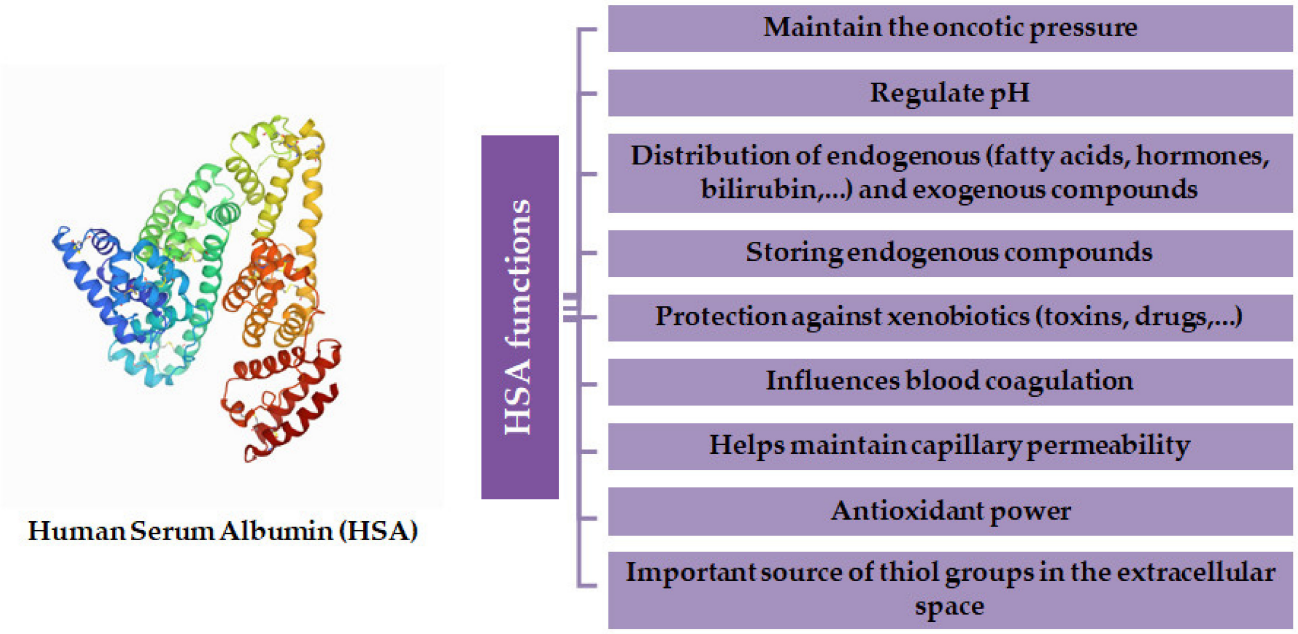

Figure 1. 3D structure of HSA (Protein Data Bank:1AO6) and its main functions in the body.

Typically, HSA shows a higher affinity for low molecular weight, hydrophobic and negatively charged compounds [58,59]. In the case of compounds with low hydrophilicity, the affinity for HSA plays a very important role, since it will prevent the aggregation of the compounds in the vascular walls and their reduced distribution in the organism. However, an excessively high affinity will make necessary to administer a higher dose of drug to reach the desired concentration of active fraction, since the distribution to biotargets will be slower and its elimination will not be as efficient [58].

The main endogenous ligands of HSA are the long chain fatty acids, for which it has seven binding sites, which are distributed in domains I–III. HSA generally transports 1 to 2 fatty acids, however, under certain conditions (very intense physical exercise, for example) there may be an increase in transport to 4 or more fatty acids per molecule of HSA. Furthermore, the binding of fatty acids to albumin can cause conformational changes in the plasma protein and may expose the free thiol group that gives it some antioxidant power [56,57]. Fatty acids are not the only endogenous ligands with the ability to bind to HSA [57]; bilirubin, ascorbate, calcium, copper, zinc, chloride, steroid hormones, nitric oxide (NO) are other examples.

As previously mentioned, there are several exogenous compounds that are able to bind to fatty acid binding sites and competition may occur. If the exogenous compounds have a higher affinity for the binding site, they will displace the HSA-bound fatty acids to occupy that site. Table 1 shows some exogenous ligands that bind to fatty acid binding sites [56].

Table 1. Example of exogenous ligands that compete for HSA fatty acid binding sites [56].

	HSA Binding Sites	
	Sudlow's Site I	**Sudlow's Site II**
Exogenous albumin ligands	Warfarin Myristate Thyroxine Indometacin Phenylbutazone Diflunisal	Ibuprofen Diazepam Propofol Myristate Halothane Thyroxine

HSA is also used as a therapeutic agent in cases of hypovolemia, shock, blood loss and surgical trauma, where it is necessary to re-establish a patient's oncotic pressure, as this plasma protein is primarily responsible for maintaining oncotic pressure [61]. Recently, it was used as a biomarker for the diagnosis of certain types of cancer, rheumatoid arthritis, post-menopausal obesity, ischemia, severe acute graft-versus-host disease, and diseases that need monitoring of glycemic control [62–64]. Another application concerns the generation of long-lasting biotherapeutics, with improved pharmacokinetic properties and superior efficacy in the clinic, by non-covalent albumin-binding ligands [65]. Adding HSA to cell cultures to increase cell growth and productivity has also been described [66]. Moreover, it is known that HSA has a great potential in terms of biopharmaceuticals, since it can be isolated, fused or in complex with other proteins or even drugs in the form of nano-formulations [55,67].

3. Determination of Protein Binding Affinity of Drugs

In recent decades, drug–protein interactions have been widely studied and several methods for analysis of these phenomena have been developed and improved (Figure 2) [53,68–71].

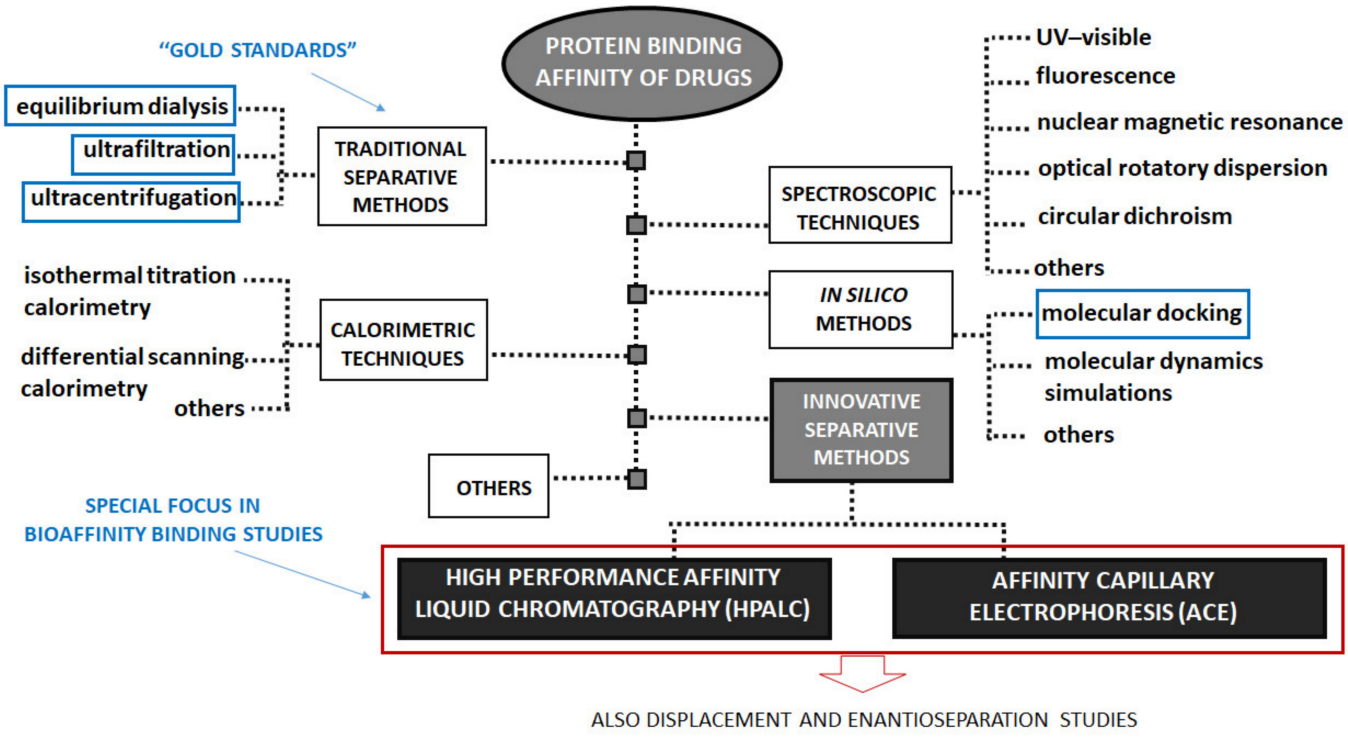

Figure 2. Methods for determination of protein binding affinity of drugs.

The methods discussed in this review include the traditional separative methods of equilibrium dialysis, ultrafiltration and ultracentrifugation, that were widely used and are considered the "gold standards". Special focus will be done to chromatographic and electrophoretic methods as innovative tools for studying the interaction of drug/ligand with plasma proteins which, in addition to the assessment of protein binding affinity, also allow other applications including the enantioseparation of chiral compounds. Besides, in silico methods, such as the docking approach, also proved to be convenient tools to estimate protein binding affinity. Some very valuable protein binding spectroscopic techniques (UV–visible, fluorescence, nuclear magnetic resonance, optical rotatory dispersion, circular dichroism) or calorimetric techniques (isothermal titration calorimetry and differential scanning calorimetry) will not be covered. We choose to limit the scope of the review in this manner since, Siddiqui et al. [72] recently reviewed those techniques.

3.1. Equilibrium Dialysis, Ultrafiltration and Ultracentrifugation

All these methods are based on the phenomenon of physical separation and measurement of the bound and unbound fraction of a drug. In the case of equilibrium dialysis and ultrafiltration, the physical separation is performed through the use of semipermeable membranes that allow the separation of the two fractions. After separation of the fractions, a quantitative analysis is generally performed using appropriate analytical techniques, such as liquid/gas chromatography coupled to a mass spectrometer (LC-MS/GC-MS) [70,73].

Equilibrium dialysis has been the method most widely used to study drug–protein interactions, being regarded as the reference method [69]. In an equilibrium dialysis experiment, an apparatus comprising two chambers separated by a semipermeable membrane is used. The plasma sample containing the drug is placed in one chamber and a buffer solution in the other. The membrane separating the two chambers is impermeable to the bound fraction and slightly permeable to the free fraction and, consequently, the latter is able to pass through the membrane. Equilibrium is reached when the concentration of free fraction is equal in the two chambers [3,7,70,73]. Compounds with higher molecular weights and highly bound to plasma proteins will take longer to reach equilibrium [70]. Once equilibrium is established, the solutions of the two chambers are collected, and the solution of the plasma chamber will allow the determination of the total concentration of the drug, while the solution of the buffer chamber will allow the determination of the free fraction of the compound. The bound fraction is determined from the subtraction of the total concentration by the free fraction [70]. More recently, the performance of this method was improved by using a 96-well plate, thus allowing up to 20 dialyses to be performed in a single experiment. However, this equipment is more expensive and the set-up is more time-consuming [74,75].

As in equilibrium dialysis, ultrafiltration also uses an apparatus comprising two chambers but, in this case, placed one on top of the other, and separated by a semipermeable membrane. In the upper chamber the plasma with drugs is placed and a centrifugal force (~2000$\times$ g, comparable to the pressure of renal filtration) is applied that promotes the passage of the free fraction through the membrane into the lower chamber containing a buffer solution. The concentration of the free fraction is obtained in the lower chamber after centrifugation, while the total drug concentration is obtained in the upper chamber before centrifugation. The calculation of the concentration of the bound fraction is similar to that performed in equilibrium dialysis as well as the membranes used that are often of the same type. However, they must be able to withstand the pressure applied during the filtration process. If the pH and temperature are adequately controlled, ultrafiltration is indeed a rapid and simple method of determining the binding of compounds to plasma proteins under physiological conditions [3,7,70,73]. Ultrafiltration is a faster and simpler method than equilibrium dialysis which is, therefore, widely used for clinical drug monitoring [7,70]; however, the latter is considered to be more accurate [7,70,75].

In ultracentrifugation, unlike equilibrium dialysis and ultrafiltration, a membrane is not used, since the separation of the free fraction from the bound fraction is done by

centrifugal force. In this method, a plasma solution containing the drug is subjected to a high centrifugal force (~500,000–625,000× *g*) for a long period of time (10 to 24 h). After centrifugation, the fraction bound to plasma proteins (HSA and AGP) and HDL will sediment to the bottom of the tube, while VLDL, LDL and chylomicrons will form a lipid layer on the surface of the tube. The free fraction is found in the supernatant immediately below the lipid layer. For determination of the concentration of the free fraction, a sampling of the supernatant has to be performed, while the total concentration of the compound has to be determined before centrifugation is carried out. Based on these measurements, the concentration of the bound fractions is determined [3,7,70]. By ultracentrifugation method, it is possible to avoid one of the main disadvantages of conventional methods (equilibrium dialysis, ultrafiltration) which is the non-specific binding of compounds to membranes, particularly those which are strongly bound to proteins. In addition to non-specific binding, the solubility of the compounds may also be a limitation, since in conventional methods it is necessary that the compounds are soluble in the buffer [70,73,76].

Table 2 summarizes advantages and disadvantages of these three methods that could influence the decision of choosing one of them for a particular analysis [69,70].

Table 2. Advantages and disadvantages of equilibrium dialysis, ultrafiltration and ultracentrifugation methods [69,70].

Method	Advantages	Disadvantages
Equilibrium dialysis	Easy procedure; As the standard method, it facilitates cross-comparison; Determination is done at equilibrium, reducing the effect of non-specific binding.	Nonspecific membrane binding may occur; Time-consuming procedure, depending on the characteristics of the compounds; Number of samples analyzed is reduced; Compounds with higher molecular weights and highly bound to plasma proteins will take longer time to reach equilibrium and errors may occur due to bacterial growth phenomena, changes in pH, or free fatty acid levels.
Ultrafiltration	Simple and fast procedure; The effect of non-specific binding is reduced since determination is done at equilibrium.	Nonspecific binding to the membrane or chamber walls may occur; Sieve effects can occur, in which water present in the plasma passes the membrane more rapidly than the drug molecules with a risk of determining a lower free fraction if not all molecules are allowed to pass through the membrane; Gibbs–Donnan effects and leakage of proteins through the membrane may occur contaminating the chamber.
Ultracentrifugation	Absence of membrane-related difficulties; Equipment allows better temperature control; Fast procedure since there is no need to establish an equilibrium.	Sampling of the supernatant may be difficult, due to the lipid layer formed by centrifugation; Sampling of the supernatant may favor contamination of the free fraction by the fraction bound to VLDL, LDL and chylomicrons; Sedimentation of the free fraction may occur due to the shape and size of the compound (especially for those with molecular weights > 400 Da) or due to the influence of temperature; The supernatant must be sampled as quickly as possible, as the free compounds diffuse as soon as the centrifuge stops; Equipment is more expensive; Time-consuming procedure; Processing a small number of samples.

3.2. High-Performance Affinity Liquid Chromatography

HPALC is a chromatographic technique that has become increasingly relevant and widely used to study the binding affinity between biomolecules, such as plasma proteins, and drugs or other bioactive compounds [1]. This method uses a biologically-related selector that selectively retains analytes, such as drugs, based on its affinity to the biomolecule. By examining the retention and competition of drugs as they pass through the protein-based column is possible to determine the association constants [77].

HPALC has several advantages, some of them related to the LC technique, such as high speed and sensitivity, remarkable precision and reproducibility, vast applicability, automatization, possibility of coupling to different detection equipments, availability of LC apparatus in almost all industrial and academic laboratories, among others [78,79]. In addition, this method allows a fast and convenient determination of drug-protein interactions by using small amounts of immobilized protein [79], having the possibility of working under near-physiological conditions (buffer pH, ionic strength and temperature). Additionally, by using a chiral stationary phase (CSP), it is possible to analyze the behavior of both enantiomers of racemic or enantiomeric mixtures in the same run [80–82]. Another advantage is the possibility to perform other type of analyses in addition to bioaffinity binding studies, including: displacement studies with the presence of a competitive agent [83], thermodynamic and variability studies of chromatographic conditions [84], quantitative structure–retention relationship studies [85], and enantioseparation of racemic or enantiomeric mixtures [2].

The commercial and scientific interest on plasma proteins, enzymes, and other biomolecules as CSPs increased due to their ability of chiral recognition [86,87]. On their large surface, proteins present different binding sites which allow for multiple possibilities of intermolecular interactions with small molecules [88]. On the other hand, as the CSPs are based on biomolecules, they proved to be very helpful to study intermolecular interactions (binding affinity) between biomolecules and drugs [1].

The first report demonstrating that a protein has different binding properties in a LC analysis was in 1973, for tryptophan resolution using bovine serum albumin (BSA) coupled to an agarose support [89]. After that, Allenmark [90], Haginaka [88], Hage [80,81], and other groups have remarkably contributed to the development of several and exhaustive studies to assess the ability of various proteins to be used as CSPs, and also in biological assays. The most used proteins, such as HSA, AGP, crude ovomucoid (OVM) and cellobiohydrolase I (CBH I), have been well documented as chiral selectors for chromatographic enantioseparation for a wide range of chiral compounds and for binding affinity studies [71,91–93].

Protein-based CSPs proved to be able to directly analyze a broad range of chiral compounds, most of them without the need for derivation. The possibility of using aqueous or aqueous–organic as mobile phases was pointed out as another advantage of protein-based CSPs considering its compatibility with mass spectrometric detection [71,88,92,94].

Recently, several protein-based CSPs were reviewed by Bocian et al. [95], including the most common (HSA, AGP) as well as the more unusual, namely, avidin and fatty acid binding proteins. Nevertheless, only a limited number of proteins are commercially available as CSPs (Table 3) [86,88,92,94,96].

The immobilization process of the protein to a chromatographic support can bring some variations comparatively to the protein in soluble form, namely, conformational changes, denaturation, steric hindrance at the binding sites, nonspecific binding by the support and anchor matrix [78,97]. There are various approaches, which can counterbalance this disadvantage, namely, covalent immobilization, biospecific adsorption and entrapment [71]. As a consequence, a high concordance of this method compared to other techniques, such as ultrafiltration, is found [76]. Effectively, the results obtained using this methodology generally correlate well with what happens in vivo [98,99].

Table 3. Protein-based CSPs, column trade names and type of resolved racemates [86,88,92,94,96].

Protein	Mr (kDa)	Type of Analytes	Column Tradename
Serum albumin			
Human (HSA)	67	Acidic and neutral	CHIRAL-HAS® [a] CHIRAL HSA® [b] KeyStone HSA® [c]
Bovine (BSA)	68	Acidic and neutral	CHIRAL-BSA® [a] Ultron ES-BSA® [d] Resolvosil BSA® [e]
α1-Acid glycoprotein (AGP)	44	Basic, neutral and acidic	CHIRAL-AGP® [a] EnantioPac® [f]
Ovomucoid (OVM)	28	Basic, neutral and acidic	Ultron ES-OVM® [d] TSKgel Enantio-OVM® [g]
Cellobiohydrolases (CBH)	60–70	Basic and neutral	CHIRAL-CBH® [a]
Avidin	68	Basic, neutral and acidic	Bioptic AV-1® [h]
Pepsin	34.6	Basic and neutral	Ultron ES-Pepsin [d]
α-Chymotrypsin	25	Amino acids and derivatives	-
Penicillin G Acylase	90	Acidic	-
Antibodies	150	Aliphatic and aromatic	-
Lysozyme	14	Basic and neutral	-

[a] ChromTech Lda., Congelton, Cheshire, UK; [b] Shandon Scientific, Pittsburgh, PA, USA; [c] Thermo Hypersil, Bellefonte, PA, USA; [d] Shinwa Chemical Industries Lda., Fushimi-ku, Kyoto, JP; [e] Macherey Nagel, Duren, Germany; [f] LKB Pharmacia, Bromma, Sweden; [g] Tosoh, Tokyo, Japan; [h] GL Sciences Inc., Torrance, CA, USA.

Protein binding studies by HPALC can be carried out based on two types of analysis: frontal and zonal [2,100].

3.2.1. Frontal Analysis

Frontal analysis is frequently used in HPALC as a tool allowing the characterization of the interactions and binding sites of compounds with plasma proteins [2]. In this type of analysis, the analyte is injected continuously into the column mixed with a mobile phase that has the most suitable pH, ionic strength and composition for the study. During the analysis, the analyte binds to the plasma proteins, leading to saturation of the column and, consequently, the amount of analyte that elutes will gradually increase. The elution of the analyte will be observed by the appearance of characteristic curves in the chromatogram (Figure 3). It is possible to determine the affinity and number of plasma protein binding sites by examining the position of the curve of a solute as its concentration is changed. In addition, it is also possible to obtain some information about the nature and type of interaction that occurs between a compound and protein by varying the temperature, protein type and solvent of the mobile phase [2,100].

In this type of analysis, both the fraction bound to plasma proteins and the free fraction of an analyte are retained on the stationary phase, until they are eluted from the column to originate a single chromatographic peak. From the peak area or peak height, the total analyte concentration (bound + free fraction) can be calculated. In principle, the free fraction of the analyte will elute first, followed by the bound fraction, since the latter will be retained longer in the column. It is possible to calculate from this data the bound percentage of the compound analyzed to plasma proteins using the following Equation [3]:

$$Bound\ fraction\ \% = \left(1 - \frac{Free\ fraction\ concentration}{Total\ compound\ concentration}\right) * 100 \quad (1)$$

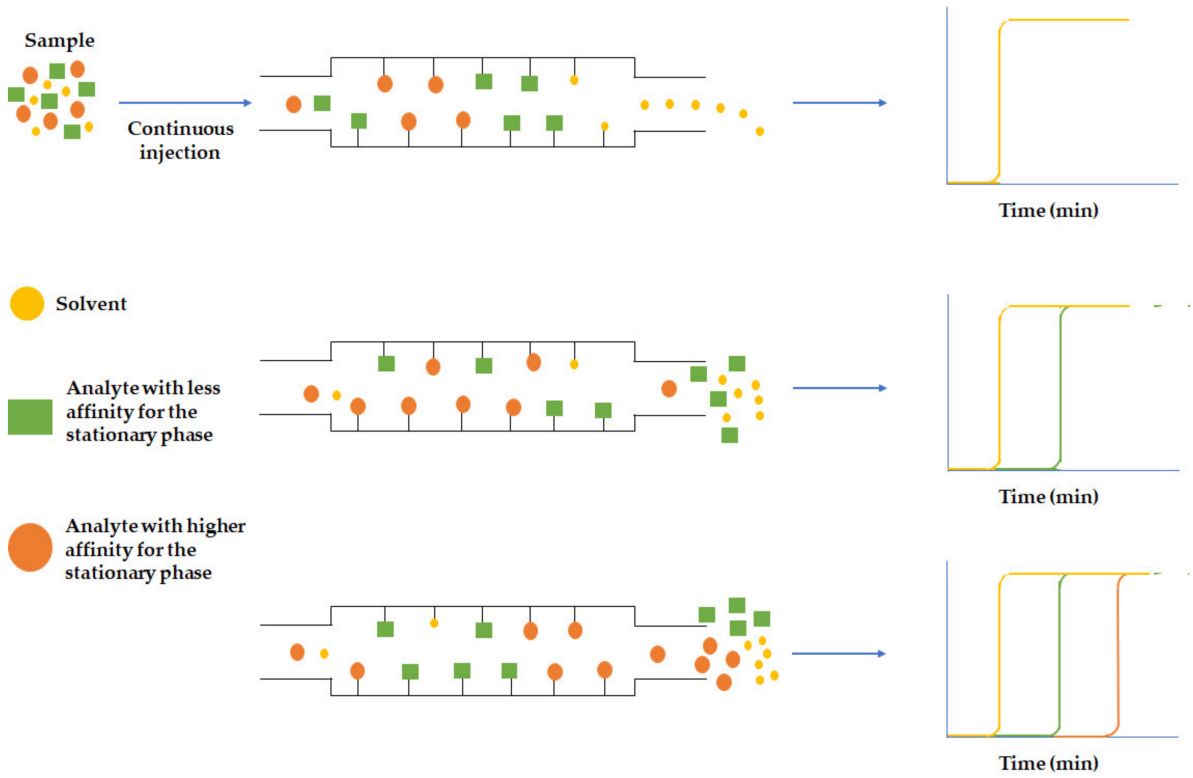

Figure 3. General principles of HPALC by frontal analysis.

In addition, with this technique it is possible to obtain important information for determination of binding constants, as well as the number and types of binding sites involved [3,101]. This method has several advantages [2,3,101], namely: errors due to non-specific binding hardly occur, unlike in ultrafiltration and equilibrium dialysis; it allows the simultaneous determination of the total drug concentration and the bound fraction; it is possible to recover plasma proteins, thus avoiding losses of material.

Frontal analysis is especially used for plasma protein binding studies of hydrophobic compounds, for small samples and for binding studies on scarce and difficult-to-obtain plasma proteins, such as glycoproteins and lipoproteins [101].

3.2.2. Zonal Analysis

Zonal analysis is the most commonly used methodology in HPALC to study the binding of compounds to plasma proteins. Moreover, it is also used to study the interactions between drugs and plasma proteins as well as for displacement studies [2].

Unlike frontal analysis, the analyte is injected in a small quantity into the column, in which a mobile phase is eluting continuously and which may contain in its composition a competing agent. The injected analyte interacts with the protein immobilized on the column and is subsequently eluted by the mobile phase and detected. The particularity of zonal analysis is that the concentration of the competing agent can be changed in the mobile phase. Thus, the analyte retention factor is calculated as the concentration of the competing agent changes. The change in retention as a function of the concentration of the agent present in the mobile phase is adjusted to various (established) models that allow to understand how the analyte under study binds to plasma proteins and to what extent this interaction is affected by the components of the mobile phase. In principle, the retention of the test analyte will decrease with increasing concentration of the competing agent. Since the analyte is less retained, it will elute more rapidly, i.e., in greater quantity; in the chromatogram this will be reflected by increased absorbance (Figure 4) [2]. However, if a competing agent is not added to the mobile phase, the retention factor is directly proportional to the binding affinity of the compound for the immobilized protein on the

column [102]. Therefore, with this analysis, it is possible to obtain some information about the equilibrium and competition constant for certain protein–analyte interactions [2,102].

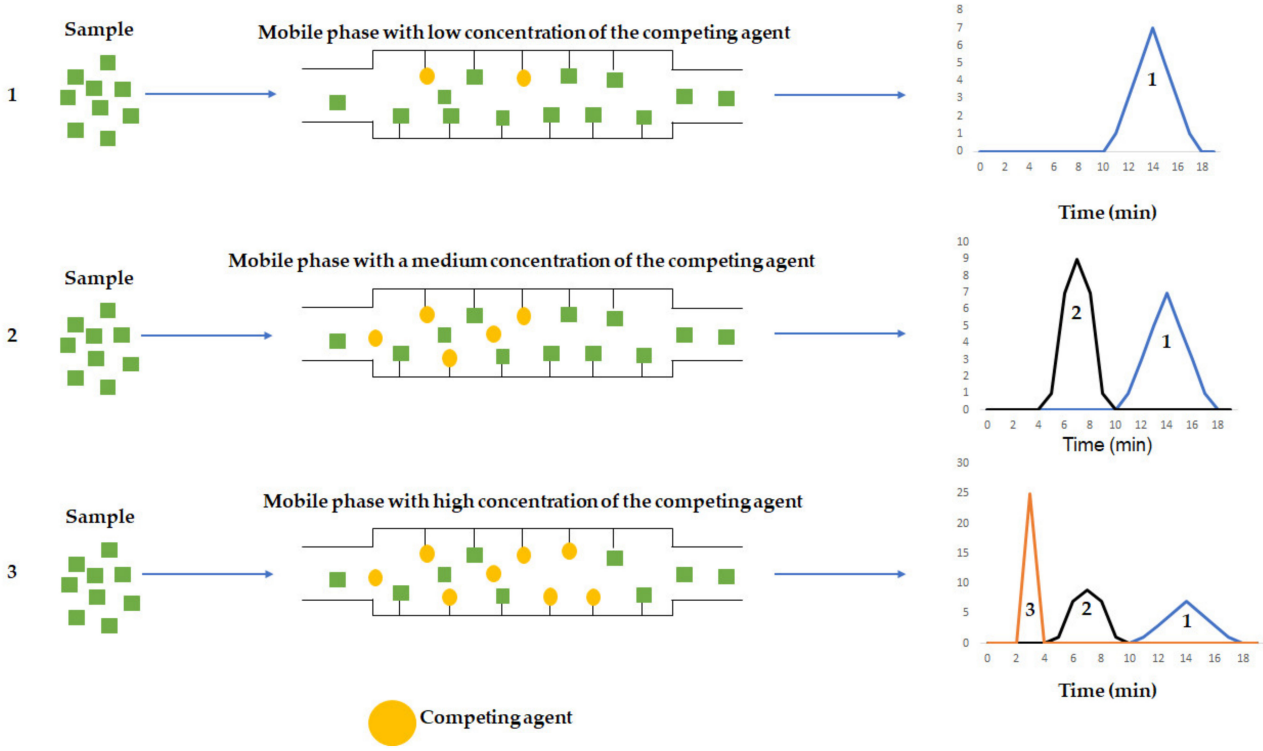

Figure 4. General principles of HPALC by zonal analysis.

A major advantage of zonal elution analysis on plasma protein columns, such as HSA, is that it allows the simultaneous study of both enantiomers of chiral compounds whenever enantioseparation occurs [79]. Enantioselectivity can occur in these three situations: (1) a different affinity of the two enantiomers for the same protein binding site; (2) the interaction of the enantiomers can take place at different binding sites; (3) the interaction can involve more than one binding site, for which the enantiomers can interact differently [79].

From the chromatographic results, the protein bound percentage (%*b*), which is the data one seeks to obtain in binding studies, can be determined using the following Equation [103]:

$$\%b = \frac{k}{1+k} \quad (2)$$

where k is the retention factor.

The association constant is also widely used to express the affinity of a compound for a plasma protein (K_a), which is calculated using the following Equation [104]:

$$K_a = \frac{[AB]}{[A] * [B]} \quad (3)$$

where (AB) is the concentration of the bound fraction, (A) is the concentration of the free solute and (B) is the concentration of free protein.

The plasma protein binding affinity of a significant number of drugs and other bioactive compounds have been studied by HPALC on HSA-based CSPs by both frontal and zonal analysis. Some examples comprise benzodiazepines [105,106], *S*/*R*-ibuprofen [107], amino acids (e.g., D/L-tryptophan) [108], hormones (e.g., D/L-thyroxine) [109,110], among others.

Updated examples of HSA binding studies are presented in Table 4. The main objective of this type of study is to understand and express the affinity of a compound for the protein,

in this specific case, for HSA. Consequently, the results are expressed as protein bound percentage (%*b*) and/or association constants (*Ka*).

Table 4. Binding affinity studies on HSA by HPALC.

Analyte	Type of Analysis	Chromatographic Conditions	Binding Affinity to HSA		Ref
			Association Constant (M^{-1})	Bound Percentage (%)	
Tocainide analogues	Frontal	Mobile phase: Solutions of L-tryptophan prepared in PPB (0.067 M, pH 7.4) ranging from 12.5 µM to 100 µM Flow rate: 1.0 mL/min Detection: UV; Spectra recorded at a scan rate of 20 nm/min Column: Silica monolithic column (50 mm × 4.6 mm i.d., 20 nm) immobilized with HSA Temperature: 28 °C	-	34.7–98.7	[111]
Imipramine	Frontal and zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.3 mL/min Detection: UV; λ = 251 nm Column: Homemade column with immobilized HSA Temperature: 37 °C	$1.6\ (\pm 1.0) \times 10^5$	-	[112]
Warfarin Gliclazide	Frontal and zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.1 mL/min Detection: UV; λ = 308, 250 nm Column: Immunoextraction microcolumns containing adsorbed HSA (1.0 cm × 2.1 mm i.d.) Temperature: 37 °C	$2.4\ (\pm 0.4) \times 10^5$ $4.1\ (\pm 0.5) \times 10^4$	-	[113]
Glipizide	Frontal and zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.5 mL/min Detection: UV; λ = 254 nm Column: Nucleosil Si-300® silica with immobilized HSA (2.0 cm × 2.1 mm i.d.) Temperature: 37 °C	$2.4–6.0 \times 10^5$	-	[114]
Tolazamide	Frontal and zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.5 mL/min Detection: UV; λ = 254 nm Column: Nucleosil Si-300® silica microcolumns with immobilized HSA (1.0 or 2.0 cm × 2.1 mm i.d.) Temperature: 37 °C	$4.3–6.0 \times 10^4$	-	[115]
Phenytoin	Frontal and zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.2 mL/min Detection: UV; λ = 205 nm Column: Nucleosil Si-300® silica column with immobilized HSA (5 cm × 4.6 mm i.d.) Temperature: 37 °C	$8.8\ (\pm 2.0) \times 10^3$	-	[116]
HIV protease inhibitors	Frontal and zonal	Mobile phase: PPB (0.067 M, pH 7.4):1-PrOH (100:0 to 90:10, *v*/*v*) Flow rate: 1.0 mL/min Detection: UV; λ = 228, 231 or 308 nm Column: Kromasil® silica column with immobilized HSA (50 × 4.0 mm i.d.) Temperature: 28 °C		70.45–98.84	[117]

Table 4. *Cont.*

Analyte	Type of Analysis	Chromatographic Conditions	Binding Affinity to HSA		Ref
			Association Constant (M^{-1})	Bound Percentage (%)	
Timolol	Zonal	Mobile phase: AAB (0.050 M, pH 7.4): *n*-PrOH (94:6, *v*/*v*) Flow rate: 0.8 mL/min Detection: MS and UV; λ = 10 nm Column: HSA column by Hypersil® (50 mm × 4.6 mm i.d.) Temperature: 37 °C	-	14.0	[118]
Pindolol			-	39.0	
Alprenolol			-	40.0	
Pentobarbital			-	35.0	
Amobarbital			-	79.0	
Methohexital			-	36.0	
Desipramine			-	83.0	
Chlorpromazine			-	43.0	
Clozapine			-	93.0	
Bromazepam			-	57.0	
Alprazolam			-	70.0	
Flunitrazepam			-	66.0	
Lorazepam			-	80.0	
Midazolam			-	92.0	
Acetaminophen	Zonal	Mobile phase: AAB (0.050 M, pH 7.4):2-PrOH (70–100: 0–30) Flow rate: 1.8 mL/min Detection: UV; λ = 230 and 254 nm Column: Chromtech® Immobilized HSA HPLC column (50 × 3 mm) Temperature: 30 °C	-	14.0	[119]
Acyclovir			-	5.4	
Amoxicillin			-	16.4	
Aspirin			-	37.3	
Carbamazepine			-	83.8	
Diazepam			-	93.2	
Ibuprofen			-	99.5	
Propanolol			-	62.0	
Warfarin			-	97.9	
Ketoconazole			-	93.0	
Clonidine			-	28.0	
Diclofenac			-	99.0	
Flumazenil			-	28.8	
Fluoxetine			-	97.0	
Naproxen			-	99.0	
m-HPPH (E1)	Zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.1–2.0 mL/min Λ = 203 nm Column: Nucleosil Si-300® immobilized with HSA (10 × 4.6 mm i.d.) Temperature: 37 °C	3.0 (±1.1) × 10^3	-	[120]
m-HPPH (E2)			3.4 (±1.3) × 10^3	-	
p-HPPH (E1)			5.1 (±0.7) × 10^3	-	
p-HPPH (E2)			6.3 (±0.9) × 10^3	-	
Dietary flavonoids	Zonal	Mobile phase: PPB (0.050 M, pH 7.0): ACN (85:15, *v*/*v*) Flow rate: 0.35 mL/min Detection: UV; λ = 210, 270, 280 and 360 nm Column: CHIRALPAK® HSA (150 × 40 mm, i.d., 5 μm) Temperature: 37 °C	-	15.36–91.79	[121]
Taxane derivatives	Zonal	Mobile phase: PPB (0.050 M, pH 7.4): 1-PrOH (100-80:0-20, *v*/*v*) Flow rate: 0.8 mL/min Detection: UV; λ = 230 nm Column: HSA Shandon Scientific® (15 × 0.4 cm i.d.) Temperature: 25 °C	-	40.2–91.9	[122]
Tocainide	Zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 1.0 mL/min Detection: UV Column: Silica column (50 mm × 4.0 mm i.d.) immobilized with HSA Temperature: 28 °C	-	25.4	[123]

Table 4. *Cont.*

Analyte	Type of Analysis	Chromatographic Conditions	Binding Affinity to HSA		Ref
			Association Constant (M^{-1})	Bound Percentage (%)	
Xanthone derivatives	Zonal	Mobile phase: PPB (pH 7.4; 0.067 M) and different percentages ACN ranging from 25 to 2% Flow rate: 0.9 mL/min Detection: UV; λ = 254 nm Column: CHIRALPAK® HSA (150 × 40 mm, i.d., 5 µm) Temperature: 37 °C	-	79.02–99.99	[98]
Universal cancer peptide 2 Universal cancer peptide 4	Zonal	Mobile phase: PPB (0.050 M, pH 7.4) Flow rate: 100–300 nL/min Detection: UV; λ = 210 nm Column: poly (GMA-EDMA) monolithic capillary column immobilized with HSA (150 mm × 75 µm i.d.) Temperature: 37 °C	0.94×10^5 1.32×10^5	- -	[124]
Acetohexamide Glibenclamide Glipizide Tolbutamide	Zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.25 mL/min Detection: UV; λ = 252, 290, 226 nm Column: Hydrazide-activated silica column with entrapped HSA (1.0 cm × 2.1 mm i.d.) Temperature: 37 °C	$1.75(\pm0.31) \times 10^5$ $1.98(\pm0.35) \times 10^6$ $2.03(\pm0.33) \times 10^5$ $1.22(\pm0.22) \times 10^5$	-	[125]
Repaglinide Nateglinide	Zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.5 mL/min Column: Nucleosil Si-300® silica column with immobilized HSA (1.0 cm × 2.1 mm i.d) Detection: UV; λ = 308, 280 nm Temperature: 37 °C	$6.1(\pm0.2) \times 10^4$ $7.1(\pm0.8) \times 10^5$	-	[126]
2-Imidazoline drugs	Zonal	Mobile phase: PPB (0.067 M, pH 7.4) Flow rate: 0.1–0.5 mL/min Detection: UV; λ = 210–283 nm Column: Nucleosil Si-300® silica column with immobilized HSA (25 mm × 2.1 mm i.d.) Temperature: 37 °C	1.62×10^3–1.07×10^4	-	[127]

AAB: Ammonium acetate buffer; ACN: Acetonitrile; i.d.: Internal diameter; HSA: Human serum albumin; MS: Mass spectrometry; Poly (GMA-EDMA): Poly (glycidyl methacrylate-ethylene dimethacrylate); PPB: Potassium phosphate buffer; PrOH: Propanol; UV: Ultraviolet.

As shown in Table 4, frontal and zonal analysis were used to evaluate the binding affinity to HSA of various drugs and bioactive compounds. Nevertheless, as expected, zonal analysis continues to be the most commonly used approach in HPALC. In some studies, both frontal and zonal analysis were applied; however, with different aims. For example, by utilizing frontal analysis, the association equilibrium constants and number of binding sites for imipramine with HSA were first estimated. Then, competition studies based on zonal elution were performed to identify the location of the major binding site for imipramine on HSA [112]. The same strategy was used for the drugs warfarin, gliclazide [113] and phenytoin [116], as well as HIV protease inhibitors [117]: frontal analysis to measure the overall affinities of these drugs to HSA, and zonal elution competition studies to examine the specific interactions of drugs at Sudlow sites I and II of the protein. Moreover, aiming to characterize the high affinity binding sites of tocainide analogues to the protein, displacement experiments were carried out for the analogues by increasing concentrations in the mobile phase of competitors, known to bind selectively to the main binding sites of HSA [111].

Commercial HSA-immobilized [98,121,122] and homemade columns [111,112,123], comprising different sizes and diameters, were used (Table 4). Over the years, different

synthetic approaches have been applied for preparation of CSPs, which embrace coating and immobilization methods. In addition to the classical and broadly applied coating and immobilization procedures, innovating strategies have been introduced [128]. The approaches applied for preparation of the homemade columns to perform the binding affinity studies shown in Table 4 were diverse, in which HSA was immobilized [111,112,123], adsorbed [113] or entrapped [125] on the chromatographic support. Regarding the chromatographic support, most of the studies used silica gel or derivatives [112,115,123,129]; however, also monoliths were explored, including monolithic epoxy-silica [111] and organic polymer monoliths [124]. Regardless of the method chosen to prepare the HSA-column, the procedure has to ensure that the binding properties of the protein are maintained unaltered. Therefore, the next step is the column characterization and preliminary studies have to be performed to check the amount of protein correctly immobilized. For example, some immobilized-HSA columns were characterized by frontal analysis of L-tryptophan [111,117].

In addition to the traditional-sized LC columns, microcolumns (i.e., columns with volumes in the low microliter range, and often with lengths of 1–5 cm or less) were described for the analysis of drug–protein interactions. For example, microcolumns of 1.0 cm × 2.1 mm i.d. were used for the analysis of warfarin, gliclazide [113], tolazamide [115], acetohexamide, glibenclamide [125], among others. The microcolumns have several advantages, which include their low back pressures and compatibility with miniaturized systems; that they only need small amounts of protein, analytes and reagents; their short analysis times; and their ability to be used in some assay formats that are not possible with traditional-sized columns [130,131].

On an even smaller scale, binding data for universal cancer peptides were obtained on a nano–organic monolithic HPLC capillary column with immobilized-HSA (150 mm × 75 µm i.d.) [124].

As shown in Table 4, the analyses were carried out under chromatographic conditions that mimic physiological environments (buffer pH, ionic strength and temperature). Since the physiological pH of human plasma is considered as 7.4 and it is assumed that HSA has a higher stability in this condition, in all the studies shown in Table 4, the buffer pH of the mobile phase was 7.4; with only one exception, in which the buffer pH was a near-physiological value (7.0) [121]. One reason that can justify the selection of that pH may be related to the fact that variations of mobile phase pH higher than 7.0 are responsible for shortening the life of the CSP. The same happens for buffer pH values lower than 5.0 [132].

Regarding the column temperature, it was found that most of the studies were carried out at 37 °C. However, in some studies, the analyses were performed at 30 °C or at room temperature (20–25 °C). Taking into account the aim of these studies, it would be expected that all the binding affinity analyses were performed at 37 °C or, in alternative, at very near temperature to mimic physiological conditions as much as possible. Nevertheless, the column temperature is another factor that may be responsible for damaging the protein-based columns [132]. Thus, aiming to protect the columns, some studies were conducted at lower temperature. Actually, one of the disadvantages of protein-based columns is their limited stability and lack of column ruggedness [88,92].

Different buffers can be used as aqueous phase for a chromatographic analysis using a protein-based CSP, namely potassium phosphate, sodium phosphate, ammonium acetate and sodium acetate [92]. Among them, for binding affinity studies, typically the potassium phosphate buffer is widely used. In the recent studies presented in Table 4, this trend has also been found. The choice of phosphate buffers is intrinsically related to the stability of the HSA as chiral selector, and also because this type of buffers are preferable to mimic the physiological conditions of human plasma [133].

As shown in Table 4, ammonium acetate buffer was also described, in two different studies, for determination of HSA bound percentage of a large number of drugs demonstrating good applicability [118,119]. A great advantage of acetate buffers compared to phosphate buffers is the possibility to be used as a component of the mobile phase for LC-

MS analysis [134]. In fact, although the vast majority of the studies used UV detection, one of them reported the application of MS detection in addition to UV [118]. In that study, an ammonium acetate buffer was used as a component of the mobile phase. Good agreement was observed for retention factors and, consequently, HSA bound percentage, with MS detection, using acetate buffer as component of the mobile phase, and UV detection, using a mobile phase containing phosphate buffer [118].

It is important to emphasize that the compatibility of HPALC with MS is one great advantage since it offers the benefits of higher sensitivity and selectivity for the method [71]. The improved sensitivity should enable analysis of a smaller amount of each compound, which would minimize errors from competitive displacement or column overloading [19]. In addition, it increases throughput by combining several compounds in one cassette injection [118]. Actually, identical data were achieved for compounds analyzed in a single or cassette for both slightly or highly bound compounds. Moreover, the column overload did not occur after analysis of cassettes containing compounds that have a high binding affinity (%*b* > than 90), even at compound concentrations up to 100 µg/mL [118].

Most of the drug–protein binding studies used the buffer concentration of 67 mM to mimic the physiological conditions [135,136]. That concentration is also used for immobilization procedures of HSA to a chromatographic support, for packing the CSP into columns as well as for in their storage [78].

In some cases, an organic modifier was added to the mobile phase to overcome the low solubility of analytes in aqueous solution and mainly for analysis of compounds with very strong affinity [69]. The 2-propanol is known to be the organic modifier that most closely matches physiological conditions. In addition to that solvent, *n*-propanol, 1-propanol and acetonitrile were also employed. In most of the studies presented in Table 4, the percentage of organic modifiers varied between 0–30%. Actually, columns with immobilized HSA are very susceptible to the presence and variation of organic modifiers [132]. They may differentially affect the reversible spatial conformation of the protein leading to changes in the number of available selective and non-selective binding sites, and may further accelerate protein denaturation leading to a significant shortening of the CSP lifetime [92].

It should be noted that, in some studies, the calculation of the binding percentage of the compounds was determined by linear regression at 100% aqueous buffer solution, through the retention times obtained using mobile phases with different proportions of organic modifier [98,117,122].

Through the analysis of the data presented in Table 4, it is possible to observe that a large number of compounds have high association constants or binding percentages to HSA, showing a good affinity for this protein. For example, high affinity for HSA-CSP was obtained for xanthone derivatives, with bound percentages ranging from 79.02 to 99.99% in 100% aqueous buffer [98]. Moreover, the higher affinity values were achieved for the xanthones comprising bulky alkyl or aryl groups in the side chain, suggesting that the hydrophobicity of the compounds was important for HSA binding process [98]. The same conclusion was inferred for taxane derivatives, in which the hydrophobicity of the drugs was fundamental in the binding process. Actually, the affinity resulted significantly affected by the nature of the isoserine side chain, the presence of the 1,14-carbonate moiety and the substituent at C-7 [122].

Most of the compounds that presented low affinity for HSA structurally presented functional groups that confer some degree of basicity (amines) or present high hydrophilicity. Acyclovir (%*b* = 5.4) [119] and timolol (%*b* = 14.0) [118] (Figure 5) are examples of drugs demonstrated to have low affinity for HSA.

Nevertheless, even for structurally similar compounds, a significant difference of the binding percentages between the various derivatives can be observed. As examples, the %*b* of taxane derivatives ranged from 40.2 to 91.9 [122] and of dietary flavonoids from 15.36 to 91.79 [121].

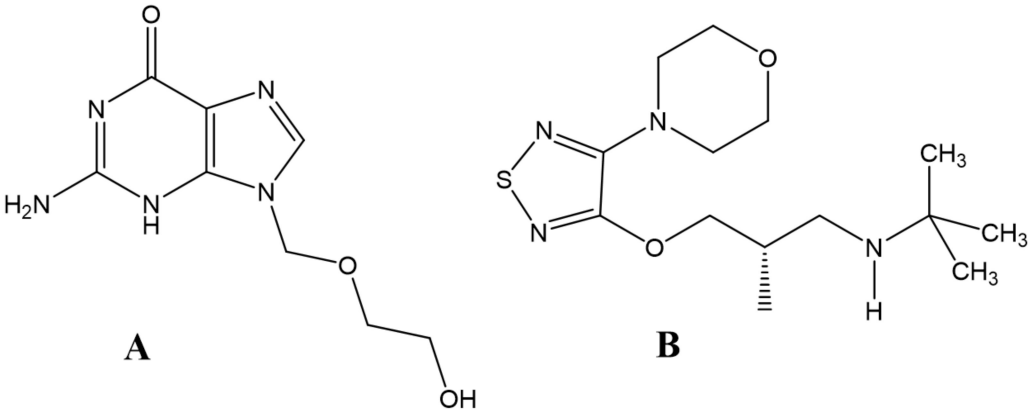

Figure 5. Examples of drugs with low affinity for HSA: acyclovir (**A**) and timolol (**B**).

Finally, it is important to infer that some works described not only the determination of binding affinity by HPALC but also included other methods to allow comparative studies. For example, in addition to the chromatographic method, docking studies [98] and circular dichroism analysis [111,117,122,123] were described, and good correlation was found between the different approaches.

In all studies, HPALC proved to be a fast and convenient method for determination of drug–protein interactions by using small amount of immobilized protein, which is one of the main advantages of this method. Moreover, HPALC allowed to determine the HSA binding affinity both for drugs that have high and low affinity to the protein.

3.3. Capillary Electrophoresis

In the last 20 years, the use of chiral selectors as buffer additives or immobilized ligands have been growing in CE for the analysis of drug enantiomers. Various types of additives or ligands can be used, such as polysaccharides, proteins, polymers, chiral micelles, among others [137]. This separation technique presents a series of advantages such as a fast method development, short analysis time, low consumption of analytes and reagents and, when compared to LC, a lower use of organic solvents, being considered a "greener" technique. Moreover, for chiral separation, CE provides a high separation efficiency, a wide variety and flexibility of chiral selectors as well as a lower consumption of them [138].

As previously demonstrated, the protein binding affinity studies became more convenient as affinity ligands were combined with LC columns. A similar trend has occurred by using affinity ligands for biological interaction studies in electrophoresis with the development of affinity capillary electrophoresis (ACE) [139]. This technique was first reported in 1992 as a CE separation technique for the study of ligand and receptor interactions [140].

ACE is based on changes in the electrophoretic mobility caused by interactions between an analyte and a specific ligand and it has been widely used to analyze biomolecules, mostly proteins, polysaccharides and hormones [141]. ACE can, for instance, be applied to measure the affinity and the binding constant (K_b) between an analyte and an associated protein, the rate constant of association (K_{on}) and dissociation (K_{off}) as well as the investigation-binding stoichiometry [139].

Three different modes have been described for ACE. Firstly, both the analyte and ligand can be premixed in the sample being designed as the pre-equilibrium mode. Secondly, for the dynamic equilibrium mode, the ligand is placed in the electrophoresis separation buffer while the analyte is dissolved in the sample solution. Lastly, in the third mode, the ligand is immobilized on the capillary wall [139].

In ACE, since separations can be performed under physiological conditions, the analyte can be preserved in a native state which retains its molecular function. Another advantage of this method is the direct analysis of the interactions of the drug and protein in

solution. Additionally, since ACE can separate the analyte from other sample components, it allows the use of impure samples [142].

Furthermore, this technique has been widely used for the separation of chiral drugs. In this case, the separation of the enantiomers will occur by the interaction that they have with the immobilized protein similar to HPALC analysis using a CSP [137]. ACE allows not only the separation of enantiomers but also the evaluation of their differences in binding affinity by the respective mobility differences [139]. Apart from trypsin and α-chymotrypsin, most proteins used in the development of CSPs, can be also used for CE being HSA and BSA the most commonly found. Additionally, other proteins have been successfully used as chiral selectors in CE such as serum albumins from other species, fungal cellulase, casein and human serum transferrin [137].

Numerous studies have been reported describing different ACE techniques for HSA binding studies with several drugs and other bioactive compounds [143–148]. Updated examples of these studies are summarized in Table 5. In addition to CE, some studies of electrokinetic chromatography (EKC), in which the chiral selector was added to a background electrolyte without the presence of further pseudostationary phases [149], were also presented.

Table 5. Binding affinity studies on HSA by electrophoretic methods.

Analyte	Technique	Electrophoretic Conditions	Binding Affinity to HSA		Ref
			Association Constant (M^{-1})	Bound Percentage (%)	
Mexiletine Chlorpheniramine Propranolol	CE	Agilent CE3D CE system Detection: DAD; λ = 200 nm for mexiletine and chlorpheniramine, λ = 214 nm for propranolol Running buffer: Phosphate buffer (67 mM, pH 7.4) Chiral selector: 0.4 M HSA Voltage: 8–16 kV Capillary temperature: 25 °C	$0.20–0.38 \times 10^3$ $0.83–1.24 \times 10^3$ $1.00–1.16 \times 10^3$	- - -	[150]
(*R*)-Amlodipine (*S*)-Amlodipine	CE	HPE-100 CE system Detection: DAD; λ = 214 nm Incubation with 300 μM HSA Separation buffer: Sodium dihydrogen phosphate buffer (60 mM, pH 3.7) Chiral selector: hydroxypropyl-β- cyclodextrin Voltage: 12 kV Capillary temperature: 23.5 °C	$0.991–1.12 \times 10^4$ $0.902–1.04 \times 10^5$	- -	[151]
Nuarimol	CE	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 220 and 278 nm Background electrolyte: Sodium phosphate (67 mM, pH 7.4) Chiral selector: 160 μM HSA solution Voltage: 15 kV Capillary temperature: 30 °C	$(9.7 \pm 0.3) \times 10^3$	75.2 ± 2	[152]
Imazalil (E1) Imazalil (E2)	CE	Beckman P/ACE MDQ CE system Detection: DAD; λ = 200 nm Incubation with 530 μM HSA Background electrolyte: Sodium dihydrogen phosphate buffer (50 mM, pH 7.0) Incubation buffer Sodium dihydrogen phosphate buffer (50 mM, pH 7.4) + NaCl (9.2 g/L) Chiral selector: Highly sulfated β-cyclodextrin Voltage: 15 kV Capillary temperature: 40 °C	- -	54.4–57.1 38.2–40.0	[153]

Table 5. *Cont.*

Analyte	Technique	Electrophoretic Conditions	Binding Affinity to HSA		Ref
			Association Constant (M^{-1})	Bound Percentage (%)	
(*S*)-Omeprazole (*R*)-Omeprazole	CE	Agilent 7100 CE system Detection: DAD; λ = 302 nm Background electrolyte: Disodiumhydrogen phosphate and sodium dihydrogen phosphate (20 mM):1-propanol (95:5, *v*/*v*, pH 7.4) Chiral selector: Different concentrations of HSA Voltage: 20 kV Capillary temperature: 25 °C	3.18×10^3 5.36×10^3	- -	[154]
R-(+)-Verapamil *S*-(-)-Verapamil *R*-(+)-Amlodipine *S*-(-)-Amlodipine	CE	PrinCE CEC- 760 system Detection: DAD; λ = 240 and 250 nm Background electrolyte: Phosphate buffer (20 mM, pH 7.4) Incubation buffer: Phosphate buffer (20 mM, pH 7.4) Chiral selector: 30–110 μM HSA Voltage: 15 kV Capillary temperature: 25 °C	1.844×10^3 0.066×10^3 22.62×10^3 25.073×10^3	- - - -	[155]
Methoxatin disodium salt	CE	Agilent CE 1600A system Detection: DAD; λ = 249 nm Incubation with HSA (0–475 μM) Background electrolyte: Phosphate buffer (67 mM, pH 7.4) Voltage: 6 kV Capillary temperature: 293–310 K	1–1.06×10^3	48.80–53.70	[156]
Captopril	CE	Agilent CE 1600A system Detection: DAD; λ = 210 nm Incubation with 400 μM HSA Background electrolyte: Phosphate buffer (67 mM, pH 7.4) Voltage: 8 kV Capillary temperature: 37 °C	$2.63 (\pm 0.21) \times 10^3$	66 ± 6	[157]
Loureirin B	CE	P/ACE MDQ CE system Detection: DAD; λ = 280 nm 30 μM HSA (injected into the capillary or mixed with Loureirin B) Background electrolyte: Phosphate buffer (50 mM, pH 7.4) Voltage: 18 kV Capillary temperature: 37 °C	2.78–13.14×10^4	-	[158]
Lidocaine Tryptophan Diclofenac Ibuprofen Chlorpropamide Tolbutamide	CE	Agilent 3D CE system Detection: DAD; λ = 214, 220, 276 nm 25 or 75 μM HSA (added to samples) Background electrolyte: Borate buffer (pH 8.5) Voltage: 14 kV Capillary temperature: 25 °C	$1.96 (\pm 0.32) \times 10^3$ $8.82 (\pm 0.08) \times 10^3$ $2.56 (\pm 0.08) \times 10^4$ $8.82 (\pm 0.60) \times 10^3$ $8.04 (\pm 0.04) \times 10^3$ $1.19 (\pm 0.08) \times 10^4$	-	[159]
Brompheniramine (E1) Brompheniramine (E2) Chlorpheniramine (E1) Chlorpheniramine (E2) Hydroxyzine (E1) Hydroxyzine (E2) Orphenadrine (E1) Orphenadrine (E2)	EKC	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 225 nm Background electrolyte: *Tris*–(hydroxymethyl)-aminomethane (50 mM, pH 8.5, 8.25, 7.00, 7.80) Chiral selector: 180, 160 μM HSA solution Voltage: 15 kV Capillary temperature: 30 °C	$(9.39 \pm 0.10) \times 10^2$ $(2.60 \pm 0.17) \times 10^3$ $(9.20 \pm 0.20) \times 10^2$ $(1.69 \pm 0.17) \times 10^3$ $(5.3 \pm 0.5) \times 10^3$ $(6.3 \pm 0.4) \times 10^3$ $(1.26 \pm 0.13) \times 10^3$ $(1.67 \pm 0.11) \times 10^4$	- - - - - - - -	[160]

Table 5. *Cont.*

Analyte	Technique	Electrophoretic Conditions	Binding Affinity to HSA		Ref
			Association Constant (M^{-1})	Bound Percentage (%)	
(*R*)-Zopiclone (*S*)-Zopiclone	EKC	Beckman P/ACE MDQ CE system Detection: DAD; λ = 220 nm Incubation with 475 μM HSA Separation buffer: *Tris*-(hydroxymethyl)-aminomethane (50 mM, pH 6.0) Incubation buffer: Phosphate buffer (67 mM, pH 7.4) Chiral selector: carboxymethylated-β-cyclodextrin Voltage: 15 kV Capillary temperature: 25 °C	-	49 ± 6 45 ± 3	[161]
Nomifensine (E1) Nomifensine (E2)	EKC	Beckman P/ACE MDQ CE system Detection: DAD; λ = 220 nm Incubation with HSA Separation buffer: *Tris*-(hydroxymethyl)-aminomethane (50 mM, pH 6.0) Incubation buffer: Phosphate buffer (67 mM, pH 7.4) Chiral selector: heptakis-2,3,6-tri-*O*-methyl-β-cyclodextrin Voltage: 15 kV Capillary temperature: 50 °C	-	40 ± 5 63 ± 4	[162]

CE: Capillary electrophoresis; DAD: Diode array detection; EKC: Electrokinetic chromatography; HSA: Human serum albumin.

Table 5 showed different studies that have been reported over the years for the evaluation of the binding affinity of HSA of numerous drugs and other bioactive compounds using CE techniques. Many studies focused on the individual binding parameters for each compound/enantiomer [153,155,161] while others also described competitive studies between compounds to the binding sites of HSA [156,157,159]. Furthermore, most of these studies were enantioselective studies where the differences in binding of each enantiomer of the analyzed compounds to HSA were evaluated and compared using, many of them, HSA as a chiral selector [150,152,154,155]. However, a few studies used cyclodextrins for this purpose while HSA was incubated with the samples before the analysis [151,153,161,162].

It was found that different employed techniques within CE or EKC were used. One of the most common was the partial filling technique (PFT). In this analysis, the capillary is only partially filled with the chiral selector while the rest of the capillary contains the electrophoretic buffer. After optimization, compounds can be detected out of the protein zone avoiding the UV interference usually caused by proteins [150,152].

The enantiomeric separation of three basic drugs (mexiletine, chlorpheniramine and propranolol) was performed by CE-PFT with human and porcine serum albumins (HSA and PSA) as chiral selectors [150]. The aim was to study and compare the chiral recognition ability of these proteins. Although chlorpheniramine and propranolol were better separated by PSA, HSA showed a better chiral recognition ability to mexiletine [150]. Another study showed the first evidence of the enantioselective binding of antihistamines to HSA using an EKC-PFT technique [160]. Furthermore, EKC-PFT was used for chiral separation and HSA binding studies of zopiclone and nomifensine enantiomers [161,162]. A significant enantioselectivity to HSA was found in these studies; for instance, for zopiclone, the (*S*)-enantiomer displayed more affinity (by a factor of 2) to HSA than the (*R*)-enantiomer [161]. These studies showed the various advantages of the PFT technique, not only the removal of the UV interference caused by proteins but also allowing a simpler performance with lower reagent consumption [150,160–162].

Along with CE/EKC-PFT, another of the most used techniques in the compiled studies was CE coupled with frontal analysis (CE-FA). In CE-FA, a large volume of an equilibrated mixture of the analyte and protein is injected resulting in a flat plateau, the height of which is proportional to the concentration of free (unbound) analyte. In the presence of the protein

molecule, the height of the plateau peak decreases with the decrease in the concentration of unbound analyte. Thus, the amount of bound analyte to the protein can be quantified [156].

A flow injection (FI)-CE coupled with FA was used to the study of stereoselectivity binding of the amlodipine enantiomers to HSA [151]. Although the racemate is used, previous studies showed that (*S*)-amlodipine is responsible for the vasodilating properties of this drug while (*R*)-amlodipine displays side effects. The results showed that the (*S*)-amlodipine binds to HSA more strongly ($0.902–1.04 \times 10^5$ M^{-1}) than its antipode ($0.991–1.12 \times 10^4$ M^{-1}) [151]. CE-FA was also used to study the binding of L-tryptophan and lidocaine to HSA as well as the effect of several drugs on the binding affinity through displacement studies [159]. A decrease in the bound of L-tryptophan was observed in the presence of the other drugs indicating that these molecules share the same binding site while no difference was observed for lidocaine [159]. Additionally, this technique was used to evaluate the binding of nuarimol, methoxatin disodium salt and captopril to HSA [152,156,157]. All these studies described the several advantages of CE-FA such as simplicity, flexibility, high speed and the possibility to use near physiological conditions [151,152,156,157,159].

Although CE/EKC-PFT and CE-FA were the most reported techniques for binding studies with HSA, other techniques were also found. A complete filling technique (CFT) was applied to evaluate the enantioselective binding of imazalil to HSA [153]. CE-CFT involves the filling of the capillary completely with the chiral selector before the sample injection. The results showed that the binding of imazalil to HSA was enantioselective which provided the first evidence of enantioselective toxicokinetic data for this compound [153]. Lastly, a mobility shift-affinity capillary electrophoresis method was also reported for the study of the binding of verapamil and amlodipine to HSA [155]. In this technique, the capillary and running background electrolyte vials are filled with background electrolyte containing the chiral selector, HSA, in this case, at various concentrations. The other binding partner is, then, injected in the capillary in a small volume. Regarding the results, the study found stronger binding of (*S*)-amlodipine and (*R*)-verapamil to HSA in comparison to their antipodes [155].

Regarding the pH, as previously mentioned for the HPALC studies, most CE studies were also performed using a pH of 7.4. However, some exceptions were observed and pH ranging from 6.0 to 8.5 were also reported [151,159,160]. In addition, although the physiological temperature is considered to be around 37 °C, only two studies used this temperature [156,157]. The most common temperature was 25 °C, one study used a lower temperature of 23.5 °C [151] while the rest used higher temperatures.

Various buffers can be used as a background electrolyte for CE analysis. As shown in Table 5, phosphate and sodium buffers were the most described. A *tris*-(hydroxymethyl)-aminomethane solution was also reported in the three EKC studies [160–162] and, lastly, one study reported a borate buffer [159].

The analysis of the results of the binding parameters showed association constants in the 10^2–10^4 range. Although in HPLAC bound percentages (%b) closer to 100 were found for some compounds, in CE, the highest percentage was 75.2% for nuarimol [152]. However, since the lowest value found was 38% for one of the enantiomers of imazalil [153], it is possible to infer that the majority of the analyzed compounds present a significant binding affinity to HSA.

CE proved to be a valuable technique for binding affinity studies for numerous compounds. It is a simple but highly versatile technique since it can be adapted to different modes with different advantages mentioned above, and can be used for several types of compounds with promising results.

3.4. In Silico Methods

Over the last years, in silico tools have been employed to elucidate the binding mechanism of drugs and other exogenous ligands with HSA [53,72]. Docking analysis is a widely applied approach, which uses algorithms to predict how small molecules

interact with different proteins at an atomic level. In this way, it is possible to predict and characterize the behavior of small molecules at the binding site of the protein under study [163]. These studies are based on two fundamental points: the prediction of the conformation, position and orientation of the ligand in the protein binding sites and the estimation of the binding affinity [164].

This type of analysis can play an important role in guiding new studies, since the production of complex molecular models can provide important information about the nature of the molecular recognition mechanism of biomacromolecules, and is, therefore, most often used at an early stage of a binding study as a guideline [164–166]. However, it is important to bear in mind that the presumed main binding site of a molecule is not the only site where interactions may occur and, therefore, the results obtained must be tested and validated. A major limitation of this method is that molecules can change their three-dimensional structure depending on their binding state. Actually, biomolecules as plasma proteins in their free form may present different conformations from those observed when they are complexed with other molecules. Besides, some programs used for this type of studies consider that the molecules in question have fixed and rigid structures. Thus, by not considering flexible molecules, there is a risk of establishing erroneous results [164–166].

First, the three-dimensional protein data bank (PDB) structure of macromolecule was obtained from the RCSB protein database or other databases. PubChem or ChemSpider database can be used to obtain the PDB structure of drug/ligand. Various softwares are available to perform docking between protein and drug/ligand, such as AutoDock 4.0, AutoDock 4.2, AutoDock vina, Hex 8.0, BSP SLIM online, and others. Among all, AutoDock is commonly used to perform docking [72]. Then, the lowest binding energy docking poses of each compound are chosen for performing the docking analysis [167]. The lower the docking score, the more stable is the complex [168].

Computational studies, using molecular docking approach, have been shown to play an important role to decipher the molecular recognition mechanisms and previewing of binding affinity of drugs and other bioactive compounds on plasma proteins. Recent docking studies for drug–protein interactions analysis encompass, for example, oral anticoagulants [169], the anticancer drug gemcitabine [170], the antihypertensive drug telmisartan [171], among others. Regarding other bioactive compounds, some recent examples include the dihydroxy-phenyl-thiazol-hydrazinium chloride (antioxidant and antiradical activities) [172], hydrazone ligand derived Cu(II) and VO(IV) complexes (antibacterial and antifungal activities) [173], uranyl complexes of alkyl substituted isothiosemicarbazone (anticancer features) [174], 7-amino coumarin derivative (analgesic, anticancer, and anticoagulant activities) [175], among others. Our group also, recently, evaluated the recognition mechanisms and protein binding affinity of chiral derivatives of xanthones on HSA by diverse methods, including docking approach (Figure 6) [98,163].

The overall results indicate that xanthone derivatives fit within the hydrophobic pocket of subdomain IIIA, presenting low negative docking scores. In order to understand the binding energy scores, a visual inspection of the binding conformations was performed for the bioactive small molecules on HSA [98,163]. Representative examples of the most stable docked conformations for different enantiomers complexes with the HSA are illustrated in Figure 6. It was found that the docking poses of the enantiomers of the xanthone derivatives were diverse, leading to different binding interactions [98].

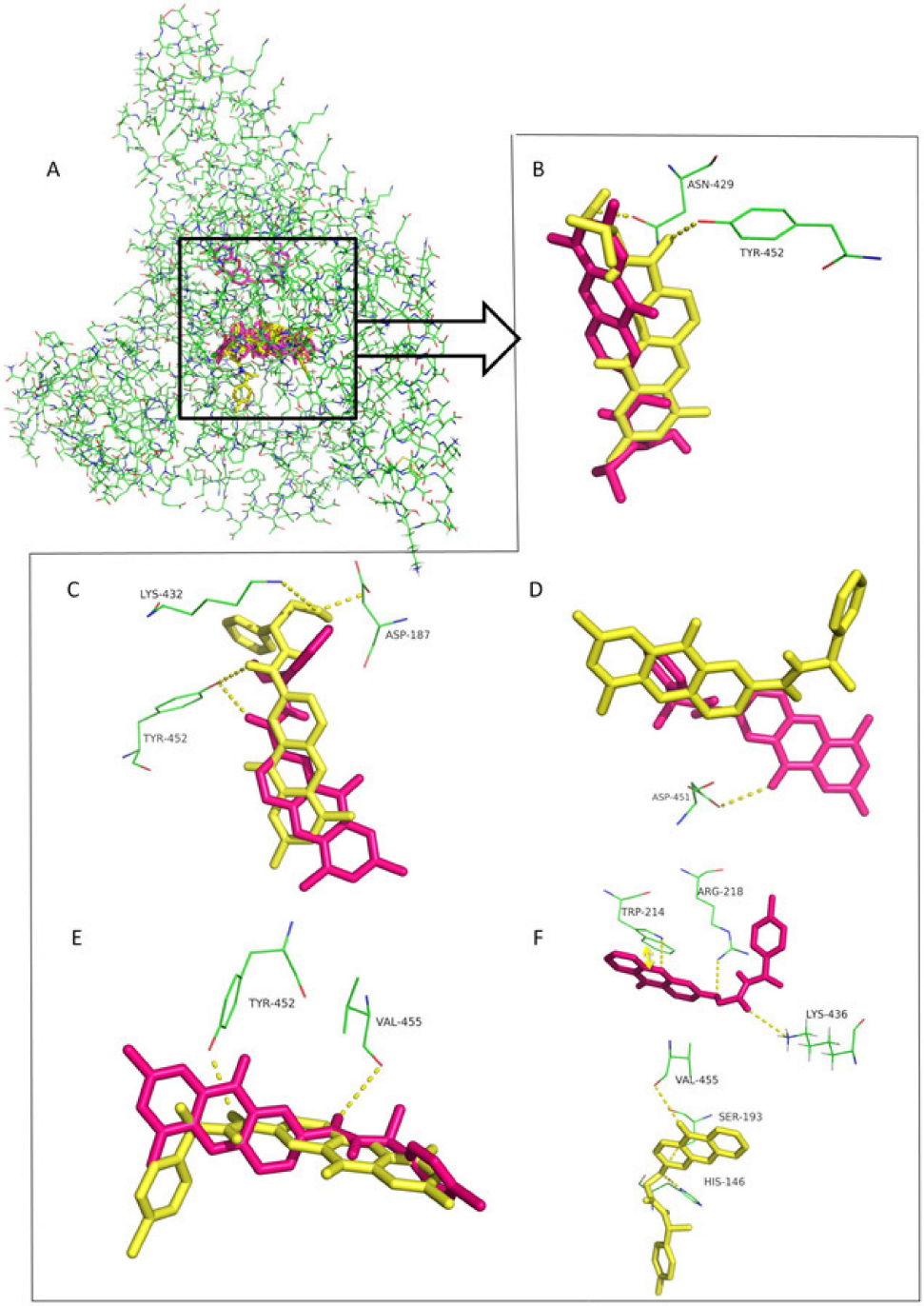

Figure 6. The most stable docked conformations for different enantiomers complexes with the HSA. (**A**) HSA; (**B**–**F**) both enantiomers of diverse chiral derivatives of xanthones. Carbons, nitrogens and oxygens in the chiral selector are represented as green, blue and red lines, respectively. (*S*) and (*R*) enantiomers are represented as magenta and yellow sticks, respectively. Hydrogen interactions are represented as a yellow broken line. π–stacking interactions are represented as a yellow doble arrow. Residues evolved in those interactions are labeled. Reprint permission from Ref. [98].

Docking studies, by providing valuable data on the interaction mechanism of various drugs and therapeutically active ligands, also make a significant contribution to the design and development of new promising drugs [176].

4. Enantioresolution Studies by Chromatographic and Electrophoretic Methods Using HSA as Chiral Selector

Separation of enantiomers is undoubtedly a matter of crucial importance in diverse research areas [44], allowing to achieve different goals, such as preparative enantioresolution of chiral drugs and other analytes [177,178], monitoring enantiomeric reactions [167,179], evaluation of enantiomeric purity in quality control [180,181], food analysis [182,183], stereochemistry determination of natural compounds [184,185], pharmacokinetic [25] and environmental studies [186,187], among other applications.

Protein-based CSPs for LC are very useful for analytical work; however, due to their low loading capacity, they are not generally applicable for preparative purposes [88,92].

In addition to protein binding affinity studies, enantioseparation of chiral compounds by chromatographic and electrophoretic methods using HSA as chiral selector continues to be reported. Actually, the interactions of chiral drugs or other bioactive compounds with proteins are often stereoselective [29]. Examples are summarized on Table 6.

Table 6. Enantioresolution studies by chromatographic and electrophoretic methods using HSA as chiral selector.

Analyte	Technique	Chromatographic/Electrophoretic Conditions	Chromatographic Parameters		Ref
			α	R_s	
Cetirizine	HPALC	Mobile phase: 2-Propanol: phosphate buffer (10 mM, pH 7.0) (10:90, *v*/*v*) Flow rate: 0.9 mL/min Detection: UV; λ = 227 nm Column: CHIRALPAK® HSA (100 × 4 mm)	1.43	1.82	[188]
Xanthone derivatives	HPALC	Mobile phases: Sodium/potassium phosphate buffer or ammonium/sodium acetate buffer (10 mM, pH 5.0 and pH 7.0): acetonitrile (90–76: 10–24, *v*/*v*) Flow rate: 0.9 mL/min Detection: UV; λ = 254 nm Column: CHIRALPAK® HSA (150 × 40 mm)	1.40–9.16	1.51–4.97	[98]
Atenolol Labetalol Nadolol Pindolol	HPALC	Mobile phase: Phosphate buffer (0.01 M, pH 7.0) Flow rate: 0.5 mL/min Column: Chrom Tech® Chiral-HSA (100 × 4 mm)	2.40 1.26 1.38 1.14	1.25 1.87 - 0.65	[189]
α-Adrenoreceptor antagonists	HPALC	Mobile phase: 1-Propanol: potassium phosphate buffer (0.1 M, pH 7.4) (10:90 or 5:95, *v*/*v*) Flow rate: 1 mL/min Detection: UV; λ = 220 nm Column: mixed HSA and AGP (50:50) column (150 × 4.6 mm i.d.)	1–1.57	-	[190]
Warfarin Temazepam Temazepam hemisuccinate Oxazepam hemisuccinate	HPALC	Mobile phase: 1-Propanol: phosphate buffer (0.1 M, pH 7.0) (5:95, *v*/*v*) Flow rate: 1.0 mL/min Detection: UV; λ = 260 nm Column: HSA Shandon Scientific® (15 × 0.4 cm)	1.16 1.43 1.89 1.75	- - - -	[191]
Ketoprofen Fenoprofen Suprofen Naproxen	HPALC	Mobile phase: 1-Propanol: phosphate buffer (0.1 M, pH 7.0) (10:90, *v*/*v*) Flow rate: 1.0 mL/min Detection: UV; λ = 260 nm and λ = 300 nm Column: HSA Shandon Scientific® (15 × 0.4 cm)	1.38 2.17 4.87 1.43	- - - -	[191]
Hydroxypropionic acids	HPALC	Mobile phase: 1-Propanol: potassium phosphate buffer (0.1 M, pH 7.0) (10:90, *v*/*v*) containing 5 mM octanoic acid Flow rate: 1.0 mL/min Detection: UV; λ = 220 nm and λ = 275 nm Column: HSA Shandon Scientific® (150 × 4.6 mm)	1–2.17	-	[192,193]
Tofisopam Oxazepam acetate Temazepam acetate Lorazepam acetate Oxazepam hemisuccinate Oxazepam methylsuccinate Lorazepam hemisuccinate Warfarin Phenprocoumon Acenocoumarol	HPALC	Mobile phase: Phosphate buffer (0.1 M, pH 7.0) with 2-propanol or acetonitrile modifiers Flow rate: 0.9 mL/min Detection: UV; λ = 310 nm Column: HSA Chiral Protein-2® (150 × 4 mm)	3.15 5.42 19.86 2.00 14.30 5.39 3.25 1.30 1.80 1.73	- - - - - - - - - -	[194]

Table 6. *Cont.*

Analyte	Technique	Chromatographic/Electrophoretic Conditions	Chromatographic Parameters		Ref
			α	R_s	
Tryptophan Metoprolol Esmolol Bisoprolol Azelastine Warfarin Labetalol Atenolol Terazosin Citalopram	CEC	Mobile phase: Phosphate buffer (10 mM, pH 7.0) without organic modifier for tryptophan, with acetonitrile (80:20, *v*/*v*) for warfarin and 2-propanol (90:10, *v*/*v*) for others Flow rate: 3 µL/min Detection: UV; λ = 215, 225, 240 and 254 nm Column: HSA–cellulase@poly(GMA–EDMA) monolith column Voltage: 10 and 15 kV Capillary temperature: 20 °C	- - - - - - - - - -	2.13 1.87 1.60 1.72 1.92 1.48 0.76, 1.79 1.07 0.42 0.45	[195]
(*R*)-Propranolol (*S*)-Propanolol	CE	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 220 nm Background electrolyte: Phosphate buffer (67 mM, pH 7.4) Chiral selector: 100 µM HSA solution Voltage: 20 kV Capillary temperature: 35 °C	1.083	1.06	[196]
(*R*)- Oxprenolol (*S*)- Oxprenolol	EKC	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 220 nm Background electrolyte: *Tris*–(hydroxymethyl)-aminomethane (50 mM, pH 8.5) Chiral selector: 190 µM HSA solution Voltage: 15 kV Capillary temperature: 35 °C	-	1.47	[197]
Promethazine Alprenolol Oxprenolol Propanolol	EKC	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 200, 220, 240 and 274 nm Background electrolyte: *Tris*–(hydroxymethyl)-aminomethane (50 mM, pH 7.6, 8.4, 8.8, 8.0) Chiral selector: 170, 167 and 190 µM HSA solution Voltage: 15 kV Capillary temperature: 30 °C	- - - -	2.01 ± 0.15 1.85 ± 0.13 1.5 ± 0.2 1.5	[198]
Brompheniramine Chlorpheniramine Hydroxyzine Orphenadrine	EKC	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 225 nm Background electrolyte: *Tris*–(hydroxymethyl)-aminomethane (50 mM, pH 8.5, 8.25, 7.00, 7.80) Chiral selector: 180, 160 µM HSA solution Voltage: 15 kV Capillary temperature: 30 °C	- - - -	2.50 1.49 1.41 1.12	[160]
Trimeprazine Prometazine	EKC	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 240 and 278 nm Background electrolyte: *Tris*–(hydroxymethyl)-aminomethane (50 mM, pH 7.6 and 7.5) Chiral selector: 170 µM HSA solution Voltage: 15 kV Capillary temperature: 30 °C	- -	1.74 2.01	[199]
Nuarimol	EKC	Hewlett-Packard HP 3D CE system Detection: DAD; λ = 220 and 278 nm Background electrolyte: *Tris*–(hydroxymethyl)-aminomethane (50 mM, pH 7.3) Chiral selector: 160 µM HSA solution Voltage: 15 kV Capillary temperature: 30 °C	-	1.1	[152]
(*R*)-Zopiclone (*S*)-Zopiclone	EKC	Beckman P/ACE MDQ CE system Detection: DAD; λ = 220 nm Incubation with 475 µM HSA Separation buffer: *Tris*-(hydroxymethyl)-aminomethane (50 mM, pH 6.0) Incubation buffer: Phosphate buffer (67 mM, pH 7.4) Chiral selector: carboxymethylated-β-cyclodextrin Voltage: 15 kV Capillary temperature: 25 °C	1.95	2.1	[161]
Nomifensine (1st enantiomer) Nomifensine (2nd enantiomer)	EKC	Beckman P/ACE MDQ CE system Detection: DAD; λ = 220 nm Incubation with HSA Separation buffer: *Tris*-(hydroxymethyl)-aminomethane (50 mM, pH 6.0) Incubation buffer: Phosphate buffer (67 mM, pH 7.4) Chiral selector: heptakis-2,3,6-tri-O-methyl-β-cyclodextrin Voltage: 15 kV Capillary temperature: 50 °C	2.7 ± 0.1	1.7	[162]

Table 6. *Cont.*

Analyte	Technique	Chromatographic/Electrophoretic Conditions	Chromatographic Parameters		Ref
			α	R_s	
Imazalil (1st enantiomer) Imazalil (2nd enantiomer)	CE	Beckman P/ACE MDQ CE system Detection: DAD; λ = 200 nm Incubation with 530 μM HSA Background electrolyte: NaH_2PO_4 (50 mM, pH 7.0) Incubation buffer NaH_2PO_4 (50 mM, pH 7.4) + NaCl (9.2 g/L) Chiral selector: Highly sulfated β-cyclodextrin Voltage: 15 kV Capillary temperature: 40 °C	2.0	4.4	[153]
R-(+)-Verapamil *S*-(-)-Verapamil *R*-(+)-Amlodipine *S*-(-)-Amlodipine	CE	PrinCE CEC- 760 system Detection: DAD; λ = 240 and 250 nm Background electrolyte: HSA (30–110 μM) + phosphate buffer (20 mM, pH 7.4) Incubation buffer: Phosphate buffer (20 mM, pH 7.4) Voltage: 15 kV Capillary temperature: 25 °C	- - -	2.7–1.0 3.6–1.1	[155]

In most studies presented in Table 6, the optimization of the chromatographic or electrophoretic conditions was performed to achieve the best conditions to obtain a higher number of separations of enantiomeric mixtures, with good enantioselectivity and resolution in a short analysis time. Therefore, diverse conditions were explored taking into account the type and proportion of organic modifier of the mobile phase (commonly isopropanol and acetonitrile), the type, concentration and pH of the mobile phase buffer, the temperature of analysis, charged additives as well as the flow rate (HPALC) or voltage (CE and EKC). Under the optimized analysis conditions, the majority of the analytes were successfully enantioseparated.

Moreover, it is also important to note that for most of the analyzed compounds, a significant enantioselective binding to HSA was found.

5. Conclusions

The importance of plasma protein binding in drug discovery and development is unquestionable and several studies have been described over the years. The demand for plasma protein binding data for a large number of compounds has motivated efforts to optimize existing technology as well as to develop new methods. Nowadays, there are various methods available for measuring protein binding affinity. In addition to the traditional approaches, such as equilibrium dialysis, ultrafiltration and ultracentrifugation, some innovative tools are significantly being used in detriment of the previous methods.

To define the most appropriate technique for a particular analysis, it is important to consider several factors, such as the procedure facilities, assay resolution and productivity. The type of information required, the number and physicochemical properties of the compounds to test and the amount of material available may also determine the choice of the method. The traditional techniques are labor-intensive and time consuming, which require a long assay preparation time and an additional analytical method. Moreover, they are difficult to become automated. The challenge of determining the concentration of the free drug can also be highlighted, since the concentrations of the free fraction of the compounds can be in the ng/mL range, especially for drugs that bind with high affinity to plasma proteins. These difficulties and challenges have triggered the development of other analytical alternatives, such as chromatographic and electrophoretic methods. It was found that HPALC and ACE using proteins as chiral selectors are of special interest and have been widely explored, as illustrated by the examples given within this review, taking into account their various advantages. In addition to bioaffinity binding studies, both HPALC and ACE, allow to perform other type of analyses, namely, displacement studies and enantioseparation of racemic or enantiomeric mixtures for a broad range of compounds. Actually, proteins, such as HSA, used as chiral selectors in chromatographic and electrophoretic methods, have unique enantioselective properties demonstrated to be suitable for the enantioseparation of a large variety of chiral drugs or other bioactive compounds.

Moreover, since the interest in the role of protein binding in drug action has increased in the medicinal chemistry field, computational medicinal chemists have become interested in plasma protein binding, and in silico methods, such as the docking approach, were explored in an attempt to elucidate and predict the molecular determinants of binding to HSA.

Future work in this area should lead to an increased understanding of the significance of plasma protein binding to drug action. ACE and HPALC with HSA as selector should allow the continued development of improved chiral separations for drug discovery or development and also pharmaceutical research. All these efforts will continue in the future.

Author Contributions: Conceptualization: C.F. Data collection and analysis: T.C., A.S.A. and C.F.; Writing—Original draft preparation: T.C., A.S.A. and C.F.; Writing—Reviewing and Editing: F.R. and C.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by national funds by FCT through the projects UIDB/04423/2020 and UIDP/04423/2020 (Group of Natural Products and Medicinal Chemistry— CIIMAR) and ERDF, through the COMPETE-POFC program in the framework of the program PT2020; the Project No. POCI-01-0145-FEDER-028736, co-financed by COMPETE 2020, under the PORTUGAL 2020 Partnership Agreement, through the ERDF; CHIRALBIOACTIVE-PI-3RL-IINFACTS-2019 and CHIRALSINTESE-APSFCT-IINFACTS_2021.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: This work received support from CIIMAR and the Amadeu Dias Foundation through a BYT-Plus 2021/22 scholarship granted to A.S.A.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hage, D.S.; Jackson, A.; Sobansky, M.R.; Schiel, J.E.; Yoo, M.J.; Joseph, K.S. Characterization of drug-protein interactions in blood using high-performance affinity chromatography. *J. Sep. Sci.* **2009**, *32*, 835–853. [CrossRef]
2. Li, Z.; Hage, D.S. Analysis of stereoselective drug interactions with serum proteins by high-performance affinity chromatography: A historical perspective. *J. Pharm. Biomed. Anal.* **2017**, *144*, 12–24. [CrossRef]
3. Zhang, F.; Xue, J.; Shao, J.; Jia, L. Compilation of 222 drugs' plasma protein binding data and guidance for study designs. *Drug Discov. Today* **2012**, *17*, 475–485. [CrossRef]
4. Smith, S.A.; Waters, N.J. Pharmacokinetic and Pharmacodynamic Considerations for Drugs Binding to Alpha-1-Acid Glycoprotein. *Pharm. Res.* **2018**, *36*, 30. [CrossRef]
5. Yamamoto, H.; Takada, T.; Yamanashi, Y.; Ogura, M.; Masuo, Y.; Harada-Shiba, M.; Suzuki, H. VLDL/LDL acts as a drug carrier and regulates the transport and metabolism of drugs in the body. *Sci. Rep.* **2017**, *7*, 633. [CrossRef]
6. Mehvar, R. Role of protein binding in pharmacokinetics. *Am. J. Pharm. Educ.* **2005**, *5*, 1–8.
7. Bohnert, T.; Gan, L.S. Plasma protein binding: From discovery to development. *J. Pharm. Sci.* **2013**, *102*, 2953–2994. [CrossRef]
8. López-Yerena, A.; Perez, M.; Vallverdú-Queralt, A.; Escribano-Ferrer, E. Insights into the binding of dietary phenolic compounds to human serum albumin and food-drug interactions. *Pharmaceutics* **2020**, *12*, 1123. [CrossRef]
9. Lin, Z.J.; Musiano, D.; Abbot, A.; Shum, L. In vitro plasma protein binding determination of flunarizine using equilibrium dialysis and liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2005**, *37*, 757–762. [CrossRef] [PubMed]
10. Roberts, J.A.; Pea, F.; Lipman, J. The clinical relevance of plasma protein binding changes. *Clin. Pharmacokinet.* **2013**, *52*, 1–8. [CrossRef]
11. Li, Z.; Beeram, S.R.; Bi, C.; Suresh, D.; Zheng, X.; Hage, D.S. High-Performance Affinity Chromatography: Applications in Drug-Protein Binding Studies and Personalized Medicine. In *Personalized Medicine*; Donev, R., Ed.; Academic Press Inc.: Cambridge, MA, USA, 2016; pp. 1–39.
12. Hughes, J.P.; Rees, S.; Kalindjian, S.B.; Philpott, K.L. Principles of early drug discovery. *Br. J. Pharmacol.* **2011**, *162*, 1239–1249. [CrossRef]
13. Gomes, A.S.; Brandão, P.; Fernandes, C.S.G.; Da Silva, M.R.P.C.; De Sousa, M.E.D.S.P.; De Magalhães Pinto, M.M. Drug-like properties and ADME of xanthone derivatives: The antechamber of clinical trials. *Curr. Med. Chem.* **2016**, *23*, 3654–3686. [CrossRef] [PubMed]
14. Di, L. An update on the importance of plasma protein binding in drug discovery and development. *Expert Opin. Drug Discov.* **2021**, 1–13. [CrossRef] [PubMed]
15. Benet, L.Z.; Hoener, B.A. Changes in plasma protein binding have little clinical relevance. *Clin. Pharmacol. Ther.* **2002**, *71*, 115–121. [CrossRef]

16. Schmidt, S.; Gonzalez, D.; Derendorf, H. Significance of protein binding in pharmacokinetics and pharmacodynamics. *J. Pharm. Sci.* **2010**, *99*, 1107–1122. [CrossRef]
17. Rolan, P.E. Plasma protein binding displacement interactions—Why are they still regarded as clinically important? *Br. J. Clin. Pharmac.* **1994**, *37*, 125–128. [CrossRef]
18. Christensen, H.; Baker, M.; Tucker, G.T.; Rostami-Hodjegan, A. Prediction of plasma protein binding displacement and its implications for quantitative assessment of metabolic drug-drug interactions from in vitro data. *J. Pharm. Sci.* **2006**, *95*, 2778–2787. [CrossRef]
19. McElnay, J.C.; D'Arcy, P.F. Protein binding displacement interactions and their clinical importance. *Drugs* **1983**, *25*, 494–513. [CrossRef] [PubMed]
20. O'Reilly, R.A.; Goulart, D.A. Comparative interaction of sulfinpyrazone and phenylbutazone with racemic warfarin: Alteration in vivo of free fraction of plasma warfarin. *J. Pharmacol. Exp. Ther.* **1982**, *219*, 691–694.
21. Perucca, E.; Hebdige, S.; Frigo, G.M.; Gatti, G.; Lecchini, S.; Crema, A. Interaction between phenytoin and valproic acid: Plasma protein binding and metabolic effects. *Clin. Pharmacol. Ther.* **1980**, *28*, 779–789. [CrossRef]
22. Sekhon, B.S. Exploiting the power of stereochemistry in drugs: An overview of racemic and enantiopure drugs. *J. Mod. Med. Chem.* **2013**, *1*, 10–36. [CrossRef]
23. Silva, B.; Fernandes, C.; Guedes de Pinho, P.; Remião, F. Chiral Resolution and Enantioselectivity of Synthetic Cathinones: A Brief Review. *J. Anal. Toxicol.* **2018**, *42*, 17–24. [CrossRef] [PubMed]
24. Smith, S.W. Chiral toxicology: It's the same thing only different. *Toxicol. Sci.* **2009**, *110*, 4–30. [CrossRef]
25. Coelho, M.M.; Fernandes, C.; Remião, F.; Tiritan, M.E. Enantioselectivity in drug pharmacokinetics and toxicity: Pharmacological relevance and analytical methods. *Molecules* **2021**, *26*, 3113. [CrossRef] [PubMed]
26. Fernandes, C.; Carraro, M.L.; Ribeiro, J.; Araújo, J.; Tiritan, M.E.; Pinto, M.M.M. Synthetic chiral derivatives of xanthones: Biological activities and enantioselectivity studies. *Molecules* **2019**, *24*, 791. [CrossRef]
27. Mannschreck, A.; Kiesswetter, R.; von Angerer, E. Unequal activities of enantiomers via biological receptors: Examples of chiral drug, pesticide, and fragrance molecules. *J. Chem. Ed.* **2007**, *84*, 2012–2017. [CrossRef]
28. Tiritan, M.E.; Ribeiro, A.R.; Fernandes, C.; Pinto, M. *Chiral Pharmaceuticals. Kirk-Othmer Encyclopedia of Chemicl Technology*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2016.
29. Shen, Q.; Wang, L.; Zhou, H.; Jiang, H.D.; Yu, L.S.; Zeng, S. Stereoselective binding of chiral drugs to plasma proteins. *Acta Pharmacol. Sin.* **2013**, *34*, 998–1006. [CrossRef]
30. Chuangl, V.T.G.; Otagiri, M. Stereoselective binding of human serum albumin. *Chirality* **2006**, *18*, 159–166. [CrossRef]
31. Zielinski, K.; Sekula, B.; Bujacz, A.; Szymczak, I. Structural investigations of stereoselective profen binding by equine and leporine serum albumins. *Chirality* **2020**, *32*, 334–344. [CrossRef]
32. Liu, C.; Guo, J.; Cui, F. Study on the stereoselective binding of cytosine nucleoside enantiomers to human serum albumin. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2020**, *224*, 117452. [CrossRef] [PubMed]
33. Mu, H.; Chen, S.; Liu, F.; Xiao, J.; Huang, H.; Zhang, Y.; Sun, Y.; Gao, X.; Lei, H.; Yuan, X. Stereoselective interactions of lactic acid enantiomers with HSA: Spectroscopy and docking application. *Food Chem.* **2019**, *270*, 429–435. [CrossRef]
34. Li, H.; Xu, Q.; Zheng, X.L.; Xu, H.; Chen, G.; Meng, Q.G. Study of Stereoselective Interaction Between Ginsenoside Rh2 and Serum Albumin by Spectroscopic Methods and Molecular Docking. *Spectrosc. Spectr. Anal.* **2018**, *38*, 3839–3845.
35. Gellad, W.F.; Choi, P.; Mizah, M.; Good, C.B.; Kesselheim, A.S. Assessing the chiral switch: Approval and use of single-enantiomer drugs, 2001 to 2011. *Am. J. Manag. Care* **2014**, *20*, e90–e97. [PubMed]
36. Calcaterra, A.; D'Acquarica, I. The market of chiral drugs: Chiral switches versus de novo enantiomerically pure compounds. *J. Pharm. Biomed. Anal.* **2018**, *147*, 323–340. [CrossRef]
37. FDA. FDA's policy statement for the development of new stereoisomeric drugs. *57 Fed. Reg.* **1992**, *22*, 249.
38. Investigation of Chiral Active Substances. Directive 75/318/EEC. 1993. Available online: https://www.ema.europa.eu/en/documents/scientific-guideline/investigation-chiral-active-substances_en.pdf (accessed on 14 September 2021).
39. Ball-Jones, N.R.; Badillo, J.J.; Franz, A.K. Strategies for the enantioselective synthesis of spirooxindoles. *Org. Biomol. Chem.* **2012**, *10*, 5165–5181. [CrossRef] [PubMed]
40. Borie, C.; Ackermann, L.; Nechab, M. Enantioselective syntheses of indanes: From organocatalysis to C-H functionalization. *Chem. Soc. Rev.* **2016**, *45*, 1368–1386. [CrossRef]
41. Quasdorf, K.W.; Overman, L.E. Catalytic enantioselective synthesis of quaternary carbon stereocentres. *Nature* **2014**, *516*, 181–191. [CrossRef]
42. Wang, W.; Liu, X.; Lin, L.; Feng, X. Recent Progress in the Chemically Catalyzed Enantioselective Synthesis of Cyanohydrins. *Euro. J. Org. Chem.* **2010**, *2010*, 4751–4769. [CrossRef]
43. Ribeiro, A.R.; Maia, A.S.; Cass, Q.B.; Tiritan, M.E. Enantioseparation of chiral pharmaceuticals in biomedical and environmental analyses by liquid chromatography: An overview. *J. Chromatogr. B* **2014**, *968*, 8–21. [CrossRef]
44. Pinto, M.M.M.; Fernandes, C.; Tiritan, M.E. Chiral separations in preparative scale: A medicinal chemistry point of view. *Molecules* **2020**, *25*, 1931. [CrossRef] [PubMed]
45. Fernandes, C.; Tiritan, M.E.; Pinto, M.M.M. Chiral separation in preparative scale: A brief overview of membranes as tools for enantiomeric separation. *Symmetry* **2017**, *9*, 206. [CrossRef]

46. Agranat, I.; Wainschtein, S.R. The strategy of enantiomer patents of drugs. *Drug Discov. Today* **2010**, *15*, 163–170. [CrossRef] [PubMed]
47. Agranat, I.; Caner, H.; Caldwell, J. Putting chirality to work: The strategy of chiral switches. *Nat. Rev. Drug Discov.* **2002**, *1*, 753–768. [CrossRef] [PubMed]
48. Caner, H.; Groner, E.; Levy, L.; Agranat, I. Trends in the development of chiral drugs. *Drug Discov. Today* **2004**, *9*, 105–110.
49. Brocks, D.R. Drug disposition in three dimensions: An update on stereoselectivity in pharmacokinetics. *Biopharm. Drug Dispos.* **2006**, *27*, 387–406. [CrossRef]
50. Kratochwil, N.A.; Huber, W.; Francis, M.; Kansy, M.; Gerber, P.R. Predicting plasma protein binding of drugs: A new approach. *Biochem. Pharmacol.* **2002**, *64*, 1355–1374. [CrossRef]
51. Fournier, T.; Medjoubi-n, N.; Porquet, D. Alpha-1-acid glycoprotein. *Biochim. Biophys. Acta* **2000**, *1482*, 157–171. [CrossRef]
52. Carter, D.C.; Ho, J.X. Structure of serum albumin. *Adv. Protein Chem.* **1994**, *45*, 153–203.
53. Lambrinidis, G.; Vallianatou, T.; Tsantili-Kakoulidou, A. In Vitro, in silico and integrated strategies for the estimation of plasma protein binding. A review. *Adv. Drug Deliv. Rev.* **2015**, *86*, 27–45. [CrossRef]
54. Bi, C.; Zheng, X.; Azaria, S.; Beeram, S.; Li, Z.; Hage, D.S. Chromatographic studies of protein-based chiral separations. *Separations* **2016**, *3*, 27. [CrossRef] [PubMed]
55. Dos Santos, R.; Figueiredo, C.; Viecinski, A.C.; Pina, A.S.; Barbosa, A.J.M.; Roque, A.C.A. Designed affinity ligands to capture human serum albumin. *J. Chromatogr. A* **2019**, *1583*, 88–97. [CrossRef] [PubMed]
56. More, J.; Bulmer, M. Human serum albumin: A multifunctional plasma protein. In *Production of Plasma Proteins for Therapeutic Use*; 2013; pp. 159–183.
57. Ascenzi, P.; Bocedi, A.; Notari, S.; Fanali, G.; Fesce, R.; Fasano, M. Allosteric modulation of drug binding to human serum albumin. *Mini-Rev. Med. Chem.* **2006**, *6*, 483–489. [CrossRef] [PubMed]
58. Ghuman, J.; Zunszain, P.A.; Petitpas, I.; Bhattacharya, A.A.; Otagiri, M.; Curry, S. Structural basis of the drug-binding specificity of human serum albumin. *J. Mol. Biol.* **2005**, *353*, 38–52. [CrossRef]
59. He, X.M.; Carter, D.C. Atomic structure and chemistry of human serum albumin. *Nature* **1992**, *358*, 209–215. [CrossRef] [PubMed]
60. Curry, S.; Brick, P.; Franks, N.P. Fatty acid binding to human serum albumin: New insights from crystallographic studies. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **1999**, *1441*, 131–140. [CrossRef]
61. Evans, T.W. Review article: Albumin as a drug—Biological effects of albumin unrelated to oncotic pressure. *Aliment Pharmacol. Ther. Suppl.* **2002**, *16*, 6–11. [CrossRef]
62. Gupta, D.; Lis, C.G. Pretreatment serum albumin as a predictor of cancer survival: A systematic review of the epidemiological literature. *Nutr. J.* **2010**, *9*, 69. [CrossRef]
63. Sbarouni, E.; Georgiadou, P.; Voudris, V. Ischemia modified albumin changes—Review and clinical implications. *Clin. Chem. Lab. Med.* **2011**, *49*, 177–184. [CrossRef]
64. Koga, M.; Kasayama, S. Clinical impact of glycated albumin as another glycemic control marker. *Endocr. J.* **2010**, *57*, 751–762. [CrossRef]
65. Zorzi, A.; Linciano, S.; Angelini, A. Non-covalent albumin-binding ligands for extending the circulating half-life of small biotherapeutics. *Med. Chem. Comm.* **2019**, *10*, 1068–1081. [CrossRef]
66. Fanali, G.; di Masi, A.; Trezza, V.; Marino, M.; Fasano, M.; Ascenzi, P. Human serum albumin: From bench to bedside. *Mol. Aspects Med.* **2012**, *33*, 209–290. [CrossRef]
67. Elsadek, B.; Kratz, F. Impact of albumin on drug delivery–new applications on the horizon. *J. Control. Release* **2012**, *157*, 4–28. [CrossRef] [PubMed]
68. Wanat, K.; Brzezińska, E.; Sobańska, A.W. Aspects of drug-protein binding and methods of analyzing the phenomenon. *Curr. Pharm. Des.* **2018**, *24*, 2974–2985. [CrossRef] [PubMed]
69. Vuignier, K.; Schappler, J.; Veuthey, J.L.; Carrupt, P.A.; Martel, S. Drug-protein binding: A critical review of analytical tools. *Anal. Bioanal. Chem.* **2010**, *398*, 53–66. [CrossRef]
70. Howard, M.L.; Hill, J.J.; Galluppi, G.R.; McLean, M.A. Plasma protein binding in drug discovery and development. *Comb. Chem. High Throughput Screen.* **2010**, *13*, 170–187. [CrossRef]
71. Hage, D.S.; Anguizola, J.A.; Bi, C.; Li, R.; Matsuda, R.; Papastavros, E.; Pfaunmiller, E.; Vargas, J.; Zheng, X. Pharmaceutical and biomedical applications of affinity chromatography: Recent trends and developments. *J. Pharm. Biomed. Anal.* **2012**, *69*, 93–105. [CrossRef] [PubMed]
72. Siddiqui, S.; Ameen, F.; ur Rehman, S.; Sarwar, T.; Tabish, M. Studying the interaction of drug/ligand with serum albumin. *J. Mol. Liq.* **2021**, *336*, 116200. [CrossRef]
73. Cheng, Y.; Ho, E.; Subramanyam, B.; Tseng, J.L. Measurements of drug-protein binding by using immobilized human serum albumin liquid chromatography-mass spectrometry. *J. Chromatogr. B* **2004**, *809*, 67–73. [CrossRef] [PubMed]
74. Banker, M.J.; Clark, T.H.; Williams, J.A. Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. *J. Pharm. Sci.* **2003**, *92*, 967–974. [CrossRef] [PubMed]
75. Wan, H.; Rehngren, M. High-throughput screening of protein binding by equilibrium dialysis combined with liquid chromatography and mass spectrometry. *J. Chromatogr. A.* **2006**, *1102*, 125–134. [CrossRef] [PubMed]
76. Singh, S.S.; Mehta, J. Measurement of drug-protein binding by immobilized human serum albumin-HPLC and comparison with ultrafiltration. *J. Chromatogr. B* **2006**, *834*, 108–116. [CrossRef] [PubMed]

77. Vuignier, K.; Guillarme, D.; Veuthey, J.L.; Carrupt, P.A.; Schappler, J. High performance affinity chromatography (HPAC) as a high-throughput screening tool in drug discovery to study drug-plasma protein interactions. *J. Pharm. Biomed. Anal.* **2013**, *74*, 205–212. [CrossRef] [PubMed]
78. Kim, H.S.; Kye, Y.S.; Hage, D.S. Development and evaluation of N-hydroxysuccinimide-activated silica for immobilizing human serum albumin in liquid chromatography columns. *J. Chromatogr. A.* **2004**, *1049*, 51–61. [CrossRef]
79. Kim, H.S.; Wainer, I.W. Rapid analysis of the interactions between drugs and human serum albumin (HSA) using high-performance affinity chromatography (HPAC). *J. Chromatogr. B* **2008**, *870*, 22–26. [CrossRef]
80. Loun, B.; Hage, D.S. Thermodynamic studies of (R)- and (S)-warfarin binding to immobilized human serum albumin. Chiral separation mechanisms in protein-based HPLC columns. 661994. *Anal. Chem.* **1994**, *66*, 3814–3822. [CrossRef] [PubMed]
81. Loun, B.; Hage, D.S. Kinetic studies of (R)- and (S)-warfarin binding to immoblized human serum albumin. Chiral separation mechanisms in protein-based HPLC columns. 681996. *Anal. Chem.* **1994**, *66*, 1218–1225. [CrossRef]
82. Patel, S.; Wainer, I.W.; Lough, W. Affinity based chiral stationary phases. In *Handbook of Affinity Chromatography*; CRC: Boca Raton, FL, USA, 2006.
83. Aubry, A.F.; Markoglou, N.; McGann, A. Comparison of drug binding interactions on human, rat and rabbit serum albumin using high-performance displacement chromatography. *Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol.* **1995**, *112*, 257–266. [CrossRef]
84. Esmaeilzadeh, S.; Valizadeh, H.; Zakeri-Milani, P. The effects of pH, temperature and protein concentration on the in vitro binding of flutamide to human serum albumin. *Pharm. Dev. Technol.* **2017**, *22*, 982–991. [CrossRef]
85. Ashton, D.S.; Beddell, C.; Ray, A.D.; Valkó, K. Quantitative structure-retention relationships of acyclovir esters using immobilised albumin high-performance liquid chromatography and reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* **1995**, *707*, 367–372. [CrossRef]
86. Lämmerhofer, M. Chiral recognition by enantioselective liquid chromatography: Mechanisms and modern chiral stationary phases. *J. Chromatogr. A* **2010**, *1217*, 814–856. [CrossRef] [PubMed]
87. Scriba, G.K.E. Chiral recognition mechanisms in analytical separation sciences. *Chromatographia* **2012**, *75*, 815–838. [CrossRef]
88. Haginaka, J. Protein-based chiral stationary phases for high-performance liquid chromatography enantioseparations. *J. Chromatogr. A* **2001**, *906*, 253–273. [CrossRef]
89. Stewart, K.K.; Doherty, R.F. Resolution of DL-tryptophan by affinity chromatography on bovine serum albumin agarose columns. *Proc. Natl. Acad. Sci. USA* **1973**, *70*, 2850–2852. [CrossRef] [PubMed]
90. Allenmark, S.; Bomgren, B.; Borén, H. Direct liquid chromatographic separation of enantiomers on immobilized protein stationary phases. III. Optical resolution of a series of N-aroyl d,l-amino acids by high-performance liquid chromatography on bovine serum albumin covalently bound to silica. *J. Chromatogr. A* **1983**, *264*, 63–68. [CrossRef]
91. Cavazzini, A.; Pasti, L.; Massi, A.; Marchetti, N.; Dondi, F. Recent applications in chiral high performance liquid chromatography: A review. *Anal. Chim. Acta* **2011**, *706*, 205–222. [CrossRef]
92. Haginaka, J. Recent progresses in protein-based chiral stationary phases for enantioseparations in liquid chromatography. *J. Chromatogr. B* **2008**, *875*, 12–19. [CrossRef]
93. Teixeira, J.; Tiritan, M.E.; Pinto, M.M.M.; Fernandes, C. Chiral stationary phases for liquid chromatography: Recent developments. *Molecules* **2019**, *24*, 865.
94. Millot, M.C. Separation of drug enantiomers by liquid chromatography and capillary electrophoresis, using immobilized proteins as chiral selectors. *J. Chromatogr. B* **2003**, *797*, 131–159. [CrossRef]
95. Bocian, S.; Skoczylas, M.; Buszewski, B. Amino acids, peptides, and proteins as chemically bonded stationary phases—A review. *J. Sep. Sci.* **2016**, *39*, 83–92.
96. Anderson, J.; Berthod, A.; Pino, V.; Stalcup, A.M. *Analytical Separation Science*; Wiley: Hoboken, NJ, USA, 2016; Volume 5.
97. Millot, M.C.; Servagent-Noinville, S.; Taleb, N.L.; Baron, M.H.; Revault, M.; Sébiller, B. Structural changes of human serum albumin immobilized on chromatographic supports: A high-performance liquid chromatography and Fourier-transform infrared spectroscopy study. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *753*, 101–113. [CrossRef]
98. Do Carmo, J.P.; Phyo, Y.Z.; Palmeira, A.; Tiritan, M.E.; Afonso, C.; Kijjoa, A. Enantioseparation, recognition mechanisms and binding of xanthones on human serum albumin by liquid chromatography. *Bioanalysis* **2019**, *11*, 1255–1274. [CrossRef] [PubMed]
99. Kamble, S.; Loadman, P.; Abraham, M.H.; Liu, X. Structural properties governing drug-plasma protein binding determined by high-performance liquid chromatography method. *J. Pharm. Biomed. Anal.* **2018**, *149*, 16–21. [CrossRef] [PubMed]
100. Hage, D.S. High-performance affinity chromatography: A powerful tool for studying serum protein binding. *J. Chromatogr. B* **2002**, *768*, 3–30. [CrossRef]
101. Shibukawa, A.; Kuroda, Y.; Nakagawa, T. High-performance frontal analysis for drug-protein binding study. *J. Pharm. Biomed. Anal.* **1999**, *18*, 1047–1055. [CrossRef]
102. Bertucci, C.; Domenici, E. Reversible and Covalent Binding of Drugs to Human Serum Albumin: Methodological Approaches and Physiological Relevance. *Curr. Med. Chem.* **2002**, *9*, 1463–1481.
103. Cass, Q.B.; Degani, A.L.G. *Desenvolvimento de Métodos por HPLC: Fundamentos, Estratégias e Validação*; Editora da UFCar: São Carlos, Brazil, 2001.
104. Talbert, A.M.; Tranter, G.E.; Holmes, E.; Francis, P.L. Determination of Drug-plasma Protein Binding Kinetics and Equilibria by Chromatographic Profiling: Exemplification of the Method Using L-Tryptophan and Albumin. *Anal. Chem.* **2002**, *74*, 446–452.

105. Noctor, T.A.G.; Pham, C.D.; Kaliszan, R.; Wainer, I.W. Stereochemical aspects of benzodiazepine binding to human serum albumin. I. Enantioselective high performance liquid affinity chromatographic examination of chiral and achiral binding interactions between 1,4- benzodiazepines and human serum albumin. *Mol. Pharmacol.* **1992**, *42*, 506–511.
106. Kaliszan, R.; Noctor, T.A.G.; Wainer, I.W. Stereochemical aspects of benzodiazepine binding to human serum albumin. II. Quantitative relationships between structure and enantioselective retention in high performance liquid affinity chromatography. *Mol. Pharmacol.* **1992**, *42*, 512–517.
107. Hage, D.S.; Noctor, T.A.G.; Wainer, I.W. Characterization of the protein binding of chiral drugs by high-performance affinity chromatography interactions of R- and S-ibuprofen with human serum albumin. *J. Chromatogr. A* **1995**, *693*, 23–32.
108. Yang, J.; Hage, D.S. Characterization of the binding and chiral separation of d- and l-tryptophan on a high-performance immobilized human serum albumin column. *J. Chromatogr. A* **1993**, *645*, 241–250. [CrossRef]
109. Loun, B.; Hage, D.S. Characterization of thyroxine-albumin binding using high-performance affinity chromatography. I. Interactions at the warfarin and indole sites of albumin. *J. Chromatogr. B Biomed. Sci. Appl.* **1992**, *579*, 225–235. [CrossRef]
110. Loun, B.; Hage, D.S. Characterization of thyroxine-albumin binding using high-performance affinity chromatography II. Comparison of the binding of thyroxine, triiodothyronines and related compounds at the warfarin and indole sites of human serum albumin. *J. Chromatogr. B Biomed. Sci. Appl.* **1995**, *665*, 303–314. [CrossRef]
111. Pistolozzi, M.; Fortugno, C.; Franchini, C.; Corbo, F.; Muraglia, M.; Roy, M.; Felix, G.; Bertucci, C. Species-dependent binding of tocainide analogues to albumin: Affinity chromatography and circular dichroism study. *J. Chromatogr. B* **2014**, *968*, 69–78. [CrossRef] [PubMed]
112. Yoo, M.J.; Smith, Q.R.; Hage, D.S. Studies of imipramine binding to human serum albumin by high-performance affinity chromatography. *J. Chromatogr. B* **2009**, *877*, 1149–1154. [CrossRef]
113. Matsuda, R.; Jobe, D.; Beyersdorf, J.; Hage, D.S. Analysis of drug-protein binding using on-line immunoextraction and high-performance affinity microcolumns: Studies with normal and glycated human serum albumin. *J. Chromatogr. A* **2015**, *1416*, 112–120. [CrossRef]
114. Matsuda, R.; Li, Z.; Zheng, X.; Hage, D.S. Analysis of glipizide binding to normal and glycated human serum albumin by high-performance affinity chromatography. *Anal. Bioanal. Chem.* **2015**, *407*, 5309–5321. [CrossRef]
115. Tao, P.; Li, Z.; Woolfork, A.G.; Hage, D.S. Characterization of tolazamide binding with glycated and normal human serum albumin by using high-performance affinity chromatography. *J. Pharm. Biomed. Anal.* **2019**, *166*, 273–280. [CrossRef]
116. Chen, J.; Ohnmacht, C.; Hage, D.S. Studies of phenytoin binding to human serum albumin by high-performance affinity chromatography. *J. Chromatogr. B* **2004**, *809*, 137–145. [CrossRef]
117. Bertucci, C.; Pistolozzi, M.; Felix, G.; Danielson, U.H. HSA binding of HIV protease inhibitors: A high-performance affinity chromatography study. *J. Sep. Sci.* **2009**, *32*, 1625–1631. [CrossRef]
118. Buchholz, L.; Cai, C.-H.; Andress, L.; Cleton, A.; Brodfuehrer, J.; Cohen, L. Evaluation of the human serum albumin column as a discovery screening tool for plasma protein binding. *Euro. J. Pharm. Sci.* **2002**, *15*, 209–215. [CrossRef]
119. Valko, K.; Nunhuck, S.; Bevan, C.; Abraham, M.H.; Reynolds, D.P. Fast Gradient HPLC Method to Determine Compounds Binding to Human Serum Albumin. Relationships with Octanol/Water and Immobilized Artificial Membrane Lipophilicity. *J. Chromatogr. B* **2003**, *877*, 2236–2248. [CrossRef] [PubMed]
120. Tong, Z.; Hage, D.S. Characterization of interaction kinetics between chiral solutes and human serum albumin by using high-performance affinity chromatography and peak profiling. *J. Chromatogr. A* **2011**, *1218*, 6892–6897. [CrossRef] [PubMed]
121. Cao, H.; Liu, X.; Ulrih, N.P.; Sengupta, P.K.; Xiao, J. Plasma protein binding of dietary polyphenols to human serum albumin: A high performance affinity chromatography approach. *Food Chem.* **2019**, *270*, 257–263. [CrossRef] [PubMed]
122. Bertucci, C.; Cimitan, S.; Riva, A.; Morazzoni, P. Binding studies of taxanes to human serum albumin by bioaffinity chromatography and circular dichroism. *J. Pharm. Biomed. Anal.* **2006**, *42*, 81–87. [CrossRef]
123. Pistolozzi, M.; Franchini, C.; Corbo, F.; Muraglia, M.; De Giorgi, M.; Felix, G.; Bertucci, C. Tocainide analogues binding to human serum albumin: A HPLAC and circular dichroism study. *J. Pharm. Biomed. Anal.* **2010**, *53*, 179–185. [CrossRef] [PubMed]
124. Claire, A.; Lethier, L.; Guillaume, Y.C. An organic monolithic capillary column functionalized with human serum albumin and its application for the nano—Chromatography study of its binding with universal cancer peptides and its impact on immunogenicity. *J. Liq. Chromatogr. Relat. Technol.* **2020**, *43*, 777–783. [CrossRef]
125. Rodriguez, E.L.; Poddar, S.; Choksi, M.; Hage, D.S. Development of an on-line immunoextraction/entrapment system for protein capture and use in drug binding studies by high-performance affinity chromatography. *J. Chromatogr. B* **2020**, *1136*, 121812. [CrossRef]
126. Ovbude, S.T.; Tao, P.; Li, Z.; Hage, D.S. High-Performance affinity chromatographic studies of repaglinide and nateglinide interactions with normal and glyoxal- or methylglyoxal-modified human albumin serum. *J. Pharm. Biomed. Anal.* **2021**, *201*, 114097. [CrossRef]
127. Woolfork, A.G.; Suh, K.; Weigand, M.; Hage, D.S. Studies of binding by 2-imidazolines to human serum albumin and alpha1-acid glycoprotein by high-performance affinity chromatography. *J. Pharm. Biomed. Anal.* **2021**, *202*, 114135. [CrossRef]
128. Fernandes, C.; Teixeira, J.; Pinto, M.M.M.; Tiritan, M.E. Strategies for Preparation of Chiral Stationary Phases: Progress on Coating and Immobilization Methods. *Molecules* **2021**, *26*, 5477. [CrossRef]

129. Matsuda, R.; Anguizola, J.; Hoy, K.S.; Hage, D.S. Analysis of drug–protein interactions by high-performance affinity chromatography: Interactions of sulfonylurea drugs with normal and glycated human serum albumin. In *Methods in Molecular Biology*; Humana Press Inc.: Totowa, NJ, USA, 2015; pp. 255–277.
130. Anguizola, J.; Joseph, K.S.; Barnaby, O.S.; Matsuda, R.; Alvarado, G.; Clarke, W.; Cerny, R.L.; Hage, D.S. Development of affinity microcolumns for drug-protein binding studies in personalized medicine: Interactions of sulfonylurea drugs with in vivo glycated human serum albumin. *Anal. Chem.* **2013**, *85*, 4453–4460. [CrossRef] [PubMed]
131. Zheng, X.; Li, Z.; Beeram, S.; Podariu, M.; Matsuda, R.; Pfaunmiller, E.L.; Ii, C.J.W.; Carter, N.; Hage, D.S. Analysis of biomolecular interactions using affinity microcolumns: A review. *J. Chromatogr. B* **2014**, *968*, 49–63. [CrossRef]
132. Daicel-Corporation. INSTRUCTION MANUAL FOR CHIRALPAK®HAS. 2018. Available online: http://chiraltech.com/wp-content/uploads/2018/09/CHIRALPAK-HSA-2018.pdf (accessed on 14 September 2021).
133. Bertucci, C.; Tedesco, D. Human serum albumin as chiral selector in enantioselective high-performance liquid chromatography. *Curr. Med. Chem.* **2017**, *24*, 743–757. [CrossRef] [PubMed]
134. Eberherr, W.; Buchberger, W.; Hertsens, R.; Klampfl, C.W. Investigations on the Coupling of High-Performance Liquid Chromatography to Direct Analysis in Real Time Mass Spectrometry. *Anal. Chem.* **2010**, *82*, 5792–5796. [CrossRef]
135. Mallik, R.; Jiang, T.; Hage, D.S. High-performance affinity monolith chromatography: Development and evaluation of human serum albumin columns. *Anal. Chem.* **2005**, *76*, 7013–7022. [CrossRef]
136. Maddi, S.; Scriba, G.; Yamsani, M.R. Stereoselective binding of chiral anti-diabetic drug nateglinide to plasma proteins. *Drug Metabol. Drug Interact.* **2011**, *26*, 81–86. [CrossRef]
137. Haginaka, J. Enantiomer separation of drugs by capillary electrophoresis using proteins as chiral selectors. *J. Chromatogr. A* **2000**, *875*, 235–254. [CrossRef]
138. Hancu, G.; Orlandini, S.; Papp, L.A.; Modroiu, A.; Gotti, R.; Furlanetto, S. Application of Experimental Design Methodologies in the Enantioseparation of Pharmaceuticals by Capillary Electrophoresis: A Review. *Molecules* **2021**, *26*, 4681. [CrossRef] [PubMed]
139. Albishri, H.M.; El Deeb, S.; AlGarabli, N.; AlAstal, R.; A Alhazmi, H.; Nachbar, M.; El-Hady, D.A.; Wätzig, H. Recent advances in affinity capillary electrophoresis for binding studies. *Bioanalysis* **2014**, *6*, 3369–3392. [CrossRef] [PubMed]
140. Chu, Y.H.; Avila, L.Z.; Biebuyck, H.A.; Whitesides, G.M. Use of affinity capillary electrophoresis to measure binding constants of ligands to proteins. *J. Med. Chem.* **1992**, *35*, 2915–2917. [CrossRef]
141. Olabi, M.; Stein, M.; Wätzig, H. Affinity capillary electrophoresis for studying interactions in life sciences. *Methods* **2018**, *146*, 76–92. [CrossRef]
142. El-Hady, D.; Kühne, S.; El-Maali, N.; Wätzig, H. Precision in affinity capillary electrophoresis for drug-protein binding studies. *J. Pharm. Biomed. Anal.* **2010**, *52*, 232–241. [CrossRef]
143. Zhu, X.; Ding, Y.; Lin, B.; Jakob, A.; Koppenhoefer, B. Study of enantioselective interactions between chiral drugs and serum albumin by capillary electrophoresis. *Electrophoresis* **1999**, *20*, 1869–1877. [CrossRef]
144. Yang, J.; Hage, D.S. Chiral separations in capillary electrophoresis using human serum albumin as a buffer additive. *Anal. Chem.* **1994**, *66*, 2719–2725. [CrossRef]
145. Ding, Y.; Zhu, X.; Lin, B. Study of interaction between drug enantiomers and serum albumin by capillary electrophoresis. *Electrophoresis* **1999**, *20*, 1890–1894. [CrossRef]
146. Ding, Y.S.; Zhu, X.F.; Lin, B.C. Capillary electrophoresis study of human serum albumin binding to basic drugs. *Chromatographia* **1999**, *49*, 343–346. [CrossRef]
147. Jia, Z.; Ramstad, T.; Zhong, M. Determination of protein-drug binding constants by pressure-assisted capillary electrophoresis (PACE)/frontal analysis (FA). *J. Pharm. Biomed. Anal.* **2002**, *30*, 405–413. [CrossRef]
148. Kim, H.S.; Austin, J.; Hage, D.S. Identification of drug-binding sites on human serum albumin using affinity capillary electrophoresis and chemically modified proteins as buffer additives. *Electrophoresis* **2002**, *23*, 956–963. [CrossRef]
149. Scriba, G.K.E.; Harnisch, H.; Zhu, Q. Enantiomer separations by capillary electrophoresis. In *Methods in Molecular Biology*; Humana Press Inc.: Totowa, NJ, USA, 2016; pp. 277–299.
150. Xu, H.; Yu, X.; Chen, H. Enantiomeric separation of basic drugs with partially filled serum albumin as chiral selector in capillary electrophoresis. *Anal. Sci.* **2004**, *20*, 1409–1413. [CrossRef]
151. Liu, X.; Song, Y.; Yue, Y.; Zhang, J.; Chen, X. Study of interaction between drug enantiomers and human serum albumin by flow injection-capillary electrophoresis frontal analysis. *Electrophoresis* **2008**, *29*, 2876–2883. [CrossRef] [PubMed]
152. Martínez-Gómez, M.A.; Escuder-Gilabert, L.; Villanueva-Camañas, R.M.; Sagrado, S.; Medina-Hernández, M.J. Enantioseparation of nuarimol by affinity electrokinetic chromatography-partial filling technique using human serum albumin as chiral selector. *J. Sep. Sci.* **2008**, *31*, 3265–3271. [CrossRef]
153. Asensi-Bernardi, L.; Martin-Biosca, Y.; Escuder-Gilabert, L.; Sagrado, S.; Medina-Hernandez, M.J. Evaluation of the enantioselective binding of imazalil to human serum albumin by capillary electrophoresis. *Biomed. Chromatogr.* **2015**, *29*, 1637–1642. [CrossRef]
154. Xu, Y.; Hong, T.; Chen, X.; Ji, Y. Affinity capillary electrophoresis and fluorescence spectroscopy for studying enantioselective interactions between omeprazole enantiomer and human serum albumin. *Electrophoresis* **2017**, *38*, 1366–1373. [CrossRef]
155. Ratih, R.; Watzig, H.; Stein, M.; El Deeb, S. Investigation of the enantioselective interaction between selected drug enantiomers and human serum albumin by mobility shift-affinity capillary electrophoresis. *J. Sep. Sci.* **2020**, *43*, 3960–3968. [CrossRef]

156. Zhao, L.; Chen, D. Characterization of interactions between methoxatin disodium salt and human serum albumin by pressure-assisted capillary electrophoresis/frontal analysis and circular dichroism spectroscopy. *Biomed. Chromatogr.* **2015**, *29*, 123–128. [CrossRef]
157. Liu, T.T.; Xiang, L.L.; Wang, J.L.; Chen, D.Y. Application of capillary electrophoresis-frontal analysis for comparative evaluation of the binding interaction of captopril with human serum albumin in the absence and presence of hydrochlorothiazide. *J. Pharm. Biomed. Anal.* **2015**, *115*, 31–35. [CrossRef]
158. Zhang, Y.; Sha, Y.; Qian, K.; Chen, X.; Chen, Q. Comparison of three methods for analyzing loureirin B and human serum albumin interaction using capillary electrophoresis. *Electrophoresis* **2017**, *38*, 1038–1043. [CrossRef]
159. Nevídalová, H.; Michalcová, L.; Glatz, Z. Applicability of capillary electrophoresis-frontal analysis for displacement studies: Effect of several drugs on l-tryptophan and lidocaine binding to human serum albumin. *J. Sep. Sci.* **2020**, *43*, 4225–4233. [CrossRef]
160. Martínez-Gómez, M.A.; Villanueva-Camañas, R.M.; Sagrado, S.; Medina-Hernández, M.J. Evaluation of enantioselective binding of antihistamines to human serum albumin by ACE. *Electrophoresis* **2007**, *28*, 2635–2643. [CrossRef]
161. Asensi-Bernardi, L.; Martin-Biosca, Y.; Medina-Hernandez, M.J.; Sagrado, S. On the zopiclone enantioselective binding to human albumin and plasma proteins. An electrokinetic chromatography approach. *J. Chromatogr. A* **2011**, *1218*, 3111–3117. [CrossRef]
162. Asensi-Bernardi, L.; Martin-Biosca, Y.; Sagrado, S.; Medina-Hernandez, M.J. Electrokinetic chromatographic estimation of the enantioselective binding of nomifensine to human serum albumin and total plasma proteins. *Biomed. Chromatogr.* **2012**, *26*, 1357–1363. [CrossRef] [PubMed]
163. Fernandes, C.; Palmeira, A.; Ramos, I.I.; Carneiro, C.; Afonso, C.; Tiritan, M.E.; Cidade, H.; Pinto, P.C.A.G.; Saraiva, M.L.M.F.S.; Reis, R. Chiral derivatives of xanthones: Investigation of the effect of enantioselectivity on inhibition of cyclooxygenases (COX-1 and COX-2) and binding interaction with human serum albumin. *Pharmaceuticals* **2017**, *10*, 50. [CrossRef]
164. Meng, X.-Y.; Zhang, H.-X.; Mezei, M.; Cui, M. Molecular docking: A powerful approach for structure-based drug discovery. *Curr. Comput. Aided Drug Des.* **2011**, *7*, 146–157. [CrossRef]
165. Halperin, I.; Ma, B.; Wolfson, H.; Nussinov, R. Principles of docking: An overview of search algorithms and a guide to scoring functions. *Proteins* **2002**, *47*, 409–443. [CrossRef]
166. Moreira, I.S.; Fernandes, P.A.; Ramos, M.J. Protein-protein docking dealing with the unknown. *J. Comput. Chem.* **2010**, *31*, 317–342. [CrossRef]
167. Phyo, Y.Z.; Cravo, S.; Palmeira, A.; Tiritan, M.E.; Kijjoa, A.; Pinto, M.M.M.; Fernandes, C. Enantiomeric Resolution and Docking Studies of Chiral Xanthonic Derivatives on Chirobiotic Columns. *Molecules* **2018**, *23*, 142. [CrossRef]
168. Phyo, Y.Z.; Teixeira, J.; Gonçalves, R.; Palmeira, A.; Tiritan, M.E.; Bousbaa, H.; Pinto, M.M.M.; Fernandes, C.; Kijjoa, A. Chiral derivatives of xanthones and benzophenones: Synthesis, enantioseparation, molecular docking, and tumor cell growth inhibition studies. *Chirality* **2021**, *33*, 153–166. [CrossRef]
169. De Simone, G.; Pasquadibisceglie, A.; di Masi, A.; Buzzelli, V.; Trezza, V.; Macari, G.; Polticelli, F.; Ascenzi, P. Binding of direct oral anticoagulants to the FA1 site of human serum albumin. *J. Mol. Recogn.* **2021**, *34*, e2877. [CrossRef]
170. Ali, M.S.; Muthukumaran, J.; Jain, M.; Al-Lohedan, H.A.; Farah, M.A.; Alsowilem, O.I. Experimental and computational investigation on the binding of anticancer drug gemcitabine with bovine serum albumin. *J. Biomol. Struct. Dyn.* **2021**, 1–14. [CrossRef]
171. Bratty, M.A. Spectroscopic and molecular docking studies for characterizing binding mechanism and conformational changes of human serum albumin upon interaction with Telmisartan. *Saudi Pharm. J. SPJ Off. Publ. Saudi Pharm. Soc.* **2020**, *28*, 729–736. [CrossRef]
172. Mic, M.; Pîrnău, A.; Floare, C.G.; Marc, G.; Franchini, A.H.; Oniga, O.; Vlase, L.; Bogdan, M. Synthesis and molecular interaction study of a diphenolic hidrazinyl-thiazole compound with strong antioxidant and antiradical activity with HSA. *J. Mol. Struct.* **2021**, *1244*, 131278. [CrossRef]
173. Thirugnanasundar, A.; Kesavan, M.P.; Kumar, S.M.; Ravi, L.; Bhaskar, R.; Rajagopal, G.; Rajesh, J. Synthesis, structure, DNA/protein molecular docking and biological studies of hydrazone ligand derived Cu(II) and VO(IV) complexes. *Inorg. Chim. Acta* **2021**, *526*, 120543. [CrossRef]
174. Yasrebi, S.A.; Takjoo, R.; Riazi, G.H. HSA-interaction studies of uranyl complexes of alkyl substituted isothiosemicarbazone. *J. Mol. Struct.* **2019**, *1193*, 53–61. [CrossRef]
175. Bapli, A.; Chatterjee, A.; Gautam, R.K.; Pandit, S.; Jana, R.; Seth, D. Interaction of a hydrophilic molecule with bovine serum albumin: A combined multi-spectroscopic, microscopic and isothermal calorimetric study in the presence of graphene oxide. *J. Mol. Liq.* **2021**, *323*, 114618. [CrossRef]
176. Ferreira, L.G.; Dos Santos, R.N.; Oliva, G.; Andricopulo, A.D. Molecular docking and structure-based drug design strategies. *Molecules* **2015**, *20*, 13384–13421. [CrossRef]
177. Leek, H.; Andersson, S. Preparative scale resolution of enantiomers enables accelerated drug discovery and development. *Molecules* **2017**, *22*, 158. [CrossRef]
178. Silva, B.; Pereira, J.A.; Cravo, S.; Araújo, A.M.; Fernandes, C.; Pinto, M.M.M.; de Pinho, P.G.; Remião, F. Multi-milligram resolution and determination of absolute configuration of pentedrone and methylone enantiomers. *J. Chromatogr. B* **2018**, *110*, 158–164. [CrossRef]

179. Carraro, M.L.; Palmeira, A.; Tiritan, M.E.; Fernandes, C.; Pinto, M.M.M. Resolution, determination of enantiomeric purity and chiral recognition mechanism of new xanthone derivatives on (S,S)-Whelk-O1 stationary phase. *Chirality* **2017**, *29*, 247–256. [CrossRef]
180. Silva, B.; Fernandes, C.; Tiritan, M.E.; Pinto, M.M.; Valente, M.J.; Carvalho, M.; de Pinho, P.G.; Remião, F. Chiral enantioresolution of cathinone derivatives present in "legal highs", and enantioselectivity evaluation on cytotoxicity of 3,4-methylenedioxypyrovalerone (MDPV). *Forensic Toxicol.* **2016**, *34*, 372–385. [CrossRef]
181. Fernandes, C.; Brandão, P.; Santos, A.; Tiritan, M.E.; Afonso, C.; Cass, Q.B.; Pinto, M.M. Resolution and determination of enantiomeric purity of new chiral derivatives of xanthones using polysaccharide-based stationary phases. *J. Chromatogr. A* **2012**, *1269*, 143–153. [CrossRef]
182. Rocco, A.; Aturki, Z.; Fanali, S. Chiral separations in food analysis. *TrAC-Trends Anal. Chem.* **2013**, *52*, 206–225. [CrossRef]
183. Alvarez-Rivera, G.; Bueno, M.; Ballesteros-Vivas, D.; Cifuentes, A. Chiral analysis in food science. *TrAC Trends Anal. Chem.* **2020**, *123*, 115761. [CrossRef]
184. Phyo, Y.; Ribeiro, J.; Fernandes, C.; Kijjoa, A.; Pinto, M.M.M. Marine natural peptides: Determination of absolute configuration using liquid chromatography methods and evaluation of bioactivities. *Molecules* **2018**, *23*, 306. [CrossRef]
185. Zin, W.W.M.; Buttachon, S.; Dethoup, T.; Fernandes, C.; Cravo, S.; Pinto, M.M.M.; Gales, L.; Pereira, J.A.; Silva, A.M.S.; Sekeroglu, N.; et al. New cyclotetrapeptides and a new diketopiperzine derivative from the marine sponge-associated fungus Neosartorya glabra KUFA 0702. *Mar. Drugs* **2016**, *14*, 136. [CrossRef]
186. Barreiro, J.C.; Tiritan, M.E.; Cass, Q.B. Challenges and innovations in chiral drugs in an environmental and bioanalysis perspective. *TrAC Trends Anal. Chem.* **2021**, *142*, 116326. [CrossRef]
187. Ribeiro, A.R.L.; Maia, A.S.; Ribeiro, C.; Tiritan, M.E. Analysis of chiral drugs in environmental matrices: Current knowledge and trends in environmental, biodegradation and forensic fields. *TrAC Trends Anal. Chem.* **2020**, *124*, 115783. [CrossRef]
188. Chmielewska, A.; Konieczna, L.; Baczek, T. A Novel Two-Step Liquid-Liquid Extraction Procedure Combined with Stationary Phase Immobilized Human Serum Albumin for the Chiral Separation of Cetirizine Enantiomers along with M and P Parabens. *Molecules* **2016**, *21*, 1654. [CrossRef]
189. Barbato, F.; Carpentiero, C.; Grumetto, L.; La Rotonda, M.I. Enantioselective retention of β-blocking agents on human serum albumin and α1-acid glycoprotein HPLC columns: Relationships with different scales of lipophilicity. *Euro. J. Pharm. Sci.* **2009**, *38*, 472–478. [CrossRef]
190. Andrisano, V.; Gotti, R.; Cavrini, V.; Tumiatti, V.; Felix, G.; Wainer, I.W. Capillary electrophoretic and high-performance liquid chromatographic studies of the enantioselective separation of α1-adrenoreceptor antagonists. *J. Chromatogr. A* **1998**, *803*, 189–195. [CrossRef]
191. Bertucci, C.; Andrisano, V.; Gotti, R.; Cavrini, V. Modulation of Chromatographic Performances of HSA-Based HPLC Column by Reversible Binding of Lithocholic Acid. *Chromatographia* **2001**, *53*, 15–18. [CrossRef]
192. Andrisano, V.; Bertucci, C.; Cavrini, V.; Recanatini, M.; Cavalli, A.; Varoli, L. Stereoselective binding of 2,3-substituted 3-hydroxypropionic acids on an immobilised human serum albumin chiral stationary phase: Stereochemical characterisation and quantitative structure-retention relationship study. *J. Chromatogr. A* **2000**, *876*, 75–86. [CrossRef]
193. Andrisano, V.; Gotti, R.; Recanatini, M.; Cavalli, A.; Varoli, L.; Bertucci, C. Stereoselective binding of 2-(4-biphenylyl)-3-substituted-3-hydroxy-propionic acids on an immobilised human serum albumin chiral stationary phase. *J. Chromatogr. B* **2002**, *768*, 137–145. [CrossRef]
194. Fitos, I.; Visy, J.; Simonyi, M. Species-dependency in chiral-drug recognition of serum albumin studied by chromatographic methods. *J. Biochem. Biophys. Methods* **2002**, *54*, 71–84. [CrossRef]
195. Xu, S.; Wang, Y.; Tang, Y.; Ji, Y. A protein-based mixed selector chiral monolithic stationary phase in capillary electrochromatography. *New J. Chem.* **2018**, *42*, 13520–13528. [CrossRef]
196. Martínez-Pla, J.J.; Martín-Biosca, Y.; Sagrado, S.; Villanueva-Camañas, R.M.; Medina-Hernández, M.J. Fast enantiomeric separation of propranolol by affinity capillary electrophoresis using human serum albumin as chiral selector: Application to quality control of pharmaceuticals. *Anal. Chim. Acta* **2004**, *507*, 171–178. [CrossRef]
197. Martínez-Gómez, M.A.; Martínez-Pla, J.J.; Sagrado, S.; Villanueva-Camañas, R.M.; Medina-Hernández, M.J. Chiral separation of oxprenolol by affinity electrokinetic chromatography-partial filling technique using human serum albumin as chiral selector. *J. Pharm. Biomed. Anal.* **2005**, *39*, 76–81. [CrossRef]
198. Martinez-Gomez, M.A.; Villanueva-Camañas, R.M.; Sagrado, S.; Medina-Hernández, M.J. Multivariate optimization approach for chiral resolution of drugs using human serum albumin in affinity electrokinetic chromatography-partial filling technique. *Electrophoresis* **2005**, *26*, 4116–4126. [CrossRef]
199. Martínez-Gómez, M.A.; Sagrado, S.; Villanueva-Camañas, R.M.; Medina-Hernández, M.J. Enantioseparation of phenotiazines by affinity electrokinetic chromatography using human serum albumin as chiral selector: Application to enantiomeric quality control in pharmaceutical formulations. *Anal. Chim. Acta* **2007**, *582*, 223–228. [CrossRef]