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Analysis of Drug Interactions with Serum Proteins and Related Binding Agents by Affinity Capillary Electrophoresis: A Review

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Abbreviations: AGP, alpha$_1$-acid glycoprotein; **CE FA**, frontal analysis ACE; **ECEEM**, equilibrium capillary electrophoresis of equilibrated mixtures; **FTPFACE**, flow through partial filling ACE; **HD ACE**, Hummel-Dreyer ACE; **ms ACE**, mobility shift ACE; **MSLIACE**, multiple steps ligand injection ACE; **NECEEM**, non-equilibrium capillary electrophoresis of equilibrium mixtures; **PACE**, pressure mediated ACE; **PFACE**, partial-filling ACE; **TDLFP**, transverse diffusion of laminar flow profiles; **VACE**, vacancy ACE; **VP ACE**, vacancy peak ACE

Keywords: Affinity capillary electrophoresis; Drug-protein binding; Hummel-Dreyer method; Mobility shift assay; Serum proteins

Abstract

Biomolecules such as serum proteins can interact with drugs in the body and influence their pharmaceutical effects. Specific and precise methods that analyze these interactions are critical for drug development or monitoring and for diagnostic purposes. Affinity capillary electrophoresis (ACE) is one technique that can be used to examine the binding between drugs and serum proteins, or other agents found in serum or blood. This article will review the basic principles of ACE, along with related affinity-based CE methods, and examine recent developments that have occurred in this field as related to the characterization of drug-protein interactions. An overview will be given of the various formats that can be used in ACE and CE for such work, including the relative advantages or weaknesses of each approach. Various applications of ACE and affinity-based CE methods for the analysis of drug interactions with serum proteins and other binding agents will also be presented. Applications of ACE and related techniques that will be discussed include drug interaction studies with serum agents, chiral drug separations employing serum proteins, and the use of CE in hybrid methods to characterize drug binding with serum proteins.

1. Introduction

When a drug enters the blood circulatory system, it may interact with serum proteins or other carrier agents in blood as the drug is transported to its target tissue or receptors to create a therapeutic response [1–3]. Because the binding of some drugs to proteins can be stereoselective in nature, it is also possible that these interactions may play a role in determining the fate of the different forms of a chiral drug within the body [4–7]. In addition, the non-bound drug (or free form) in the blood circulation is often influenced by interactions of the drug with serum proteins, which can affect the drug's pharmacokinetic and pharmacodynamic properties. The binding of a drug with serum agents and the size of the resulting non-bound drug fraction can further be affected by competition between the drug and endogenous compounds (e.g., bilirubin, fatty acids) in the blood circulatory system [8–11]. Similar effects can occur when a mixture of drugs is administered and direct or indirect competition occurs between two drugs for the same binding proteins [1,12]. Thus, the characterization of drug-protein interactions in serum has great importance during the discovery or development of new drugs and for monitoring and understanding the effect of drugs during their use to treat disease [12].

There are many methods that have been used to examine drug interactions with proteins and other agents that occur in serum and blood. Two common reference methods for such studies are equilibrium dialysis and ultrafiltration [5]. Other approaches for this work have included spectroscopic methods based on the measurement of surface plasmon resonance, UV/Vis or infrared absorbance, fluorescence, circular dichroism, and nuclear magnetic resonance [13–19]. Additional methods that can be used for such studies are mass spectrometry, affinity chromatography, electrophoresis, partial artificial membrane permeability assays, and isothermal

calorimetry [13,17–20]. However, many of these techniques are slow, require a relatively large amount of sample or binding agent, or are expensive to perform [21].

Affinity capillary electrophoresis (ACE) is one method that has been developed and explored as a means to overcome many of the limitations in other techniques for drug-protein binding studies [20,22–25]. ACE combines the use of a system for capillary electrophoresis (CE) with a biologically-related binding agent that is used to capture or separate analytes as they are resolved from other sample components based on their differences in electrophoretic mobility [26]. For instance, if the binding agent is present in the BGE and the charge-to-size ratio of the analyte changes when it interacts with this binding agent, there will be a shift in the observed mobility for the analyte as it goes from its free to bound forms [27,28]. This allows the binding agent to affect the separation of the analyte from other components in the sample but also provides a means for characterizing the analyte-binding agent interaction [28].

CE can be carried out by using a binding agent in either a soluble form or in an immobilized form, as shown in Figure 1 [29]. When the target binding agent is present in a soluble form, the analytes and binding agent interact within the BGE as they pass through the CE system [7,30]. In this method, the separation depends on the mobilities of the free analyte vs the analyte-binding agent complex [7,31]. Alternatively, the binding agent may be immobilized or adsorbed within the capillary, resulting in a separation technique based on electrochromatography [26,30,32,33].

ACE and related CE methods can provide various types of information on drug-protein interactions. Examples of this information are the binding constant between a drug and protein, the rate constants for this interaction, and the binding stoichiometry [34-36]. This information is crucial for the evaluation of drug affinity to a given target protein and in understanding the mechanism by which a drug associates with a protein [5,13]. In addition, CE can provide

information on the charge of the complex that is formed by this process [27,36]. CE-based techniques have many potential advantages over alternative methods for drug binding studies. For instance, these CE methods frequently require only a small amount of binding agent and have sample injection volumes in the pL-nL range [37–39]. CE is also noted for having short separation times and high separation efficiencies [37]. In addition, ACE and related techniques often do not require prior purification of the sample, which means that biological fluids can be injected directly into the capillary [38]. Affinity-based CE approaches can also be used in some cases to examine binding by multiple solutes to a given agent, as demonstrated in the use of such methods to screen a library of candidates for binding to the same target as part of combinatorial studies [34,40].

As is shown in Figure 2, over 2500 articles have appeared since 1990 on the use of ACE and related methods to study drug-protein binding or to use these interactions as part of a chemical separation. This review will examine the principles and applications of ACE and affinity-based CE, with an emphasis on systems that involving drug interactions with serum proteins or related binding agents and on recent developments that have appeared in this area. The analysis formats that have been used in this field will be discussed, along with their advantages or potential limitations. Applications of ACE and related techniques that will be described will include drug-protein interaction studies, chiral drug separations that employ serum proteins, and the use of CE in hybrid methods to characterize drug binding with serum agents.

2. Serum binding agents used in ACE and drug interaction studies

Several types of serum binding agents have been used in ACE or other forms of CE for separations and drug binding studies. Two examples are the major serum proteins human serum albumin (HSA) and bovine serum albumin (BSA) [14,41]. HSA is a single chain, non-glycosylated protein with a molar mass of 66.5 kDa. HSA contains 585 amino acids and has an isoelectric point of 4.7

[42]. BSA has a similar molar mass and composition to HSA but only contains 583 amino acids [43]. Both HSA and BSA have two major drug binding sites: Sudlow site I, which is located in subdomain IIA, and Sudlow site II, which is in subdomain IIIA (see Figure 3) [41]. Sudlow site I has a hydrophobic cavity with two clusters of ionic/polar residues. This site tends to bind to drugs that are bulky, heterocyclic compounds with negative or electronegative groups, such as warfarin, azapropazone, phenylbutazone, and salicylate [42,44]. Sudlow site II has a cluster of cationic residues near its hydrophobic pocket and tends to bind drugs through a combination of hydrophobic, hydrogen bonding and electrostatic interactions. Examples of compounds that bind to Sudlow site II are aliphatic and aromatic carboxylates such as benzodiazepines, ketoprofen, and L-tryptophan [42,44].

Another important plasma protein for the transport of drugs is α_1-acid glycoprotein (AGP) [42]. Human AGP is a heavily glycosylated protein that has a single chain of 183 amino acids and a typical molar mass between 41 and 43 kDa [42,45]. AGP has a low isoelectric point (2.8-3.8) due to the sialic acid groups in its carbohydrate chains [42]. A heterogenous mixture of bi-, tri-, and tetra-antennary glycans are attached to five N-linked glycosylation sites on AGP [42,45]. AGP tends to bind to neutral or cationic drugs and has a high affinity, low-capacity binding site with three lobes. This site includes a hydrophobic lobe that is surrounded by two negatively charged lobes, which can provide strong binding by drugs to this region [42,45].

An additional set of serum binding agents for drugs and small organic solutes are lipoproteins [46,47]. Lipoproteins consist of a hydrophobic core, containing materials such as cholesterol ester and triglycerides, that is surrounded by a shell of phospholipids and free cholesterol that also includes apolipoproteins [46,47]. Plasma lipoproteins are classified based on their densities, giving categories such as high-density lipoprotein (HDL) and low-density

lipoprotein (LDL) [46,48]. Plasma lipoproteins bind and transport cholesterol and triglycerides, as well as some hydrophobic and/or basic drugs, in the bloodstream [48].

3. General principles of ACE in the study of drug-protein interactions

A typical system that is used for ACE and other forms of CE is illustrated in Figure 4 [37,38]. This system requires a BGE that is placed in the capillary of the CE system and held in two reservoirs that are in contact with the electrodes (i.e., the anode and cathode). The electrodes are used to apply an electric field across the capillary [37,38]. Other components of this system include the separation cartridge (i.e., which houses the capillary), a power supply, an injection system, a computer for data acquisition and system control, and a detector [37,38]. The detector is placed at the opposite end of the capillary to the injection site. Modern CE systems can include online detectors based on UV-vis absorbance, laser-induced florescence, electrochemical detection, or mass spectrometry [37,38].

The capillary in CE has a typical inner diameter of 20-100 μm and a length of 20-100 cm. The use of a capillary with a small internal diameter provides a high surface-to-volume ratio and efficient dissipation of Joule heat, thus allowing use of a high applied electric potential (i.e., up to 25-30 kV) [38,49]. Both coated and uncoated capillaries are used in CE [23–25,50–55], with the uncoated capillaries typically being made from bare fused silica [23–25]. Polyimide is usually employed as a coating on the outside of silica capillaries to make them less fragile; however, part of this coating is removed near one end of the capillary to provide a window when using optical detection for analytes [27]. Capillaries with either permanent or dynamic coatings have also been used in CE and ACE [50–53]. A permanent coating consists of a chemical layer that is covalently bound to the interior of the capillary, while a dynamic coating makes use of non-covalent adsorption to reversibly place a coating material onto the inner wall of the capillary [50–52].

Injection of the analytes into a CE system is performed by temporarily replacing one of buffer reservoirs with a sample vial [38]. A defined amount of sample, often in the range of pL-nL, is introduced into the capillary by either using hydrodynamic flow or electromigration [38]. In the hydrodynamic mode, the sample is injected into the capillary by applying a pressure difference between the two ends of the capillary [56]. This injection mode is often chosen when low viscosity buffers are used for CE or ACE [56,57]. For the electromigration or electrokinetic mode of injection, sample is introduced into the capillary in the presence of an applied potential [56]. In this method, the amount of an injected substance will depend on the mobility of this compound and can vary between different analytes in the sample [56,58].

Electroosmotic flow (EOF) is an important phenomenon that must be considered in CE and ACE. If a silica capillary is used, EOF is produced when a negative charge is present on the interior wall of the capillary due to the acid dissociation of silanol groups [50,51]. This negative charge attracts cations from the BGE, creating a fixed Helmholtz plane and a mobile outer layer of charge [50,51]. In the presence of an applied potential, cations in the outer mobile layer are pulled towards the negative electrode (or cathode) [51]. This situation results in net movement of the BGE and its solutes towards the cathode, where a detector can be located to monitor analytes that are migrating with or against the EOF [51]. The size of the EOF will depend on such factors as temperature, the amount of charge on the interior surface of the capillary, and the pH of the BGE [59–61].

CE can be conducted in either a normal polarity mode or reversed polarity mode [37,38,49–51,62]. In the normal polarity mode, the sample is injected at the anode end of a silica capillary, with the sample components then migrating with different migration velocities and in the presence of the EOF to a detector located near the cathode end [37,38]. In the reversed polarity mode, the

CE separation is carried out in the presence of suppressed or absent EOF. Under these conditions, the sample is injected at the electrode with same relative charge as the analytes, with these analytes then traveling through electromigration to the electrode of the opposite charge [62]. For instance, in the reversed polarity mode a sample containing anions would be injected at the cathode end of the capillary and detected as they migrate to the anode end [62].

4. Principles of ACE for studying drug-protein interactions

The binding of many drugs with serum proteins and related agents can be described as a reversible and non-covalent process [39]. This process can be represented by the following reaction and equations, where a drug (D) binds to a serum protein (P) or similar agent to form a drug-protein/binding agent complex (C) [5].

$$\mathrm{D} + \mathrm{P} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{C} \tag{1}$$

$$K_a = \frac{k_{on}}{k_{off}} = \frac{[\mathrm{C}]}{[\mathrm{D}][\mathrm{P}]} \tag{2}$$

$$K_d = \frac{1}{K_a} = \frac{k_{off}}{k_{on}} = \frac{[\mathrm{D}][\mathrm{P}]}{[\mathrm{C}]} \tag{3}$$

In these relationships, [D] and [P] are the equilibrium molar concentrations of the non-bound forms of the drug and the protein or binding agent, respectively, and [C] is the equilibrium molar concentration of their complex. The terms k_{on} and k_{off} are the association and dissociation rate constants for this reaction, while K_a and K_d are the corresponding association and dissociation equilibrium constants for this process. This description is a simplification of the overall steps that are involved as a drug forms a reversible complex with a serum protein or related agent. However, eqs. (1-3) usually provide a good description of the overall extent and net reaction rate of such a process [5,39,63]. One possible exception occurs when allosteric interactions are present, for which more complex reaction models should instead be employed [5].

One method that can be used in ACE to study drug-protein interactions is a mobility shift assay (see Section 5.1.1). This method is used when a drug and protein/binding agent have fast association/dissociation kinetics during their reaction and there is a significant difference in mobilities for the non-bound form of the drug and drug-binding agent complex [5,39,63,64]. Under these conditions, a shift should be seen in the overall position of the drug's peak during CE as the concentration of the binding agent within the BGE and capillary is varied (see Figure 5A) [65]. The apparent mobility (μ_{app}) of the drug is calculated from the experimental data by using eq. (4) along with the known total length of the capillary (L_{tot}), the effective length of the capillary from the injection end to the location of the detection window (L_{eff}), the measured migration time for the drug (t), and the applied voltage (V) [5].

$$\mu_{app} = \frac{L_{eff} L_{tot}}{t V} \tag{4}$$

When ACE is used to estimate association or dissociation equilibrium constants, the mobility of the drug (μ) can be obtained from the drug's apparent electrophoretic mobility by making a separate measurement of the mobility due to EOF (μ_{EOF}) and using the relationship shown in eq. (5).

$$\mu = \mu_{app} - \mu_{EOF} \tag{5}$$

In the case where a drug's observed mobility changes as the binding agent concentration is varied, the binding constant for this system can be obtained by using the following equation [63].

$$\mu = \frac{\mu_f + \mu_C K_a[P]}{1 + K_a[P]} \tag{6}$$

In this relationship, μ_C and μ_f are the effective electrophoretic mobilities of the complex and the free, non-bound drug [5,39,63,64]. Factors such as temperature, ionic strength, change in

solution viscosity, and interactions of proteins with the capillary wall, all of which may affect the observed mobilities, should be taken in account when using such equations [66].

Non-linear least squares regression based on eq. (6) or related expressions is the current preferred approach for obtaining binding constants from ACE data [63,67,68]. However, eq. (6) can also be rearranged into a linear form by using one of the following relationships [5,69].

$$\frac{(\mu-\mu_f)}{[P]} = -K_a(\mu-\mu_f) + K_a(\mu_c-\mu_f) \quad (7)$$

$$\frac{[P]}{(\mu-\mu_f)} = \frac{1}{(\mu_C-\mu_f)}[P] + \frac{1}{(\mu_C-\mu_f)K_a} \quad (8)$$

$$\frac{1}{(\mu-\mu_f)} = \frac{1}{(\mu_C-\mu_f)K_a}\frac{1}{[P]} + \frac{1}{(\mu_C-\mu_f)} \quad (9)$$

When using eq. (7), a plot of $\frac{(\mu-\mu_f)}{[P]}$ vs $(\mu-\mu_f)$ should produce a best-fit line with a slope that gives the value of the association equilibrium constant (K_a) [5,69]. One advantage of this equation is it does not have any co-dependence in the left- and right-hand terms on the value of [P] [5]. Several reports have suggested the use of such data treatment to evaluate the binding constants of drug-protein interactions [30,36,40,64,70]. In addition, analysis based on eq. (7) can be valuable when work is being carried out with a large or a highly charged binding agent (e.g., protein) that does not exhibit a significant mobility shift upon binding to the drug of interest [5].

Reciprocal plots can also be prepared based on eqs. (8) and (9). For eq. (8), a plot of $\frac{[P]}{(\mu-\mu_f)}$ vs [P] can provide the value of K_a from the ratio of the slope to the intercept. For eq. (9), a plot of $\frac{1}{(\mu-\mu_f)}$ vs $\frac{1}{[P]}$ gives the value of K_a from the ratio of the intercept to the slope [5,69,70]. The same type of relationships, as given in eqs. (7-9), can be employed to estimate a binding constant by reversing the role of drug and protein. For instance, this can be done by using [D] in place of [P] in eqs. (7-9) [40]. This approach is useful in situations where only small amounts of a protein or peptide are available or in which a mixture of multiple proteins and peptides is present in the

sample [40,68,69]. A statistical advantage of using non-linear regression based on eq. (6) instead of linear regression with eqs. (7-9) is the former approach avoids a dependent variable appearing in both the *x*- and *y*-axis values that are used for data analysis [63,67,68,72].

If variations in the EOF are present during the study of drug-protein binding, a correction for these variations can be made through the use of mobility ratios [23–25,27,39,73–75]. For instance, a mobility ratio (M) can be calculated by using the following equation [73–75].

$$M = \frac{\mu+\mu_{EOF}}{\mu_{EOF}} \tag{10}$$

Based on the definition of μ that was given in eq. (4), factors such as L_{tot}, L_{eff}, and V that are the same for the analyte and EOF will appear in both the numerator and denominator in eq. (10) and be removed as sources of variation when using a mobility ratio. This is indicated more clearly by an equivalent expression for the mobility ratio that is provided in eq. (11) [73–75],

$$M = \frac{t_{EOF}}{t_P} + 1 \tag{11}$$

where t_{EOF} is the migration time of an EOF marker and t_P is the migration time of the analyte. The fact that mobility ratios are independent of capillary length, applied voltage, and variations in the EOF makes these values a more reproducible means of estimating binding constants by ACE than the direct use of absolute mobilities or migration times [73–75].

5. Approaches of ACE used in the study of drug-protein interactions

ACE can be performed in several modes to study drug-protein binding or other types of biomolecular interactions. The first mode is used when the kinetics of the interaction are relatively fast on the time scale of the CE separation, creating a dynamic equilibrium in the system [30,40]. The second mode is based on the use of pre-equilibration of these components [25,30,40]. The third mode is known as kinetic ACE and is used for systems with intermediate reaction rates that

are similar to the CE separation time [69]. Each of these modes is described in more detail in this section.

5.1. Dynamic equilibrium mode of ACE

There are several approaches that can be used when two interacting components have a relatively fast rate of binding and dissociation, thus allowing a dynamic equilibrium to be created as these components pass through a CE system. Techniques that can be employed in this situation include mobility shift ACE, ACE assays based on vacancy peaks, and ACE techniques that use the Hummel-Dreyer method [7,69,71,76–81].

5.1.1 Mobility shift ACE

Mobility shift ACE (ms ACE) has been used in many studies to examine drug-protein interactions [2,35,39,82–88]. In this method, several concentrations of one of the interacting agents (i.e., the binding target, T) are placed into the BGE of the CE system. A fixed concentration of the complementary agent (i.e., the drug or analyte, A) is dissolved in BGE, usually with an EOF marker, and injected as a sample. The apparent mobility of the injected agent and the mobility of the EOF marker are then determined from the corresponding electropherograms (see Figure 5A). The observed mobility shift of the injected agent is then related to the concentration of the complementary component in the BGE and used to find the binding constant for the system [89].

This type of analysis is typically carried out by using a concentration for the binding target in the BGE that is 10-100 times higher than the concentration of the injected analyte [90]. The temporal and spatial variations in the concentration of the binding target along the zone of the injected analyte are generally negligible under these conditions because the BGE contains a large excess of the target [2]. Careful analysis of peak shapes and widths along with the determination

of mobility and the elution profile can be used in ms ACE to provide information on both equilibrium and kinetic parameters for a biological interaction [91–98].

The simplicity of ms ACE and its ease of data evaluation make this method appealing for the study of drug-protein interactions [2,99]. However, ms ACE does have disadvantages. For instance, the use of a high concentration of the target can produce systematic errors (e.g., by changing the viscosity and ionic strength of the BGE) when estimating a binding constant [85,86]. Corrections for this must be made to minimize any associated errors in the analysis of binding [74,90,100]. In addition, factors such as temperature, buffer electrolysis, and the characteristics of the capillary surface (e.g., if one of the two binding partners can adsorb onto the capillary) must be considered as these can affect the reproducibility of the migration times [74]. The use of a relatively high concentration of the injected analyte in ms ACE may be needed to obtain a sufficient signal but can introduce deviations in the local concentration of the binding target in the BGE [91,92,101]. Use of an analyte concentration that is too high can also result in shifts in the observed mobility for this agent [102].

ms ACE has been used to examine many types of drug-protein interactions and related systems [6,61,75,103]. For example, ms ACE has been used to analyze the interactions of heparin with antithrombin variants [104]. Other reports have examined factors that can be varied to improve the precision of binding constants that are determined by ms ACE, as applied to research examining the interactions of tryptophan with HSA, warfarin with BSA, and quercetin with beta-lactoglobulin [6,103]. ms ACE has been used to investigate how the N- and S-homocysteinylation of HSA affects binding by this protein to several catechins [105]. Another study employed ms ACE to investigate the interactions of polysulfate sodium with HSA and BSA [106].

Pressure mediated ACE (PACE) is a sub-category of ms ACE that has been used to study weak non-covalent interactions, such as those between BSA and some drugs [107]. This method has also been employed to determine binding affinities for the enantiomers of amlodipine and verapamil with HSA, where accurate mobility estimates were obtained by using on a nonlinear mobility function [68,93].

5.1.2 Vacancy peak methods in ACE

In vacancy ACE (VACE), the BGE is filled with both the analyte and binding target. The concentration of either component may be fixed while the other is varied [79]. A small plug of pure BGE is then injected into the CE system. The injection of this blank sample results in two vacancy peaks that have a lower response at the detector than the BGE containing the added components (see Figure 5B). These two negative peaks appear due to depletion in the local free concentrations of the analyte (A) and free target (T) as the sample passes through the system. The binding of the analyte to the target can be studied by using the mobility shift of the negative analyte peak as the target concentration is varied [77–79]. This approach can be used with analytes that have low solubilities in water, as the binding target is also present in the BGE and can assist in dissolving such compounds [79]. VACE is also useful in examining the competitive interaction by several analytes to the same site on a binding target [77]. In addition, it is possible to determine the number of active binding sites by an analyte on a target by using VACE [78,86].

The vacancy peak method of ACE (VP ACE) uses a similar set up to VACE and again produces two negative, vacancy peaks that are created by a local depletion in the concentrations of the free binding target and free analyte. The concentration of free analyte can be found from the peak for A by comparing its area to those obtained for injected samples of the BGE plus known concentrations of the analyte [69,86]. It is important in this method to optimize the concentration

of the detected species in the BGE to obtain good sensitivity. For instance, when using absorbance detection too little background signal due to the components in the BGE can result in a poor dynamic range for this method, while a high background absorbance may produce a non-linear response and result in poor assay sensitivity [69].

5.1.3 ACE and the Hummel-Dreyer method

In the Hummel-Dreyer method of ACE (HD ACE), the capillary is filled up with BGE that contains the analyte, and the injected sample consists of the BGE that contains the binding target [69,80]. If the analyte and binding target have relatively fast interactions, this method will result in two observed peaks, a vacancy peak for the analyte and a positive peak for the injected target (see Figure 5C). The positive peak will correspond to the free target and the complex that is formed between the analyte and target (which, in this case, often possess similar mobilities). The vacancy peak is produced by local depletion of analyte in the BGE as some this analyte binds with the target [69]. The change in the area of the vacancy peak is then used to find the amount of analyte that was bound to the injected target [86].

HD ACE has been used to examine the binding of several drugs with serum proteins. For instance, this method has been employed to study the binding of BSA with salicylic acid [108]. This method has also been used to characterize the interactions between HSA and transferrin with the platinum-containing drugs cisplatin and oxaliplatin; however, the HD ACE technique used in this case had to be modified because the binding of cisplatin to HSA did not reach equilibrium within the time-frame of a typical CE analysis [109]. In a typical HD ACE experiment, BGE containing the binding target is used as the injected sample. The work with cisplatin and HSA instead used an injected sample that contained an excessive amount of the drug (i.e., up to 20-fold) vs binding agent and that was subjected to incubation before injection [109].

5.2 Pre-equilibrium mode of ACE

If two interacting components have an intermediate rate of binding and/or dissociation, there may not be sufficient time to establish a local equilibrium as these components pass through a CE system. In this situation, the pre-equilibrium mode of ACE can be used. In this mode, the analyte and binding target are premixed in the sample before they are injected for separation by CE [30,40,110].

Frontal analysis ACE (i.e., CE FA, also known as FA ACE or FACE) is a type of pre-equilibrium mode of ACE that has been used in several studies to study drug interactions with serum protein or other binding agents [89,111]. In this approach, a moderately large plug of a pre-equilibrated mixture containing known concentrations of the binding target and drug/analyte is injected into a CE system [12,17,70,82,83,86,108,111–120]. This experiment is done for several samples usually containing a fixed total concentration of the binding target and several total concentrations of the analyte. When a potential is applied, the components of the samples are partially separated based on their electrophoretic mobilities [70,82,86,120]. The sample bands that are produced will consist of a series of plateaus that correspond to the free binding target (often overlapped with the analyte-target complex) and the free analyte [90,102]. The free analyte concentration in the sample is then determined from its plateau in the observed sample band, which in turn makes it possible to determine the ratio of the bound analyte per target [17,70,76,120,121]. This ratio is measured for several sample mixtures of the binding agent and drug/analyte and plotted against the free analyte concentration to determine the binding constant for the target-analyte interaction [76].

An advantage of this approach is that a local equilibrium is maintained in the overlapping zones of the sample band, allowing interactions that have rapid association and dissociation

kinetics to be examined [70]. It is also possible to evaluate the stoichiometry for this interaction by carrying out experiments with samples that contain various concentrations of the analyte vs binding target [86]. However, CE FA does have some drawbacks. For example, this method requires a suitable difference in the mobilities of the bound vs unbound target and analyte to form observable and measurable plateaus for these chemical species [70,120]. This also means that relatively pure binding agents or analytes are required to avoid creating extra peaks or bands [70].

There are several reports in which CE FA has been used to characterize the binding of drugs with serum proteins [89,111,122]. For instance, this technique has been used to examine the interactions of AGP and BSA with alprenolol, oxprenolol, pindolol, propranolol, carbamazepine, diclofenac, salicylic acid, and warfarin [89]. CE FA has also been employed to study the binding of glycated HSA and unmodified HSA with acetohexamide, carbutamide, chlorpropamide, and tolbutamide [111]. Furthermore, CE FA has been used to analyze the interactions of loureirin B with HSA [122] and to investigate the binding by the nonsteroidal anti-inflammatory drugs flurbiprofen, ibuprofen, and naproxen with HSA and BSA [123]. The interaction of dexamethasone with BSA and HSA under simulated physiological conditions has also been studied by using CE FA [117].

A number of modified forms of CE FA have been reported for use in binding studies. For instance, the similar mobilities that are often present for a drug-protein complex and free protein in CE FA has been noted to limit the range of applications for this method [85,118]. To broaden the scope of applications, an electrophoretic mobility-based correction was utilized to examine the binding of ibuprofen with hydroxypropyl-β-cyclodextrin [90,118]. A CE FA method has also been developed by changing from offline mixing of sample components to a procedure that uses online

transverse diffusion of laminar flow profiles (TDLFP) for mixing; this method has been used to obtain the binding parameters for BSA with propranolol, lidocaine, and phenylbutazone [124].

5.3 Kinetic mode of ACE

The third general format of ACE is the kinetic mode. This format is used when intermediate reaction rates are present that produce a relaxation time that is similar to the CE separation time [69]. Approaches that can be used in this format include non-equilibrium ACE, equilibrium CE, partial-filling ACE, and multiple-step ligand injection ACE.

5.3.1 Non-equilibrium and equilibrium CE of equilibrium mixtures

In non-equilibrium CE of equilibrium mixtures (NECEEM), a short plug of a pre-equilibrated mixture is injected into a capillary that is filled with a BGE [31,125–130]. A separation is then carried out during which there is continuous dissociation of the injected complex when potential gradient is applied and with both the inlet and outlet reservoirs containing BGE [131]. In this approach, reassociation of the binding agent and analyte is assumed to be neglected. The elution profile that is obtained will contain a total of five regions: three peaks for the binding target, target-drug complex and drug, and two exponential smears produced by the binding agent drug after the dissociation of their complex [131]. In NECEEM, complex dissociation overpasses complex formation, creating conditions in which it is favorable to obtain the binding constant and dissociation rate constant for the system [31,131,132].

NCEEM has been used mostly to study protein-protein, protein-DNA interactions, and aptamer-protein interactions [31,127–131]. For the analysis of drug-protein binding, NECEEM has been used with mass spectrometry to develop label-free and solution-based methods to study the kinetics of protein–small molecule interactions [133,134]. However, this approach is limited

to systems where the analyte and binding target have similar electrophoretic mobility [133]. One example of where NECEEM has been employed is to examine the interactions of isoprenaline hydrochloride with BSA [88].

In equilibrium CE of equilibrated mixtures (ECEEM), a short plug of a mixture at equilibrium is injected into the inlet of a capillary filled with a solution of binding target that is at the same concentration as the target in the equilibrium mixture. A separation of the sample components is carried out by CE with both the inlet and outlet reservoirs containing the same solution of target [132]. This method has been shown to be useful to examine the kinetics of noncovalent interactions for systems with complicated stoichiometry, such as may occur during the binding of some proteins with peptide-based drugs [132].

5.3.2 Partial-filling ACE

Partial-filling ACE (PFACE) is also known as plug-plug kinetic CE (ppKCE). In this method, the analyte and binding target are injected as separate bands and then allowed to interact [131]. This is a variant of traditional ms ACE method where only part of the capillary is filled with the BGE containing the binding target and the rest of the capillary is filled with pure buffer solution [83,84,135–140]. This approach has been used in the separation of chiral substances and in the determination of equilibrium constants based on measurements of mobility [137,138,140–147]. An example in which PFACE has been used is to measure the binding of loureirin B with HSA [122]. Drug displacement studies involving both retinol and retinoic acid with HSA and BSA were conducted by this approach by employing ibuprofen as a site-selective probe and displacing agent [148].

A benefit of PFACE over traditional ms ACE is it consumes a smaller amount of a sample. In addition, detection of the analyte can be done in pure BGE with a low background signal, which

can result in an improved signal-to-noise ratio [84,137,138]. PFACE can also be coupled with a wide range of detectors, including MS [138]. However, PFACE can only be used when certain criteria are met for the mobilities of analyte and binding agent [84,139,141,147]. For instance, the binding target should ideally be uncharged and have no appreciable mobility [139] or should have a mobility that is in the opposite direction of travel from the analyte [141]. Finally, mobility of the analyte should be high enough to allow this agent to completely pass through the target plug before reaching the detector [139]. These conditions can be maintained by using an EOF marker in the BGE or by conducting the experiment under pressure [6,147]. For example, pressure mediated PFACE was used to investigate interactions between the human insulin hexamer and low mass compounds such as serotonin, dopamine, L-arginine, and phenol in aqueous alkaline media (see Figure 6) [143].

Another type of PFACE is flow through PFACE (FTPFACE). In this method, the capillary is filled with a plug of analyte, and a sample containing the target and non-interacting standards is then injected. When a potential is applied to the system, the sample and sections containing binding target and analyte will begin to overlap, allowing a local equilibrium to be established [131,149]. As the potential is applied for a longer duration, the target will flow through the region that contains the analyte plug [131,149]. The relative migration time ratio of the target vs the non-interacting standards is then determined and used with Scatchard analysis to obtain the binding constant for the analyte with the target [149]. This method may be employed in situations where the analyte and target plugs do not elute simultaneously at the point of detection. FTPFACE has been used with both charged and neutral agents, as demonstrated in binding studies for mesityl oxide, benzenesulfonamide, and *p*-toluenesulfonamide with carbonic anhydrase B [149].

5.3.3 Multiple-step ligand injection ACE

Multiple steps ligand injection ACE (MSLIACE) is another kinetic mode of ACE. This technique has been used to estimate binding constants for the interactions of drugs with a set of small peptides that possess similar masses and charges to each other [150,151]. In this method, separate plugs of peptides and inert standards are injected into a capillary which contains a known concentration of the drug in the BGE. The change in migration time of a peptide as it interacts with the drug is then determined and used with techniques such as Scatchard analysis to obtain the binding constant for this system [150,151]. This approach is faster than the conventional ACE techniques for determining binding constants of some drugs with peptides and uses less material [151].

6. Applications of ACE and affinity-based CE related to drug-protein binding

The previous sections have provided several examples of how ACE has been employed in studies of the interactions of drugs with serum proteins or related binding agents. A summary of such applications is also provided in Table 1. The previous examples that have been described in this review have involved the use of homogeneous methods where the interaction between the target binding agent and analyte occurs within a solution, such as within a sample or a running buffer [5,26,30,152,153]. However, as shown earlier in Figure 1, it is also possible in CE to use heterogeneous methods in which the binding agent is immobilized onto the inner surface of a capillary or onto a support that is placed into a CE system and used to capture the analyte [26,30,153,154]. This latter form of affinity-based CE can also be viewed as a type of electrochromatography [7,26]. In this section, both homogeneous and heterogenous applications of CE will be considered as related to the analysis or use of interactions between drugs and serum proteins or related binding agents. The use of CE-based immunoassays to examine drugs, chiral

separations in CE based on serum proteins, and hybrid CE methods to examine drugs or drug-protein interactions will be also considered.

6.1 Homogeneous methods used in CE for studying drug-protein interactions

As can be seen from the methods described in Section 5 and the examples provided in Table 1, the use of ACE in a zonal elution format is a common approach for the analysis of drug-protein interactions in the solution phase [5]. For instance, this combination has been used to investigate the binding of anionic carbohydrates and oligonucleotides to synthetic peptides derived from the heparin-binding region of human serum amyloid P component [155,156]. This format has also been used to characterize the binding of procainamide and its derivatives to hemoglobin and histone proteins [157]. The interactions between HSA and several fluoroquinolones were studied by ACE using zonal elution and a homogeneous method [158]. A method that used chemically modified forms of HSA as buffer additives in ACE was developed and used to screen the binding of flurbiprofen, ibuprofen, suprofen, and warfarin at Sudlow sites I and II of this protein [61,159].

Zonal elution and a homogeneous format have also been used in CE-based immunoassays to measure the free and bound fractions of drugs in samples [5,26,160,161]. For instance, this approach has been employed with CE in immunoassays for hormones, insulin, glucagon, cortisol, digitoxin, opiates, chloramphenicol as well as the tumor marker alpha-fetoprotein [131–142]. This form of immunoassay has also been used in CE with laser-induced fluorescence detection to estimate the binding constants of anti-insulin antibodies with insulin [174].

Drugs have been used in some studies as buffer additives in ACE to measure the equilibrium constants of drug-protein interactions [5]. Early work with this approach used it to simultaneously determine the binding of multiple enantiomeric peptides to vancomycin [71]. The binding interactions of 4-alkylbenzyne sulfonamides with carbonic anhydrase A and B have also

been investigated by employing this method [40]. Modified versions of this approach have been used to determine the peptides in a peptide library with the highest affinities to vancomycin and to estimate the maximum number of binding agents that can be simultaneously screened by this approach [175].

6.2 Chiral separations in CE using serum proteins

A number of studies have been conducted by CE in which enantioselective selectors have been used as buffer additives or immobilized binding agents to achieve separate chiral drugs [176]. This work has used buffer additives that have included proteins, polysaccharides, organic polymers, and chiral micelles, among others [176–178]. Chiral separations based on CE can provide short analysis times, high efficiency, and low consumption of analytes, reagents, and solvents compared to liquid chromatography [176,178].

The use of serum proteins as buffer additives for the separation of chiral drugs has been described in various studies [7,88,179]. For instance, HSA has been used as a buffer additive to separate enantiomers of verapamil and amlodipine [179]. BSA has been utilized as a buffer additive for the chiral separation of ephedrine enantiomers by CE [180]. ms ACE has been used with laser induced fluorescence detection and HSA as a chiral selector to both separate omeprazole enantiomers and to measure the binding constants of these enantiomers with HSA [181].

Several studies have reported the use of electrochromatography to study the binding of drug enantiomers with HSA [176,182–186]. For instance, a partial filling technique was used in this technique to investigate the binding of HSA with the enantiomers of brompheniramine, chlorpheniramine, hydroxyzine, and orphenadrine [182]. Moreover, a similar approach was employed for the enantiomeric separation of zopiclone using carboxymethylated-β-cyclodextrin as a chiral selector [183]. The method was used in the same study to evaluate the binding of the

zopiclone enantiomers to HSA [183]. Another study employed electrokinetic chromatography and a partial filling technique to study the binding of nomifensine enantiomers with HSA, using heptakis-2,3,6-tri-O-methyl-β-cyclodextrin as a chiral selector [184]. The chiral separation of nuarimol enantiomers by electrokinetic chromatography and a partial filling technique using HSA as chiral selector has also been reported [185].

Several additional reports have used ACE to examine the binding between serum agents and chiral drugs. For instance, ACE was used to estimate the conditional association constants between AGP and the enantiomers of disopyramide and remoxipride using a partial filling technique [137]. The conformational change of HSA upon binding to the basic drug mexiletine was examined by ACE, as well as the effects of pH, temperature, and other parameters on the chiral separation of this drug [187]. A related study developed a method for the enantiomeric separation of mexiletine, propranolol and chlorpheniramine by using HSA and porcine serum albumin as chiral selectors [188]. CE FA was used to study the stereoselective binding of amlodipine enantiomers with HSA under physiological conditions [189].

6.3 Heterogeneous CE methods based on drug interactions with serum proteins

Heterogeneous methods based on CE have also been used with serum proteins to study drug interactions or to carry out chiral separations. In these methods, the serum protein may be placed within a gel, coupled or coated onto a capillary wall, or coupled to a support that is packed within the capillary [103,190–196]. The chiral separation of D- and L-tryptophan by CE using BSA immobilized in a gel has been described [191]. BSA has been coated onto polystyrene nanoparticles and used in CE for the separation of D- and L-tryptophan [196]. Another study utilized CE and BSA that was immobilized onto silica monoliths for the separation of D- and L-tryptophan [197]. A dynamic coating of HSA onto a capillary has been used in CE to separate *R*-

and *S*-warfarin [103]. In addition, a mixture of cellulose and HSA immobilized in an organic monolith has been used in ACE to resolve various chiral analytes [195].

6.4 Hybrid methods using CE for studying drug-protein interactions

Recent studies have combined the use of CE with affinity-based liquid chromatography to characterize the binding by several drugs with serum proteins [198,199]. For instance, CE has been used with on-line immunoextraction and high-performance affinity chromatography to study the interactions of AGP with chlorpromazine, disopyramide, imipramine, propranolol, and warfarin [198]. CE was used in this work for the measurement and glycoform analysis of normal AGP and AGP that had been derived from serum from systemic lupus erythematosus (SLE) patients (see Figure 7). Affinity microcolumns containing immobilized polyclonal anti-AGP antibodies were then employed in the frontal analysis and zonal elution formats to investigate the binding of these drugs with the same samples of AGP [198]. A similar hybrid CE method was developed and used to characterize the binding of aprindine, disopyramide, imatinib, mepivacaine and propranolol with both AGP and HSA [199].

6.5 Use of CE to examine interactions by drugs with other binding agents

The use of ACE to study the interaction of drugs with other agents that can be found in blood or serum has also been reported [83,200,201]. For instance, the interaction of heparin with antithrombin III was studied by using ACE [200]. Another related study reported the use of ACE to study the binding of antithrombin III with low molecular weight heparins and fondaparinux [175]. Moreover, ACE has been used to study the interactions of heparin with intact low-density lipoprotein and peptide fragments from apolipoproteins [194]. CE FA was employed to gain insight on how the chemical properties of dipeptide β-naphthylamide derivatives and seven

cationic amino acids affect their binding with the glycosaminoglycan hyaluronic acid and HSA [115]. PFACE was used to study the binding of a chondroitin-6-sulfate with low-density lipoprotein and with a peptide fragment from apolipoprotein B-100 [145].

6.6 Application of computational simulations in ACE

Computer simulations have been used in several reports to help describe and predict the behavior of analytes in ACE [90,92,98,106,203,204]. For instance, the migration and band broadening of an analyte in ACE can be described through the use of appropriate differential equations [205]. However, solving these equations and placing them into forms that can be used experimentally to obtain parameters such as equilibrium constants and mobilities for analyte-binding agent complexes can be quite challenging [206]. Computer simulations can be used with these differential equations and finite difference schemes to describe the behavior of analytes in a capillary. Data processing and underlying calculation methods for this approach have been substantially improved for methods such as ms ACE, ppKCE, and CE FA [90,92,98,106,203,204], as described in recent reviews [90,207]. However, applications in which computer simulations have been used specifically to examine drug interactions with serum proteins or related materials in ACE are not yet common [98], making this a potential area of interest for future studies.

7. Concluding remarks

This review examined the use of ACE and related CE method to examine the interactions of drugs with serum binding agents. This review also described recent developments in this field. An emphasis was placed on the serum proteins such as HSA, BSA, and AGP, although other binding agents found in serum or blood were also considered, such as lipoproteins, antithrombin and antibodies. Various assay formats were described that can be used for systems with fast,

intermediate, or slow interaction kinetics. Examples of specific formats that were discussed in this review were ms ACE, vacancy-based ACE techniques, use of the Hummel-Dreyer method in ACE, and frontal analysis in ACE, along with method such as NECEEM, ECEEM, partial filling ACE, and multistep ligand injection ACE. Heterogeneous methods in CE that use immobilized binding targets for drug-protein interaction studies or drug analysis were also described. The mathematical approaches that are used to analyze data from such techniques were considered as well. Applications of ACE and related techniques that were discussed include the study of drug-protein interactions, chiral drug separations, and the use of CE in hybrid methods to characterize drug binding with serum proteins. Additional applications are expected in the future as research continues in the development of new tools for ACE and related methods as related to drug interactions with serum proteins and related binding targets.

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The authors have no conflicts of interest to declare for this work.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. General schemes for the study or use of drug-protein binding in CE by (A) ACE as a homogeneous format in which the drug (or analyte) and binding agent (e.g., a serum protein) are both present in solution or (B) the use of an immobilized binding agent within the CE capillary in a heterogenous format.

Figure 2. Number of publications that have appeared between 1990 and 2022 on the use of ACE and related affinity CE methods for the analysis of drug-protein interactions. This plot is based on a search that was conducted on SciFinder in May 2022.

Figure 3. Structure of human serum albumin (HSA), including the location of Sudlow sites I and II. Reproduced with permission from Ref. [41].

Figure 4. The general instrumentation for ACE and affinity-related CE methods, as shown for a system where a sample is injected or applied to a silica capillary at the end of the positive electrode (anode).

Figure 5. Approaches used based on ACE and a dynamic equilibrium mode: (A) mobility shift ACE, (B) vacancy ACE, and (C) use of the Hummel-Dreyer method with ACE. Terms:

A, analyte; T, binding target; EOF, electroosmotic flow. The solid and dashed lines show how the results of these methods change as experimental conditions are varied for use in binding studies. For instance, the dashed lines in (A-C) show how the peaks for the analyte change as the concentration of target is varied within the BGE and capillary. More details on these methods are provided in Section 5.

Figure 6. Use of partial-filling ACE to study the interactions of phenol with the human insulin (HI), using DMSO as a marker for EOF. The injections time for the target zone into the BGE are shown on the left. Reproduced with permission from Ref. [143].

Figure 7. (A) Electropherograms and (B) distribution of glycoform bands showing a shift in the profiles for normal AGP and AGP isolated from serum of patients with systemic lupus erythematosus (SLE). The horizontal and vertical error bars in (B) represent a range of ± 1 S.D. in the migration time or % total peak area values, respectively. The data was obtained for triplicate injections and are similar in size to the symbols used in this plot. Reproduced with permission from Ref. [198].

Figure 1.

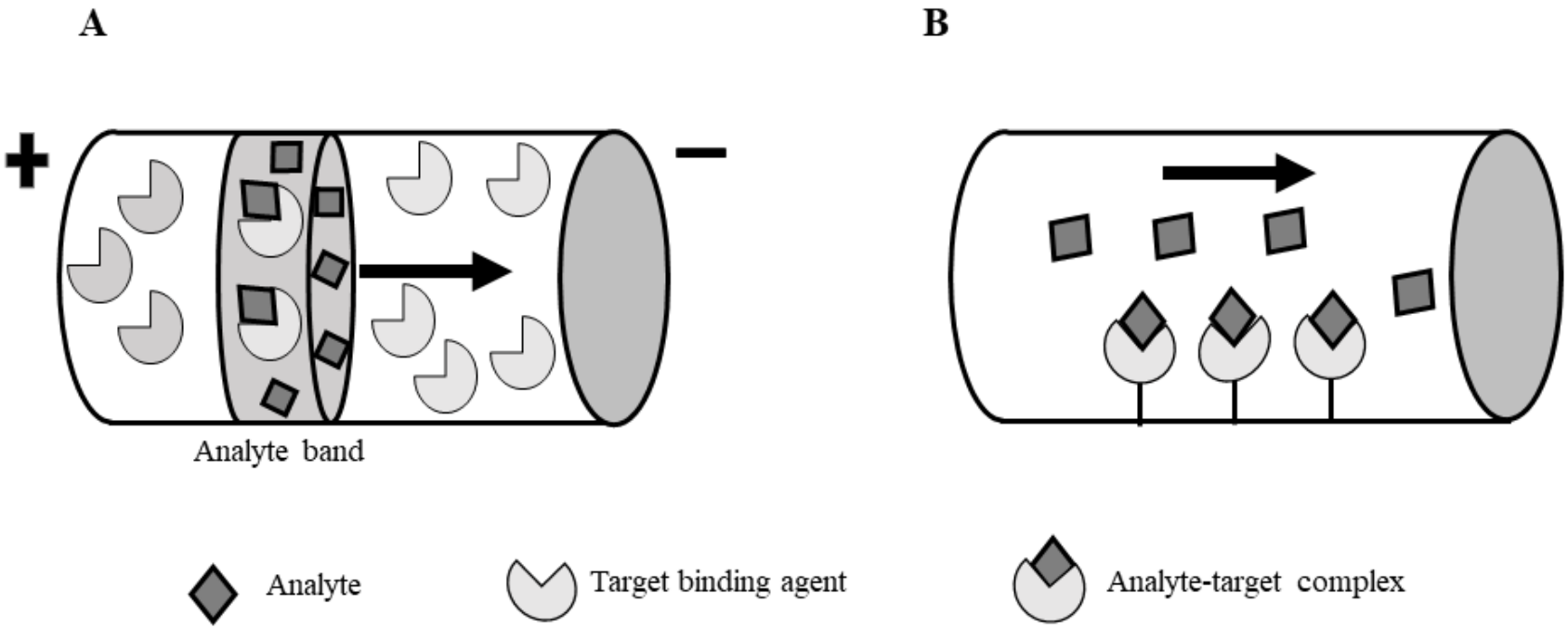
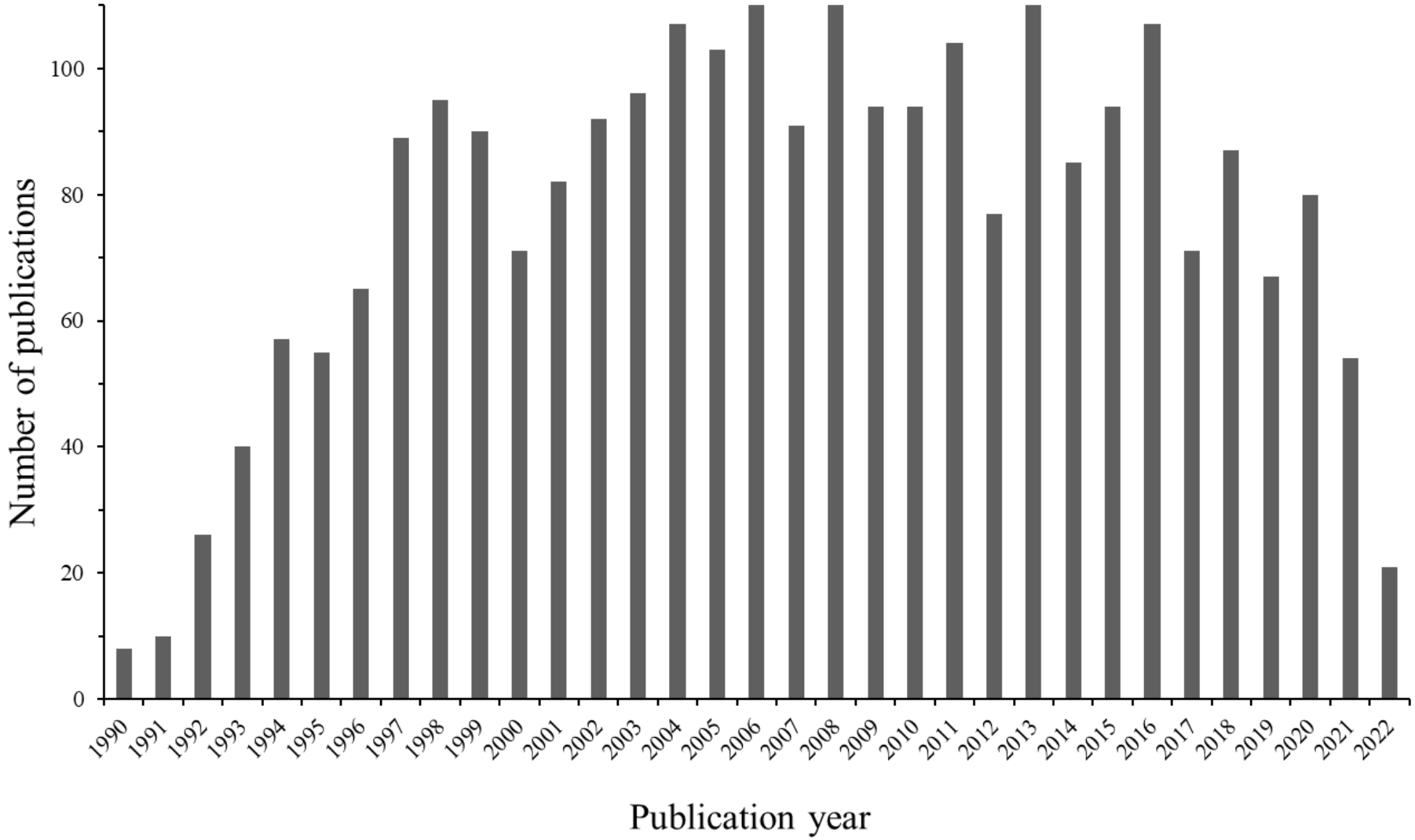

A
B
+
−
Analyte band
Analyte
Target binding agent
Analyte-target complex

Figure 2.


Number of publications
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20
40
60
80
100
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
Publication year

Figure 3.

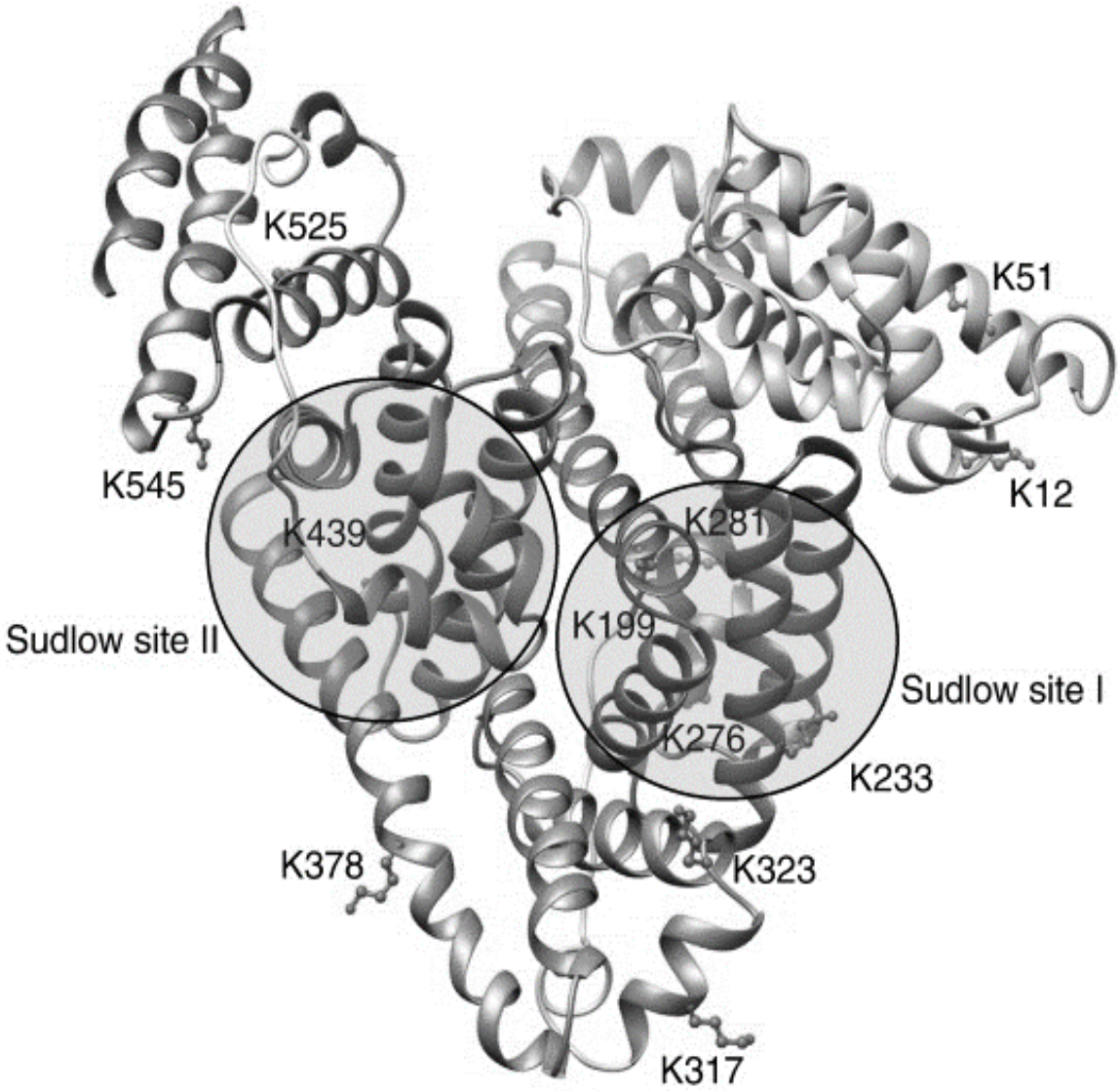
K525
K51
K545
K12
K439
K281
Sudlow site II
K199
Sudlow site I
K276
K233
K378
K323
K317

Figure 4.

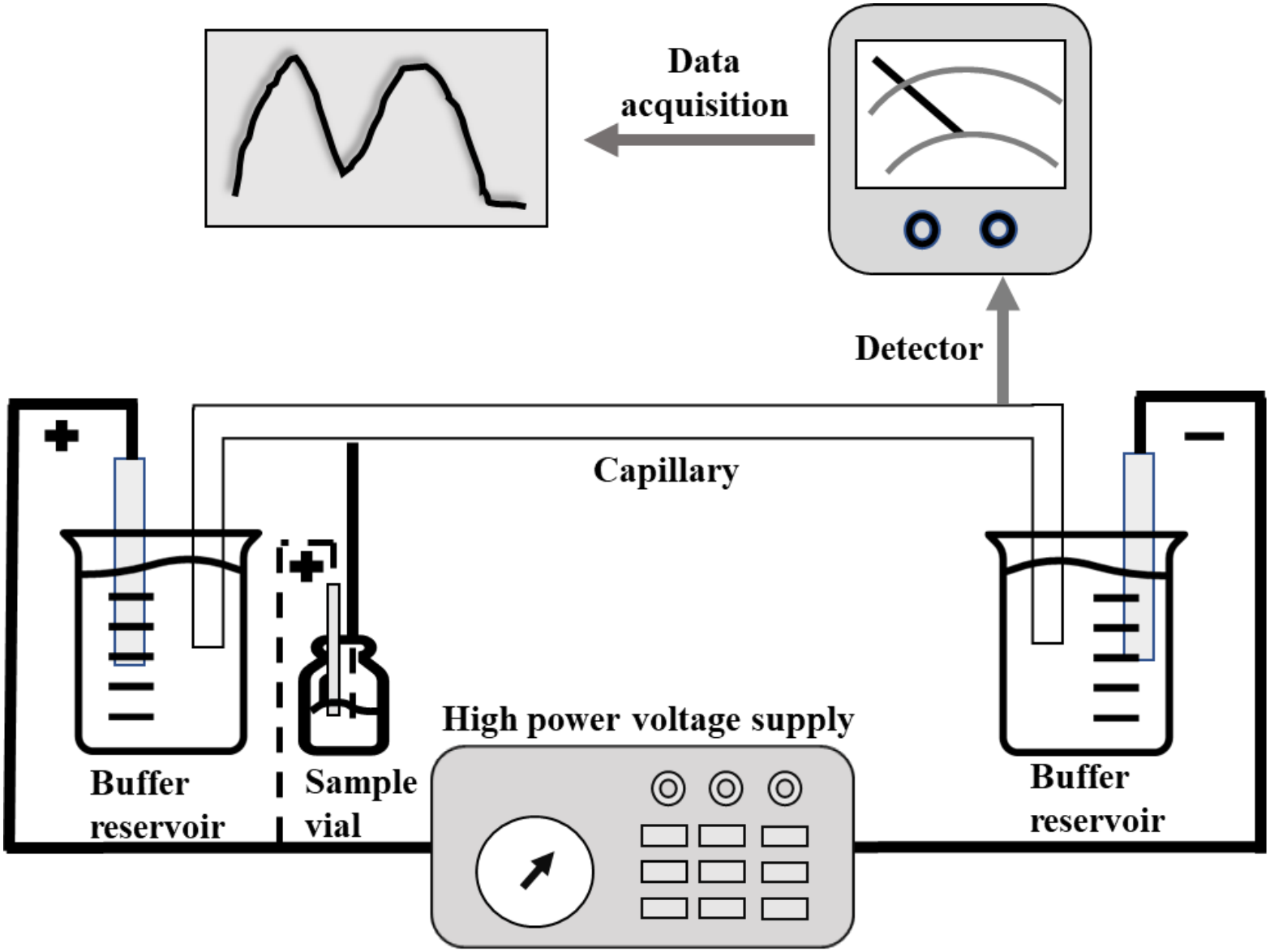
Data
acquisition
Detector
Capillary
High power voltage supply
Buffer
reservoir
Sample
vial
Buffer
reservoir

Figure 5.

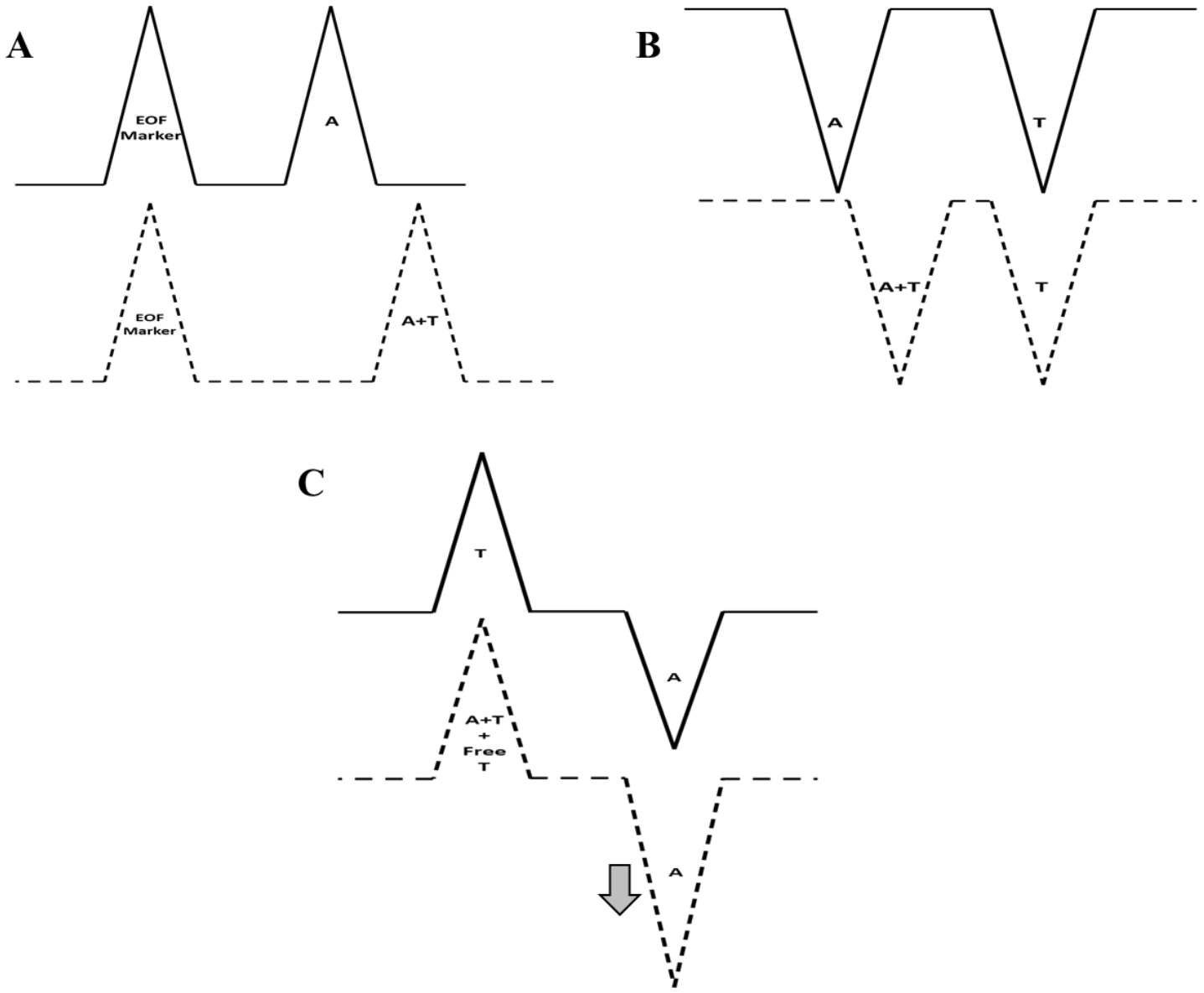

Figure 6.

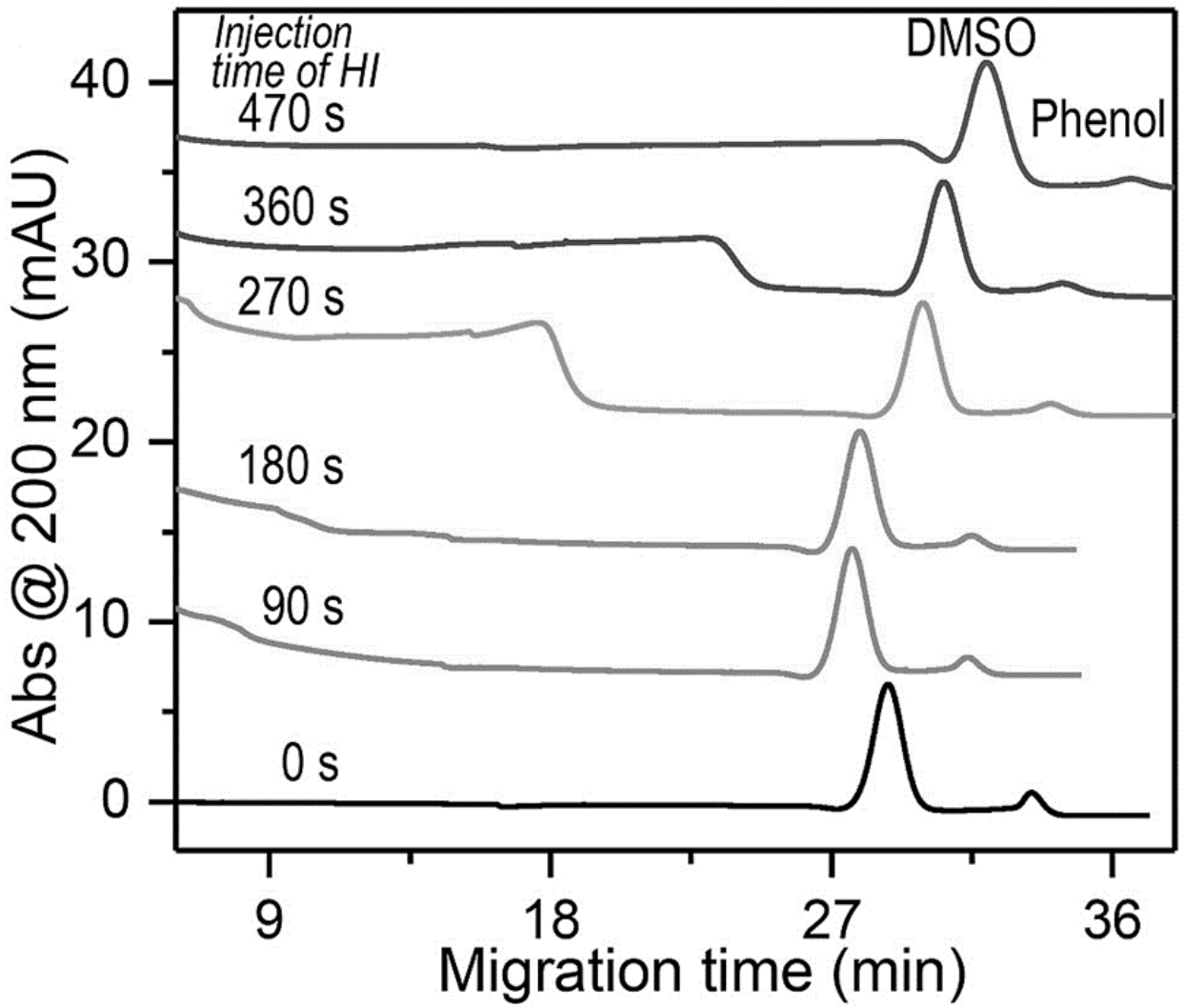
Injection
time of HI
470 s
360 s
270 s
180 s
90 s
0 s
DMSO
Phenol
Abs @ 200 nm (mAU)
0
10
20
30
40
9
18
27
36
Migration time (min)

Figure 7.

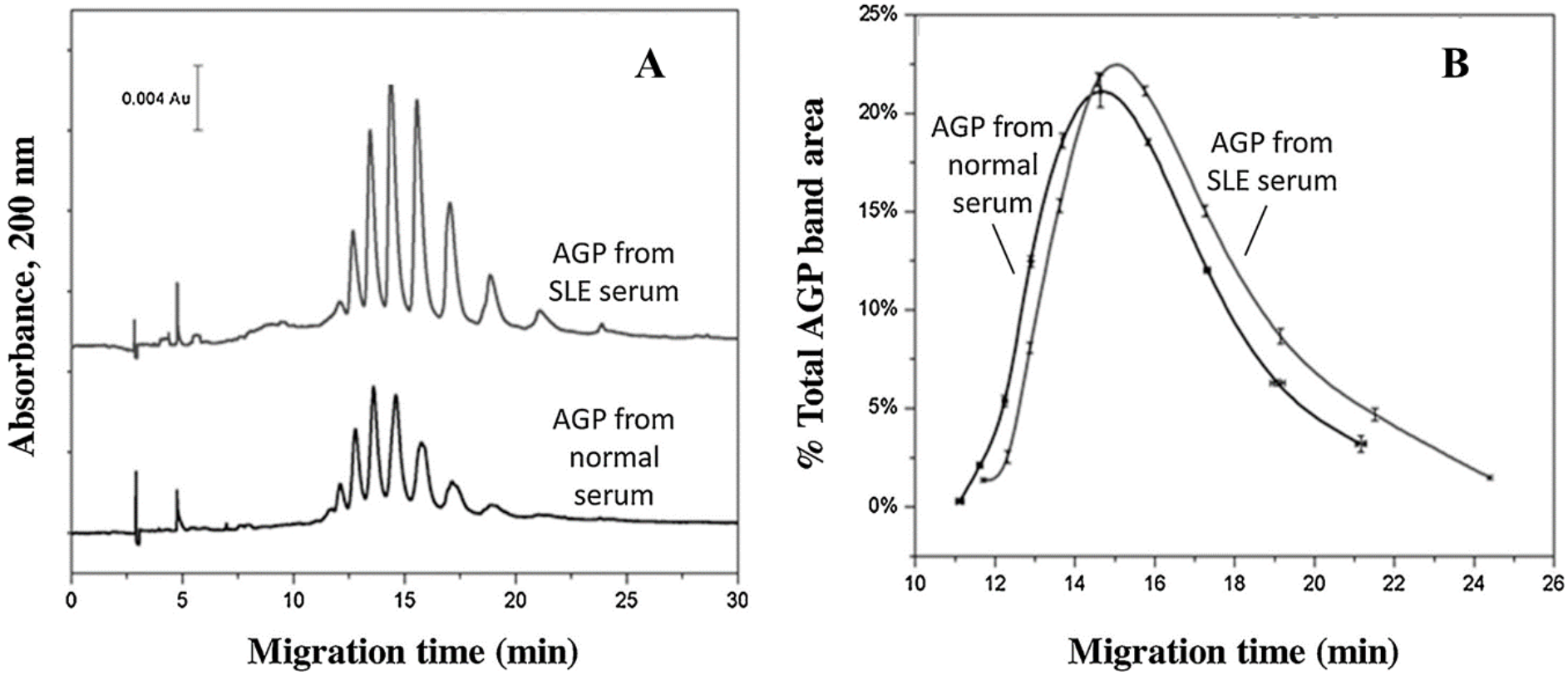
A
0.004 Au
AGP from SLE serum
AGP from normal serum
Absorbance, 200 nm
0
5
10
15
20
25
30
Migration time (min)
B
AGP from normal serum
AGP from SLE serum
% Total AGP band area
25%
20%
15%
10%
5%
0%
10
12
14
16
18
20
22
24
26
Migration time (min)

Table 1. Examples of applications for ACE and affinity-based CE methods to the study or use the interactions of drugs with serum proteins and related binding agents

	General format	Drug/solute	Protein/binding agent	Refs.
Homogenous formats	Direct separation	Anionic carbohydrates and oligonucleotides	Synthetic peptides from human serum amyloid P	[156]
		Procainamide and its derivatives	Hemoglobin and histone proteins	[157]
		Methionyl recombinant human growth hormone	Anti-human growth hormone	[162]
		Alpha-fetoprotein and tetraiodothyronine (T_4)	Anti-T_4 antibody	[163]
		Insulin and glucagon	Anti-insulin antibodies and anti-glucagon antibodies	[169]
		Fluoroquinolones	HSA	[158]
	Mobility shift assay	Tryptophan, warfarin, and quercetin	HSA, BSA, and β-lactoglobulin	[6]
		Catechins	HSA	[105]
		Heparinoids	HSA, BSA	[106]
	Enantioseparation and equilibrium	Ephedrine and its derivatives	BSA	[180]
		Isoprenaline hydrochloride	BSA	[88]

constant measurements using proteins as buffer additives	Verapamil and amlodipine enantiomers	HSA	[179]
	Omeprazole enantiomers	HSA	[181]
	Flurbiprofen, ibuprofen, suprofen, and warfarin	Chemically-modified HSA	[61,159]
	Enantiomers of brompheniramine, chlorpheniramine, hydroxyzine, and orphenadrine	HSA	[182]
	Enantiomers of zopiclone	Carboxymethylated-β-cyclodextrin as a chiral selector to separate zopiclone, HSA as a binding agent	[183]
	Nomifensine enantiomers	Heptakis-2,3,6-tri-O-methyl-β-cyclodextrin as a chiral selector to separate nomifensine enantiomers, HSA as a binding agent	[184]
	Nuarimol enantiomers	HSA as a chiral selector	[185]
	Imazalil enantiomers	Highly-sulfated β-cyclodextrin as a chiral selector, HSA as a binding agent	[186]
	Enantiomers of disopyramide and remoxipride	AGP as a chiral selector and binding agent	[137]

	Enantiomers of mexiletine, propranolol, and chlorpheniramine	HSA and PSA as chiral selectors	[188]
	Amlodipine enantiomers	Hydroxypropyl-β-CD as a chiral selector, HSA as a binding agent	[189]
Equilibrium constant measurements using drug as buffer additives	Vancomycin as buffer additive	Enantiomeric peptides	[71]
	4-Alkylbenzyne sulfonamides	Carbonic anhydrase A and B	[40]
	Vancomycin	Peptide library	[175]
	Alprenolol, oxprenolol, pindolol propranolol, carbamazepine, diclofenac, salicylic acid, and warfarin	AGP and BSA	[89]
	Loureirin B	HSA	[122]
	Acetohexamide, carbutamide, chlorpropamide, and tolbutamide	Normal and glycated HSA	[111]
	Flurbiprofen, ibuprofen, and naproxen	BSA and HSA	[123]
	Dexamethasone	HSA	[117]
	Ibuprofen	Hydroxypropyl-β-cyclodextrin	[118]
Equilibrium saturation method	Warfarin	BSA	[77,85]

Hummel-Dreyer method	Salicylic acid	BSA	[108]
	Cisplatin and oxaliplatin	HSA and transferrin	[109]
Heterogeneous formats	D/L-Tryptophan	BSA immobilized in a gel	[191]
	R/*S*-Warfarin	HSA adsorbed onto a capillary wall	[103]
	D/L-Tryptophan	BSA coated onto polystyrene nanoparticles	[196]
		BSA immobilized in silica monoliths	[197]
	Enantiomers of atenolol, azelastine, bisoprolol, citalopram esmolol, labetalol, metoprolol, terazosin, tryptophan, and warfarin	Cellulose and HSA immobilized in an organic monolith	[195]
Hybrid methods	Chlorpromazine, disopyramide, imipramine, propranolol, and warfarin	AGP	[198]
	Aprindine, disopyramide, imatinib, mepivacaine, and propranolol	AGP and HSA	[199]
Interactions of drugs with other biologically important molecules	Heparin	Antithrombin III	[200]
	Heparin	Low-density lipoprotein and peptide fragments of apolipoproteins	[202]

	Dipeptide β-naphthylamide derivatives and seven cationic amino acids	Hyaluronic acid and HSA	[115]
	Chondroitin-6-sulfate	Low-density lipoprotein and a peptide fragment of apoB-100	[145]