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Improvement of a capillary electrophoresis/frontal analysis (CE/FA) method for determining binding constants: Discussion on relevant parameters

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ABSTRACT

Drug–plasma protein interactions have a significant impact on both pharmacokinetics (i.e., absorption, distribution, metabolism, and excretion) and pharmacodynamics (pharmacological effects). Therefore, it is of high interest to evaluate this binding during the drug development process. Capillary electrophoresis (CE) is an interesting analytical tool for drug–protein binding characterization because it consumes a relatively low amount of reagents and enables assays that can be carried out under near-physiological conditions. The most interesting mode of CE for the study of biomolecular interactions is CE/frontal analysis (CE/FA). However, some confusion in how to conduct CE/FA experiments has emerged in the literature. The present study examines, using research into drug–albumin interactions as an example, the most important steps to take into consideration when building up new CE/FA binding assays. These include the following: choosing the buffer and applied voltage; evaluating protein adsorption onto the capillary wall; choosing the injection volume; choosing the drug and protein concentrations; and, finally, verifying the co-migration of the protein and drug–protein complex. The experimental part of the present report can serve as a checklist for developing the key parameters that need to be addressed for successful and reliable interaction studies. In a second time, short-end injection was used to enhance throughput. The strengths of the binding constants (K_a) for nine selected drugs (basic, neutral, and acidic substances) to albumin, which is the most important plasma protein, were from log K_a 2.9 to 5.4. These values were compared to those obtained with validated methods and good agreement was achieved.

1. Introduction

Reversible interactions play a key role in many biochemical and physiological processes. The assessment of these interactions in terms of affinity constants (K_a) and stoichiometry is an important part in describing and understanding such systems. In this context, drug–plasma protein binding is a critical feature because of its significant impact on both the pharmacokinetics and pharmacodynamics of drugs [1,2]. It is widely accepted that only the free drug fraction can cross membrane barriers and be distributed to tissues. Also, it is accepted that the effect of a drug (pharmacological or toxicological) is related to the exposure of a patient to the free drug in plasma rather than to the total drug concentration [3,4]. Data on plasma protein binding are extensively used as a parameter in pharmacokinetic modeling to predict absorption, distribution, metabolism, and excretion (ADME) of drugs in humans [5,6]. The binding of a drug to plasma proteins, by regulating the free drug fraction, is thus considered an important parameter to be determined in the research and development process.

To characterize these interactions, many methods have been proposed, as recently reviewed in Refs. [7,8]. Equilibrium dialysis (ED) is considered the reference method for this application, but it suffers from many drawbacks [9–14], such as low-throughput, protein and/or drug adsorption onto the dialysis membrane, volume changes in the cells, and the Donnan effect. Spectroscopic [12,15,16] and calorimetric techniques [17–20] are the preferred approaches to get a clear view of the binding mechanism. However, these methods have high sample consumption rates. Chromatographic [21,22] and biosensor-based assays [23–26] require the

Abbreviations: ACE, Affinity capillary electrophoresis (mobility shift assay); ADME, Absorption, distribution, metabolism, excretion; BGE, Background electrolyte; BSA, Bovine serum albumin; CE, Capillary electrophoresis; CE/FA, Capillary electrophoresis/frontal analysis; CZE, Capillary zone electrophoresis; ED, Equilibrium dialysis; EOF, Electroosmotic flow; FACCE, Frontal analysis continuous capillary electrophoresis; FS, Fused silica; FWHM, Full width at half maximum; H_f, Height of the free drug plateau; H_s, Height of the drug standard plateau; HSA, Human serum albumin; K_a, Thermodynamic affinity constant; K_d, Dissociation constant; K_{ss}, Steady state affinity constant; ℓ_{eff}, Effective capillary length; ℓ_{tot}, Total capillary length; M, Mobility ratio; m, Number of different classes of binding sites; n, Number of binding sites with the same affinity per protein molecules; r, Number of total drugs bound per protein; RSD, Relative standard deviation; t_m, Migration time; V, Voltage; μ, Electrophoretic mobility.

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immobilization of the protein on a support, which might alter the binding properties of the protein. In this context, capillary electrophoresis (CE) is proposed as a satisfactory alternative to ED, thanks to its numerous advantages. These include the following: (i) high efficiency and separation selectivity, (ii) low reagent and sample consumption, (iii) high speed of analysis, (iv) ease of automation, and (v) possibility to work under near-physiological conditions (in terms of buffer pH and ionic strength) [27–29]. Different CE setups have been reported, although affinity capillary electrophoresis (ACE) and capillary electrophoresis/frontal analysis (CE/FA) are the most widely used methods [30,31]. In ACE, systems with $K_a > 10^5\ M^{-1}$ (~95% of binding to albumin) are difficult to characterize due to the large protein/drug ratio required, leading to sensitivity issues [29]. Moreover, ACE cannot yield the necessary reaction stoichiometry and has difficulty dealing with multiple equilibria. On the contrary, CE/FA can handle higher affinity systems and multiple equilibria and can assess the stoichiometry of the studied system. Because of these advantages, CE/FA is an interesting tool for drug–plasma protein binding characterization.

Many research papers have been published that demonstrate the use of CE/FA to study drug–protein interactions [30,32–43]. However, no complete method developments or analyses of the important parameters to take into consideration have been presented. Papers dealing with CE/FA have mainly considered the application of this method toward different drugs or proteins (e.g., plasma proteins [36,44] or antibodies [45]), the development of new equations to analyze CE/FA data [32], methods for achieving better plateau separation [35,46], or sensitivity enhancement [47,48]. The method development, however, comes down to the injection of a sufficiently large volume that gives plateaus instead of peaks. Nevertheless, no mention has been given in the literature regarding the choice of the amount of sample to inject. In fact, when describing the principle of the CE/FA method, most often only vague terms are used, such as: “a large sample plug is injected into the capillary”; “it is recommended that a large injection up to 5% of the total capillary volume should be injected”; or “large injection, i.e., 5–20% of the total capillary volume” [30,41,49–51]. Concerning the interaction of drugs with human serum albumin (HSA), volumes as low as 5% of the effective capillary volume have been reported [33,42]; others have used 5–10% [34,35,41] or up to 20% [30,36–38,40]. Moreover, other parameters have not been evaluated, such as the choice in drug and protein concentrations, or the effect of having different drug–protein complex and protein mobilities. The present paper thus proposes to settle these issues by describing the important steps that should be taken into consideration when conducting CE/FA binding assays. The throughput of the CE/FA method was also enhanced by using short-end injection. Binding association constants and stoichiometries obtained by CE/FA for nine drugs with albumin were also compared to ED results to confirm the reliability of this methodology.

2. Materials and methods

2.1. Chemicals

All investigated drugs were obtained from Sigma–Aldrich (Steinheim, Germany), except for phenobarbital, which was obtained from Lipomed (Arlesheim, Switzerland). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (Steinheim, Germany). Methanol and acetonitrile were purchased from Panreac (Castellar del Vallès, Barcelona, Spain). Isopropanol and sodium hydroxide were purchased from Acros Organics (Geel, Belgium). DMSO and hydrochloric acid were obtained from Riedel-de Haën (Seelze, Germany). Sodium dihydrogen phosphate dihydrate, potassium dihydrogen phosphate, and disodium phosphate were obtained from Sigma–Aldrich (Steinheim, Germany). Ultrapure water was supplied by a Milli-Q gradient A10 purification unit from Millipore (Bedford, MA, USA).

2.2. Solution and sample preparation

The buffer (BGE) was a 67 mM sodium phosphate buffer at pH 7.4. For CE experiments, the buffer was prepared from sodium dihydrogen phosphate dihydrate and the pH was adjusted via the addition of NaOH. For ED experiments, the buffer was prepared from potassium dihydrogen phosphate and disodium phosphate. The pH values were measured with a Mettler-Toledo SevenMulti pH meter (Schwerzenbach, Switzerland). Stock solutions of albumin and the investigated drugs were prepared daily in phosphate buffer, except for the low-soluble drugs, in which acetonitrile or DMSO was used. Samples were prepared so that the organic solvent content was <2% (v/v). For CE assays, drug (up to 800 μM) and protein (40–440 μM) samples at the desired concentrations were mixed and then directly injected into the capillary. For ED experiments, protein and drug samples were prepared separately. Albumin concentration was 300 μM and drug concentrations ranged up to 1000 μM.

2.3. Instrumentation

2.3.1. Capillary electrophoresis (CE)

CE experiments were performed with an HP ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a power supply able to deliver up to 30 kV. CE ChemStation software (Agilent, Waldbronn, Germany) was used for instrument control, data acquisition, and data handling. The separations were performed in uncoated fused silica (FS) capillaries (G1600-60232, BGB Analytik AG, Böckten, Switzerland) with a 50 μm I.D., an extended light path and a total length of 48 cm. New FS capillaries were rinsed with MeOH, 1 M HCl, water, 0.1 M NaOH, water, and BGE at 1 bar for 5 min each. At the beginning of each day, the capillary was flushed for 5 min each with MeOH, water, and BGE. Then the separation voltage was applied for 5 min. When not in use, the capillary was rinsed with water and stored dry. After each sample injection, the capillary was rinsed at 1 bar for 2 min each with 0.1 M NaOH and fresh BGE. The capillary was thermostated at 25 °C by a high velocity air stream. UV/vis detection was performed at the wavelength that gave the best sensitivity and specificity for each compound (200 nm for lidocaine, bupivacaine, salicylic acid, and diclofenac; 215 nm for propranolol; 240 nm for phenobarbital; 236 nm for carbamazepine; 220 nm for L-tryptophan; 310 nm for warfarin).

2.3.1.1. Long-end injection. In long-end injection, the capillary effective length was 40 cm. Samples were injected in normal polarity. In other words, the anode was situated at the inlet and the cathode was positioned at the outlet (detector side). Hydrodynamic injection was performed at the inlet end at 50 mbar × 80 s (equivalent to 18.3% of the capillary effective length). During the analyses, a voltage of +12 kV was applied.

2.3.1.2. Short-end injection in reversed polarity. In short-end injection, the capillary effective length was 8 cm. Samples were injected in reversed polarity. In this case, the cathode was situated at the inlet and the anode was positioned at the outlet (detector side). Hydrodynamic injection was performed at the outlet end at 50 mbar × 20 s (equivalent to 22.8% of the capillary effective length). During the analyses, a voltage of −12 kV was applied.

2.3.1.3. Short-end injection in normal polarity. In this case, the capillary effective length was 8 cm. Samples were injected in normal polarity, where the anode was situated at the inlet and the cathode

at the outlet (detector side). Hydrodynamic injection was performed at the outlet end at 50 mbar × 20 s (equivalent to 22.8% of the capillary effective length). During the analyses, a voltage of +12 kV and an external pressure of −50 mbar were applied.

2.3.2. Equilibrium dialysis (ED)

ED experiments were performed with an equilibrium dialyzer (Dianorm; ScienceTec, Les Ulis, France) equipped with 20 Teflon cells, as described elsewhere [52]. Each cell was divided into two 1 mL compartments. The cuttoff-mass of the dialysis membranes (Diachema dialysis membranes; Dianorm GmbH, Munich, Germany) was 5 kDa. First, the membranes were soaked in buffer for 30 min. One compartment was then filled with the albumin sample, while the drug sample was introduced into the other compartment. An initial set of studies was performed for each compound to determine the time necessary for the system to reach equilibrium. Afterwards, drugs at different concentrations (0–1000 μM) were dialyzed at room temperature against the protein sample until equilibrium was reached. At the end of dialysis, the free drug fraction was quantified by UV/vis using a Lambda 35 UV/Vis spectrometer from PerkinElmer (Waltham, MA, USA).

2.4. Data processing

2.4.1. Procedure for adsorption determination

Irreversible protein adsorption onto the capillary surface was evaluated by monitoring the stability of the electroosmotic flow (EOF) during a series of ten BSA-acetone injections (5 μM and 1%, v/v, respectively) [53]. Because acetone is a neutral compound, this molecule was used as an EOF marker. Another series of ten acetone injections (1%, v/v) was performed before and after the BSA-acetone sample series was injected. Another indicator of irreversible adsorption is a change in the protein baseline.

Reversible adsorption was evaluated by monitoring the full width at half maximum (FWHM) of the BSA peak in a series of ten injections. Relative standard deviations (RSD, $N = 10$) of protein migration times for ten successive injections were also used to evaluate reversible adsorption.

2.4.2. Procedure for CE/FA experiments

The 1:1 binding of a drug to a protein to form a complex can be described by the following equation, where [D], [P], and [DP] are the free drug, free protein, and drug–protein complex concentrations, respectively [54]:

$$[D] + [P] \rightleftarrows [DP] \tag{1}$$

The equilibrium association constant, K_a, is used to characterize this reaction, which is governed by the law of mass action:

$$K_a = \frac{[DP]}{[D][P]} \tag{2}$$

The number of total drugs bound per protein, r, is expressed as shown in Eq. (3), where n, [B] and [P_{tot}] are the maximum number of binding sites on the protein, the concentration of bound drug and the total concentration of protein, respectively [14]:

$$r = \frac{[DP]}{[P] + [DP]} = \frac{[B]}{[P_{tot}]} = \frac{n \cdot K_a \cdot [D]}{1 + K_a \cdot [D]} \tag{3}$$

The 1:1 binding is often an oversimplification of the reality. Therefore, a more complex model, including multiple interaction types and binding sites, is frequently required. This case is described in Eq. (4), where m is the total number of different classes of binding sites and n_i is the number of binding sites possessing the same affinity constant for a drug [55]:

$$r = \sum_{i=1}^{m} \frac{n_i \cdot K_{ai} \cdot [D]}{1 + K_{ai} \cdot [D]} \tag{4}$$

Binding constants and stoichiometries were estimated by nonlinear least-square fitting of the experimental data to the binding isotherm (Eq. (4)) using GraphPad Prism 5 software (La Jolla, CA, USA). The free drug concentration was calculated based on the external drug standard in the absence of protein (Eq. (5)), where H_f is the height of the free drug plateau in the presence of protein, and H_s and [D_s] are the height and concentration of a pure drug standard, respectively:

$$[D] = \frac{[D_s]}{H_s} H_f \tag{5}$$

As the total protein concentration, [P_{tot}], is known and the concentration of bound drug, [B], corresponds to the subtraction of the total drug concentration and the free drug concentration, the parameter r could be calculated ($r = [B]/[P_{tot}]$). This parameter was then plotted against [D] to get the binding isotherm.

All experiments were performed in duplicate.

2.4.3. Procedure for determination of complex mobility

The electrophoretic mobility of BSA was first determined by injecting (50 mbar × 5 s, short-end injection) a sample containing 5 μM BSA and 1% (v/v) acetone (EOF marker) into the capillary filled with neat buffer. The apparent mobility, μ_{app}, was calculated via Eq. (6) where ℓ_{tot}, ℓ_{eff} are the total and effective capillary length, V is the applied voltage, and t_m is the compound migration time:

$$\mu_{app} = \frac{\ell_{tot} \cdot \ell_{eff}}{V \cdot t_m} \tag{6}$$

To calculate the apparent mobility of BSA, BSA migration time (t_m) was used.

In the same manner, the mobility of the EOF, μ_{EOF}, was calculated according to Eq. (7), where t_{EOF} is the EOF marker migration time:

$$\mu_{EOF} = \frac{\ell_{tot} \cdot \ell_{EOF}}{V \cdot t_{EOF}} \tag{7}$$

The electrophoretic mobility of drug–BSA complexes was determined by ACE [40]. Samples containing 5 μM BSA and 1% (v/v) acetone (EOF marker) were injected into the capillary (50 mbar × 5 s) with the short-end injection procedure. The capillary was filled with the drug added at high concentration (500 μM) to the electrophoretic buffer. As BSA entered the capillary, it encountered the drug dissolved in the running buffer, and could interact with it (complex formation). The apparent mobility of the complex was thus calculated via Eq. (6), where t_m stands here for the complex migration time.

In order to get rid of any fluctuations of the EOF, mobility ratios (M), instead of apparent mobilities μ_{app}, were used to compare the difference in mobility between the BSA and drug–BSA complex (Eq. (8)):

$$M = \frac{\mu_{app}}{\mu_{EOF}} \tag{8}$$

The mobility ratio of BSA was then compared to the mobility ratios of the different complexes.

If the mobility of the complex, μ_{DP}, differs from the mobility of the protein, μ_P, then the association constant obtained is a steady state constant, K_{ss}, as opposed to the thermodynamic binding constant of the equilibrium, K_a. In this case, Eq. (9) should be applied to calculate the thermodynamic binding constant [56]:

$$K_a = K_{ss} \frac{2(\mu_D - \mu_P) - (n-1)(\mu_{DP} - \mu_P)}{2(\mu_D - \mu_P) - (n+1)(\mu_{DP} - \mu_P)} \tag{9}$$

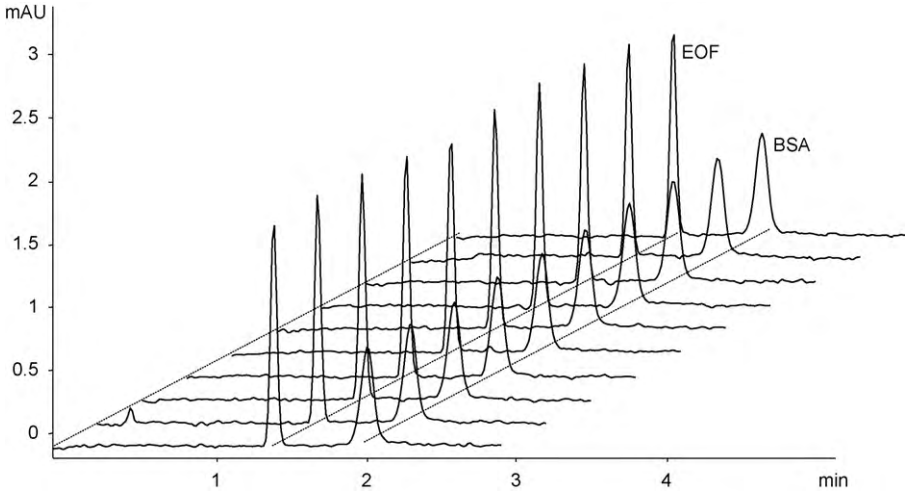

Fig. 1. Electropherograms of 10 consecutive injections of 5 μM BSA and acetone (1%, v/v) (injection 50 mbar × 5 s) in NaH_2PO_4 buffer, pH 7.4. FS capillary, total capillary length 48 cm, capillary effective length 8 cm, 50 μm ID with an extended light path, detection wavelength 280 nm.

3. Results and discussion

3.1. Key parameters in CE/FA experiments

3.1.1. General considerations

To consider the different parameters important in CE/FA, a model system was chosen that consisted of bovine serum albumin (BSA) and warfarin, an acidic compound that interacts strongly with albumin. Because warfarin can be detected at a specific wavelength (310 nm), this compound allows easy visualization of the free and bound drug, as well as free protein. The different parameters that must be taken into consideration when conducting CE/FA assays are described below. These include: the choice of the buffer (composition and pH), the evaluation of the time required

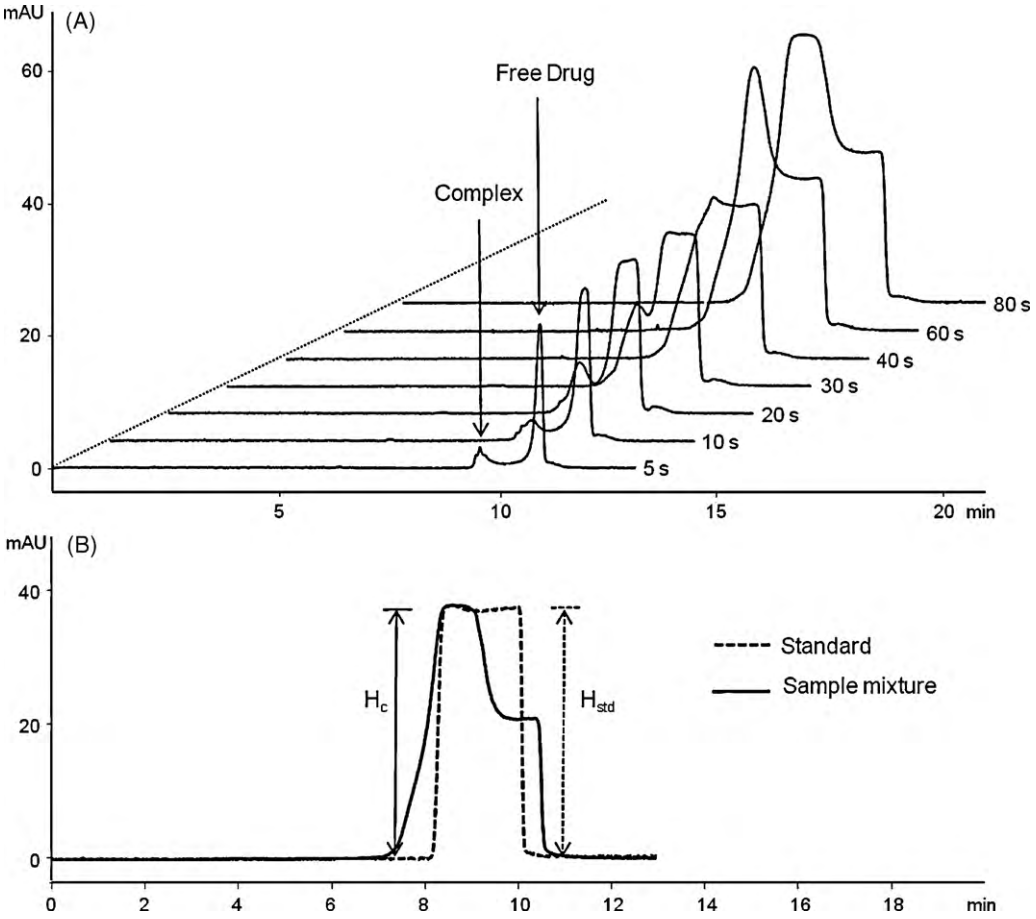

Fig. 2. (A) Electropherograms of 200 μM warfarin – 40 μM BSA samples in 67 mM phosphate buffer (pH 7.4) showing the effect of the injection volume. Hydrodynamical injections: 50 mbar × 5–80 s (1.1–18.3% of the capillary effective length, i.e., 9–143 nL). (B) Superposition of the warfarin standard (200 μM) (dashed line) and warfarin-BSA (200–40 μM) (continuous line) electropherograms obtained for an injection of 50 mbar × 80 s. H_c and H_{std} stand for the height of the complex region plateau and the height of the standard plateau, respectively. Detection wavelength 310 nm, capillary total length 48 cm, capillary effective length 40 cm.

Table 1
Binding percentage of drugs (100 μM) to BSA measured at different BSA concentrations by CE/FA.

[BSA] (μM)	1	2	3	4	5	6	7
40	3	3	4	54	10	26	10
50	5	3	6	62	–	–	–
60	7	4	6	73	–	43	14
75	8	4	11	80	–	48	17
100	9	6	12	88	20	61	19
150	14	11	17	96	31	71	27
225	21	–	25	98	41	84	39
300	28	17	30	–	46	89	48
440	–	27	–	–	–	–	–

1, propranolol; 2, lidocaine; 3, bupivacaine; 4, warfarin; 5, phenobarbital; 6, salicylic acid; 7, carbamazepine.

Table 2
Electrophoretic mobility ($\mu \pm$ SD) and mobility ratio ($M \pm$ SD) of BSA and drug–BSA complexes determined by ACE[a].

Compound	μ[b] ($cm^2/min\,V$)	M[b]
BSA	$1.58\ (\pm0.01) \times 10^{-2}$	$7.04\ (\pm0.03) \times 10^{-1}$
Propranolol	$1.64\ (\pm0.01) \times 10^{-2}$	$6.88\ (\pm0.10) \times 10^{-1}$
Propranolol[c]	$1.66\ (\pm0.11) \times 10^{-2}$	$6.95\ (\pm0.20) \times 10^{-1}$
Warfarin	$1.62\ (\pm0.10) \times 10^{-2}$	$6.95\ (\pm0.15) \times 10^{-1}$
Lidocaine	$1.71\ (\pm0.14) \times 10^{-2}$	$7.03\ (\pm0.20) \times 10^{-1}$
Diclofenac	$1.54\ (\pm0.12) \times 10^{-2}$	$6.48\ (\pm0.14) \times 10^{-1}$
L-tryptophan	$1.52\ (\pm0.02) \times 10^{-2}$	$7.08\ (\pm0.06) \times 10^{-1}$
Bupivacaine	$1.46\ (\pm0.02) \times 10^{-2}$	$6.88\ (\pm0.05) \times 10^{-1}$
Salicylic acid	$1.59\ (\pm0.01) \times 10^{-2}$	$6.88\ (\pm0.06) \times 10^{-1}$
Phenobarbital	$1.61\ (\pm0.01) \times 10^{-2}$	$6.93\ (\pm0.03) \times 10^{-1}$
Carbamazepine	$1.50\ (\pm0.02) \times 10^{-2}$	$6.87\ (\pm0.03) \times 10^{-1}$

[a] Injection of 5 μM BSA + acetone 1% (v/v) (50 mbar × 5 s) in the capillary filled with the drug sample (500 μM).
[b] $n = 5$.
[c] 800 μM.

to reach the binding equilibrium, the choice of the applied voltage, the evaluation of protein adsorption onto the capillary wall, the maintenance of the binding equilibrium throughout the whole run, the choice of the drug and protein concentrations, and finally the verification of protein and drug–protein complex co-migration.

3.1.2. Buffer composition and pH

The pH of the buffer can affect drug–protein interactions by changing the conformation of the protein or the net charge of either the drug or the protein, which may alter coulombic interactions. The ionic strength of the buffer can also have an effect on the interaction of the drug with the protein. For example, an increase in ionic strength tends to decrease coulombic interactions through a shielding effect [55,57]. Experiments were therefore conducted at near-physiological conditions (i.e., with a phosphate buffer at pH 7.4, 67 mM). Sodium dihydrogen phosphate (NaH_2PO_4) buffer was chosen instead of potassium dihydrogen phosphate (KH_2PO_4) buffer because the electrophoretic current generated with Na^+-ions was lower than that with K^+-ions for a given voltage.

3.1.3. Time required to reach the binding equilibrium

Another parameter is the determination of the time required to reach the binding equilibrium. For this purpose, the free calculated concentration of a series of consecutive injections of a sample, incubated for different periods of time, should remain constant. For drug–plasma protein binding, equilibrium is reached almost instantaneously because the association and dissociation rates of the drug–protein complex are very high. Their half-times are in the range of 0.1 s and 0.1–0.001 s, respectively [14]. Thus, no specific incubation time was necessary for the present study.

3.1.4. Voltage and electric field

According to Ohm's law, the maximum voltage that could be applied without creating any Joule effect was 12 kV. Another point of concern arose regarding the potential influence of the high electric field on the studied molecular interaction. A calculation made by Shimura and Kasai [58] has considered the effect of an electric field ($500\ V\,cm^{-1}$) on the interaction between a singly charged

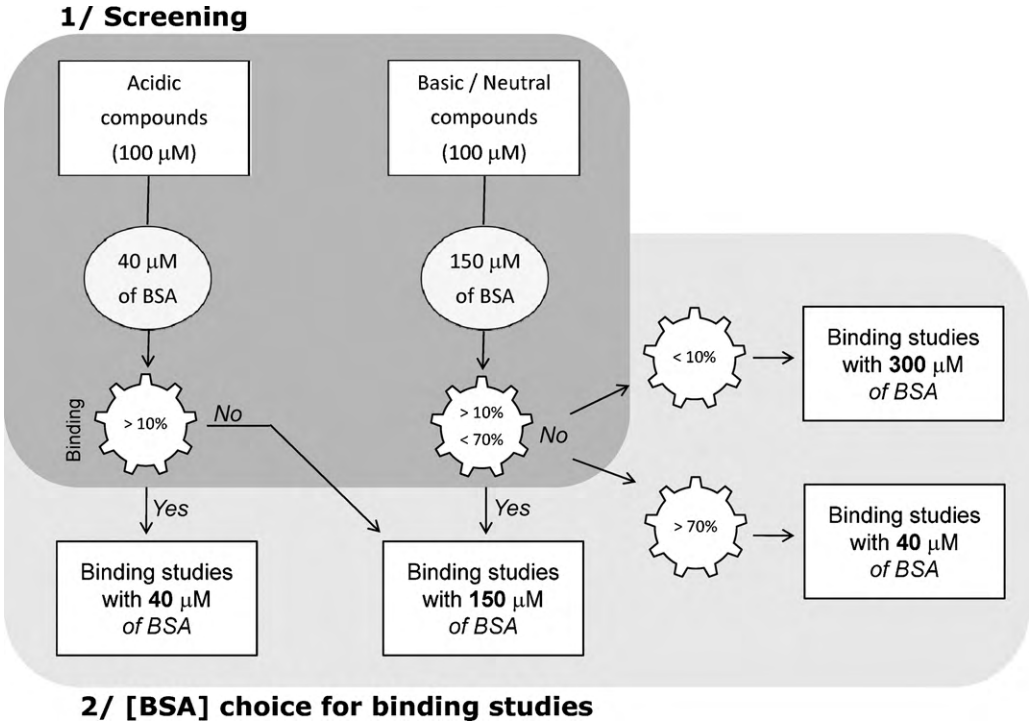

Fig. 3. Flowchart for determining the BSA concentration to use when performing drug–BSA interaction studies by CE/FA.

Table 3
Comparison of the binding parameters ($\log K_a$ and n, given with their 95% confidence interval) of drug–BSA interactions obtained by CE/FA and literature or in-house ED values.

Compound	CE/FA results		Comparison values	
	$\log K_{a1}$ $\log K_{a2}$	n_1 n_2	$\log K_{a1}$ $\log K_{a2}$	n_1 n_2
Warfarin[a]	5.39 ± 0.07	1.09 ± 0.40	5.29 ± 0.08[b,c]	1.32 ± 0.51[b,c]
	3.61 ± 0.18	1.92 ± 0.30	3.77 ± 0.29[b,c]	1.33 ± 0.35[b,c]
Salicylic acid	4.36 ± 0.09	1.61 ± 0.05	4.33 ± 0.12[d] [60]	2.2 ± 0.10[d] [60]
Carbamazepine[e]	3.51 ± 0.01		3.48 ± 0.03[b,c]	
Propranolol[e]	3.10 ± 0.01		3.13 ± 0.03[b,c]	
Lidocaine[e,f]	2.92 ± 0.02		2.78[b] [61]	
Bupivacaine[e]	3.19 ± 0.01		3.50[g] [62]; 3.76[g] [62]	
L-tryptophan	4.30 ± 0.02	1.00 ± 0.05	4.30[b]	0.94[b]
Diclofenac	4.94 ± 0.01	4.05 ± 0.10	5.04[b] [63]; 4.21[b] [64]	3.20[b] [63]; 4.21[b] [64]
Phenobarbital[e]	3.46 ± 0.08			
(long-end injection)		3.40[b] [65]		

[a] Warfarin possesses two classes of binding sites with different affinity towards BSA ($\log K_{a1}$ and $\log K_{a2}$).
[b] ED.
[c] In-house values.
[d] ITC.
[e] Insaturable behavior, so that the result obtain is the total affinity: $\log(n \cdot K_a)$.
[f] References values obtained for lidocaine correspond to the binding to HSA because no values was found in the literature for BSA.
[g] UF.

ligand and a binding protein. This study has highlighted that the impact of the electric field on the equilibrium constant can be as small as 0.04%. Even for an interaction involving multiply charged molecules, the effect of the electric field on the interaction seemed negligible. This issue was experimentally evaluated by varying the applied voltage and comparing the binding percentage obtained. With 4 kV, the binding percentage between warfarin (100 μM) and BSA (40 μM) was 55 ± 1% while it was 54 ± 1% (N = 4) with 12 kV. The effect of the electric field can thus be considered as insignificant.

3.1.5. Protein adsorption onto the capillary wall

Another issue was the putative protein adsorption onto the capillary wall. Lucy et al. [53] have reviewed the parameters used to monitor protein adsorption onto the capillary surface. First, the stability of the EOF during a series of protein injections could serve as a simple and effective means of monitoring irreversible protein adsorption. This is because the adsorption of proteins onto the capillary wall alters the EOF velocity by altering the zeta potential at the capillary inlet. Ten consecutive injections of a BSA–EOF marker sample (5 μM BSA and acetone 1%, v/v) yielded a variation of EOF mobility <1% (Fig. 1). The EOF mobility RSD (N = 10) obtained before, during, and after this series of BSA-acetone sample injections was <3%, suggesting that protein adsorption, if it occurred, was negligible. A second indicator of irreversible adsorption is a change in baseline after the migration time of the protein. This is not what happened for BSA in the conditions presented here (Fig. 1). The reversible adsorption of protein onto the capillary surface was evaluated by monitoring the FWHM value of the protein peak and the protein migration time for ten consecutive injections. The variation of the FWHM of BSA was <4% (N = 10) and the RSD for the protein migration time was <1%. According to all of these results, BSA adsorption onto the capillary surface can be considered negligible in our conditions.

3.1.6. Maintenance of the binding equilibrium

One of the most important issues to face when using CE/FA (i.e., a mixture of drug and protein samples followed by its injection into a capillary containing only neat buffer) concerns the maintenance of the binding equilibrium during electrophoresis. For systems with a $K_a < 10^7\ M^{-1}$ (the case of most drug–plasma protein interactions), if plugs that are too small are injected into the capillary, the equilibrium is not sustained during the separation step (i.e., the complex dissociates). This is because the electrophoresis buffer does not contain the interacting species. Therefore, to maintain the equilibrium during the separation process, one of the interacting species could be added directly to the electrophoresis buffer, as in ACE. Alternatively, the introduction of a sample volume that is sufficiently large to maintain the equilibrium during the whole process could be carried out. This technique is what gives specificity to the CE/FA method.

The effect of varying the volume introduced into the capillary is depicted in Fig. 2A. The injection time at 50 mbar was varied between 5 and 80 s (1.1–18.3% of the capillary effective length, i.e., 9–143 nL). When small plugs were injected (injection at 50 mbar for 5 s), the electropherograms consisted of thin peaks. On the contrary, the larger the injection volume, the larger the peaks became, and for injections greater than 20 s, plateaus instead of peaks were obtained for the free drug. The first (9.5 min) and second (11 min) peaks represented the characteristic zone of the complex and free warfarin, respectively. The height of the peak or plateau representing the complex zone increased with the injected volume, with a maximum height reached for a 60 s injection time. The equilibrium was thus sustained only with an injection volume greater than 50 mbar × 60 s (14% of the capillary effective length). For the following experiments, an injection time of 80 s was chosen to prevent injection variability and keep the measured plateau height constant. Another important aspect that has been pointed out by Winzor [56], and is based on the theory originally developed for moving boundary electrophoresis, is the necessity of generating an electrophoretic profile that contains a plateau of original composition, as illustrated in Fig. 2B. This means the height of the complex region must be similar to the height of the standard sample (containing the drug without any protein). This ensures that the equilibrium was maintained during the electrophoretic run. To validate that enough sample was introduced into the capillary for any drug–BSA system, another compound was chosen. This compound, lidocaine (basic compound with very low affinity towards BSA), differs from warfarin in terms of binding properties and electrophoretic behavior. As lidocaine does not possess any specific wavelength detection characteristics, frontal analysis continuous capillary electrophoresis (FACCE) was used to compare results obtained with the setup developed for warfarin (injection at 50 mbar for 80 s). In FACCE, the capillary is filled and equilibrated with buffer. Then, the inlet end of the capillary is immersed in the sample vial and migration is carried out by applying the voltage across the capillary. Equilibrium is ensured because of the contin-

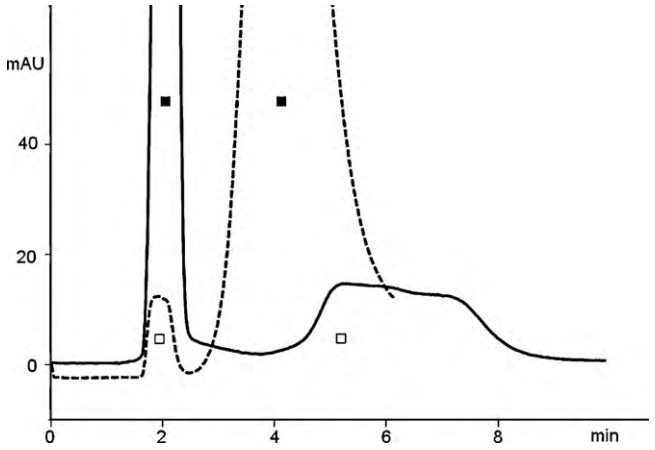

Fig. 4. CE/FA electropherograms obtained in short-end reversed polarity mode (injection 50 mbar × 20 s, voltage − 12 kV) (continuous line) and normal polarity coupled to external pressure (−50 mbar) (injection 50 mbar × 20 s, voltage + 12 kV) (dashed line). Sample: benzoic acid (□, 200 μM)–BSA (■, 40 μM).

uous injection of the sample. From a practical viewpoint, this setup is nevertheless less practical than CE/FA and was therefore used only to validate results obtained for CE/FA. The binding percentage obtained at high (500 μM) and low (50 μM) lidocaine concentrations was identical for both methods (16.1 ± 0.9% vs. 16.3 ± 1.0% and 16.5 ± 0.6% vs. 16.2 ± 1.8% for CE/FA and FACCE at high and low concentrations, respectively). Therefore, the quantity of sample introduced in CE/FA (50 mbar × 80 s) was validated as being sufficient.

3.1.7. Drug and protein concentrations

The reciprocal of the association constant (K_a), i.e., the dissociation constant (K_d), is the drug concentration that occupies half of the maximum number of binding sites on the protein. This value allows to evaluate a range of drug concentrations that should be used to build the desired part of the binding curve. At a concentration of 10 K_d, the drug occupies about 91% of the binding sites, and at 100 K_d this value goes up to 99% [14]. In practice, a range of total drug concentrations extending from 0.1 to 10 K_d seems to be a reasonable drug range concentration for practical use. According to the experience of our laboratory in working with various drugs, for compounds with unknown binding properties, drug concentrations range up to 800 μM should be used.

To choose an adequate protein concentration, numerous pharmaceutical compounds (Table 1) with different affinities towards BSA were analyzed. For each compound, the binding percentage (bound drug/bound + free drug) obtained for a low drug concentration in the presence of different concentrations of BSA (40–440 μM) was measured (Table 1). To obtain the most accurate data, a protein concentration where the binding percentage is at least 10% is recommended. The protein concentration must be sufficiently high to obtain a high binding percentage. On the other hand, if the protein concentration is too high, carry-over issues can arise. According to the results obtained, 40 μM of BSA produced enough binding for acidic compounds, which are mainly strongly bound to BSA. However, 150 μM was needed for most basic and neutral drugs, which are bound to a lesser extent to BSA. For further experiments [i.e., complete binding study to assess K_a (see Section 3.2)], BSA concentrations of 40 and 150 μM for acidic and basic/neutral compounds, respectively, should be used as a first attempt (only a standard and sample solution injection is needed to get a binding percentage). If the measured binding percentage is too low (<10%), an upper BSA concentration should be used (150 or 300 μM, according to the acid/base properties of the compound). On the contrary, if this parameter is too high, a lower protein concentration should be used. A flowchart of the recommended BSA concentrations to use is given in Fig. 3.

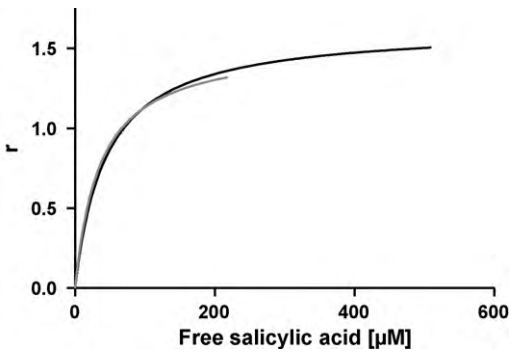

Fig. 5. Comparison of binding isotherms obtained for salicylic acid–BSA interaction with short-end reversed polarity (black line) and normal polarity mode (grey line).

3.1.8. Protein and drug–protein complex co-migration

Another important requirement of CE/FA is the co-migration of the protein and the drug–protein complex. In fact, based on the mass conservation law [56,59], unless the mobility of the protein and the drug–protein complex are equal, the free drug plateau does not reflect the true free drug equilibrium concentration. Rather, it reflects a steady state instead of a binding equilibrium in the thermodynamic sense. The mobility ratios (M) (Eq. (8)) of BSA and the warfarin–BSA complex were 7.04 (± 0.03) × 10^{-1} and 6.95 (± 0.15) × 10^{-1}, respectively. Thus, co-migration of the protein and the drug–protein complex was valid for the warfarin-BSA system. This parameter must, nevertheless, be evaluated for each drug–BSA system, because different migration times are not improbable.

3.1.9. Conclusion concerning the important parameters in CE/FA

After selection of the buffer according to the interacting system studied and the analytical method used, the maximum voltage that can applied must be adapted to remain in the linearity domain of Ohm's law. Then, protein adsorption onto the capillary wall should be evaluated. If adsorption is an issue, coated capillary can be used, such as polyacrylamide- or polyvinylalcohol-coated capillaries. For rapid equilibrating systems, such as drug–plasma proteins interactions, the equilibrium is reached almost instantaneously so that no specific incubation time is necessary. For unknown systems, the drug–protein mixture should be incubated for at least one hour in a first attempt to be sure the equilibrium is reached. Once the injection volume has been decided, the incubation time can be reduced if the free drug concentration calculated does not vary between the two measures. In the next step the injection volume should be varied in order to choose a volume large enough to ensure the equilibrium is kept during the whole electrophoretic run. The protein concentration must be selected to produce enough binding. However if the protein concentration is too high, carry-over issues can arise. Finally, in order to calculate reliable K_a, the mobility of the protein and drug–protein complex should be equal. If this is not the case, Eq. (9) must be applied.

3.2. Method improvement using short-end injection

The developed CE/FA method was then modified to improve the throughput by using a short-end injection. In this setup, the polarity was reversed and samples were injected at the detector side. The injected volume was adapted to the shorter capillary effective volume in the same manner as presented for long-end injection (i.e., by varying the injected volume between 1 and 20 s at 50 mbar).

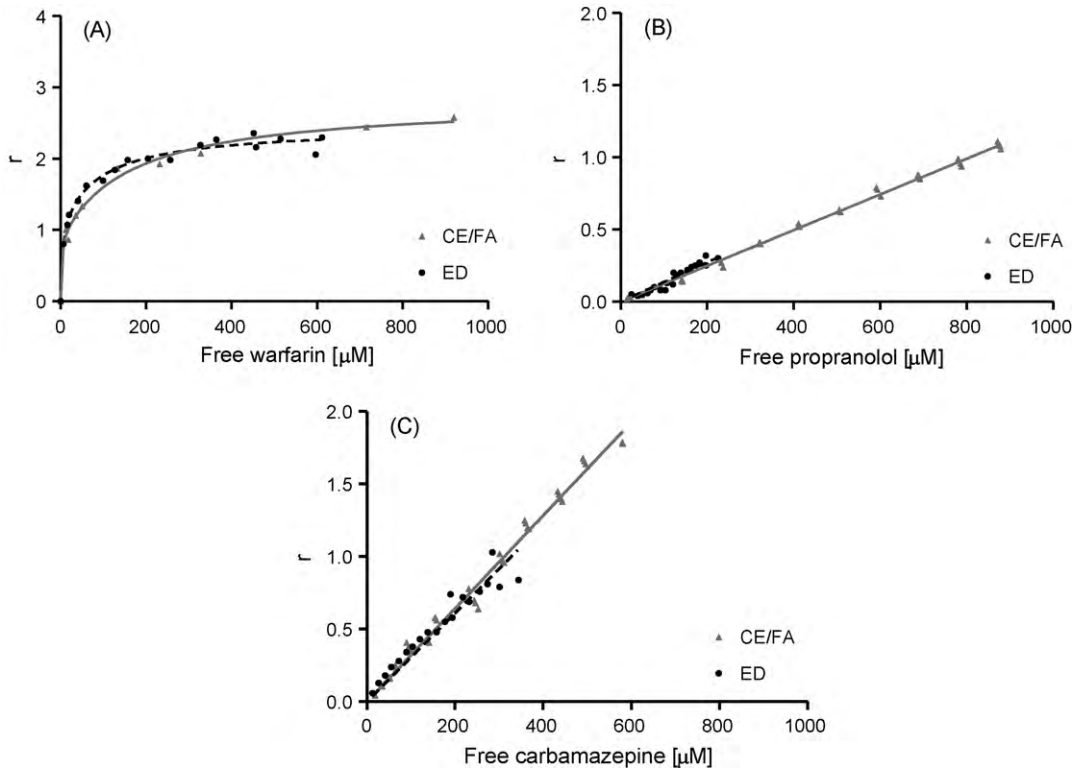

Fig. 6. Comparison of binding isotherms obtained for warfarin-BSA (A), propranolol-BSA (B), and carbamazepine-BSA (C) interactions by ED and CE/FA.

The optimal injection volume was 50 mbar for 20 s (i.e., 22.8% of the effective capillary length).

Nine drugs (acidic, neutral, and basic compounds) (Tables 2 and 3) displaying different affinities towards BSA and electrophoretic behaviors were selected to undergo complete interaction studies. Studies were performed at a BSA concentration selected according to the developed flowchart (Fig. 3). The drug concentration ranged up to 800 μM. For phenobarbital only, a too small difference in electrophoretic mobilities between the drug and the protein was observed. Therefore, the use of the long-end injection to improve the resolution between the two species was required. As demonstrated in this case, there might be a limitation in the use of short-end injection for compounds with mobilities close to that of the protein (mainly acidic compounds). Another issue that may arise for acidic compound determinations is the instable plateau (no constant plateau height) that was generated for some compounds, as illustrated in Fig. 4 for benzoic acid. This hindered quantitation in the present work because a smooth plateau could not be obtained. To circumvent this problem, short-end injection in the normal polarity mode was used. In this setup, because the EOF swept the analytes up towards the injection side, an additional pressure (varied between −10 and −50 mbar) was applied during the analysis to enable analyte detection. With −10 mbar applied during electrophoresis, no analytes were detected. A value of −50 mbar was the best alternative to obtain a smooth plateau, as well as to achieve a rapid analysis. Better plateau shapes and shorter analysis times were thus achieved with normal polarity mode coupled to external air pressure. Whereas quantitation was not possible in reversed polarity mode, this enhanced setup enabled easy benzoic acid quantitation. To investigate if the applied pressure was detrimental for the binding equilibrium, results obtained in reversed and normal polarities coupled to external pressure were compared for salicylic acid, which is a compound that could be easily analyzed with both setups. The pressure applied during the analysis did not affect the binding of salicylic acid to BSA, as demonstrated by their similar binding curves (Fig. 5). As previously mentioned (Section 3.1.8), it was also necessary to evaluate the co-migration of the protein and drug–protein complex for each drug–BSA system. The electrophoretic mobility and mobility ratio (M) of BSA and drug–BSA complexes were measured by ACE, as explained in Section 2.4.3. These values are reported in Table 2. From this table, it is obvious that the addition of the drug to the running buffer did not affect the mobility of BSA in a significant manner, except for diclofenac. For the latter drug, the largest change in mobility ratio (i.e., the largest mobility difference between the protein and drug–protein complex) was observed (4%). To check the impact of this change in mobility on K_a, values obtained with (according to Eq. (9)) and without correction of the difference in mobility between BSA and the drug–BSA complex were compared. Without correction, the $\log K_a$ obtained for diclofenac was 4.94. With correction, the $\log K_a$ value was 4.91. Thus, the small difference between the two values obtained was considered negligible.

Results obtained (K_a, n) for all compounds are displayed in Table 3 along with K_a-values found in the literature or analyzed by ED in our laboratory. A good correlation between the values obtained by CE/FA and the reference values was obtained with a slope close to one (0.97 ± 0.06) and a statistical Y-intercept value of zero (0.13 ± 0.22). Moreover, binding isotherms with values obtained by CE and ED for three compounds [i.e., warfarin (acidic), propranolol (basic), and carbamazepine (neutral compound)] are presented in Fig. 6. Results were similar using CE/FA and ED, the reference method, emphasizing the pertinence of the CE/FA method developed for measuring binding affinities. Furthermore, in CE/FA with short-end injection, the total analysis time was reduced by a factor 3–5 compared to long-end injection, and even more compared to ED (approx. 3 h *vs.* approx. 20 h, respectively).

4. Conclusion

Because some confusion has been pointed out in the literature when conducting CE/FA binding assays, the aim of the present work was to present all important steps that should be taken into consideration when developing such binding studies. A model system consisted of warfarin and bovine albumin was selected to study the different parameters important in CE/FA. A description on how to proceed (which parameter, in which order) when building up new CE/FA binding studies was presented. The most critical point is the maintenance of the binding equilibrium during the electrophoretic run. In fact, if a too small plug of a drug–protein mixture is injected into the capillary, the complex formed will dissociate, leading in an undervaluation of the binding strength. A procedure to evaluate protein adsorption onto the capillary wall was also presented. A flowchart of the recommended BSA concentrations to use was next proposed as well as the range of drug concentrations to use for complete binding studies. The user that wants to start with CE/FA binding studies should first select the composition of the buffer, then evaluate if protein adsorption on the capillary wall occurs, and next choose an injection volume large enough to maintain the binding equilibrium during the whole run. If the time required to reach the binding equilibrium is short, as for drug–plasma protein binding, no specific incubation time is necessary. In other cases, this parameter should also be evaluated. The next step is the choice of the drug and protein concentrations. The protein concentration must be large enough to produce enough binding but not too important in order to avoid carry-over issues. The last part should be dedicated to the evaluation of the protein and drug–protein complex mobilities, which must be as close as possible (difference < 5%) to avoid introducing errors in the estimation of K_a. The method was then developed using short-end injection to increase the throughput of the technique (three times for acidic compounds and five times for basic compounds). This approach was applied to a set of well-balanced compounds. Results (K_a, n) obtained by CE/FA were found to be in good agreement with reference values obtained by ED or other validated techniques. This study thus confirms that CE/FA, when conducted in a proper manner, is a suitable technique for the characterization of drug–protein interactions. This methodology presents some advantages in comparison to traditional ED for assessing binding constants and interaction stoichiometries, such as (i) low sample consumption (a few μg in CE/FA vs. a few mg in ED), (ii) short analysis time (approx. 3 h for CE/FA vs. approx. 20 h or more for ED), and (iii) easy automation. The strength of the binding constants obtained in this work ranged from $\log K_a$ 2.9 to 5.4, which includes most of the drug–albumin interaction systems. Acidic compounds may present issues, such as chaotic plateau shapes. Short-end injection in normal polarity mode coupled with the application of external pressure during the analysis was used to improve the quality of plateau shapes. Whereas quantitation was not possible in reversed polarity, this latter setup allowed easy quantitation and shorter analysis times. The most important limitations that can occur for high-affinity acidic compounds are the lack of sensitivity of the CE/FA-UV/vis method (i.e., difficulty in characterizing the lower part of the binding isotherm) and the co-migration of the free drug and protein. The use of an extended light path reduced the sensitivity limits. However, a mass spectrometer interfaced with CE/FA could afford more sensitivity as well as selectivity, which in turn could widen the accessible K_a-range.

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