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Precision in affinity capillary electrophoresis for drug–protein binding studies

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ABSTRACT

In order to achieve excellent precision in the estimation of binding constants by affinity capillary electrophoresis (ACE), electroosmotic flow (EOF) stability is the key parameter, especially when using proteins in binding assays. Appropriate rinsing protocols are mandatory. In our study, the capillary was rinsed after each run with 0.1 mol/L sodium hydroxide for 2.0 min, with water for 2.0 min followed by running electrolyte (phosphate buffer at pH 7.4) for 3.0 min (pressure = 3000 mbar each). Tryptophan-human serum albumin, warfarin-bovine serum albumin and quercetin-β-lactoglobulin were used as ACE models. Further improvements in precision have been obtained by avoiding a complete standstill of liquid within the capillary and flushing the capillary with buffer for 25 min after each 30 consecutive runs. The precision of measurements is further improved by the use of mobility ratios to report mobility changes (RSD% less than 0.5% in a long-term measurement, $n = 300–600$). Apart from the importance of a stable EOF, other ACE key parameters include protein concentration, drug plug length, applied voltage, and the choice of the regression method. In the present work, useful protocols and templates are provided in order to allow users a quick and efficient start with ACE methods. The comprehensive experimental part can serve as a checklist, which parameters need to be addressed for successfully applying ACE. Here, the suggested experimental design allows for the determination of binding constants within a couple of hours using standard instrumentation. This time could still be decreased by orders of magnitude using capillary arrays or miniaturized systems.

1. Introduction

Affinity capillary electrophoresis (ACE) may often be used advantageously for studying affinity interactions compared to other well-established techniques [1–13]. Among the virtues that make ACE an attractive platform are low sample and ligand consumption, relatively short analysis times, high efficiency and suitability for probing high and weak affinity interactions [14]. In contrast to chromatographic and surface plasmon resonance-based methods, ACE does not require immobilization on a support with the risk of alteration of binding properties and can often be performed without derivatization of the interacting species [15,16]. Fluorescence-based binding assays have been very successful during the last years, because they are fast and can provide binding constants within 10–20 min. However, please keep in mind that in order to provide this speed, preparations are needed, which usually take weeks or even months. For example, appropriate reagent labelling must be provided, e.g. for FRET experiments. Still, for repeated binding investigations for a series of compounds, fluorescence-based binding assays are often the method of choice. However, in some cases these assays produce false-positive results, e.g. from aggregation of ligands, which can cause allosteric inhibition. Further, light-absorbing or emitting interferences have been observed for several examples [17,18]. Hence, in some cases fluorescence-based assays are not suitable. Maybe even more important, they all need reference methods for validation.

In ACE, separations can be performed in solution under physiological buffer conditions, it is – as a rule – possible to preserve the analyte in a native state and hence to maintain its molecular function. Therefore, ACE has been used quite broadly in analytical chemistry and the biological sciences, integrated in many functional biology studies e.g. the functional and structural assessment of drugs and novel drug candidates.

There are a number of formats to measure binding parameters for affinity interactions [19,20]. ACE has been demonstrated to be a

Abbreviations: ACE, affinity capillary electrophoresis; HSA, human serum albumin; BSA, bovine serum albumin; βLG, β-lactoglobulin; Trp, tryptophan; War, warfarin; Qu, quercetin; Ac, acetanilide; SDS, sodium dodecyl sulphate; HTS, high-throughput screening; RSD, relative standard deviation; EOF, electroosmotic flow; p*I*, isoelectric point; *M*, mobility ratio; t_{eof}, migration time of EOF marker; t_{drug}, migration time of drug; BGE, background electrolyte; R_i, mobility ratio of the drug (t_{eof}/t_{drug}) measured in the presence of protein; R_f, mobility ratio of the drug (t_{eof}/t_{drug}) measured in the absence of protein; R_c, mobility ratio of the drug (t_{eof}/t_{drug}) measured at saturated protein concentration; $c(L)$, micro-molar concentration of the protein.

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useful approach for the determination of binding constants based on mobility measurements [21]. In ACE, one of the two binding species is injected to form a narrow plug into the capillary filled with a buffer containing the other binding species at varying concentrations [20].

The high throughput capabilities of ACE are still challenged by technical limitations. Today, up to one week is needed to reliably determine a binding constant. However, miniaturization is a continued focus for technological innovations to the extent that development of a simple, fast and precise analysis, i.e., high-throughput screening (HTS) together with binding data, is attractive but still in the prototype phase.

ACE as a probe for studying drug–protein interactions has gained increasing popularity in the last few years due to its advantages compared to other techniques [22]. One benefit of ACE is that it directly examines the interactions of the drug and protein in solution. Since the ACE also acts to separate the analyte from other sample components, this method can often be used with impure samples. As well, ACE requires a single experiment to yield dissociation or binding rate and equilibrium binding constants using, e.g., simple EXCEL™ programs for the computing. This makes it simple to carry out series of experiments in various scientific environments.

Although the use of ACE for drug–protein binding measurements has many potential advantages, there is still relatively little information regarding the experimental conditions that are most appropriate for improving the precision of determined binding constants. Up to now, precision has been reported only for short measurement series ($n = 4$) with RSD% more than 8% [23–25]. There are several factors affecting the precision of measurements in ACE. One of the major challenges faced by mobility-based ACE is the fluctuation of EOF. Protein interactions with the inner surface of a capillary may contribute to changes in mobility that can be understood from the Gouy–Chapman–Stern–Grahame model [26,27]. However, coating of the capillary inner wall or developing rinsing strategies can reduce protein adsorption. The use of small multivalent ions like phosphate under physiological conditions as a running electrolyte and solvent for drug and protein materials could reduce the imprecision in measurements resulting from conductivity and/or pH inhomogeneities or sticking of the capillary wall [27]. The mobility ratio [28] of the drug related to the mobility of a nonreacting neutral marker can be used to compensate for fluctuations of the EOF.

Other experimental conditions affect the precision of measurements such as buffer viscosity, protein concentration, sample plug length, and applied voltage. Moreover, there are four linear and nonlinear regression methods for estimation of ACE binding constants. The choice of the proper regression method is an essential factor for providing optimal precision of the measurements of binding constants.

The current work will examine the influence of such factors on the precision of ACE measurements for long analysis time using well-known models, including the interactions of tryptophan (Trp) with human serum albumin (HSA) and the interaction of warfarin (War) with bovine serum albumin (BSA). These systems were chosen as models since: (1) they have known stoichiometries and equilibrium constants for their binding [29,30]; (2) they have relatively fast association and dissociation processes [11]; and (3) they represent two different classes of drug–protein systems, i.e., one system (Trp-HSA) with large difference in mobilities and another with small difference in mobilities (War-BSA). The observations made with these models will be compared to results obtained from other ACE experiments described in the literature. Subsequently general experimental guidelines, protocols and checklists will be developed that can be applied to other drug–protein systems with affinity interactions.

To the best of our knowledge, there is no ACE study on the binding of quercetin (Qu) with β-lactoglubolin (βLG; Qu-βLG system). As a final test of the derived concepts, this system will be investigated following the derived protocols and checklists, in order to make ACE acceptable for routine drug–anionic protein analysis.

2. Materials and methods

2.1. Materials

Human serum albumin (99% agarose gel electrophoresis, p*I* 4.70 [23], M_r 66.48 kDa), bovine serum albumin (98% agarose gel electrophoresis, p*I* 4.70 [20], M_r 66.00 kDa), β-lactoglubolin (80% bovine milk, p*I*: 4.83–5.40 [31], M_r 18.40 kDa), L-tryptophan (Trp, 98% TLC, M_r 204.23) and warfarin (War, 98%, M_r 308.33) were purchased from Sigma–Aldrich (Steinheim, Germany). Quercetin (Qu, 99% HPLC, M_r 338.27) was obtained from Fluka (Buchs SG, Switzerland). All chemicals were used as received. Disodium hydrogen phosphate-2-hydrate (analytical reagent) and potassium dihydrogen phosphate (analytical reagent) were purchased from Riedel-deHaën (Sigma–Aldrich, Seelze, Germany). Acetanilide (Ac), ethanol and sodium hydroxide were obtained from Fluka (Sigma–Aldrich, Steinheim, Germany). Sodiumdodecyl sulfate (SDS for biochemistry) and hydrochloric acid were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

ACE experiments were carried out on a UniCAM Crystal 310 CE System (UniCAM Ltd., Cambridge, UK) equipped with a Spectra Physics Spectra 100 UV detector (typically, the wavelength of 280 nm was monitored). Bare fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA) with 50 μm ID and used with 58 cm total length, which corresponds to an effective length of 48 cm to the detector window. Electropherograms were monitored using a Crystal CE program V.1.3. The collected data were integrated by a homemade integration program K.I.S.S. [32].

The pH measurements were performed on a pH meter 761 Calimatic Knick (Kirchnüchel, Germany). Rotilabos-syringe filters were obtained from Carl Roth (PVDF, 0.22 mm, Karlsruhe, Germany).

2.3. Preparation of solutions

The buffer system was 12.5 mmol/L phosphate buffer at pH 7.4. This buffer was prepared by dissolving 2.1775 g disodium hydrogen phosphate in bi-distilled water and adjusting the pH to 7.4 using 12.5 mmol/L potassium dihydrogen phosphate solution and then made up to 1000 mL with water. The buffer is stable for one week when stored at 4 °C. All drug and protein solutions were freshly dissolved in phosphate buffer. The concentrations of HSA, BSA and βLG were prepared in the ranges of 5–200, 20–100 and 1–80 μmol/L, respectively. Stock solutions of Trp and War (2500 μmol/L) were freshly prepared in 25 mL phosphate buffer. A stock solution of Qu (1000 μmol/L) was prepared in 10 mL ethanol. Acetanilide (Ac, 1500 μg/mL) was prepared in 25 mL phosphate buffer. Series of Trp, War and Qu ranged between 100–1000, 50–1000 and 1–20 μmol/L, respectively. They have been prepared by pipetting the appropriate volume from stock solution and diluted by phosphate buffer in 5 mL measuring flasks. Before dilution, 1 mL of acetanilide solution was added into each sample solution in order to give a concentration of 300 μg/mL. Ten different concentrations of studied drugs and proteins have been used in each binding study. The running electrolyte and injected sample solutions were filtered through 0.22 μm syringe filters, degassed and sonicated for 10 min prior to their application onto the CE system.

2.4. Rinsing protocol and separation conditions

The uncoated fused-silica capillaries were conditioned by flushing at 1000 mbar with 1.0 mol/L sodium hydroxide solution for 40 min and water for 10 min before first use. At the beginning of each analysis, the capillary was rinsed with 0.1 mol/L sodium hydroxide at 3000 mbar for 2.0 min, with water at 3000 mbar for 2.0 min followed by running electrolyte (phosphate buffer at pH 7.4) at 3000 mbar for 3.0 min. At the end of the analysis day, the capillary was flushed at 1000 mbar with sodium hydroxide for 5 min and water for 10 min; then, both capillary ends were kept immersed in water vials. For long-period continued measurements, the capillary was flushed by pressuring phosphate buffer at 3000 mbar for 25 min after each 30 subsequent runs in order to avoid the formation of noise in the background.

Drugs were injected hydrodynamically at 25 mbar for 9 s. The separations were performed by applying a voltage of 20 kV giving a typical current of 19 μA. All separations were carried out at 23 °C with detection at 280 nm for Trp-HSA and War-BSA or at 210 nm for Qu-βLG systems. All experiments were performed by injecting a series of 5–10 different concentrations of drug in the presence of a constant concentration of protein. Each concentration was repeatedly measured 6 times and consequently the number of runs ranged between 30 and 60. Each study was repeated with ten different concentrations of protein. Consequently, the total number of runs for each studied drug–protein model ranged between 300 and 600.

The net electrophoretic mobility of the drugs was determined from their migration times using the fraction $(L_{eff}L_{tot})/(tV)$, where L_{eff} is the effective capillary length from the injection end to the detector, L_{tot} is the total length of the capillary, and V is the applied voltage. The mobility ratio (R) of the drug was calculated by using the equivalent relationship $R = t_{eof}/t_{drug}$ [22], where t_{eof} is the migration time for a neutral marker (Ac) analyzed during the same run used to measure t_{drug}.

2.5. Software

Microsoft EXCEL™ program (Microsoft Corporation, Version 2007) was used to perform the statistical analysis of the regressions.

3. Results and discussion

In order to establish a quick and efficient start with ACE-based binding assay for anionic protein interaction with drug molecules, we initially evaluated the most straightforward and well-known model system, Trp-HSA. This model is relatively easy to handle due to the pronounced difference in mobility with a relatively fast association and dissociation processes [7,30]. As well, HSA is one of the main proteins involved in the binding of drugs in blood or serum [30]. An uncoated, bare fused-silica capillary was used because the inherent negatively charged wall resulting from dissociation of silanol groups under physiological conditions could reduce the adsorption of anionic proteins. Further, this capillary type is available at low costs compared to coated capillaries. A buffer system was chosen similar to the physiological conditions (phosphate buffer at pH 7.4), in order to optimally stabilize the native structure of the protein ligand. Further, a low buffer concentration, 12.5 mM phosphate buffer, was used, which proved beneficial in an earlier work [33].

3.1. Adsorption of protein

In ACE, protein adsorption often causes low precision of binding data. Protein interactions with the inner surface of the capillary may contribute to a change in mobility [26]. EOF neutral marker can be used as a measure of the mobility shift [34]. In the present work,

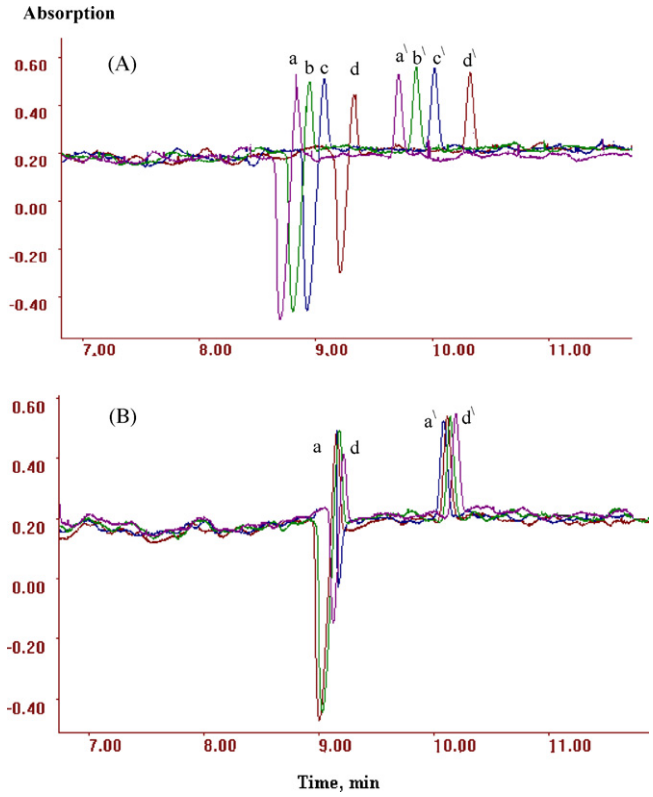

Fig. 1. The effect of rinsing regime using SDS (A) and the proposed protocol (B) on the repeatability of Ac (a–d) and Trp (a\–d\) measurements in the presence of 200 μM HSA as buffer additive.

acetanilide (Ac, 300 μg/mL) was successfully used as EOF marker without any difficulty for its solubility in the electrolyte. Moreover, this substance remained uncharged under the applied conditions.

In order to sufficiently reduce protein adsorption, appropriate rinsing procedures are required to obtain good results. From earlier works rinsing regimes are known to wash protein off the uncoated capillary making use of SDS containing buffers [35,36]. In the present work, we also tried to use SDS rinsing procedures (Fig. 1A) but the repeatability remained poor (RSD% > 8%, $n = 4$). This unfavorable result could be due to the use of pH 7.4, in contrast to the higher pH (above 9.5) used in earlier works. Probably SDS can only be effective with some hydroxide activity. Therefore, other rinsing procedures were investigated using hydrochloric acid or sodium hydroxide in various concentrations. It was found that 0.1 mol/L sodium hydroxide rinsing for 2 min at 3000 mbar was successful in removing adsorbed protein from the capillary wall. The rinsing procedure was efficiently completed by water for 2 min at 3000 mbar and with running electrolyte for 3 min after each run (Fig. 1B). However, it was obvious that the mobility of analytes changed dramatically after 30 consecutive runs. For example, the mobility of acetanilide (EOF mobility) shifted about 23% after 30 subsequent measurements in the presence of 200 μM HSA. Further, additional background noise built up over time (Fig. 2A). The baseline noises could be due to the aging of small amounts of protein which may be adsorbed on the inner wall after 30 consecutive runs. These particles have been mechanically removed from the wall by flushing the capillary with buffer for 25 min under 3000 mbar (high streaming velocity) as shown in Fig. 2B. This rinsing protocol was used in experiments with long period-continued measurements.

It was still desirable to identify other key parameters that can be useful for releasing adsorbed protein molecules. The effect of separating voltage was investigated by repeating the measurements at 5, 10, 15, 20, 25 and 30 kV five times in the presence of 100 μM HSA.

Table 1
Migration time and mobility ratio of Ac and Trp in plain phosphate buffer after finishing runs in the presence of variable concentrations of HSA.

Parameter	Migration time of Ac (min); RSD% (n = 5)	Migration time of Trp (min); RSD% (n = 5)	Mobility ratio; RSD% (n = 5)
At the beginning	4.418; 1.113	4.468; 1.101	0.989; 0.013
After 100 μM HSA (1st cycle)	4.588; 1.147	4.638; 1.135	0.989; 0.012
After 200 μM HSA (1st cycle)	4.698; 1.982	4.744; 2.030	0.990; 0.107
After 100 μM HSA (2nd cycle)	4.660; 0.868	4.711; 0.857	0.989; 0.049
After 200 μM HSA (2nd cycle)	4.714; 0.786	4.767; 0.752	0.989; 0.073
Mean; RSD% (n = 25)	4.616; 2.650	4.666; 2.629	0.989; 0.079

The results indicated that the precision was improved from RSD 1.53% at 5 kV to 0.48% at 20 kV (n = 5) and maintained unchanged up to 30 kV. Therefore, applied voltage 20 kV was chosen for further experiments. The used buffer favourably provided rather high resistance and thus just a low current generation of 19 μA, which corresponds to an electrical power of 380 mW, and to 655 mW/m considering the capillary length of 58 cm. This value is uncritical being far below the generally accepted threshold of 5 W/m for 50 μm i.d. capillaries. Thus, heat generation should not influence the estimation of the binding constant here. This was also indicated by almost the same results whether we worked with 20 or 30 kV, These results are very close to the work by Yang and Hage [33] that examined the stability of the adsorbed HSA with various voltages.

A test program was established to check the nature of the surface wall under the above optimal conditions. This test consisted of injecting 500 μmol/L Trp and 300 μg/mL Ac in plain phosphate buffer (n = 5) followed by five injections of analytes in the presence of 100 μmol/L HSA as a buffer additive, and then injecting analytes again in plain phosphate buffer (n = 5). The test was subsequently continued with the injection of analytes in the presence of 200 μmol/L HSA (n = 5) and then again in plain phosphate buffer (n = 5). This program was continually cycled two times. The measurements of migration times of Ac and Trp in plain phosphate buffer after finishing runs with 100 μmol/L and 200 μmol/L HSA are given in Table 1.

Throughout this study, a slight change in the migration time of the EOF marker could be observed, since the amount of HSA was increased in the buffer system. There was a shifting in the migration time of Ac from 4.418 min (1.113% RSD) at the beginning of the program to 4.588 min (1.147% RSD) and 4.698 min (1.982% RSD) after finishing the measurements with 100 μmol/L HSA and 200 μmol/L HSA, respectively for the first cycle. The migration time of Trp was shifted from 4.468 min (1.101% RSD) at the beginning of the program to 4.638 min (1.135% RSD) and 4.744 min (2.030% RSD) after finishing the measurements with 100 μmol/L HSA and 200 μmol/L HSA, respectively. For the second cycle, there is no large change in mobility comparing to the data in the first cycle as shown in Table 1. These results indicated that the percent change in the migration times of Ac and Trp were 6.34 and 6.18%, respectively after finishing the first cycle of the test program and 6.69 and 6.69%, respectively after finishing the second cycle. This was presumably just due to the changes in the buffer viscosity because of adding protein to the running electrolyte. However, such shifts affect all measurements of drug mobility and consequently the precision of ACE based binding constant calculations. Thus, the mobility of the drug was normalized vs. that measured for the EOF marker. The resulting mobility ratio (Ac/Trp) showed an almost constant value of 0.989 with RSD% (n = 5) ranged between 0.012% and 0.107%. This agrees with previous works, which also demonstrated that the use of such ratios could almost cancel out any effects that may be caused by changes in the viscosity of the running buffer [11,26]. Because of these results, the mobility ratio was used in all later experiments as the preferred means for describing drug mobility during ACE binding studies. These results proved that our proposed rinsing protocol and other key parameters are effectively working to improve the precision of ACE measurements to be less than 0.2% (n = 25) comparing to the higher RSD% values (more than 8%, n = 5) obtained in previous works [23–25]. As well, the proposed rinsing protocol was tested for long-term precision with subsequently repeating the measurements up to n = 60 giving RSD% less than 0.5%.

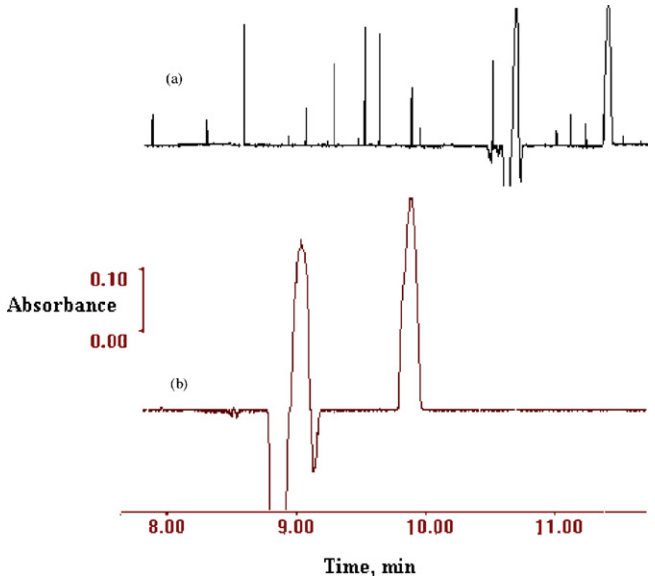

Fig. 2. The effect of rinsing protocol on the mobility of Ac and Trp after 30 consecutive runs without further flushing (A) and with further flushing by phosphate buffer for 25 min under pressure 3000 mbar (B).

The same program was tested for the other studied model systems (War-BSA and Qu-βLG) under the optimal conditions. For the War-BSA system, in the presence of 75 μmol/L BSA, the precision (RSD%) of the measurements (n = 45) for the migration time of Ac and the mobility ratio (Ac/War) were 1.784 and 0.467%, respectively. In the case of Qu-βLG system, the precision (RSD%, n = 50) for the migration time of Ac and the mobility ratio (Ac/Qu) were 1.781 and 0.467%, respectively in the presence of 40 μmol/L βLG. These results assured that our proposed rinsing protocol is very useful to reduce the capillary wall–anionic protein interaction and to increase the precision of measurements for long-term study with RSD less than 0.5%.

3.2. Drug sample concentration and plug length

Another requirement of ACE binding constant measurements is that the migration of the drug must be independent of the drug's initial concentration [28]. This was tested by varying the concentration of Trp in the range between 100 and 1000 μmol/L at a given HSA level and determining the mobility ratio (Ac/Trp). When we started the test by applying injection pressure 200 mbar for 30 s, a broad peak of Trp was obtained, due to the high sample plug length (≈86.6 mm) and consequently sample overload (sample plug length ≈ 18% of the effective length of the capillary). Therefore,

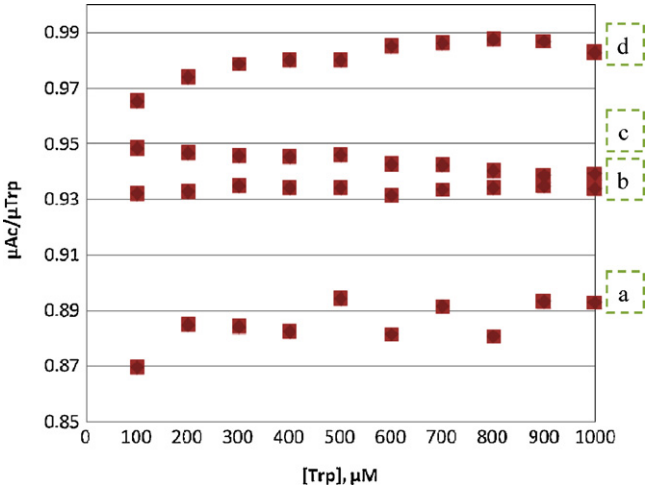

Fig. 3. The effect of injected drug (Trp) amount on the mobility ratio (Ac/Trp) in the presence of (a) 10 μM, (b) 50 μM, (c) 150 μM and (d) 200 μM HSA as buffer additives under the optimal conditions as indicated in the text.

it is necessary to optimize the injection pressure and time before going to varying the concentration of Trp. The effect of injection time was studied in the range of 3–20 s with constant injection pressure at 50 mbar. The best peak shape was achieved in the presence of 9 s injection time giving sample plug length ≈ 6.5 mm (≈1% of the effective length of the capillary). Afterwards, the effect of injection pressure was studied in the range of 25–200 mbar. Good peak shapes were observed up to 75 mbar injection pressure (sample plug length ≈ 9.7 mm ≈ 2% of the effective length of the capillary) but the repeatability of the data was the best at 25 mbar (sample plug length ≈ 3.2 mm ≈ 0.7% of the effective length of the capillary). Therefore, the optimal parameters 25 mbar and 9 s were employed throughout the current work. These observations are in agreement with the concept described in previous work [37].

After these optimizations, the main investigation started. Fig. 3 indicates the obtained mobility ratio (Ac/Trp) values with changing the injected amount of Trp from 100 to 1000 μmol/L at four fixed HSA concentrations. It was observed that a noticeable change in the mobility ratio occurred at high and low protein levels. At high level (200 μmol/L, Fig. 3d), the mobility ratio ranged from 0.97 to 0.99 with precision 2.31% (RSD%, $n = 50$). The reasons could be due to the possibilities for achievement of complex saturation or poor solubility of the protein under these experimental conditions. In the case of low-level protein (10 μmol/L, Fig. 3a), the mobility ratio changed from 0.87 with 100 μmol/L Trp to 0.90 with 1000 μmol/L Trp giving precision 2.11% (RSD%, $n = 50$). This could be due to the difficulty to imply the assumption of the equivalence of the protein concentrations in the injected drug plug and in the background electrolyte (BGE) when the concentration of protein is small [37]. Either of these situations (Fig. 3b and c) produced a case in which the amount of injected drug approached the amount of protein ligand, thus resulting in nonlinear isotherm conditions. The precision of the mobility ratio measurements with varying Trp concentration from 100 to 1000 μmol/L within the amount range of HSA 20–150 μmol/L as buffer additives gave RSD% ranged between 0.14 and 0.99% ($n = 50$). From these results, it was obvious that the injected drug sample could not be produced large changes in the mobility ratio inside the proper protein concentration range and did not strongly affect the precision of ACE measurements.

3.3. Protein ligand concentration

After appropriate experimental conditions had been established for ACE systems, the migration of drugs was analyzed at different protein concentrations. There are several practical considerations limiting the choice of protein concentrations range for the estimation of ACE binding constant [11]. The most effective parameters were the protein's solubility in the running electrolyte, the detector response at the selected wavelengths and the saturation of the drug–protein complex. For the protein's solubility in phosphate buffer under physiological conditions, there was no problem when working with HSA, BSA and βLG, where studies were done well below the solubility limit of these proteins. A response at the detector due to the protein was a second practical item that affected the ACE experiments. The background signal increased at higher ligand concentrations, as proteins absorb at the wavelengths we used for detection, viz. 210 and 280 nm. Therefore, ACE experiments were limited to work up to 200 μmol/L for HSA and BSA and up to 80 μmol/L for βLG. Some typical electropherograms obtained with the studied systems at different protein concentrations were shown in Fig. 4. Trp-HSA system was indicated in Fig. 4A. As noticed by this figure, the migration time of HSA when injected as a sample was about 7.0 min (curve a) in the presence of Ac at about 4.6 min. The migration time of Trp was about 4.7 min, which was very close to Ac (curve b) in the absence of HSA. The difference between the migration times of free HSA and free Trp was about 2.3 min, which was considered an advantage facilitating the ACE precision study of such system. The migration time of Trp shifted as more HSA ligand was added to the running electrolyte (curves c–f). As higher concentrations of protein were used, the extent of drug–protein binding increased and a larger shift in mobility was observed. These shifts in mobility and their relation to binding affinity made such studies useful in the determination of drug–protein equilibrium constants. Each protein concentration was repeatedly measured 6 times. The precision of the measurements was achieved giving RSD% ranged between 0.038 and 0.158%.

In the case of War-BSA (Fig. 4B), the migration times of free protein and free drug were about 6.9 min (curve a) and 7.3 min (curve b), respectively. Two parameters of War-BSA system different from Trp-HSA system that the difference in the migration time between free War and free BSA was relatively small (about 0.4 min) and the mobility of War was smaller than BSA as indicated in Fig. 4B. These parameters might retard the high precision of ACE based mobility ratio measurements. The application of our proposed protocol proved the reverse that RSD% values of repeating each protein concentration 6 times were ranged from 0.173 to 0.466%. As we can see from curves c–e (Fig. 4B), the migration time of War shifted as more BSA ligand was added to the running electrolyte. As well, the negative peaks could be due to the depletion of protein at the injection sample plug that moves quicker than the drug. The presence of such negative peaks cannot influence on the ACE measurements that are based on mobility ratio [30].

For Qu-βLG, there was another challenge faced ACE precision due to the low solubility of drug in an aqueous phosphate buffer. Qu proved to be soluble in phosphate buffer on adding a small amount of an ethanolic stock solution of Qu (3.4 mg/mL EtOH) to the buffer. The EtOH concentration had to be small because βLG denaturates very fast when the EtOH amount exceeds 5% in the injected drug solutions [38]. Different percents (1–5%) of ethanolic Qu solution prepared in the buffer and injected to the ACE system were studied. It was found that good peak shape of Qu without the possibility of protein denaturing during the analysis was achieved in the presence of 2% ethanol content in the injected Qu solution. Fig. 4C indicated the analyses of Qu-βLG system under the optimal conditions. As we can see, the electrophoretic mobility of Qu was decreasing while the EOF remained relatively constant with increasing protein concentration in the buffer (curves a–d). The shift in the migration time of Qu was substantial and can be measured. The precision of each βLG measurement was achieved giving RSD less than 0.5%.

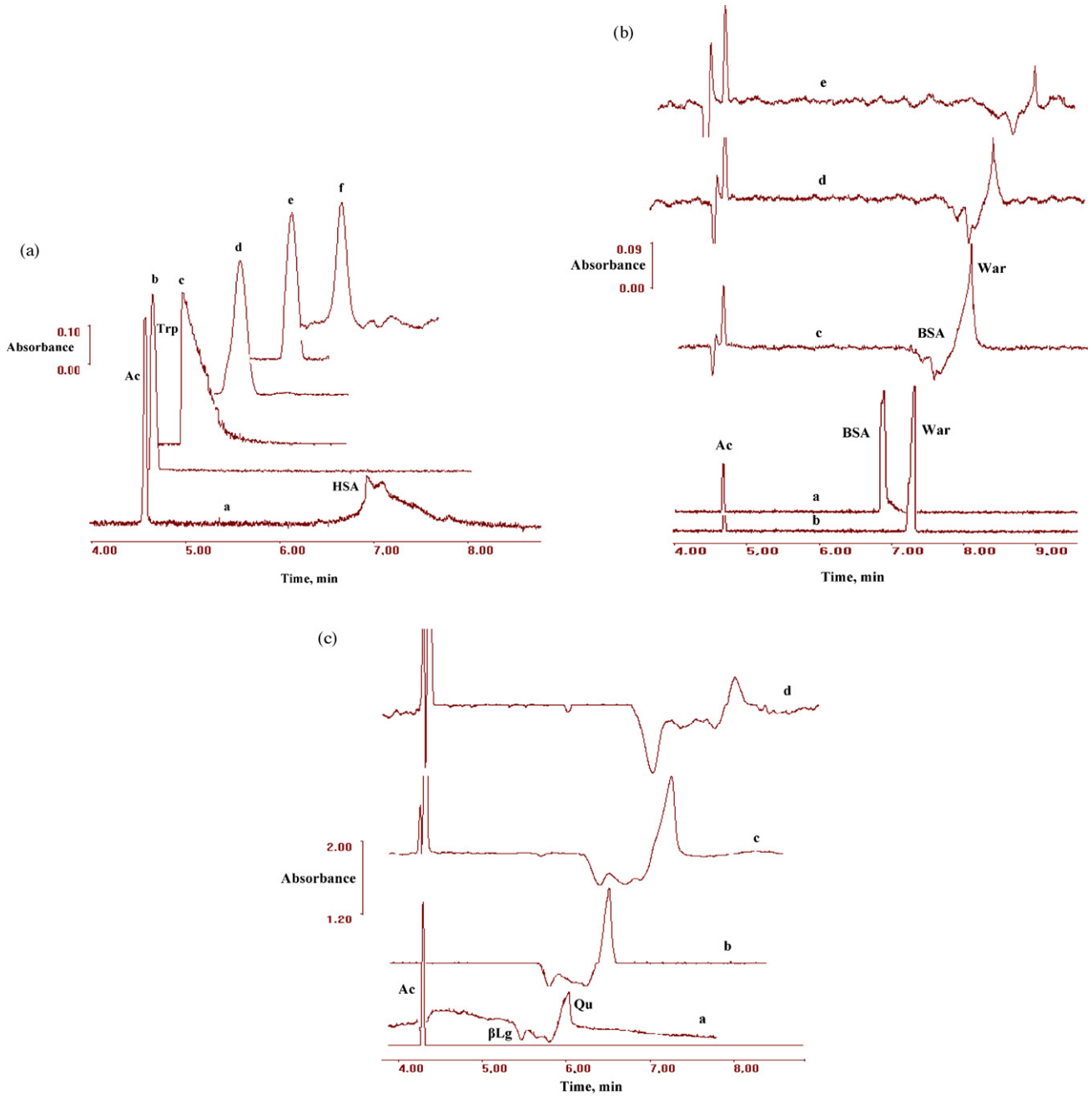

Fig. 4. (A) Electropherograms of the free HSA (a), free Trp in the absence of HSA (b), and Trp in the presence of 50 μmol/L (c), 75 μmol/L (d), 100 μmol/L (e) and 150 μmol/L (f) HSA under optimal conditions as described in the text. (B) Electropherograms of the free BSA (a), free War in the absence of BSA (b) and in the presence of various BSA concentrations 30 μmol/L (c), 50 μmol/L (d) and 75 μmol/L (e) under the optimal conditions as described in the text. (C) Electropherograms of Qu-βLG system in the presence of βLG as buffer additive: 2 μmol/L (a), 5 μmol/L (b), 20 μmol/L (c) and 40 μmol/L (d) under the optimal conditions as described in the text.

Plotting the shifts in mobility ratio difference ($R_i - R_f$) *versus* the protein ligand concentration [39] results in binding curves like shown in Fig. 5A–C, where R_i is the mobility ratio (Ac/drug) measured in the presence of protein and R_f is the mobility ratio (Ac/drug) measured in the absence of protein. A binding curve gives an idea of how far complexation was proceeded. Linear behavior was achieved over a range of HSA concentrations up to 100 μmol/L (Fig. 5A). It is obvious that saturation occurred at HSA concentrations starting from 125 μmol/L. Further, the high precision of Trp-HSA measurements (RSD less than 0.5%) was achieved in the proper linear HSA concentration range up to 100 μmol/L. It is necessary to say that the number of data points included in the linear region of binding curve should vary from 4 to 7 in order to achieve high precision of ACE binding constant.

In the case of War-BSA system, the same protein concentration range up to 100 μmol/L as described in the previous model was applied. This is due to the similarity between HSA and BSA as albumin proteins. It was noticed from the binding curve (Fig. 5B) that the linearity in the studied range was also achieved with RSD less than 0.5%.

When we dealt with another type of anionic protein like βLG by varying its concentration between 1 and 80 μmol/L in the running electrolyte, the linearity range has been changed. Fig. 5C indicated that the linearity limit was achieved at 50 μmol/L. Therefore, the proper βLG concentration range was up to 50 μmol/L that can be further used in the calculations of Qu-βLG ACE binding constant.

From the above results, we can say that the main factor limiting the useful range of protein concentrations was the resolution

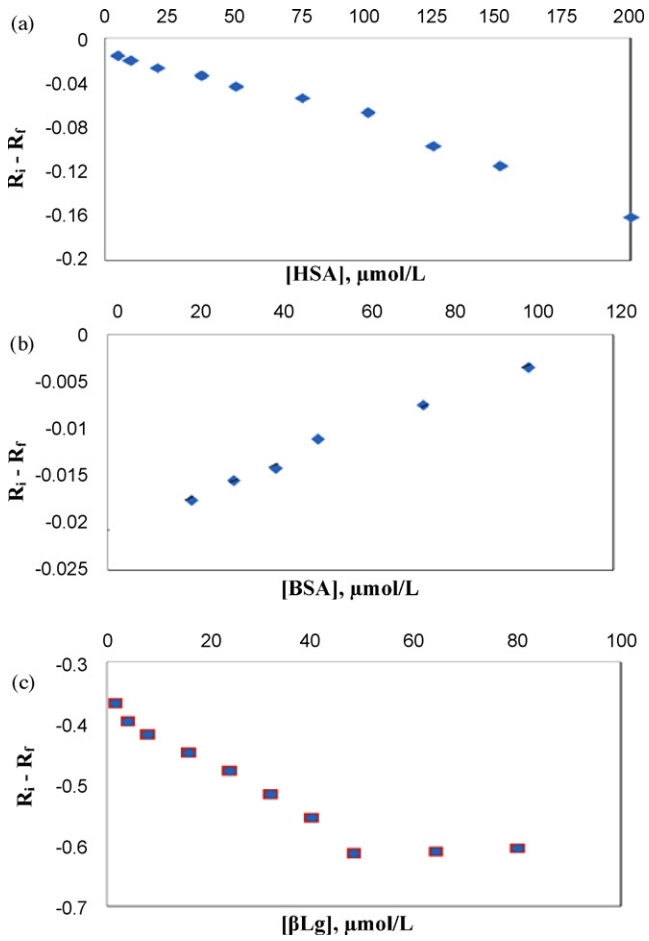

Fig. 5. Binding curves of Trp-HSA (A), War-BSA (B) and Qu-βLG (C) systems. Data shown in Fig. 5A were used in the calculation of binding constant in the spreadsheet at http://www.pharmchem.tu-bs.de/forschung/waetzig/support/.

obtained between the peak for the partially complexed drug (R_i) and that for the free drug (R_f). This item was capable of causing problems at either low or high protein concentrations, and was found to be a function of the precision of the mobility measurements. Such resolution is completely depend on the type of the studied protein. The construction of the binding curve with data points not less than 7 for the studied drug–protein system could be an useful tool to know the linearity mobility limit of the studied complex. Further, repeating the measurements ($n \geq 6$) especially in the lower protein concentrations was necessary. By collecting these results, we can choose the proper protein concentration range in order to achieve high precision ACE binding constant.

3.4. Calculation of binding constants

The analysis of data for the calculation of binding constants was achieved by using four mathematical plotting models [19,39]: nonlinear regression, *x*-reciprocal, *y*-reciprocal and double reciprocal. All these plotting methods have different statistical treatment of data points, which are shown in Table 2. An example spreadsheet can be found at http://www.pharmchem.tu-bs.de/forschung/waetzig/support/, based on the following equations (for the plotting forms, compare Table 2):

1. nonlinear regression: $Kc(L) = \frac{R_f - R_i}{R_i - R_c}$ (Eq. 1 in [39])
2. *x*-reciprocal: $\frac{1}{R_i - R_f} = \frac{1}{(R_c - R_f)K}\frac{1}{c(L)} + \frac{1}{R_c - R_f}$ (Eq. 12 in [39])
3. *y*-reciprocal: $\frac{c(L)}{R_i - R_f} = \frac{1}{R_c - R_f}c(L) + \frac{1}{(R_c - R_f)K}$ (Eq. 13 in [39])
4. double-reciprocal: $\frac{R_i - R_f}{c(L)} = -K(R_i - R_f) + K(R_c - R_f)$ (Eq. 14 in [39])

This spreadsheet can directly be used to calculate binding constants from one's own experiments. Further spreadsheets with binding data are available upon request.

A previous study [38] showed that a major advantage of the nonlinear regression method is the elimination of the cumbersome weighting procedure necessary in the statistical analysis of the linearized plots (*x*-reciprocal, *y*-reciprocal and double reciprocal). The main difficulty for the calculation of a binding constant using the nonlinear regression method is the estimation of the R_c value. This value can be measured in a few ways. A marker can be used which binds completely to the protein, or the maximum mobility of the complex measured at high protein concentration that is equal to the mobility ratio of the free protein (R_L) [20]. Currently, no markers are available for anionic protein systems. The binding constant of Trp-HSA was calculated by the nonlinear regression method where $R_c = R_L$; the results are cited in Table 3. It was found that the calculated binding constant was 3.674 ± 0.925 mmol/L ($n = 300$) by nonweighted data analysis and 2.021 ± 0.115 mmol/L ($n = 300$) by weighted data analysis. There was no large difference between weighted and nonweighted data analysis but still was far from the literature results measured at different temperatures [7,11] as indicated in Table 3. It is necessary now to modify the analysis of data. We tried to calculate the binding constant under the case of R_c equal to the mobility ratio of the drug measured at saturated protein concentration (R_{sat}). From our previous results in the binding curve of Trp-HSA, the saturation was achieved at 125 µmol/L. By putting the mobility ratio measured at such saturated protein concentration in the nonlinear regression analysis, the binding constant was 14.071 ± 1.759 ($n = 300$) by nonweighted data analysis and 12.213 ± 0.803 mmol/L ($n = 300$) by weighted data analysis (Table 3). When we compared the binding constant value with literature values, the measured value showed good agreement with those previous results obtained when using comparable temperatures (Table 3). These results were very close to the data analysis by other linearized plotting methods (*x*-reciprocal and *y*-reciprocal). This could be attributed to the large difference in the mobility between free Trp and free HSA (2.3 min) as indicated in Fig. 4A. The standard deviation (SD) in the case of weighted nonlinear regression method were relatively low (less than 1.0, $n = 300$) comparing to other data analysis methods as cited in Table 3. Therefore, nonlinear regression analysis was considered more precise than other plotting linear methods. The general binding constant ($n = 3000$) for the Trp-HSA system was calculated by the four mathematical plotting methods and the results were cited in Table 4. It was found that the calculated binding constant under our optimal parameters was very close to the values calculated previously by other techniques [7,11]. The slight difference in the comparable values was due to the difference in temperature between the controlled temperature around the capillary and the internal temperature of the capillary. Despite the temperature was controlled at 23 °C, the internal temperature was estimated to be 37 °C by Bose et al. [23] using the electrophoretic mobility method of Burgi et al. [40]. Such a problem was true for all existing CE instruments because the water or air cooling circulation could not completely dissipate the internally generated temperature.

In the case of War-BSA and Qu-βLG models, the general binding constants were calculated under our optimal conditions and the results were shown in Table 4. When we compared the binding constant values with literature results, the measured values using nonlinear regression method showed good agreement with those previous results obtained at different temperatures [20,41,42]. The presence of slight difference between the comparable results

Table 2
Plotting forms of binding constant (K).

Plotting method	K	Ref.
1. Nonlinear regression: $\frac{R_f-R_i}{R_i-R_c}$ vs. $c(L)$	Slope	Eq. 1 [39]
2. x-Reciprocal: $\frac{R_i-R_f}{c(L)}$ vs. $R_i - R_f$	−Slope	Eq. 8 [19] and Eq. 12 [39]
3. y-Reciprocal: $\frac{c(L)}{R_i-R_f}$ vs. $c(L)$	Slope/intercept	Eq. 7 [19] and Eq. 13 [39]
4. Double-reciprocal: $\frac{1}{R_i-R_f}$ vs. $\frac{1}{c(L)}$	Intercept/slope	Eq. 6 [19] and Eq. 14 [39]

R_i is the mobility ratio of the drug (t_{Ac}/t_{drug}) measured in the presence of definite protein concentration; R_f is the mobility ratio of the drug (t_{Ac}/t_{drug}) measured in the absence of protein; R_c is the mobility ratio of the drug (t_{Ac}/t_{drug}) measured at saturated protein concentration; $c(L)$ is the micro-molar concentration of the protein.

Table 3
Binding constant of Trp-HSA system.

c(Trp), μmol/L	Binding constant, mmol/L					
	Nonlinear ($R_c = R_L$)	Nonlinear ($R_c = R_{sat}$)	c(Trp), μmol/L	y-Reciprocal	Double-reciprocal	Literature values
100	5.960[a];	17.594;	17.210;	45.748;	82.187;	
	1.958[b]	11.249	57.354	34.989	183.967	
200	4.521;	16.530[c];	14.881;	35.745;	16.669;	
	1.810	13.311[c]	46.209	33.311	80.507	
300	3.892;	15.248;	12.011;	27.660;	50.108;	
	1.854	12.991	30.742	27.027	39.600	13.0 at 37 °C [7]
400	3.376;	13.497;	8.566;	14.813;	10.250;	
	2.161	11.861	16.739	14.450	34.090	
500	3.338;	13.236;	8.801;	16.163;	47.154;	
	2.043	12.789	17.525	15.740	34.925	
600	3.246;	13.261;	5.934;	10.896;	36.483;	
	2.100	11.376	9.586	10.238	30.710	
700	3.236;	13.257;	5.040;	8.296;	22.388;	
	2.025	12.768	7.546	7.320	22.416	
800	3.047;	12.707;	1.991;	1.172;	21.981;	
	2.063	12.826	2.328	0.916	21.547	
900	3.049;	12.755;	2.115;	2.552;	29.948;	27.0 at 25 °C
	2.059	11.744	2.498	2.145	26.472	[11]
1000	3.079;	12.626;	5.444;	11.217;	50.137;	
	2.139	11.210	8.431	10.915	26.839	
Mean ± SD	3.674 ± 0.925;	14.071 ± 1.759;	8.199 ± 5.155;	17.426 ± 14.533	36.731 ± 21.361;	
(n = 300)	2.021 ± 0.115	12.213 ± 0.803	19.896 ± 18.945	15.705 ± 12.190	50.107 ± 49.500	

[a] In all fields, the upper value was obtained using nonweighted data analysis (mean value).
[b] In all fields, the bottom value was calculated using weighted data analysis.
[c] These values have been calculated as indicated in the spreadsheet at http://www.pharmchem.tu-bs.de/forschung/waetzig/support/.

Table 4
The general binding constant values of the studied models.

Model	$K_{general}$, mmol/L (n = 300)				
	Nonlinear	x-Reciprocal	y-Reciprocal	Double-reciprocal	Literature value
Trp-HSA	12.225	19.916	15.832	50.112	13.0 at 37 °C [7] 27.0 at 25 °C [11]
War-BSA	4.153	32.293	31.911	33.382	2.2 at 37 °C [41] 7.4 at 27 °C [20]
Qu-βLg	1.689×10^3	0.585×10^3	0.254×10^3	0.002×10^3	2.083×10^3 [42]

could be attributed to the temperature change as described above. Another reason for Qu-βLG that βLG had a purity of at least 80% according to the manufacturer. This could introduce an uncertainty about the exact protein concentration in the buffer. Consequently, this can lead to an uncertainty in the estimated binding constant. It is difficult to evaluate theoretically the exact impact of this on the binding constant values obtained with the different plots. However, by simulating a decrease and increase of 20% in the mean value of nonlinear regression analyses, the variation in the resulting binding constant appeared to be more close to the literature result. This would lead to the conclusion that a minor presence of impurities in the protein does not significantly influence the estimated binding constant (Table 4). The estimated general binding constants of War-BSA and Qu-βLG using x-reciprocal, y-reciprocal and double reciprocal were apart from the literature results and different from the nonlinear regression values as indicated in Table 4. This could be due to the sometimes observed ill-conditioning of linear regression methods, that means their high sensitivity to random error. Such problems did not affect the estimation of binding constant using nonlinear regression analysis.

Generally, nonlinear regression should provide the most accurate (very close to the reference values) and precise (low standard deviation values) for the estimation of ACE binding constants than linear regressions following algebraic manipulation. These results support the demonstration showed that the use of nonlinear regression with ACE reduces error in the calculation of dissociation or binding constants [19,43]. The high efficiency and ease of affinity capillary electrophoresis combined with nonlinear regression plotting method makes the estimation of binding constants a simple and straightforward process.

The remaining error is not so much due to t_M measurements but rather due to the lack of fit between the measured data and the used regression model. This lack of fit is possibly due to aggregation at higher concentrations and several effects including ligand losses at lower concentrations. Further, the numerical effect of extinction followed by division by very small numbers during the course of some of the calculations may play a role. This lack of fit results in variations of approximately 10% in estimated binding constants, depending on the ligand concentrations that are taken into account. At the present time, it is difficult to avoid these lack of fit variations by a refined model function, since we do not completely understand which effects are relevant and which are most influential. In future works, at first the effect of extinction shall be thoroughly analysed. Guidance needs to be derived under which circumstances measured values should be excluded from the data sets due to numerical extinction. Further, more sophisticated binding models shall be derived and employed to reduce the lack-of-fit error.

Approximately seven different protein concentrations are advisable to estimate properly the binding constants. These could be arranged on a pseudo-logarithmic scale, e.g. 2, 5, 10, 20, 50, 100 and 200 μmol/L, in order to hit the linear range of mobility ratio difference $(R_i - R_f)$ and concentrations at least to a sufficient extend.

Every analysis including rinsing procedures needs approximately 10–20 min, depending on the capillary length. Considering 7 concentrations, 2 fold measurements and 20 min analysis time results in 4 h/binding constant. For screenings of e.g. substance collections one measurement at each concentration could suffice, if the measurements were later repeated for interesting substances. Then a binding constant can be determined in 2 h or less. This speed can be further increased by capillary arrays [44–46]. Reported instrumentation allows for a 100-fold throughput [46] but it is expensive to use especially in small laboratories. A multiplexed CE [47] could be used to improve the throughput seven fold with a cost similar to that of our proposed system.

4. Conclusion

This paper demonstrates how precise ACE binding constants of drug–anionic protein systems can be achieved. Using an elaborated rinsing protocol, freshly adsorbed protein molecules were effectively removed. Rinsing with sodium hydroxide followed by water on application of high pressure and voltage is the best option to remove adsorbed molecules on the uncoated fused silica capillaries. Flushing the capillary with phosphate buffer after 30 consecutive runs and avoiding capillary storage during routine analysis provide further improved precision of measurements with RSD% values less than 0.5%. The use of mobility ratios was shown to be clearly superior to other possibilities to report mobility changes caused by the presence of proteins, because these ratios can compensate for changes in the viscosity of the running buffer.

The optimal additive protein concentration range is related to the affinity strength of the drug–protein interaction to be studied. Approximately 7 different protein concentrations are advisable to properly estimate binding constants. The accuracy of general binding constant was often dictated by the degree of linear fit of the binding curve. Calculation and statistical analysis of the binding constant was best done with nonlinear regression analysis, which is less sensitive to the unavoidable rest of random error of measured mobility ratios. In the future, the estimation of binding constants will further be improved by reducing the effects of numerical extinction and optimizing the binding models employed. The high efficiency of affinity capillary electrophoresis combined with nonlinear regression method makes the estimation of binding constants a simple, precise, accurate and straightforward process.

For repeated binding investigations for a series of compounds, fluorescence-based binding assays are often the method of choice. However, in some cases they are not suitable due to artifacts. Then ACE becomes the best option. ACE is also often preferable if just a single binding experiment is intended, because this approach does not need time-consuming preparations such as producing or labeling appropriate reagents, which may easily use up the speed advantages of fluorescence-based binding assays for short measurement series. Furthermore, ACE always comes into play as attractive reference method for validation of any proposed methodology.

Recently, CE once more proved advantageous as a rapid and simple screening method [48], which provides quantitative results of the interactions of drugs with metal ions. For high throughput screenings of several drugs with one protein, the present work suggests a reduced experimental design in which single experiment could suffice, later repeating the experiments for substances with interesting binding properties. Then, the realistic duration of one binding constant determination, including analysis and rinsing times, does not need to be more than a couple of hours, and this speed could be further multiplied by capillary arrays or miniaturized systems.

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