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Determination of protein–drug binding constants by pressure-assisted capillary electrophoresis (PACE)/frontal analysis (FA)

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Abstract

A simple method for the determination of binding constants of drugs to human serum albumin (HSA) and α_1-acid glycoprotein (AGP) was developed by pressured-assisted capillary electrophoresis (PACE) based on the principle of frontal analysis (FA). The free drug concentration was measured from the height of the frontal peak and calculated based on the external drug standard in the absence of protein. With a known concentration of total drug, the percentage of drug bound to HSA or AGP was then determined. The binding constants of drug to HSA or AGP were obtained from non-linear curve fitting of the percentage of bound drug as a function of total protein concentration or total drug concentration. The sample was prepared by mixing known concentrations of drug and protein in phosphate buffered saline (PBS) and equilibrated for 30 min. A large volume of sample solution ($\sim$80 nl) was injected at 1.0 psi for 40 s into the fused silica capillary, which was filled with PBS buffer. Due to the difference in charge/size ratio, the free drug was separated from the protein/protein–drug complex when 15–25 kV voltage and 0.5–1.5 psi air pressure were applied. External air pressure was used to improve the throughput, prevent protein loss, and achieve a better drug plateau. By modifying experimental conditions, a wide range of binding constants could be measured. This PACE/FA method works well for basic, neutral, and weakly acidic compounds.

Keywords: Capillary electrophoresis; Frontal analysis; Protein–drug binding constant

1. Introduction

Drug–protein binding is an important process in determining the activity and fate of a pharmaceutical agent once it enters the body [1–3]. It is well recognized that the pharmacological activity of a drug is closely related to the free drug concentration in the blood. In order to be able to adjust the optimum therapeutic dose of a drug, it is therefore necessary to know the extent of drug–protein binding. Human serum albumin (HSA; 35–50 mg/ml in plasma; MW: 66 500) and α_1-acid glycoprotein (α_1-AGP; 0.5–1.0 mg/ml in plasma; MW: 40 000) are the two most important drug binding proteins in plasma [4–6]. HSA is largely responsible for the binding of acidic drugs, where

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both electrostatic and hydrophobic interactions are involved. AGP has a single binding site and binds mainly basic and neutral drugs, where hydrophobic interactions dominate.

A variety of methods have been used for protein–drug binding measurement, such as equilibrium dialysis [7,8], ultrafiltration [8,9], ultracentrifugation [8], calorimetry [10], tryptophan fluorescence quenching (TFQ) [11–13], surface plasmon resonance (BIACORE) [14], liquid chromatography [15–17], and capillary electrophoresis (CE) [18–27]. Each method has advantages and disadvantages. Equilibrium dialysis and ultrafiltration, both membrane based separation methods, are the two most commonly used techniques. Oravcová and co-workers [28] made a comparison among equilibrium dialysis, ultrafiltration, and ultracentrifugation in terms of interfering factors and shortcomings. The adsorption of ligands to the dialysis membrane and device is a serious problem for equilibrium dialysis. In ultrafiltration, the binding equilibrium (i.e. the ratio of protein–bound drug to unbound protein) may change during the separation process. Even though ultracentrifugation eliminates the problems associated with membrane effects, it suffers from sedimentation and back diffusion during the separation process, which will alter the binding equilibrium and cause errors in the measurement of free drug concentration. Calorimetric methods can provide information about stoichiometry, the number of binding sites, and the binding constant as well as enthalpy and entropy of interaction. However, buffer for drug and protein must match, which is problematic for drugs with poor aqueous solubility. TFQ is a domain specific binding assay that is susceptible to interference from fluorescent compounds. Therefore, it is difficult to measure the binding constants for compounds that bind at sites far removed from the tryptophan site or fluorescence. However, TFQ is suitable for high-throughput screening in a multiwell format [13]. BIACORE technique can provide useful information about on- and off- rates in addition to the binding constants. The limitations of BIACORE are large non-aqueous solvent effects and lack of ruggedness.

The most recently developed techniques are based on chromatographic and electrophoretic systems. Several review papers [28–34] have been published regarding the principles, limitations, and advances in chromatographic and electrophoretic methods for the determination of drug–protein binding constants. Affinity chromatographic method, employing immobilized HSA or AGP columns, is used for measuring drug bound percentage based on the correlation of the solute retention on the stationary phase. It is required to use internal standards, such as diazepam, for calibration. Based on our in-house experience, it is difficult to determine compounds with very low affinity ($< 50\%$) and very high affinity ($> 95\%$). The reason for the former is the short retention time, and for the later is non-specific binding (NSB) of drug to the column. We have encountered many compounds without positive identification by mass spectrometry within one run. On the other hand, HPLC method is a well established high-throughput assay that can be used to rank order or to identify high serum protein binding liabilities. CE is an orthogonal method that can be used to complement HPLC assay. It is accurate for low affinity compounds, and NSB is less a problem. Advantages of CE methods over chromatographic methods include its resolving power and small amounts of proteins and drugs are required. However, the CE method does offer advantage over HPLC method in terms of throughput. The major limitation of the CE methods is its low sensitivity. Five CE methods have been used for drug–protein binding studies: affinity capillary electrophoresis (ACE), Hummel-Dreyer, frontal analysis (FA), vacancy peak, and vacancy affinity electrophoresis. FA appears to be the preferred method [29]. It is simple, robust, easy to implement, can deal with multiple equilibra, and requires less reagents (nl) than all the other methods [30]. There are also some issues facing the CE methods in general, e.g. adsorption of protein and drug to the capillary wall, throughput, reproducibility, sensitivity, and the range of binding constants. In this work, we sought to address these issues in developing a system for use in pharmaceutical screening. To this end, we developed a pressure-assisted capillary electrophore-

sis/frontal analysis (PACE/FA) method for protein–drug binding studies.

2. PACE/FA method

The schematic representation of CE/FA method is shown in Fig. 1, [32]. In the CE/FA method, the capillary is filled with buffer and subsequently a large sample plug consisting of known concentrations of total drug and total protein in the buffer is injected. In the sample, the drug, protein, and drug–protein complex are in rapid equilibrium. For the application of the FA method, it requires that the mobilities of the protein and the complexes are approximately the same and the mobility of the drug differs sufficiently from those of the protein and complexes in order to be separated. Due to the difference in mobility, the free drug leaks out of the plug and forms a plateau. The height of the plateau is a measure of the free drug concentration in the injected sample. The free drug concentration, $[D]_f$, can be calculated based on the external drug standard in the absence of protein (Eq. (1)), where H_f is the height of the free drug plateau in the presence of proteins, H_s and $[D]_s$ are the plateau height and concentration of a pure drug standard, respectively.

$$[D]_f = \frac{[D]_s}{H_s} H_f \quad (1)$$

For 1:1 protein–drug binding equilibrium in Eq. (2), the protein–drug binding constant, K_{PB}, is defined as in Eq. (3), where $[PD] = [D]_b$, $[P]_f = [P]_t - [D]_b$, and $[D]_f = [D]_t - [D]_b$. The subscript b represents bound drug concentration, subscript f represents free drug or protein concentration, and subscript t represents total concentration. Substitution of $[PD] = [D]_b$, $[P]_f = [P]_t - [D]_b$, and $[D]_f = [D]_t - [D]_b$ into Eq. (3) and rearrangement will give Eq. (4). The drug bound concentration, $[D]_b$, can be obtained by solving the quadric Eq. (4). The percentage of drug bound to a protein can be calculated from Eq. (5). The relationship among the percentage of drug bound, protein–drug binding constant K_{PB}, total drug concentration $[D]_t$, and total protein concentration $[P]_t$ is given in Eq. (6) by substituting $[D]_b$ obtained from Eq. (4) into Eq. (5) [25]. The protein–drug binding constants can be obtained by non-linear curve fitting of the drug bound percentage as a

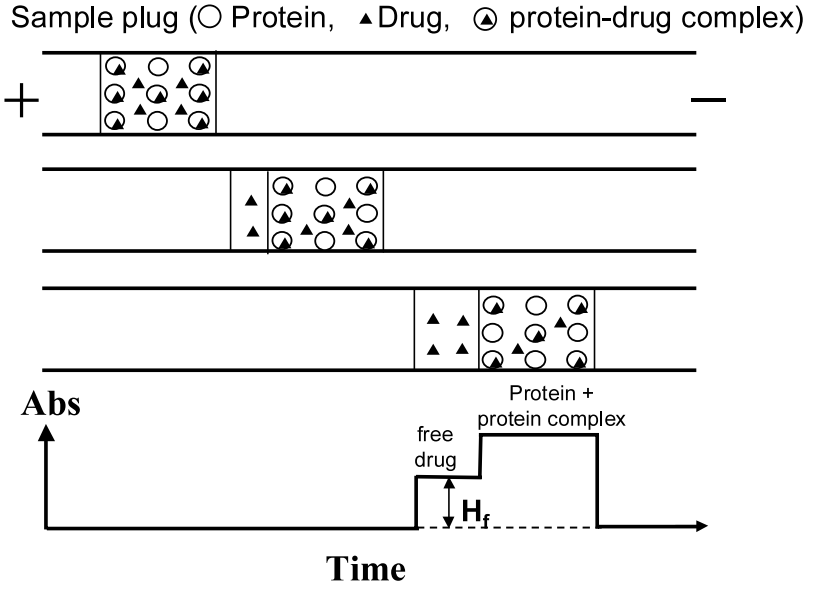

Fig. 1. Schematic representation of CE/FA method (modified from Ref. [32]).

function of total drug concentration when total protein concentration is constant or as a function of total protein concentration when total drug concentration is constant.

$$P + D \overset{K_{PB}}{\leftrightarrow} PD \tag{2}$$

$$K_{PB} = \frac{[PD]}{[P]_f[D]_f} \tag{3}$$

$$K_{PB}[D]_b^2 - K_{PB}([D]_t + [P]_t + 1)[D]_b + K_{PB}[P]_t[D]_t = 0 \tag{4}$$

$$\%\ \text{Drug bound} = \frac{[D]_b}{[D]_t} \times 100 \tag{5}$$

$$\%\ \text{Bound drug} = \frac{(K_{PB}[D]_t + K_{PB}[P]_t + 1) - \sqrt{(K_{PB}[D]_t + K_{PB}[P]_t + 1)^2 - 4K_{PB}^2[P]_t[D]_t}}{2K_{PB}[D]_t} \times 100 \tag{6}$$

3. Materials and methods

3.1. Materials

HSA (fatty acid free and globulin free) and α_1-AGP (human purified from Cohn fraction VI) were purchased from Sigma. Dulbecco's phosphate buffered saline (PBS; pH 7.4; ionic strength 0.17 M) from Life Technologies was used for sample preparation and run buffer. Stock solutions of drugs and proteins were prepared in PBS buffer and filtered through a 0.22 μm sterilizing cellulose acetate membrane. The samples for protein–drug binding assays were prepared by mixing known concentrations of drug and protein in PBS buffer and equilibrated at 25.0 °C for 30 min before injection. Single concentration drug standard in the absence of protein was used for calibration.

3.2. Apparatus and methods

The CE experiments were performed on a Beckman P/ACE MDQ CE system with a diode array UV/Vis detector. The uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ) of 60 cm total length (50 cm to the detector) × 50 μm ID × 360 μm OD was used throughout the experiments. The capillary was thermostated at 25.0 °C. In most cases, a voltage of +15 kV plus 0.5 psi external air pressure was applied for separation. The external air pressure was applied by a built-in air pump to shorten the migration time, to reduce the loss of drug and protein, and to achieve better drug plateau. The observed currents were about 95 μA. Prior to the first run of each day, the capillary was rinsed with 0.1 M NaOH for 20 min and H_2O for 5 min at 30 psi. Before injection of each sample, the capillary was rinsed with 60 mM sodium dodecyl sulfate (SDS) in PBS buffer for 1.5 min, H_2O for 1 min, and PBS buffer for 2 min at 30 psi. New PBS buffers were used for each separation to avoid cross contamination. The samples were injected at 1.0 psi for 40 s (~ 80 nl). Triplicate runs were performed for each protein–drug sample. The drug standard was injected 4 times before the batch runs, in the middle of the batch runs, and after the batch runs. The average plateau height was used for free drug calculation. The protein–drug binding constants were obtained from non-linear regression fitting with SigmaPlot (V 4.0) and reported with one standard deviation at 25.0 °C.

4. Results and discussion

4.1. Adsorption, throughput, and reproducibility

Protein adsorption to the bare silica wall in CE can cause problems in separation and loss of protein and drug. A number of studies have been conducted on protein adsorption to minimize this phenomenon [35–39]. Compared to commonly adopted washing procedures with 1 M NaOH or 1 M HCl, SDS was proved to be more effective for desorbing bound protein [35,39], which may be due to the combination of micellar effect and denaturation of proteins. In this work, 60 mM SDS in PBS buffer was used for rinsing the capillary.

Because of the high ionic strength of PBS buffer, high current was generated when 30 kV was applied, which may cause joule heating. In order to minimize joule heating, a separation

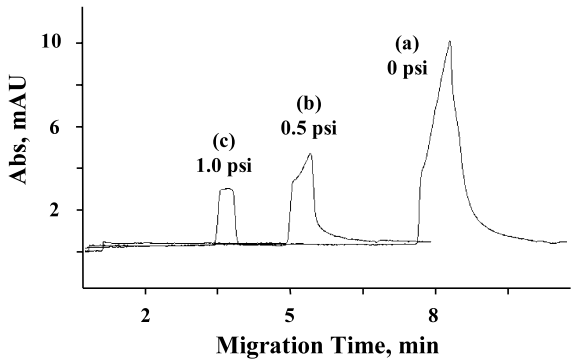

Fig. 2. Electrophoregrams of desipramine at different air pressures. Capillary: $L_d = 50$ cm, $L_t = 60$ cm, ID = 50 μm; injection: 1.0 psi, 40 s; $[D]_t = 50$ μM, $[HSA]_t = 0$ μM; $\lambda = 214$ nm; separation: (a) + 15 kV, 0 psi; (b) + 15 kV, 0.5 psi; (c) + 15 kV, 1.0 psi.

voltage of 15 or 25 kV was applied. However, the migration time is long at low voltage. PACE has been successfully applied for screening of acid dissociation constants [40]. The electrophoretic mobility measurement is very reproducible with PACE (RSD < 3%). With external pressure, the migration time of the drug plateau is reduced to less than 10 min. For protein–drug binding studies, external air pressure not only reduces the analysis time but also prevents the loss of protein during CE separation. At pH 7.4, HSA and α_1-AGP have multiple negative charges, which may cause them to migrate back to the inlet buffer vial in the absence of air pressure. Another benefit of using air pressure is that better-defined drug plateaus can be achieved for some strongly basic drugs. For example, impramine (pK_a 9.52) and desipramine (pK_a 10.28) adsorb strongly on the capillary wall and form tailing peaks (Fig. 2(a)). External air pressure was applied to minimize the drug-to-wall interaction and achieve better plateau peaks (Fig. 2(b) and (c)). However, higher pressure would result in more serious longitudinal diffusion and lower its UV sensitivity as shown in Fig. 2.

Poor quantitative reproducibility is a concern for CE. Several approaches on how to improve reproducibility were discussed in the literature [41–43]. Among the parameters, the injection process is one of the most important factors for reproducibility. Hydrodynamic injection is more precise and robust than electrokinetic injection, especially for protein–drug studies. It was reported that the injection repeatability of short time injections at high pressure is better than long time injections at lower pressure [42]. We also found that injection at 1.0 psi for 4 s (STD = 0.9%) was more reproducible than injections at 0.5 psi for 5 s (STD = 1.4%) and 0.1 psi for 10 s (STD = 1.7%) with internal correction. The injection conditions (1.0 psi/40 s) applied in this work gave a stable and reproducible free drug plateau. The PACE/FA method has a wide linearity range of plateau height vs. concentration. For propranolol, the linearity range is 20–1000 μM, which means that the plateau height is proportional to the drug concentrations. In this study, the binding constants are relatively low ($< 10^6$ M^{-1}). In most protein binding experiments, the binding curve studied is less than 50%. Multiple concentration calibration is time consuming and not necessary. Therefore, single concentration drug standard with multiple injections was used in this work.

4.2. HSA–drug binding

Electrophoregrams for HSA–diphenhydramine are shown in Fig. 3. The height of the free drug plateau decreases with increasing total HSA concentration because more drugs bind to the protein. The plateau height at [HSA] = 0 μM was used as the drug standard. The bound percentage

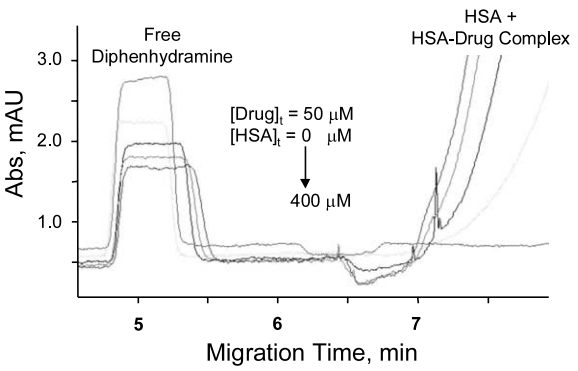

Fig. 3. Electrophoregrams of HSA–diphenhydramine. Capillary and injection as in Fig. 2; separation: + 15 kV, 0.5 psi; $[D]_t = 50$ μM.

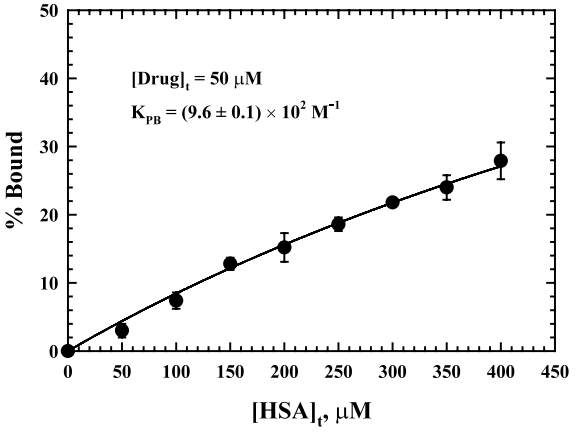

Fig. 4. Binding curve of HSA–diphenhydramine. Conditions are the same as in Fig. 3.

was calculated according to Eq. (5). The binding curve was generated by plotting the percentage of drug bound vs. HSA concentration (Fig. 4). The HSA–drug binding constant is obtained by non-linear curve fitting according to Eq. (6). In order to obtain high percentage of drug bound for weak protein–binding drugs like diphenhydramine ($K_{PB} < 10^5$ M^{-1}), the percentage of drug bound should be measured as a function of total protein concentration while the total drug concentration is kept constant. For strong protein–binding drugs like diazepam ($K_{PB} > 10^5$ M^{-1}), the free drug concentration will be too small to measure with increasing total protein concentration. Therefore, in the case of diazepam the percentage of drug bound should be measured by increasing the total drug concentration while the total protein concentration is kept constant (Fig. 5).

Protein–drug binding constants (K_{PB}) obtained by PACE/FA in this work are summarized in Table 1 and compared with the literature values [4,11,14,15,26]. Caution should be taken when making comparisons among the techniques because each method measures specific parameters under certain conditions and correlates them to the protein binding constants [4]. Different types of HSA or plasma used also make a difference. Non-defatted HSA gives much higher binding constants than defatted HSA because fatty acids also bind to the drugs. There is a bias in some protein–drug binding measurements among the techniques. However, rank order can be made within one method, such as CE. The current PACE/FA method can measure the protein binding constants for basic, neutral, and weakly acidic drugs as shown in Table 1. The CE/FA method was mostly applied to basic drugs [24,25]. In this work, the protein binding constants for weak acids such as indapamide ($pK_a = 9.16$) can also be measured. For strongly acidic drugs such as warfarin ($pK_a = 5.15$), it is challenging because of the difficulties in separating the negatively charged free acidic drugs from the negatively charged HSA.

4.3. α_1-AGP–drug binding

The PACE/FA method is more suitable for α_1-AGP–drug binding studies than for HSA because α_1-AGP binds mainly to basic and neutral drugs [5]. Fig. 6 is the binding curve for α_1-AGP–impramine. Impramine adsorbs strongly to the capillary wall and migrates as a tailing peak in CE/FA separation. High air pressure (1.5 psi) was used to achieve a better drug plateau. Because high air pressure will reduce the peak resolution of drug and protein, high voltage (25 kV) was applied to get better separation. The α_1-AGP–drug binding constants measured by PACE/FA are summarized in Table 2 along with the litera-

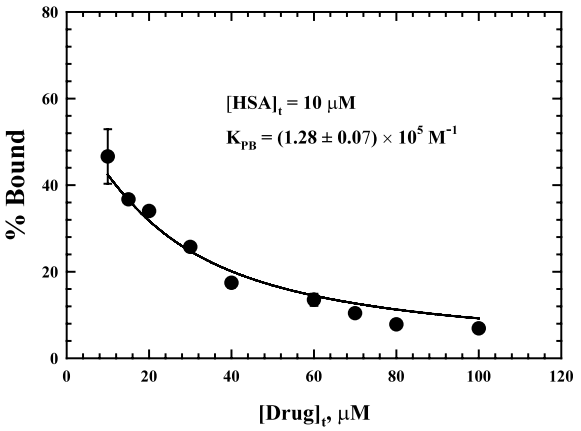

Fig. 5. Binding curve of HSA–diazepam. Capillary, separation, and injection as in Fig. 3; $[HSA]_t = 10$ μM.

Table 1
Protein binding constants (K_{PB}) from PACE/FA vs. literature values [4,11,14,15,26]

Compound	K_{PB}, M^{-1} (PACE/FA) ($n = 3$)	K_{PB}, M^{-1} (literature)	% Bound (in vitro) (literature)	pK_a (PACE)[b]
Diltiazem	$(5.9 \pm 0.3) \times 10^2$		75	7.75 (base)
Lidocaine	$(7 \pm 2) \times 10^2$	1.3×10^5 (ED)[a]		7.79 (base)
Bupropin	$(7.1 \pm 0.5) \times 10^2$		80	8.30 (base)
Diphenhydramine	$(9.6 \pm 0.1) \times 10^2$		63	9.12 (base)
Verapamil	$(1.10 \pm 0.03) \times 10^3$	1.8×10^3 (CE/FA)		8.78 (base)
Chlorpheniramine	$(1.43 \pm 0.01) \times 10^3$		70	3.86; 9.18 (base)
Impramine	$(1.8 \pm 0.3) \times 10^3$	2.39×10^4 (FLU)[a]	90	9.52 (base)
Propranolol	$(2.2 \pm 0.2) \times 10^3$	1.7×10^3 (CE/FA)		9.55 (base)
Rifampicin	$(2.97 \pm 0.08) \times 10^3$	5.1×10^3 (BIA)		7.58 (acid/base)
Promazine	$(3.0 \pm 0.4) \times 10^3$	8.5×10^4 (FLU)[a]		9.09 (base)
Indapamide	$(3.6 \pm 0.3) \times 10^3$		75	9.16 (acid)
Doxepin	$(3.6 \pm 0.3) \times 10^3$		83	9.16 (base)
Desipramine	$(3.7 \pm 0.1) \times 10^3$	7.02×10^4 (FLU)[a]	82	10.28 (base)
Chlorpromazine	$(1.13 \pm 0.09) \times 10^4$	4.2×10^4 (DSP)		9.12 (base)
Triflupromazine	$(2.1 \pm 0.3) \times 10^4$	5.5×10^4 (DSP)		8.56 (base)
Omeprazole	$(4.6 \pm 0.3) \times 10^4$		95	6.15 (base)
Diazepam	$(1.28 \pm 0.07) \times 10^5$	1.14×10^5 (TFQ)		7.63 (base)

HSA, human serum albumin; TFQ, tryptophan fluorescence quenching; BIA, biacore; DSP, difference spectrophotometry; FLU, fluorescence; ED, equilibrium dialysis.

[a] Non-defatted HSA.

[b] Acid dissociation constants were measured by PACE [40].

ture values [3,5]. The level of α_1-AGP in plasma can vary considerably under different physiological and pathological conditions [5]. As in HSA–drug binding, discrepancies also occur for α_1-AGP–drug binding measurements among the techniques. Lidocaine, impramine, propranolol, and chlorpromazine bind more strongly to α_1-AGP than to HSA by at least one order of magnitude (Tables 1 and 2). For strong binders, such as chlorpromazine, the free drug concentration is too low to measure, which causes large errors in the protein binding constant measurement. Therefore, the PACE/FA method is best suited for drugs with binding constants less than 10^6 M^{-1}.

4.4. Reproducibility of the PACE/FA method

With an effective SDS washing cycle, reproducible injection, and PACE separation, the average relative standard deviation for plateau height measurement is less than 5% ($n = 3$). HSA–drug binding constants for five compounds were measured twice on different days and show good reproducibility (Table 3).

5. Conclusions

A PACE/FA method for protein–drug binding studies has been developed. It is simple and can directly measure free drug concentrations under near physiological conditions. A wide range of binding constants (10^2–10^6 M^{-1}) can be determined and only a small amount of drug and protein is required. With external air pressure, the throughput and the quality of the drug plateau are much improved. It has been demonstrated that this assay is applicable to basic, neutral, and weakly acidic drugs and has potential applications

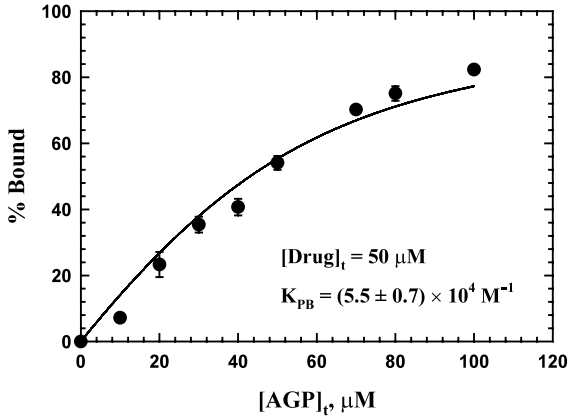

Fig. 6. Binding curve of α_1-AGP–impramine. Capillary and injection as in Fig. 2; separation: +25 kV, 1.5 psi; $[D]_t = 50$ μM.

in the drug candidate selection process. It will be difficult for strongly acidic drugs because of poor separation. This assay is not suitable for non-UV absorbing or poorly soluble compounds.

Table 2
Comparison of α_1-AGP–drug binding constants from PACE/FA with literature values [3,5]

Compound	K_{PB}, M^{-1} (PACE/FA) ($n = 3$)	K_{PB}, M^{-1} (literature)
Lidocaine	$(3.2 \pm 0.2) \times 10^4$	1×10^5
Impramine	$(5.5 \pm 0.7) \times 10^4$	4×10^4
Propranolol	$(4.7 \pm 0.5) \times 10^5$	3×10^4
Chlorpromazine	$(5 \pm 16) \times 10^6$	1×10^6

Table 3
Reproducibility of PACE/FA method for HSA–drug binding constant measurement

Compound	K_{PB}, M^{-1} (Day 1, $n = 3$)	K_{PB}, M^{-1} (Day 2, $n = 3$)
Verapamil	$(1.1 \pm 0.1) \times 10^3$	$(1.1 \pm 0.1) \times 10^3$
Propranolol	$(2.1 \pm 0.2) \times 10^3$	$(2.1 \pm 0.2) \times 10^3$
Rifampicin	$(3.0 \pm 0.1) \times 10^3$	$(2.2 \pm 0.1) \times 10^3$
Indapamide	$(3.7 \pm 0.6) \times 10^3$	$(3.6 \pm 0.3) \times 10^3$
Chlorpromazine	$(1.0 \pm 0.1) \times 10^4$	$(1.1 \pm 0.1) \times 10^4$

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