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Review

Estimation of binding constants by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) has become a useful technique for measuring binding constants. This review is focused on recent trends in the estimation of binding constants by affinity CE. First, we introduce several mathematical equations in which it is assumed that the stoichiometry of the binding between drug and protein is 1:1 as a simple model. In order to calculate accurate binding constants by affinity CE, several experimental considerations are described in this review. In addition, some recent methodologies, such as partial filling technique and multiple-step ligand injection method, are introduced. Among research publications within 3 years, recent applications for determining binding constants are reviewed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Binding constant; Frontal analysis; Reviews

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1. Introduction

Noncovalent molecular interactions widely exist in nature and their binding constants are fundamentally important parameters in binding studies. For in-

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stance, the measurement of binding constants between plasma protein and drugs is essential for drug development because the concentration of unbound drugs shows a better correlation to the pharmacological activity [1]. Over the last two decades, a number of techniques have been developed to measure binding constants [1]. Most of them are based on the use of separation techniques under equilibrium conditions. Among these separation techniques, capillary electrophoresis (CE) has been proven a new attractive analytical tool because of its speed of analysis, high efficiency and high resolving power [2]. Currently, several CE methods are available to measure binding constants, e.g. affinity capillary electrophoresis (ACE), Hummel–Dreyer method (HD), vacancy affinity capillary electrophoresis (VACE), vacancy peak method (VP) and frontal analysis (FA) [3–5]. With the exception of VACE, they were primarily developed in high-performance liquid chromatography (HPLC) and transferred to CE [6,7]. In this review, we will first describe the mathematical equations for the estimation of binding constants by CE and briefly introduce some CE methods. In addition, we will describe recent advances in the methodology and representative applications from recent publications within 3 years because several reviews cover methodologies and applications of binding studies by CE [2,8–10].

2. Estimation of binding constants

The CE methods for the estimation of binding constants and their experimental setups are summarized in Table 1. The principle and the procedure of each method have been introduced in some previous papers [3–5,11]. In order to estimate the binding constants, a series of experiments where the concentration of one component is varied while keeping that of the other component constant should commonly be performed. The binding constants are calculated from the dependence of either the electrophoretic mobility or the concentration ratio of unbound (free) and bound analytes. Up to now, various affinity interactions, such as drug–protein, protein–protein, protein–DNA, protein–carbohydrate, peptide–antibiotic, enzyme–cofactors, lectin–sugar, antigen–antibody and cyclodextrins (CDs)–enantiomer have been investigated by CE [8,12,13, and references cited therein]. Several research papers have described the mathematical equations of each affinity interaction [4,10,14,15]. In all cases of interactions, however, the calculation of binding constants can be considered to be similar. Therefore, we will consider a drug–protein binding as an example to discuss the principle of binding studies and will denote the two components as D and P , e.g. a drug and a protein in this review.

Table 1
CE methods and their experimental setups for the estimation of binding constants

Method	Sample	Running solution	Estimation of K
ACE	D	Buffer + P	Change in mobility of D
	P	Buffer + D	Change in mobility of P
VACE	Buffer	Buffer + $D + P$	Change in mobility of D or P (vacancy peak)
HD	Buffer + $D + P$	Buffer + P (or D)	Peak area of vacancy peak corresponding to $[DP]$
VP	Buffer	Buffer + $D + P$	Peak area of vacancy peak corresponding to $[D_f]$
FA	Buffer + $D + P$	Buffer	Plateau height corresponding to $[D_f]$
FACCE	Buffer + $D + P$	Buffer	Plateau height corresponding to $[D_f]$

Abbreviations: ACE, affinity capillary electrophoresis; VACE, vacancy affinity capillary electrophoresis; HD, Hummel–Dreyer method; VP, vacancy peak method; FA, frontal analysis; FACCE, frontal analysis continuous capillary electrophoresis; D , ligand such as drug; P , receptor such as protein; $[DP]$, concentration of bound drug; $[D_f]$, concentration of free drug.

When we assume that the equilibrium is established very quickly, the reversible binding between D and P is expressed by the following equation [1,3,4]:

$$r = \frac{[DP]}{[P_{\text{total}}]} = \sum_{i=1}^m n_i \frac{K_i [D_f]}{1 + K_i [D_f]} \quad (1)$$

where r is the ratio of number of bound drug (drug–protein complex) molecules to total protein molecules; $[D_f]$, $[DP]$ and $[P_{\text{total}}]$ are the concentrations of free drug, drug–protein complex and total protein, respectively; n_i is the number of sites of class i and K_i is the corresponding binding constant. However, most of publications in binding studies with CE assume that the stoichiometry of the binding between D and P is 1:1 to establish a simple model [4,8,14]. In the following session, therefore, we will also assume the equations for the simple model to estimate binding constants.

2.1. Calculation from the change in peak area or plateau height

In HD and VACE, the peak area linearly depends on the concentration of the analyte. Therefore, $[D_f]$ is calculated from the peak area as a function of $[P_{\text{total}}]$. On the other hand, a broad peak with a plateau is obtained in FA because a relatively larger volume (typically 100–200 nl) of the sample solution is introduced into the capillary [16]. The peak height of the plateau zone is linearly related to the concentration of the analyte. When we assume that the stoichiometry of the binding between D and P is 1:1, the reversible reaction between D and P is expressed by Eq. (2) and the binding constant (K) is given by Eq. (3):



$$K = \frac{[DP]}{[D_f][P_f]} \quad (3)$$

where $[D_f]$, $[P_f]$ and $[DP]$ are the concentrations of free drug, free protein and drug–protein complex, respectively. For Scatchard analysis, Eq. (3) can be traditionally rearranged as below and the linear least squares method can be adopted for data fitting [10,17]:

$$\frac{[DP]}{[D_f]} = -K[DP] + K[P_{\text{total}}] \quad (4)$$

or

$$\frac{r}{[D_f]} = -Kr + K \quad (5)$$

Consequently, the binding constant is calculated from the slope ($-K$). On the contrary, a non-linear slope is observed when multiple binding sites having different binding constants exist on P . Although each binding constant can be calculated from each linear slope as shown in Fig. 1 in some cases [18], we must usually consider a non-linear regression curve fitting of acquired experimental data with Eq. (1) [1,3,4].

2.2. Calculation from the change in electrophoretic mobility

In ACE and VACE, K value is calculated from the dependence of the electrophoretic mobility on the free and bound analyte concentrations. When we assume that the stoichiometry of the binding between D and P is 1:1 and that the analyte is D , the apparent electrophoretic mobility of D , μ_i , is expressed by Eq. (6) [19]:

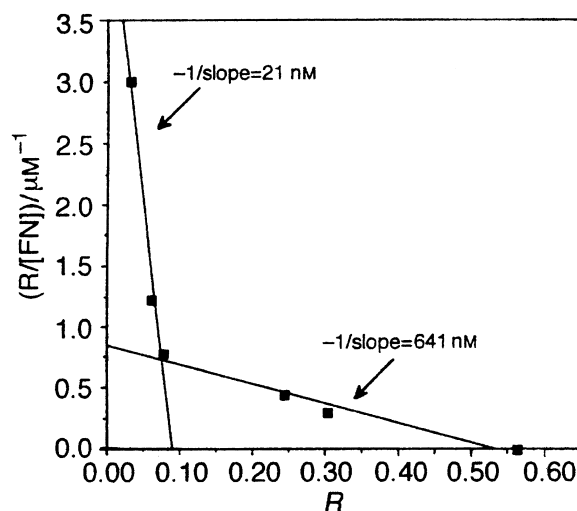


Fig. 1. Scatchard plot of fibronectin–heparin interaction with a stoichiometry of 2:1. From Ref. [18], with permission.

$$\mu_i = \frac{[D_f]}{[D_f] + [DP]} \mu_f + \frac{[DP]}{[D_f] + [DP]} \mu_c \quad (6)$$

where μ_f and μ_c are the electrophoretic mobilities of free D and DP complex, respectively. Eq. (6) is further rearranged as below:

$$\frac{[DP]}{[D_f]} = K[P_f] = \left(\frac{\mu_f - \mu_i}{\mu_i - \mu_c} \right) \quad (7)$$

Eq. (7) has been traditionally used for the calculation of binding constants with ACE [1]. However, the mobility of the DP complex, μ_c , must be experimentally measured for the calculation of binding constants with Eq. (7). In micelle electrokinetic chromatography (MEKC), several markers, such as sudan IV, timepidium, are available to measure the μ_c [20,21]. Otherwise, the μ_c is also obtained by assuming that the μ_c is similar to the mobility of free P since the binding of D with a low molecular mass does not affect the mobility of P with high molecular mass in ACE. However, to know the real mobility of DP complex is almost impossible. A non-linear curve fitting of acquired experimental data, therefore, should be employed with Eq. (7). On the other hand, the linear plotting methods do not require the measurement of μ_c . From Eqs. (3) and (6), the following equations are obtained [14]:

$$\mu_i = \frac{\mu_f + \mu_c K[P_f]}{1 + K[P_f]} \quad (8)$$

$$(\mu_i - \mu_f) = \frac{(\mu_c - \mu_f) K[P_f]}{1 + K[P_f]} \quad (9)$$

Eq. (9) can be further rearranged into different forms [14]:

$$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f) K} \frac{1}{[P_f]} + \frac{1}{(\mu_c - \mu_f)} \quad (10)$$

$$\frac{[P_f]}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f) K} [P_f] + \frac{1}{(\mu_c - \mu_f) K} \quad (11)$$

$$\frac{(\mu_i - \mu_f)}{[P_f]} = -K(\mu_i - \mu_f) + K(\mu_c - \mu_f) \quad (12)$$

Eqs. (10)–(12) have been called the double re-

ciprocal, y-reciprocal and x-reciprocal forms, respectively. They have appeared in the publications with different names. The double-reciprocal plot is known as the Benesi–Hildebrand plot in spectrophotometry and the Lineweaver–Burk plot in enzyme studies, while the x-reciprocal plot is called the Eadie plot in enzyme kinetics or the Scatchard plot in protein binding studies [14]. Consequently, Eq. (12) is the same as the Eqs. (4) and (5). Fig. 2 shows their corresponding plots [22].

The above equations assume a 1:1 binding stoichiometry. The assumption may be supported by the linearity of the plots. In fact, the above linear equations have been practically used in various binding studies (see Table 2). However, two or multiple binding sites may exist on the receptors; for drug–human serum albumin and antibody–antigen binding, multiple stoichiometry is well known [3,17]. In these cases, the binding constants cannot be calculated from the plots according to Eqs. (10)–(12) because some plots are non-linear. It is said that the non-linearity shows up most readily in the x-reciprocal plot [23]. A non-linear model should be considered for the calculation of the binding constants in multiple complex formation according to Eq. (1) [15]. Recently, more complicated interactions, such as more than one complexation additive or multiple stoichiometry, have been described theoretically in several papers [24,25]. Each binding constant to the multiple interactions, such as K_A and K_B , should be considered in the above equations. Although the confirmation of the stoichiometry is important to estimate the accurate binding constants, this review does not deal with the scenario of the complicated interactions. Different studies, such as NMR, UV-spectra and MS, have been also practically useful to obtain further information on stoichiometry for the affinity interactions of the complexes. For instance, the stoichiometry of some analyte–CD complexes has been investigated with the spectroscopic methods [26].

3. Affinity capillary electrophoresis (ACE)

ACE is the most popular method for measuring the binding constants in CE. A capillary is filled with buffer containing P in varying concentrations and a

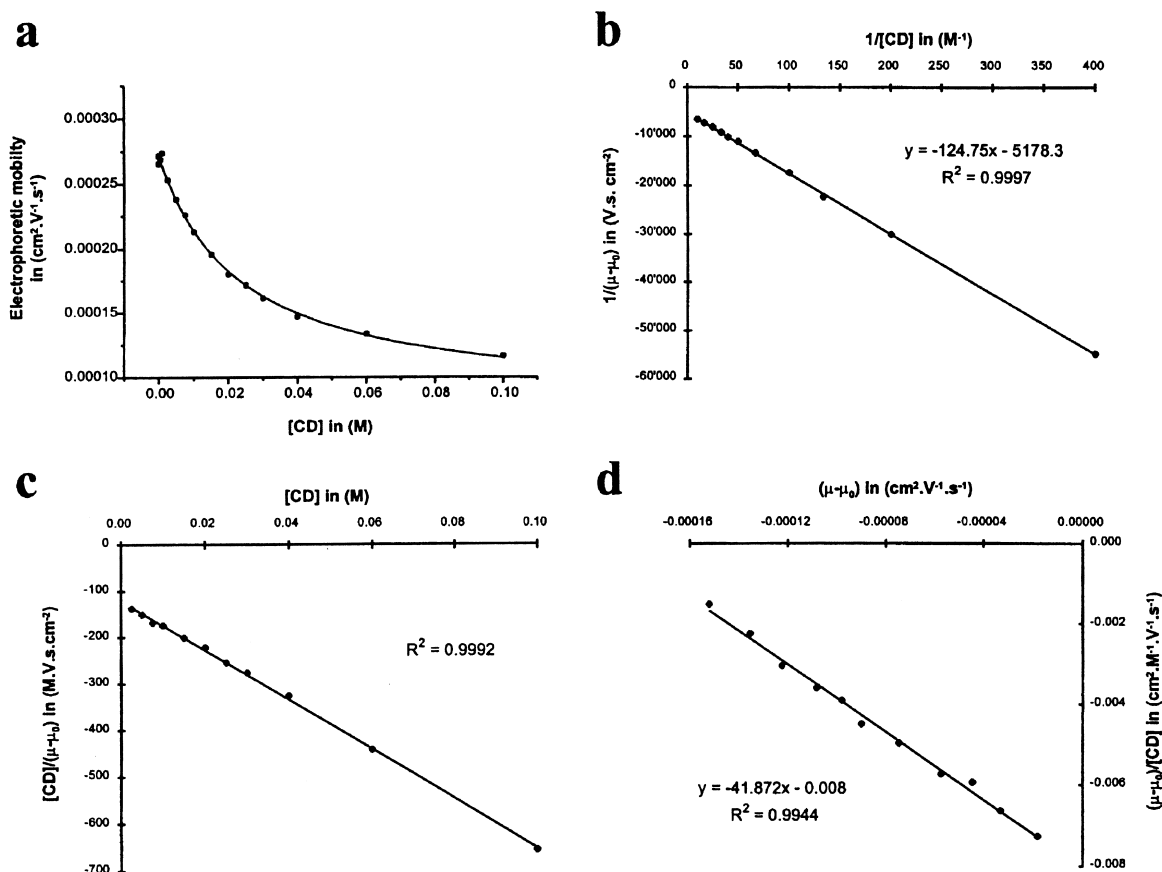


Fig. 2. Representative plots for the estimation of binding constant with various equations. (a) Non-linear regression, (b) double reciprocal plot, (c) y-reciprocal plot, (d) x-reciprocal plot. From Ref. [22], with permission.

small amount of D is injected. Since the equilibrium between D and P is established in the capillary, the apparent mobility of D depends on the binding constant and the difference between μ_f and μ_c . The binding constant is calculated from the change in electrophoretic mobility of D . On the contrary, D can be also added on the buffer instead of P . In this case, a small amount of P is injected as the sample. Various applications have been reported over the last decade and recent studies are listed in Table 2. ACE has several potential advantages as follows: (1) only a small amount of P and D is required, (2) the injection sample need not be highly purified, and (3) binding constants of several samples can be simultaneously determined. For instance, plasma binding of racemic drugs is potentially stereoselective. Each drug enantiomer frequently exhibits the different

pharmacological activities and/or the different side effects. The estimation of each binding constant is essential for stereoselective drug developments. For this purpose, ACE can be performed by injecting the racemic mixture directly. The enantiomer separations are performed by dissolving P in the running buffer [27].

ACE is greatly utilized for the analysis of enantiomers as well as the estimation of binding constants. Enantiomer separation is one of the most successful fields in CE. Numerous successful separations have been reported by many research groups [27,28]. In the enantiomer separations by CE, however, CDs and their derivatives are most widely used as chiral selectors [12]. This technique is commonly called CD-capillary zone electrophoresis mode [29] and the theoretical model was described previously by

Table 2
Recent examples for the estimation of binding constants with CE

Method	Ligand	Receptor	Equation	Ref.
ACE (Receptor in the running buffer)	Disopyramide,	Human α_1 -AGP	(9), Partial filling technique	[51]
	Remoxipride			
	Peptides	Vancomycin	(12)	[57]
	Polypeptides	Ribose	(10)	[61]
	Peptides	DNA	(5)	[62]
	Cyclosporin A	Enzyme cyclophilin	Other	[63]
	Various drugs	Transthyretin	(12)	[64]
	Drug enantiomers, Tris	18C6	Other	[65]
ACE (Ligand in the running buffer)	Heparin	Fibronectin	(5)	[18]
	Kedarcidin chromophore	Kedarcidin apoprotein	(5)	[18]
	Sulfonamides	Vancomycin,	(12)	[43]
		Carbonic anhydrase B		
	Ca	Casein phosphopeptides	(7)	[45]
	Heparin	Heparin-binding peptides	(4)	[66]
	Zinc	NCp7	(5)	[67]
	Dipeptides,	Vancomycin,	(12), Partial filling	[44]
	Sulfonamides	Carbonic anhydrase B	technique	
	Bilirubin	HSA	(5)	[68]
	Antigen	Antibody	(11)	[69]
	Dipeptides,	Vancomycin,	(12)	[54]
	Sulfonamides	Carbonic anhydrase B		
	Dipeptides,	Vancomycin,	(12)	[55]
	Sulfonamides	Carbonic anhydrase B		
Porphyrin	HSA	(12)	[70]	
CD–CZE	Benzoates,	CDs	(11)	[36]
	Salicylate,			
	Ibuprofen etc.			
	Phenols	CDs	(9)–(12)	[25]
	Imidazole derivatives	CDs	Non-linear regression	[15]
	Amphetamines	CDs	(7), (10)–(12)	[22]
	Dipeptide stereoisomers	CDs	(10)	[37]
	Propranolol,	CDs	(7),	[38]
	Tetracycline,		(10)–(12),	
	Salicylic acid,		Non-linear regression	
	Ibuprofen etc.			
	Tripeptide enantiomers	CDs	(10)	[39]
Econazole enantiomers etc.	CDs	Other linear regression	[40]	
EKC	BNDHP enantiomers	Bile salt micelle	Non-linear regression	[71]
	Aromatic anions	Nonionic surfactant micelles	Non-linear regression	[72]
	Triazines	Cationic surfactant micelles	(8)	[73]
	Triazines	Cationic surfactant monomer	(10)	[74]
	Nitrophenols	TESMR	(9)	[75]
	Nitrophenols	TESMR + α -CD	(9)	[75]

Table 2. Continued

Method	Ligand	Receptor	Equation	Ref.
VACE	Warfarin	BSA	(1)	[58]
FA	β -Blockers	Human serum proteins	Non-linear regression	[59]
	Verapamil enantiomers	HSA	(3)	[60]
	Propranolol,	HSA	(5), Non-linear regression	[76]
	Verapamil			
	Nilvadipine	Lipoproteins	Not described	[77]
	Various drugs	Transthyretin	(5)	[64]

Abbreviations: α_1 -AGP, α_1 -acid glycoprotein; 18C6, (+)-(18-crown-6)-tetracarboxylic acid; NCp7, highly basic nucleocapsid protein of HIV-1; HSA, human serum albumin; CD-CZE capillary zone electrophoresis employing cyclodextrin; CDs, cyclodextrins; EKC, electrokinetic chromatography; BNDHP, 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate; TESMR, tetraethylsulfonate derivative of 2-methylresorcinarene; BSA, bovine serum albumin; other abbreviations, see Table 1.

Wren and co-workers [19,30,31]. However, the mathematical model of CD-analyte complex is the same as that of drug-protein binding. Rawjee and co-workers [32,33] subsequently developed a multiple-equilibrium model to account for separation of chiral weak acids and bases as a function of both pH and CD concentration. The formation constants between CD and various enantiomers have been also obtained [15,22,34–40].

3.1. Technical issues

As compared to the other methods such as HPLC and non-chromatographic methods, approximate binding constants can be estimated easily and rapidly with ACE [1]. However, we should be careful when attempting the estimation of accurate binding constants due to several disadvantages of ACE. First, the fluctuation of the electroosmotic mobility may adversely influence the observed electrophoretic mobility. Although the effect of the electroosmotic flow on the velocity of the complex is mostly compensated for the effective mobility of the complex, change on the electroosmotic flow during a single run will adversely affect the measurement of the effective mobility. The conditioning of the capillary surface is important to obtain the reproducibility of the electroosmotic mobility. Second, changes in the magnitudes of both electrophoretic and electroosmotic mobilities are observed due to the viscosity variation with increasing concentration of the buffer additive such as *P* [41]. Unless the

concentration of the buffer additive is low, the viscosity variations should be corrected for accurate measurement of electrophoretic mobilities. This can be accounted for by introducing a correction factor ν ($=\eta/\eta^\circ$, where η is the viscosity of the running buffer and η° is the viscosity of the neat buffer solution) to normalize the experimental electrophoretic mobilities [23]. For example, Eq. (6) can be shown as follows:

$$\nu\mu_i = \frac{[D_f]}{[D_f] + [DP]} \mu_f + \frac{[DP]}{[D_f] + [DP]} \mu_c \quad (13)$$

Otherwise, the mobility ratio (*M*) has been also used to estimate *K* values for providing a more accurate equation for analyte migration in CE [42–44]. When the observed electrophoretic, net electrophoretic and electroosmotic mobilities are represented μ , μ_{net} , and μ_{eo} , respectively, the *M* is defined as follows:

$$M = \mu_{\text{net}}/\mu_{\text{eo}} = (\mu + \mu_{\text{eo}})/\mu_{\text{eo}} \quad (14)$$

The change in the viscosity is adequately corrected using *M* without the direct measurement of the viscosity in the running buffer. Third, some components such as proteins, peptides and basic analytes, may adsorb on the inner wall of the capillary. This causes peak broadening and inaccurate mobility measurements. In order to avoid the adsorption, we must carefully select the ionic strength and pH of the running buffer. Alternatively, a neutral coated capillary, e.g. a linear polyacrylamide-coated capillary or a polyvinylalcohol-coated one, is frequently useful

for this purpose [45]. By using the neutral coated capillary, the change in the electroosmotic mobility can be also avoided due to the almost complete suppression of the electroosmotic flow. Fourth, the concentration of unbound P , $[P_f]$, in the above equations is not equal to the concentration of P dissolved in the running buffer. In order to calculate K values, however, we usually substitute the total protein concentration, $[P_{\text{total}}]$, for the $[P_f]$ in the equations. When the $[P_{\text{total}}]$ is much higher than the total drug concentration or the binding constants are not large, it does not significantly affect the K value because the $[P_f]$ can be approximated by $[P_{\text{total}}]$. On the contrary, the correction is necessary to estimate the accurate K value with the equation of $[P_f] = [P_{\text{total}}] - [DP]$ when the difference between $[P_f]$ and $[P_{\text{total}}]$ is not negligible [14]. In practical experiments, the concentration range of the P is also important. To minimize the error in the estimation of the binding constant, the necessity of matching the concentration range of P is demonstrated using Monte Carlo simulation [46,47].

3.2. Partial filling technique

The use of proteins or the other UV-absorbing components in the running buffer causes a detection problem. The partial filling technique has been an attractive procedure to solve this problem [48–50]. In this technique, the running buffer is partially filled in the capillary containing the UV-absorbing components and the remaining portion is filled with the neat running buffer without such components. The analyses are performed under the condition where the UV-absorbing components do not migrate toward the detection end of the capillary or migrate at a velocity slower than the analyte. Thus, the analyte can be detected in the neat running buffer without the interference of the UV-absorbing components. Several research groups applied this technique to the estimation of binding constants [44,51,52]. Because the binding equilibrium is only established in the partial plug containing a protein, the ratio between the effective plug length and the effective length of the capillary must be included in the equations for the calculation of binding constants. Therefore, an additional advantage is obtained using the partial filling technique as well as the improvement of

detection sensitivity. By changing the effective plug length of the protein containing buffer, the multiple experiments for the estimation of binding constants can be performed without varying the concentration of the components [44]. Such a technique will expand the applicability of ACE in binding studies.

3.3. Multiple-step ligand injection method

In the conventional ACE method, multiple experimental runs at different concentrations of D or P should be performed to obtain the binding constants. In addition, several repeat experimental runs are often performed under the same conditions in which the different concentration of D or P is kept constant to obtain the mean value of the electrophoretic mobilities. However, it is time-consuming and the stable electroosmotic flow is required during the runs. To overcome the above drawback, Gomez and co-workers [53,54] developed the multiple-plug binding assay method. Several sample plugs are injected into the capillary discontinuity and their determinations are simultaneously performed in a single run. More recently, they expanded this technique to the multiple-step ligand injection as shown in Fig. 3 [55]. In this technique, a sample plug containing P (receptor) and non-interacting standards is injected and electrophoresed in a buffer containing a given concentration of D (ligand). Because the sequence is repeated at increasing concentrations of D , the ligand concentrations of each ligand plug in Fig. 3 are different. Consequently, multiple electropherograms at the different concentration of D are obtained in a single run as shown in Fig. 3. In order to obtain the binding constant, it is impossible to relate changes in μ at different ligand concentrations, hence the migration time ratio relative to the non-interacting standards is adopted. This technique has an advantage of much faster analyses than the conventional ACE method. In addition, the electroosmotic mobility is constant in the series of the experiments at the different concentrations of D .

3.4. Affinity capillary electrophoresis–mass spectrometry (ACE–MS)

Combinatorial technologies have been of great interest in drug discovery in recent years. In order to

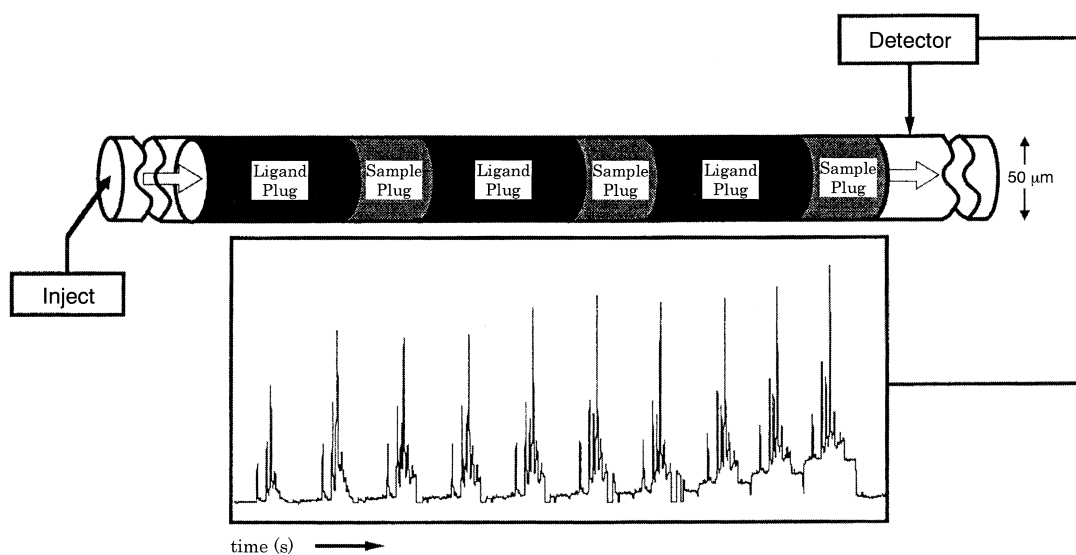


Fig. 3. Schematic illustration of a multiple-step ligand injection ACE. From Ref. [55], with permission.

identify active ligands from combinatorial mixtures, mass spectrometry (MS) is a promising detector. ACE has been already combined with MS for screening combinatorial libraries [56,57]. Even if the migration times of the ligands are the same, their binding constants can be easily measured with MS detection due to the different molecular masses. However, the current CE–MS instruments are insufficient for the determination of accurate binding constants as yet. In CE–MS, the detection end of the capillary must be introduced into the electrospray interface and cannot be immersed in the running buffer. Therefore, the reproducibility of the migration time may be worse as compared to conventional ACE with UV detection. However, the technology of the analytical instruments has made remarkable progress. As the performance of commercial CE–MS instruments is improved, ACE–MS will be a promising technique in binding studies.

4. Vacancy affinity capillary electrophoresis (VACE)

VACE is a relatively new method in CE for the estimation of binding constants [7,58]. The capillary is filled with a running buffer containing both D and P . The concentration of either D or P is fixed and

that of the other component is varied. The binding constants are estimated from the migration behavior of D as well as ACE. An advantage of the VACE is the fact that the absolute number of the binding sites, n_i , can be calculated from the change in μ_i and $[D]$ as compared to ACE [7]. In addition, VACE is particularly convenient for the estimation of binding constant of a weakly soluble D in water because the solubility should be increased in the running buffer containing P [7].

5. Frontal analysis (FA)

In frontal analysis (FA), the equilibrium between D and P is obtained in the sample vial. After injection of the equilibrated sample into the capillary filled with buffer electrolytes, the free and bound components are separated in the capillary.

McDonnell et al. [59] speculate that FA can be applied to the estimation of K between 10^3 – 10^8 M^{-1} . For weak interactions where K is approximately less than 10^3 M^{-1} , it will be experimentally difficult to measure the accurate height difference between the frontal zones of drug plus protein and drug. In this case, ACE may be preferable for the estimation of K . On the other hand, when K is larger than 10^8 M^{-1} , the $[D_f]$ will be low in comparison to the $[DP]$

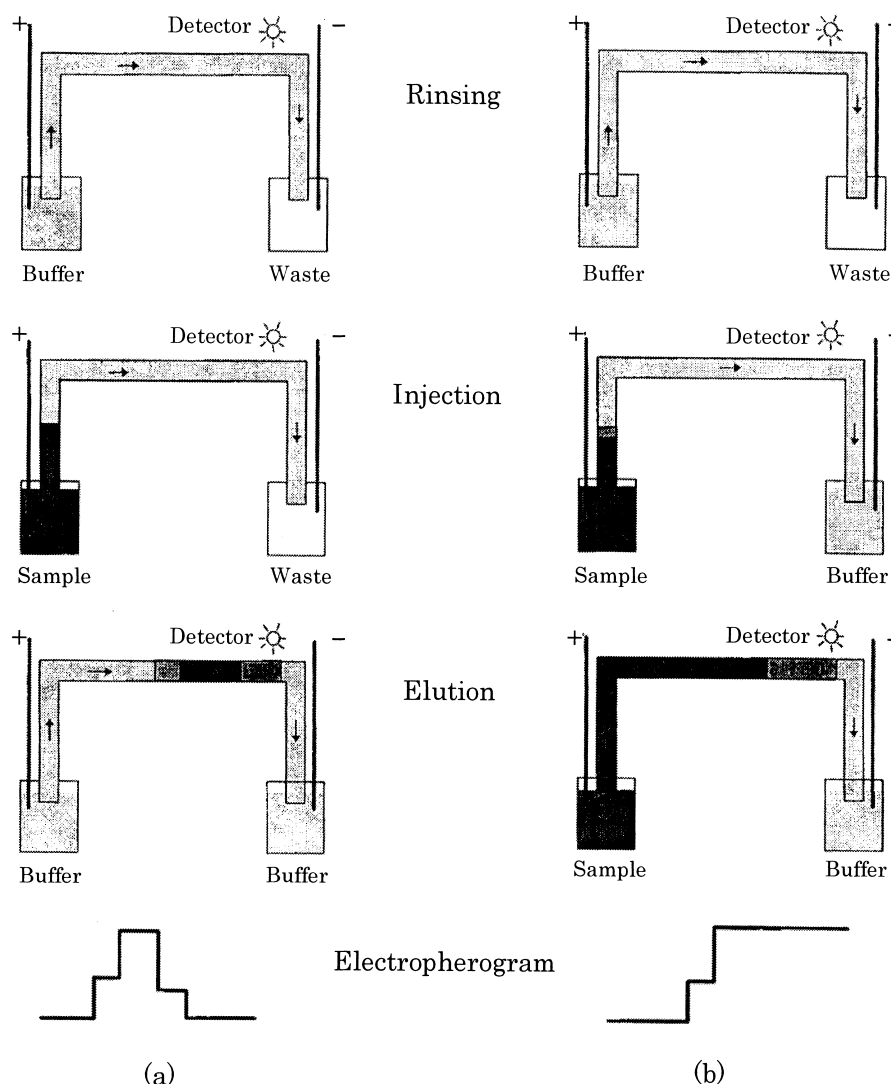


Fig. 4. Schematics of (a) conventional FA and (b) FACCE. From Ref. [11], with permission.

and the height of free D in the frontal peak will be too small.

FA has been also used for enantiomer separations [16,60]. As well as the ACE method, racemic drugs can be used for the estimation of each enantiomer's binding constant in FA. First, the enantiomer discrimination is achieved in the sample vial due to the enantioselective equilibrium between each enantiomer and P . Therefore, the different $[D_f]$ of each enantiomer is obtained as the function of the different K value. However, since both enantiomers are

simultaneously injected in the capillary, the free enantiomers must be subsequently separated with another chiral selector, such as CDs, dissolved in the running buffer. According to the successful enantiomer separation, the different elution peaks of each enantiomer are obtained. The plateau height of the elution peak is depended on the $[D_f]$ in the sample solution.

Recently, frontal analysis continuous capillary electrophoresis (FACCE) has been developed by Gao et al. [11] as a novel CE method. The principle

of FACCE and its schematic electropherogram are illustrated in Fig. 4. The capillary is filled and equilibrated with buffer containing P prior to the run, and the inlet end of the capillary is immersed in the sample solution containing D and P . In this method, a voltage is applied for the analysis keeping the sample solution as the inlet vial. The sample is continuously introduced into the capillary during the analysis and the separation process progresses at the same time. When the mobility of D is higher than that of P , the first plateau in the electropherogram is due to the elution of free D and the second plateau is due to free D and DP . A disadvantage of this method is greater sample consumption than the other CE methods. However, FACCE offers lower detection limits and is free from the reproducibility problems arising from slow binding kinetics as compared to the other CE methods, such as HD and conventional FA [11].

6. Conclusion

CE has been the most rapidly-growing analytical technique to study affinity interactions in recent years and the analytical methodology progresses continuously. As for the estimation of binding constants, a number of publications have been published in the last few years. Since CE features quick analysis, high efficiency, high resolving power, low sample consumption and wide range of possible analytes, it is beneficial for the estimation of binding constants such as drug–plasma binding. In this review, the mathematical equations for the estimation of binding constants and some recent advances in this field are described. In drug development, the investigation of drug binding affinity to the biomolecules will be increasingly important. The applicability of CE will be also expanded in the field of binding studies.

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