Physicochemical Profiling by Capillary Electrophoresis

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Abstract: The physicochemical properties of pharmaceuticals such as acid dissociation constant (pK_a), octanol-water partition coefficient (logP_{ow}), protein binding constant, inclusion complex constant with cyclodextrin (CD), and self-association are very important in drug design, candidate selection, and drug delivery. Capillary electrophoresis (CE) is a simple, versatile, automated, and powerful separation technique and widely applied in physicochemical profiling for pharmaceuticals. It has advantages over traditional potentiometric, spectrophotometric, chromatographic, and other methods, as CE requires very small amounts of sample and can measure compounds with impurities and low aqueous solubility. Principles and applications of CE in profiling various physicochemical properties will be reviewed.

Keywords: Capillary electrophoresis, Acid dissociation constant, Octanol-water partition coefficient, Drug-protein binding, Inclusion complex constant, Self-association.

1. INTRODUCTION

The pharmaceutical industry has faced increased pressure to reduce the high attrition rate of development compounds. It was estimated that approximately 41% of the drug candidate molecules failed due to poor biopharmaceutical properties [1]. Physicochemical properties such as acid dissociation constant (pK_a), octanol-water partition coefficient (logP_{ow}), solubility, permeability, protein binding are closely related to drug absorption, distribution, metabolism, excretion (ADME). During the early phase and later phase of drug development, knowledge of these physiochemical properties of the compounds in a timely manner will assist in candidate selection, formulation design, and drug delivery. There are various experimental and computational approaches in physicochemical profiling [2], [3]. Capillary electrophoresis (CE) has been widely used in physicochemical profiling [4], [5] and pharmaceutical analysis [6]. The present review will only cover the applications of CE in profiling pK_a, logP_{ow}, protein binding constant, inclusion complex constant, and self association.

2. PK_A PROFILING

2.1 Traditional Methods

It was estimated that 95% of drugs are ionizable [7]. Other physicochemical properties, such as lipophilicity and solubility, are pK_a dependent. Therefore, pK_a is one of the fundamental parameters of a drug molecule. Potentiometric and spectrophotometric methods are commonly used for pK_a determination [8]. In the potentiometric method, the requirement for sample concentration is usually greater than 10^{-4} M. The pK_a values are calculated from a difference curve of average number of bound protons vs. pH derived by subtracting the blank (without sample) titration curve from the sample titration curve. For poorly water soluble compounds, the pK_a values in aqueous solution are determined in organic-water mixtures by extrapolation to zero percent of organic solvent [9]. The spectroscopic method can determine pK_a values of compounds with large molar absorptivities to as low as 10^{-6} M. The sensitivity of the pK_a measurement depends on the spectral dissimilarity of the protonated and deprotonated forms of the compound. A high-throughput multiwavelength spectrophotometric titration method has been developed for pK_a determination with 4 min/assay [10]. Microscale pH-titrimetric and spectrophotometric methods for pK_a determination have been reported using micrograms of sample with 10-100 μL of solution [11]. Both potentiometric and spectrophotometric methods have difficulties in dealing with impure and unstable compounds. The pK_a measurement of water insoluble compounds is still a challenge for potentiometric titration method, especially with pK_a of less than 3. At early stage of drug discovery, the pK_a values can be predicted using various commercial programs [12]. The predicted values sometimes could be much off and can only be used as references. For novel classes of compounds, the pK_a values of few representative structures should be measured to validate the prediction program.

2.2 Capillary Electrophoresis

2.2.1 Principles

The CE method for pK_a determination was first introduced in early 1990s [13], [14]. Its applications have constantly increased in various areas [15]-[25], especially in the pharmaceutical industry [4], [26]-[33]. The pK_a determination of acids and bases by CE is based on measuring the electrophoretic mobility of charged species associated with the acid-base equilibria as a function of pH. In CE, the effective mobility of the compound is measured, which describes the overall electrophoretic mobility contributions from all of the charged forms that are resulted from the acid-base equilibria. Variation of electroosmotic flow (EOF) at different pH is corrected by using a neutral

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compound (e.g. methanol) as neutral marker or EOF marker. The effective mobility is calculated according to Eq. (**1**), where V is the applied voltage, L_d the effective capillary length to the detector, L_t the total capillary length, t_m the migration time of the analyte, and t_o the migration time of neutral marker due to the electroosmotic flow (EOF).

$$M_e = \frac{L_d L_t}{V}\left(\frac{1}{t_m} - \frac{1}{t_o}\right) \quad (1)$$

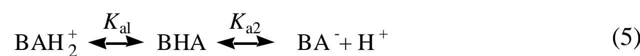
When a base, B, is protonated, the effective electrophoretic mobility of the base, M_e, is given by Eq. (**2**), where M_b is the electrophoretic mobility of the fully protonated base, BH^+. Eq. (**3**) can be derived based on Eq. (**2**), where K_a is the dissociation constant of BH^+ [14]. The third term in Eq. (**3**) is for ionic strength (*I*) correction using the Debye-Hückel equation, where Z is the valence of the ion and *a* is assumed to be 5 Å for the ion size parameter. The ionic strength correction term in Eq. (**3**) only applies to small molecules at low ionic strength [34]. For pK_a determination, the concentrations of most CE buffers are in the range of 10-50 mM. Similar to the pK_a of a protonated base (Eq. (**3**)), Eq. (**4**) can be derived for an acid (HA), where M_a is the electrophoretic mobility of the fully ionized species, A^-. Electrophoretic mobility is positive for bases and negative for acids that can be used as identification of acids and bases. However, CE is not a structure-specific technique. The assignment of pK_a to the ionization site needs common knowledge of chemistry, experience, and help from other structure-specific techniques such as ^{1}H-NMR [35]-[37].

$$M_e = \frac{[BH^+]}{[BH^+] + [B]} M_b \quad (2)$$

$$pK_a = pH + \log\left(\frac{M_e}{M_b - M_e}\right) - \frac{0.5085Z^2\sqrt{I}}{1 + 0.3281a\sqrt{I}} \quad \text{(base)} \quad (3)$$

$$pK_a = pH - \log\left(\frac{-M_e}{M_e - M_a}\right) + \frac{0.5085Z^2\sqrt{I}}{1 + 0.3281a\sqrt{I}} \quad \text{(acid)} \quad (4)$$

For compounds with multiple pK_a values, the general equations were described in the literature [16], [33]. Most pharmaceuticals have less than three measurable pK_a values. For zwitterions (Eq. (**5**)) and doubly protonated bases (Eq. (**7**)), Eqs. (**6**) and (**8**) can be derived, respectively, where M_{BAH2}, M_{BA}, M_{BH2}, and M_{BH} are the electrophoretic mobilities of the corresponding charged species; the numerical values of 0.04 and 0.16 are for the ionic strength correction when the ionic strength of the buffers is 0.01 M. For compounds with double bases and mono acid as in Eq. (**9**), the effective mobility as a function of pH is described in Eq. (**10**), where M_{BAH3} is the mobility of the double protonated form. When the number of data points is limited, the calculation of multiple pK_a values could be difficult by non-linear curve fitting due to many parameters. In this case, the number of parameters can be reduced assuming $M_{BAH2} = -M_{BA}$ and $M_{BH2} = 2M_{BH}$ based on the charge to size ratio.



$$BAH_2^+ \overset{K_{a1}}{\rightleftharpoons} BHA \overset{K_{a2}}{\rightleftharpoons} BA^- + H^+ \quad (5)$$

$$M_e = \frac{M_{BAH_2} + M_{BA}10^{(2pH - pK_{a1} - pK_{a2})}}{1 + 10^{(pH - pK_{a1} - 0.04)} + 10^{(2pH - pK_{a1} - pK_{a2})}} \quad (6)$$

$$BH_2^{2+} \overset{K_{a1}}{\rightleftharpoons} BH^+ \overset{K_{a2}}{\rightleftharpoons} B + H^+ \quad (7)$$

$$M_e = \frac{M_{BH_2} + M_{BH}\,10^{(pH - pK_{a1} + 0.04)}}{1 + 10^{(pH - pK_{a1} + 0.04)} + 10^{(2pH - pK_{a1} - pK_{a2} + 0.16)}} \quad (8)$$

$$BAH_3^{2+} \overset{K_{a1}}{\rightleftharpoons} BHA_2^+ \overset{K_{a2}}{\rightleftharpoons} BHA \overset{K_{a3}}{\rightleftharpoons} BA^- + H^+ \quad (9)$$

$$M_e = \frac{M_{BAH_3} + M_{BAH_2}10^{(pH - pK_{a1})} + M_{BA}10^{(3pH - pK_{a1} - pK_{a2} - pK_{a3})}}{1 + 10^{(pH - pK_{a1})} + 10^{(2pH - pK_{a1} - pK_{a2})} + 10^{(3pH - pK_{a1} - pK_{a2} - pK_{a3})}} \quad (10)$$

2.2.2 Experimental Conditions

The CE experiments can be performed on the commercial available CE systems mostly with a diode array UV/Vis detector [26]. The uncoated fused silica capillaries with ID of 50 and 75 μm are widely used. The temperature of the capillary is usually controlled by air- or liquid-cooling systems. For the current commercial CE systems, voltage up to +30 kV can be applied for separation. Prior to the first run of each buffer, the capillary is usually regenerated by flushing with 0.1 or 1.0 M NaOH for 15-30 min and water for 5-10 min to ensure reproducibility. Then the capillary is equilibrated with the run buffer by flushing for 2-5 min. Then each of the samples is analyzed sequentially in a given pH buffer before proceeding to the next buffer. Because only the migration time is measured not the peak area, the injection volume can be varied and should be kept as low as possible. If too much sample is injected, the local pH of the buffer in the capillary could be changed and compound precipitation may occur with changing buffer pH.

Samples are prepared in deionized water with small amount of neutral marker such as dimethyl sulfoxide (DMSO), methanol, mesityl oxide (MESO), or acetone. DMSO is mostly used as the neutral marker due to its popularity in drug discovery, strong short-wavelength of absorption (<230 nm) and low volatility [26]. The data points on the titration curve within the pH of pK_a ± 1 are very important for the pK_a determination. In most cases, twelve pH buffers ranging from pH 2 to 11 prepared from acetate, phosphate, and borate will cover the pK_a values for most pharmaceuticals with enough accuracy [26], [33]. For compounds with multiple pK_a values close to one another, more pH buffers with narrow spaced pH may be needed to obtain a good curve fitting. Commonly used CE pH buffers are listed in Table **1**. The ionic strength of the pH buffers should be controlled at constant. After filtering through a 0.22-μm membrane filter and stored in the refrigerator at 4 °C, the pH buffers are stable for few months but the pH should be checked regularly.

2.2.3 Data Analysis

After CE separation, the effective mobilities of analytes are determined at different buffer pH using Eq. (**1**). The

Table 1. CE pH buffer preparation [a)]

pH range	Constituent	Stock solution [b)]	Ionic strength
2.0−3.0	Phosphate	0.5 M NaH_2PO_4 0.5 M H_3PO_4	0.01 M
3.8−5.8	Acetate	0.5 M NaOAc 0.5 M HOAc	0.01 M
6.0−8.0	Phosphate	0.1 M Na_2HPO_4 0.5 M NaH_2PO_4	0.01 M
8.5−9.5	Borate	0.05 M $Na_2B_4O_7$ 0.2 M H_3BO_3	0.01 M
9.5−11.0	Borate	0.05 M $Na_2B_4O_7$ 0.10 M NaOH	0.01 M

[a)] reference [26].
[b)] Stock solutions were mixed in appropriate ratios and diluted to I = 0.01 M.

thermodynamic pK_a values can be obtained from non-linear regression of effective mobility, M_e, as a function of pH in accord with model equations such as Eqs. (**3**), (**4**), (**6**), or (**8**). A representative plot of effective mobility as a function of pH is shown in Fig. (**1**) for the determination of pK_a of a Roche compound that has two bases and one acid. Non-linear curve fitting was performed by SigmaPlot program according to Eq. (**10**). The selection of the model equation is critical for data analysis and should be based on the M_e-pH curve and the structure of the compound. The results obtained from non-linear curve fitting should also be a reasonable match for the structure. For compounds with similar molecular weight, their effective mobilities with the same charge are similar. Semi-empirical relationships between effective mobility, charge (Z_c), and molecular weight (MW) have been reported: $M_e = 0.1853(Z_c/MW^{0.4920})$ (for anions) and $M_e = 0.3888(Z_c/MW^{0.6330})$ (for cations) [38]. These equations can only be applied to pH buffers with ionic strength of 0.05 M. The effective mobility is very sensitive to ionic strength of the pH buffers. For pH buffers with ionic strength of 0.01 M, we found that $M_e = 0.523/MW^{0.58}$ for mono-anions and $M_e = 0.433/MW^{0.52}$ for mono-cations based on the pK_a measurement of hundreds pharmaceuticals.

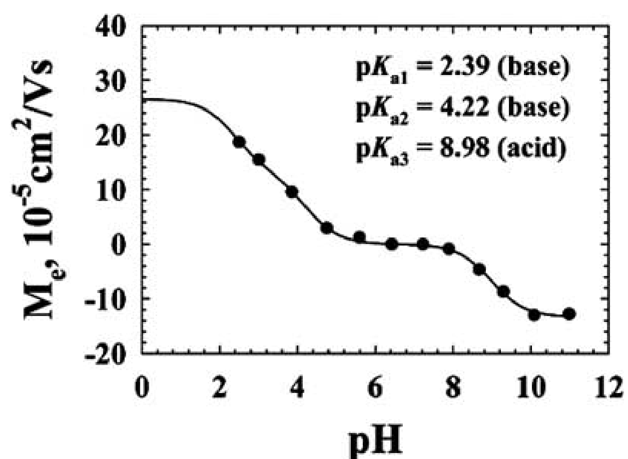


Fig. (1). Effective electrophoretic mobility as a function of pH for a Roche compound with 3 pK_a values (two bases and one acid). Solid circle (•): experimental data; solid line (-): non-linear curve fitting.

2.2.4 Approaches for High Throughput

For pK_a profiling of pharmaceuticals in the discovery setting, throughput is critical. One major problem with conventional CE methods is the long migration time at low buffer pH due to diminished electroosmotic flow (EOF) [39]. Under this circumstance, the electrophoretic mobilities of anions may exceed the EOF in the opposite direction and, therefore, the anionic analytes may never reach the detector at the cathode. To overcome this problem, it is necessary to reverse the voltage polarity or use dynamic coating of a positively charged polymer to reverse the EOF [40], [41], which can only be used for anionic compounds. These strategies are not applicable for rapid pK_a screening since the acid-base nature is either unknown or variable.

The unique feature of the CE-pK_a method has drawn serious attention from the pharmaceutical industry in developing it into a high throughput pK_a assay. A medium-throughput pK_a screening assay was developed by Jia, Ramstad, and Zhong [26] using pressure-assisted capillary electrophoresis (PACE). The applied external pressure does not affect the effective mobility and pK_a measurement when it is less than 2 psi. The throughput of this PACE method was reported to be 20 compounds per day with an accuracy of 0.2 pK_a units comparing to literature values. The pK_a values of some compounds determined by PACE are listed in Table **2**, which are in good agreement with the literature values [26]. A similar throughput using PACE was also reported [4]. Throughput can be improved to 36 or 96 compounds per day when short capillaries and less buffers were used [28], [29]. However, the current throughput of PACE with single capillary is limited by the low applied air pressure, the number of pH buffers, and the length of capillary in order to obtain reasonable CE separation and enough points for non-linear curve-fitting of M_e-pH equations. More recently, a high throughput pK_a screening assay has been reported using multiplexed capillary electrophoresis with absorbance detection [42]. The 96-capillary array CE instrument introduced by CombiSep (Ames, IA, USA, www.combisep.com) can simultaneously determine the pK_a values for 24 compounds (12 pH points) in 1 h.

2.2.5 pK$_a$ in Non-aqueous and Organic Solvent-water Mixtures

Capillary electrophoresis using non-aqueous solvents and organic-solvent mixtures has been increasingly applied for the separation of a wide range of analytes [43]-[45]. Knowledge of the ionization behaviors (pK_a) of the analytes in non-aqueous and organic solvent-water mixtures is essential in method development and understanding of the separations in RP-HPLC [46]-[49] and CE [50]-[54]. Mobility and ionization behavior of different bases have been measured in methanol [50], [52] and acetonitrile [51] by CE using background electrolytes (BGEs) based on a conventional pH scale. The pH scale was established from acids with know conventional pK_a* values in this solvent

Table 2. Comparison of pK_a values determined by PACE with literature values [a)]

Compound	pK_{a1} pK_{a2} (PACE)	pK_{a1} pK_{a2} (Literature)	Difference
Anthranilic acid	2.30 5.02	2.09 4.79	0.21; 0.23
Tryptophan	2.30 9.43	2.38 9.39	–0.08; 0.04
Tyrosine	2.30 9.23	2.18 9.21	0.12; 0.02
Nicotine	3.00 8.29	3.12 8.02	–0.12; 0.27
2-Fluoroaniline	3.00	3.20	–0.20
Salicylic acid	3.02	2.98	0.04
2, 6-Dinitrophenol	3.78	3.71	0.07
4-Bromoaniline	3.91	3.88	0.03
Quinine	4.13 8.39	4.11 8.52	0.02; –0.13
2, 4-Dinitrophenol	4.14	4.08	0.06
Benzoic acid	4.26	4.20	0.06
trans-Cinnamic acid	4.47	4.44	0.03
Quinidine	4.50 8.57	4.00 8.54	0.50; 0.03
Aniline	4.61	4.60	0.01
Pyridoxine	4.80 8.30	4.84 8.87	–0.04; –0.57
1, 10-Phenanthroline	4.94	4.86	0.08
Quinoline	4.97	4.80	0.17
N-Methylaniline	5.02	4.85	0.17
Warfarin	5.15	5.03	0.12
2-Benzylpyridine	5.22	5.13	0.09
2, 5-Dinitrophenol	5.22	5.22	0
Pyridine	5.31	5.17	0.14
p-Anisidine	5.38	5.36	0.02
Acridine	5.52	5.60	–0.08
Benzimidazol	5.56	5.53	0.03
Isoquinoline	5.59	5.40	0.19
2, 6-Dimethylquinoline	6.01	5.46	0.55
3, 5-Lutidine	6.12	6.09	0.03
2-Methylbenzimidazole	6.20	6.19	0.01
Papaverine	6.21	6.39	–0.18
Flumequine	6.38	6.38	0
4-Chloro-2-nitrophenol	6.39	6.45	–0.06
2, 5-Lutidine	6.53	6.43	0.10
3, 4-Lutidine	6.60	6.47	0.13
2, 6-Lutidine	6.67	6.71	–0.04
2, 4-Lutidine	6.79	6.74	0.05
Pilocarpine	6.92	6.85	0.07
4-Nitrophenol	7.05	7.15	–0.10
2, 4, 6-Collidine	7.54	7.43	0.11
2-Chlorophenol	8.57	8.55	0.02
1-Phenylpiperazine	8.60	8.71	–0.11
4-Aminopyridine	9.02	9.11	–0.09
3-Chlorophenol	9.10	9.10	0
Atenolol	9.43	9.58	–0.15
4-Chlorophenol	9.44	9.43	0.01
2-Methylbenzylamine	9.48	9.48	0
3-Methylbenzylamine	9.57	9.54	0.03
2-Methoxybenzylamine	9.96	9.92	0.04

[a)] reference [26].

used as buffers. Conventional pK_a* values are measured in a cell without liquid junction by taking into account the activity of chloride ion derived from the Debye-Hückel equation. The asterisk (*) indicates that infinite dilution is selected as standard state. For example, acetic acid, chloroacetic acid, dichloroacetic acid, and trichloroacetic acid have known pK_a* values of 9.7, 7.8, 6.3, and 4.9 in methanol, respectively [51]. The BGEs were prepared by mixing equimolar of acids with their respective salts in the organic solvents [52], which should have pH values equal to the pK_a* of the acids without any further calibration. Actual mobilities (mobilities for fully charged analytes) were measured in acidic organic solvent solutions containing perchloric acid. The pK_a* values in organic solvents were derived from the measured mobilities as a function of pH of the BGEs. The pK_a* values of basic drugs in acetonitrile were reported to increase from 5.2 to 8.9 pK_a units compared to that in water, while the pK_a* values in methanol are only 1 to 2.5 pK_a units higher than in water [50]-[52]. For acids, the pK_a* values in methanol can be 5 pK_a units higher than in water. The pK_a values of organic bases were also measured in aqueous acetonitrile solution [53] and aqueous methanol solution [54]. The pK_a values of bases decrease with acetonitrile and methanol concentrations and shift from the aqueous pK_a values up to -0.8 pK_a units in 60% acetonitrile and -1.05 pK_a units in 70% methanol. The aqueous pK_a values obtained by extrapolation to zero organic solvent are in good agreement with the literature aqueous pK_a values. This could be used for aqueous pK_a determination of water-insoluble compounds. Again, multiplexed capillary electrophoresis can provide much higher throughput using co-solvent buffers, in which two compounds could be analyzed over 12 pH values and four different methanol concentrations in one CE run [42].

3. LOGP$_{OW}$ PROFILING

3.1 Traditional Methods

Hydrophobic interaction plays a significant role in partitioning into lipid bilayers of biomembranes, bioavailability, and pharmacokinetics of drugs. Solute

hydrophobicity is usually expressed by the thermodynamic 1-octanol-water partition coefficient (P_{ow}) that is defined as the ratio of the concentrations of a species in the two phases at equilibrium. The symbol P_{ow} refers to the partition of the neutral form only. When a compound is ionizable, the partition coefficient at a specific pH is represented by the symbol D that refers to the partition of all the neutral and ionized forms. Extensive data collection of log P_{ow} values can be found in the literature [55]-[57].

A number of direct and indirect methods have been applied for log P_{ow} measurement [58], [59]. Conventional shake-flask method was historically considered to be the gold standard assay for direct measurements of log P_{ow}. The conventional shake-flask method is time consuming and tedious, requires highly pure compounds, and is difficult to adjust for highly hydrophobic compounds with log $P_{ow} > 4$. A high throughput version of shake-flask method has been developed using 96-well plate technology and auto-sampling [60]. Potentiometric titration technique has also been commercialized for log P_{ow} measurement based on pK_a shift from aqueous phase to octanol/water phase [61]-[63]. An indirect method for estimation of log P_{ow} values is reversed-phase liquid chromatography (RP-LC) utilizing the linear relationship between log P_{ow} and retention factor, log k [64]-[69]. The RP-LC method has some advantages such as small sample size, speed, high sample throughput, no requirement for highly pure samples, better reproducibility, wider dynamic range, and feasibility for automation. A throughput over 1000 compounds/day using LC/UV/MS has been reported [70]. However, there are also some disadvantages in the RP-LC method. RP-LC is a two-phase separation technique and variation exists due to changes in column and mobile phase composition. It is also difficult for log P_{ow} determination of compounds with ionizable or special functional groups because of specific interactions with the free silanols on the stationary phase.

There are about 30 to 50 commercially available programs for log P_{ow} calculation [71]. These programs often generate different log P_{ow} values due to different data sets used for the calibration. The overall error in log P_{ow} calculation is in the range of 0.5 to 1.5 log units. Sometimes the calculation error is very large especially for the novel and complex structures. One of the atom/fragment contribution methods, KowWin (Syracuse Research Corp., North Syracuse, NY, USA), is able to predict log P_{ow} within 0.8 log units [72].

3.2 Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC) is an electrophoretic technique using a micellar solution as a running buffer solution [73]. It is an analytical technique with combined features of conventional chromatography and capillary electrophoresis, which enables the separation of neutral and charged analytes. Solute separation is based on the mobility differences as in CE and differential partitioning between the micelles and the BGE comparable to chromatography [74]-[77]. Sodium dodecyl sulfate (SDS) is the most widely used surfactant in MEKC because of its high aqueous solubility, low critical micelle concentration (CMC), low Kraft point, low UV molar absorptivity, availability, and low cost. Micellar pseudo-stationary phases are considered to be more structurally similar to biomembranes than 1-octanol or RP-LC stationary phases and their compositions can be easily adjusted. MEKC has been recently used for hydrophobicity/lipophilicity assessment [78]-[86]. Herbert and Dorsey [82] have measured log k values for over 100 compounds by MEKC that correlate well with log P_{ow} ($R^2 = 0.835$) for 9 orders of magnitude in log P_{ow}. A number of papers [78]-[81] have also reported good correlation of log k values measured by MEKC with log P_{ow}. However, congeneric behavior was observed in the estimation of log P_{ow} for different groups of solutes by MEKC [84], [85].

3.3 Microemulsion Electrokinetic Chromatography

Microemulsion electrokinetic chromatography (MEEKC) is also one of the electrokinetic chromatography (EKC) techniques like MEKC. Similar to MEKC, the separation principle of MEEKC is based on differential partitioning into the oil droplets [87]-[90]. In MEEKC, the microemulsions are solutions containing dispersed nanometer-sized oil droplets of a water-immiscible liquid such as heptane. The oil droplets are coated with a surfactant (e.g., SDS) and a co-surfactant (e.g., 1-butanol) to reduce the surface tension between the oil droplets and water that allows the emulsion to form. This microemulsion system was more similar to the phospholipid vesicle than the octanol-water and SDS micellar systems. More recently, research interests have grown in the use of MEEKC for log P_{ow} estimation [91]-[102]. It was reported that MEEKC using the SDS/butanol/heptane microemulsion system provided better estimation of log P_{ow} than MEKC using the SDS micellar system [91]. Even though the partition mechanism in EKC was not fully understood [75], [76], results from linear solvation energy relationship (LSER) analysis suggested that the SDS/butanol/heptane microemulsion system is a good model for octanol-water partition [94]. Literature results also demonstrated that log k values measured by MEEKC in the SDS/butanol/heptane microemulsion system were highly correlated with log P_{ow} ($R^2 > 0.96$) over 5–8 orders of magnitude in log P_{ow} [91], [93], [96], [97].

A typical microemulsion in MEEKC for log P_{ow} measurement of bases consists of 50 mM SDS, 0.87 M 1-butanol, 82 mM heptane, and 50 mM borate-phosphate (2:3) at pH 10 [101]. High pH buffers are used to generate high EOF and ensure charge neutrality of weakly basic compounds. For weakly acidic compounds, a pH 3 and a sulfonated or dynamically coated silica capillary are required to ensure charge neutrality of the weakly acidic compounds and maintain a strong EOF [96], [100]. The separation window in MEEKC is defined by using a double internal marker approach. The samples are prepared with the two markers in microemulsion. Highly water-soluble neutral compounds such as DMSO are used as the EOF marker, which will predominantly reside in the aqueous phase and will be swept by the EOF. A highly water-insoluble compound such as dodecanophenone is used as the micelle marker, which will predominantly partition into the negatively charged oil droplets and move with the oil droplets. The compound will migrate between the DMSO

Table 3. MEEKC calibration standards [a)]

Standard Compound	log *k* (MEEKC)	log P_{ow} (literature)	pK_a (CE)
Pyrazine	–1.25	–0.26	< 2
Benzamide	–0.49	0.64	None
Nicotine	–0.12	1.17	3.2; 8.75
Indazole	0.13	1.77	~ 1.6
Benzocaine	0.22	1.86	2.63
4-Chloroaniline	0.29	1.88	3.98
Lidocaine	0.61	2.26	7.92
Pyrilamine	0.96	3.27	3.99; 9.18
Impramine	1.71	4.42	9.21
Pyrene	2.17	4.88	None

[a)] reference [101].

peak and the dodecanophenone peak depending on its lipophilicity.

For log P_{ow} measurement of bases, a set of standard compounds (Table **3**) with know log P_{ow} values are used for constructing the calibration curve of log P_{ow} against log *k* [101]. A list of standard acidic compounds has been used for log P_{ow} measurement of acids [4]. A typical electropherogram is shown in Fig. (**2**) for the separation of the 10 standard basic compounds [101]. The MEEKC retention factor, *k*, is calculated according to Eq. (**11**), where t_R, t_{EOF}, and t_{MC} are the migration time of the compound, the EOF marker, and the micelle marker, respectively. The log P_{ow} values for the compounds of interest were calculated based on the measured log *k* and the calibration curve.

$$k = \frac{t_R - t_{EOF}}{(1 - \frac{t_R}{t_{MC}})t_{EOF}} \tag{11}$$

For log P_{ow} determination in MEEKC, it is very important that the compounds are neutral in the microemulsion and only the partitioning of the neutral form is involved. For strongly basic compounds with pK_a 10, large errors (0.5–1.0 log units) were observed due to a mixed partitioning mechanism [101]. In the microemulsion at pH 10, these basic compounds are partially protonated. In addition to partitioning into the oil droplets, the positively charged solutes will have ion-pair interactions with the negatively charged oil droplets and surfactants to increase the migration time and also have electrophoretic mobility to reduce the migration time. Correction of the charged fraction based on electrophoretic mobility measurement has been applied when ion-pair interaction is not involved or very weak [95]. Surface active compounds such as chlorpromazine may form micelles that could change partition mechanism [101]. Errors could also be caused due to poor separation with the markers if the compounds have very low or very high log P_{ow} values. The log P_{ow} values of some compounds measured by MEEKC are listed in Table **4** and compared with the literature values [101]. The range of log P_{ow} values that MEEKC can be measured is usually 0-5 [101], [102].

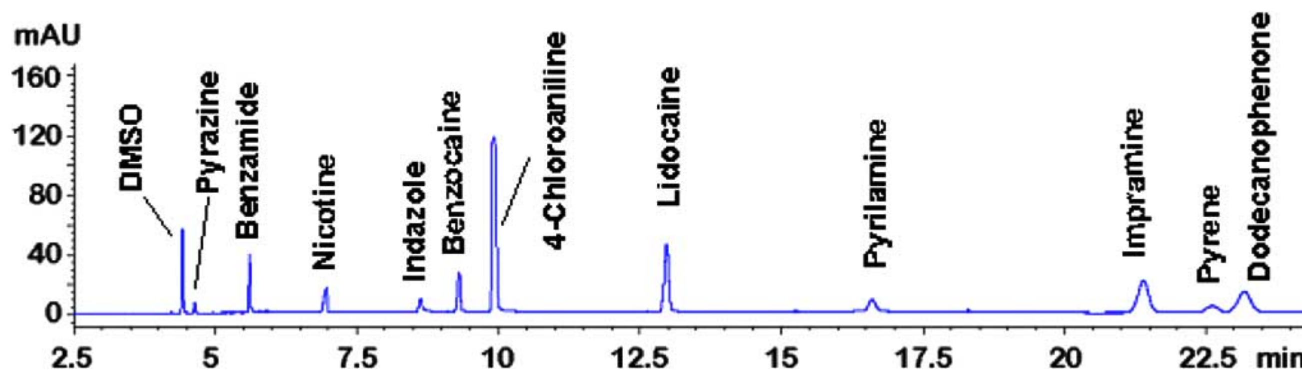


Fig. (2). Electropherogram obtained by MEEKC for the mixture of 10 standard compounds. Capillary, L_d = 40 cm, L_t = 50 cm, ID = 50 µm; separation, +20 kV, 5 mbar; 25.0 °C; injection, 25 mbar, 3 s; detection, 200 nm; microemulsion, 50 mM SDS, 0.87 M 1-butanol, 82 mM heptane, 50 mM borate/phosphate (2:3), pH 10; EOF marker, 0.2% (v/v) DMSO; micelle marker, 0.5 mg/mL dodecanophenone. Reference [101].

Table 4. log P_{ow} values measured by MEEKC *vs*. literature values [a)]

Compound	log *k* (MEEKC)	Log P_{ow} (MEEKC)	log P_{ow} (literature)	log P_{ow}	pK_a (CE)
Atenolol	–0.67	0.49	0.15	0.34	9.58
Pilocarpine	–0.57	0.66	0.20	0.46	7.08
Aniline	–0.43	0.88	0.94	–0.07	4.61
N-methylaniline	–0.07	1.46	1.65	–0.20	4.86
Acebutolol	0.14	1.79	1.75	0.04	9.41
Procaine	0.29	2.02	2.03	–0.01	9.04
Quinoline	0.22	1.91	2.15	–0.24	4.97
Quinidine	0.71	2.71	2.64	0.07	4.5; 8.57
Buspirone	0.71	2.70	2.78	–0.08	7.6
Papaverine	0.61	2.54	2.91	–0.37	6.38
3-bromoquinoline	0.82	2.88	2.91	–0.03	2.74
Propranolol	1.06	3.25	3.35	–0.10	9.53
Chlorpromazine	1.88	4.56	5.34	–0.78	9.24

[a)] reference [101].

The CE procedures for rinsing, injection, and separation are similar to the pK_a measurement. In MEEKC separations, the migration behavior of a solute is very sensitive to the surface condition of the capillary. Preconditioning of the capillary is very important for reproducibility. It was reported that variation of the slope and intercept is within 10% from day-to-day measurements [101]]. Therefore, calibration standards should be run in the same sequence with the samples to minimize errors. Pressure-assisted MEEKC using external air pressure can improve throughput to 2 samples/h using a single capillary system [101]. For high throughput, 96-capillary multiplexed microemulsion electrokinetic chromatography (MMEEKC) is the method of choice and can provide a throughput of 46 samples/h [102].

Other EKC techniques using liposome (LEKC) [103] and vesicles (VEKC) [104], [105] have also been applied for lipophilicity estimation. Because liposome and vesicles are difficult to make, they are only used for special cases.

4. DRUG-PROTEIN BINDING CONSTANT

4.1 Overview

Drug-protein binding is an important process in determining the activity and fate of a pharmaceutical agent once it enters the body [106]-[108]. It is also a complex issue that can have both positive (protection from first-pass metabolism, carrier to target tissues) and negative (restricted access to target tissues, drug-drug interaction) implications in drug discovery. The degree of drug-protein binding can have significant impact on a number of critical parameters, including determination of margins in safety assessments/toxicology studies, the efficacy of the drug, drug metabolism and pharmacokinetics, drug-drug-interactions, blood-brain barrier penetration. It is well recognized that the pharmacological activity of a drug is 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: 66500) and $_1$-acid glycoprotein ($_1$-AGP; 0.5-1.0 mg/mL in plasma; MW: 40000) are the two most important drug binding proteins in plasma [109]-[111]. HSA has two major binding sites (warfarin-azapropazone site and indole-benzodiazepine site) and is largely responsible for the binding of acidic drugs, where 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 has been used for protein-drug binding measurement, such as equilibrium dialysis [112]-[114], ultrafiltration [113], [115], ultracentrifugation [113], calorimetry [116], tryptophan fluorescence quenching (TFQ) [117]-[119], surface plasmon resonance (BIACORE) [120], liquid chromatography [121]-[123], and capillary electrophoresis (CE) [124]-[139]. Each method has advantages and disadvantages. However, caution should be taken when comparing the results among the techniques because each method measures specific physical properties under certain conditions and correlates them to the protein binding parameters. The 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.

Equilibrium dialysis and ultrafiltration, both membrane based separation methods, are the two most commonly used conventional techniques. The drug concentrations on both sides of the membrane are directly measured. Equilibrium dialysis is often regarded as the reference method for the

determination of drug-protein binding. Oravcová, Bohs, and Liondner [140] made a comparison among equilibrium dialysis, ultrafiltration, and ultracentrifugation in terms of interfering factors and shortcomings. The adsorption of proteins to the membrane and leakage of bound drug through the membrane are problematic for equilibrium dialysis and ultrafiltration. 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. A high-throughput assay based on the distribution of drugs between plasma water, plasma proteins, and solid-supported lipid membranes was reported for the determination of free fraction of drugs strongly bond to plasma proteins [141]. By measuring changes in heat flow that occur for binding interactions, 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, the buffer for drug and protein must match, which is problematic for drugs with poor aqueous solubility. HSA contains a single tryptophan (Trp) residue which is conserved among species. In tryptophan fluorescence quenching (TFQ), the Trp is excited at 285 nm and the emission fluorescence is monitored as the drug is titrated into the system. The fluorescence of the intrinsic Trp in HSA is quenched by the drugs that interact closely to the Trp. The binding constant is measured by fitting the drug concentration vs. fluorescence profile. TFQ is a domain specific binding assay that is susceptible to interference from fluorescent compounds. It cannot measure the binding constants for compounds that bind at sites distant from the Trp such that quenching is not detected. However, TFQ is suitable for high-throughput screening in a multiwell format [119]. The BIACORE technique measures the refractive index changes at a solution/surface interface and 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.

Chromatographic methods can be divided into two main groups: affinity chromatography using immobilized protein stationary phases [121], [141]-[146] and size-exclusion chromatography using soluble proteins [122], [123], [147]. These techniques have been extensively reviewed previously [134], [148]. The affinity chromatography is used for measuring drug bound percentage based on a correlation of the chromatographic retention on the immobilized protein stationary phase and the percentage of drug bound. A set of standards with known percentage bound are used for calibration. 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 of drug to the column. The affinity chromatographic method has high-throughput capability and can be used for ranking order or identifying high serum protein binding liabilities. Several variants of the size-exclusion chromatographic methods have been described: frontal analysis (FA), Hummel-Dreyer (HD), vacancy peak (VP), and retention analysis. Frontal analysis and Hummel-Dreyer methods are the two most used size-exclusion chromatographic methods. In the frontal analysis method, a large volume of drug-protein mixed solution is directly injected on to a restricted-access HPLC column which excludes large molecule of plasma protein but retains the drug of small molecule size [130], [147]. An equilibrium zone is generated near the top of the column, where the chromatographic partition equilibrium inside the micropores and the drug-protein binding equilibrium outside the micropores are established simultaneously. The unbound drug is separated from the protein and eluted as a trapezoidal peak with a plateau region. From the plateau height that corresponds to the unbound drug concentration and the known total drug concentration, the relative amount of drug bound per protein can be calculated. In the Hummel-Dreyer method, a know amount of protein is injected onto a size-exclusion HPLC column and eluted with a mobile phase that contains a constant concentration of drug [148]-[150]. If the protein and drug have rapid association/dissociation kinetics and the protein-drug complex and free drug have different retention times, a trough will be generated in the drug concentration of the mobile phase. A positive peak corresponding to the protein and the protein-drug-complex followed by a negative peak representing the free drug will appear on the chromatogram. Both frontal analysis and Hummel-Dreyer methods can measure drug-protein binding constant by varying the drug/protein molar ratio.

4.2 Capillary Electrophoresis

The electrophoretic techniques have been reviewed periodically regarding the principles, limitations, and advances for the determination of drug-protein binding constants [140], [150]-[158]. Advantages of CE methods over chromatographic methods include its resolving power and small amounts of proteins and drugs required. The major limitation of the CE methods is its low sensitivity. In the electrophoretic techniques, the separation of the free drug, the free protein and the drug-protein complex is based on their differences in electrophoretic mobilities due to the net charge and size differences. Analogous to the chromatographic methods, five CE methods have been used for drug-protein binding studies: affinity capillary electrophoresis (ACE), Hummel-Dreyer (HD), frontal analysis (FA), vacancy peak (VP), and vacancy affinity electrophoresis (VACE). Frontal analysis appears to be the preferred method [151]. It is simple, robust, easy to implement, can deal with multiple equilibria, and requires less reagents than all the other methods [152].

4.2.1 Frontal Analysis

The schematic representation of the CE/FA method is shown in Fig. (**3**) [135]. 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 free 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

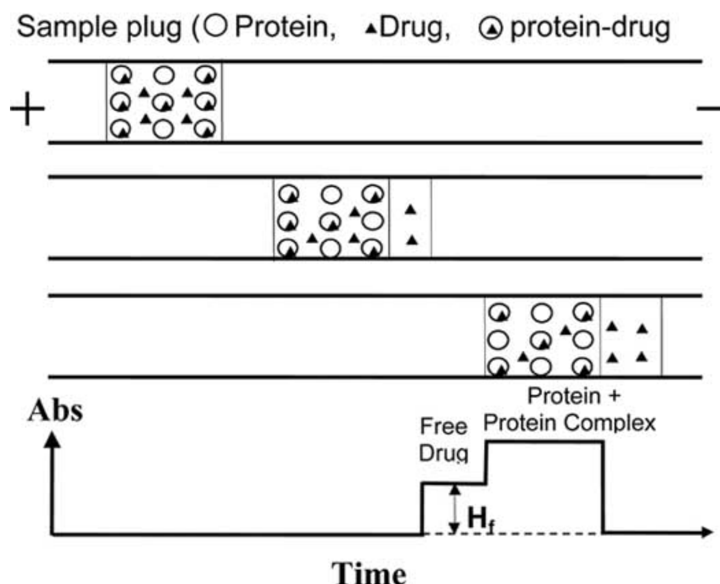


Fig. (3). Schematic representation of CE-FA method. H_f, height of the free drug plateau.

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. (**12**)), 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.

$$[D]_f = \frac{[D]_s}{H_s} H_f \tag{12}$$

For 1 : 1 protein-drug binding equilibrium in Eq. (**13**), the protein-drug binding constant, K_{PB}, is defined as in Eq. (**14**), 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. (**14**) and rearrangement will give Eq. (**15**). The drug bound concentration, $[D]_b$, can be obtained by solving the quadric Eq. (**15**). The percentage of drug bound to a protein can be calculated from Eq. (**16**). 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. (**17**) by substituting $[D]_b$ obtained from Eq. (**15**) into Eq. (**16**). The protein-drug binding constants can be obtained by non-linear curve fitting of the drug bound percentage as a 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. A typical binding curve of HSA-diazepam with increasing diazepam concentration is shown in Fig. (**4**) [135].

$$P + D \overset{K_{PB}}{\longleftrightarrow} PD \tag{13}$$

$$K_{PB} = \frac{[PD]}{[P]_f [D]_f} \tag{14}$$

$$K_{PB}[D]_b^2 - K_{PB}([D]_t + [P]_t + 1)[D]_b + K_{PB}[P]_t[D]_t = 0 \tag{15}$$

$$\%\ \text{Drug Bound} = \frac{[D]_b}{[D]_t} \times 100 \tag{16}$$

The CE-FA method has been widely used in drug-protein binding studies for various proteins and drugs [130]-[139]. Protein binding constants measured by CE-FA for various compounds and proteins are listed in Table **5**. The CE-FA method is mainly used for basic, neutral, and weakly acidic drugs because these drugs have positive or zero charges and are readily separated from the negative charged proteins under physiological pH [130], [135]. Strong acidic drugs are difficult to separate from the proteins because they are all negatively charged and have similar electrophoretic mobilities. Cyclodextrins (CD) modified EKC was applied for protein binding studies of acidic drugs [136]. The acidic drugs are able to separate from the proteins by interacting with the CD to cause mobility shift. McDonnell, Caldwell, and Masucci applied CE-FA to determine the binding capacity of -adrenoceptor blocking drugs to individual serum proteins, serum mixtures, and human serum [131]. The results show that HSA, AGP, high density lipoprotein (HDL), and low density lipoprotein (LDL) are the main contributors to serum binding for these drugs. In CE-FA,

$$\%\ \text{Drug Bound} = \frac{(K_{PB}[D]_t + K_{PB}[P]_t + 1) - \sqrt{(K_{PB}[D]_t + K_{PB}[P]_t + 1)^2 - 4K_{PB}^2[P]_t[D]_t}}{2K_{PB}[D]_t} \times 100 \tag{17}$$

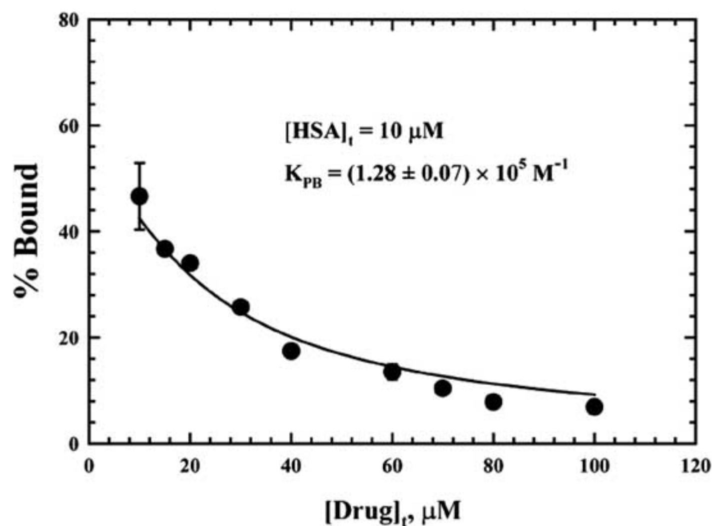


Fig. (4). Binding curve of HSA-diazepam. Capillary: L_d = 50 cm, L_t = 60 cm, ID = 50 µm; injection: 1.0 psi, 40 s; separation: +15 kV, 0.5 psi; $[HSA]_t$ = 10 µM; = 214 nm. Reference [135].

Table 5. Protein binding constants (K_{PB}) measured by CE-FA and other methods

Compound	Protein	K_{PB}, M^{-1} (CE-FA)	Reference (CE-FA)	K_{PB}, M^{-1} (Literature)	% Bound (*in vitro*) (Literature)
Diltiazem	HSA	5.9×10^2	[135]		75
Lidocaine	HSA	7×10^2	[135]	1.3×10^5 (ED) [a)]	
Bupropin	HSA	7.1×10^2	[135]		80
Diphenhydramine	HSA	9.6×10^2	[135]		63
Verapamil	HSA	1.10×10^3	[135]	1.8×10^3 (CE/FA)	
Chlorpheniramine	HSA	1.43×10^3	[135]		70
Impramine	HSA	1.8×10^3	[135]	2.39×10^4 (FLU) [a)]	90
Propranolol	HSA	2.2×10^3	[135]	1.7×10^3 (CE/FA)	
Rifampicin	HSA	2.97×10^3	[135]	5.1×10^3 (BIA)	
Promazine	HSA	3.0×10^3	[135]	8.5×10^4 (FLU) [a)]	
Indapamide	HSA	3.6×10^3	[135]		75
Doxepin	HSA	3.6×10^3	[135]		83
Desipramine	HSA	3.7×10^3	[135]	7.02×10^4 (FLU) [a)]	82
p-Nitrophenol	HSA	5.5×10^3	[137]		
Benzoic acid	HSA	6.2×10^3 (K_1) 1.7×10^2 (K_2)	[137]	1.7×10^4 (K_1) 4.5×10^3 (K_2) (ED)	
L-Tryptophan	HSA	6.7×10^3	[137]	1.03×10^4 (ED)	
Quindine	HSA	8.2×10^3	[137]	3.6×10^4 (ED)	
Chlorpromazine	HSA	1.13×10^4	[135]	4.2×10^4 (DSP)	
Triflupromazine	HSA	2.1×10^4	[135]	5.5×10^4 (DSP)	
Salicylic acid	HSA	2.4×10^4 (K_1) 6.2 (K_2)	[137]	4.0×10^4 (ED)	
Omeprazole	HSA	4.6×10^4	[135]		95
(R/S)-Warfarin	HSA	1.2×10^5 (K_1) 1.2×10^4 (K_2)	[137]	3.04×10^5 (K_1) 2.92×10^4 (K_2) (ED)	
Diazepam	HSA	1.28×10^5 2.18×10^5	[135] [137]	1.14×10^5 (TFQ)	

Table 5 (contd….)

Compound	Protein	K_{PB}, M^{-1} (CE-FA)	Reference (CE-FA)	K_{PB}, M^{-1} (Literature)	% Bound (*in vitro*) (Literature)
(S)-Flurbiprofen	HSA	4.7×10^5 (K_1) 1.1×10^4 (K_2)	[137]	1.38×10^6 (K_1) 8.98×10^4 (K_2) (ED)	
(R/S)-Ibuprofen	HSA	7.1×10^5 (K_1) 1.4×10^4 (K_2)	[137]	3.56×10^6 (K_1) 1.78×10^3 (K_2) (ED)	
Lidocaine	$_1$-AGP	3.2×10^4	[135]	1×10^5	
Oxprenolol	$_1$-AGP	8×10^4	[131]		
Alprenolol	$_1$-AGP	5×10^4	[131]		
Impramine	$_1$-AGP	5.5×10^4	[135]	4×10^4	
Propranolol	$_1$-AGP	4.7×10^5 1×10^5	[135] [131]	3×10^4	
Verapamil	$_1$-AGP	1.13×10^6	[130]	9.46×10^5 (ED)	
Chlorpromazine	$_1$-AGP	5×10^6	[135]	1×10^6	
(R)-Propranolol	HDL LDL	2.38×10^4 4.01×10^5	[134]		
(S)-Propranolol	HDL LDL	2.43×10^5 4.02×10^5	[134]		

HSA: human serum albumin; $_1$-AGP: $_1$- acid glycoprotein; HDL: high density lipoprotein; LDL: low density lipoprotein; TFQ: tryptophan fluorescence quenching; BIA: biacore; DSP: difference spectrophotometry; FLU: fluorescence: ED: equilibrium dialysis. [a)] Non-defatted HSA.

protein binding measurement is usually conducted for individual proteins such as HSA, AGP, and lipoproteins [134], [135]. Binding constants of 10^2 to 10^8 M^{-1} could be determined by CE-FA [131], [135].

4.2.2 Hummel-Dreyer Method

In the Hummel-Dreyer method, the capillary is filled with a buffer containing the drug with known concentration [152], [154]. Then a small plug containing the same buffer and drug plus a known amount of protein is injected. If the electrophoretic mobilities of the free protein and drug-protein complex are equal and larger than that of the free drug, they will migrate faster than the drug. Therefore, a positive peak corresponding to the drug-protein complex and free protein will appear on the electropherogram followed by a negative peak corresponding to the bound drug concentration due to the deficiency of the drug concentration in the sample plug (Fig. (**5**)). During the CE separation, the drug-protein complex is always in equilibrium with the free drug and protein. The bound drug concentration from the negative peak can be quantified using internal or external calibration. In the internal calibration, a series of samples with a fixed protein concentration and incremental concentration of drug are injected. The amount of drug required to fill the vacancy peak can be determined by extrapolating to zero absorbance. The internal calibration method requires many sample injections to obtain one data point. An alternative one is the external calibration, in which only two injections are needed: the protein sample and the blank buffer. The amount of bound drug is calculated from the difference of the trough areas of the protein sample and the blank buffer. Because the bound drug concentration is measured, the HD method could determine the number of drug molecules bound to a protein molecule from the ratio of bound drug concentration to the protein concentration in the sample.

4.2.3 Vacancy Peak Method

In the vacancy peak method, the capillary is filled with a buffer containing both the drug and protein. One has a constant concentration and the other has varied concentration [151], [152]. A small plug of blank buffer is injected. Two negative peaks will appear on the electropherogram as shown in Fig. (**5**). The first negative peak is due to the vacancy in the drug-protein complex and the free protein and the second negative peak is due to the vacancy in the free drug concentration. The area of the second negative peak is usually used for the determination of the free drug concentration. A similar internal calibration method as in the HD method is used to determine the free drug concentration from the second negative peak.

4.2.4 Affinity Capillary Electrophoresis

The experimental set-up of the ACE method is the same as the HD method [124]-[126], [128], [129], [152], [155]. In ACE, however, the mobility of one component (e.g. protein) is monitored by injecting a small amount of this component into the capillary filled with the buffer containing the other component (e.g. drug) as shown in Fig. (**5**). If the drug and drug-protein complex have fast associa-tion/dissociation kinetics and different electrophoretic mobilities, the average mobility of the monitored component will shift with increasing the concentration of the other component. The mobility shift is related to the bound percentage as shown in Eq. (**18**), where $M_{P,D}$, $M_{P,O}$, and M_C are the average mobility of protein, the mobility of free protein, and the mobility of protein-drug complex, respectively, and is the fraction of

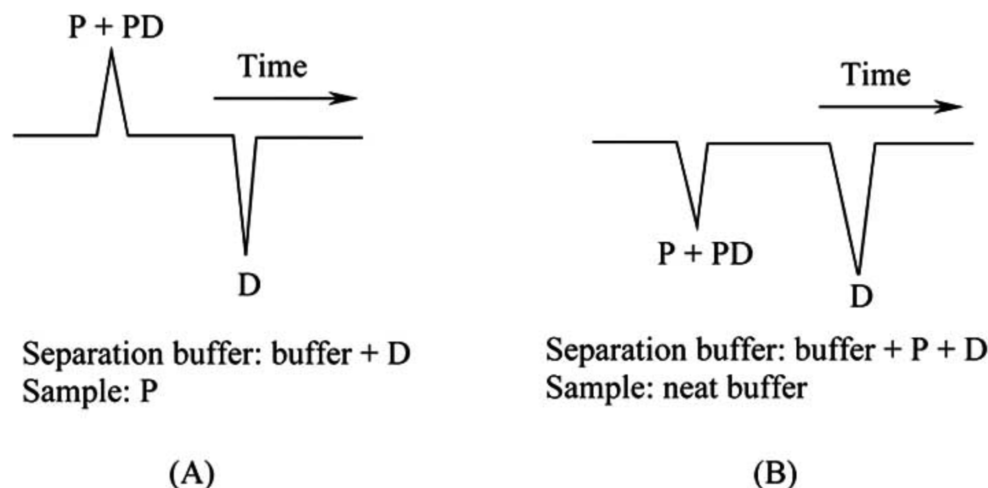


Fig. (5). Schematic elution profiles of the CE methods for drug-protein binding. (A) HD, ACE; (B) VP, VACE; D, drug, P, protein, PD, drug-protein complex.

protein bound [152]. In ACE, the free drug concentration can not be measured directly. Other factors such as protein adsorption on the capillary and viscosity changes in the buffer could also cause mobility shift and measurement errors. Guidelines for selecting the ligand concentrations were proposed for the determination of binding constants using ACE [159]. Other types of ACE using immobilized proteins and microchip technology are also developed [160].

$$M_{P,D} = (1 - \quad) M_C + \quad M_{P,O} \qquad (18)$$

4.2.5 Vacancy Affinity Capillary Electrophoresis

The vacancy affinity capillary electrophoresis method uses the same experimental set-up as the VP method and the same mobility shift analysis as the ACE method [161], [162]. In the VACE method, the mobility shift of both the drug negative peak and protein negative peak are used for the estimation of binding constants (Fig. (**5**)). The number of binding sites can be estimated from VACE, which is considered to be an advantage over the ACE method. However, VACE and ACE share the similar limitations.

4.2.6 Adsorption, Throughput, and Reproducibility

Protein adsorption to the bare silica wall in capillary electrophoresis 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 [163]-[167]. Compared to the commonly adopted washing procedures with 1 M NaOH or 1 M HCl, SDS was shown to be more effective for desorbing bound protein [163], [167]. This maybe due to the combination of micellar effect and protein denaturation. Because of the high ionic strength of PBS buffer, joule heating should be avoided by using low voltage. External air pressure is usually applied to shorten the separation time. In the PACE-FA method, the separation can be completed in 10 min [135]. External air pressure not only reduces the analysis time but also prevents the loss of protein during CE separation. Another benefit of using external air pressure is that better-defined drug plateaus can be achieved for some strongly basic drugs. High throughput could be achieved using capillary array system.

Several approaches on how to improve reproducibility were discussed in the literature [[168]-[170]. Among the parameters, the injection process is one of the most important factors for reproducibility. Hydrodynamic injection is preferred over the electrokinetic injection for protein binding studies because it is reproducible, robust, and unbiased. It was reported that the injection repeatability of short time injections at high pressure is better than long time injections at lower pressure [169].

5. INCLUSION COMPLEX CONSTANT OF CYCLODEXTRINS

Cyclodextrins (CDs) are toroidally-shaped polysaccharides formed from D-glucose monomers. The -, -, and -CDs consist of 6, 7, and 8 glucose units, respectively. They have a hydrophobic cavity and a hydrophilic exterior. The native and modified CDs can form inclusion complexes with a wide range of molecules. Cyclodextrins are used in drug formulations to increase solubility and improve bioavailability [171] and chiral separation [172]-[178]. Knowledge of formation constants of CD with drugs can assist drug formulation design and CE separation. Various approaches have been used for the determination of inclusion complex constants of CD such as NMR [179], [180], sepctrophotometric and electrochemical methods [181], conductometry [182].

The inclusion complex constants of CD with various compounds have been measured by ACE method [183]-[193]. Native CDs are non-ionic compounds which do not appreciably absorb UV above 200 nm or visible light. When the drug (D) and CD form a inclusion complex (DCD) (Eq. (**19**)) with a formation constant of K (Eq. (**20**)), the effective mobility of the drug (M_e) is described in Eq. (**21**) that can be rearranged to Eq. (**22**), where M_D is the mobility of free ionized drug and M_C is the mobility of the drug-CD complex. The effective mobility is measured according to Eq. (**1**) by using DMSO as neutral marker. Non-linear regression of mobility changes of the drug with increasing concentration of CD according to Eq. (**22**) will give the formation constant of inclusion complex. With increasing CD concentration, the viscosity of the buffer will increase.

The electrophoretic and electroosmotic mobilities are inversely proportional to the viscosity of the buffer. Therefore, mobility correction due to viscosity changes should be made. It has been found that current ratio could be used for the correction as shown in Eq. (**23**), where i and i_o are the measured current of the buffer in the presence and in the absence of CD, respectively [191], [192].

$$D + CD \overset{K}{\longleftrightarrow} DCD \tag{19}$$

$$K = \frac{[DCD]}{[D][CD]} \tag{20}$$

$$M_e = \frac{[D]}{[D]+[DCD]} M_D + \frac{[DCD]}{[D]+[DCD]} M_C \tag{21}$$

$$M_e = \frac{M_D + M_C K[CD]}{1 + K[CD]} \tag{22}$$

$$M_{e,corr} = M_e \frac{i}{i_o} \tag{23}$$

Eq. (**22**) is only used for measuring the inclusion complex constant of CD with the fully ionized form of the drug at extreme pH. The unionized form of the drug also forms complex with CDs and the inclusion complex constant cannot be measured by CE because both CDs and the unionized drug do not have charges. An equilibrium model has been developed to describe the pH and CD concentration dependence of the electrophoretic mobilities of the drug as shown in Eq. (**24**), where K_1 and K_2, respectively, are the inclusion complex constants of CD with the unionized and the ionized forms of the drug, and K_a is the acid dissociation constant of the drug [194], [195]. The effective mobilities of the drug are measured by changing both pH and the CD concentrations. The K_1 and K_2 values can be obtained simultaneously from non-linear regression of mobilities as a function of pH and CD concentrations according to Eq. (**24**). The inclusion complex constants of native -CD with fenoprofen, ibuprofen, and naproxen were measured both in ionized and unionized forms according to Eq. (**24**), which are in the range of 10^2-10^3 M^{-1} [194], [195]. The influences of several factors such as temperature and pH on the inclusion constant have also been discussed [192].

$$M_e = \frac{M_D + M_C K_2[CD]}{1 + K_2[CD] + ([H^+]/K_a)(1 + K_1[CD])} \tag{24}$$

6. SELF-ASSOCIATION

It is well known that some organic molecules are surface active and can form aggregates or micelles in their uncharged or charged forms. Chlorpromazine, promazine, desipramine, impramine, propranolol, acetobutolol, diphenhydramine, amphotericin B, dibucaine, chlorphenoxamine, and penicillin G are typical non-peptide surface active drugs [196]. The aggregation of surface active drugs follows the same principles as the classical detergents. The aggregation number of the surface active drugs is usually small (3-19) and drug self-association is also temperature, ionic strength, and pH dependent [196]. Drug in monomeric or aggregate states can have significantly different physicochemical properties such as solubility, dissociation constant (pK_a), reactivity, permeability, biological activity [2], [196]. The formation of aggregates and micelles can greatly increase the solubility. In the study of pH-solubility profile, it has been observed that many drug molecules exhibited much higher solubility than expected if only pH and salt effects are considered. One explanation is that drug undergoes self-association to form aggregates or micelles [197]. The understanding of drug self-association phenomenon may also explain the activity of many nonspecific inhibitors and may account for the activity of many promiscuous inhibitors in high-throughput screening [198]. Self-association has been studied using solubility, conductivity, calorimetry, surface tension, osmometry, and pH measurements [197], [199], [200].

Various CE based approaches have been applied to determine the CMC values of surfactants [201]. There are three major CE methods: MEKC, ACE, and electric current measurement. In the MEKC method, the CMC is measured based on the linear relationship of the retention factor of a solute with the total surfactant concentration [73]. In the ACE method, the effective electrophoretic mobilities of a neutral compound are measured as a function of total surfactant concentration [202], [203]. The CMC value is the surfactant concentration where a sharp change in the slope occurs. However, the partition of the neutral compound may alter the CMC value to some extent in ACE. The third method is based on the measurement of the electric current of micellar electrolyte solutions as a function of surfactant concentration at a given voltage [204], which is a CE version of the traditional conductivity measurement. The CMC values are obtained from the intersection of two straight lines corresponding to the monomeric state of the surfactant below CMC and the micellar state of the surfactant above CMC, respectively. Recently, a CE-FA method has also been applied for the determination of CMC values [205], [206]. For CMC measurement of surfactants, the ACE method is preferable because the CMC can be precisely determined by curve-fitting procedures [201]. Most surface active drugs have a CMC value of 1-100 mM [196] and are also UV-absorbing. Therefore, the electric current measurement may be more applicable for surface active drugs because of its simplicity and non-absorbance measurement.

CONCLUSION

Capillary electrophoresis is a simple and powerful one-phase separation technique based on the size and charge ratio of the analytes. If the equilibrium or interactions involve a change in overall charge of the molecule, CE can be used for determining the equilibrium constants. The various separation modes make CE a versatile technique for measuring physicochemical properties such as pK_a, logP, protein binding, inclusion complex constant, and CMC. The small sample size is also a very attractive feature for CE. With new technologies of CE in separation and detection such as multiplexed-CE system and CE-MS, CE will be a

valuable tool in physicochemical profiling for pharmaceuticals with higher throughput and better sensitivity.

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