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RESEARCH ARTICLE

Comparative Study of Three Methods for Affinity Measurements: Capillary Electrophoresis Coupled with UV Detection and Mass Spectrometry, and Direct Infusion Mass Spectrometry

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Abstract

We present affinity capillary electrophoresis and mass spectrometry (ACE-MS) as a comprehensive separation technique for label-free solution-based affinity analysis. The application of ACE-MS for measuring affinity constants between eight small molecule drugs [ibuprofen, s-flurbiprofen, diclofenac, phenylbutazone, naproxen, folic acid, resveratrol, and 4,4'-(propane-1,3-diyl) dibenzoic acid] and β-cyclodextrin is described. We couple on-line ACE with MS to combine the separation and kinetic capability of ACE together with the molecular weight and structural elucidation of MS in one system. To understand the full potential of ACE-MS, we compare it with two other methods: Direct infusion mass spectrometry (DIMS) and ACE with UV detection (ACE-UV). After the evaluation, DIMS provides less reliable equilibrium dissociation constants than separation-based ACE-UV and ACE-MS, and cannot be used solely for the study of noncovalent interactions. ACE-MS determines apparent dissociation constants for all reacting small molecules in a mixture, even in cases when drugs overlap with each other during separation. The ability of ACE-MS to interact, separate, and rapidly scan through *m/z* can facilitate the simultaneous affinity analysis of multiple interacting pairs, potentially leading to the high-throughput screening of drug candidates.

Key words: Affinity Capillary Electrophoresis-Mass Spectrometry (ACE-MS), Direct Infusion Mass Spectrometry (DIMS), Affinity interactions, Dissociation constant, Cyclodextrin, Ibuprofen, S-flurbiprofen, Diclofenac, Phenylbutazone, Naproxen, Folic acid, Resveratrol, 4,4'- (propane-1,3-diyl) dibenzoic acid (PDDA)

Introduction

Kinetic capillary electrophoresis (KCE) [1] is a separation of species that interact during electrophoresis, and is applied for measuring rate and equilibrium constants of molecular noncovalent interactions [2, 3], assessing thermodynamics [4], and performing affinity purification of both

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DNA aptamers [5] and DNA-tagged drugs [6]. The development of KCE started with pioneering work of Whitesides on affinity capillary electrophoresis (ACE) [7–9] and established a new paradigm that separation can be used as comprehensive kinetic tool for the study of drug actions and screening [10].

While UV absorption and laser-induced fluorescence detection have been successfully used in conjunction with capillary electrophoresis (CE), the ability to acquire accurate molecular mass and structural information about interacting molecules is highly desirable. Applications of capillary electrophoresis coupled with mass spectrometry (CE-MS) started over 20 years ago [11] and made significant advancements in the field of analytical chemistry. The mass spectrometer can provide information regarding the structure of known and unknown components present in a complex mixture with high specificity and high sensitivity.

The study of noncovalent molecular interactions is of great interest for designing and screening new drugs. Recently, several reviews were published on this topic [12, 13]. While there are different techniques for affinity measurements, such as MS [14], NMR [15], spectroscopy [16], SPR [17], stop-flow [18], and HPLC [19], only CE provides a possibility to study simultaneously multiple analytes in a liquid and homogenous phase due to their spatial separation.

In this work, we coupled on-line ACE with MS to combine separation and binding capability of ACE together with molecular weight and structural elucidation of MS in one system. The potential advantages of ACE-MS are that (1) analytes interact with each other in a homogeneous reaction at near physiologic conditions (pH and ionic strength), and binding parameters are measured in solution; (2) analytes do not require special labeling for the MS detection; and (3) interactions of multiple analytes are studied together in one capillary microreactor.

To understand the benefits of ACE-MS, we compare three methods: direct infusion mass spectrometry (DIMS), ACE with UV detection (ACE-UV), and ACE-MS for finding the affinity constants of noncovalent interactions between β-cyclodextrin (CD) and eight small molecule drugs (SMs) in the following equilibrium reaction:

$$SM + CD \rightleftarrows Complex$$

where SM denotes the drug, CD is the cyclodextrin, and Complex is the drug-cyclodextrin complex. The apparent dissociation constant of drug–cyclodextrin complex, K_d, is defined according to the mass action law for the reaction above:

$$K_d = \frac{[SM] \times [CD]}{[SM]_0 - [SM]} \quad (1)$$

where $[SM]_0$ and $[SM]$ are the total and unbound drug concentrations, respectively. [CD] is the unbound cyclodextrin concentration. SMs are five non-steroidal anti-inflammatory drugs: ibuprofen, s-flurbiprofen, diclofenac, phenylbutazone, naproxen, as well as three other small molecules: folic acid, resveratrol, and 4,4'-(propane-1,3-diyl) dibenzoic acid (PDDA).

The experimental model involving the formation of inclusion complexes between CD and SMs is chosen as an important example of fast affinity noncovalent interactions [20]. It is also a complicated model because of high stoichiometry of complexes and different ionization levels for SM, CD, and their complexes. SMs form a host–guest complex with CD. The formation of inclusion complexes modifies the physical and chemical properties of guest SMs and significantly increases their water solubility. This is the reason why CDs have attracted interest in pharmaceutical applications [21]. CDs enhance the bioavailability of poorly soluble drugs by delivering a hydrophobic drug to a lipid cell membrane, where the drug can penetrate inside a cell [22]. Only the unbound (free) drug is available for diffusion across membranes, which results in absorption and distribution, and eventually in reaching the activity site. The unbound drug fraction, $F_u(SM)$, is defined as the ratio of the unbound drug concentration [SM] to the total drug concentration $[SM]_0$, and depends on the apparent dissociation constant.

$$F_u(SM) = \frac{[SM]}{[SM]_0} = \frac{1}{1 + [CD]_0/K_d} \; when \; [CD]_0 \gg [SM]_0 \quad (2)$$

In DIMS, ion intensities of free SMs are used for finding apparent dissociation constants in several titration experiments with different CD concentrations (Figure 1). This is the simplest method, but its major problem is the difference in electrospray ionization efficiency of each compound in a mixture when they are injected all together without prior spatial separation.

ACE-UV is a CE-based separation technique with a universal and nonspecific UV detection. We applied an ACE method called equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM) [23]. In ECEEM, an equilibrium mixture (EM) of β-cyclodextrin with all small molecules is prepared and equilibrated. A plug of EM is injected into a capillary prefilled with a run buffer containing CD with a total concentration identical to EM and is subject to a high electric field along the capillary (Figure 1). EM is separated while quasi-equilibrium is maintained between CD, SMs, and their complexes inside the capillary during electrophoresis. Two features are characteristic for ECEEM: (1) the migration time of the EM peak depends on concentration of CD in the run buffer; therefore, SMs with different K_ds migrate with different velocities (Figure 2a), and (2) a free SM and its complex migrate as a single EM peak due to fast exchange between them.

When ACE is combined with MS detection (Figure 1), it tracks abundance of all SMs and determines dissociation

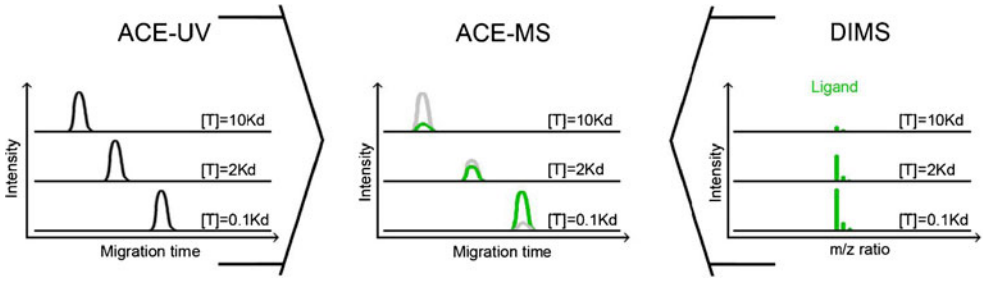

Figure 1. Schematic representation of titration experiments using three methods for affinity measurement: ACE-UV, ACE-MS, and DIMS. Grey color represents the complex between a small molecule ligand and cyclodextrin as a target (T). Green color represents the free ligand. Concentration of the target varies from 0.1 K_d to 10 K_d. In DIMS experiments, only ion intensity of a free ligand is used for calculation of K_ds. In ACE-UV, migration times of an equilibrium mixture between the ligand and the target are used. ACE-MS combines the information from migration times and ion intensities

constants for more small molecules than ACE-UV and DIMS. In this work, we show that this label-free, homogeneous, and multiplexed method, ACE-MS, significantly reduces the error in all K_d measurements and calculates K_d for a non-shifting drug as well.

Materials and Methods

Reagents were purchased from Sigma-Aldrich (Canada) unless otherwise noted. One hundred mM ammonium acetate buffer, pH 6.5, was prepared by dissolving 1.92 g of ammonium acetate powder in 250 mL of distilled deionized water (ddH_2O). Ten mM ammonium acetate was used as a running/incubation buffer in all experiments. First, all SMs were dissolved in methanol (HPLC grade) to create a stock solution with a concentration of 10 mM. Final equilibrium mixtures of SMs and CD were prepared in the incubation buffer with the following concentrations of all SMs: 15, 50, and 100 μM, and CD in a range of 10 μM to 15 mM. All solutions were filtered through 0.22-μm pore size membrane filters (Millipore, Nepean, ON, Canada).The bare-silica capillary was purchased from Polymicro (Phoenix, AZ, USA).

All MS experiments were done in negative polarity mode. For integration the following *m/z* ratios were used with 0.02 Da window: ibuprofen, 205.12; s-flurbiprofen, 243.08; resveratrol, 227.07; PDDA, 283.09; folic acid, 440.13; naproxen, 229.08; diclofenac, 315.99; phenylbutazone, 307.14 (Figure S1, Supplemental Data).

Experimental Conditions for ACE-UV

ACE-UV experiments were carried out with a PA800 plus Pharmaceutical Analysis CE system (Beckman Coulter, Brea, CA, USA) equipped with either a UV or PDA detector. The sample storage and capillary temperature was maintained at 25±0.5 °C. An electric field of 325 V/cm was applied inside the capillary with a positive electrode at the

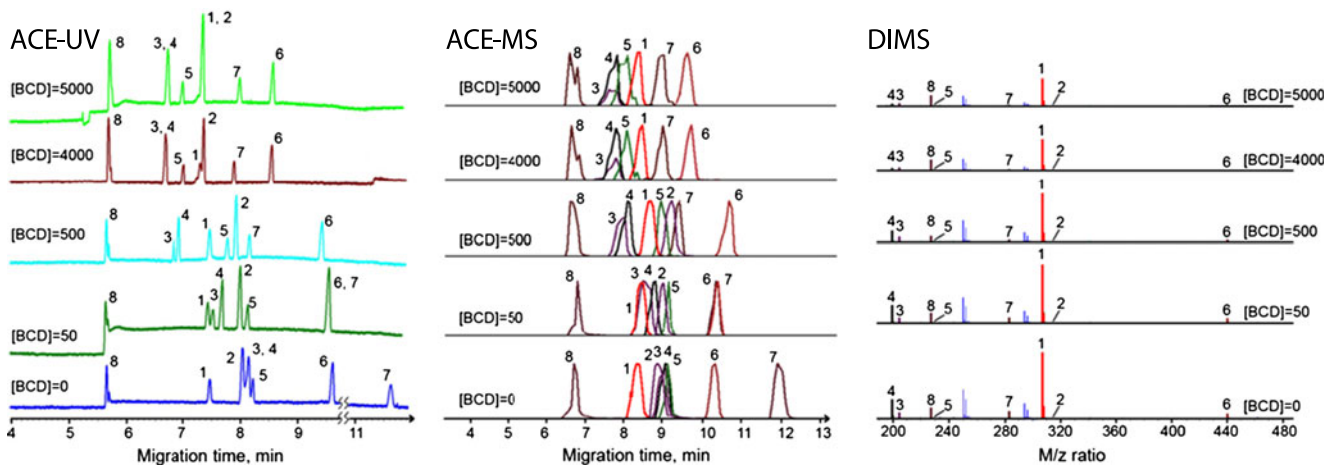

Figure 2. Experimental data from three methods for affinity measurements. Small molecule compounds: 1 – phenylbutazone, 2 – diclofenac, 3 – ibuprofen, 4 – s-flurbiprofen, 5 – naproxen, 6 – folic acid, 7 – PDDA, 8 – resveratrol. Titration experiments were performed with a fixed concentration of a SM (15 μM each) and different concentrations of β-cyclodextrin (BCD) from 0 to 5000 μM

injection end (an inlet) and a ground electrode at the detection end (an outlet). The inlet vial was filled with the run buffer containing one of the cyclodextrins, and the outlet vial contained the run buffer only. The concentration of CD in the equilibrium mixture and the run buffer was the same for individual ACE-UV experiments. The capillary was 89 cm long (80 cm to the detection window) with an inner diameter of 50 μm and an outer diameter of 360 μm. The equilibrium mixture was injected into the capillary from the inlet end by a pressure pulse of 8 s×0.5 psi. Before each experiment, the capillary was rinsed by 20 psi pressure with: 0.1 M HCl for 3 min, 0.1 M NaOH for 3 min, ddH_2O for 3 min, 10 mM ammonium acetate buffer for 5 min, and the incubation/run buffer with CD for 1 min. The output data was absorbance intensity in the detection point, as a function of time passed since the application of the electric field.

Experimental Conditions for ACE-MS

SYNAPT G2 High Definition Mass Spectrometer from Waters (Milford, MA, USA) was coupled on-line with PA800plus Pharmaceutical Analysis CE system (Beckman Coulter, Brea, CA, USA) through the CE-ESI sprayer from Micromass (Manchester, UK). Ionization conditions were as follows: capillary voltage 3 kV, sampling cone voltage 45 V, extraction cone voltage 3 V, source temperature 100 °C, cone N_2 gas 0 L/h, nano flow N_2 gas 0.5 Bar, purge N_2 gas 3 L/h. Sheath liquid—80:20 isopropanol:ddH2O 2 mM ammonium acetate—was delivered with a flow rate of 1.5 μL/min. All CE conditions were the same as for ACE-UV experiments.

Experimental Conditions for DIMS

Source and MS conditions were the same as for ACE-MS. All samples were injected into MS by applying a constant pressure (2 psi) through a capillary (89 cm long and an inner diameter of 50 μm).

Experimental Conditions for MS/MS and Kinetic Stability of Gas Phase Complexes

Kinetic stabilities of β-CD/SM noncovalent complexes were measured using an ESI-MS/MS and RRKM unimolecular rate modeling method explained in detail elsewhere [24, 25]. Briefly, using a Waters Q-TOF 1 with MassLynx 4.1 for analysis and data processing, (capillary voltage was 3 kV, cone voltage was 45 V) breakdown diagrams were generated by measuring the percentage of complex survival as a function collision energy.

$$k(E) = \frac{\sigma N^{\ddagger}(E - E_o)}{h\rho(E)}$$

The theoretical breakdown curve is found using the unimolecular rate *k(E)* constant, which is calculated using the transition state sum-of-states above the 0 K activation energy [$N^{\ddagger}(E\text{-}E_0)$], the density of states of the reactant ion [$\rho(E)$], at an internal energy, which are themselves calculated from the molecular and transition state vibrational frequencies (Gaussian 03, AM1 level) [26] using the direct count method. The transition state vibrational frequencies and activation energy (E_0) are scaled until the best possible theoretical-experimental match is obtained.

Results and Discussion

Measuring Affinity by DIMS

Direct infusion of equilibrium mixtures of SMs with different CD concentration were performed by applying constant pressure to create stable nano-electrospray ionization (nano-ESI) of analytes and following with MS detection. No CE separation of drugs and the cyclodextrin was performed prior to ionization. Three concentrations of SMs (15, 50, and 100 μM of each SM) and a range of CD concentrations from 10 to 5 mM were used.

All eight SMs were detected and identified by characteristic m/z ratios (Figure 2c and Figure S1, Supplemental Data). The main complex was 1SM-1CD. Nevertheless, multiple noncovalent complexes of SMs with CD were also observed with stoichiometry 1SM-2CD, 1SM-3CD (Figure S2, Supplemental Data), though some of them possibly were adducts formed during ionization than specific complexes. Ion mobility mass spectrometry (IM-MS) experiments confirmed inclusion nature of 1SM-1CD complex as shown in Figure S3, Supplemental Data.

Due to non-simple stoichiometry, we measured the apparent constant, K_d, by using changes in ion intensity of free SMs. We plotted the unbound drug fraction, F_u(SM), versus concentration of cyclodextrin in the run buffer. F_u(SM) is defined as the ratio of the intensity of free SM ions in the presence of CD, I(SM), to the intensity of free SMs without CD, I_0(SM):

$$F_u(SM) = \frac{I(SM)}{I_0(SM)} \quad (3)$$

The DIMS detection showed ion suppression of all analytes with the increase of CD concentration (Figure 4). Even for phenylbutazone, which does not interact with CD, the ion suppression was significant at [CD]>200 μM. It is important to note that ion intensities of SMs were in linear correlation with their concentration at zero concentration of CD. In the presence of CD, it is almost impossible to distinguish complexes with different stoichiometry and salt adducts, and calculate the unbound fraction of SMs. To compensate the effect of ion suppression with increasing [CD] for affinity calculations, we normalized F_u(SM) of all interacting SMs by the ion intensity of phenylbutazone, which does not bind CD and works as an internal standard (Figure 3). Concentration of CD when half of a small

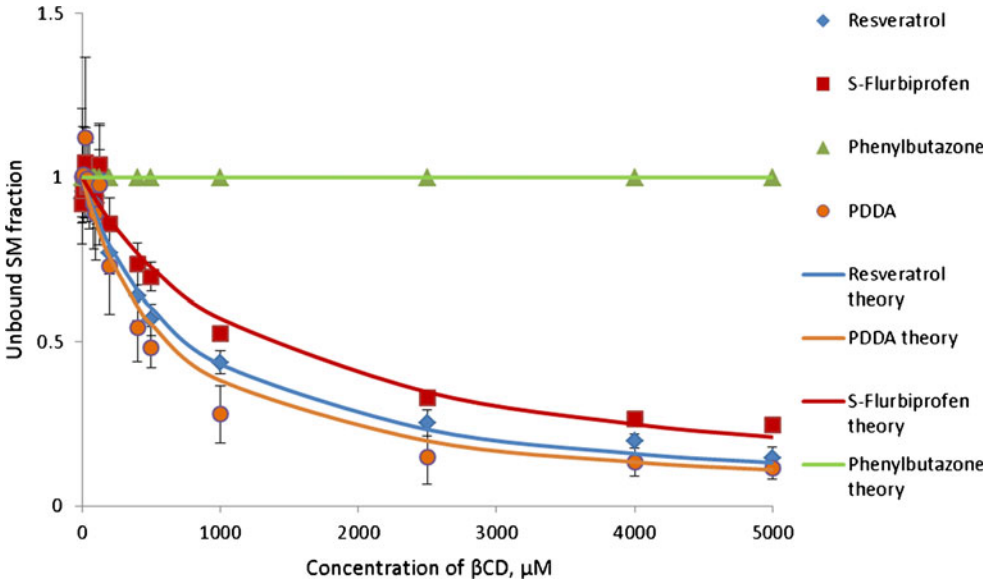

Figure 3. Correlation of the normalized unbound SM fraction from concentration of βCD. Normalization was done by phenylbutazone's ion intensity. Apparent K_d for SM equals the CD concentration when a half of small molecules is bound

molecule's amount is bound (F_u(SM)=0.5) gives the apparent K_d. All K_ds found by DIMS are presented in Table 1. Affinity constants only for three SMs (s-flurbiprofen, resveratrol, and PDDA) out of seven reactive drugs were calculated. For folic acid, diclofenac, and naproxen, K_ds lay in range of CD concentrations where ionization suppression becomes overwhelming. Ibuprofen has an impurity with the same mass that made it impossible to calculate K_d without prior separation.

Measuring Affinity by ACE-UV

In ACE-UV, the determination of the apparent dissociation constant for each small molecules is dependent on electrophoretic mobility and can be calculated using the following equation:

$$K_d = [CD]\left(\frac{t_{EM} - t_0}{t_0 - t_C}\right) \quad (4)$$

where [CD] is the concentration of β-cyclodextrin in the running buffer, t_{EM} is migration time of EM, t_0 and t_C are migration times of free SM and its complex, respectively. Migration time of the complex can be estimated from experiments with high concentration of CD when most of SM is bound. All migration times were normalized by the migration time of one of the internal standards such as phenylbutazone or an electro-osmotic flow (EOF) peak.

The apparent K_d constant for a SM can be found by two ways. The first way is similar to the method described above for DIMS. The relative migration time of EM peak and CD concentration is plotted (Figure 4), where K_d equals the concentration of CD at 50 % of the total shift of EM peak. The second more precise method requires fitting of experimental data using equation 4, while minimizing the error, *H*, between theoretical and experimental migration time of EM:

$$H = \sum \ln\left(1 + \frac{1}{Std(t)_i^2}\left(t_i^{exp} - t_i^{theory}\right)^2\right) \quad (5)$$

where $t^{exp} - t^{theory}$ is the difference between experimental and theoretical migration time of SM for an experiment with i-th CD concentration, *Std(t)* is standard deviation of t^{exp}.

Table 1. Apparent K_d Values for Affinity Interactions between SMs and β-cyclodextrin Measured by DIMS, ACE-UV, and ACE-MS Methods

	DIMS, μM	ACE-UV, μM	ACE-MS, μM	Reference values, μM
Resveratrol	760±78	N/D	559±35	520[30], 992[31]
S-flurbiprofen	1329±161	183±14	186±14	455[32], 224[33], 167[34]
Ibuprofen	N/D	131±12	116±9	114[35], 94[36], 1170[32]
Folic acid	N/D	1181±335	837±132	N/F
Phenylbutazone	N/R	N/R	N/R	N/F
PDDA	619±102	66±6	69±2	N/F
Naproxen	N/D	979±89	1066±244	680[37], 590[38], 475[32]
Diclofenac	N/D	2421±676	1503±111	13000[39], 9940[40]

N/R=a SM does not bind β-cyclodextrin, N/D=K_d was not determined, N/F=no data found.

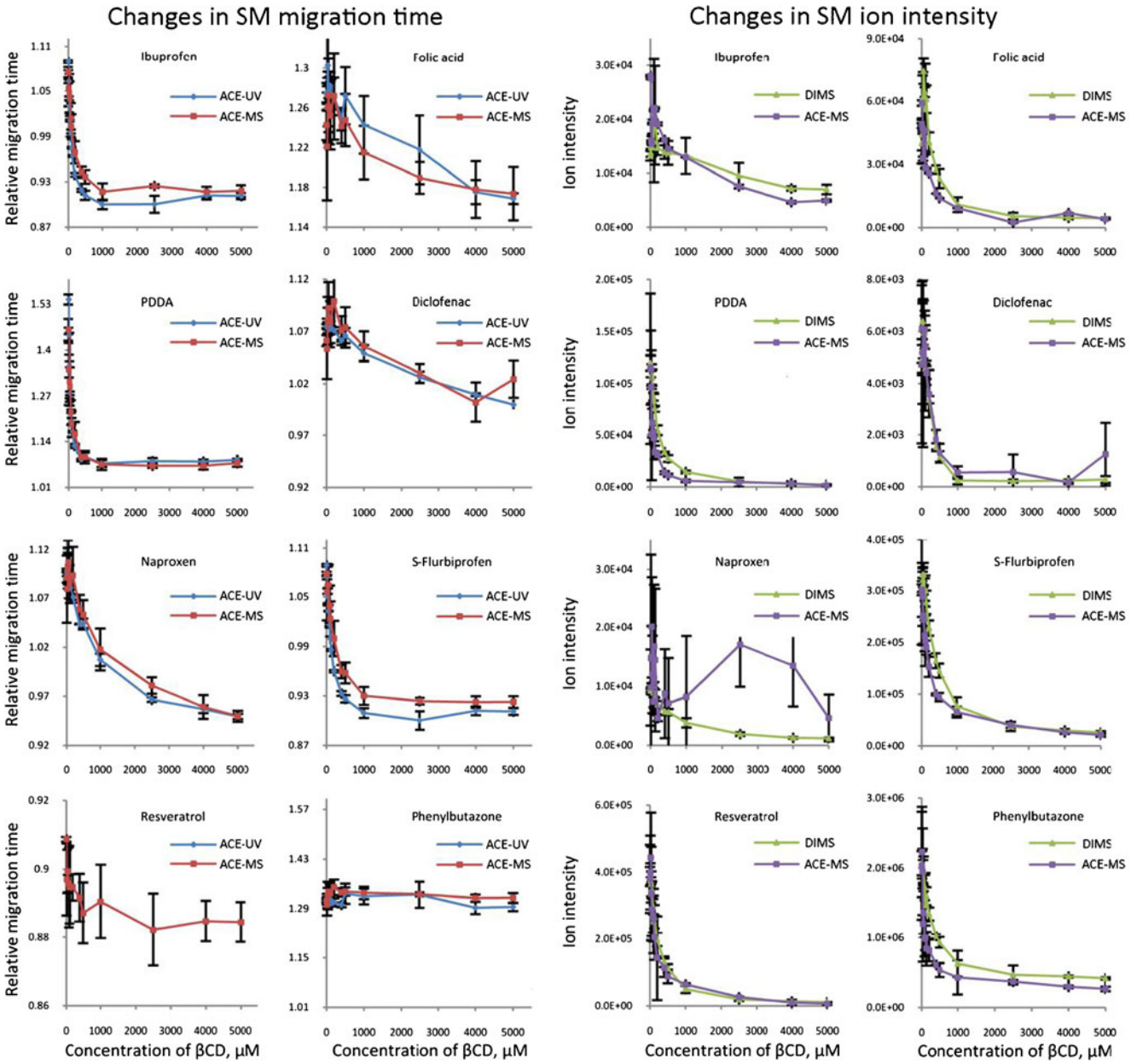

Figure 4. Correlation between migration time and ion intensity of SMs from the concentration of βCD based on ACE-UV, ACE-MS, and DIMS experiments. Experiments were repeated three times with [SMs]=100 μM and [CD]=0–5000 μM

The constants found by fitting are presented in Table 1. They were found based on 15 experiments repeated three times each with a fixed concentration of SMs (15, 50, and 100 μM of each SM) and varying concentrations of CD from 10 to 5 mM. The representative ACE-UV electropherograms are displayed in Figure 2. Six compounds out of 8 show significant shift of EM peak with the increase of CD concentration in the run buffer. Resveratrol and phenylbutazone peaks are not shifted with the increase of CD. Resveratrol is neutral at the experimental conditions and, thus, it migrates with EOF and makes the calculation of K_d impossible by the mobility change. In addition to the problem of the neutral drug, overlapping peaks complicate the calculation of migration times of individual components and affinity constants for them.

Measuring Affinity by ACE-MS

ACE-MS brings the advantages of both DIMS and ACE-UV methods, where ion intensity and CE mobility for every small molecule drug are determined. Therefore, both ion intensities and migration time shifts can be combined into one math model to measure K_d for all reacting SMs. Changes in ion intensities and migration times for each SM

upon increasing CD concentration are plotted in Figure 4. Dissociation constants can be found by fitting equations 2 and 4 while minimizing the combined error, H:

$$H = \sum \ln\left(1 + \frac{1}{Std(I)_i^2}\left([SM_i]\cdot a - I(SM_i)\right)^2\right) + \sum \ln\left(1 + \frac{1}{Std(t)_i^2}\left(t_i^{\exp} - t_i^{theory}\right)^2\right)$$

where $[SM_i]$ is the theoretical concentration of a SM for an experiment with i-th type of conditions, $I(SM_i)$ – an experimental ion intensity signal of the SM, $Std(I)_i$ is standard deviation of $I(SM_i)$, a is a transformation coefficient between concentration and ion intensity signal. K_ds for all seven reacting compounds were measured with better accuracy than DIMS and ACE-UV and in good agreement with references presented in Table 1. Overlapping peaks were well resolved by multiplexed MS detection of ions with different *m/z* ratios. Neutral resveratrol did not show a CE mobility shift, so the binding was measured by the change of its ion intensity in MS.

Small broadening of the peaks of SMs was observed in ACE-MS compare to ACE-UV experiments. The peak broadening happens because of a suction effect caused by evaporation of liquid at the outlet of the capillary and a gas flow during electrospray ionization. It creates a laminar flow with a parabolic profile inside the capillary and disperses peaks. However, the peaks becoming wider does not have any considerable effect on reproducibility of migration time of SMs. Also, the ion intensities were less reproducible in ACE-MS experiments compared with DIMS for naproxen and diclofenac because of Taylor's effect causing peak dispersion and less stable ionization by the fluctuation of an ion current in CE separation, as seen in Figure 4.

Comparing an Apparent Dissociation Constant with an Activation Energy of 1:1 Complex in MS/MS Experiments

Many different tandem mass spectrometry techniques are used to investigate stability of noncovalent complexes, and although sometimes there are examples of gas-phase binding values coinciding with the relative solution phase affinities, there are a plethora of cases where they do not. A previous study ranking the gas phase stabilities of α-, β-, and γ-cyclodextrin with rutin showed good correlation with solution phase association constants [27]. Here we employed Rice-Ramsperger-Kassel-Marcus (RRKM)-based breakdown diagram modeling approach [24]; taking into account the differences in vibrational frequencies, cross sections, and degrees of freedom. All singly deprotonated, CD-SM complexes showed a single dissociation pathway involving the loss of neutral SM. There were some exceptions, including folic acid, which retained the charge, and phenylbutazone, which had two dissociation pathways where the negative charge could be retained by CD or phenylbutazone and was not included in ranking the relative activation energy (E_0). Unlike the aforementioned CD MS/MS study, we found poor correlation between the solution phase K_d values and the gas phase complex stabilities.

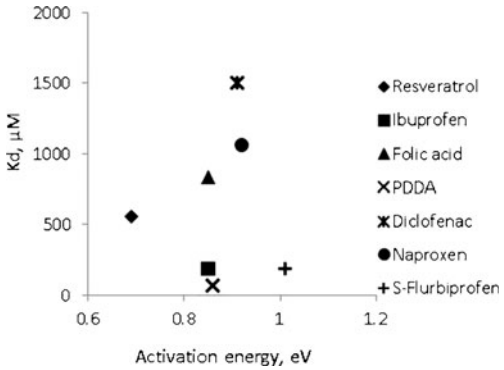

Figure 5. Comparative plot of activation energies for seven SMs versus their apparent K_ds. The activation energy was calculated for each SM by CID experiments and RRKM theory. K_d was found from ACE-MS experiments

Interestingly, the E_0 values for ibuprofen, PDDA, folic acid, diclofenac, and naproxen were quite similar, between 0.84 and 0.91 eV. Outliers included resveratrol, which has the lowest E_0 value of 0.69 eV, and s-flurbiprofen, which has the highest E_0 value of 1.01 eV. There is very little correlation between the K_d and E_0 values (Figure 5), attesting to the difference between solution-phase and gas-phase complexes. The E_0 measurements point to the electrostatic interactions as being the major factor in determined the gas phase stabilities of these systems. Resveratrol differs from the other SM because it lacks a carboxylic acid moiety, explaining its much lower activation energy. PDDA and folic acid possess two carboxylic acid groups, yet have very similar E_0 to SM, which possess only one carboxylic acid. Because there is only one charge bearing site within these gas-phase complexes, this suggests that ionic H-bonds are the most important influence upon the stabilities of these singly deprotonated gas phase complexes.

Conclusion

DIMS analysis is fast and simple, and requires only a MS instrument, but the major disadvantage of DIMS is the lack of spatial separation between drugs before MS analysis. Therefore, it is impossible to eliminate competitive binding of SMs to cyclodextrin, difficult to distinguish specific binding from nonspecific, and to analyze compounds with the similar masses. High concentrations of CD (>200 μM) suppress ionization of SMs. K_d values measured by DIMS differ significantly from more reliable separation-based techniques. The advantage of ACE-UV is in spatial separation of small molecules at near physiologic pH with

high concentration of salts and additives (up to 100 mM). The main disadvantages are that small molecules must be detectable in UV or VIS region and separated from each other as individual peaks.

Beneficially, ACE-MS provides an opportunity to estimate apparent K_d, even if a SM has the same mobility as CD, and its complex by tracking ionization intensity of the free SM. It is seen from experiments with resveratrol. In addition, ACE-MS separates and detects analytes from impurities. Unfortunately, MS detection does not work well in high concentrations of CD (>1 mM) in the run buffer. The high concentration of CD decreases electro-osmotic flow during CE separation and suppresses ionization of small molecules. The ACE-MS approach is also applicable for off-line connection CE and MS, when ACE and MS data are obtained separately on two different instruments at different times [28].

To summarize, ACE-MS is a comprehensive platform for the development of label-free solution-based methods for studying the affinity of molecule interactions. This technique shows the migration profile of small molecules in ACE by multiplex MS detection. In this work, we interfaced ACE and MS on-line, which allowed us to identify all small molecules and their complexes directly by MS without any intermediate steps (desalting or buffer-exchange) between ACE and MS. The method works well with a mixture of small molecules and allows the determination of K_ds for all reacting small molecules, even in cases when peaks overlap with each other during capillary electrophoresis separation. The range of K_ds that can be measured by ACE-MS will depend on the efficiency of ionization of small molecules and the MS detection limit. Instrumentation for ACE-MS used in this study can measure K_d values from 1 μM to 2.5 mM. Measuring nanomolar K_d values will require a different CE method called non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) [29], which is more suitable for stable complexes with slow dissociation rates. Advantageously, ACE-MS does not need MS detection of an intact cyclodextrin–small molecule complex, which can be very challenging because of the stoichiometry different from 1:1 and a decay of the complex during ionization. The ability of MS to rapidly scan through *m/z* facilitates the simultaneous analysis of the interaction between a cyclodextrin with several small molecules; this would potentially lead to high-throughput screening of panels of new binding candidates. In the future, ACE-MS can be used for a wide range of applications outside the cyclodextrin–small molecule model such as nucleic acid–metal complexes, protein and DNA/RNA conformational changes, high-throughput screening, and discovery of new ligands.

Acknowledgments

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