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Simultaneous Evaluation of Ligand Binding Properties and Protein Size by Electrophoresis and Taylor Dispersion in Capillaries

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The interplay between biophysical characteristics such as protein size and shape and protein function is difficult to ascertain using simple methods. Here, we present an approach for characterizing both protein−ligand binding as well as protein hydrodynamic radius in one operation combining electrophoresis and size measurement by dispersion using capillaries. The methodology is based on the integration of Taylor dispersion analysis and capillary electrophoresis and is here demonstrated using commercially available capillary electrophoresis instrumentation modified with a pixel sensor UV area imager, allowing two detection points along the capillary. Analytes are the human serum proteins α_1-acid glycoprotein and albumin interacting with the drug propranolol in a frontal analysis mode. Upon introduction of the propranolol−protein sample, voltage is initially applied to facilitate electrophoretically mediated separation of ligand and protein and frontal analysis. Then a pressure mobilization step is used whereby Taylor dispersion can be characterized online based on the signal from the UV area imager. Estimates of ligand binding and values for hydrodynamic radii agree with values obtained by independent methods.

Characterization of proteins and their interactions is fundamental to the understanding of biochemical processes since most biomolecular processes involve molecular recognition and ligand binding by proteins. In addition, an increasing fraction of the new pharmaceuticals reaching the market place are biological drugs, i.e., based on peptides and proteins, which are challenging compared to traditional small-molecule drugs in terms of characterization and evaluation for safety and efficacy.[1,2] As an example, proteins are flexible macromolecular assemblies and their structural integrity is uniquely sensitive to conditions encountered during formulation, production, and storage. Further, the therapeutic efficacy of biopharmaceuticals is usually closely related to molecular function, i.e., molecular conformation. Thus, successful development of protein drugs requires a detailed knowledge of chemical and physical stability in addition to pharmacokinetics and immunogenicity.[1−3] Consequently, there is a widespread interest in methods for characterizing and probing the structural integrity and functioning of proteins. Methods for characterization of protein size and oligomerization/aggregation state include size exclusion chromatography, sedimentation velocity, and dynamic light scattering. Likewise, several analytical methods, including capillary electrophoresis (CE),[4−8] are used for investigating the ligand binding properties of proteins. CE is widely used in biomolecular interaction studies due to low sample and reagent consumption and due to the versatility of substances amenable for study. It would be beneficial if biophysical and functional characterization could be combined into one method, and we focus on using micromethods such as CE for this purpose. We take advantage of the fact that the laminar steady-state flow through a capillary tube follows a parabolic function over the tubular cross section. The combined action of convection and diffusion leads to a specific form of solute dispersion (Taylor dispersion) when the liquid is subject to a hydrodynamic flow through the capillary.[9−11] Using Taylor dispersion analysis (TDA) based on the variances associated with the solute peak broadening or boundary sharpness, diffusion coefficients, D, and hence hydrodynamic radii, R_h, of analytes can be determined. Such values should be useful for determining the size of stable complexes of proteins with large ligands as well as evaluating aggregation state, e.g., the presence of monomeric, dimeric, or oligomeric species, of proteins. Capillary electrophoresis instrumentation has been found to be well-suited for performing dispersion analyses of single model molecules; examples include phenylalanine, proteins, DNA, magnetic beads, polymers, and col-

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loidal particles.[11−17] By applying an initial electrophoretic separation step prior to generating a laminar flow by application of pressure, Le Saux and Cottet[18] were able to perform diffusion coefficient measurements in sample mixtures. The first step in the analysis provided separation of the analyte mixture by capillary zone electrophoresis (CZE), whereas the second step facilitated online measurement of the diffusion coefficients of the individual analytes by TDA.

Here we demonstrate a method for the simultaneous measurement of protein hydrodynamic radius and ligand binding properties in thin capillaries. This constitutes a convenient approach for efficient protein characterization where protein function (ligand binding), as well as protein size, is estimated concurrently. The approach is based on the combination of TDA and CE. Similar to the work of Le Saux and Cottet,[18] our method can be considered a two-step approach in which CE is followed by online TDA. In the present work, however, affinity CE in the frontal analysis format precedes the TDA-based measurement of diffusion coefficients. Utilizing commercial CE instrumentation hyphenated with a pixel sensor UV area imager providing two detection points along the capillary, we show that the interaction between the low-molecular-weight drug propranolol and the human serum proteins α_1-acid glycoprotein (AGP) and albumin (HSA) can be investigated quantitatively at the same time as the protein hydrodynamic radii (diffusion coefficients) are measured.

TAYLOR DISPERSION ANALYSIS

The velocity of a laminar steady-state flow of fluid through a capillary tube varies over the capillary cross section according to a parabolic function. Thus, the flow velocity is zero at the capillary wall and has the maximum value at the capillary axis. Analyte molecules introduced into the flow move along the capillary with different velocities according to the position of the molecules in the capillary cross section.[9,10] Molecular diffusion occurs over the capillary cross section as well as along the capillary axis. The combined action of molecular diffusion and convection leads to a special form of dispersion termed Taylor dispersion.[11] The local concentration C of an analyte in the capillary is a function of the radial position r, axial position x, and time t:

$$\frac{\partial C}{\partial t} + 2u\left(1 - \frac{r^2}{{R_C}^2}\right)\frac{\partial C}{\partial x} = D\left(\frac{\partial^2 C}{\partial^2 x} + \frac{1}{r}\frac{\partial}{\partial r}r\frac{\partial C}{\partial r}\right) \quad (1)$$

where u is the mean fluid velocity, R_C is the capillary radius, and D is the diffusion coefficient.

By assuming that diffusion along the capillary axis is negligible, analytical solutions for the convection−diffusion equation (eq 1) for concentration profiles of fronts and pulses were obtained:[16]

$$\frac{\bar{C}}{\bar{C}_0} = \frac{1}{2} \pm \frac{1}{2}\,\mathrm{erf}\left(\frac{(t - t_R)}{\sigma\sqrt{2}}\right) \quad (2)$$

$$\bar{C} = \frac{M}{2\pi^{3/2}{R_C}^2\sqrt{kt_R}}\exp\left(-\frac{(t - t_R)^2}{2\sigma^2}\right) \quad (3)$$

where $\bar{C}$ is the analyte concentration across the cross section of the capillary, $\bar{C}_0$ is the concentration of the front, t_R is the mean residence time (the time required for the analyte to reach the detector when moving with the mean fluid velocity u), M is the mass of solute in the sample, k is the dispersion coefficient, and σ^2 is the temporal variance of the concentration profile related to the dispersion coefficient k by

$$\sigma^2 = \frac{2kt_R}{u^2} \quad (4)$$

The analytical expression for the dispersion coefficient was given by Aris:[9]

$$k = D + \frac{{R_C}^2u^2}{48D} \quad (5)$$

Under conditions where $D \ll {R_C}^2u^2/48D$, the analyte diffusivity can be determined from

$$D = \frac{{R_C}^2}{24\sigma^2}t_R \quad (6)$$

obtained from combining eqs 4 and 5. The dimensionless residence time τ and the Peclet number (Pe) given by eqs 7 and 8, respectively, can be used to evaluate the validity of eq 6.

$$\tau = \frac{Dt_R}{{R_C}^2} \quad (7)$$

$$Pe = \frac{uR_C}{D} \quad (8)$$

Equation 6 is valid when τ is greater than the time it takes to decrease variations in radial concentration by a factor of e ($\tau \gg 0.14$) and diffusion along the axial direction is negligible as compared to convection ($Pe \gg 7$).[19] Following previous practice,[15,16] Taylor's condition is considered to be satisfied when $\tau > 1.4$ and $Pe > 69$.

Using the UV area imager with two detection points along the capillary, the diffusion coefficient can be calculated from

$$D = \frac{{R_C}^2(t_2 - t_1)}{24({\sigma_2}^2 - {\sigma_1}^2)} \quad (9)$$

where t_1 and t_2 are the times required for the analyte to reach the detection windows 1 and 2, respectively, and ${\sigma_1}^2$ and ${\sigma_2}^2$ are the temporal variances of the concentration profile at windows 1 and 2, respectively. Subsequently, protein hydrodynamic radius, R_h, may be calculated from Stokes equation:

$$D = \frac{k_BT}{6\pi\eta R_h} \quad (10)$$

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where k_B, T, and η are the Boltzmann constant, the absolute temperature, and the solution viscosity, respectively.

EXPERIMENTAL SECTION

Reagents. Human serum albumin (fraction V, essentially fatty acid and globulin free) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Human α_1-acid glycoprotein and (±)-propranolol hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Sodium dihydrogen phosphate monohydrate was obtained from Merck (Darmstadt, Germany). All chemicals were used as received. Purified water from a Milli-Q deionization unit (Millipore, Bedford, MA) was used throughout.

The sample and running buffer used was a 67 mM sodium phosphate buffer, pH 7.40, prepared from sodium dihydrogen phosphate monohydrate. The pH was adjusted by addition of 5 M NaOH.

Samples containing 5.0×10^{-5} M propranolol and 2.5×10^{-5} M AGP or 8.0×10^{-5} M HSA were prepared by mixing appropriate volumes of propranolol, AGP, and HSA stock solutions, all prepared in the 67 mM sodium phosphate buffer (pH 7.4). Standard solutions without protein were prepared in a similar fashion.

Instrumentation. Capillary electrophoresis was performed on an Agilent ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany). The Actipix D100 active pixel sensor UV area imaging detector[20,21] was obtained from Paraytec Ltd. (York, U.K.). The electropherograms were recorded with Actipix D100 control software (version 0.885 or 1.0). Uncoated fused-silica capillaries of 92 cm in total length (50 μm i.d., 192 μm o.d.) and lengths of 34.5 and 49 cm to the first and second detection window, respectively, were obtained from Polymicro Technologies (Phoenix, AZ). The windows for detection were 1.5 cm in width. A notch was made in the standard Agilent capillary cassette allowing the capillary to be lead out of the side of the cassette (Supporting Information Figure S1). The capillary was inserted and detection windows aligned in the Actipix CE102 cartridge (Paraytec) allowing two-point detection before returning it into the capillary cassette. The Actipix sensor head was placed under the cover of the CE instrument (Supporting Information Figure S2).

CE Procedures. The fused-silica capillaries were conditioned for 30 min each with 1 M NaOH and running buffer. Between the measurements, the capillaries were flushed for 3 min each with 0.1 M NaOH and running buffer. Samples were introduced by pressure injection (50 mbar) for 100 s. Following the injection, a constant voltage of +23 kV in the normal polarity mode was applied (current ~55 μA) for 2.5 min after which the voltage was shut down and pressure (50 mbar) was applied to force propranolol and protein past the detection windows. The capillary cassette temperature was set to 25 °C, and UV detection was performed at 200 nm. Analyses were performed in triplicate. The free propranolol concentration in the samples was determined from the plateau heights by comparison to standards containing a similar propranolol concentration. The electropherograms recorded with Paraytec Actipix D100 control software were exported to Microsoft Office Excel and formatted for subsequent data analysis (eq 2) using Igor Pro 5.0 (WaveMetrics, Lake Oswego, OR). In the calculations, the i.d. of the capillaries (50 μm) was taken as stated by the manufacturer and the values used for temperature and buffer viscosity were 25 °C and 8.90×10^{-4} Pa s, respectively.

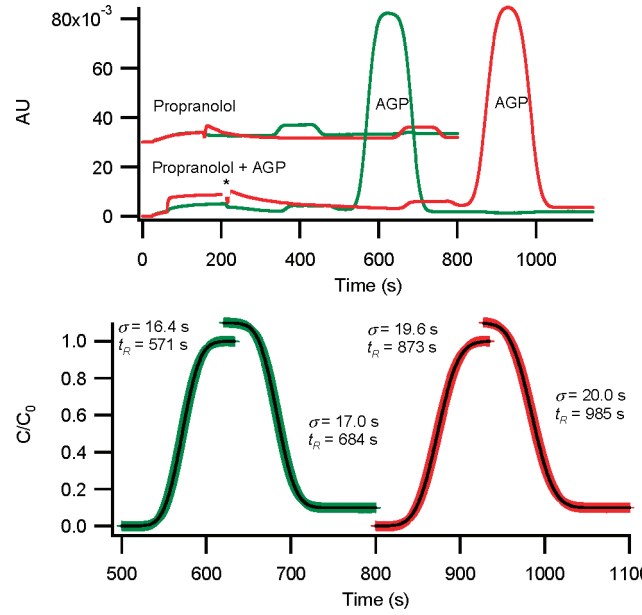

Figure 1. Combined Taylor dispersion analysis and frontal analysis capillary electrophoresis on a propranolol−AGP sample and propranolol standard solutions in 0.067 M phosphate buffer (pH 7.40). Upper panel: Electropherograms obtained by applying a voltage of 23 kV for 2.5 min followed by pressure mobilization (50 mbar) to force analytes past the two detection windows. The * denotes the time where voltage is switched off and pressure is applied. The fused-silica capillary was 92 cm in total length (50 μm i.d., 192 μm o.d.) with 34.5 and 49 cm to the first (green trace) and second (red trace) detection window; detection was at 200 nm; AGP and total propranolol (sample and standard) concentrations were 2.5×10^{-5} and 5.0×10^{-5} M, respectively. Lower panel: Taylor dispersion analysis performed on the normalized AGP traces, advancing and trailing (shifted) fronts. Broad traces show experimental data, and the solid black line was curve-fitted to eq 2.

RESULTS AND DISCUSSION

Preincubated samples of propranolol and protein in a phosphate buffer similar to the separation buffer were introduced by pressure (50 mbar for 100 s) into the capillary to achieve frontal analysis conditions.[22−24] Voltage was applied to initially separate the analyte propranolol from the ligand proteins AGP and HSA prior to performing TDA. Initial method development experiments revealed that the selected injection step (50 mbar for 100 s) together with the separation procedure described above were effective in attaining plateau peak conditions, necessary for quantification of the degree of ligand binding, and complete separation of the ligand and AGP, allowing TDA to be conducted on both the advancing and trailing fronts of the protein peak (Figure 1). The separation of ligand and protein attained during the separation step does not interfere with the use of the frontal analysis CE binding results.[23] That is, the presence of subsequent adjoining fronts is not an absolute requirement of frontal analysis

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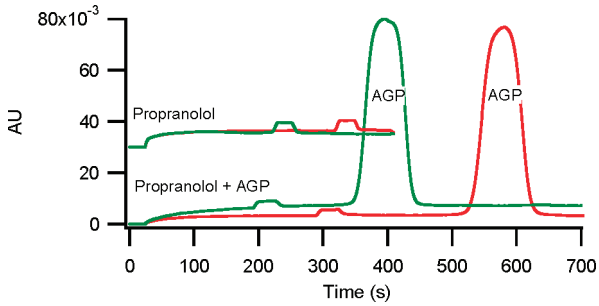

Figure 2. Frontal analysis capillary electrophoresis of a propranolol−AGP sample and propranolol standard solutions in 0.067 M phosphate buffer (pH 7.40). Electropherograms were obtained by applying a voltage of 23 kV during the entire run (pressure was not applied). The fused-silica capillary was 92 cm in total length (50 μm i.d., 192 μm o.d.) with 34.5 and 49 cm to the first (green trace) and second (red trace) detection window; detection was at 200 nm; AGP and total propranolol (sample and standard) concentrations were 2.5×10^{-5} and 5.0×10^{-5} M, respectively.

CE for binding data to be useful. Drug−plasma protein binding equilibria, as investigated here, are most often characterized by rapid on-and-off kinetics (relative to the separation time used in CE). Thus, due to the separation, the complex does not survive the electrophoretic step and consequently does not reach the detection windows. The two plateau peaks detected are due to unbound propranolol and AGP. Due to the relatively large sample volume (~94 nL) introduced into the capillary, the obtained protein plateau peaks were characterized as fronts (eq 2) rather than pulses (eq 3) in the TDA. The initial electrophoretic separation step was found not to compromise the peak shapes of AGP and HSA (e.g., through electromigration dispersion) (data not shown); thus, evaluation of the sharpness (variance) of the peak fronts through eq 2 was feasible (Figure 1). The large volumes of sample facilitated plateau peak conditions, where the propranolol peak heights are proportional to the free propranolol concentration in the sample, and thus the execution of affinity CE in the frontal analysis mode prior to the TDA step. Frontal analysis electropherograms for the propranolol−AGP system obtained after applying an initial separation step (23 kV for 2.5 min) followed by application of 50 mbar pressure needed for forcing the peaks past the detection windows are shown in Figure 1. A change in the baseline absorbance is observed at 220 s (marked with a star in the figure) when the potential is turned off, an effect presumably related to the sudden shift in Joule heating conditions. At 375 s the broad plateau peak for propranolol reaches detection window 1 prior to the much higher AGP peak (570 s). A qualitatively similar UV absorbance profile is observed for the second detection window but with a delay of ~300 s (red trace).

The effect of the pressure-driven flow (Poiseuille flow) on the AGP peak shape as compared to frontal analysis CE runs without pressure mobilization is apparent upon inspection of Figures 1 and 2. The peak shape changes (sharpness of the fronts) occurring during transport from the first to the second detection window are slightly more pronounced when pressure is applied indicating that TDA is a more sensitive approach for measurement of D values than dispersion due to electromigration because—in the latter case—dispersion is smaller. Equally important, the change in the sharpness of peak fronts can be related directly to the analyte diffusion coefficient through

Table 1. Fraction of Free Propranolol (f_{free}), α_1-Acid Glycoprotein (AGP), and Human Serum Albumin (HSA) Diffusion Coefficients (D) and Stokes Hydrodynamic Radii (R_h) Obtained Using Frontal Analysis CE and Taylor Dispersion Analysis in 0.067 M Phosphate Buffer (pH 7.40) at 25 °C (Mean ± SD; n = 3)

sample	f_{free}	$10^{11} \times D$ (m^2 s^{-1})	R_h (nm)	R_h lit. (nm)
AGP[a]	0.66 ± 0.02[b]	6.89 ± 0.14[c]	3.56 ± 0.07[c]	3.2[d]; 3.3[e]; 3.5[e]
AGP[f]	0.64 ± 0.003[b]			
AGP[a]	[g]	6.67 ± 0.15[c]	3.63 ± 0.08[c]	
HSA[a]	0.87 ± 0.001[b]	6.59 ± 0.12[h]	3.73 ± 0.07[h]	3.3−4.1[i]; 3.4−3.6[j]
HSA[f]	0.95 ± 0.002[b]			
HSA[a]	[g]	6.41 ± 0.18[h]	3.83 ± 0.11[h]	

[a] Pressure applied; combined TDA and CE. [b] AGP, HSA, and propranolol concentrations were 2.5×10^{-5}, 8.0×10^{-5}, and 5.0×10^{-5} M, respectively. [c] Advancing and trailing fronts used for determination of D and R_h ($n = 2 \times 3$). [d] Ref 30. [e] Ref 31. [f] Pressure not applied; conventional frontal analysis CE. [g] Sample without propranolol. [h] Advancing fronts only used for determination of D and R_h ($n = 3$). [i] Ref 32. [j] Ref 33.

the TDA (eqs 2−5), whereas the extraction of D values will be more complex when electrokinetic dispersion is also present.[25] Because separation between ligand and protein is achieved prior to application of pressure and because two detection points are utilized, the diffusion coefficients determined are related only to the size of the proteins, and these are not influenced by the binding interactions between ligand and protein and the effects that these interactions may have on the migration patterns. The advantages of having two detection windows include shorter analysis times and simplified data analysis. Correction of variances, discussed by Sharma et al. for capillaries with a single detection window,[16] is not required, and fewer experiments are needed for determining D values as compared to employing standard UV detection at one point at the capillary.

Frontal analysis conditions were maintained for the propranolol ligand when the pressure-driven mobilization was performed, i.e., plateau peak conditions are still prevailing, even though the peaks are less square (Figures 1 and 2). Ligand binding is apparent as a decrease in height and broadening of the propranolol sample peak as compared to the propranolol standard solution. The fraction of free propranolol determined from the peak heights of the samples relative to the propranolol peak heights of the standard solutions with similar concentration is reported in Table 1. Good agreement is observed between experiments with and without the pressure mobilization step. For affinity systems with 1:1 stoichiometry, stability constants may be estimated from the experiments such as the ones shown, although a series of measurements are to be preferred. However, due to the ability of the proteins AGP and HSA to interact with more than one ligand only the degree of binding is reported here.

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The UV absorbance and corresponding time points of the AGP peak boundaries were formatted and analyzed according to eq 2, and temporal variances, σ^2, and mean residence times, t_R, were determined for both the advancing and trailing fronts ($n = 2 \times 3$) of the AGP peaks (Figure 1). Insertion of σ and t_R values into eq 9 provided diffusion coefficients, and hydrodynamic radii were obtained from the Stokes equation (eq 10). Average D and R_h values are reported in Table 1. The Taylor dispersion analysis showed that Taylor's condition ($\tau > 1.4$ and $Pe > 69$) was satisfied for AGP (and HSA) in all experiments.

Samples without propranolol were analyzed in the combined TDA–frontal analysis mode and provided similar results with respect to D and R_h (Table 1) supporting the validity of the combined ligand binding and sizing approach. The agreement between our experimental results and literature values is satisfactory considering the temperature dependence of diffusion coefficients and the suboptimal temperature control of the sensor head placed inside the CE instrument (Supporting Information Figure S2). Samples containing propranolol and HSA could also be analyzed using the protocol developed for the propranolol–AGP system. Figure 3 shows the obtained electropherograms. UV traces for the corresponding propranolol standard solution are included in Figure 3 and show that binding of propranolol to HSA (free fraction 0.87) is significantly less than the degree of binding observed for the prepared AGP samples (Table 1). This trend is in agreement with previous reports[26,27] of propranolol possessing a larger affinity for AGP than for HSA. Frontal analysis CE has been widely utilized for studying ligand binding to biomacromolecules including plasma proteins.[4,22,24,26,27] In general good agreement between frontal analysis CE and other affinity techniques has been reported. For the low-molecular-weight drug interactions with AGP and HSA, comigration of complex and protein was assumed due to the size differences between ligand and protein. This is a necessary requirement for obtaining binding data without systematic errors.[28,29]

It is known that HSA to some extent adsorbs onto uncoated fused-silica capillaries. This is also apparent in Figure 3 where the trailing front does not decrease to the baseline level before the HSA plateau peak. This affects the temporal variances for this front, and thus only variances for the advancing fronts ($n = 3$) of the HSA plateau peak were fitted (Figure 3). The obtained D and R_h values are listed in Table 1. In agreement with literature the hydrodynamic radius of HSA ($M_r = 66\,500$) was found to be larger than that of AGP ($M_r = 40\,000$) (Table 1).

CONCLUSIONS

The present study demonstrates the first application of a TDA–CE approach facilitating simultaneous measurement of protein hydrodynamic radius (sizing) and quantification of ligand–protein affinity. For both affinity systems analyzed here, analysis times less than 20 min were obtained and absolute protein diffusion coefficients (size) and ligand binding ability were evaluated in a fast and efficient manner requiring only a minimum of sample by the use of capillaries with two detection points. It is suggested that the frontal analysis CE method with online TDA can provide important additional information on the aggregation states of proteins. Knowledge of size as well as ligand binding properties is crucial for efficient protein characterization in basic protein research and biomedical sciences.

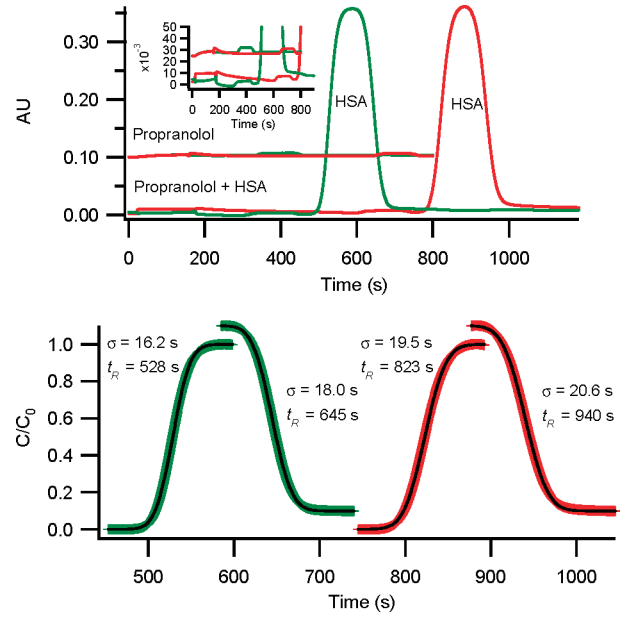

Figure 3. Combined Taylor dispersion analysis and frontal analysis capillary electrophoresis on a propranolol–HSA sample and propranolol standard solutions in 0.067 M phosphate buffer (pH 7.40). Upper panel: Electropherograms obtained by applying a voltage of 23 kV for 2.5 min followed by pressure mobilization (50 mbar) to force analytes past the two detection windows. The fused-silica capillary was 92 cm in total length (50 μm i.d., 192 μm o.d.) with 34.5 and 49 cm to the first (green trace) and second (red trace) detection window; detection was at 200 nm; HSA and total propranolol (sample and standard) concentrations were 8.0×10^{-5} and 5.0×10^{-5} M, respectively. Lower panel: Taylor dispersion analysis performed on the normalized HSA traces, advancing and trailing (shifted) fronts. Only the advancing fronts were used in the determination of the HSA diffusion coefficient. Broad traces show experimental data, and the solid black line was curve-fitted to eq 2.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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