Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Flow Induced Dispersion Analysis Rapidly Quantifies Proteins in Human Plasma Samples

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Taylor dispersion conditions

In the FIDA experiment, a pressure gradient generates the flow resulting in a parabolic flow profile. The parabolic flow profile inside the capillary distorts the indicator zone resulting in a concentration gradient between the center and the periphery of the capillary (figure S1). Equation 5 in the main manuscript is valid provided there is sufficient time for radial diffusion, and that advective mass transport dominates over diffusive mass transport in the axial direction meaning that the contribution from axial diffusion is insignificant.[1] A further prerequisite for applying the theory is that the width of the injected sample zone is sufficiently small compared to the width of the peak when detected.

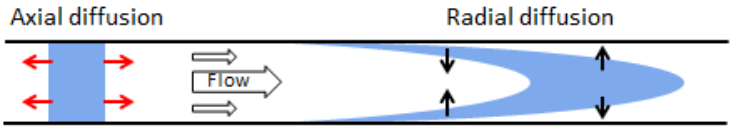

Figure S1. Schematic representation of Taylor Dispersion. The axial diffusion is represented by red arrows while the radial diffusion is represented by black arrows. The effect of the parabolic flow profile on the indicator zone distribution is represented by the axial elongation of the zone. Radial diffusion acts towards eliminating the concentration gradient generated in the radial direction. At the front of the peak the indicator will diffuse from the center of the capillary towards the edge and thus move from the faster flowing streamlines to the slower and vice versa at the back of the peak. Thus, the faster the indicator diffuses the more narrow the indicator peak will be.

The dimensionless τ parameter describes the ratio between the diffusion distance and the capillary radius. Ideally, τ must be larger than 1.4 in order to allow sufficient time for radial diffusion.

$$\tau = \frac{Dt_R}{r^2} > 1.4 \qquad \text{S1}$$

where r is the capillary radius, D is the diffusivity and t_R is the peak retention time.
The Péclet number (Pe) describes the ratio between the advective transport rate and the diffusive transport rate. When the Péclet number is sufficiently high diffusive mass transport in the axial direction can be ignored.[1]

$$Pe = \frac{u \cdot r}{D} > 69 \qquad \text{S2}$$

where u is the linear flow rate.
The present methodology is valid for a pressure driven flow having a parabolic flow profile. A similar effect is not observed for an electroosmotic flow as diffusive peak broadening only takes place in the longitudinal direction. An electroosmotic flows is therefore associated with a much smaller diffusive peak broadening.

Correcting diffusivity for changes in viscosity

During the experiments with HSA it was noted that the peak retention time for fluorescein increased with increasing HSA concentration present in the entire capillary. This was due to increased viscosity of the sample matrix. The viscosity also influences the diffusion coefficient, D. Thus it is necessary to correct the D values to the same viscosity in order to allow direct comparison between samples with different viscosity.
The Hagen–Poiseuille equation describes the relationship between volumetric flow rate, Q, and dynamic viscosity, η:

$$Q = \frac{\Delta P \cdot \pi \cdot r^4}{8 \cdot \eta \cdot L_t} \qquad \text{S3}$$

where L_t is the total length of the capillary, ΔP is the mobilization pressure, and r is the capillary radius. The peak retention time may be calculated by dividing the capillary volume from inlet to the detector with the volumetric flow speed:

$$t_R = \frac{V}{Q} = \frac{L_d \cdot L_t}{r^2} \cdot \frac{8 \cdot \eta}{\Delta P} \quad , V = L_d \cdot r^2 \cdot \pi \qquad \text{S4}$$

where L_d is the length of the capillary from the inlet to the detector. When the peak retention time is changing solely due to changes in the viscosity the following expression holds:

$$\frac{t_{R,1}}{t_{R,2}} = \frac{\eta_1}{\eta_2} \Leftrightarrow \eta_1 = \frac{\eta_2 \cdot t_{R,1}}{t_{R,2}} \quad \text{S5}$$

The Stokes-Einstein equation is used for normalization of the diffusivities according to viscocity:

$$D_1 = \frac{k_B \cdot T}{6 \cdot \pi \cdot R_H \cdot \eta_1} \quad \text{S6}$$

where k_B is the Boltzmann constant, T is temperature and R_H is hydrodynamic radius. D_1 is the diffusivity corresponding to a dynamic viscosity of η_1. Using equation S5 relating the viscosity to the peak retention time the expression may be rewritten in in terms of η_2:

$$D_1 = \frac{k_B \cdot T}{6 \cdot \pi \cdot R_H \cdot \eta_2} \cdot \frac{t_{R,2}}{t_{R,1}} \quad \text{S7}$$

Substituting D_2 into equation S7 yields:

$$D_1 = D_2 \cdot \frac{t_{R,2}}{t_{R,1}} \quad \text{S8}$$

Thus correcting the observed diffusivity for changes in viscosity is mathematically straight forward as it is simply obtained from the already available retention times.

Standard addition curve

The sensitivity of the fluorescein-HSA assay towards matrix effects in plasma was investigated by making a standard addition experiment. Increasing concentrations of HSA was added to a 2% v/v plasma sample. The standard addition curve was fitted to equation 4 in the main paper. Using the indicator diffusivity of $4.28 \cdot 10^{-10}$ m^2s^{-1} found for fluorescein, the HSA concentration in the plasma sample was found to be 10.3 µM (34 g/L in 100% plasma). In order to graphically represent the matrix effect the standard addition curve and the standard curve were overlaid as shown in figure S2. This was achieved by plotting the total concentration of HSA in the spiked samples used for the standard addition curve. The total HSA concentration was found by adding the HSA concentration from the plasma and the spiked concentration. In figure S2 it can be seen that the standard addition curve and the standard curve are similar indicating only minor matrix effects.

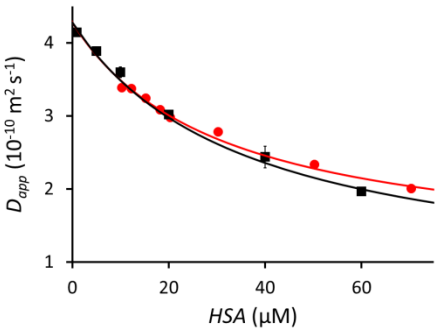

Figure S2. Standard addition curve in 2% v/v plasma plotted together with the standard curve for HSA in buffer for ease of comparison. The apparent fluorescein (indicator) diffusivity is plotted as a function of HSA (analyte) concentration in the run buffer. Red points and curve represent the standard addition data points and fit, respectively, and the black points and curve represent the standard curve. Both the standard curve and the standard addition curve have been fitted with the binding isotherm shown in equation 4 in the main paper. Fluorescein (indicator) and HSA were dissolved in 67 mM phosphate buffer pH 7.4. The plasma sample was diluted using the same buffer. All data points are triplicates with error bars showing the standard deviation. A 50 µm ID fused silica capillary with a length of 65.3 cm (47.5 cm to the detection point) was used.

Quantification of anti-HSA

The standard curve shown in figure 3 in the main manuscript was used to quantify anti-HSA in three independently prepared samples. Three different analyte concentrations were used in order to validate the standard curve in the concentration range from 35 to 350 nM. The results are shown in table S1. It can be seen that there is good agreement between the known analyte concentration and the measured concentration with an error of 11%. The relative standard deviation on the measured analyte concentration however is relatively large up to 36%. The reason for this is that small variations in diffusivity translate to large differences in the concentration when the value is approaching the edges of the dynamic range. However the dynamic range can easily be adjusted as shown in figure 4 in the main manuscript.

Table S1. Three different independently prepared samples of anti-HSA in 67 mM phosphate buffer pH 7.4 were analysed with FIDA. The concentration was determined with the standard curve in figure 3 in the main manuscript and compared with the known concentration. All experimental conditions were the same as for the standard curve. Each sample has been measured in triplicate and the given values are the average and the relative standard deviation. The deviation between the true and the measured analyte concentration is also given.

Sample	Apparent diffusivity (10^{-11} m^2 s^{-1})	Analyte concentration (nM)		Error
		True	Measured	
1	7.64 (± 2%)	35	32 (± 36%)	11%
2	7.13 (± 1%)	106	97 (± 8%)	9%
3	6.48 (± 2%)	353	316 (± 29%)	11%

Calculating the theoretical FIDA binding curves

The theoretical FIDA curves were calculated from a binding isotherm assuming 1:1 binding:

$$K = \frac{[AI]}{[A] \cdot [I]} \Leftrightarrow \frac{[AI]}{K} = [A] \cdot [I] \quad \text{S9}$$

The free concentrations of the indicator, analyte and complex are not known, but the formal concentrations are given by:

$$c_A = [AI] + [A] \quad \text{S10}$$

$$c_I = [AI] + [I] \quad \text{S11}$$

Inserting S10 and S11 into S9 results in equation S12 where only the free concentration of the analyte indicator complex is unknown:

$$\frac{[AI]}{K} = (c_A - [AI]) \cdot (c_I - [AI]) \quad \text{S12}$$

Equation S12 is a second order polynomial and solving this equation for [*AI*] allows calculating the proportion of bound indicator. From equation 3 in the main manuscript it is then possible to calculate the apparent indicator diffusivity from the bound fraction of the indicator and the diffusivity of the complex and the free indicator as shown in figure 4 in the main manuscript.

Theoretical limit of detection in FIDA

The limit of detection of the FIDA method is assessed graphically in the paper. Many parameters influence the sensitivity. It is assumed that it will be possible to distinguish the apparent indicator diffusivity at 10% binding from the diffusivity of the free indicator. The lowest analyte concentration that can bind 10% of the indicator can thus be used as an estimate of the limit of detection of the assay. This critical analyte concentration can be calculated from:

$$K = \frac{[AI]}{[A] \cdot [I]} \text{, where } [AI] = 0.1 \cdot c_I \text{, } [A] = c_A - 0.1 \cdot c_I \text{, } [I] = 0.9 \cdot c_I \quad \text{S13}$$

K is the binding constant, [*AI*], [*A*] and [*I*] are the free concentration of the complex, analyte and indicator, respectively, and c_I and c_A are the formal concentration of the indicator and analyte, respectively. Inserting and rearranging gives the following expression:

$$c_A = \frac{1}{9K} + 0.1c_I \quad \lim_{c_I \to 0} \quad c_A = \frac{1}{9K} \quad \text{S14}$$

This analyte concentration can be defined as the theoretical limit of detection for the assay. It is important to note that the lowest degree of binding that can be detected depends on the standard deviation of the measurements of D and may thus both be smaller or larger in individual cases. Also, the sensitivity of the

assay is affected by the magnitude of the difference in diffusivity of the indicator on free and complexed form. Slow binding kinetics may lower the sensitivity of the assay due to insufficient time for complex formation. If the binding is sufficiently strong, detection of the indicator may become the main factor limiting the sensitivity of the assay.

Experimental

Equipment

All FIDA experiments were performed using an Agilent ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany) coupled to a ZETALIF Evolution LIF detector. A 488 nm Melles Griot Diode laser was used for excitation and the emission light was passed through a 513 nm high pass filter before detection. A fused silica capillary (Polymicro Technologies) with an inner diameter of 50 µm (360 µm outer diameter) and a total length of 65 cm and 47.5 cm to the detection window was used. All experiments were performed at 25 °C. In the bromocresol purple assay the absorbance was measured with a Cecil Aquarius CE 7200 spectrophotometer. Curve fitting was done using the software OriginPro 9 (OriginLab Corporation).

Materials and chemicals

Ultra pure water was obtained from a Millipore Milli-Q water purification system. Human Serum Albumin (fatty acid free) was obtained from Sigma. Mix-n-Stain™ CF™488A antibody labelling kit 50-100 µg obtained from Biotium Inc. was used for fluorescently marking HSA. Monoclonal antibody against HSA was obtained from Sigma (product no A2672). Fluorescein was obtained from Sigma-Aldrich. Brij® 35 was obtained from Sigma Aldrich. Bromocresol purple was obtained from Merck. Tween 20 was obtained from Sigma Aldrich.

Procedures

Quantification of HSA by FIDA

A 67 mM phosphate buffer, pH 7.4, was prepared using Milli-Q water. Fluorescein was dissolved in the buffer at a concentration of 50 nM and used as indicator solution. The following concentrations of HSA were prepared in the phosphate buffer: 1 µM, 5 µM, 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM, and 200 µM. These albumin solutions were used as standards to create the standard curve. Phosphate buffer was used as a blank sample. Buffer, samples and indicator solutions were passed through a 0.45 µm nylon filter. Before analysis, the capillary was rinsed at 1 bar with 0.1 M NaOH for 2 min, Milli-Q water for 2 min, and the sample for 2 min. The indicator was injected into the capillary for 5 s at 50 mbar pressure giving an injection volume of 7 nL. The indicator zone was mobilized with the sample injected at a pressure of 50 mbar. All analyses were performed as triplicates by injecting the same indicator solution in the same sample three times. After three analyses the capillary was flushed with 1 M NaOH for 10 min and Milli-Q water for 10 min

with a pressure of 1 bar. The shape of the indicator peak was fitted to a Gaussian function and the resulting peak variance was extracted and the diffusivity was calculated and corrected for changes in viscosity according to equation S8. Plotting the apparent indicator diffusivity as a function of the HSA concentration in the run buffer resulted in a standard curve.

One series of experiments were carried out with an elevated mobilization pressure of 250 mbar instead of the 50 mbar. The pressure of 250 mbar was obtained by coupling an external nitrogen flask to the CE apparatus. For this series of experiments, the following HSA concentrations were used: 0.91, 4.5, 9.1, 18, 36, 54, 72, 91 and 181 µM. All other parameters were left unchanged including the injection step (50 mbar for 5 s) of the indicator solution.

The albumin content of human plasma samples was determined. Filtration of the plasma through a 0.22 µm nylon filter was necessary to avoid clogging the capillary. It was decided to dilute the plasma samples to 2% v/v, thereby placing the normal clinical concentrations in the dynamic range of the standard curve. The samples were analysed as described above. Using the above mentioned standard curve the concentration of HSA in the sample was determined based on the apparent indicator diffusivity calculated from the indicator peak variance.

FIDA standard addition curve for HSA

The FIDA standard addition curve was obtained using the same preconditioning, injection and run settings as above. A plasma sample was diluted to 2% v/v and 0, 2, 5, 8, 10, 20, 40 and 60 µM HSA were added to provide the different points on the standard addition curve.

Quantification of HSA using the bromocresol purple assay

A BCP reagent containing 0.15 M NaCl, 140 µM bromocresol purple and 0.2% w/V Brij® 35 solution dissolved in deionised water was prepared. A 0.1 M acetate buffer, pH 5.3, was also prepared in deionised water. A stock solution of HSA containing 45 mg/mL HSA in the acetate buffer was prepared and filtered through a 0.45 µm filter. For the standard curve 500 µL BCP reagent was mixed with 10, 20, 30, 40, 50, 60, 70, 80, and 90 µL of the HSA stock solution and diluted to a final volume of 600 µL with acetate buffer. The absorbance at 604 nm was measured after approximately 10 min with 500 µL BCP reagent mixed with 100 µL acetate buffer as reference. The plasma samples were filtered through a 0.45 µm nylon filter and 12 µL was mixed with 500 µL BCP reagent and 88 µL acetate buffer.

Quantification of Human Serum Albumin in stock solutions

The concentration of HSA in the stock solutions was determined by measuring the absorbance of the diluted stock solution at 289 nm with a Cecil Aquarius CE 7200 spectrophotometer. The concentration was calculated form Lambert-Beers law using an extinction coefficient of $35.3 \cdot 10^3$ L mol^{-1} cm^{-1}. [2]

Labelling of HSA

A HSA stock solution was prepared by dissolving 2 mg HSA in 2 mL 67 mM phosphate buffer pH 7.4. The concentration was determined by an absorbance measurement as described above. The stock solution was diluted to 0.65 mg/mL with 67 mM phosphate buffer, pH 7.4, and 123 µL of this solution was stained with a Mix-n-Stain™ CF™488A antibody labelling kit 50-100 µg according to the protocol in the kit. From the reaction product 75 µL was transferred to a 10 kDa cut-off filter and spun at 14000 rpm for 6 min. Subsequently 150 µL phosphate buffer was added and the filter was spun at 14000 rpm for 12 min. This step was repeated once with the purpose of removing all unreacted dye. Finally the solution was diluted to a final volume of 506 µL with the phosphate buffer. The HSA concentration in this solution was estimated to be approximately 200 nM assuming a loss of 50% of the protein in the spin filter. The exact concentration is not important for the method.

Standard curve for anti-HSA

A 67 mM phosphate buffer, pH 7.4, with a content of 0.005% v/v Tween 20 was used as run buffer and both indicator and analyte were dissolved in this buffer. Before each injection, the capillary was flushed with 1 M NaOH for 4 min and run buffer for 2 min each at a pressure of 1 bar. After each third injection the capillary was flushed with 96% ethanol for 3 min, 1 M NaOH for 3 min, and phosphate buffer for 1 minute, each at a pressure of 1 bar.

HSA covalently linked to the CF488 fluorophore was diluted to 20 nM and used as indicator. The indicator was injected at 50 mbar for 5 s resulting in an injection volume of 7 nL. The indicator plug was mobilised by injecting sample at a pressure of 50 mbar. The sample contained the antibody against HSA (Sigma product no A2672) in varying concentrations (35 pM, 0.11 nM, 0.35 nM, 1.1 nM, 3.5 nM, 7.1 nM, 18 nM, 35 nM, 71 nM, 0.11 µM, 0.18 µM, 0.35 µM, 0.71 µM, 1.8 µM). Experiments were performed in triplicates by injecting the same indicator solution in the same sample three times. The indicator peak was fitted to a Gaussian function and the resulting peak variance and residence time was extracted. The apparent diffusion constant was calculated and plotted as a function of the analyte concentration.

References

(1) G. Taylor, *P. Roy. Soc. Lond. A Mat.* **1953,** *219,* 186-203.
(2) Peters Jr. T. All About Albumin: Biochemistry, Genetics, and Medical Applications. San Diego, CA: Academic Press, 1996.