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Evaluation of Ligand-Selector Interaction from Effective Diffusion Coefficient

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We present an analytical technique for determination of ligand-selector equilibrium binding constants. The method is based on the measurements of effective molecular diffusion coefficient of the ligand during Poiseuille flow through a long (approximately 25 m), thin (0.254 mm ± 0.05 mm ID) capillary with and without the selector. The data are analyzed using the Taylor dispersion theory. Bovine Serum Albumin (BSA) and cyclodextrin (CD) were taken as model selectors. We have tested our method on the following selector-ligand complexes: BSA with warfarin, propranolol, noscapine, salicylic acid, and riboflavin, and cyclodextrin with 4-nitrophenol. The results are in good agreement with data from the literature and with our own results obtained within classical chromatography. This method works equally well for uncharged and charged compounds.

The formation of a macromolecule-ligand complex in a solution is an important process in chemistry, pharmacy, and molecular biology.[1,2] Drugs-protein binding properties[1−14] have been examined using equilibrium dialysis, ultracentrifugation and ultrafiltration. These traditional methods involve the physical separation of the free and bound analyte (drug, ligand), followed by an analysis step. Equilibrium dialysis is often considered as a reference method in drug−protein binding studies.[3] Ultrafiltration has been used as a routine method in clinical laboratories due to its simplicity.[2] Spectroscopic methods such as circular dichroism,[5] fluorescence,[6−8] UV/vis absorption[6] and also NMR[9,10] have been added to the list of applied techniques. NMR studies and one color FCS (fluorescence correlation spectroscopy) are suitable for studies of drug complexes characterized by weak interactions and fast exchange between free and complexed drug. Other recent techniques developed for characterization of ligand−receptor (protein) binding are based on chromatographic and electrophoretic methods.[11−15] Surface plasmon resonance (SPR) biosensor technology, such as BIACORE[16] can give high sensitivity and high throughput information. However conformational changes in the protein due to immobilization may cause of erroneous results when using SPR or affinity chromatography. Capillary electrophoresis suffers from protein adsorption to the capillary and at least one of the reagents in electrophoresis must carry a charge. Each technique has its own limitations and only a judicious choice of various technique can lead to reliable results. We present a new technique for determination of ligand-selector binding constants and additionally use the classical chromatography to test the method.

The Poiseulle flow, characterized by a parabolic velocity profile across the capillary, leads to the flow-induced widening of the initially narrow injection zone of the analyte. The shape of the zone is mapped on the concentration distribution of the analyte by the UV adsorption intensity of the analyte. The distribution, at the capillary end, is predicted by the Taylor theory to be Gaussian.[17,18] According to the Taylor theory the width of the concentration distribution is proportional to the square of the velocity and inversely proportional to the effective diffusion coefficient of the studied ligand (in a capillary filled with a selector). The ligand-selector binding constant influence the effective diffusion coefficient of the ligand and change the width of the concentration distribution. Therefore the latter quantity can

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(1) Bisswange, H. *Enzyme Kinetics Principle and Methods*, Chapter 3.1 "Methods for investigation of multiple equilibtia", 2nd ed.; Wiley VCH: New York, 2008.

(2) Connors, K. A. *Binding Constants The Measurements of Molecular Complex Stability*; John Wiley & Sons: New York, 1987.

(3) Lindup, W. E. In *Progressin Drug Metabolism*, Chapter 4; Bridges, J. W.; Chesseud, L. F.; Gibson, G. G., Eds.; Taylor and Francies: New York, 1987; Vol. 10.

(4) Hage, D. S.; Tweed, S. A. *J. Chromatogr., B* **1997**, *699*, 499–525.

(5) Cooper, C. L.; Dubin, P. L.; Kayitmazer, A. B.; Turksen, S. *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 52–78.

(6) Seedher, N.; Bhatia, S. *J. Pharm. Biomed. Anal.* **2005**, *39*, 257–262.

(7) Seedher, N.; Agarwal, P. *Ind. J. Pharm. Sci.* **2006**, *68*, 327.

(8) Xie, M.; Long, M.; Liu, Y.; Qin, C.; Wang, Y. *Biochim. Biophys. Acta* **2006**, *1760* (8), 1184–1191.

(9) Danel, C.; Azaroual, N.; Brunel, A.; Lannoy, D.; Vermeersch, G.; Odou, P.; Vaccher, C. *J. Chromatogr., A* **2008**, *1215*, 185–193.

(10) Zhu-Sheng, J.; Cong-Gang, L.; Xi-An, M.; Mai-Li, L.; Ji-Ming, H. *Chem. Pharm. Bull.* **2002**, *50* (8), 1017–1021.

(11) Joseph, K. S.; Moser, A. C.; Basiaga, S. B. G.; Schiel, J. E.; Hage, D. S. *J. Chromatogr., A* **2009**, *1216*, 3492–3500.

(12) Mallik, R.; Yoo, M. J.; Chen, S.; Hage, D. S. *J. Chromatogr., B* **2008**, *876*, 69–75.

(13) Sun, Y.; Cressman, S.; Fang, N.; Cullis, P. R.; Chen, D. D. Y. *Anal. Chem.* **2008**, *80*, 3105–3111.

(14) Zhao, P.; Zhu, G.; Zhang, W.; Zhang, L.; Liang, Z.; Zhang, Y. *Anal Bioanal Chem* **2009**, *393*, 257–261.

(15) Kwaterczak, A.; Duszczyk, K.; Bielejewska, A. *Anal. Chim. Acta* **2009**, *645*, 98–104.

(16) Rich, R. L.; Day, Y.S. N.; Morton, T. A.; Myszka, D. G. *Anal. Biochem.* **2001**, *296*, 197–207.

(17) Taylor, G. I. *Proc. R. Soc. London, Ser. A*, **1953**, *279*, 186−203. Aris, R. *Proc. R. Soc. London, Ser. A*, **1954**, *252*, 538−551.

(18) Aris, R. *Proc. R. Soc. London, Ser. A* **1956**, *235*, 67–77.

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be used for the determination of the constant. For the rest of this paper we will denote the method TDA (Taylor Dispersion Analysis).

TDA was used to obtain distribution of organic substrates between aqueous and micelle phases.[19,20] Based on Taylor dispersion monitored by electrospray mass spectrometry Clark et al.[21,22] identified monocovalent complexes of macromolecules. Capillary electrophoresis (CE) instrument combined with TDA was used to obtain the diffusion coefficient of phenylalanine, proteins and nanoparticles.[23−25] In this approach the first step is the separation of the studied mixture by capillary electrophoresis and the second step is online measurement of diffusion coefficient by TDA.[25,26] Propranolol and proteins were the model systems to demonstrate the first application of TDA-CE approach facilitating simultaneous measurement of protein size and quantification of ligand protein affinity.[27] Noncovalent interaction between cyclodextrin and naphthol and naproxen were quantified by TDA in Jensen and Ostergaard recent communication.[28]

In our method we use typical chromatographic equipment, where the chromatographic column is replaced by a long (around 25 m) and thin (0.254 mm ±0.05 mm ID) capillary filled with an appropriate eluent (e.g., Tris buffer, water−ethanol mixture etc.) or an eluent with a selector of interest. This eluent flows in the capillary with a typical flow rate of 0.1−0.05 mL/min. A narrow injection of the sample (eluent-ligand or eluent-selector) is applied to the system and the diffusion coefficients of the free ligand and the free selector is determined by TDA. Next we fill the capillary with the eluent with the selector of known concentration and inject ligand-selector sample. The concentration of ligand is the same as in the experiment without selector. The concentration of the selector is the same as in the eluent. The effective diffusion coefficient of the ligand during the flow in the presence of the selector depends on the diffusion coefficient of free and complexed ligand and on also stability constant of the ligand-selector complex. This diffusion coefficient is also determined by TDA.

The properties of the measuring system were checked by injection of a solution of KNO_3 as the inert substance. Albumin-warfarin, propranolol, noscapine, salicylic acid, and riboflavin experiments were used to demonstrate the usefulness of the method for studying biologically important interactions. In order to compare the new method with the classical chromatographic method, experiments with 4-nitrophenol and cyclodextrin were performed using both methods.

THEORY

We consider an eluent moving at the average velocity u along a tube of radius R. At an injection point a compound of interest is introduced into the eluent. The distance along the tube from the injection point to the detection point is L. At the detection point we measure the shape of the concentration distribution of the compound, for example, by absorbance as a function of time t. The normalized distribution inside a capillary near its end is given by the Gaussian distribution according to the Taylor theory:[17,29]

$$P(t) = \frac{1}{2\sqrt{\pi\sigma t}}\exp\left[-\frac{(L-ut)^2}{4\sigma t}\right] \quad (1)$$

The width of $P(t)$ is determined by the dispersion coefficient:

$$\sigma = \frac{u^2R^2}{48D^{\text{eff}}} \quad (2)$$

The dispersion coefficient, σ, is proportional to the square of the velocity and inversely proportional to the effective molecular diffusion coefficient D^{eff}. The former dependence is due to the shape of the velocity profile (parabolic) across the capillary. The latter dependence is due to the compound diffusion during the flow with or without complexing agent. The compound may diffuse freely without any binding or it may diffuse as a complex with the selector, added to the eluent. If there is no selector in the eluent and we monitor only one compound, say ligand, $D^{\text{eff}} = D_L$, where D_L is the molecular diffusion coefficient of the ligand. If the ligand injected into the capillary forms **L**:**S** complex with the selector present in the eluent, D^{eff} will depend on K the equilibrium constant of the reaction

$$L + S \leftrightarrows L{:}S$$

The dependence of D^{eff} on K can be determined on the basis of the following assumptions. The time of the flow of the ligand between the injection point and the detector is long and given by t_F (we use a very long capillary and a small flow rate). The reaction mixture of **L** and **S** is assumed to come to the equilibrium in a much shorter time than t_F.

During the flow the **L** compound is in a complex with **S** on average during the time t_{LS} and in the free state during the time t_L and consequently $t_F = t_L + t_{LS}$ and

$$D^{\text{eff}} = \frac{D_L t_L + D_{LS} t_{LS}}{t_L + t_{LS}} \quad (3)$$

The time spent in the free state, t_L, and in the bound state, t_{LS}, determine the ratio of the molar concentration of free, c_L and bound ligands, c_{LS}. From this simple observation we calculate the equilibrium constant K multiplied by the molar concentration of the selector, c_S, in the eluent as

$$Kc_s = \frac{c_{LS}}{c_L} = \frac{t_{LS}}{t_L} \quad (4)$$

Under the experimental condition the selector is in large excess over the ligand and can therefore be approximated by total selector concentration in the mobile phase (amount of selector,

(19) Burkey, T. J.; Griller, D.; Lindsay, D. A.; Scaiano, J. *J. Am. Chem. Soc.* **1984**, *196*, 1983–1985.
(20) Yang, X.; Matthews, M. A. *J. Colloid Interface Sci.* **2000**, *229*, 53–61.
(21) Clark, S. M.; Leaist, D. G.; Konermann, L. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1454–1562.
(22) Clark, S. M.; Konermann, L. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 430–441.
(23) Bello, M. S.; Rezzonico, R.; Righetti, P. G. *Science* **1994**, *266*, 773–776.
(24) Sharma, U.; Gleason, N. J.; Carbeck, J. D. *Anal. Chem.* **2005**, *77*, 806–813.
(25) d'Orlyé, F.; Varenne, A.; Gareil, P. *J. Chromatogr., A* **2008**, *1204*, 226–232.
(26) Le Saux, T.; Cottet, H. *Anal. Chem.* **2008**, *80*, 1829–1832.
(27) Ø´stergaard, J.; Jensen, H. *Anal. Chem.* **2009**, *81*, 8644–8648.
(28) Jensen, H.; Ø´stergaard, J. *J. Am. Chem. Soc.* **2010**, *132*, 4070–4071.
(29) Lewicz, B. G. *Fiz-Khim. Gidrodin., G.I.F-M.* **1959**, 123–127, Moskwa.

which fills the whole capillary (1.4 milliliters), is much higher than ligand injected in a small volume of 10 μL). Finally we find from eqs 3 and 4 that

$$D^{\mathrm{eff}} = \frac{D_{\mathrm{L}} + D_{\mathrm{LS}} K c_{\mathrm{S}}}{1 + K c_{\mathrm{S}}} \tag{5}$$

The molecular diffusion of ligand **L**, is much larger than the diffusion coefficient of **S** ($D_{\mathrm{L}} \gg D_{\mathrm{S}}$) if the selector **S** is a macromolecule much larger than the ligand **L**. Therefore the diffusion coefficient of the **L**:**S** complex can be approximated by the diffusion coefficient of the selector **S**, $D_{\mathrm{LS}} = D_{\mathrm{S}}$. In our experiment with albumin D_{L} and D_{S} are first determined in two separate experiments, where a single substance is used such as **L** or **S** separately. In this case $D^{\mathrm{eff}} = D_{\mathrm{L}}$ or D_{S}.

Summarizing the theoretical consideration: from the experiment we determine $P(t)$. Using eq 1 we obtain the dispersion coefficient σ from $P(t)$. In the framework of the Taylor theory we get the effective diffusion D^{eff} from σ according to eq 2. D^{eff} depends on the equilibrium constant as given in eq 5. Thus our method allows to determine the appropriate equilibrium constants from the dependence of D^{eff} on the molar concentration c_{S}. The Taylor condition for the applicability of our technique can be written as follows:

$$\frac{LD^{\mathrm{eff}}}{R^2 u} > > 1 \tag{6}$$

In all cases studied here the range of LD^{eff}/R^2u values is between 9.5 (for albumin) and 147 (for KNO_3). Therefore the condition is satisfied in our experiments.

EXPERIMENTAL SECTION

Apparatus. Our experiments were performed using a Waters (Vienna, Austria) HPLC pump model 515 and autosampler model 717 plus. The PEEK (polyether ether ketone) capillary was thermostatted at 25 ± 0.2 °C by a WO Industrial Electronics (Langenzersdorf, Austria) Jetstream 2 thermostat. The absorbance was measured by Waters UV VIS detector model 2487 and recorded by PC computer using Empower Pro build number 1154 software (Empower Software Waters Corporation).

For almost all experiments the flow rate was 0.05 mL/min and the injection volume was 10 μL. For experiments of the precision procedure the flow was 0.1 mL/min and the injection volume was 5, 10, and 20 μL.

We applied standard chromatography to the system of 4-nitrophenol with cyclodextrin and compared the obtained equilibrium constants to the ones obtained in our method based on the effective diffusion coefficient. We used the Waters (Vienna, Austria) model 590 pump, a Rheodyne type injector with 5 μL loop and a Waters UV VIS detector model 490 (detection: 254 nm). The mobile phases were water ethanol mixture (90:10 v/v) with the appropriate concentration of CDs (concentration range 5×10^{-5}–1.5×10^{-2} M). We used the column Luna 5 μ C18(2) 100A 150 × 1 mm, Phenomenexs (Aschaffenburg, Germany). The column was thermostatted at 25 ± 0.2 °C by a WO Industrial Electronics (Langenzersdorf, Austria) Jetstream 2 thermostat. The binding constants obtained as a result of these experiments were calculated as presented earlier.[30−32]

Materials and Solutions. Bovine serum albumin (BSA) was purchased from Across Organics (NJ). Warfarin, propranolol, (S,R)-noscapine base, (−) riboflavin, 4-nitrophenol, ortho-phosphoric acid (H_3PO_4), and alpha, alpha, alpha-tris-(hydroxymethyl)-methylamine (Tris) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). β-Cyclodextrin (β-CD) came from Chinoin (Budapest, Hungary). Salicylic acid and potassium nitrate (KNO_3) were from POCH (Gliwice, Poland).

Experiments. *Precision Procedure.* The PEEK capillary length was measured on a tube with known diameter. The dry PEEK capillary was connected to the end of the stainless steel capillary of the sampling device. Next the capillary was filled with a mobile phase. The PEEK capillary volume was measured by the time of the capillary filling. The pump mass outflow and mobile phase density were also measured. The dimensions of the stainless steel capillaries connected to the sampling device and the detector were taken from their specification and added to the capillary length and the volume of the PEEK capillary. The obtained total volume value was verified by experimental determination of the time of filling the capillary with the KNO_3 solution. The total capillary length was 2749.8 cm and the total capillary volume was 1.3775 cm^3, as obtained from both the calculation and the calibration experiments. These values were used in all further experiments and calculations.

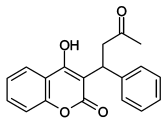
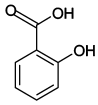
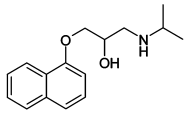
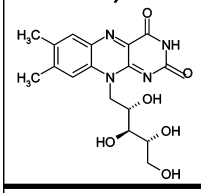
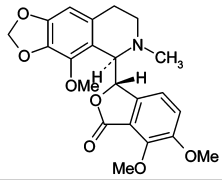
The mobile phase was prepared using an 18 mM Tris solution adjusted to pH 7.4 with H_3PO_4. The stock solution of KNO_3 was prepared by dissolving 10 mg of salt in a 10 mL volumetric flask with the mobile phase. For measurements the stock solution was diluted 10 times by the mobile phase. The sample of KNO_3 (0.1 mg/mL, 1 mM) was injected into the capillary. The injected volume was 5, 10, and 20 μL and all experiments were repeated three times. The UV absorbance was measured at 220 nm wavelength.

Experiments with BSA and Ligands. Three different pH were chosen: pH 7.4, 4.8, and 3.0. The buffer solution was prepared using 18 mM Tris solution adjusted to the appropriate pH with H_3PO_4. The albumin, propranolol and riboflavin samples were studied in all three pH. The salicylic acid and warfarin samples were studied only at pH 7.4 and noscapine only at pH 3.0. The samples were dissolved in the appropriate Tris solution and injected (three times each) into the capillary with the same Tris solution as the mobile phase. The sample concentrations were albumin, 2.3×10^{-5} M; propranolol, 9.6×10^{-5} M; riboflavin, 3.3×10^{-5} M; salicylic acid, 9.9×10^{-5} M; warfarin, 9.7×10^{-5} M; noscapine, 6.1×10^{-5} M. The chemical formulas for the model compounds are presented in Table 1. For the albumin solution the absorbance was measured at 275 nm and for the rest of the compounds at 308 nm.

To determine the BSA-ligand equilibrium binding constants, a new mobile phase was prepared by dissolving BSA in the appropriate Tris solution. For albumin warfarin complex we prepared six solution of albumin in the concentration range 0−4.5

(30) Dodziuk, H.; Demchuk, C. M.; Bielejewska, A.; Kozminski, W.; Dolgonos, G. *Supramol. Chem.* **2004**, *16*, 287–292.
(31) Fujimura, K.; Ueda, T.; Kitagawa, M.; Takayanagi, H.; Ando, T. *Anal. Chem.* **1986**, *58*, 2668.
(32) Terabe, S. *J. Chromatogr., A* **1994**, *666*, 295–319.

Table 1. Diffusion Coefficients and Stability Constants for Studied Compounds and BSA Complexes SD Standard Deviation, RSD Relative Standard Deviation (*, data taken from ref 16)[a]

Compound and	Conditions	Free Compund $D^{eff}=D_{L\ or\ S}$			Free +Complex wit BSAstate $D^{eff}=\frac{D_L + D_{LS}Kc_S}{1+Kc_S}$ 5			Stability constants
		D [cm^2s^{-1}]	SD (n=3)	RSD%	D^{eff}	SD (n=3)	RSD%	K [M^{-1}]
Warfarin 	pH=7.4	$4.75x10^{-6}$	$1.3x10^{-7}$	2.8	$1.47x10^{-6}$	$1.3x10^{-8}$	1.1	$(1.35\pm0.05)x10^5$ *$(2.7\pm0.88)x10^5$
Salicylic acid 	pH=7.4	$7.46x10^{-6}$	$4.8x10^{-7}$	0.6	$6.13x10^{-6}$	$1.9x10^{-8}$	3.2	$(5.67\pm1.06)x10^3$ *$(7.1\pm1.8)x10^3$
Propranolol 	pH=3.0	$4.86x10^{-6}$	$2.2\ x10^{-7}$	4.5	$4.61.10^{-6}$	$6.5x10^{-8}$	1.4	$(1.46\pm1.0)x10^3$
	pH=4.8	$4.78x10^{-6}$	$9.2x10^{-8}$	1.9	4.78E-06	$9.2x10^{-8}$	1.9	**No detectable interaction**
	pH=7.4	$3.28x10^{-6}$	$3.7x10^{-8}$	1.1	$2.86x10^{-6}$	$3.6x10^{-8}$	1.3	(2.36±0.3)E+03
Riboflavin, B2 	pH=3.0	$4.13x10^{-6}$	$8.1x10^{-8}$	2.0	$3.55x10^{-6}$	$2.2x10^{-7}$	6.3	$(4.51\pm2)x10^3$
	pH=4.8	$4.35x10^{-6}$	$6.4x10^{-8}$	1.5	$4.11x10^{-6}$	$1.8x10^{-7}$	4.4	$(8.48E\pm7)x10^2$
	pH=7.4	$4.20x10^{-6}$	$1.1x10^{-7}$	2.7	$3.58x10^{-6}$	$6.4x10^{-8}$	1.8	$(2.57\pm0.6)x10^3$
Noscapine								
	pH-3.0	$4.56x10^{-6}$	$1.2x10^{-7}$	2.6	$4.61x10^{-6}$	$3.0x10^{-7}$	6.5	**No detectable interaction**
BSA	pH=3.0	$7.32x10^{-7}$	$9.8x10^{-10}$	0.1				
	pH=4.8	$9.71x10^{-7}$	$3.3x10^{-8}$	3.4				
	pH=7.4	$8.94x10^{-7}$	$1.7x10^{-8}$	1.9				

[a] SD standard deviation, RSD relative standard deviation (*- data taken from ref 16). Experimental conditions: for the free compounds—mobile phase was 18 mM Tris at pH as in table, for complex study—mobile phase was 9.0×10^{-5} M or 4.5×10^{-5} M BSA (see Experimental Section) in 18 mM Tris at pH as shown in the table. Detection was 275 nm for albumin and 308 for the rest studied systems. Flow 0.05 mL/min. Temperature 25 ± 0.2 °C.

$\times 10^{-5}$ M. For the rest of compounds single concentration experiment was done (concentration of BSA 9.0×10^{-5} M for pH 7.4 and 4.8 and 4.5×10^{-5} M for pH 3.0). The absorbance was measured at 308 nm.

We also injected warfarin into serum of blood and observed a clear peak from warfarin. Serum was 20 times dissolved by Tris solution pH 7.4 and used as the mobile phase for warfarin sample.

Experiment with 4-Nitrophenol and Cyclodextrin. The mobile phases in these experiments were a water−ethanol mixture (90:10 v/v) with concentrations of CDs in the range $0-2 \times 10^{-2}$ M. We prepared eleven such solutions Additionally only for TDA analysis seven solution of CD in phosphate buffer pH 7.3 were prepared in concentration range $0-1.6 \times 10^{-2}$ M. The samples of 4-nitrophenol were prepared by dissolving 2.8 mg in 25 mL of each mobile phase and injected into the capillary (or into the column for the comparative chromatographic experiment) with the appropriate mobile phase. The absorbance was measured at 254 nm.

Calculations Applied to the Experiment. The concentration distribution of ligand is given by eq 1. After correcting eq 1 for the finite injected volume of the sample[33] we get

$$P(t)_c = \frac{1}{\sqrt{2\pi}} \frac{Ah}{\sqrt{2\sigma \cdot t + m_2}} \exp\left[-\frac{(L - u \cdot t + m_1)^2}{2(2\sigma \cdot t + m_2)}\right] \quad (7)$$

where the injection length in the capillary is $h = (v_{inj}/v_{cap})L$, where V_{inj} is the injection volume, V_{cap} is the volume of the capillary, L is its length and (m_1), (m_2) are corrections for the rectangular injection. These corrections are related to h and given by the

formulas $m_1 = h/2$ and $m_2 = h^2/12$. Finally we rewrite eq 6 with these formulas included:

$$P(t)_c = \frac{2\sqrt{3}}{\sqrt{2\pi}} \frac{Ah}{\sqrt{24\sigma t + h^2}} \exp\left[-\frac{6(L - u \cdot t + h/2)^2}{(24\sigma t + h^2)}\right] \tag{8}$$

where h is calculated from the injection volume and the only fitting parameters are: the amplitude A, the velocity u and the dispersion coefficient σ given by eq 2.

The data were collected at times t_i from t_i = 1 s up 40 min with $\Delta t = 1$ s time step. The absorbance was measured in the 10 μL absorbance cell for which the filling time is 12 s with 0.05 mL/min flow. The detector integrates the signal given by eq 8 over 12 consecutive seconds and therefore the measured absorbance is given by:

$$\mathrm{AU}(t_i) = \sum_{i-12}^{i} P(t_i) \tag{9}$$

where the times $t_i - t_{i-1} = 1$ s. Additionally the absorbance signal is recorded after a 1 s RC filter. The fit was performed only for AU larger than 0.5 $\mathrm{AU_{max}}$. The absorbance was recorded at constant wavelength. Zero drift of the detector signal was corrected before fitting. The number of points used for fitting depended on the width of the peak and changed in our experiments from 68 to 253. Least square fitting was performed. The fitting procedure was done using the Microsoft Excel Office 2007 solver.

RESULTS AND DISCUSSION

Precision of Molecular Diffusion Coefficient Measurements. The objective of the procedure was to assess the precision with which we measured the diffusion coefficients. The molecular diffusion coefficient of KNO_3 was determined for three different injection volumes of 5, 10, and 20 μL, each measured three times. The determined mean σ was 2.32 cm^2s^{-1} with the standard deviation (SD) 0.08 cm^2s^{-1} and relative standard deviation (RSD) 3.4%. The molecular diffusion coefficient for KNO_3 was 1.60×10^{-5} cm^2s^{-1} with (SD) equal to 5.0×10^{-7} cm^2s^{-1}. Thus the relative standard deviation (RSD, %) was 3.4%. From literature data the diffusion coefficient of KNO_3-H_2O solution at 25 °C measured on the volume fixed frame of reference over the concentration range 0.025−2.61 M decreased from 1.928×10^{-5} to 1.166×10^{-5} $cm^2 \cdot s^{-1}$ as the concentration increased.[34] Our value of 1.6×10^{-5} $cm^2 \cdot s^{-1}$ measured in a Tris buffer was well inside the range of the literature values.

Study of Molecular Interaction. Two types of selectors were chosen: albumin and cyclodextrin. Serum albumin is an abundant plasma protein that plays an important role in transporting compounds in blood plasma.[1] The most characteristic property of cyclodextrin is its remarkable ability to form inclusion complexes with various organic and inorganic compounds.[35]

(33) Alizadeh, A.; Nieto de Castro, C. A.; Wakeham, W. A. *Int. J. Thermophys.* **1980**, *1*, 243.
(34) Daniel, V.; Albright, J. G. *J. Solution Chem.* **1991**, *20*, 633–642.

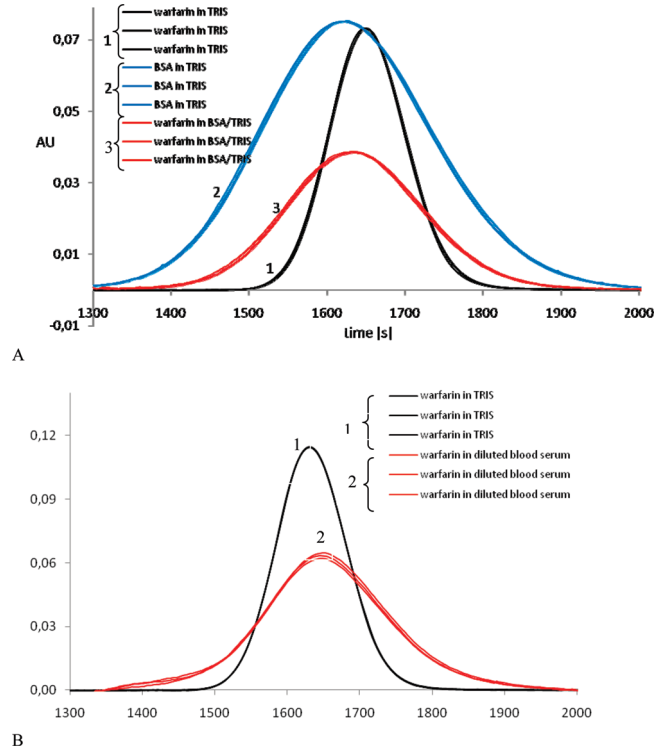


Figure 1. The peaks obtained for warfarin in Tris solution, albumin in Tris solution and warfarin in Tris solution with albumin (each measurements were repeated three times) The peaks of warfarin obtained in Tris solution and in 20× diluted serum. Experimental conditions: (A) for peaks 1 and 2-mobile phase was 18 mM Tris pH 7.3, for 3-mobile phase was 9.0×10^{-5} M BSA in 18 mM Tris pH 7.3, B) for 1-mobile phase was 18 mM Tris pH 7.3, for 2-mobile phase was serum 20× diluted with 18 mM tris pH 7.3. Detection was 275 nm for albumin and 308 nm for the rest studied systems. Flow 0.05 mL/min. Temperature 25 ± 0.2 °C.

Albumin and Drugs. The interaction between albumin and small drug molecule was determined in three experiments. From the first two experiments we obtained the molecular diffusion coefficient of the drug D_L and the albumin D_S. In the last experiment we determined the effective diffusion coefficient D^{eff} of the drug (eq 5) with the albumin present in the mobile phase.

The experimental peaks obtained for albumin, warfarin and warfarin with albumin are presented in Figure 1A. In Figure 1B the peak of warfarin obtained in 20 times diluted serum is presented. The experiment showed a possibility to use the method in medical analysis of blood samples; for example to predict concentration of the free drug in the serum. Additionally typical fits and errors are presented in Figure 2.

For all the studied compounds the stability constant was determined from eq 5, assuming that D_{LS}=D_S which is reasonable providing that the ligand **L** is much smaller than the selector **S** (albumin). The results, for warfarin, obtained for a single concentration ($K = 1.35 \times 10^5$ M^{-1}) are in good agreement with a measurement for various concentration of albumin ($K = 1.40 \times 10^5$ M^{-1}). This comparison proves that for the single concentration experiment good concentration range was chosen. However we suggest to use caution when calculating

(35) Dodziuk, H. *Cyclodextrin and Their Complexes; Chemistry, Analytical methods, Application*; John Wiley & Sons: Weinheim, 2006.

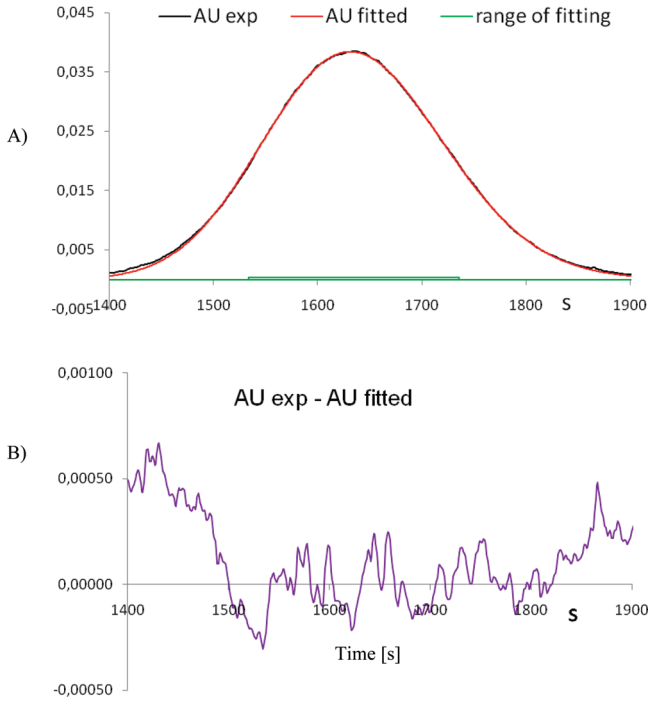


Figure 2. (A) The absorbance measured at the end of 25 m capillary by UV detector 308 nm for the warfarin-BSA complex together with theoretical fit. (B) The difference between measured and fitted absorbance Experimental conditions: mobile phase was 9.0×10^{-5} M BSA in 18 mM Tris pH 7.3. Flow 0.05 mL/min Temperature 25 ± 0.2 °C.

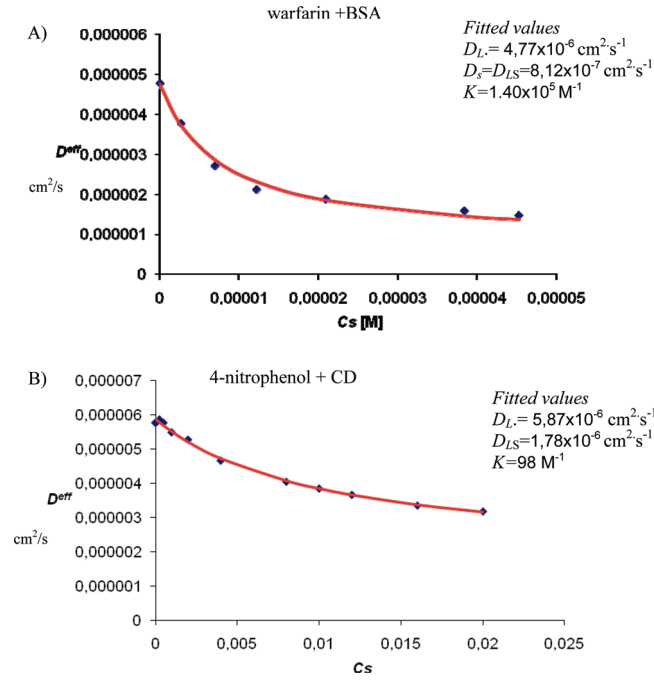


Figure 3. D^{eff} as a function of selector concentration, experimental points and fitted plots for eq 5 (A) for warfarin-BSA complex (B) for 4-nitrophenol-CD complex. Experimental conditions: flow 0.05 mL/min, temperature 25 ± 0.2 °C (A) mobile phase 18 mM Tris with appropriate concentration of BSA (B) mobile phase 90:10 v/v water: ethanol mixture with appropriate concentration of CD.

binding constants using data generated at a single concentration of substrate and ligand. In our case it did not appear to introduce significant error. However it can introduce significant error if the location of the data point on the binding isotherm is not known. You need to understand your system before you can assume that the single point is appropriate.[36] The experimental points and fitted curve are presented in Figure 3A. All results are presented in Table 1. Results obtained from the literature are also presented.[16]

We used three different pH, below, above and at the isoelectric point of BSA. The diffusion coefficient of BSA depends slightly on pH. Such dependence may be caused by the changes of conformation of albumin induced by the pH shift.[37] The value of the molecular diffusion coefficient found from our data ranged from 7.3 to 9.7×10^{-7} cm^{2s-1} depending on pH. In the literature this diffusion coefficient depends on pH, ionic strength and concentration of albumin and varies[38] between 4.9×10^{-7} cm^2s^{-1} and 9.5×10^{-7} cm^2s^{-1}.

For propranolol, salicylic acid and riboflavin, the stability constants for BSA complexes are 2 orders of magnitude lower than the BSA−warfarin complex. Near the isoelectric point of BSA the propranolol complex with BSA was not detected and for riboflavin it was weaker than for pH 3.0 and 7.4. The complex of noscapine and BSA was not detected at all. The results for warfarin and salicylic acid are in good agreement with the literature data at pH 7.4.

Cyclodextrin with 4-Nitrophenol. The cyclodextrin complex with 4-nitrophenol was studied first by the standard chromatographic method. The data were analyzed by the model described earlier.[39−41] The stability constant K was fitted by the nonlinear least-squares procedure according to the following equation:

$$\frac{1}{k^1} = \frac{1}{k_0} + \frac{K_1[\mathrm{CD}]}{k_0} + \frac{K_1K_2[\mathrm{CD}]^2}{k_0} + \frac{K_1K_2K_3[\mathrm{CD}]^3}{k_0} + \quad (10)$$

where k_0 is the retention factor without cyclodextrin (CD) and k_1 is the retention with CD. The function of $1/k_1$ versus CD concentrations provides information on the stoichiometry and stability constants of the complex. For the 4-nitrophenol complex in 10% etanol-aquous solution in the studied range of cyclodextrin concentration, 1:1 stoichiometry was found. The stability constants $K_1 = 108 \pm 11$ M^{-1} were found from the fitting procedure.

For our method, changes of the effective diffusion coefficient D^{eff} with growing concentration of CD was followed. The results are shown in Figure 3B. The stability constant was fitted using eq 5. The obtained diffusion coefficient for 4-nitrophenol and 1:1 complex with CD (in ethanol−water mixture 10:90 v/v) were 5.87×10^{-6} $cm^2 \cdot s^{-1}$ and 1.78×10^{-6} $cm^2 \cdot s^{-1}$ respectively (from literature we found the diffusion coefficient for 4-nitrophenol in aqueous equal to 6.92×10^{-6} $cm^2 \cdot s^{-1}$).[42] The obtained

(36) Bowser, M. T.; Chen, D. D. *J. Phys. Chem.* **1998**, *102*, 8063–8071.
(37) Peters, T., Jr. *All about Albumin: Biochemistry, Genetics and Medical Applications*; San Diego, CA: Academic Press, 1996.
(38) Raj, T.; Flygare, W. H. *Biochemistry* **1974**, *13*, 3336–3340.
(39) Armstrong, D. W.; Nome, F. *Anal. Chem.* **1981**, *53*, 1662–1666.
(40) Armstrong, D. W.; Gail, T. S. *J. Am. Chem. Soc.* **1983**, *105*, 2962–2964.
(41) Armstrong, D. W.; Nome, F.; Spino, L. A.; Golden, T. D. *J. Am. Chem. Soc.* **1986**, *108*, 14.
(42) Armstrong, D. W.; Ward, T. *J. Anal. Chem.* **1988**, *58*, 579–582.

stability constant $K_1 = 98 \pm 12\ M^{-1}$ were in good agreement with the results from the chromatographic method. However, the literature values obtained from calorimetric study for 1:1 complex in aqueous solution were $350 \pm 50\ M^{-1}$)[43] which seemed to disagree with our results. We found that the difference between literature and our results was due to the composition of the mobile phase which in our case contained 10% of ethanol and most probably the ethanol made also complexes with cyclodextrin.[44] Therefore we repeated our experiment without ethanol in the phosphorus buffer pH 7.3. For this buffer the diffusion coefficients for 4-nitrophenol and 1:1 complex with CD were $7.58 \times 10^{-6}\ cm^2 \cdot s^{-1}$ and $2.38 \times 10^{-6}\ cm^2 \cdot s^{-1}$, respectively, and the stability constant was $276 \pm 40\ M^{-1}$, well within the error bar of the literature data.

CONCLUSION

Our method allows to study ligand-selector interaction in a solution. Various solvents with various selectors can be used. There is no need to use derivatives of the reagents. Compounds can be neutral or charged. The range of stability constants measured using the method was between 10^2 and $10^5\ M^{-1}$. For fast search of ligand-protein interaction even one point analysis (single concentration) gives sufficient information about various ligands affinity to the protein. However we suggest to use caution when calculating binding constants using data generated at a single concentration of substrate and ligand. In our case it does not appear to introduce significant error. However it can introduce significant error if the location of the data point on the binding isotherm is not known. Additionally the study of D^{eff} as a function of selector concentration gives the possibility to measure even low stability constants with reasonable small errors as we show in the manuscript (for 4-nitrophenol cyclodextrin in ethanol−water mixture 10:90 v/v $K = 98 \pm 12\ M^{-1}$). Our additional experiments with human serum and warfarin demonstrate that technically the method can be used in medical analysis of blood samples.

(43) Bertrand, G. L.; Faulkner, J. R., Jr; Han, S. M.; Armstrong, D. W. *J. Phys. Chem.* **1989**, *93*, 6863–6867.

(44) Matsui, Y.; Mochida, K. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 2808–2811.

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GLOSSARY

u	average velocity
R	radius of the capillary
L	distance along the tube from the injection point to the detection point
t	time
t_L	time of flowing as a free **L**
t_{LS}	time of flowing as a complex **L:S**
t_F	$t_L + t_{LS}$
$P(t)$	the concentration distribution
σ	dispersion coefficient
D^{eff}	effective diffusion coefficient obtained from the experiment according to eq 2
D_L	molecular diffusion coefficient of ligand
D_S	molecular diffusion coefficient of selector
D_{LS}	molecular diffusion coefficient of complex
K	equilibrium constant
x_L, x_{LS}	molar fraction of free, and bound ligands
c_S	molar concentration
h	the injection length in the capillary $h = (v_{inj}/v_{cap})L$
V_{inj}	injection volume
V_{cap}	volume of the capillary
m_1,m_2	corrections for the rectangular injection, are given by the formulas $m_1 = h/2$ and $m_2 = h^2/12$

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