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Short communication

Enhanced albumin protein separations and protein–drug binding constant measurements using anti-inflammatory drugs as run buffer additives in affinity capillary electrophoresis

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Abstract

An affinity capillary electrophoresis (ACE) method for improving albumin protein separations has been developed. Separation efficiencies for bovine serum albumin (BSA) and human serum albumin (HSA) are dramatically improved by using anti-inflammatory compounds as run buffer additives. The anti-inflammatory drugs used as biospecific ligands to improve the protein separation include ibuprofen (IB), flurbiprofen (FL), and ketoprofen (KE). The binding constants of proteins (BSA and HSA) for the anti-inflammatory ligands (FL and IB) are estimated by ACE and compared to literature values.

1. Introduction

Capillary electrophoresis (CE), a powerful separation technique, has developed rapidly in the past years [1–5]. The method is capable of separating ionic species based on charge to mass ratio, approaching efficiencies of 1 000 000 theoretical plates. Unfortunately, the routine application of CE to proteins and other macromolecules is still hindered by the problem of solute adsorption to the fused-silica surface of the capillaries. These interactions result in band broadening and peak tailing, with greatly reduced separation efficiencies and poor separation reproducibilities. Reported attempts to eliminate sample adsorption include: (1) addition of chemical agents to the run buffer [2]; (2) deactivation of the silica surface by physically or chemically coating the capillary wall [6–11]; and (3) adjustment of run buffer pH [7,12]. Although these approaches have shown some success in reducing the adsorption problem, the improvement of protein separations is still an interesting and challenging area in CE.

Recently, affinity capillary electrophoresis (ACE), which is considered to be a branch of CE, has opened up new horizons for biospecific separations as well as for characterizing molecular properties by analyzing biospecific interactions. Guttman and Cooke [13] reported an ACE method using capillaries filled with linear polyacrylamide, in which ethidium bromide was solubilized as a ligand, for specific separation of double-stranded DNA fragments. Poly(9-vinyladenine) has been used as an affinity macroligand entrapped within a gel matrix to separate

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nucleic acids [14]. Recently, several approaches for chiral separations of various drug and amino acid samples have been developed in our laboratory using bovine serum albumin (BSA) as an affinity ligand [15–17]. Another potential of ACE is its ability to measure the binding constants of solutes for ligands [15,18,19].

In this report, we present an ACE method for protein separations. Separation of bovine serum albumin (BSA) and human serum albumin (HSA) has been improved by using different anti-inflammatory compounds as run buffer additives. Also, the mechanism of separation is used to estimate the binding constants for the ligand–protein complexes.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA, fatty-acid free), human serum albumin (HSA, fatty-acid free), α-lactoglobulin (α-LA), ibuprofen (IB), flurbiprofen (FL) and ketoprofen (KE) were purchased from Sigma (St. Louis, MO, USA). All drugs were in their racemic form. All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Apparatus

ACE separations were performed on a laboratory-constructed instrument which included a safety plexiglas box, a CZE 1000 PN 30 high power supply (Spellman, Plainview, NY, USA), a high power supply local control (Chamonix Industries, Johnson City, NY, USA), a Spectra 100 UN detector and a SP-4400 integrator (Thermo Separation Products, Freemont, CA, USA). Detection wavelength was 214 nm. The temperature inside the plexiglas box was cooled by a fan. Polymer deactivated capillaries (75 μm I.D. × 360 μm O.D., Chemical Solutions, South New Berlin, NY, USA) exhibiting no electroosmotic flow (EOF) were used for all separations.

2.3. Protein separations by ACE

Proteins were separated using 10 m*M* phosphate (pH 7.04) and the addition of different amounts of anti-inflammatory compounds as run buffers. The sample solution was prepared by dissolving proteins in a 10 m*M* phosphate buffer (pH, 7.04). Concentrations of proteins were: BSA 0.4 mg/ml; HSA 0.4 mg/ml; and α-LA 0.2 mg/ml. Syphon injection (6 cm × 6 s) was used for all separations. The effective length of the capillary was 32 cm and the electric field strength was 300 V/cm. Binding constants were measured using the method reported by Chu et al. [18,19] with minor modifications.

3. Results and discussion

3.1. Improvement of BSA and HSA separation

Fig. 1 shows the separation of a three-protein mixture sample. α-LA is completely resolved under the conditions employed, BSA and HSA cannot be baseline separated, exhibiting poor peak efficiencies and resolution. With the addition of ibuprofen (IB) to the run buffer, the separation of BSA and HSA is improved dramatically (Fig. 1b–d). It is well known that serum albumin has the important function of reversible binding with various substances such as fatty acids, tryptophan, bilirubin and many acidic drugs [20]. This characteristic of serum albumin has been used for the chiral separation of acidic drugs in HPLC [21] and ACE [15–17]. In this experiment, the electrophoretic mobilities of BSA and HSA are determined to be $1.84 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} and $1.78 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. The electrophoretic mobility of IB is $2.32 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. In a coated capillary which has no EOF, BSA and HSA will migrate at a greater rate when IB is added to the run buffer if they form complexes with the faster moving modifier IB, and the separation of these two proteins can be improved due to their different affinities for IB. As shown in Fig. 1, the separation efficiencies for BSA and HSA increase with an increase in IB concentration in the run buffer. The

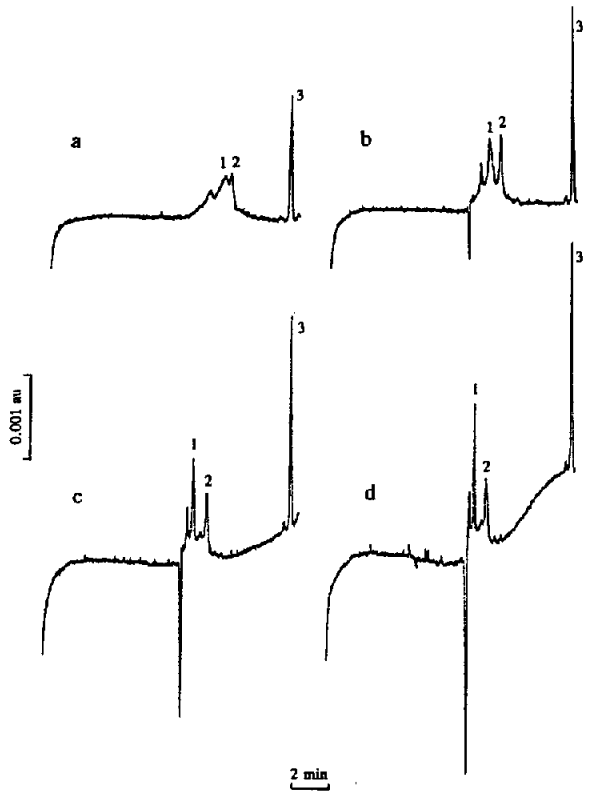

Fig. 1. Electropherograms of BSA (1), HSA (2) and α-LA (3). Conditions: capillary, polymer coated capillary (75 μm I.D. × 360 μm O.D.) with an effective length of 32 cm; electric field strength, 300 V/cm; current, 23 μA; sample injection, 6 cm × 6 s; run buffers, 10 m*M* phosphate at pH 7.04 with different amounts IB added: (a) no IB added; (b) $4.85 \cdot 10^{-5}$ *M*; (c) $24.24 \cdot 10^{-5}$ *M*; and (d) $48.5 \cdot 10^{-5}$ *M*. Peak I: unidentified peak.

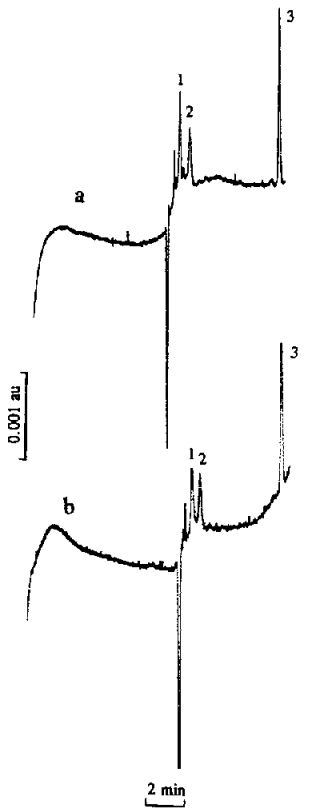

Fig. 2. Electropherograms of BSA (1), HSA (2) and α-LA (3) using FL and KE as buffer additives. Conditions are the same to those of Fig. 1 except that the run buffers are (a) 10 m*M* phosphate at pH 7.04 containing $20.5 \cdot 10^{-5}$ *M* FL; and (b) 10 m*M* phosphate at pH 7.04 containing $19.7 \cdot 10^{-5}$ *M* KE.

migration times of the two proteins decrease with an increase of IB concentration. The migration time of α-LA is independent of IB concentration in the run buffer. This suggests that α-LA does not interact with IB under the experimental conditions employed.

Similar improvement for BSA and HSA resolution has been achieved using flurbiprofen (FL) and ketoprofen (KE) as run buffer additives. Fig. 2 shows electropherograms of the protein mixture sample. An interference peak, which is suggested to originate from an impurity in the sample, is observed prior to the BSA peak in all electropherograms. Also, an inverted peak is observed when IB is added to run buffer. The source of the peak is unknown, although it may be caused by phosphate ions being injected during the sample introduction displacing IB in the run buffer.

3.2. Binding constant measurements

Binding constant measurements by ACE were first reported by Whiteside's research group [18,19]. The following equation gives a convenient form for Scratchard analysis of binding constants [18];

$$\left(\frac{\delta\,\Delta t}{\delta\,\Delta t_{max}}\right)\left(\frac{1}{[L]}\right) = K_b - K_b\left(\frac{\delta\,\Delta t}{\delta\,\Delta t_{max}}\right) \quad (1)$$

where Δt is the difference between the migration time of the protein of interest and the reference protein ($\Delta t = t_{ref} - t_{interest\ sample}$) at concentration $[L]$ of the charged ligand, $\delta\ \Delta t = \Delta t - \Delta t_0$ (Δt_0 is the value of Δt at $[L] = 0$), and $\delta\ \Delta t_{max}$ is the value of $\delta\ \Delta t$ at saturating concentrations of L. If the migration time is affected by changes in EOF, Eq. (1) is not accurate to estimate binding constants. Recently, Comez et al. [22] reported a method to correct the effect caused by changes in EOF. In our experiments, the EOF of the

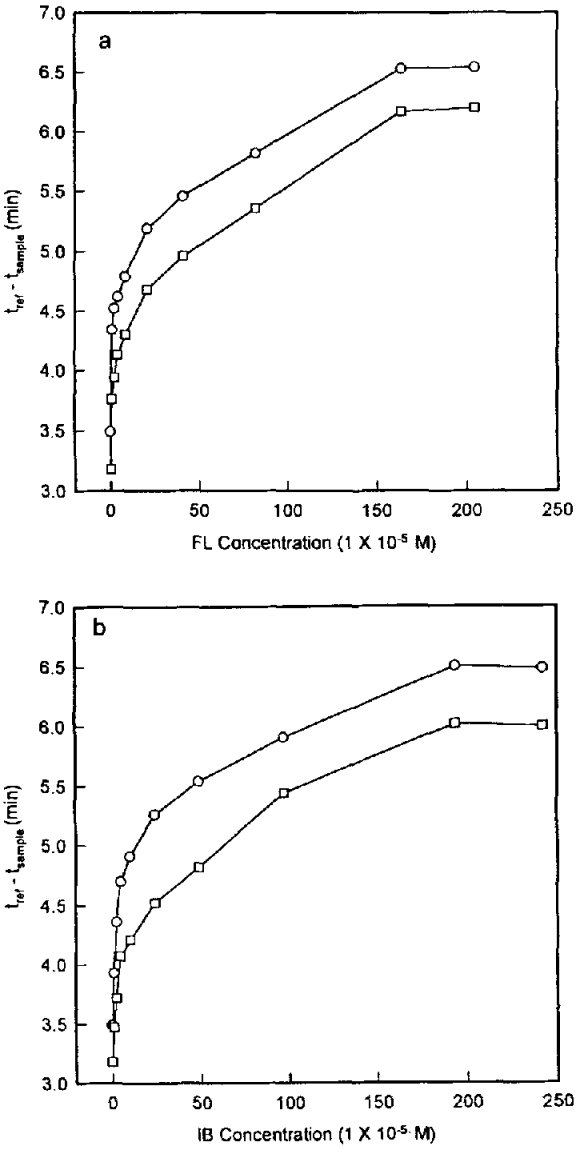

Fig. 3. Plots of Δt (the difference of migration times of the internal reference protein and interest proteins) versus the concentrations of ligands FL (a) and IB (b). (○) BSA; (□) HSA.

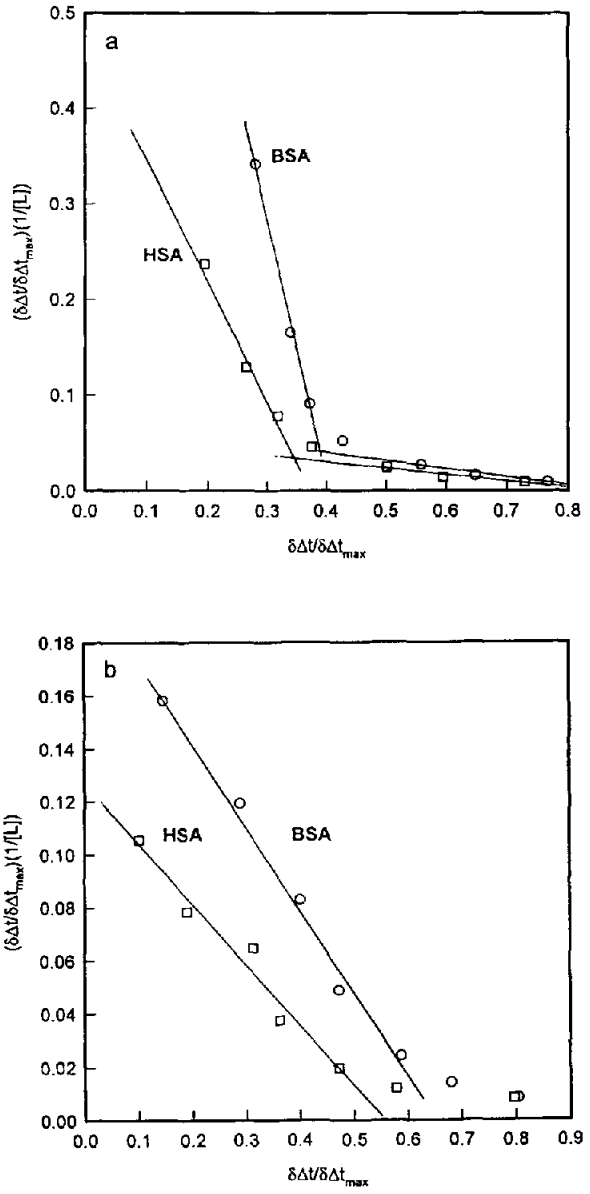

Fig. 4. Scatchard plots of experimental data according to Eq. (1). (a) FL is used as ligand; and (b) IB is used as ligand. (○) BSA; and (□) HSA.

coated capillary has been determined to be zero, therefore Eq. (1) is suitable in the estimation of binding constants.

Fig. 1 shows a representative series of electropherograms of a three-protein mixture sample in run buffers containing various concentrations of the ligand ibuprofen (IB). The migration time of α-lactoglobulin (α-LA) is not affected by the increase of the IB concentration, indicating little or no interaction with IB under the electrophoretic conditions employed. The same results are observed when flurbiprofen (FL) and ketoprofen (KE) are used as run buffer additives (Fig. 2). As a result, α-LA has been used as the internal reference protein in all binding constant measurement experiments. Migration times of BSA and HSA decrease with increasing IB concentrations. Fig. 3 shows the dependence of Δt on the concentrations of ligands FL and IB. It can be seen that Δt values for both BSA and HSA increase dramatically at low ligand concentrations. They reach maximum values at certain ligand concentration considered to be the saturating concentration.

Fig. 4 shows the plots of Eq. (1) derived from the experimental data. The curved shapes of the plots, which are similar to some regular Scatchard plots, suggest multiple class binding [23]. In Fig. 4a two sets of straight lines (first three points and last three points) are obtained for both BSA and HSA. Binding constants of FL to BSA and HSA are estimated based on the slopes. The results are listed in Table 1. In Fig. 4b the second binding constants (K_2) of IB to BSA and HSA cannot be estimated due to inadequate data points. The K_1 values of IB (based on first four points) are found to be $3.2 \cdot 10^4\ M^{-1}$ for BSA and $2.4 \cdot 10^4\ M^{-1}$ for HSA. These values are similar to those obtained by measuring nuclear magnetic resonance relaxation time [$(1.07–1.45) \cdot 10^4\ M^{-1}$] [24], but lower than the values obtained by other methods ($K_1 = 10^5–10^6\ M^{-1}$) [23,25,26]. Reasons for this are currently under investigation in our laboratory. Some possible reasons are buffer ionic strength effects, the use of high concentrations of ligand in the run buffer [24], and different experimental temperatures [23].

In conclusion, ACE can be used to improve protein separations by adding biospecific affinity agents, such as anti-inflammatory compounds, to run buffers. In addition, qualitative and quantitative information on drug–protein binding interactions can be achieved. ACE can be used as an alternative method to the conventional methods for drug–protein binding constant measurements. Major advantages of this method include: (1) simplicity, the biospecific ligands are simply added to the run buffer; (2) microscale, only nanograms of ligands and proteins required; (3) it is applicable simultaneously to several proteins in the same solution because of the high resolving power of CE; and (4) the availability of commercially automated instruments.

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Table 1
Measured binding constants by ACE

Ligand	Protein	K_1 ($1 \cdot 10^5\ M^{-1}$)	K_2 ($1 \cdot 10^5\ M^{-1}$)
Flurbiprofen	Bovine serum albumin	2.8	0.085
	Human serum albumin	1.3	0.067
Ibuprofen	Bovine serum albumin	0.32	–
	Human serum albumin	0.24	–

References

[1] J.W. Jorgenson and K.D. Lukas, *Anal. Chem.*, 58 (1981) 1298.
[2] J.W. Jorgenson and K.D. Lukas, *Science*, 222 (1983) 266.
[3] B.L. Karger, *Nature*, 39 (1990) 64.
[4] W.G. Kuhr, *Anal. Chem.*, 62 (1990) R403.
[5] W.G. Kuhr and C.A. Monnig, *Anal. Chem.*, 64 (1992) R389.
[6] S. Hjerten, *J. Chromatogr.*, 347 (1985) 191.
[7] R.M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
[8] J.K. Towns and F.E. Regnier, *Anal. Chem.*, 63 (1991) 1126.
[9] W. Nashabeh and Z. El Rassi, *J. Chromatogr.*, 559 (1991) 367.
[10] A.M. Dougherty, C.L. Woolley, D.L. Williams, D.F. Swaile, R.O. Cole and M.J. Sepaniak, *J. Liq. Chromatogr.*, 14(5) (1991) 907.
[11] T. Wang and R.A. Hartwick, *J. Chromatogr.*, 594 (1992) 325.
[12] H.H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
[13] A. Guttman and N. Cooke, *Anal. Chem.*, 63 (1991) 2038.
[14] Y. Baba, M. Tsuhake, T. Sawa, M. Akashi and E. Yashima, *Anal. Chem.*, 64 (1992) 1920.
[15] G.E. Barker, P. Russo and R.A. Hartwick, *Anal. Chem.*, 64 (1992) 3024.
[16] P. Sun, G.E. Barker, R.A. Hartwick, N. Grinberg and R. Kaliszan, *J. Chromatogr.*, 652 (1993) 247.
[17] P. Sun, N. Wu, G.E. Barker and R.A. Hartwick, *J. Chromatogr.*, 648 (1993) 475.
[18] Y.-H. Chu, L.Z. Avila, H.A. Biebuyck and G.M. Whitesides, *J. Med. Chem.*, 35 (1992) 2915.
[19] Y.-H. Chu and G.M. Whitesides, *J. Org. Chem.*, 57 (1992) 3524.
[20] T. Peters, in F.W. Putnam (Editor), *The Plasma Proteins*, Vol. 1, Academic Press, New York, NY, 1975, p. 133.
[21] K.E. Choi and R.L. Schilsky, *Anal. Biochem.*, 168 (1988) 398.
[22] F.A. Gomez, L.Z. Avila, Y.-H. Chu and G.M. Whitesides, *Anal. Chem.*, 66 (1994) 1785.
[23] J.B. Whitlam, M.J. Crooks, K.F. Brown and P.V. Pedersen, *Biochem. Pharmacol.*, 28 (1979) 675.
[24] M. Tanaka, Y. Asahi, S. Masuda and T. Ota, *Chem. Pharm. Bull.*, 39(1) (1991) 1.
[25] M. Kuchar, B. Brenova, V. Rejhohec, P. Verner and O. Numecek, *Pharmazie*, 38 (1983) 744.
[26] L. Aarons, A.Z. Khan, D.M. Grennan and M. Alam-Siddiqi, *J. Pharm. Pharmacol.*, 37 (1985) 644.