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Short communication

# Hyphenated affinity capillary electrophoresis with a high-sensitivity cell for the simultaneous binding study of retinol and retinoic acid in nanomolars with serum albumins

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ABSTRACT

Retinol and retinoic acid are Vitamin A components that are critical for many biological processes. Both of them are strongly complexing with serum albumins giving constants of the order of $10^5$ L mol$^{-1}$ or higher. With respect to this fact, affinity capillary electrophoresis (ACE) is not applicable in its commonly used form. Therefore, for the first time, the hyphenated ACE with a high-sensitivity cell was developed and employed to investigate the binding of retinol and retinoic acid in nanomolars with human serum albumin (HSA) and bovine serum albumin (BSA) under physiological conditions. ACE/high-sensitivity coupled cell had contributed to fast the association and dissociation rates of the complexes in nanomolar scale of analytes ensuring the establishment of a dynamic equilibrium within a short electrophoresis time. In addition, this hyphenation led to reduce the concentrations of serum albumins as additives in background electrolyte making a sense beside the proper rinsing protocol for the negligible possibility of their adsorption. The mobility ratio based on nonlinear regression analysis was used to deduce precise binding constants of analytes with serum albumins. The binding constants ($K$, L mol$^{-1}$) of retinol were $1.28 \times 10^5$ and $5.25 \times 10^6$ and retinoic acid were $3.29 \times 10^5$ and $2.27 \times 10^6$ with HSA and BSA, respectively. The displacement and reciprocal competitive binding of analytes were investigated and indicated that retinoic acid was able to replace retinol from HSA and vice versa in the case of BSA.



## 1. Introduction

Vitamin A components, retinol and retinoic acid, are fat-soluble micronutrients and are two physiologically important retinoids [1]. The recommended Dietary Allowance (RDA) of Vitamin A is 900 μm for men and 700 μm for women. Deficiency impairs the immunity system of human beings, causes skin rashes and typical ocular effects such as dry eyes and night blindness. As a general, the disease could be due to a deficiency in the ligand–receptor binding. Both retinol and retinoic acid are very hydrophobic; it was previously pointed out that the interaction of retinol and retinoic acid with protein serves to solubilize the water-insoluble retinoids molecule and to protect the unstable molecules against chemical degradation [2]. Serum albumin, as a transport protein, is the major target of several micronutrients in vivo. FTIR, UV–vis, CD and fluorescence spectroscopic methods were used to determine retinoid binding constant with human serum albumin (HSA) [3]. The results have pointed to that retinol and retinoic acid bound HSA with high binding constants of $K$ (retinol) = $1.32 \times 10^5$ L mol$^{-1}$ and $K$ (retinoic acid) = $3.33 \times 10^5$ L mol$^{-1}$. Another study [4] was performed by the same spectroscopic methods for the calculation of the binding constants of the investigated analytes with bovine serum albumin (BSA) resulting in high binding constants of $K$ (retinol) = $5.3 \times 10^6$ L mol$^{-1}$ and $K$ (retinoic acid) = $2.3 \times 10^6$ L mol$^{-1}$. The spectroscopic techniques such as fluorescence-based binding assays usually take weeks or even months for the preparation of solutions that need an appropriate reagent labeling [3,4] as well as in some cases these assays produce false-negative results, e.g. from aggregation of ligands, which can cause allosteric inhibition of binding to ligands [5].

Affinity capillary electrophoresis (ACE) may often be used advantageously for studying affinity interactions compared to other well-established techniques [6–14] due to the low sample and ligand consumption, relatively short analysis times, high efficiency, good precision and suitability for probing high and weak affinity interactions. In ACE, separations can be performed in

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solution under physiological buffer conditions, it is – as a rule – possible to preserve the analyte in a native state and hence to maintain its molecular function. There is a principal requirement on experimental data serving for the calculations of binding constants. These data, and their changes with the concentration of serum albumin, must result from the mutual interactions of the complex constituents only; if not, the deviations must be corrected by choosing the proper electroosmotic flow (EOF) neutral marker as described in our previous work [15]. In addition, protein interactions with the inner surface of a capillary might contribute to changes in mobility that could influence the precise measurements of binding constants. However, the reduction of the required protein additive concentrations and/or developing rinsing strategies could reduce protein adsorption.

The sensitivity of capillary electrophoresis (CE) measurements was enhanced by coupling CE with a high-sensitivity cell having a special design as elucidated in our previous works [16,17]. The light path through the cell was entirely made of black fused silica to minimize stray light and to define the aperture for diode-array detection. These properties enhanced the spectral analysis with diode-array detection as well as the flared and beveled capillaries maintained peak shape of analytes by ensuring proper alignment and coupling to the cell body.

For high stability constants in the order of $10^5$ L mol$^{-1}$ or higher between small molecules and high-mass-molecular constituent like serum albumin, the equilibrium concentration of the protein in the migrating zones of analytes has to differ markedly from its starting concentration in the background electrolyte (BGE) [18,19]. This could be reduced by injecting and detecting analytes in very low concentrations. The coupling of high-sensitivity cell with ACE enhanced the sensitivity of retinol and retinoic acid several times to be in nanomolar concentrations leading to fast the association and dissociation rates of the complexes in nanomolar scale of analytes ensuring the establishment of a dynamic equilibrium within a short electrophoresis time.

To the best of our knowledge, there is no ACE system coupled with a high-sensitivity cell dealt with the simultaneous binding of retinol and retinoic acid with serum albumins. Therefore, the aim of present study was to investigate retinol and retinoic acid binding mode in nanomolars with HSA and BSA under physiological conditions. The obtained mobility ratios were mathematically treated by using nonlinear regression analysis to give more precise binding constants. As well, the required additive protein concentrations were decreased by this hyphenation system to enlarge the precision of binding constants. In addition, the displacement binding studies of simultaneous retinol and retinoic acid on HSA or BSA were studied in the presence of ibuprofen.

## 2. Experimental

### 2.1. Materials and solutions

Retinol and all-trans-retinoic acid (97%) were obtained from Acros (Geel, Belgium). Human serum albumin (HSA), bovine serum albumin (BSA) and R-ibuprofen were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate-2-hydrate (analytical reagent) and potassium dihydrogen phosphate (analytical reagent) were purchased from Riedel-deHaën (Sigma–Aldrich, Germany). Acetanilide (Ac), ethanol and sodium hydroxide were obtained from Fluka (Sigma–Aldrich, Germany).

The running buffer was 12.50 mmol L$^{-1}$ phosphate buffer at pH 7.4, which was stable for 1 week when stored at 4 °C. HSA and BSA solutions were freshly dissolved in phosphate buffer in the concentration range of 0.00–20.00 μmol L$^{-1}$. Stock solutions of retinol and retinoic acid (100.00 nmol L$^{-1}$) were prepared in 10 mL phosphate buffer containing 40% (v/v) ethanol. The standards were stored at −20 °C and were protected from light during the analysis process. Acetanilide (Ac, 1500 ng mL$^{-1}$) was prepared in 25 mL phosphate buffer. Series of one analyte between 0.10 and 10.00 nmol L$^{-1}$ was prepared in the presence of constant concentration of another analyte by pipetting the appropriate volume from stock solution and diluted in 5 mL phosphate buffer containing acetanilide (EOF marker) solution (300 ng mL$^{-1}$). The running electrolyte and injected sample solutions were filtered through 0.22 μm syringe filters and sonicated for 10 min prior to their application onto the CE system.

### 2.2. Instrumentation

ACE experiments were carried out by using Agilent 7100 capillary electrophoresis System (Germany) equipped with a diode array detector. Bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with high-sensitivity cell (Agilent, Germany having 50 μm ID, 8.5 cm outlet and 56 cm effective length to the detector window) was used. The detection cell was constructed from silica parts, which were fused together, and had an area of 100 μm$^2$ with a path length of 1.2 mm. Electropherograms were monitored using a chemstation software. The temperature of the capillary was kept at 25 °C.

### 2.3. Rinsing protocol and separation conditions

The uncoated fused-silica capillary was conditioned by flushing at 940 mbar with 1.0 mol L$^{-1}$ sodium hydroxide solution for 40 min and water for 10.0 min before the first use. At the beginning of each analysis, the capillary was rinsed with 0.10 mol L$^{-1}$ sodium hydroxide for 3.0 min and water for 2.0 min followed by running electrolyte (phosphate buffer at pH 7.4) for 3.0 min. At the end of the analysis day, the capillary was washed with sodium hydroxide for 5.0 min and water for 10.0 min. Analytes were injected hydrodynamically at 25 mbar for 10.0 s. The separations were performed by applying a voltage of 20 kV giving a typical current of 19 μA. All separations were carried out at 25 °C with detection at 350 nm. All experiments were performed by injecting a series of 10 different concentrations of retinol in the presence of constant concentration of retinoic acid onto running buffer containing definite concentration of HSA. Another series of various concentrations of retinoic acid in the presence of constant concentration of retinol was injected. Both series were injected onto 7 different concentrations of HSA and BSA. The net electrophoretic mobility of the analyte was determined from their migration times using the fraction $(L_{eff}L_{tot})/(tV)$, where $L_{eff}$ is the effective capillary length from the injection end to the detector, $L_{tot}$ is the total length of the capillary, and $V$ is the applied voltage. The mobility ratio ($R$) of the analyte was calculated by using the equivalent relationship $R = t_{eof}/t_{analyte}$ [20], where $t_{eof}$ is the migration time for a neutral marker (Ac) analyzed during the same run used to measure $t_{analyte}$.

### 2.4. Displacement and reciprocal competitive experiments

The displacement studies were performed by increasing systematically the concentration of R-ibuprofen, serum albumin-site II, in the running buffer up to 20 nmol L$^{-1}$ with keeping the concentration of serum albumins fixed. Reciprocal competitive experiments were performed by monitoring changes in the mobility ratio of one of two analytes as a result of the addition of another analyte to the running buffer containing serum albumins. The percentage of protein binding with retinol and retinoic acid was calculated using the following equation: $P = 100(R/R + 1)$, where $R$ is the mobility ratio of analytes.

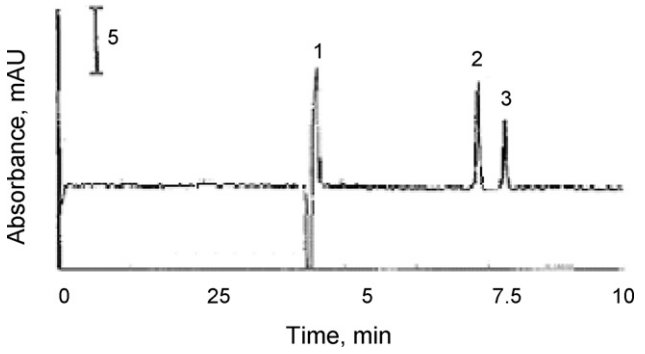


**Fig. 1.** Simultaneous separation of equimolar concentration (3.00 nmol $L^{-1}$) of retinol (2) and retinoic acid (3) in the presence of Ac (1) under the electrophoretic conditions as described in the text.

## 3. Results and discussion

### 3.1. Developing ACE experimental conditions

Meeting of the principal requirements in electrophoresis need serum albumin, analytes and their complexes should not form micelles or stick to the capillary wall [21–23].

In ACE, HSA or BSA interactions with the inner surface of the capillary may contribute to a change in mobility [24–26]. An uncoated, bare fused-silica capillary was employed because the inherent negatively charged wall under physiological conditions could reduce the adsorption of anionic HSA or BSA as well as its availability at low costs [27]. EOF neutral marker can be used as a measure of the mobility shift [28]. In the present work, acetanilide (Ac, 300 ng $mL^{-1}$) was successfully used as EOF marker without any difficulty in its solubility in the electrolyte. As well, to eliminate the adsorption of HSA or BSA, an appropriate rinsing procedure was required. It was found that rinsing the capillary with 0.10 mol $L^{-1}$ sodium hydroxide for 3.0 min and water for 2.0 min followed by running electrolyte (phosphate buffer at pH 7.4) for 3.0 min was succeeded in removing the adsorbed HSA or BSA molecules.

A buffer system was chosen meeting to the physiological conditions, 12.50 mmol $L^{-1}$ phosphate buffer at pH 7.4, in order to optimally stabilize the native structure of the protein–ligand complex [24].

Throughout this study, a slight change in the migration time of the EOF marker could be observed, since the amount of HSA or BSA was increased in the buffer system. This was presumably just due to the changes in the buffer viscosity. Thus, the mobility of the analytes was normalized in relation with that measured for the EOF marker to give the mobility ratio (Ac/analyte), as described in Section 2.

### 3.2. Hyphenation of ACE with the high-sensitivity cell

It is well-known that the concentration of sample has a significant impact on ACE [29,30]. In the present work, it was found that the working range of 0.10–10.00 nmol $L^{-1}$ prepared in phosphate buffer of both analytes was easily detected at 350 nm with correlation coefficient ($r^2$) of 0.9983 and standard deviation (SD) of 0.1538. Fig. 1 indicates the simultaneous separation of equimolar concentration (3.00 nmol $L^{-1}$) of retinol and retinoic acid at migration times of 7.3 and 8.1 min, respectively, in the presence of Ac (EOF marker) at 4.4 min. The mobility ratio shift of analytes was negligibly changed with the increase of the concentration of analytes initially within the range (0.10–10.00 nmol $L^{-1}$). When the concentrations of the analytes reached more than 10 nmol $L^{-1}$, the trend of mobility shifts turned to gradual increase, suggesting that the estimation of binding constant values could be achieved with analyte concentrations between 0.10 and 10.00 nmol $L^{-1}$. Retinol and retinoic acid gave linear calibration equations $Y = 0.981X - 0.005$ and $Y = 0.992X + 0.021$, respectively, where $Y$ is the concentration of analytes (nmol $L^{-1}$) and $X$ is the peak area of analytes. The intraday ($n = 6$) and interday ($n = 18$) precisions of equimolar concentration (1.00 nmol $L^{-1}$) of analytes under the optimal conditions were checked and found to be 0.0671 and 3.0581 nmol $L^{-1}$ standard deviations, respectively. The limit of detection (LOD, signal to noise ratio = 2) and limit of quantitation (LOQ, signal to noise ratio = 10) were found to be 0.02 and 0.16 nmol $L^{-1}$, respectively.

Therefore, the hyphenation of ACE with the high sensitivity cell led to the ease simultaneous determination of retinol and retinoic acid in nanomolar levels with good precision. This could contribute to solve the problem regarding to the difficult calculation of high binding constants of analytes by ACE because the association and dissociation rates of the complexes were too rapid at nanomolars to ensure the establishment of a dynamic equilibrium within a short electrophoresis time. In addition, it is expected that this hyphenation could lead to reduce the concentrations of serum albumins as additives in background electrolyte making a sense beside the proper rinsing protocol for the negligible possibility of their adsorption. A test program was established to check the nature of the surface wall in the protein concentration range between 0.01 and 20.00 μmol $L^{-1}$. This test consisted of injecting 10.00 nmol $L^{-1}$ of both analytes and 300.00 ng $mL^{-1}$ Ac in plain phosphate buffer ($n = 5$) followed by five injections of analytes in the presence of 10.00 μmol $L^{-1}$ HSA or BSA as a buffer additives, and then injecting analytes again in plain phosphate buffer ($n = 5$). The test was subsequently continued with the injection of analytes in the presence of 20.00 μmol $L^{-1}$ serum albumins ($n = 5$) and then again in plain phosphate buffer ($n = 5$). Throughout this study, very slight change in the migration time of the EOF marker could be observed, since the amount of serum albumins was increased in the buffer system. There was a shifting in the migration time of Ac from 3.8 min (1.12% RSD) at the beginning of the program into 3.9 min (1.13% RSD) and 4.0 min (1.982% RSD) after finishing the measurements with 10.00 μmol $L^{-1}$ and 20.00 μmol $L^{-1}$ serum albumins, respectively for the first cycle. For the second cycle, there is no large change in mobility comparing to the data in the first cycle. This slight shift was presumably just due to the changes in the buffer viscosity because of adding protein to the running electrolyte. However, such shifts affect all measurements of analyte mobility and consequently the precision of ACE based binding constant calculations. Thus, the mobility of analytes was normalized vs. that measured for the EOF marker. The resulting mobility ratios (Ac/retinol and Ac/retinoic acid) showed an almost constant value of 0.99. Because of these results, the mobility ratio was used in all later experiments as the preferred means for describing analyte mobility during ACE binding studies.

The accuracy of the regression methods for the calculations of binding constants could also improve with larger mobility differences between free analyte and free HSA or BSA [31]. In the current work, this was checked at three concentration levels 1.00, 10.00 and 50.00 nmol $L^{-1}$. The results showed that the best resolutions $R = 2.87$ and 1.89 between analytes were achieved at 1.00 and 10.00 nmol $L^{-1}$, respectively while the resolution was bad (less than 1.31) at 50.00 nmol $L^{-1}$. As well, the migration time of HSA when injected as a sample was about 8.9 min while the migration time of BSA was about 9.0 min. The difference between the migration times of free serum albumins and free retinol and retinoic acid were about 1.7 and 0.9 min, respectively, which was considered an advantage facilitating the ACE precision study of such system.

### 3.3. Estimation of the binding constants

In ACE, the binding constant ($K$) can be determined from the variation of mobility shifts of a sample as a role of additive

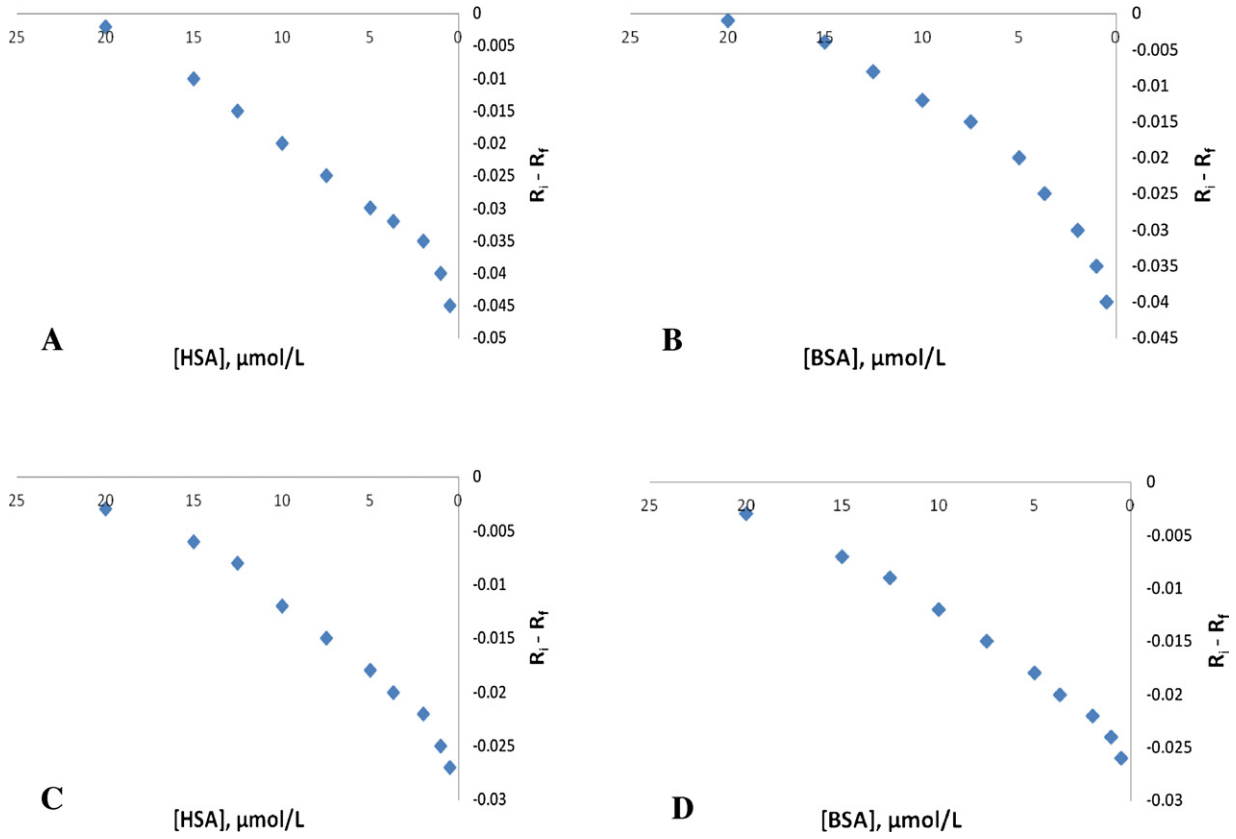


**Fig. 2.** Binding curves of retinol-HSA (A), retinol-BSA (B), retinoic acid-HSA (C) and retinoic acid-BSA (D) systems.

concentrations in the running buffer. The analysis of data for the calculation of binding constants could be achieved by using four mathematical plotting models: nonlinear regression, *x*-reciprocal, *y*-reciprocal and double reciprocal. Our previous work [15] investigated that the nonlinear regression method was the best to eliminate the cumbersome weighting procedure necessary in the statistical analysis of the linearized plots (*x*-reciprocal, *y*-reciprocal and double reciprocal). The binding constant based on nonlinear regression analysis was calculated by the following equation [32]:

$$K \cdot c(L) = \frac{R_f - R_i}{R_i - R_c}$$

where $R_f$ is the mobility ratio of the analyte ($t_{eof}/t^{\circ}_{analyte}$) measured in the absence of protein, $R_i$ is the mobility ratio of the analyte ($t_{eof}/t_{analyte}$) measured in the presence of protein, $R_c$ is the mobility ratio of the analyte ($t_{eof}/t_{analyte}$) measured at saturated protein concentration, $c(L)$ is the micro-molar concentration of the protein and $K$ is the binding constant. The main difficulty for the calculation of a binding constant using the above equation is the estimation of the $R_c$ value [15]. This value was measured from the maximum mobility of the complex measured at high protein concentration (20 μmol $L^{-1}$). The migration times of retinol and retinoic acid were shifted from 7.3 and 8.1 min, respectively, in the absence of HSA into 9.1 and 11.2 min, respectively, in the presence of high concentration HSA. In the case of BSA, the migration times were shifted into 9.3 min of retinol and 11.5 min of retinoic acid. Fig. 2 shows the binding curves of analytes with HSA (A) and (C) and with BSA (B) and (D). The general binding constants were calculated under our optimal conditions and the results were shown in Table 1. When it was compared the binding constant values with literature results, the measured values using nonlinear regression method showed good agreement with those previous results obtained. Despite the temperature was controlled at 25 °C, the internal temperature was estimated to be 37 °C by Bose et al. [33] using the electrophoretic mobility method of Burgi et al. [34]. Such a problem was true for all existing CE instruments because the water or air cooling circulation could not completely dissipate the internally generated temperature. The results demonstrated that the binding constant of retinoic acid with HSA was much greater than that of retinol, i.e., retinoic acid had a significantly larger affinity to human serum albumin than that of retinol. In the case of BSA, the situation was vice versa in which retinol had a significantly larger affinity compared to retinoic acid. This could be due to the confirmation of the presence of variations in the spatial distances of the BSA and HSA binding sites [35] and the ability of functional groups in both analytes to include inside these sites.

The reproducibility of mobility ratio from retinol and retinoic acid was determined in the presence of various concentrations of HSA or BSA (0.00–20.00 μmol $L^{-}$). The intraday relative standard deviations (RSD) for seven replicate injections was ranged between 1.3% and 4.4% and the interday RSD values was tested over a period of three consecutive days and was found to be 3.8–7.6%.

### 3.4. Displacement and competitive binding of retinol and retinoic acid

The displacement electrophoretic studies of retinol and retinoic acid were studied by increasing the concentration of R-ibuprofen up to 20 nmol $L^{-1}$ in the running buffer containing HSA or BSA at constant concentration under physiological conditions. Previous binding study performed by ACE on HSA [36] indicated that ibuprofen [2-(4-isobutylphenyl)-propionic acid] enantiomers had high affinity for HSA on site II (the indole-benzodiazepine binding site) and the binding of R-enantiomer was stronger. In the present work, it was observed that the increasing of displacer concentration

**Table 1**
Binding constants ($K$, L mol$^{-1}$) of retinol and retinoic acid with HSA and BSA.

| Analyte | With HSA | | With BSA | |
|---|---|---|---|---|
| | $K \pm$ SD ($n$ = 7), L mol$^{-1}$ | | | |
| | Found value | Literature value | Found value | Literature value |
| Retinol | $1.28 \pm 0.04 \times 10^5$ | $1.32 \times 10^5$ [3] | $5.25 \pm 0.06 \times 10^6$ | $5.30 \times 10^6$ [4] |
| Retinoic acid | $3.29 \pm 0.03 \times 10^5$ | $3.33 \times 10^5$ [3] | $2.27 \pm 0.04 \times 10^6$ | $2.30 \times 10^6$ [4] |

**Table 2**
Changes in the mobility ratios ($R$) of retinol and retinoic acid as a result of the addition of ibuprofen to the running buffer.

| Analyte | With HSA | | With BSA | |
|---|---|---|---|---|
| | Mobility ratio ($R$) $\pm$ SD ($n$ = 7) | | | |
| | Before addition | After addition | Before addition | After addition |
| Retinol | 0.67 ± 0.006 | 0.44 ± 0.003 | 0.70 ± 0.006 | 0.51 ± 0.005 |
| Retinoic acid | 0.63 ± 0.005 | 0.48 ± 0.004 | 0.68 ± 0.006 | 0.41 ± 0.002 |

led to high depression in the mobility time of retinol; e.g. 5 nmol L$^{-1}$ R-ibuprofen produced a decrease in the mobility ratio value about 65.13% (Table 2) compared to the value in the absence of displacer. The mobility time of EOF marker (Ac) was remained constant during these studies. Table 2 shows the observed values for the displacement study of retinoic acid on HSA or BSA. Therefore, these results indicated the possibility of direct competitive interaction between R-ibuprofen and retinol or retinoic acid on HSA-site II or BSA-site II.

The reciprocal competitive electrophoretic studies of retinol on BSA or HSA were performed by increasing the concentration of retinoic acid up to 10.0 nmol L$^{-1}$ in the running buffer containing a constant concentration of serum albumins. The vice versa was performed in the case of competitive studies of retinoic acid in which it was injected onto running buffer containing performed binary complex of retinol to HSA or BSA. Results (not shown) indicated the possibility of competitive binding interaction between retinol and retinoic acid on HSA or BSA. In the case of HSA retinoic acid had the ability to replace retinol while retinol was replaced by retinoic acid in the case of BSA.

Quantitatively, when retinoic acid was injected into the binary system of serum albumin and retinol, the first binding constant of retinoic acid-HSA decreases from $3.29 \times 10^5$ L mol$^{-1}$ to $6.85 \times 10^4$ L mol$^{-1}$ while the binding constant of retinoic acid-BSA decreases from $2.27 \times 10^6$ L mol$^{-1}$ to $7.56 \times 10^5$ L mol$^{-1}$. For retinol, its binding constant with HSA decreases from $1.28 \times 10^5$ L mol$^{-1}$ to $4.39 \times 10^4$ L mol$^{-1}$ while with BSA decreases from $5.25 \times 10^6$ L mol$^{-1}$ to $0.42 \times 10^6$ L mol$^{-1}$. The percentage of serum albumins binding with retinol and retinoic acid was calculated using the following equation: $P = 100\,(R/R + 1)$ and was found to be about 39% for retinol and 61% for retinoic acid on HSA and 59% for retinol and 41% for retinoic acid on BSA. These results indicate how the functional groups of analytes and the affinity abilities of serum albumins affect the extent of binding.

## 4. Conclusion

A simple, rapid and reliable affinity capillary electrophoresis (ACE) coupled with a high-sensitivity cell (special design) was developed to calculate the simultaneous high binding constants based on nonlinear regression analysis of retinol and retinoic acid with HSA and BSA under physiological conditions. The binding of retinoic acid with serum albumins was affected by retinol when they were simultaneously binding to serum albumins, demonstrating that both analytes shared the same binding site in serum albumins. The displacement studies investigated that competition occurred in the high-affinity binding site II. The reciprocal competitive experiments indicated that retinoic acid replaced retinol from HSA while retinol replaced retinoic acid from BSA. Compared with other methods, the hyphenated ACE/high-sensitivity cell was a convenient tool to study the high binding properties of small molecules in nanomolar scale with protein. Especially, it can be used as an alternative method to quantitatively analyze the competitive binding of two small molecules which simultaneously bind to serum albumins. As well, the proposed hyphenation system was made a sense beside the proper rinsing protocol to reduce the interaction of protein with the capillary wall by decreasing the required additives concentrations in the running buffer.

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