Study on the Protein Binding of Ketoprofen Using Capillary Electrophoresis Frontal Analysis Compared with Liquid Chromatography Frontal Analysis

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Abstract

A method of capillary electrophoresis frontal analysis (CEFA) is developed for the first time to study the binding of ketoprofen to human serum albumin (HSA) and compared with high-performance liquid chromatography frontal analysis (LCFA). The separation is performed in an uncoated fused-silica capillary (60-cm × 75-µm i.d., 50-cm effective length) with a phosphate buffer (pH 7.4, ionic strength of 0.17M) as the running buffer. The applied voltage is 13 kV and the detection is set at 254 nm. A trapezoidal peak of the unbound ketoprofen appears after HSA elution in the electropherogram. The plateau height of the peak is employed to determine the unbound concentration of ketoprofen in the HSA equilibrated sample solution. The CEFA method provides the advantages of small sample injection volume and rapidity and the disadvantage of low sensitivity compared with LCFA. CEFA is applicable to the binding parameter estimation of ketoprofen to the secondary binding site; an association constant (K_2) of 0.24 × $10^6 M^{-1}$ and the number for the binding site per molecule HSA of 2.54 is estimated. In contrast, LCFA measures parameters for both primary and secondary sites, which are 1.05 × $10^6 M^{-1}$ and 0.94 for K_1 and n_1, respectively, and 0.12 × $10^6 M^{-1}$ and 3.16 for K_2 and n_2, respectively. It is found that ketoprofen binds mainly at the primary site at a molecular ratio of ketoprofen versus HSA lower than 0.75, and the binding at the secondary site occurs at a higher ratio.

Introduction

In circulation, drugs are usually bound to proteins such as serum albumin, α_1-acid glycoprotein, lipoproteins, and other blood constituents. They exist in the blood in two forms, free (unbound) and protein-bound forms, and only the free form is pharmacologically effective (1–3). The plasma concentration of an unbound drug shows better correlation to the pharmacological activity than the total concentration (4). The protein binding of drugs has a significant effect on their clinical pharmacokinetics and pharmacodynamics. The study of drug–protein binding is therefore fundamentally important in the development of more active and better-tolerated new drugs and in therapeutic drug monitoring.

Ketoprofen is a nonsteroidal anti-inflammatory drug. It binds very strongly with albumin. Some methods such as continuous ultrafiltration (5), high-performance frontal analysis (FA) (HPFA) (6), and microdialysis–liquid chromatography (7) have been developed for the investigation of ketoprofen binding to human serum albumin (HSA) or plasma protein. Two classes of binding sites on HSA have been reported for ketoprofen in one study (7), and others have presented only one (5,6). Although it is administered clinically as a racemate, the stereoselectivity of ketoprofen enantiomers in protein binding has been studied with HPFA coupled with chiral chromatography (6) and other methods (8–11). However, no capillary electrophoresis (CE) method has been reported for determining the protein binding of ketoprofen.

CE has proven to be an attractive analytical technique because of its high efficiency, high resolving power, high speed, and small volume of sample required. Recently, CE methods were reported for the investigation of drug–protein binding. The methodologies employed included affinity CE (ACE), the Hummel–Dreyer method, vacancy ACE, the vacancy peak method, and FA (13–19). When the mobility of the protein is equal to that of the drug–protein complex, FA is the most favorable technique to study drug–protein binding.

Capillary electrophoresis frontal analysis (CEFA) has been used in a study of the protein binding of the basic drug verapamil (18). With a chiral selector (such as cyclodextrins) in the running buffer, CEFA can also be used for the enantioselective study of drug–protein binding (16). In this study, CEFA was employed to study the binding between ketoprofen and HSA. This method was compared with the same methodology using high-performance liquid chromatography FA (LCFA).

Experimental

Reagents and materials

HSA (fatty acid free) was purchased from Sigma (St. Louis, MO). Ketoprofen was supplied by the Institute of Pharmaceutical

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and Biological Product Control (Beijing, China). All other chemicals were of analytical grade and obtained from Shenyang Chemicals (Shenyang, China). Redistilled water was used in the experiments. An uncoated fused-silica capillary (75-µm i.d.) was purchased from Ruifeng Chromatographic Product Co. (Yongnian, Hebei, China). An ISRP column (GFF II-S5-80, 15-cm × 4.6-mm i.d., 5 µm) was obtained from Regis Chemical Company (Morton Grove, IL).

Apparatus

All of the CE experiments were performed on a CAPEL-103R CE System (Lumex, St. Petersburg, Russia) with a UV detector fixed at 254 nm. The electrophoretic data were acquired with a Jiangshen (Dalian, China) workstation. LCFA was performed on a Jasco (Tokyo, Japan) chromatograph with a PU 980 HPLC pump and a UV 975 detector. The samples were injected using a Rheodyne (Cotati, CA) 7125 injector with a loop of 1 mL. The chromatographic data were acquired with a Ckchrom Star workstation (Scientific Development, Tianjin, China).

Preparation of sample solutions

A phosphate buffer was prepared by mixing 67mM disodium hydrogen phosphate and 67mM sodium dihydrogen phosphate (10:1), yielding an ionic strength of 0.17M and a pH of 7.4. The buffer was degassed by sonication in an ultrasonic water bath (DL 180, Shipuhaitian Electrical Instrument Co., Zhejiang, China) for 15 min and filtered through a 0.45-µm membrane filter before use. An HSA solution (40µM) was prepared by dissolving 26.6 mg HSA in 10 mL phosphate buffer. Ketoprofen was dissolved in methanol providing a stock solution of 2000µM. Appropriate volumes of the stock solution were added in a glass tube, and the methanol was evaporated to dryness. The HSA solution was then added into the tube and gently mixed. The ketoprofen–HSA solution was equilibrated for 1 h before analysis using CEFA or HPFA.

Determination of unbound ketoprofen by CEFA

An uncoated fused-silica capillary (60-cm × 75-µm i.d., 50-cm effective length) was filled with a phosphate buffer (pH 7.4, 0.17M ionic strength). The column temperature for separation was maintained at 25°C. The UV detector was set at 254 nm. The samples were injected at 30 mbar pressure for 24 s. The injection end of the capillary was then immersed into the running buffer and a voltage of 13 kV was applied between both ends. The capillary was cleaned between runs by running 30mM of sodium dodecyl sulfonate for 3 min and the buffer for another 3 min. The unbound concentrations of ketoprofen were determined by comparing the plateau height of the HSA-equilibrated sample with that of a neat ketoprofen solution.

Determination of unbound ketoprofen by LCFA.

The LCFA procedure for determining the unbound concentration of ketoprofen was similar to that described in our previous studies (20,21). Briefly, 950 µL of a ketoprofen solution mixed with HSA was injected onto the ISRP column using the injector reswitching technique (22). The phosphate buffer of pH 7.4 and 0.17M ionic strength were used as the mobile phase. The flow rate was maintained at 0.2 mL/min. Ketoprofen eluted as a zonal peak after the HSA. The plateau height was used for the determination of the free drug concentration in the sample.

Determination of unbound ketoprofen by ultrafiltration

Ultrafiltration followed by CE was used as a reference method. To a disposable ultrafiltration kit (NANOSEP Pall Filtron, Northborough, MA) with a molecular weight cutoff of 10000, 300 µL of the ketoprofen–HSA solution was added and centrifuged at 7100 rpm. The second filtrate (200–300 nL) was injected at 30 mbar for 4 s and analyzed by CE as described previously. The peak height as the measure of ketoprofen concentration in the filtrate was compared with that of a standard solution.

Estimation of protein binding parameters

The protein binding parameters, association constant (K), and number of drug molecules bound to a particular class of binding sites that are present on the protein molecule (n) have the following relationship with the unbound concentration (C_f) and binding rate (r):

$$r = \frac{C_b}{[P_t]} = \sum_{i=1}^{m} n_i \frac{K_i C_f}{1 + K_i C_f} \qquad \text{Eq. 1}$$

where C_b and $[P_t]$ are the concentrations of bound drug and total protein, respectively, n_i is the number of sites of class i, and K_i is the corresponding association constant. In the case of ketoprofen, two classes of binding sites ($m = 2$) were reported (9). The parameters were therefore estimated with several sets of r and C_f values by using the following equation:

$$r = \frac{n_1 K_1 C_f}{1 + K_1 C_f} + \frac{n_2 K_2 C_f}{1 + K_2 C_f} \qquad \text{Eq. 2}$$

A linear relationship between r/C_f and r exists when there is only one class of a binding site in the protein ($m = 1$), which is expressed as the Scatchard equation:

$$\frac{r}{C_f} = nK - Kr \qquad \text{Eq. 3}$$

A Scatchard graph was performed to estimate the binding parameters in this study.

Results and Discussion

CEFA of ketoprofen–HSA

The electropherograms of a 125µM ketoprofen solution mixed with 40µM HSA (Figure 1A) and a 125µM ketoprofen solution (Figure 1B) are shown in Figure 1. From the electropherograms, it can be observed that a plateau was formed corresponding with the free ketoprofen.

The plateau height (Figure 1A) of the ketoprofen peak of the HSA-equilibrated sample was obviously lower than that of the pure ketoprofen solution (Figure 1B). In this particular case, free drug migrated slower than HSA or the complex of ketoprofen–HSA under the electrophoretic conditions. Unbound ketoprofen might therefore leak out of the sample plug at the rear edge in the capillary. Because protein binding is a reversible interaction, bound ketoprofen would quickly release from the protein. Because the sample volume was large enough, the binding equi-

librium in the mixed zone remained the same as in the sample solution; therefore, the height of the trapezoidal peak reflected the unbound concentration of ketoprofen in the sample solution.

In FA, a large volume of sample solution is required to produce a zonal peak. In electrophoresis, the injection volume is controlled by the injection time. In order to find the appropriate injection time, a 125µM ketoprofen solution without HSA was injected for 5, 10, 15, 20, and 25 s under a pressure of 30 mbar. It was found that an injection time of 20 s or longer provided a plateau peak in the electropherogram. The injection time was therefore controlled at 24 s in all of the experiments, which injected a volume of approximately 72 nL (much smaller than that in LCFA).

LCFA of ketoprofen–HSA

A typical chromatogram of a ketoprofen–HSA solution is shown in Figure 2. By comparing this chromatogram with the electropherogram in Figure 1A, it can be seen that CEFA is rapid and LCFA provides better separation between unbound ketoprofen and HSA.

Method validation

The developed CEFA method was compared with ultrafiltration–CE. The unbound ketoprofen concentrations determined by the two methods are listed in Table I. The unbound ketoprofen concentrations calculated from trapezoidal peak heights agreed well with those determined with ultrafiltration–CE. No significant differences ($p > 0.05$) were observed by using a paired *t*-test. Both within- and between-day relative standard deviations (RSDs) were less than 8% ($n = 5$) for CEFA, which indicated a reasonable reproducibility of the CEFA method, though they were slightly higher than those in LCFA (RSD < 5%) (20).

The linearity of CEFA was investigated by introducing a series of ketoprofen standard solutions with no protein into the capillary for 24 s at a pressure of 30 mbar. Electrophoresis was performed as described in the Experimental section. The plateau height of the trapezoidal peak was plotted against the ketoprofen concentration. Linear calibration curves were obtained at the concentrations of 3.82, 7.66, 15.3, 30.5, 61.0, and 91.5µM. An average slope of 91.5 ± 3.8 (standard deviation) and an intercept of 21.2 ± 0.9 ($r = 0.9990$) were calculated from 3 runs. The limit of detection (LOD) was 2µM.

The validation of LCFA as a method for determining drug–protein interactions has been reported in our previous study (20). A linear relationship between the peak height and ketoprofen concentration was established in the range of 0.5 to 50µM with a regression equation of $h = (4260 \pm 126)\ C - (105 \pm 20)$ ($r = 0.9999$). The LOD was 0.1µM.

Binding parameters of ketoprofen to HSA

Solutions of ketoprofen at various concentrations that equili-

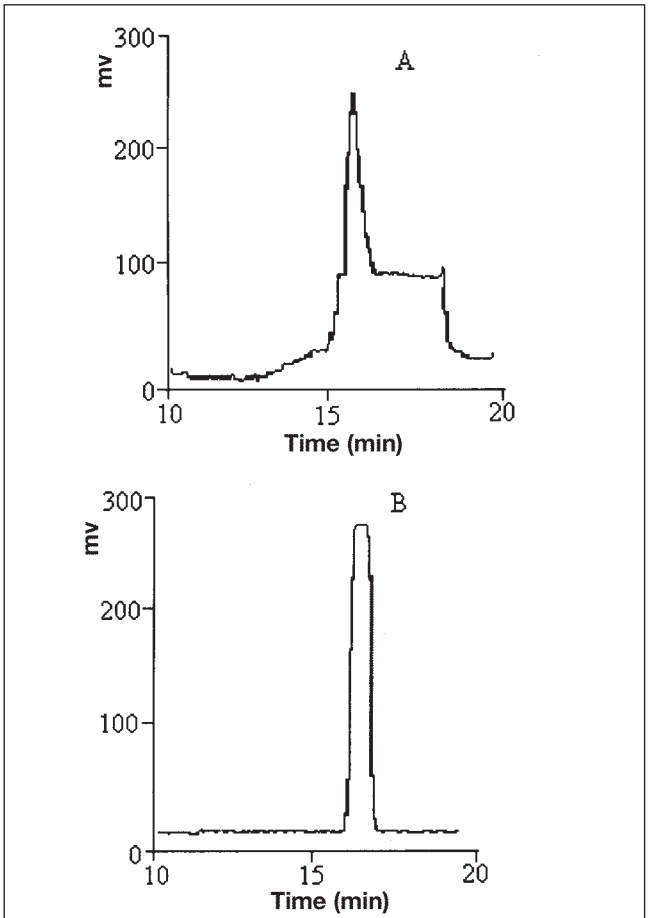

Figure 1. CEFA electropherograms of (A) 125µM ketoprofen equilibrated with 40µM HSA and (B) 125µM neat ketoprofen.

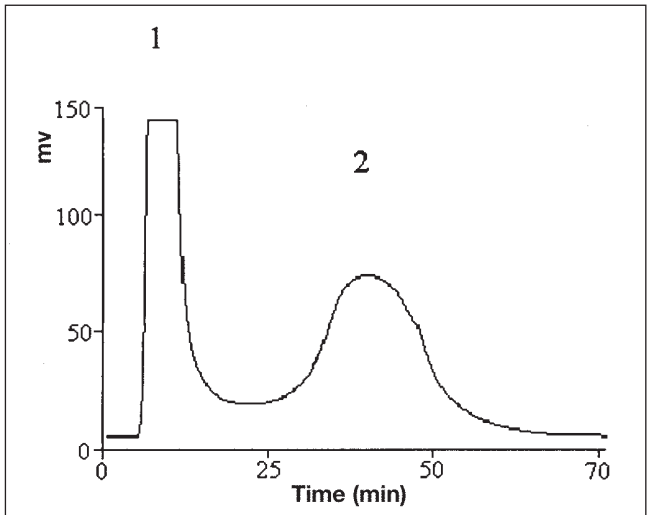

Figure 2. LCFA elution profile of 75µM ketoprofen equilibrated with 40µM HSA. The peaks represent HSA (1) and ketoprofen (2).

Table I. Unbound Concentrations of Ketoprofen in HSA Solutions Determined by CEFA and Ultrafiltration–CE*

	Unbound concentration	
Ketoprofen–HSA (µM)	CEFA	Ultrafiltration–CE
62.5–40	4.92 ± 0.37	4.90 ± 0.34
83.3–40	10.67 ± 0.86	10.32 ± 0.45
93.8–40	17.00 ± 0.50	16.78 ± 0.53
125–40	31.14 ± 0.93	30.96 ± 0.74

* All data are expressed as mean ± standard deviation ($n = 5$).

brated with 40µM HSA were analyzed by the CEFA method to estimate the binding parameters. The Scatchard plot is shown in Figure 3 with a linear regression equation of $y = -0.2353x + 0.6062$ and a correlation coefficient of 0.9338. The K value and the number of the binding site per molecule HSA estimated by this plot were $0.24 \times 10^6 M^{-1}$ and 2.54, respectively.

The Scatchard graph that was plotted by using the data from the LCFA measurement is shown in Figure 4. Two lines with different slopes appeared in the plot, which reflected two types of binding sites existing on the HSA molecule for ketoprofen. The correlation coefficients of the two lines were 0.9780 and 0.9922,

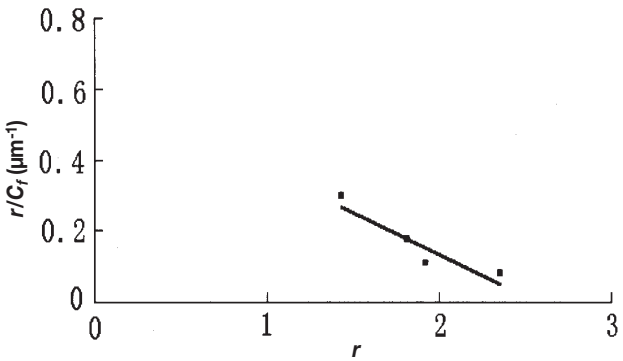

Figure 3. Scatchard plot for the ketoprofen–HSA interaction measured by CEFA.

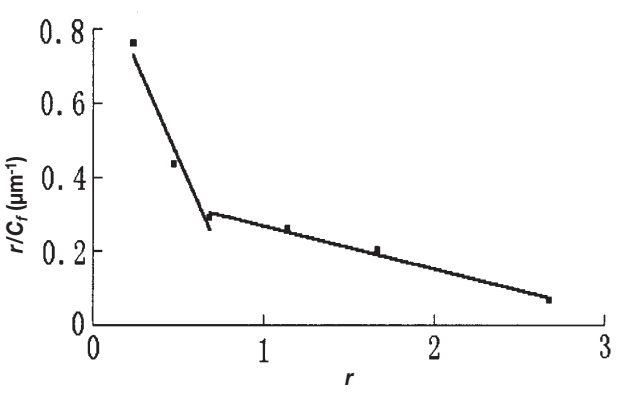

Figure 4. Scatchard plot for the ketoprofen–HSA interaction measured by LCFA.

respectively. The estimated n_1, K_1 and n_2, K_2 from the plot were 0.94, $1.05 \times 10^6 M^{-1}$ and 3.16, $0.12 \times 10^6 M^{-1}$, respectively (Table II). From the data shown in Table II, it can be found that LCFA was able to measure the parameters of ketoprofen binding to two classes of sites on HSA, and CEFA measured only those to one class. Because CEFA was performed with solutions at a high concentration ratio of ketoprofen versus HSA, the determined binding site was designated as the secondary one.

Carefully examining the data in Table II and the Scatchard graphs in this study and in the literature, the following observation was obtained. When the binding study was performed with a large range of a molecular ratio of ketoprofen versus HSA (such as 0.25–3.75 in the this study and 0.1–2.4 in reference 7), binding parameters were estimated for two classes of sites. The reflective point of the Scatchard graph was at a molecular ratio of approximately 0.75 (30µM ketoprofen with 40µM HSA in this study and 36.65µM ketoprofen with 50µM HSA in reference 7). Parameters were obtained for only the primary binding site at lower molecular ratios such as 100–300µM ketoprofen to 550µM HSA (6) and the secondary binding site at higher ratios such as in this study's CEFA measurement.

The following characteristics are therefore proposed for the binding of ketoprofen to HSA. Two classes of binding sites are available for ketoprofen on the HSA molecule. When the molecular ratio for ketoprofen versus HSA is between 0.1 and 0.75, ketoprofen binds mainly at the primary binding site. When the ratio is higher than 0.75, binding at the secondary site occurs.

Comparison of CEFA with LCFA

In this study, CEFA was not applicable to the parameter estimation of the primary binding site of HSA for ketoprofen, mainly because of its relatively high detection limit. Under the particular experimental conditions described previously, the LOD at a signal-to-noise ratio of 3 was 2µM for CEFA and 0.1µM for LCFA. The sensitivity of CEFA was obviously insufficient for the analysis of samples at a high binding rate (low unbound concentration). It might therefore be difficult to determine binding parameters with high affinity.

One of the advantages of the CEFA method is the small sample volume required. In LCFA using an ISRP column packed with restricted-access packing materials, the drug–protein mixed zone under the same binding equilibrium as that in the sample is formed in the interstices. In order to create this zone, a large volume of sample injection is needed. As the unbound fraction of a drug in the sample solution increases, the sample injection volume necessary to produce a trapezoidal peak becomes larger. In contrast, CEFA uses a very small volume of sample injection because it does not need extra sample volume to release a drug from protein and fill the microspores as in LCFA, and the injection volume is essentially the same for all samples having various unbound fractions. In this study, approximately 72 nL of a sample injection volume was used for CEFA, which was much smaller than that in LCFA (950 µL). Therefore, CEFA could be useful in a protein-binding study with precious samples and drugs highly sensitive to this type of detection.

Table II. The Binding Parameters of Ketoprofen to HSA

HSA (µM)	Total concentration range of ketoprofen (µM)	Method	Parameters T (°C)	$K_1 \times 10^6$ (M^{-1})	n_1	$K_2 \times 10^6$ (M^{-1})	n_2	Reference
40	62 ~ 125	CEFA	25			0.24	2.54	This work
40	10 ~ 150	LCFA	25	1.05	0.98	0.12	3.16	This work
50	4.6 ~ 122	Microdialysis–HPLC	37	3.18	0.799	0.201	2.15	(7)
550	100 ~ 300	HPFA	25	2.33	1.12			(6)
550	100 ~ 300	Ultrafiltration–HPLC	25	2.37	1.13			(6)
		Continuous ultrafiltration		1.37				(5)

It is known that the separation of unbound drugs from their bound form and protein in CEFA is based on their differing electrophoretic mobility and not on the molecule size as in LCFA (18). This characteristic provides another advantage to CEFA in the possibility of studying the interaction between molecules of similar size with different electrophoretic behaviors.

Conclusion

CEFA has been developed for the first time to determine the binding parameters of ketoprofen to HSA. The method has the advantages of small sample injection volume and rapidity; however, it measures only the parameters for the secondary binding site for this drug because of its relatively low sensitivity. The binding parameters of ketoprofen for two classes of sites can be determined using LCFA. The K_1 and n_1 values are $1.05 \times 10^6 M^{-1}$ and 0.94, respectively, and the K_2 and n_2 values are $0.12 \times 10^6 M^{-1}$ and 3.16, respectively, determined by LCFA and $0.24 \times 10^6 M^{-1}$ and 2.54, respectively, by CEFA. When the molecular ratio of ketoprofen versus HSA is lower than 0.75, ketoprofen may bind mainly at the primary binding sites, and when the ratio is higher then secondary binding occurs.

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