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RESEARCH ARTICLE

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Tudy on drug displacement interactions by capillary electrophoresis-frontal analysis

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Abstract The interaction between 18-methyl norethindrone and ketoprofen, including the displacement of ketoprofen from human serum albumin binding sites, was investigated by the capillary electrophoresis-frontal analysis method (CE-FA) at room temperature. A very large sample plug was introduced hydrostatically into the capillary (65 cm × 50 μm i.d.; effective length of 35 cm) over 80 s at a height difference of 11 cm. The working conditions for CE-FA separation are as follows: operating voltage, 10 kV; running buffer, 67 mmol·L^{-1} phosphate, pH 7.4. The unbound ketoprofen concentration was directly measured from the height of the frontal peak. When the concentration of 18-methyl norethindrone was increased from 0 to 200 μmol/L, the unbound ketoprofen concentration was found to increase from 22.4 to 26.4 μmol/L at 100 μmol/L total ketoprofen concentration and from 82.1 to 106.2 μmol/L at 200 μmol/L total ketoprofen concentration. From these data, it may be deduced that the administration of high concentration of 18-methyl norethindrone can displace ketoprofen from its secondary binding site.

Keywords capillary electrophoresis-frontal analysis, human serum albumin, displacement interaction

1 Introduction

The extent to which drugs bind to plasma proteins, particularly the albumin, varies enormously, but some drugs bind intensively. Binding is a reversible equilibrium that is established between bound molecules and unbound molecules. Only the unbound molecules remain free and pharmacologically active, while those that are bound form a pharmacologically inactive reservoir that is not immediately exposed to metabolism [1].

Depending on the concentrations and their relative affinities for the binding sites, one drug (displacer) may successfully compete with another (target drug) and displace it from the binding sites. The increase of unbound target drug concentration may result in possible increase of side effects, even toxicity. Especially, for target drugs having a narrow therapeutic index, this interaction may be clinically significant.

There are numerous methods that may be applied in the study of drug displacement interactions [2–7]. Current high performance capillary electrophoresis is the most dynamically growing analytical technique in the study of molecular interactions because of its speed, efficiency and selectivity. CE offers several modes for the quantitative assessment of protein-drug interactions depending on the stability of the complex or the on-and-off kinetics of binding reaction [8]. When the mobility of the protein is equal to the mobility of the complex, CE-FA is the most favorable method among them [9]. The principle and main features of the CE-FA method have been described in the literature [6].

Ketoprofen (Fig.1 (a)), a nonsteroidal anti-inflammatory drug (NSAID), is known as a clinically effective agent for the treatment of inflammatory diseases such as rheumatoid and osteoarthritis, and its side effects include gastrointestinal disturbances and cartilage. Since it strongly binds to human serum albumin (HSA) in the circulation, the effects of concomitant drugs on its plasma pharmacokinetics were widely studied [2,10,11]. 18-methyl norethindrone (Fig.1 (b)) is an oral contraceptive highly (greater than 80%) bound to proteins [12]. In order to predict possible plasma proteins displacement interactions for ketoprofen and its concomitant oral contraceptive (18-methyl norethindrone), this work investigated displacement interaction of ketoprofen by 18-methyl norethindrone from the HSA molecule using the CE-FA method.

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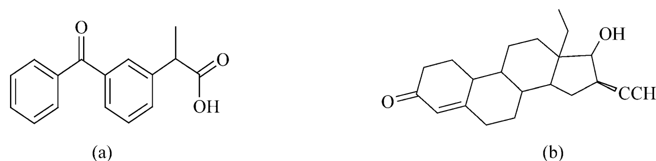

Fig. 1 Chemical structures of ketoprofen (**a**) and 18-methyl norethindrone (**b**)

2 Experimental

2.1 Materials and reagents

Human serum albumin (HSA, fatty acid unbound) was purchased from Sigma (St. Louis, MO, USA). Ketoprofen was supplied by the China Institute of Pharmaceutical and Biological Product Control (Beijing, China), and 18-methyl norethindrone was kindly donated by Family Plan Research Institute (Shanghai, China). All other chemicals were of analytical grade and were obtained from Shenyang Chemicals (Shenyang, China). The water was always double distilled. Phosphate buffer (I=0.17 mol/L) of physiological pH 7.4 was used as the sample solvent. Uncoated fused silica capillary (75 μm i.d.) was purchased from Ruifeng Chromatographic Product Co. (Yongnian, Hebei, China).

2.2 CE conditions

All CE experiments were performed in a HV-301 HVPS Capillary Electrophoresis System (Younite Unimicro Technologies, Co. Ltd, Tongliao, Inner Mongolia, China) with a VUV-22 UV detector（Younite Unimicro Technologies, Co. Ltd, Tongliao, Inner Mongolia, China） fixed at 214 nm. The power supply was operated in a voltage-controlled mode. The operating parameters of CE are given in Table 1. The electrophoretic data were acquired with a Jiangshen chromatographic workstation (Dalian, China).

Table 1 Operating parameters of CE

Parameter	Setting
Fused-silica Capillary	65 cm (Lc) × 75 μm I.D; L_E = 35 cm
Analyte	Ketoprofen, 18-methyl Norethindrone and 40μmol/L HSA
Injection method	hydrostatic injection; Δh = 11 cm, 30 s
Detection	UV at 214 nm
Voltage applied	10 kV
Temperature	room temperature
BGE	Potassium Phosphate buffer (pH 7.4, 67 mmol/L)

2.3 Displacement experiment

2.3.1 Preparation of sample solutions

The running phosphate buffer was prepared by mixing 10 parts of 67 mmol/L dipotassium hydrogen phosphate and one part of 67 mmol/L potassium dihydrogen phosphate. Isotonic phosphate buffer was prepared by mixing 10 parts of 67 mmol/L dipotassium hydrogen phosphate and one part of 67 mmol/L potassium dihydrogen phosphate with the isotonicity of each solution being adjusted with approximately 5 and 4 mg/mL of NaCl, respectively, yielding an ionic strength of 0.17 mol/L and pH 7.4. The obtained running phosphate buffer and isotonic phosphate buffer were degassed by sonication in an ultrasonicate water bath (DL_180, Shipuhaitian Electronical Instrument Co., Xiangshan, Zhejiang, China) for 25 min and filtered through a 0.45 μm membrane filter before use. HSA solution of (80 μmol/L) in 67 mmol/L potassium phosphate buffer (pH 7.4; I = 0.17 mol/L) was prepared by adding the appropriate volume of buffer to a weighed amount of HSA. Ketoprofen, the target drug, was dissolved in isotonic phosphate buffer thereby yielding a stock solution of 2 mmol/L, and working solutions of required concentration were prepared by diluting the stock solutions with pH 7.4 isotonic phosphate buffer. The samples were prepared as follows: parallel six aliquots of 300 μL ketoprofen working solution of 200 μmol/L were pipetted into six marked tubes, one was mixed with 300 μl isotonic phosphate buffer, the remaining five aliquots was gently mixed with 300 μl HSA solution (80μmol/L), respectively. After being equilibrated at room temperature for 2 h, one was analyzed for unbound drug concentration by direct injection into the CE-FA system; the remaining was used for displacement interaction study.

2.3.2 Preparation of sample solutions containing displacer

18-methyl norethindrone, the displacer, was dissolved in ethanol thereby providing a stock solution. A series of parallel volumes, 8, 16, 24 and 40 μL, of the stock solution were pipetted into different marked tubes, evaporated under N_2 stream to dryness and redissolved with the ketoprofen-HSA equilibrium solutions that were prepared as described above. As a result, the final concentrations of 18-methyl norethindrone in mixture solutions were 40 μmol/L, 80 μmol/L, 120 μmol/L and 200 μmol/L,

respectively. The tubes were gently shaken at room temperature for 2 h to allow the dissolving completeness of 18-methyl norethindrone and then were analyzed for unbound ketoprofen concentration by direct injection into the CE-FA system.

2.4 Determination of unbound ketoprofen by CE-FA

Uncoated fused silica capillary (65 cm × 75 μm i.d., effective length of 35 cm) was filled with phosphate buffer (pH 7.4, 67 mmol/L). The column temperature for separation was maintained at room temperature. The UV detector was set at 214nm. The sample solutions were introduced into the separation capillary using a hydrostatic method with a height of 11 cm for 80 s. The injection end of the capillary was then immersed into the running buffer and a voltage of 10 kV was applied between both ends. The unbound concentrations of ketoprofen were determined by comparing the plateau height of the equilibrated samples with that of the neat ketoprofen solution.

2.5 Data analysis

All statistical analyses were performed using the software package SAS (Version 8.0, SAS Institute Inc. Cary, NC, USA.) An ANOVA test was used to compare the different unbound ketoprofen concentrations according to the initial concentration of ketoprofen.

3 Results and discussion

The CE-FA method has successfully been applied to drug displacement interaction from HSA molecule [13] and the validation of CE-FA for the study of displacement study has been reported [14].

In this study, no detectable response of 18-methyl norethindrone was recorded; it is therefore used as a displacer for ketoprofen-HSA interaction.

The capability of 18-methyl norethindrone to displace ketoprofen from HSA molecule using the CE-FA method is shown in Fig. 2 (a). Table 2 summarizes the unbound concentrations of ketoprofen in 40 μmol/L HSA equilibrium solution in the absence and presence of 18-methyl norethindrone determined by the CE-FA method. The change in plateau height of unbound ketoprofen (as is clear by comparing Figs. 2 (b) and 2 (c)) demonstrates the occurrence of displacement. The protein binding rate of ketoprofen decreased from 77.6% to 59.1% while the total concentration of ketoprofen increased from 100 μmol/L to 200 μmol/L in 40 μmol/L HSA equilibrium solution. This suggests that the ketoprofen bind to HSA is concentration-dependent. It can be seen in Table 2 that when the total concentration of ketoprofen in 40 μmol/L HSA equilibrium solution is 100 μmol/L, the unbound ketoprofen concentration in the presence of 18-methyl norethindrone (0~200 $\mu mol \cdot L^{-1}$ concentration range) increase from 22.4 to maximal of 27.3 μmol/L. While the total concentration of ketoprofen is 200 μmol/L, the unbound ketoprofen concentration increased from 82.1 to 106.2 μmol/L.

Table 2 Concentrations of unbound ketoprofen in HSA equilibrated solutions in the absence and presence of 18-methyl norethindrone as determined by the CE-FA method (n = 3) μmol/L

Total concentration of ketoprofen	C (18-Methyl norethindrone)				
	0	40	80	120	200
100	22.4±0.6	23.6±0.8 $^{1)}$	27.3±0.8 $^{1)}$	26.6±0.7 $^{1)}$	26.4±0.9 $^{1)}$
200	82.1±2.3	84.7±2.4 $^{2)}$	87.5±2.5 $^{2)}$	91.7±2.8 $^{2)}$	106.2±2.7 $^{2)}$

1) $P < 0.05$, 2) $P < 0.001$

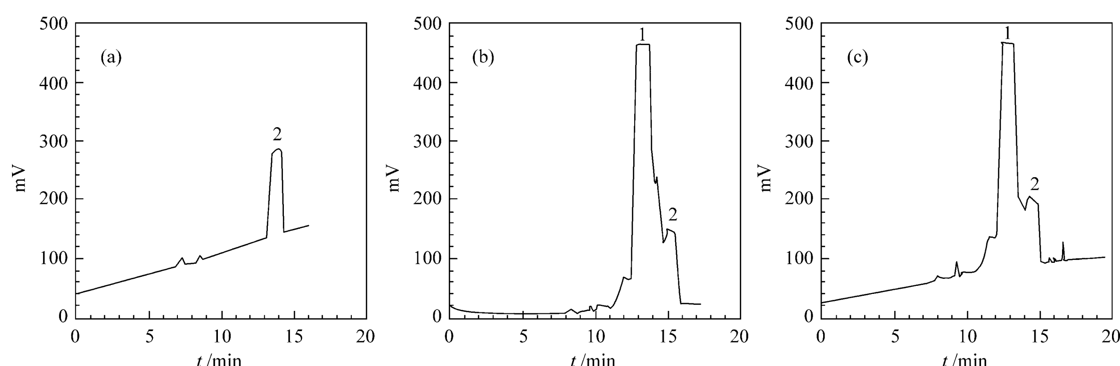

Fig. 2 Electropherograms of 200 μmol/L ketoprofen (**a**) 200 μmol/L ketoprofen and 40 μmol/L HSA (**c**) 200 μmol/L ketoprofen, 200 μmol/L 18-methyl norethindrone, 40 μmol/L HSA (**b**) CE-FA displacement studies in 67 mmol/L phosphate buffer (pH 7.4).1. HSA; 2. ketoprofen.

According to our previous study using HPLC-FA [15], two classes of binding sites are available for ketoprofen on the HSA molecule. When the molecular ratio of ketoprofen to HSA is not higher than 2.4, ketoprofen binds mainly at the primary binding site (high affinity binding site); as the molecular ratio of ketoprofen to HSA increases, however, the binding (low affinity binding site) occurs at the secondary site. On carefully examining the data in Table 2, it is clear that when the total concentration of ketoprofen in 40 μmol/L HSA equilibrium solution is 100 μmol/L, ketoprofen would bind mainly at the primary binding site. Little change of unbound ketoprofen concentration was observed even at 200 μmol/L of the displacer—18-methyl norethindrone—which indicated that 18-methyl norethindrone hardly displaced ketoprofen from its primary binding site. When the total concentration of ketoprofen in 40 μmol/L HSA equilibrium solution was 200 μmol/L, both the primary and secondary binding sites of ketoprofen on HSA were occupied. The unbound ketoprofen concentration increased significantly in the presence of 18-methyl norethindrone at 200 μmol/L, which showed that ketoprofen could be displaced by 18-methyl norethindrone, at high concentration, from its secondary binding site.

The only requirement for the use of the CE-FA method as a technique to study displacement is that either the mobility of the target or that of the displacer should differ from each other or the displacer should not respond to the detector. Favorable reproducibility and small sample injection volume are the advantages of this method. Therefore this method has great potential in the study of clinically significant ligand displacement interactions for a variety of endogenous and exogenous ligands.

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