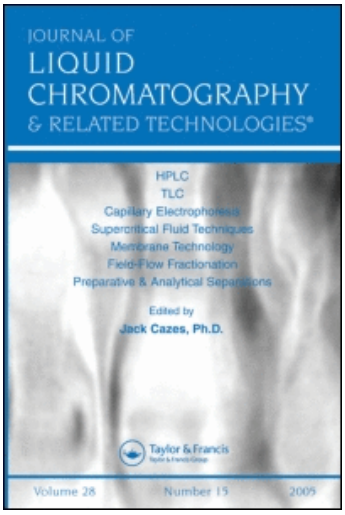

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Hanfa Zou[a]; Hailin Wang[a]; Yukui Zhang[a]
[a] National Chromatographic R & A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, P. R. China

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STEREOSELECTIVE BINDING OF WARFARIN AND KETOPROFEN TO HUMAN SERUM ALBUMIN DETERMINED BY MICRODIALYSIS COMBINED WITH HPLC

Hanfa Zou,* Hailin Wang, Yukui Zhang

National Chromatographic R & A Center
Dalian Institute of Chemical Physics
Chinese Academy of Sciences
Dalian 116011, P. R. China

ABSTRACT

Microdialysis sampling technique combined with high performance liquid chromatography (HPLC) was utilized to determine the stereoselective binding of warfarin and ketoprofen to human serum albumin (HSA) in solution. The HPLC conditions for separation of warfarin and ketoprofen enantiomers on HSA column were optimized. The unbound enantiomers in the mixed buffer (0.067 M phosphate and pH 7.4) solution of the drug racemate with HAS were sampled with microdialysis probe, and the amount of them was determined by HPLC on HSA column. It was observed that the unbound concentrations of R-warfarin and R-ketoprofen are about 1.08~1.34- and 1.08~1.15-fold of S-warfarin and S-ketoprofen, respectively, which indicated that the S-enantiomers bind to HSA more strongly than R-enantiomers to HSA. The interaction parameters for binding of warfarin and ketoprofen enantiomers to HSA including binding constants and number of binding site were obtained by the Scatchard analysis of

experimental data, and the stereoselectivity (α) calculated by the binding constants of warfarin enantiomers to HSA is 1.92, but that of ketoprofen enantiomers is very close. HSA exhibits stronger stereoselectivity to warfarin racemate than to ketoprofen racemate.

INTRODUCTION

It is a common case for an optically active drug that the pharmacological activity, side effect and /or drug disposition are different between the enantiomers. This is because the interactions between a drug and biopolymers such as receptors, enzymes, and proteins affect the activity and disposition of the drug, and are highly stereoselectivity.[1,2] In plasma, a drug binds, more or less, with proteins such as albumin and α_1 acid glycoprotein. Protein binding is a rapid and reversible interaction, and the concentrations of the drug and the protein are in an equilibrium state. Protein binding potentially behaves as the stereoselective process, and plays an important role in the pharmacokinetics and pharmacodynamics of the drugs.[3] Therefore, investigation of the stereoselective binding of chiral drugs to proteins is important for understanding the pharmacokinetic and pharmacodynamic process of the drugs. For this purpose, the development of the simple methods for the determination of molecular interactions between drug enantiomers and proteins is inevitable.

Clinically, warfarin is administered to patients as a racemic mixture. It has been known that hypoprothrombinemic activity of the S-enantiomer is 2~5 times[4-6] more potent than that of the R-enantiomer, and S-enantiomer binds to human serum albumin (HSA) more strongly than the R-enantiomer does.[7,8] Ketoprofen is a non-steroidal anti-inflammatory drug with an asymmetric carbon atom, and is administered clinically also as a racemate; the S-enantiomer is more potent than the R-enantiomer. The irreversible metabolic~inversion from R-enantiomer to S-enantiomer, which is a unique feature of profens (2-arypropionic acid derivatives), was observed in the rat and the rabbit.[9] Ketoprofen binds very strongly with albumin, and the binding is enantioselective. However, the lack of an adequate method to determine a low level of the unbound concentration of ketoprofen enantiomers has prevented progress in the analysis of the stereoselective pharmacokinetics of ketoprofen.

Recently, a method of microdialysis combined with high-performance liquid chromatography was developed to determine the interactions of various drugs with proteins. This method shows a number of advantages such as fast, simple, and easy to be automated and hyphenated with other analytical techniques.

In this paper, we utilized microdialysis combined with high performance liquid chromatography with HSA stationary phase packed column to study the stereoselective binding of warfarin and ketoprofen to HSA.

EXPERIMENTAL

Reagent and Materials

The racemates of warfarin and ketoprofen (racemates) and the HSA (fatty acid and globulin free) were purchased from Sigma (St. Louis, MO, USA). A CMA/20 microdialysis probe was purchased from CMA/Microdialysis (Acton, MA, USA), the length of the dialysis membrane is 4 mm.

Preparation of the Sample Solution

The drug stock solutions were made up in ethanol with concentration of 10 mM. HSA was dissolved in a potassium phosphate buffer (pH 7.4, 0.067M) at concentration of 200 μM. Appropriate volumes of warfarin or ketoprofen racemate stock solutions were put in a 2 mL open vial by the electric pipette, and the ethanol was evaporated in air. 1.25 mL of the prepared HSA solution (in phosphate buffer of pH 7.4, and 0.067 M) was added to the vial to obtain the mixture solutions of drugs with HSA.

Microdialysis Sampling

The microdialysis system comprises a FAMILIC-100N microsyringe pump (JASCO, Japan) and a microdialysis probe (CMA, USA). The perfusion solution is a 0.067 M potassium phosphate buffer of pH 7.4; perfusion rate is of 1 μL/min. The microsyringe was filled with a perfusion solution.

The drugs-HSA mixed solution was incubated at 37°C in a water-bath for more than 10 minutes before the probe was put into this solution, and sampling from the solutions was started. After 12 minutes, the dialysate was collected for 30 minutes. The collected dialysate was handled for HPLC analysis.

The probe must be washed by the perfusion solution at a rate of 5 μL/min for several minutes before the probe was put into the mixed solution to get rid of air in the probe and of organic solvents, which are used for protection of the dialysis membrane.

Apparatus and Instruments

The present HPLC system comprises a LC-10A pump (Shimadzu, Kyoto, Japan), a Rheodyne-type injector valve with a 50 μL loop, a SPD-l0AV UV detector (Shimadzu, Kyoto, Japan), and a WDL-95 chromatographic workstation (National Chromatographic R. & A. Center, Dalian, China). The Hypersil HSA column with the dimension of 150x 4.6 mm I.D. packed by the HSA immobilized silica (7 μm) was used for separation of the warfarin and ketoprofen enantiomers. The mobile phases used were acetonitrile/ water/phosphate buffer and isopropanol added. The warfarin and ketoprofen enantiomers were detected at UV wavelength of 280 nm and 232 nm, respectively. The flow-rate of the mobile phase was from 0.6 to 0.8 mL/min. The column temperature was controlled by enclosing the column in a homemade thermosetting jacket (±0.2°C).

Recovery of Microdialysis

The recovery (R), also called the dialysate extraction fraction, was defined as the concentration ratio of the drug in dialysate (C_d) to the unbound fraction in drug-protein solution. The dialysate was collected by microdialysis sampling in a standard solution with 0.067 M potassium phosphate buffer of pH 7.4 and then analyzed by high performance liquid chromatography with HSA immobilized silica as the stationary phase. The standard solution of drug enantiomers was also analyzed by the same liquid chromatographic method. The recovery of the individual drug was calculated by the ratio of peak area for drug enantiomer in microdialysate to that in the standard solution.

RESULTS AND DISCUSSION

Optimization of the Chiral Separation Conditions

A number of factors affecting the enantioselectivity for separation of warfarin enantiomers on an HAS column were investigated. The capacity factors of warfarin enantiomers decreased with increasing concentration of acetonitrile, while the enantioselectivity decreased slightly. The capacity factors of warfarin enantiomers were measured at different pH values of the eluent by maintaining column temperature at 35°C and keeping other conditions at constant, and the obtained results were shown in Figure 1. It can be seen that the capacity factors of enantiomers decreased with increasing pH value of the eluent. For an example, the capacity factors of enantiomers at pH 7.4 were about 2.0-fold higher than that at pH 5.5.

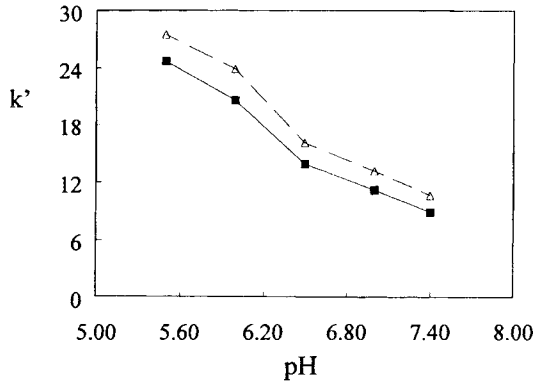

Figure 1. Effect of pH on the retention of R, S-warfarin. Solutes: ■, R-warfarin; Δ S-warfarin. Chromatographic conditions are as follows: Column, Hypersil HSA (7 μm, 150 mm x 4.6 mm I.D.); mobile phase, 50 μM potassium phosphate buffer including 15% acetonitrile (v/v); flow rate, 0.8 mL/min; detection, 260 nm; temperature, 35°C.

Such a result seems contrary to that obtained by B. Lound and D. S. Hage,[10] who reported that the capacity factors of warfarin enantiomers decreased with decreasing pH. The enantioselectivity of warfarin enantiomers at different pH values of eluent was also calculated, and it has been found that the enantioselectivity of warfarin enantiomers on an HSA column increased with increasing pH value of the eluent. For an example, the enantioselectivity (α) of warfarin enantiomers increased from 1.13 to 1.28 with a change of the eluent pH value from 5.5 to 7.4. Therefore, a high pH value of the eluent favors fast separation of warfarin enantiomers. Both the capacity factors and stereoselectivity of warfarin enantiomers largely depends upon column temperature; they decreased with increasing column temperature. An equation has been used for describing the relationship of the capacity factor and column temperature as follows:

$$\ln k' = A - \frac{\Delta H}{RT} \qquad (1)$$

where k' represents the capacity factor, R is the ideal gas law constant, T is the absolute temperature. The term ΔH represents the associated changes in enthalpy. Equation 1 predicts that a plot of lnk' versus 1/T will yield a linear relationship with the slope of -ΔH/R, which provides the value of ΔH. The ΔH of R- and S-warfarin experimentally measured are about -15.08 KJ/mol and -20.53 KJ/mol, respectively. It can be seen that the absolute ΔH value of R-warfarin is lower than that of S-warfarin, which means that the capacity factor

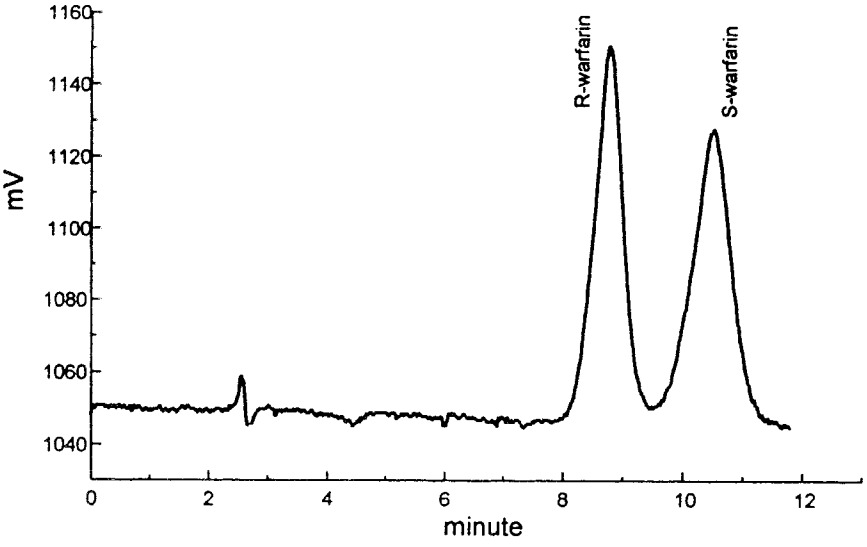

Figure 2. Chromatogram for the separation of R, S-warfarin enantiomers. Experimental conditions: column, Hypersil HSA (7 μm, 150 mm x 4.6 mm I.D.); mobile phase, water/100 mM phosphate buffer, pH 7.4/AcCN = 7/10/3; column temperature, 35°C; flow rate, 0.8 mL/min. For other experimental conditions, see text

of S-enantiomer on an HSA column decreased more rapidly with increasing of the column temperature than that of R-enantiomer did, the stereoselectivity of enantiomers might be governed by enthalpy change. It should be noted that the added amount of organic modifiers in the eluent and pH value of the eluent should be limited in a certain range in order to protect the activity of HSA from denaturation and the stability of silica from damage. Based on the above results, the HPLC conditions were selected for determination of warfarin enantiomers in microdialysis samples as follows: the mobile phase was 50 mmol/L phosphate buffer (pH 7.4) mixed with acetonitrile (85/15,V/V); the column temperature was at 35°C, and the flow rate was 0.8 mL/min. The baseline separation of warfarin enantiomers within 12 min was achieved and shown in Figure 2 under the above experimental conditions.

In the same way as for warfarin, the retention behavior of ketoprofen enantiomers on an HSA column was studied. It was observed that the stereoselective retention of ketoprofen enantiomers on the HSA stationary phase was some different from that of warfarin. Although the capacity factors of ketoprofen enantiomers decreased with increasing organic solvent, the stereoselectivity increased with increasing acetonitrile and isopropanol in mobile

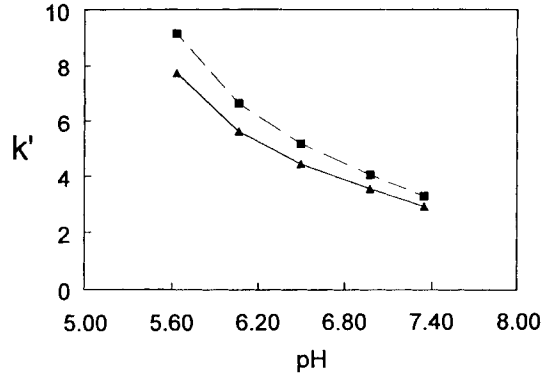

Figure 3. Effect of pH on retention of R, S-ketoprofen. Solutes: ▲ R-ketoprofen; ■ S-ketoprofen; chromatographic conditions are as in Figure 1.

phase. Their capacity factors decreased with increasing pH as shown in Figure 3, but the stereoselectivity slightly decreased. The baseline separation of ketoprofen enantiomers could not be achieved until 15% acetonitrile (V/V) was added into 50 mmol/L phosphate buffer of pH 7.4. If the concentration of phosphate buffer decreased from 50 mmol/L to 25 mmol/L, then both 3.5% isopropanol and 15% acetonitrile should be added into the phosphate buffer for baseline separation of ketoprofen enantiomers at 28°C. Figure 4 showed the separation of ketoprofen enantiomers on HSA column at the selected HPLC conditions. In Figure 4, the flow rate of mobile phase was set at 0.6 mL/min in order to improve the column efficiency due to slow dynamic process for binding of ketoprofen enantiomers to HSA on the stationary phase.

Binding of Warfarin and Ketoprofen Enantiomers to HSA

The recoveries of microdialysis sampling (R_1) for warfarin and ketoprofen enantiomers measured were 46.5% and 43.4%, respectively. It should be mentioned that there is no difference between the recoveries of a pair of isomers. The unbound concentration of drug in drug-protein mixed solution can be calculated by following equation:

$$C_f = C_d/R_1 \qquad (2)$$

Where C_f and C_d represent concentration of unbound drug in drug-protein mixed solution and that of drug in microdialysate, respectively. The microdialysis samplings of the mixed solutions of warfarin with HAS by keeping HSA

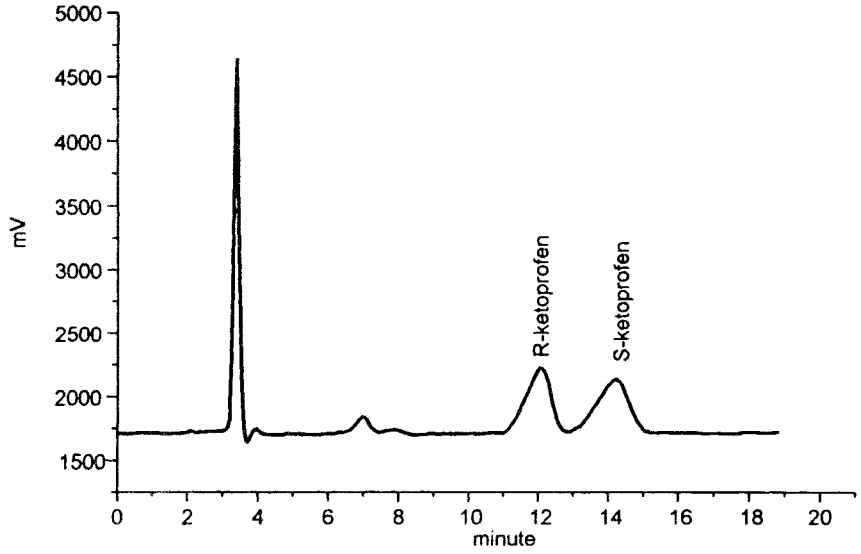

Figure 4. Chromatogram for separation of R, S-ketoprofen enantiomers. Experimental conditions: mobile phase, water/50 mM phosphate buffer, pH 7.4/AcCN/isopropanol = 31/50/15/4; column temperature, 28°C; flow rate, 0.60 mL/min.; for other experimental conditions, see text.

concentration at 200 μM and warfarin concentration at 160, 151, 80, 60, 40 μM, respectively, were carried out. Microdialysate fractions were analyzed on an HSA column at the separation conditions obtained above. The unbound concentration of drug enantiomers (drug ref. to ketoprofen or warfarin) in the drug racemate-HSA solutions were sampled by a microdialysis probe, then determined by HPLC using an HSA column. The relative errors of sampling for warfarin were between 0.44%~7.18% and those for ketoprofen were between 1.5%~6.4 %. It has been found that the unbound concentrations of the R-ketoprofen and R-warfarin are higher than those of S-warfarin and S-ketoprofen, respectively. The ratio of unbound concentration of R-warfarin to that of S-warfarin is changed from 1.08 to 1.34 with decreasing concentration of warfarin racemate from 160 to 40 μM when HSA was kept at 200 μM. However, the ratio of unbound concentrations of the R-ketoprofen to that of S-ketoprofen is only changed in the range from 1.09~1.15 by change of the ketoprofen racemate concentration from 100 to 40 μmol/L. These results indicated that the S-warfarin and S-ketoprofen bind more strongly to HSA than R-warfarin and R-ketoprofen do, and HSA exhibits stronger stereoselectivity to warfarin racemate than to ketoprofen racemate.

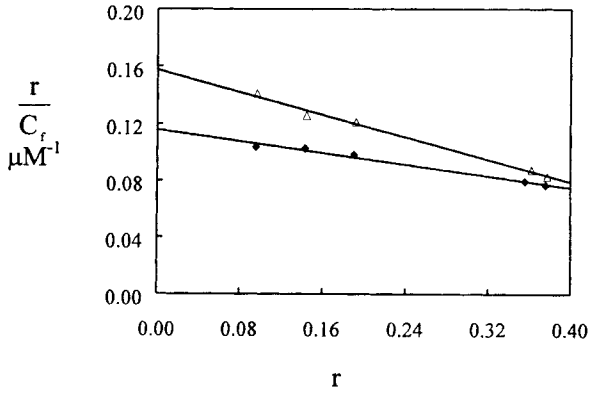

Figure 5. Scatchard plot of R, S-warfarin - HSA interaction. Solutes: ◆ R-warfarin; Δ S-warfarin.

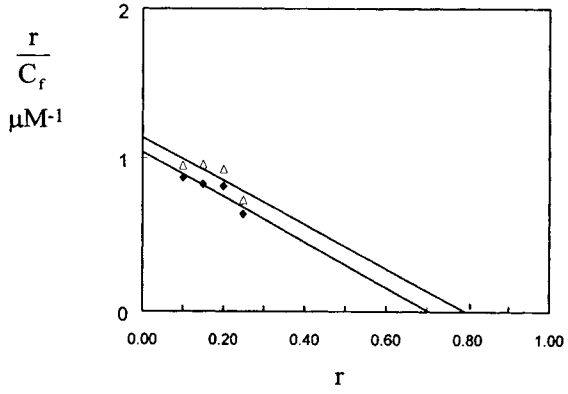

Figure 6. Scatchard plot of R, S-ketoprofen - HSA interaction. Solutes: ◆ R-ketoprofen; Δ S-ketoprofen.

The interaction parameters for the enantiomers of warfarin and ketoprofen were estimated by Scatchard analysis, and the obtained plots were shown in Figures 5 and 6. It can be seen that the results of warfarin could be approximated by linear relationship with correlation coefficients of 0.991 for R-warfarin and 0.996 for S-warfarin. The interaction parameters for binding of warfarin enantiomer to HSA estimated by Scatchard analysis in this work and obtained by other methods were listed in Table 1.

Table 1

Comparison of Association Constants Measured for the Binding of (R)- and (S)-Warfarin to HSA at 37°C

Status of HAS	Binding Constant ($M^{-1} \times 10^5$)[a] K_R	K_s	Analysis	Ref.
In solution	1.02	1.96	microdialysis equilibrium	this work
In solution	2.5	5.69	dialysis equilibrium	11
In solution	2.06(0.02)[a]	2.44	dialyis	12
Immobilized	2.1 (0.2)	2.6 (0.4)	front analysis	10
Immobilized	3.3	4.4	zone elution	13

[a] Values in parenthesis represent ± SD. All binding constant was measured at pH 7.4 except that from ref. 12 was determined at pH 10.0.

It can be seen that the binding constants of R-warfarin to HSA in solution or immobilized HAS, measured by different methods, varied from 1.02x105 to $3.3x10^5$ M^{-1}, but those of S-warfarin varied from $1.96x10^5$ to $5.69x10^5$ M^{-1}, respectively; this means that all of those interaction parameters may be acceptable.

According to our result, the stereoselectivity for warfarin enantiomers in 50 mmol/L phosphate buffer with pH 7.4 is about 1.92. It is higher than that obtained by HPLC on an HSA column shown in the above section. On the other hand, it is difficult to make a difference of the stereoselective binding of a drug to protein in aqueous solution from that to immobilized protein, although the stereoselectivity might be different due to the change of the binding property of protein caused by immobilized process or organic modifier present in mobile phase. The correlation coeffficients of the Scatchard analysis for R- and S-ketoprofen were 0.90 and 0.82, respectively. The values of nK obtained for R- and S-ketoprofen were $1.04x10^6$ and $1.14x10^6$ M^{-1} and the values of K were $1.44x10^6$ and $1.48x10^6$ M^{-1}, respectively.

This indicated that the binding constants of ketoprofen enantiomers to HSA are much higher than those of the warfarin enantiomers to HAS; but the binding constant of R-ketoprofen is very close to that of S-ketoprofen, and the stereoselectivity of HSA to ketoprofen racemate in phosphate solution is quite small.

CONCLUSION

The technique of microdialysis combined with HPLC has been utilized to determine the stereoselective binding of the warfarin and ketoprofen enantiomers to HSA in solution. The HPLC conditions for separation of warfarin and ketoprofen enantiomers on HSA columns have been optimized. It has been observed that S-warfarin and S-ketoprofen bind to HSA in solution more strongly than the R-warfarin and R-ketoprofen do. The binding constant and the number of binding sites for molecular interaction between the enantiomers and HSA in solution have been determined. It has been observed that HSA exhibits stronger stereoselectivity to warfarin racemate than to ketoprofen racemate.

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