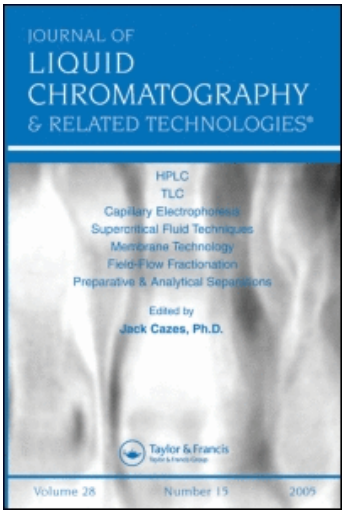

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Protein Binding Study of Perillyl Alcohol Enantiomers by High Performance Frontal Analysis

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ABSTRACT

High-performance frontal analysis (HPFA) was used for protein binding study of perillyl alcohol (POH) enantiomers to human serum albumin (HSA). The analysis was performed on a Develosil 100-Diol-5 (10 cm × 4.6 mm I.D.) column. Sodium phosphate solution (pH 7.4, ionic strength 0.17) was used as the mobile phase at a flow rate of 1 mL/min. UV wavelength was set at 205 nm. An injection volume of 600 μL was chosen to ensure the drug to be eluted as a trapezoidal peak with a plateau. Experimental data were fitted by Scatchard analysis. The binding constant (K) and binding affinity (nK) of POH enantiomers to HSA were: $K = 5.20 \times 10^6$ (1/mol),

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$nK = 1.74 \times 10^4$ (1/mol) for (*S*)-POH and $K = 3.71 \times 10^6$ (1/mol), $nK = 1.51 \times 10^4$ (1/mol) for (*R*)-POH, respectively.

Key Words: Protein binding; Perillyl alcohol enantiomers; Frontal analysis.

INTRODUCTION

Binding of drugs to plasma proteins, mostly to serum albumin and α-acid glycoprotein, is one of many factors that influences drug disposition. It is widely accepted that the effect of a drug is related to the exposure of a patient to the unbound concentration of drug in plasma rather than total concentration.[1,2] Unbound drugs in plasma can easily reach the target organ, whereas bound drugs are hard to pass through the blood capillary wall. Consequently, unbound drug concentrations show better correlation to the pharmacological activity than the total drug concentrations. Also, pharmacokinetic properties such as hepatic metabolism rate, renal excretion rate, biomembrane partition rate, and steady state distribution volume are functions of unbound drug fractions (unbound/bound concentration ratio). Considering the high concentration of albumin, for example human serum albumin (HSA), it is the most abundant protein in blood plasma, and the free concentration available for therapeutic action can be effectively reduced for drugs with high binding to plasma proteins, the quantitative investigation of drug-binding is essential in pharmacokinetic study and clinicial applications.[3]

Equilibrium dialysis and ultrafiltration, followed by HPLC analysis methods, have been widely used for this purpose. However, the conventional analytical methods are limited for the drug adsorption onto the membrane and the leakage of the bound drug from the membrane, as well as the difficulty in determining low concentrations of unbound drugs. To overcome this problem, high-performance frontal analysis (HPFA), which allows simple and easy determination of unbound drug concentrations after direct sample injection had been reported.[4–8] This method is free from the problems mentioned above and the bound drug is transformed into the unbound form in the HPFA column, which improves the measurement of low levels of unbound drugs, so it specially fits for the analysis of strongly bound drugs.[8]

Almost half of the chiral drugs are clinically used as recemates, whereas it is often the case that either one of the enantiomers shows pharmaceutical activity. The protein binding property of a racemic drug is often different between the enantiomers, which may cause the difference in their pharmacokinetic character.[9,10] A stereoselective protein binding study is, hence, essential for the good understanding of the racemic drugs and the safety in their clinical use.

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Perillyl alcohol (POH) is a naturally occurring monocyclic monoterpene found in essential oils of numerous species of plants including mint, cherry, and celery seed (chemical structure of POH can be seen in Fig. 1). This monoterpene shows chemopreventive and therapeutic activities in skin,[11] lung,[12] liver,[13] pancreatic,[14] and colon tumor models.[15,16]

Here, we present a work of protein binding study of POH enantiomers to HSA by HPFA. This work may provide useful information for the understanding of pharmacokinetics and clinical applications of POH.

EXPERIMENTAL

Reagents and Materials

Human serum albumin (fatty acid free), sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$), and sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) were purchased from Sigma (St. Louis, MO). Perillyl alcohol enantiomers were from Aldrich (Milwaukee, WI). The diol-silica column (Develosil 100Diol5, 100×4.6 mm I.D.) was purchased from Phenomenx (Japan). Water was twice distilled and filtered by using decompressing pump (Division of Millipore, Waters) and filter (FH-0.45 μm).

Instrumentation and Method

Instrumentals

The instruments used in this study were as follows: M930 solvent delivery pump (Young Lin Co.), UV detector (M720 Absorbance Detector, Young-In

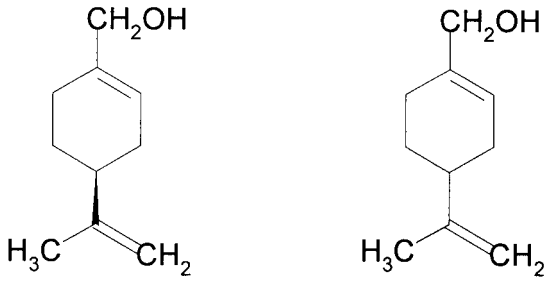

Figure 1. Chemical structures of POH enantiomers. (a) *S*-POH; (b) *R*-POH.

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Scientific Co.), column oven (CTS30 HPLC Column Oven, Young Lin Co.), a Reodyne injection valve with a 5 mL sample loop, and integrated data system (Autochrowin. Ver. 1.42, Young Lin Co.).

Preparation of Sample Solutions

First, sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) were dissolved in water to make solutions of 0.2 M, respectively. Then the two solutions were mixed together (19% $NaH_2PO_4 \cdot 2H_2O$ and 81% $Na_2HPO_4 \cdot 7H_2O$). In this way, phosphate solution of pH 7.4, ionic strength 0.17 was made and it was used as the mobile phase in HPFA analysis. Perillyl alcohol (3 μM) and HSA solution (700 μM) was prepared by dissolving the samples into phosphate solution. Sample solutions were kept at 37°C in a column oven for 3 hours before being injected into the HPFA column.

Determination of POH by HPFA Analysis

After incubation at 37°C for 3 hours, the POH–HSA solution was injected into the diol-silica column without another pretreatment. According to the principle of HPFA, the drug profile should be eluted as a trapezoidal peak, so an injection technique called "injector-reswitching technique"[4–8] was used: First a certain volume of the sample solution was loaded into the 5 mL loop and connected with mobile phase for a certain period by switching the injector valve, which resulted in a sample injection of desired volume, then the injector valve was reswitched to the load position so the loop was detached from mobile phase. By this technique, the diffused tail of the sample, which was connected with mobile phase and diluted was cut off, and the injected sample could be regarded as an ideal rectangular. When the injection was about 600 μL, POH was eluted as a trapezoidal peak with a plateau region after HSA elution. The height of the plateau region corresponds to the unbound drug concentration in the sample solution.[9]

RESULTS AND DISCUSSION

High Performance Frontal Analysis Profile

In HPFA, drug–protein mixture is injected directly into a restricted-access type HPLC column, which excludes large molecules such as proteins, but retains drugs of small molecular size on a stationary ligand in the micropores

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of the packing materials.[17,18] When the injection is small, the drug will be eluted as a normal HPLC peak following the protein peak. If the injection volume is large enough, the release of bound drug from protein is apparently suppressed. Then in the interstices of packing material near the top of the column, an equilibrium zone, in which the drug reaches the same protein binding equilibrium as in the sample solution is generated. The drug concentration in the micropores becomes equal to the unbound drug concentration in the interstices, and reaches chromatographic partition equilibrium. The protein peak is eluted first from the column and the unbound drug is eluted later as a trapezoidal peak having a plateau region. This plateau drug region is formed due to the elution of the unbound drug in the equilibrium zone. Therefore, the unbound drug concentration can be determined from the plateau height of the plateau region.[19] According to this principle, different injection volumes (in the range of 50–800 μL) were investigated with the sample solution (1.5 μM of *S*-POH in 350 μM HSA) maintained constant. Figure 2 shows the effect of injection volume on the eluted profile. It can be seen that when the injection volume is below 400 μL, (*S*)-POH can only be eluted as an ordinary HPLC peak; a zontal profile with an obvious plateau appears when injection volume is above 600 μL. Further increasing of the injection volume can only result in a longer plateau, but the peak height hardly changes, which can be seen in Fig. 3. Hence, an injection volume of 600 μL was chosen for further experiments.

Determination of the Unbound Drug

Different concentrations of (*R*)- and (*S*)-POH changing from 0.5 to 1.5 μM with the HSA concentration maintained at 350 μM, were injected to the HPFA system. From the heights of the peak plateaus, the unbound drugs could be determined (see Fig. 4). For quantitative determinations, calibration curves were carried out under the same condition as that in HPFA, but only standard (*R*)- and (*S*)-POH samples in the absence of protein were injected. The injection volume was 600 μL. The concentration ranges of (*R*)- and (*S*)-POH were all from 0.1 μM to 2 μM. By plotting of peak height vs. concentrations, the regression equations were obtained and they were: $y = -201.51x^2 + 910.6x + 29.72$, $R^2 = 0.9993$ for (*S*)-POH and $y = -206.12x^2 + 946.12x + 31.87$, $R^2 = 0.9987$ for (*R*)-POH, respectively. The determined unbound drugs for (*R*)- and (*S*)-POH are listed in Table 1. It can be seen that the binding rates (C_B/C_{total}) of the two isomers are nearly at the same degree, which indicates that they may show similar affinity to the protein.

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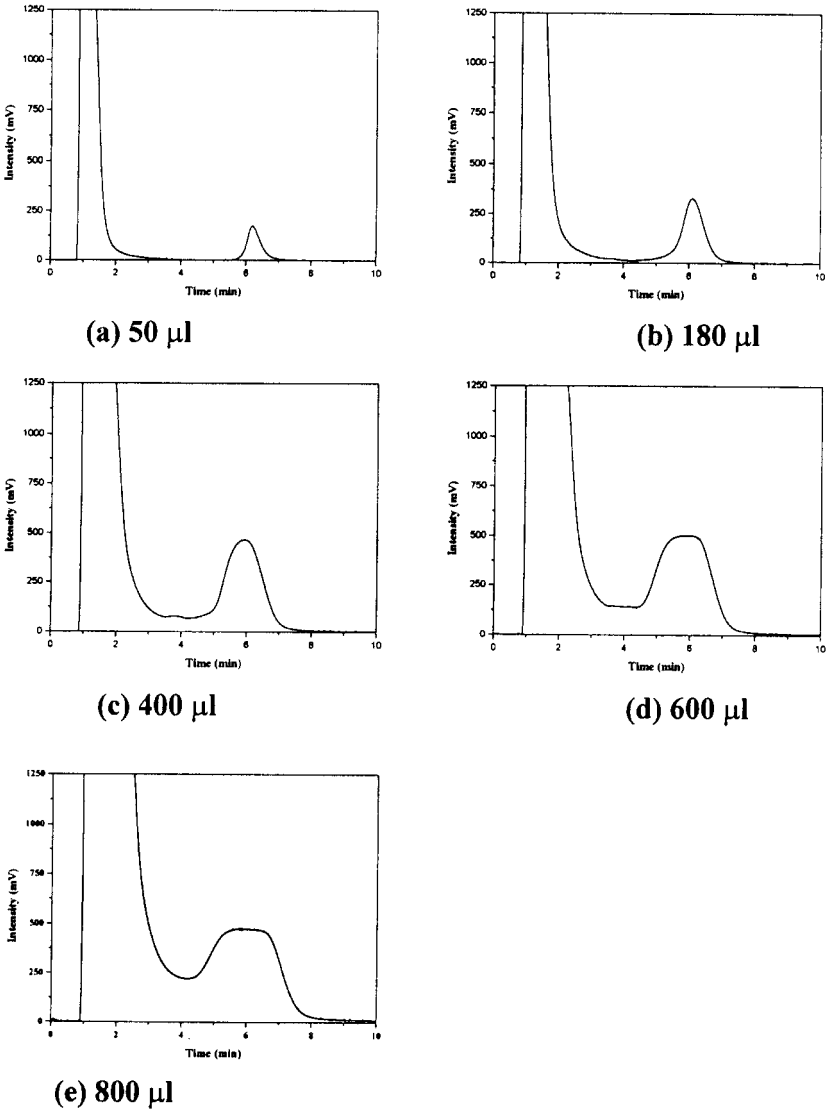

Figure 2. The effect of injection volume on elution profile of 1.5 μM (*S*)-POH and 350 μM HSA mixed solution [mobile phase, sodium phosphate buffer (pH = 7.4, $I = 0.17$); flow rate, 1 mL/min; UV wavelength, 205; temperature, 37°C; injection volume, 50–800 mL].

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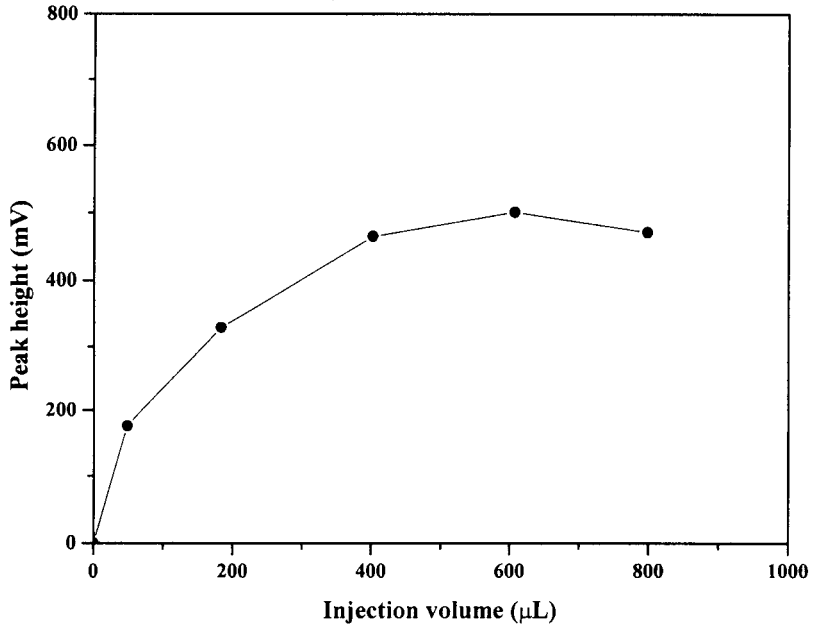

Figure 3. Relationship between injection volume of POH–HSA solution and peak height.

Determination of the Binding Parameters

The binding parameters were determined by fitting the experimental data to the Scatchard equation:

$$\frac{r}{C_u} = -Kr + nK$$

where r, C_u, K and n represent the number of moles of bound drug per mole of HSA, the unbound drug concentration, the binding constant, and the number of binding sites on one HSA molecule, respectively. Figure 5 illustrates the Scatchard plots of (*R*)- and (*S*)-POH. The correlation coefficient of the line was 0.985 for *S*-POH and 0.967 for *R*-POH, respectively, which indicates a good agreement of the experimental data to the theoretical equation. From the slope and intercept of the Scatchard plots, the binding parameters of the two enantioisomers are: $K = 5.20 \times 10^6$ (1/mol), $nK = 1.74 \times 10^4$ (1/mol), for (*S*)-POH and $K = 3.71 \times 10^6$ (1/mol), $nK = 1.51 \times 10^4$ (1/mol) for (*R*)-POH, respectively.

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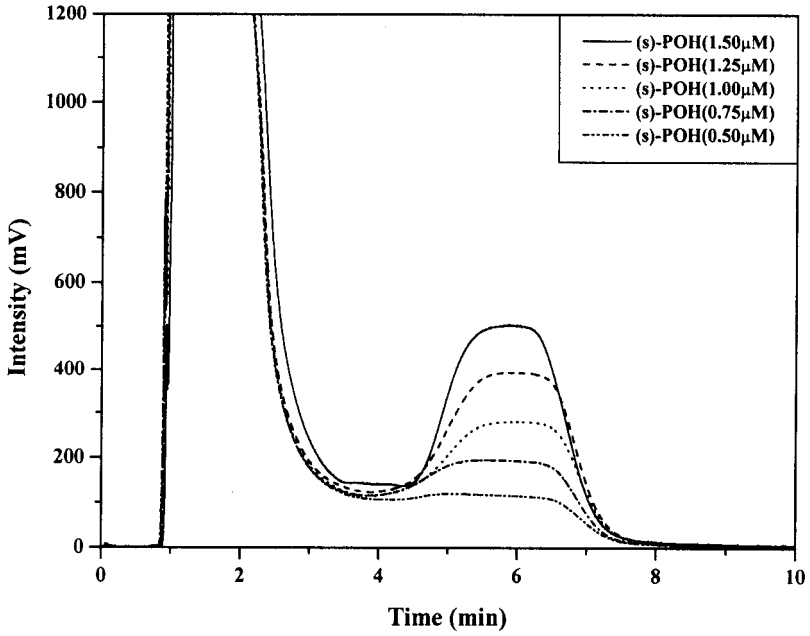

Figure 4. Chromatogram of (*S*)-POH with different concentrations in 350 μM HSA by HPFA [injection volume, 600 mL, flow rate, 1 mL/min, UV, 205, mobile phase, sodium phosphate (pH = 7.4, $I = 0.17$), column temperature was constant at 37°C].

Table 1. Determination of unbound drugs of (*S*)-POH and (*R*)-POH by HPFA.

Sample	Concentration (μM)	Unbinding drug (μM)	Binding drug (μM)
(*S*)-POH	0.50	0.0973 ± 0.004	0.403
	0.75	0.188 ± 0.005	0.562
	1.00	0.293 ± 0.011	0.707
	1.25	0.440 ± 0.043	0.810
	1.50	0.597 ± 0.036	0.903
(*R*)-POH	0.50	0.101 ± 0.003	0.399
	0.75	0.181 ± 0.007	0.569
	1.00	0.279 ± 0.023	0.721
	1.25	0.398 ± 0.030	0.852
	1.50	0.529 ± 0.048	0.971

Note: Concentration of HSA was fixed at 350 μM.

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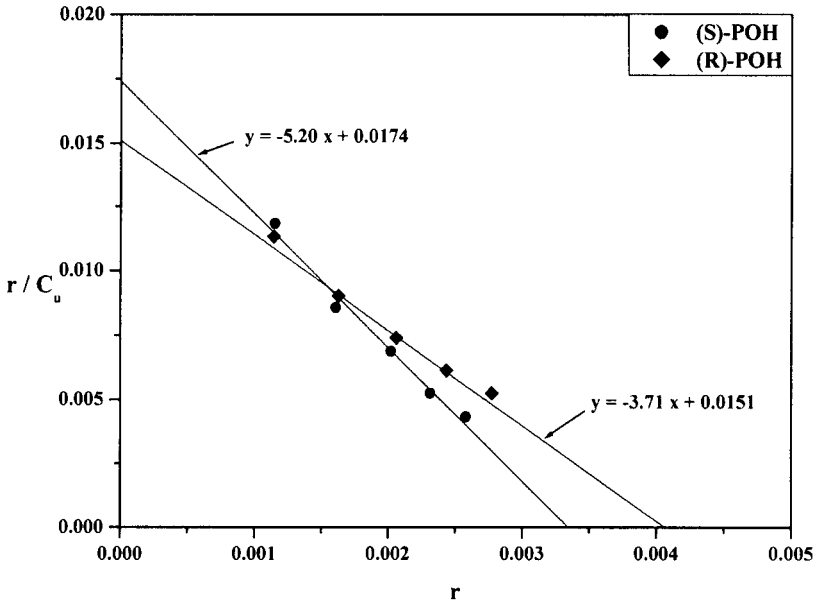

Figure 5. The Scatchard plots of (*S*)-POH isomer-HSA binding and (*R*)-POH isomer-HSA binding. The correlation coefficient of the line was 0.985 for (*S*)-POH, and 0.967 for (*R*)-POH, respectively.

CONCLUSIONS

High performance frontal analysis has been successfully applied for the protein binding study of POH enantiomers with HSA. This method is simple and precise, and has been used for the quantitative binding determination of low concentration of POH with HSA. From the experiment result, the binding constant (K) of (*S*)-POH is approximately 1.40 times larger than (*R*)-POH, the nK values (1.74×10^4 (1/mol) for (*S*)-POH and 1.51×10^4 (1/mol) for (*R*)-POH, respectively) are similar, which indicates that they may show similar affinity to the protein. This work may provide useful information for clinical application of this drug.

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