DRUG-PROTEIN INTERACTION STUDIED BY TECHNIQUE OF MICRODIALYSIS COMBINED WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Hanfa Zou*, Hailin Wang, Yukui Zhang

National Chromatographic R. & A. Centre, Dalian Institute of Chem. Phys., Chinese Academy of Sciences, Dalian 116011, China

SUMMARY

Drug-protein binding is an important process in determining the activity and fate of a pharmaceutical agent once it has entered the body. This review examines the method of microdialysis combined with high-performance liquid chromatography (HPLC) that has been developed by ours to study such interactions, in which the microdialysis was applied to sample the free drug in the mixed solution of drug with protein, and HPLC to quantify the concentration of free drug in the microdialysate. This technique has successfully been used for determining various types of binding interactions between the low affinity drugs, high affinity drugs and enantiomers to HSA. For the case of competitive binding of two drugs to a protein in solution, a displacement equation has been derived and examined with four nonsteroidal anti-inflammatory drugs and HSA as model drugs and protein, respectively. Microdialysis with HPLC was adopted to determine simultaneously the free solute and displacing agent in drug-protein solutions. The method is able to locate the binding site and determine affinity constants even up to 10^7 L/mol accurately.

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* Correspondence author:
Prof. Hanfa Zou
National Chromatographic R. & A. Centre, Dalian Institute of Chem. Phys.,
Chinese Academy of Sciences, Dalian 116011, China
Phone No: +86-411-3693409, Fax No: +86-411-3693407
E-mail: zouhfa@pub.dl.lnpta.net.cn

INTRODUCTION

It is well known that a drug in blood is bound to a greater or lesser extent to plasma proteins such as albumin and α_1-acid glycoprotein and that the concentrations of bound and free species are in equilibrium. Studies on drug-protein binding are important in pharmacology and pharmacokinetics /1-3/, because drug-protein interaction affects the pharmacological activities and side effects of the drug as well as the drug distribution and elimination. The unbound drug alone is supposed to diffuse from the blood to the extravascular active sites and to exhibit the pharmacological activity and/or the side-effect. Some important pharmacokinetic properties, such as hepatic metabolism rate, renal excretion rate, biomembrane permeation rate, and steady state distribution volume, also depend on the free drug fraction. The development of a simple and easy method to determine the concentration of free drug serves to promote progress in these studies.

Two common methods that are used in evaluating the binding of drugs to proteins include equilibrium dialysis and ultrafiltration /4-6/. Equilibrium dialysis is considered by many to be the reference method for such analyses; however, it does suffer from several disadvantages. Perhaps its greatest disadvantage is the long periods of time that are typically required to establish an equilibrium during the dialysis process /4/. Ultrafiltration is similar in its operation to equilibrium dialysis but requires much less time to perform (i.e., typically less than 30 min) /4,5/. However, like dialysis, it still requires the use of labeled drug and/or an additional analysis step for the actual measurement of the final free drug concentration. The other problems associated with this method include difficulties with temperature control during the separation.

Because of these limitations, there has been continuing research to find better, faster and more convenient approaches for the analysis of drug-protein binding. Many of the newer techniques developed for this purpose are based on chromatographic or electrophoretic systems /7-10/. Many chromatographic methods for solution-phase studies are based on columns that contain a size-exclusion /11-13/ or internal surface reversed-phase (ISRP) /14-16/ supports; both types of columns provide a means for resolving low to intermediate molecular mass drugs from proteins or drug-protein complex /7/. Such supports can be used in three general formats to investigate the binding of soluble drugs and proteins. The first of these formats is zonal elution, which includes the techniques of direct drug and protein separation,

peak-splitting measurements and the use of proteins as mobile phase additives. The second format is frontal analysis, and last format is that of the vacancy techniques, which includes both the Hummel-Dreyer method and the equilibrium saturation (or vacancy peak) method. However, the chromatographic approaches for the determination of drug protein-binding based on size-exclusion chromatography (SEC) requires a relatively large volume of sample solution to achieve a clear γ plateau, and that based on internal surface reversed-phase support requires good skill of operator and a column with suitable hydrophobic strength to allow the mobile phase to be used without adding any organic modifiers and elution of drug from the column. Another promising method involves the correlation of the degree of protein binding of a drug with its retention on a chiral stationary phase (CSP), derived from bovine serum albumin (BSA) /17/, human serum albumin (HSA) /18/, or α_1-acid glycoprotein /19/ for liquid chromatography. This method is able to reveal competitive and anticooperative interactions between ligands simultaneously bound to HSA or other plasma proteins, but it is difficult to find a satisfying correlation between the degree of protein binding and retention for all drugs or most of drugs which are not similar in structure /20/.

Like the chromatography, electrophoresis can be used as a tool to study drug-protein interactions. Many of the same approaches such as zonal elution, frontal analysis or vacancy in chromatography can be used in electrophoresis /9,10/. A relatively new development in the study of biomolecular interactions by electrophoresis has been in the development of the technique known as affinity capillary electrophoresis(ACE) /21-23/. As its name implies, this method is performed as part of a capillary electrophoresis (CE) system, with the ligand of interest being placed within the capillary as a running buffer additive. Advantages of ACE versus traditional electrophoresis include its speed, resolving power and ability to work with small amounts of ligand or analyte. Since CE system also acts to separate the analyte from other sample components, this method can often be used with impure samples or it can be used to simultaneously study the binding of several different compounds with the ligand of interest. The practical problems associated with CE or ACE methods include the adsorption of proteins or ligands on the capillary wall, the change in the binding constants for the drug-protein by Joule heating within the electrophoretic system as well as the difficulty to keep the running buffer as similar to the physiological solution /24-26/.

Microdialysis has been extensively applied to monitor continuously the

concentration of unbound drug and neurotransmitter *in vivo* /27-30/. Microdialysis sampling allows the determination of the concentrations of unbound drugs after a dialysis membrane has been placed in the drug-protein mixed solution. The technique is based on the kinetic dialysis principle in which substances diffuse down their concentration gradient. The microdialysis probe is usually a tubular membrane mounted on a double cannula made of the fused silica and plastic. A perfusion solution is pumped at a low-rate (1~5 μL/min) through the inlet of the probe and collected at the outlet, yielding a sample ready for analysis. The dimensions of the probe, i. e. the membrane length, diameter, and molecular weight cut-off, can be varied according to the requirements of application. The method is time-saving and even simpler than equilibrium dialysis. Microdialysis also has the advantage that the technique is easy to automate and can be on-line hyphenated with many analytical techniques such as LC, capillary electrophoresis (CE), flow injection analysis (FIA) and mass spectrometry (MS) etc. Recently, the microdialysis sampling has also been used for determination of binding degree of drugs to plasma protein *in vitro* /31,32/. In our laboratory, a combined technique of microdialysis with HPLC has been used for studying the interaction of various drugs to human serum albumin, here we will review the recent progress on this subject.

THEORY

The binding studies involve the determination of the parameters, such as the binding constants, the maximum number of drug molecules bound to a protein molecule and classes of binding sites on a protein. The reversible binding of a drug to a protein is governed by the multiple equilibria theory expressed by the following equation /33/:

$$r = \frac{C_b}{[P]} = \sum_{i=1}^{m} \frac{n_i K_i C_f}{1 + K_i C_f} \qquad (1)$$

where m is the number of classes of independent adsorption site, n_i is the number of sites in a class i with an association constant of K_i and C_b is the concentration of bound drug, C_f is the free concentration of drug and [P] represents the total concentration of protein. This equation can be converted

to a simple formula if m=1, which means that the protein just has one class binding site for the drug, as follows:

$$r = \frac{nKC_f}{1 + KC_f} \tag{2}$$

Eqn.(2) describes a Langmuir isotherm, and is usually transformed to eqn.(3) or (4), described as follows:

$$r/C_f = -Kr + nK \tag{3}$$

$$1/r = 1/n + (1/nK)(1/C_f) \tag{4}$$

where K is the association constant, and n is the number of the binding site on one protein molecule. Eqn. (3) is used for Scatchard analysis /34/ and eqn. (4) for a Klotz plot /35/.

INSTRUMENTATION AND RECOVERY OF MICRODIALYSIS

Figure 1 shows a schematic diagram of the present microdialysis system for the study of drug-protein interaction. The system consists of a microinjection pump, a microdialysis probe, the drug-protein mixed solution and collection vials. The microinjection pump is filled with perfusion solution, which is a phosphate buffer with same pH and ion strength as the drug-protein mixed solution, which simulates physiological condition. The solution in the collection vial is sampled from the drug-protein mixed solution by microdialysis and named microdialysate, which can be handled for analysis by chromatography, UV spectrometry and other techniques. In our studies, the microdialysates are usually determined directly by HPLC.

The recovery (R), also called the microdialysate extraction fraction, defined as the concentration ratio of the drug in dialysate (C_d) to the unbound fraction in drug-protein solution, is determined by placing the microdialysis probe in 0.067 mmol/L potassium phosphate buffer at pH 7.4 by adding drug with known concentration (standard solution). R is a key parameter to the microdialysis method for the determination of drug-protein interaction. We investigated the influences of perfusion rate and temperature on the relative recovery, and showing that the operation condition for microdialysis sampling

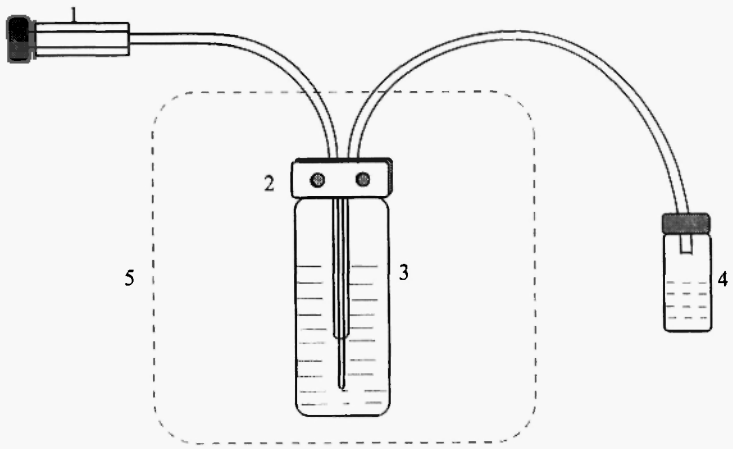

Fig. 1: Schematic illustrution of microdialysis system
(1) microinjecting pump filled with perfusion solution; (2) microdialysis probe; (3) sample vial filled with drug-protein solution; (4) collection vial and (5) temperature controlled region

such as perfusion rate, temperature must be controlled strictly /36,37/, because these two factors have a serious influence on precision and accuracy of R. R is expressed as follows:

$$R\ \% = (A_d/A_m)\ 100\ \% \qquad (5)$$

where A_d and A_m represent peak areas of drug in microdialysate and in standard solution, and are proportional to their concentrations, respectively. R changes with the change of perfusion rate. Recovery decreases when perfusion rate increases, reversal, recovery increases when perfusion rate decreases. Usually, the perfusion rate used set to 1 or 2 µL/min. At lower perfusion rate, the sampling process becomes time-consuming in spite of improvement of the relative recovery.

The disturbance of the equilibrium between free and bound drug must be also considered when determining the possibilities of this method. The fraction A_1 of drug sampled from a mixed drug-protein solution disturbed by microdialysis sampling can be calculated according to the following equation /38/:

$$A_1\,(\%) = [(v \times R \times t \times C_f)/(V \times C_{tot})] \times 100 \qquad (6)$$

where v is the perfusion rate, R is relative recovery, V is the volume of the mixed solution sampled, and C_{tot} and C_f are the total and free concentration of drug in the drug-protein solution, respectively; t is the period of time for sampling. It is assumed that t = 30 min, R is about 40% at perfusion rate (v) of 1 μL/min, V is high than 1.0 mL and the value of C_f/C_{tot} is larger than 0.5; then A_1 calculated by use eqn.(6) is much lower than 1%. Because A_1 is a parameter which represents the degree of disturbance, it appears that the effect of sampling disturbance on the equilibrium between drug and protein can be neglected.

The average recovery of several drugs by microdialysis sampling with dialysis membrane of 4 mm long and 0.5 mm O.D. as probe at perfusion rate of 1 or 2 μL/min was determined as shown in Table 1. These results demonstrate that a good precision and reproducibility can be achieved under strictly controlled experimental conditions. If longer dialysis membrane is used, recovery may be improved and sampling becomes faster. We also found that there is no difference in their recovery between the enantiomers of ketoprofen and warfarin. Then the free drug concentration determined by the technique of microdialysis combined with HPLC was quantified by the calibration curve or the single point in time. The free drug concentration in drug-protein mixed solution can be calculated as follows:

$$C_f = C_d/R \qquad (7)$$

where C_d is the concentration of drug in microdialysate, and R is the relative recovery. Furthermore, if the free drug concentrations in the drug-protein solution with different ratio of drug to protein are determined, then the drug-protein interaction parameters including binding constant and number of binding site can also be determined by eqns. (1-4).

MONO-SITE BINDING OF DRUGS TO PROTEIN

Some drugs only have low affinity interaction with proteins, which are difficult to be determined by the direct chromatographic or electrophoretic separation methods /7/. We applied the technique of microdialysis with HPLC to study the interaction between low affinity drug and protein with the

Table 1
The recoveries of 7 drugs by microdialysis sampling

Drug	Recovery (%)	RSD(%)
Carbamazepine	42.7 (6)*	1.85
Sulfamethoxazole	41.8 (4)*	2.30
Ketoprofen	43.4	-
Warfarin	46.5	-
Fenoprofen	56.0 (3)*	1.11
Ibuprofen	45.2	-
Naproxen	51.6	-

* Number in parentheses is the times of microdialysis samplings.

carbamazepine (CBZ) /38/ and sulfamethoxazole (SMZ) /39/ as model of drugs and human serum albumin (HSA) as the model of protein. Figures 2 and 3 showed the Scatchard plots for the CBZ-HSA and SMZ-HSA interactions experimentally measured. The calculated number of binding site and the binding constant of CBZ on a HSA molecule are 0.88 and 1.06×10^4 L/mol, respectively; which are values in agreement with literature determined by the high performance frontal analysis /40/. The calculated interaction parameters between SMZ and HSA are 3.04 and 3.24×10^3 L/mol, respectively; as we know, which are not reported yet. These results indicated that CBZ and SMZ are the low and moderately binding drugs, respectively; and they have much lower affinity to HSA than some high affinity drugs such as warfarin and ibuprofen with nK of 10^6 L/mol and endogenous compounds such as fatty acids with nK of 10^8 L/mol.

MULTI-SITE BINDING OF DRUGS TO PROTEIN

Another type of drugs having a high affinity interaction with proteins, fenoprofen and ketoprofen was chosen as the model compounds binding with high affinity to HSA in multiple equilibria to HSA to demonstrate the unique capacity of microdialysis in the study of the complex interactions between small molecules and macromolecules /37,41/. Due to the strong binding of this type of drugs to proteins, the free drug concentration in mixed solution of

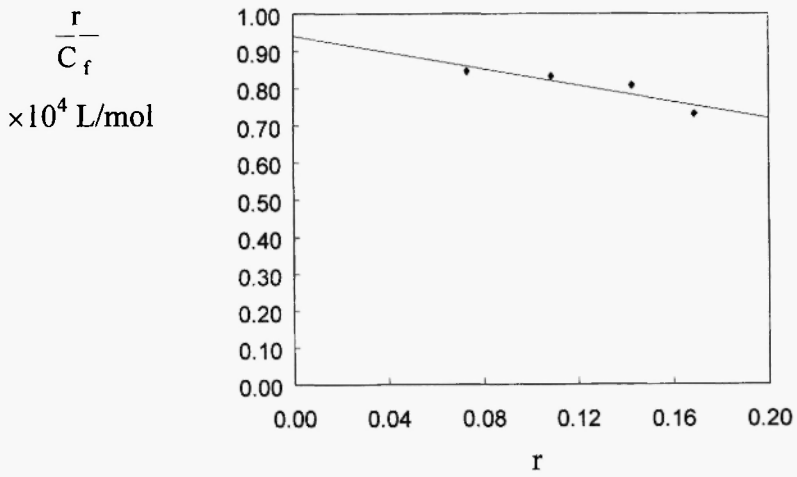

Fig. 2: Scatchard plot of CBZ-HSA interaction

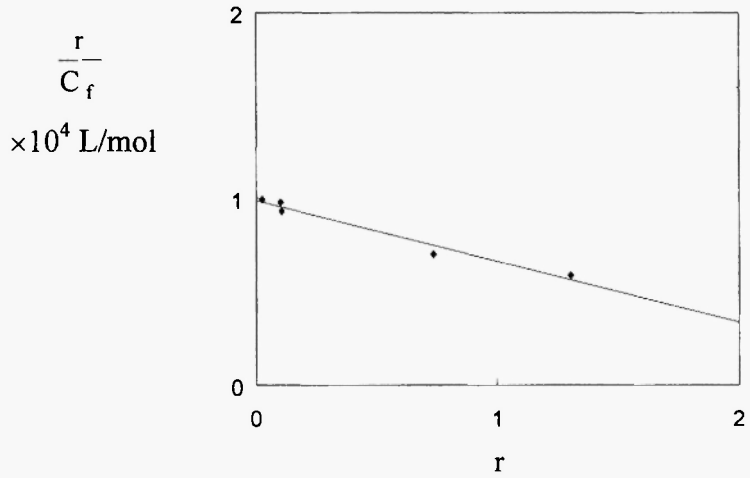

Fig. 3: Scatchard plot of SMZ-HSA interaction

drug-protein is quite low. Accurate quantitation of free drug will be a problem because of limitation of detection limit by high performance liquid chromatography. The large volume of microdialysate collected and utilization of short HPLC column are necessary. For example, 60 μL of microdialysate was collected from fenoprofen-HSA solutions with total concentration of

fenoprofen below 50 μmol/L, and 10 cm length of column was adopted for determination of fenoprofen in microdialysate.

The Scatchard plots for binding of the fenopoprofen and ketoprofen to HSA were illustrated in Figures 4 and 5 according to the data experimentally measured. It can be seen that the experimental data could be best

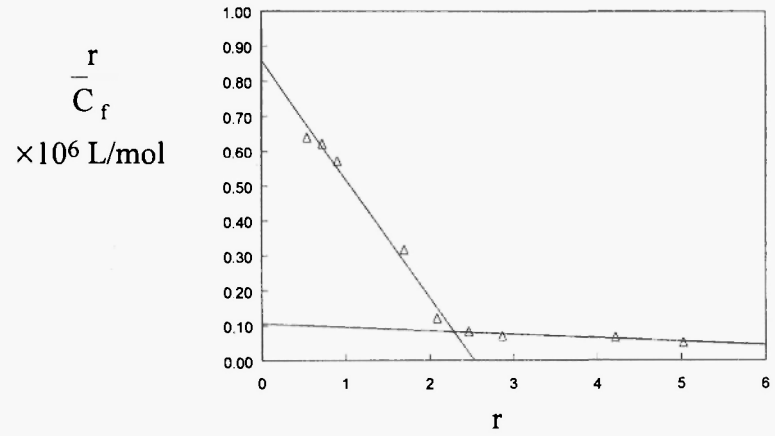

Fig. 4: Scatchard plot of fenoprofen and HSA interaction

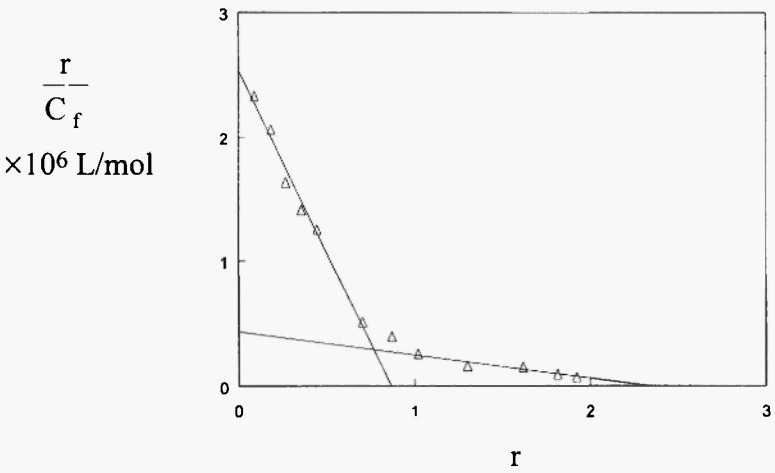

Fig. 5: Scatchard plot of ketoprofen and HSA interaction

approximated by two straight lines with linear regression analysis, suggesting two independent and nonequivalent classes of binding sites on HSA for both fenoprofen and ketoprofen. One is primary sites with high affinity and the other is secondary sites with low affinity. The binding parameters including association constants (K) and the numbers of the binding sites were shown in Table 2. It can be seen that the correlation coefficient for high affinity sites is much higher than that for low affinity sites.

Table 2

Interaction parameters between the high affinity drugs and the HSA measured by microdialysis with HPLC

Drug-Protein	n	K (L/mol)	γ*
Fenoprofen-HSA			
Primary binding site	2.5	3.4×10^5	0.99
Secondary binding site	10.0	1.0×10^4	0.92
Ketoprofen-HSA			
Primary binding site	0.8	3.2×10^6	0.99
Secondary binding site	2.2	2.0×10^5	0.96

* γ is the correlation coefficient for the Scatchard analysis

STEREOSELECTIVE BINDING OF DRUGS TO PROTEIN

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 /42,43/. The microdialysis with HPLC by the use of a suitable chiral column or additives in mobile phase for resolving the enantiomers has been applied to study the stereoselective binding of drug to proteins with the warfarin and ketoprofen as the model of enantiomers /44/. Figures 6 and 7 showed the separation of warfarin and ketoprofen enantiomers on HSA column at the optimized conditions. It has been found that the free concentrations of the R-ketoprofen and R-warfarin

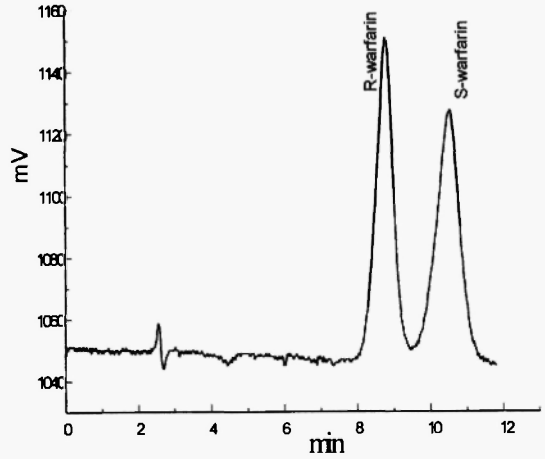

Fig. 6: Chromatogram for the separation of R, S-warfarin enantiomers. Experimental conditions: column, Hypersil-HSA (7 μm, 150×4.6 mm I.D.); mobile phase, water/100 mmol/L phosphate buffer (pH 7.4)/ACN=7/10/3; column temperature, 35°C; flow rate, 0.8 mL/min; detection wavelength, 280 nm.

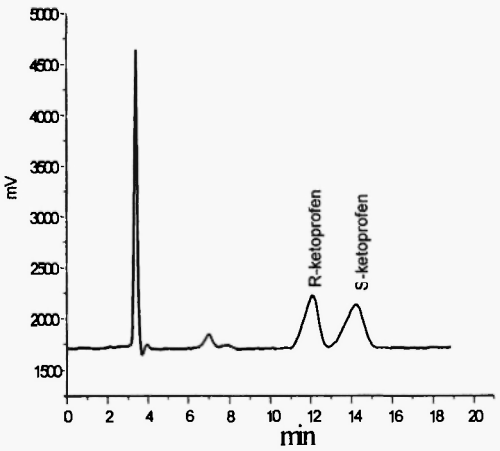

Fig. 7: Chromatogram for the separation of R, S -ketoprofen enantiomers. Experimental conditions: column, Hypersil-HSA (7 μm, 150×4.6 mm I.D.); mobile phase, water/50 mmol/L phosphate buffer (pH 7.4)/ACN/isopropanol=31/50/15/4; column temperature, 28°C; flow rate, 0.60 mL/min; detection wavelength, 232 nm.

are higher than those of S-warfarin and S-ketoprofen, respectively. The ratio of free 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 μmol/L when HSA was kept at 200 μmol/L. However, the ratio of free 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 behaves stronger stereoselectively to warfarin racemate than to ketoprofen racemate.

The interaction parameters for the enantiomers of warfarin and ketoprofen were estimated by Scatchard analysis, and the obtained plots were shown in Figures 8 and 9. 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 method and obtained by other methods were listed in Table 3. It can be seen that the binding constants of R-warfarin to HSA in solution or immobilized HSA measured by different methods were varied from 1.02×10^5 to 3.3×10^5 L/mol, but those of S-warfarin from 1.96×10^5 to 5.69×10^5 L/mol, respectively, which 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 HSA column shown in Table 3. On the other hand, it is difficult to make a difference of the stereoselective binding of drug to protein in aqueous solution from that to immobilized protein, although the stereoselectivity might be different due to the change of binding property of protein caused by immobilized process or organic modifier present in mobile phase. The correlation coefficients 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 1.04×10^6 and 1.14×10^6 L/mol and the values of K were 1.44×10^6 and 1.48×10^6 L/mol, respectively, which indicated that also the binding constants of ketoprofen enantiomers to HSA are much higher than those of the warfarin enantiomers to HSA, 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.

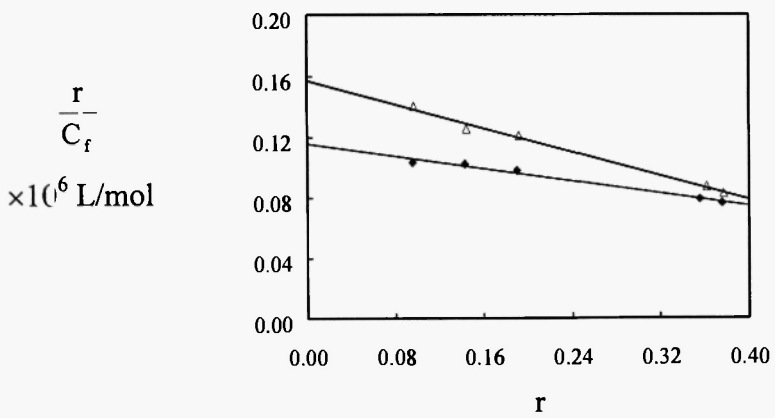

Fig. 8: Scatchard plot of R, S-warfarin enantiomers- HSA interaction. Solutes: ♦. R-warfarin; Δ, S-warfarin

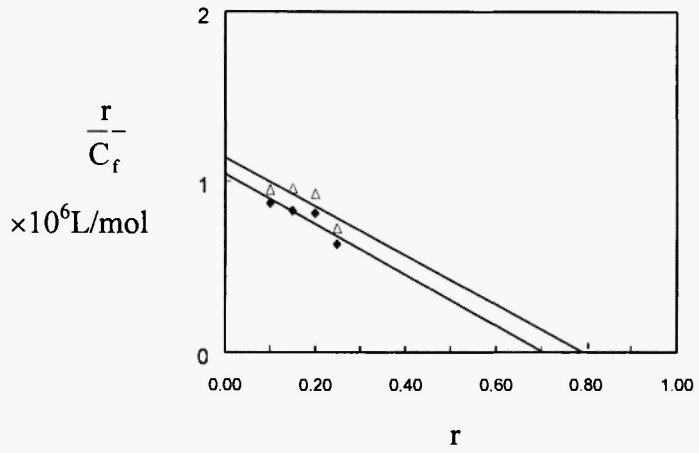

Fig. 9: Scatchard plot of R, S-ketoprofen enantiomers- HSA interaction. Solutes: ♦. R-ketoprofen; Δ, S-ketoprofen

COMPETITIVE BINDING OF DRUGS TO PROTEIN

Displacement studies with endogenous and/or exogenous compounds as competitive agents have been utilized to provide additional information on the mechanism of the binding and possible interactions, and help in the

Table 3
Comparison of Association Constants Measured for the Binding of (R)- and (S)-Warfarin to HSA at 37°C

Status of HSA	Binding Constant (L/mol 10^5)[a]		Analysis Method	Ref.
	K_R	K_S		
in solution	1.02	1.96	microdialysis	44
in solution	2.5	5.69	equilibrium dialysis	45
in solution	2.06(0.02)[a]	2.44	equilibrium dialysis	46
immobilized	2.1(0.2)	2.6(0.4)	front analysis	47
immobilized	3.3	4.4	zone elution	48

[a] Values in parentheses represent SD. All binding constants were measured at pH 7.4 except that from ref.46 which was determined at pH 10.0

further characterization of drug and macromolecule interactions. Such information is of special importance, especially to improve the safety of therapy with combinations of drugs that are highly protein bound and might compete for binding sites. It is well known that competitive binding of cobinding solutes to a protein will elevate free concentrations of both drugs, which maybe easy to be detected accurately. The microdialysis sampling has been applied to examine the drug displacement effect /41/. The displacement of fenoprofen by palmitic acid and ibuprofen taken as a model case, and three kinds of mixed solutions of fenoprofen (60 μmol/L) and HSA (20 μmol/L) by addition of palmitic acid (180 μmol/L, solution B) or ibuprofen (180 μmol/L, solution C) and without either of displacers (solution A) were prepared. Free drugs such as fenoprofen, ibuprofen and palmitic acid can enter the microdialysis membrane and be collected in microdialysate. The effect of displacement interaction can be investigated by means of HPLC determination of free drug concentration in microdialysates, and obtained results were illustrated in Table 4. Evidently, the displacement of drug from protein by both of the palmitic acid and ibuprofen raised the free fenoprofen concentration significantly, the binding degree of fenoprofen to HSA was

Table 4

Effects of displacer added to 60 μmol/L fenoprofen-20 μmol/L HSA mixed solution on the binding of fenoprofen to HAS

Displacer added	C_f (FP)*	Binding degree (%)	$C_{f(displacer)}$* (μmol/L)
-	15.8	73.3	-
180 μmol/L ibuprofen	55.9	5.45	122
180 μmol/L palmitic acid	51.7	12.4	-

* C_f (FP) and $C_{f(displacer)}$ represent molar concentration of free fenoprofen and displacer, respectively

decreased about 5-fold. Chromatograms A, B and C in Figure 10 showed the results obtained by the HPLC analyses of the microdialysates from fenoprofen-HSA solutions A, B and C, respectively. Ibuprofen appeared on Figure 10C, but palmitic acid did not appear on Figure 10B because of poor ultra-violet adsorption. Ibuprofen and fenoprofen belong to nonsteroidal antiflammatory drugs /49/, reported that the primary sites of both drugs are the same, so there is a competitive interaction between ibuprofen and fenoprofen, when overweighed ibuprofen added to fenoprofen-HSA solution, free fenoprofen is raised greatly. Palmitic acid is a long chain fatty acid, it is known that the binding affinities of the free fatty acids at the strong binding sites are much higher than those of most drugs /50,51/, but when it was added to fenoprofen-HSA solution, the released fenoprofen concentration is lower than that by ibuprofen with the same concentration used. That agree well with observed results by Chakrabarti /52/ and Sjodin /53/, the secondary sites of long chain fatty acid are the same as the primary sites of nonsteroidal anti-flammatory drugs.

In order to quantitatively study the competitive binding of drugs to protein, based on two assumptions that (1) the binding of drugs to a protein according to site-binding model, two drugs binding to same sites of protein will elevate the free fraction of both drugs; and (2) the binding of drugs to a

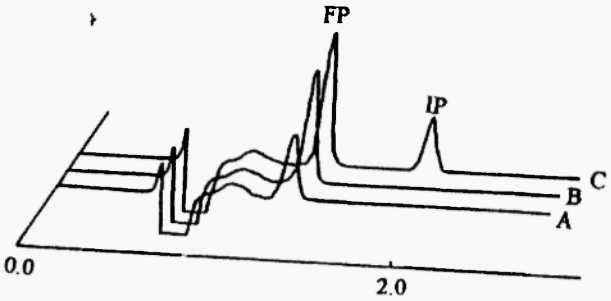

Fig. 10: Comparative chromatograms of the drug displacement effect. Experimental conditions: column, 4.6×100 mm I.D. packed with 5 μm of Hypersil BDS C_{18}; mobile phase, methanol/water/acetic acid (80/20/1.5, v/v/v); flow rate, 1.0 ml/min; detection wavelength, 232 nm.
Chromatograms: 60 μmol/L fenoprofen-20 μmol/L HSA mixed solutions (A) without any displacer, (B) with addition of 180 μmol/L of palmitic acid and (C) with addition of 180 μmol/L of ibuprofen.

protein conforms the stoichiometric relationship, a displacing equation for describing the competitive binding of two drugs to a protein has been derived as follows /54/:

$$\frac{1}{k'-X_0} - \frac{[A]}{n_1 \cdot C_P} = \frac{1}{K_1 \cdot n_1 \cdot C_P} + \frac{K_B}{K_1 \cdot n_1 \cdot C_P} \cdot [B] \qquad (8)$$

$$X_0 = \frac{n_2 \cdot K_2 \cdot C_P}{1 + K_2 \cdot [A]}$$

where K_1 and K_2 are the constants for the binding of drug A to the primary and secondary binding sites of protein P, n_1 and n_2 are the number of the primary and secondary binding sites on one protein molecule, C_P is the total concentration of protein P, K_B is the constant for binding of displacing drug B to primary binding sites of protein P, k' is defined as the ratio of the bound concentration to free one of drug A and named the apparent capacity factor, and [A] and [B] represent the free concentrations of drugs A and B, respectively. Eqn. (8) describes a complex and general case in the binding of

drug to protein, in which the two drugs competitively bind to the primary site on a protein, but only the solute binds to the secondary binding sites, unaffected by the displacing agent. There are other cases in the binding of drug to protein. One is the simplest is that in which two drugs only competitively bind to a single site without solute binding at the secondary site, which means n_2=0. Then, eqn. (8) is simplified as /54/:

$$\frac{1}{k'} - \frac{[A]}{n_1 \cdot C_P} = \frac{1}{K_1 \cdot n_1 \cdot C_P} + \frac{K_B}{K_1 \cdot n_1 \cdot C_P} \cdot [B] \qquad (9)$$

If there are three or more drugs competitively bind to the primary site on protein, an equation was also obtained based on an analogous treatment for eqn.(8), as follows /54/:

$$\frac{1}{k'-X_0} - \frac{[A]}{C_P} = \frac{1}{K_A \cdot C_P} + \frac{K_B}{K_A \cdot C_P} \cdot [B] + \frac{K_C}{K_A \cdot C_P} \cdot [C] + \bullet\bullet\bullet \qquad (10)$$

In eqn.(10), C,••• represents the additional competing agents, and K_C,• • • represents the association constants of C,•••. This equation is useful for quantitatively dealing with the competitive binding of multiple drugs to a protein and in evaluating the association constants of multiple drugs simultaneously by nonlinear regression.

In drugs and protein mixed solution, if there are two drugs competitively bound to the protein, one of the eqns. (8) and (9) might be applied, then a plot of $\frac{1}{k'-X_0} - \frac{[A]}{C_P}$ against [B] will be linear, the slope divided by the intercept will give K_B, which is affinity constant for the binding of the competing agent to the site in question; the intercept is equal to $\frac{1}{K_A \cdot C_P}$, so the affinity constant of solute A can also be obtained from intercept. If three or more drugs cobinding to a protein in solution, eqn. (10) might be applied to obtain the interaction parameters of the drugs to the protein.

The competitive binding experiments were performed by keeping the total concentrations of solute and HSA at constant with change of ibuprofen concentration in solutions. Each of ketoprofen, fenoprofen and naproxen has been used as solute with ibuprofen used as displacing agent, and their free

concentrations were determined by means of microdialysis with HPLC. Table 5 showed the interaction parameters obtained from the displacement of the nonsteroidal anti-flammatory drugs (NSAIDs) by ibuprofen after having plotted $\frac{1}{k'-X_0}-\frac{[A]}{C_P}$ versus free concentration of ibuprofen [B] by employing eqn. (8). All compounds tested gave linear relationships as shown in Figure 11. Linear regression analysis on the data gave correlation coefficients above 0.9976 for all compounds. X_0 was obtained by an iterative test, which reveals the contribution to k' from the sites unaffected by ibuprofen /54/. The affinity constants of ibuprofen (K_B) for the sites from which it displaced NSAIDs was obtained by calculating the ratio of the slope to the intercept for each curve; K_B is about $(7.7\pm1.6)10^5$ L/mol. The primary association constants of the solutes were obtained from the intercept because C_P is known, and they are also listed in Table 5. The total affinity constants of secondary binding were calculated from X_0 and were listed in Table 6. The primary binding constants of ketoprofen and fenoprofen are similar, which are determined directly by the conventional method, and that of naproxen is slightly higher than those of ketoprofen and fenoprofen, but their capacity

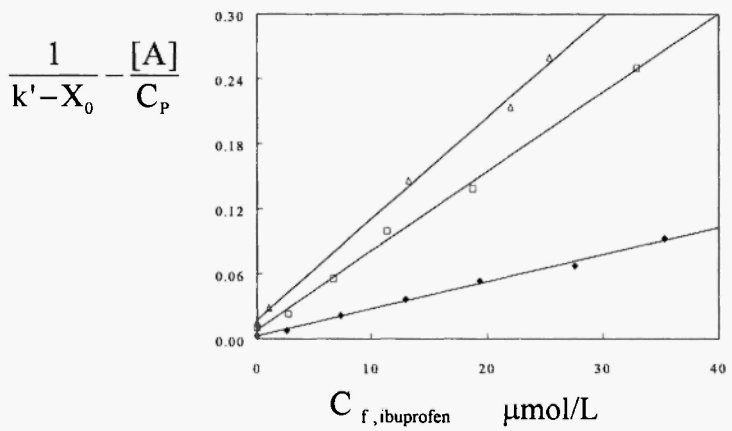

Fig. 11: The influence of ibuprofen on the binding of ketoprofen (Δ), fenoprofen (□) and naproxen (♦), plotted according to a model describing competition at a single site with further binding of the solute at other site(s), which are unaffected by ibuprofen. X_0 is the contribution to k' from such unaffected sites.

Table 5
Parameters obtained from the displacement of NSAIDs by ibuprofen

solute	X_0	Intercept	slope	γ	K_I (L mol)	K_B (L/mol)
ketoprofen	1.162	0.01746	0.009321	0.9983	$1.21\ 10^6$	$5.34\ 10^5$
fenoprofen	2.2182	0.008216	0.007299	0.9982	$2.43\ 10^6$	$8.88\ 10^5$
naproxen	1.693	0.002841	0.002495	0.9976	$7.00\ 10^6$	$8.78\ 10^5$

Note: X_0 is the contribution of k' to binding at sites unaffected by ibuprofen; column 2 and 3 summarize the slope and intercept of the regression curves, respectively; γ is correlation coefficient, K_I is the affinity constant of the solutes for the site from which ibuprofen displaces it, K_B is the affinity constant of ibuprofen for the site from which it displaces the NSAIDs.

Table 6
Secondary affinity constants of solutes calculated from X_0 obtained from displacing equation.

compounds	ketoprofen	fenoprofen	naproxen
n_2K (10^4 L/mol)	2.4	4.4	3.8

factors on HSA chiral stationary phase are 2 times higher. This phenomenon is contradictory. The direct determination of the interaction of drug and protein with high affinity by conventional methods may be subject to significant errors because the free drug concentrations are very low and impurities interfere with the measurement.

Study on the competitive binding of drugs to protein can also be used to identify the location of binding sites on a protein to interact with drugs. Ketoprofen, fenoprofen and naproxen were significantly displaced by ibuprofen, confirming that they bind to indole-benzodiazepine sites of HSA (site II). In addition, data from Table 5 reveal that the values of X_0 are not zero, suggesting that they bind to the secondary sites on HSA in addition to the primary binding site. The association constants of ketoprofen, fenoprofen, and naproxen correlated very well with their capacity factors on HSA chiral stationary phase, as shown in Figure 12, which were proportional to the total affinity of the drugs, suggesting that the sites from which ibuprofen displaces them are their primary binding sites on HSA[55]. Those results agree well with those of Rahim and Aubry /49/, who reported that site II of HSA may be composed of two subsites to which (R)-ibuprofen binds with higher and lower affinity, and (R)-ibuprofen displaces fenoprofen A from the lower affinity site. It has been concluded that ketoprofen, fenoprofen, naproxen and ibuprofen predominantly bind to indole-benzodiazepine sites of HSA (site II). Maybe the sites from which ibuprofen displaces ketoprofen, fenoprofen and naproxen are lower affinity subsites of site II.

The validity of eqn. (10) for the evaluation of competitive binding of three or more drugs with protein has not been confirmed experimentally yet. We will examine this possibility by a multidrug competitive binding experiment in future

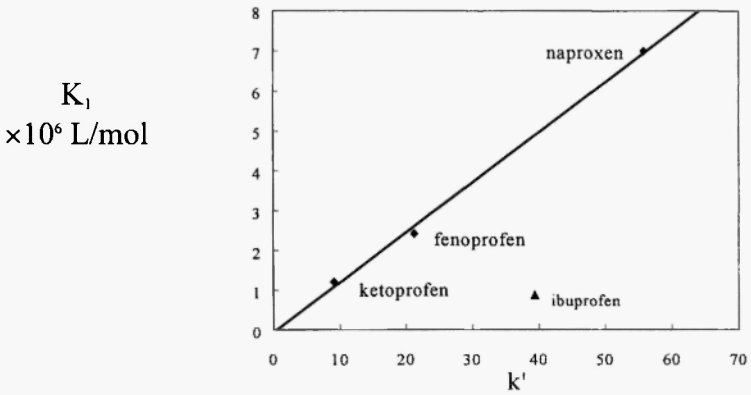

Fig. 12: Plot of the drug-HSA association constant, K, determined by displacement equation against the capacity factor k' on HSA-CSP obtained from ref. 55.

- ♦ data on linear line, ($K = -7.1\ 10^4 + 1.26\ 10^5$ L/mol, $\gamma = 0.9986$)
- ▲ data deflect seriously from linear line

CONCLUSION

The technique using microdialysis combined with HPLC demonstrates its potential in the drug-protein interaction study based on the kinetic dialysis in which substances diffuse down their concentration gradient. The method developed can be applied to the determination of the binding degree, affinity constant and binding number for small molecules such as drug, toxin and other biochemicals to macromolecules. Various types of interactions between the low affinity drugs, high affinity drugs and enantiomers and the human serum albumin were measured successfully. A displacement equation describing competitive binding of drugs to protein in solutions was derived and examined experimentally. The method developed is simple, time-saving and easy to automate by on-line hyphenation with an analytical technique such as HPLC, HPCE, FIA and MS.

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