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Protein Binding Study of Isoflavone, Perillyl Alcohol and S-Ibuprofen by High-Performance Frontal Analysis

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Abstract–High-performance frontal analysis (HPFA) was used for a protein binding study of isoflavones (daidzein, genistin, and genistein), enantiomers of perillyl alcohol and S-ibuprofen to human serum albumin (HSA). The analyses were performed on a Develosil and Inertsil 100-Diol-5 column (10 cm×4.6 mm). Sodium phosphate solution (pH 7.4, ionic strength 0.17) was used as the mobile phase at a flow rate of 1 ml/min. To ensure the drug to be eluted as a trapezoidal peak with a plateau, injection volumes were each fixed up the zonal profile with an evident plateau appears. The unbound drug concentration was determined from a plateau height of the plateau region after that experimental data were fitted by Scatchard equation. The binding constants (K) and total binding affinities (nK) of drugs to HSA were calculated, respectively.

Key words: High-Performance Frontal Analysis, Isoflavones, S-Ibuprofen, Enantiomers of Perillyl Alcohol, Human Serum Albumin, Binding Parameter

INTRODUCTION

Plasma protein binding has a significant effect on the pharmacokinetic and pharmacodynamic properties of a drug. Plasma protein binding consists of variable and intricate binding equilibrium. Several plasma proteins, such as albumin and lipoproteins, possibly are simultaneously involved in the plasma protein binding of drugs, and the overall binding property in plasma is the sum of each protein binding [Shibukawa et al., 2002a]. Unbound drug concentrations show better correlation to the pharmacological activity than the sum drug concentration. Also, pharmacokinetic properties such as hepatic metabolism rate, renal excretion rate, biomembrane partition rate and steady state distribution volume are a function of unbound drug fraction (unbound/bound concentration ratio). In the case of racemic drugs, the binding affinity is potentially different between the enantiomers, which may cause the difference in pharmacokinetic properties. These effects further enhance the complexity in plasma protein binding property. Therefore, quantitative and enantioselective binding studies of individual proteins and drug-metabolite interaction are important in developing racemic drugs and their safe and reasonable use [Shibukawa et al., 2002b].

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 drug. To overcome this problem, high-performance frontal analysis (HPFA), a chromatographic method that allows simple and easy determination of unbound drug concentrations after direct sample injection [Qiao et al., 2002; Shibukawa et al., 1999a], has been reported. This method is free from the problems arising from using a membrane and the bound drug is transformed into the unbound form in the HPFA column, which improves the measurement of low levels of unbound drug, so it specially fits for the analysis of strongly bound drug [Shibukawa et al., 1995, 1999a].

Isoflavones including daidzein and genistein are found almost exclusively in soybeans. Soy isoflavones are known to exhibit various health-beneficial effects [Bahram and Brenda, 2002; Messina et al., 1994] including relief of menopausal symptoms and preventive effects in the development of cardiovascular diseases and hormone-dependent cancers [Kurie et al., 2003].

Perillyl alcohol (POH) has chemotherapeutic activity against chemically induced rat mammary tumors with little toxicity to the host, which inhibits the proliferation of cultured human colon carcinoma cells. Moreover, perillyl alcohol is not only a potent breast anti-cancer agent but also an effective chemotherapeutic agent against advanced mammary tumors [Jung and Row, 1998].

Ibuprofen [(±)-(*R*, *S*)-2-(4-isobutylphenyl)propionic acid] (IBU), is a chiral nonsteroidal anti-inflammatory drug common used for the treatment of several rheumatisms. Ibuprofen is extensively metabolized via the oxidation and glucuronidation routes showing selectivity for (+)-(*S*)-ibuprofen (s-ibuprofen) [Pierina et al., 2003].

It is useful to know the pharmacological behavior of each component involved in the mixture so that we can understand the therapeutic and pharmacological activities of our research drugs. Here we present a new work of protein binding study of isoflavones, enantiomers of perillyl alcohol and S-ibuprofen to HSA by high-performance frontal analysis. This work may provide useful information for the understanding of pharmacokinetics and clinical applications.

EXPERIMENTAL

1. Materials

The standard chemicals, human serum albumin (HSA, fatty acid free), daidzein, genistein, genistin, POH (S-POH, R-POH), s-ibu-

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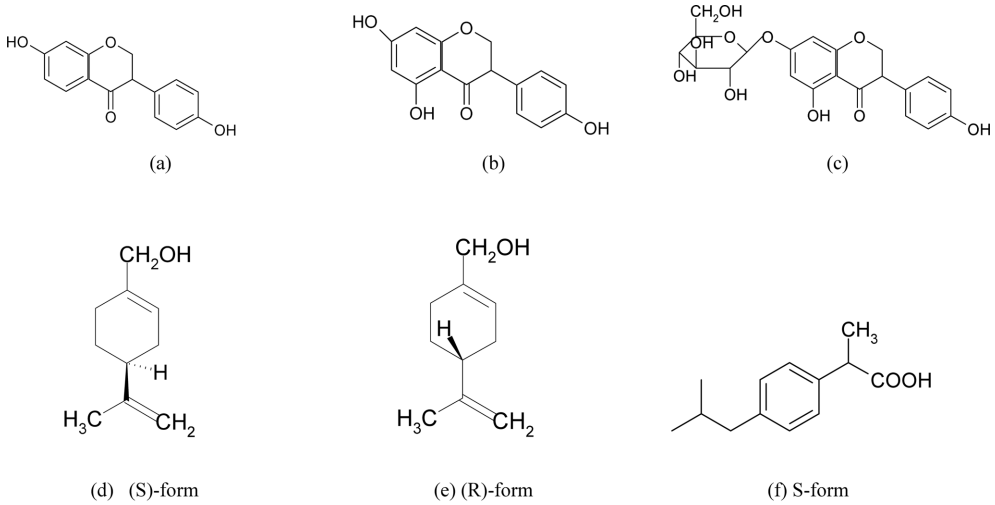

Fig. 1. Chemical structures of drug compounds.
(a) daidzein (b) genistein (c) genistin (d) S-POH (e) R-POH (f) S-IBU.

profen, 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, USA). The diol-silica columns, Develosil 100 Diol 5 (100×4.6 mm) and Inertsil Diol 5 (100×4.6 mm) were purchased from Phenomenex and GL Science Inc. (Japan). Water was twice distilled and filtered by using a decompressing pump (Division of Millipore, Waters) and filter (FH-0.45 μm).

2. Instruments

The instruments used in this study were as follows: M930 solvent delivery pump (Young Lin Co.), UV detector (M 720 Absorbance Detector, Young-In 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.).

3. 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% $Na_2HPO_4 \cdot 7H_2O$). By 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. Isoflavones (30 μM), POH (3 μM) and HSA solution (700 μM), and S-ibuprofen (120 μM) and HSA (140 μM) were 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.

RESULTS AND DISCUSSIONS

1. Selecting of Injection Volume

After incubation at 37 °C for 3 hours, the drug-HSA mixed solution was directly injected into the HPFA column. According to the principle of HPFA, the drug should be eluted as a zonal peak with a plateau after the non-retained HSA. Hence a restricted injection method called "injection-re-switching technique" was used. The procedure was as follows: the injection loop was loaded with a certain volume (it must be larger than actual injection volume) of the sample solution and connected with mobile phase for a certain period. Then the injector valve was re-switched, which resulted in a sample injection of desired volume, and the loop was detached from mobile phase flow. By this injection re-switching technique, the diffused portion of the sample in the injection loop was not introduced into column and the injection could be regarded as an ideal rectangular. 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 a plateau height of the plateau region [Pinkerton, 1991; Rosas et al., 1997].

Fig. 1 shows the chemical structures of daidzein, genistein, genistin, S-POH, R-POH, S-IBU, respectively. According to the theoretical background, the different injection volumes about each drug were investigated with the each sample solution. Fig. 2 shows the effect of injection volumes on the eluted profiles; it can be seen that a zonal profile with an obvious plateau appears when injection volumes are above (a) 700 μl, (b) 900 μl, (c) 700 μl, (d) 600 μl, (e) 200 μl. For all the cases, further increasing of the injection volume can only result in a longer plateau but the peak heights hardly change. The height of the plateau region corresponds to the unbound drug concentration in the sample solution. Based on this result, the injection volumes of these analyses were each fixed at selected injection volumes for further experiments.

2. Determination of Unbound Drugs

In HPFA, the release of bound drug from protein is seemingly suppressed, and finally an equilibrium zone is generated near the top of the column. In that zone, two different equilibrium states can be established simultaneously. One is the chromatographic partition equilibrium inside the micropores, and the other is drug-protein binding equilibrium in the interstices (outside the micropores). The drug concentration in the sluggish flow of mobile phase in the micropores is equal to the unbound drug concentration in the bulk mobile phase in the interstices. Therefore, the unbound drug concentration in the mobile phase is equal to that in the initial sample solution.

After sample injection, drug and protein are separated from each other, keeping the binding equilibrium. Finally, the unbound drug in this zone is eluted as a trapezoidal peak with a plateau region, being separated from protein. The drug concentration in the plateau region becomes equal to the unbound drug concentration in the initial sample solution, and the plateau height and the peak area correspond to the unbound drug concentration and total drug concentration, respectively [Shibukawa et al., 1999a, b].

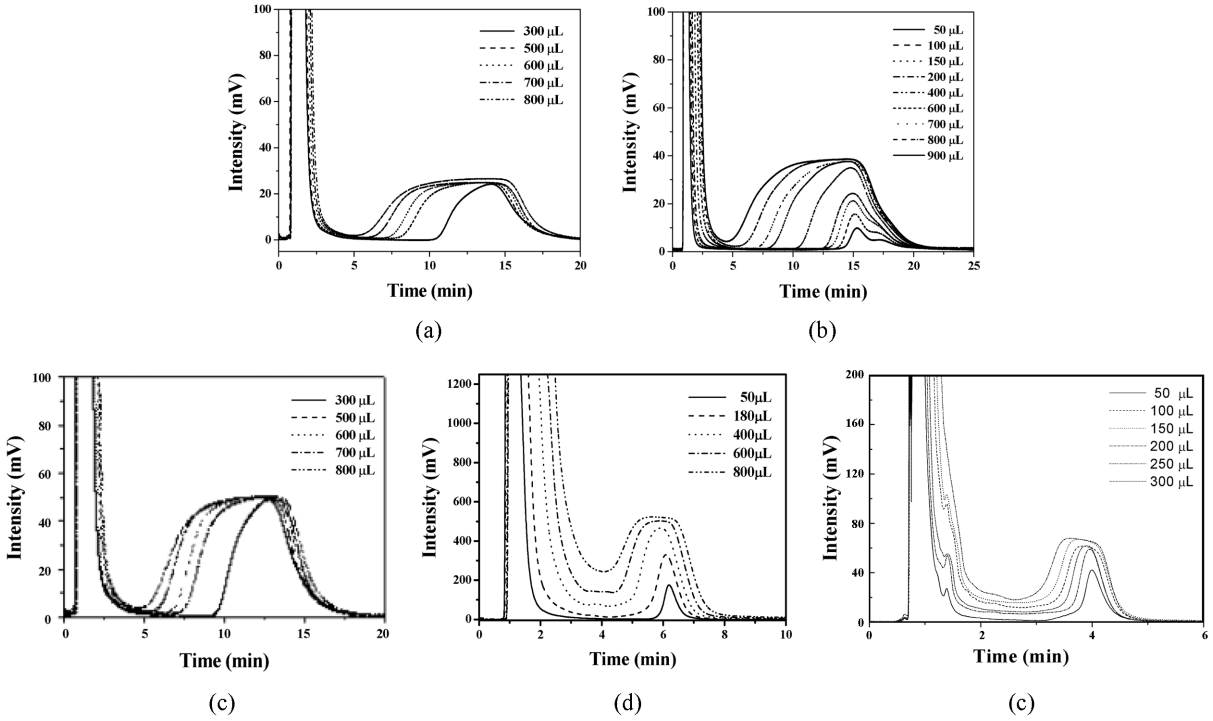

Fig. 2. The effect of injection volume on elution profile of drugs and 350 μM HSA mixed solution.
(a) daidzein (b) genistein (c) genistin (d) S-POH (e) S-IBU (70 μM HSA) (UV wavelength 260 nm (a-c), 205 nm (d) and 220 nm (e))

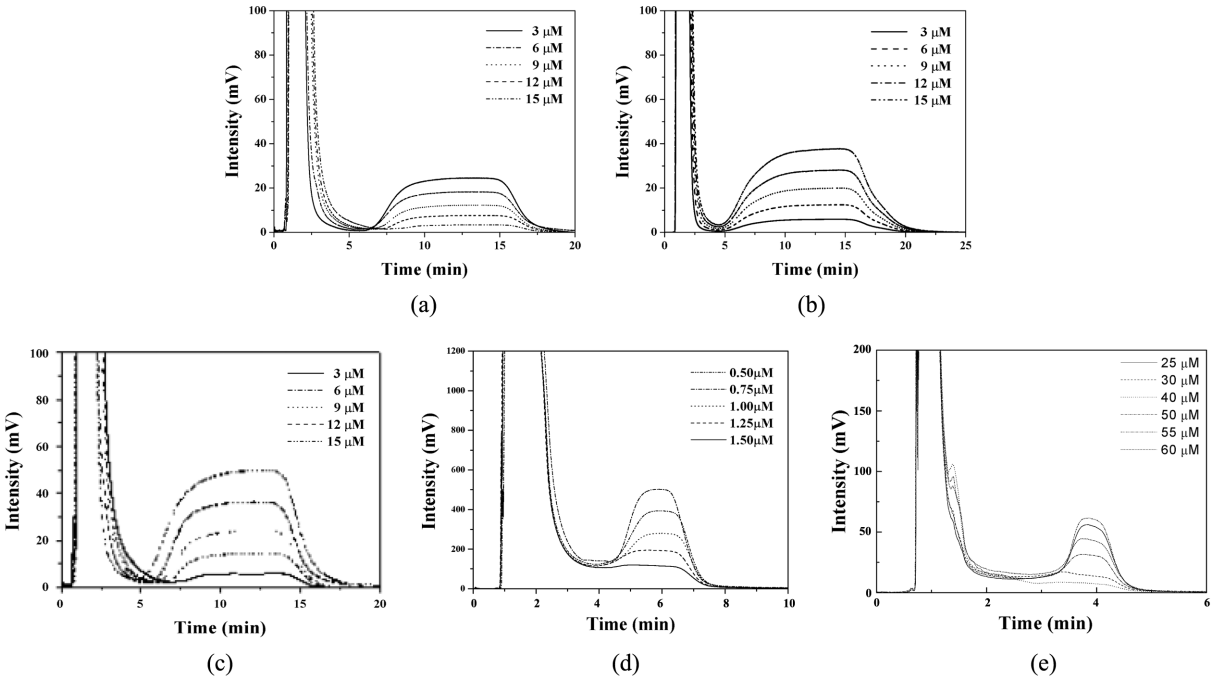

Fig. 3. Chromatograms of drugs with different concentrations in 350 μM HSA by HPFA.
(a) daidzein (Inj. Vol. 700 μl) (b) genistein (Inj. Vol. 900 μl) (c) genistin (Inj. Vol. 700 μl) (d) S-POH (Inj. Vol. 600 μl) (e) S-IBU (70 μM HAS, Inj. Vol. 200 μl) (UV wavelength 260 nm (a-c), 205 nm (d) and 220 nm (e))

Different concentrations of drugs changing (daidein, genistein and genistin; 3.0-15 μM at 350 μM HSA, POH; 0.5-1.5 μM at 350 μM HSA, S-ibuprofen; 25-60 μM at 70 μM HSA) were injected to the HPFA system. From the heights of the peak plateaus, the unbound drugs could be determined, as one see in Fig. 3. Standard drug samples in the absence of HSA were directly injected into the column. By plotting of peak height vs. concentrations, the regression equation of the calibration curve was y=0.2612x and correlation coefficient (r^2) was obtained 0.9992 for daidzein, y=0.2622x (0.9992, genistein), y=0.1948x (0.9997, genistin), $y=-201.51x^2+910.6x+29.72$ (0.9993, S-POH), $y=-206.12x^2+946.12x+31.87$ (0.9987, R-POH) and y=0.2202x+4.3474 (0.9941, s-ibuprofen), respectively. The determined unbound drugs for all the drugs are listed in Table 1. It can be seen from Table 1 that in the equilibriums of HSA and isoflavones the unbound drugs were about 30-65% in the investigated concentration range, POH were 20-40%, S-ibuprofen was 20-30%, which contributes to the parts that can easily transfer from blood into the target organ to extend the pharmaceutical activity for isoflavones. Although it is the same as non-glycoside, the performance was differentiating daidzein (30-42%) from genistein (50-65%) according to the number of carbons. As the differences in the structures of the drugs lie in C5 and C7, the polarity and hydrophobicity may change accordingly, which may be the reason that affects the binding rates of the drugs.

Table 1. Determination of unbound drugs of drugs by HPFA

Sample	Concentration [μM]	Unbinding drug [μM]	Binding drug [mM]
Daidzein	3	0.889±0.027	2.111
	6	2.987±0.060	4.013
	9	3.178±0.095	5.802
	12	4.767±0.143	7.233
	15	6.398±0.192	8.602
Genistein	3	1.551±0.042	1.449
	6	3.261±0.098	2.739
	9	5.224±0.157	3.776
	12	7.350±0.201	4.650
	15	9.859±0.296	5.141
Genistin	3	1.063±0.038	1.937
	6	2.774±0.088	3.226
	9	4.627±0.134	4.373
	12	7.053±0.194	4.947
	15	9.654±0.288	5.346
S-POH	0.50	0.097±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
S-Ibuprofen	25	6.319	18.681
	30	7.877	22.123
	40	11.076	28.924
	50	14.471	35.529
	55	16.456	38.544
	60	18.199	41.800

3. Estimation of Binding Parameters

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

$$r/C_u = nK - Kr \quad (1)$$

where r and C_u express the number of moles of bound drug per mole of HAS and the unbound drug concentration, respectively. Also, K and n represent the binding parameter constant and the number of binding sites on one HSA molecule. Since K and n values could not be estimated individually, the total binding affinity (nK) was calculated according to Eq. (2).

$$nK = C_b/(C_p C_u) \quad (2)$$

where C_b, C_u and C_p represent bound drug concentration, unbound drug concentration and HSA concentration, respectively. Especially,

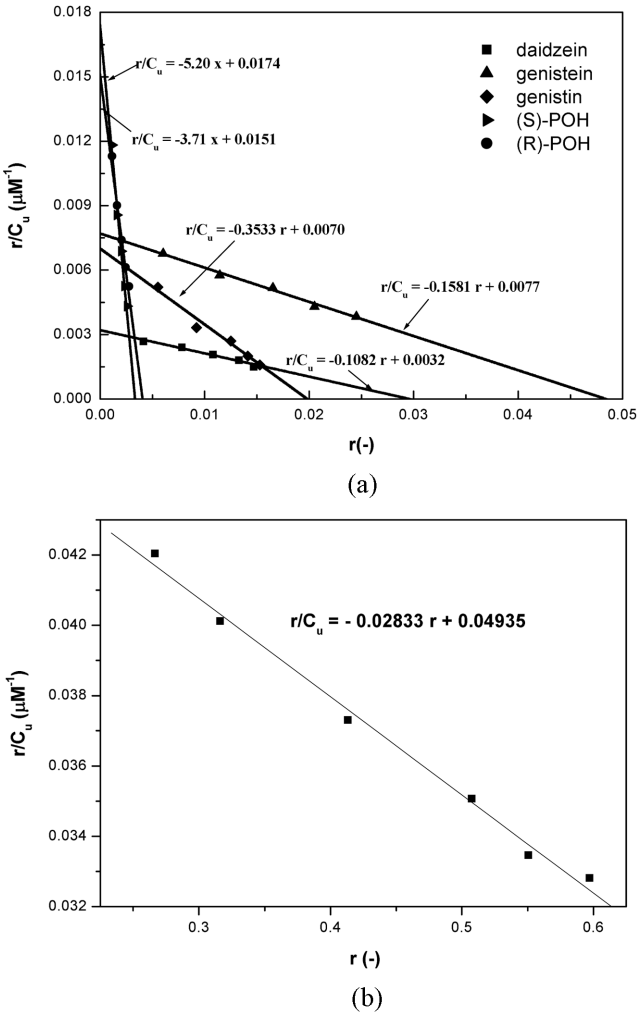

Fig. 4. The Scatchard plots of drug-HSA binding. The correlation coefficients of the lines were 0.993, 0.971, 0.974, 0.985, 0.967 (a) and 0.996 (b) for daidzein, genistein, genistin, S-POH, R-POH (a) and S-IBU (b).

Table 2. Total binding affinities (nK), binding parameter constants (K) and correlation coefficients (r^2) of drugs

Sample	nK [M^{-1}]	K [M^{-1}]	r^2
Daidzein	0.771	1.581	0.993
Genistein	0.321	1.082	0.971
Genistin	0.701	3.533	0.974
S-POH	1.741	5.201	0.985
R-POH	1.511	3.711	0.967
S-Ibuprofen	4.935×10^4	2.833×10^4	0.996

rate of bound drug concentration and HSA concentration indicate r, the bound drug amount per one protein molecule.

Fig. 4 illustrates the Scatchard plots of sample drugs. The correlation coefficients of the lines are organized in Table 2. They indicate good agreement of the experimental data to the theoretical equation. From the slope and intercept of the Scatchard plots, the calculated binding parameters of sample drugs. As one can see, the total binding affinities and binding parameter constants are arranged in Table 2. The total binding affinities (nK) of daidzein and genistin are nearly the same, while that of genistein is two times lower. POH are two or three times larger for nK. The binding constant of S-POH shows the higher value than isoflavones, while that of genistein is the lowest. The value of S-Ibuprofen is the largest.

CONCLUSIONS

High-performance frontal analysis has been successfully applied for a protein binding study of drugs to human serum albumin. This simple and precise method has been used for a quantitative binding determination of low concentration of drugs with HSA. From the experimental results, the binding constants (K) and total binding affinities (nK) of the six sample drugs show some difference according to their molecular structures but belong to the same magnitude grade. This work may provide useful information for clinical application of this drug.

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