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SHORT COMMUNICATION

Protein binding study of catechin hydrate and genistein by high-performance frontal analysis

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Abstract−High-performance frontal analysis (HPFA) was used for the protein binding study of catechin hydrate and genistein to human serum albumin (HSA). The experiment was performed on a Develosil 100Diol-5 column, and sodium phosphate buffer (pH 7.4 and ionic strength of 0.17) was used as the mobile phase. The mixtures of the drug-HSA solution were directly injected into the HPFA column, the HSA was eluted first and the unbound drugs were eluted out as a trapezoidal peak with a plateau region. The unbound drug concentration was determined from a plateau height of the plateau region and the experimental data were fitted by Scatchard equation. The binding constants (K) and binding affinities (nK) of the drug to HAS were $K=1.32\times10^4$ (L mol^{-1}), $nK=0.47\times10^4$ (L mol^{-1}) for catechin hydrate, and $K=5.17\times10^4$ (L mol^{-1}), $nK=2.14\times10^4$ (L mol^{-1}) for genistein.

Key words: High-performance Frontal Analysis, Catechin Hydrate, Genistein, Human Serum Albumin, Binding Parameters

INTRODUCTION

When a drug is administered, it enters the blood stream and is bound to plasma proteins such as albumin and α-acid glycoprotein. Protein binding is a reversible and kinetically rapid process, and the concentrations of unbound drug are in an equilibrium state [1]. Unbound drug concentrations show better correlation to the pharmacological activity than the sum drug concentration [2,3].

Several methods have been developed for determining the concentration of unbound drug, such as equilibrium dialysis [4], ultrafiltration [5], ultracentrifugation [6], and gel filtration [7]. However, these methods have potential problems such as the adsorption of drug onto membranes and the leakage of bound drug through membranes [8,9]. To overcome these problems, high-performance frontal analysis (HPFA), a chromatographic method which allows simple and easy determination of unbound drug concentrations after direct sample injection has been reported [10,11]. 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 [12,13].

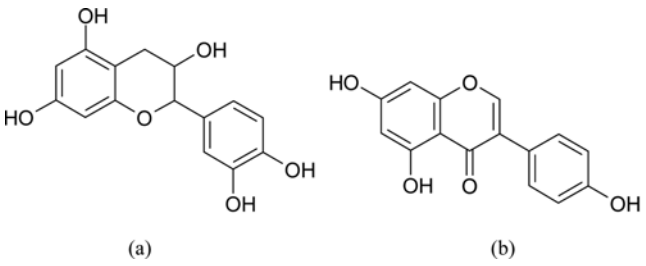

Fig. 1. Molecular structures of Catechin hydrate and Genistein.

Catechin hydrate (Fig. 1(a)), a strong antioxidant that scavenges radicals [14], is phenolic compound extracted from plants and present in natural food and drinks, such as green tea or red wine. Catechin also shows a protective effect against cerebral ischemic damage [15,16].

Genistein (Fig. 1(b)) is a natural isoflavone compound found in soy products [17]. It is associated with a broad variety of beneficial properties on human health. After consumption, primary isoflavonoids are metabolized in the gut and transformed into active aglycones, some of which are absorbed as free isoflavones [18,19].

In this paper we present a new work of protein binding study of catechin hydrate and genistein to HSA by high-performance frontal analysis. The unbound drug concentration was determined from a plateau height of the plateau region and the experimental data were fitted by Scatchard equation. This work may provide useful information for the understanding of pharmacokinetics and clinical applications.

EXPERIMENT

1. Materials

Human serum albumin (HSA), catechin hydrate, genistein, were purchased from Sigma (St. Louis, MO, USA), sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$), were purchased from Duksan (Korea). The Develosil 100 Diol 5 column (100 mm×4.6 mm) was purchased from GL Science Inc. (Japan). Water was twice distilled and filtered (FH-0.45 μm, Advantec MFS, Inc., Japan) by using a decompressing pump (Division of Millipore, Waters).

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 Rheodyne injection valve with a 5 ml sample loop, and integrated data system (Autochrowin.

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Ver. 1.42, Young Lin Co.).

3. Preparation of Sample Solutions

First, $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ were dissolved in water to make solutions of 66.7 mM, respectively. Then the two solutions were mixed together (19% $NaH_2PO_4 \cdot 2H_2O$ and 81% $Na_2HPO_4 \cdot 12H_2O$). By this way, a phosphate solution of pH=7.4, ionic strength 0.17 was made and it was used as the mobile phase in HPFA analysis. Catechin hydrate (65 μM) and genistein (74 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 the Injection Volume

After being kept at 37 °C for 3 hours, the drug-HSA mixed solution was directly injected into the HPFA column. According to the principle of HPFA [8], 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 [20]. Fig. 2(a) shows the elution profiles of 40 μM HSA with 32.5 μM catechin hydrate mixed solution with different injection volume. And Fig. 2(b) shows the elution profiles of 40 μM HSA with 8 μM genistein.

It can be seen that the injection volumes above 800 μL to catechin hydrate and 1 mL to genistein result in trapezoidal peak with a plateau region. The height of the plateau region corresponds to the unbound drug concentration in the sample solution. Further increasing of the injection volume can only result in a longer plateau but the peak heights hardly change. Based on these results, the best injection volumes were fixed at 2.4 mL and 2.0 mL for further experiments.

2. Determination of Unbound Drugs

Different concentrations of the drugs mixed with 40 μM HSA solution were injected to the HPFA system. From the heights of the peak plateaus, the unbound drugs could be determined (Fig. 3).

By plotting of peak height vs. concentration, the regression equation of the calibration curve was y=1.831xg−2.565 and correlation

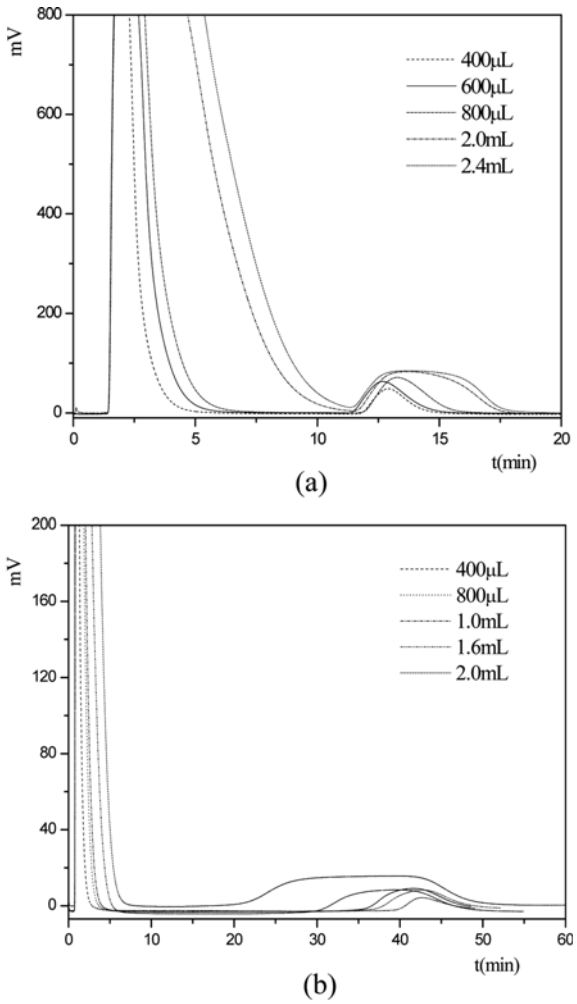

Fig. 2. The effect of injection volume on elution profile of drug and 40 μM HSA mixed solution.
(a) Catechin hydrate (32.5 μM) (b) Genistein (8 μM) (UV wavelength 280 nm (a), 260 nm (b)).

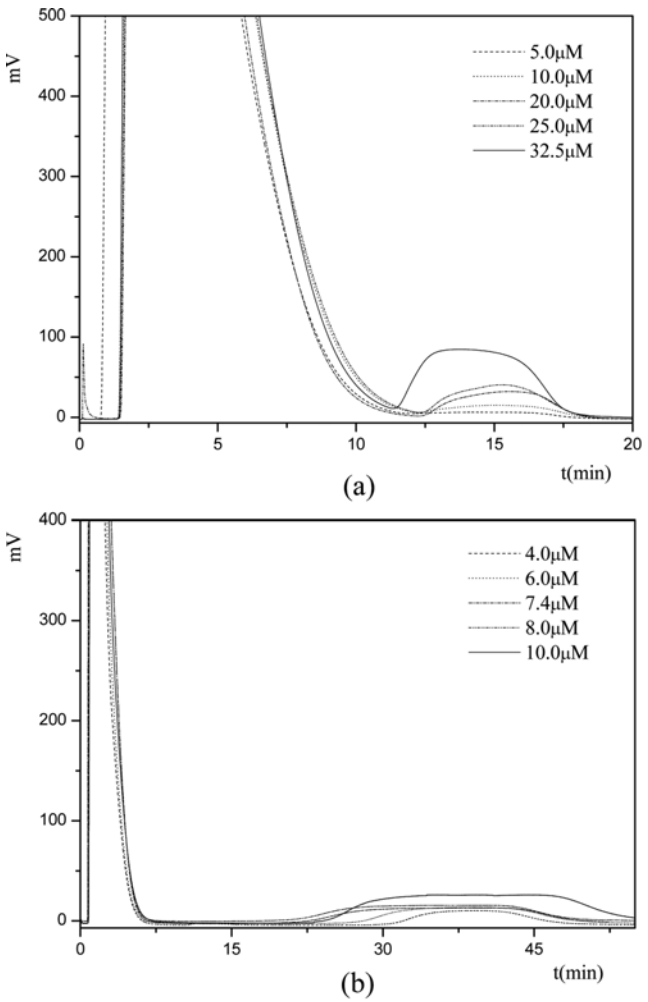

Fig. 3. Chromatograms of drugs with different concentrations in 40 μM HSA by HPFA.
(a) Catechin hydrate (2.4 mL) (b) Genistein (2 mL) (UV wavelength 280 nm (a), 260 nm (b)).

coefficient (r^2) was 0.997 for catechin hydrate, y=2.826x−1.439 and r^2 was 0.988 for genistein. The results of unbound drugs concentration are listed in Table 1. It can be seen form Table 1 that in the equilibriums of HSA and drugs, the unbound drugs of catechin hydrate were 75%-93% in total drug concentration, genistein were 56%-61%.

3. Estimation of Binding Parameters

Table 1. Determination of unbound drugs by HPFA

Sample	Total concentration [μM]	Unbound drug [μM]	Bound drug [μM]
Catechin hydrate	5.0	3.755	1.245
	10.0	8.117	1.883
	20.0	17.955	2.045
	25.0	22.879	2.121
	32.5	30.302	2.198
Genistein	4.0	2.247	1.753
	6.0	3.535	2.465
	7.4	4.395	3.005
	8.0	4.786	3.214
	10.0	6.109	3.891

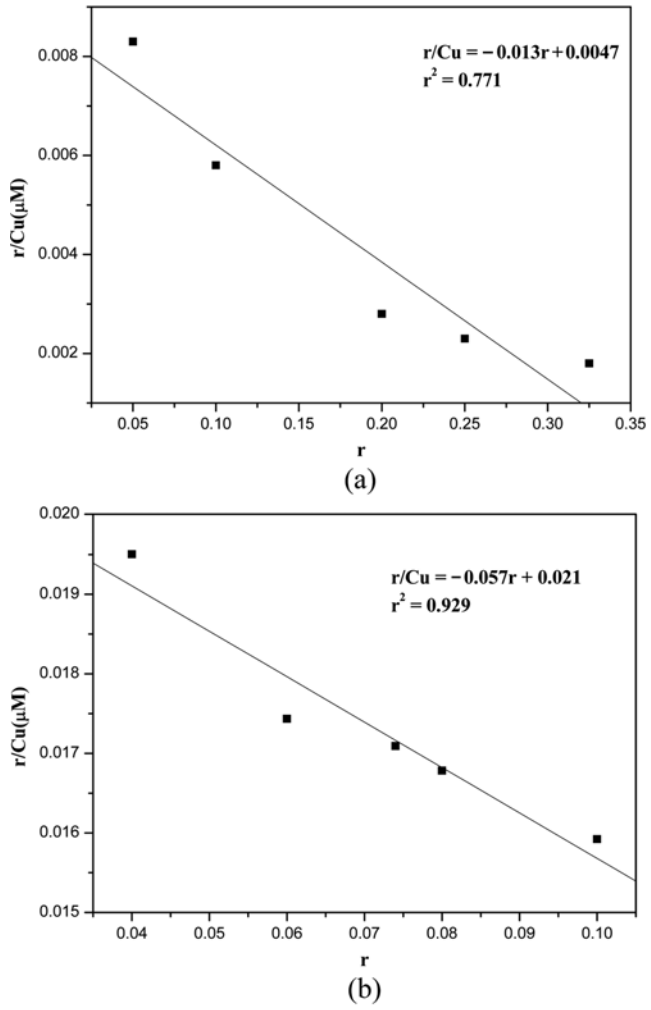

Fig. 4. The Scatchard plot for drug-HSA binding.

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

Sample	nK [M^{-1}]	K [M^{-1}]	r^2
Catechin hydrate	0.47×10^4	1.32×10^4	0.771
Genistein	2.14×10^4	5.17×10^4	0.929

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

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

Where r, Cu, 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.

Fig. 4 illustrates the Scatchard plots of drugs. From the slope and intercept of the Scatchard plots, the calculated binding parameters of catechin hydrate are arranged in Table 2.

CONCLUSIONS

HPFA method has been successfully applied for the protein binding of drug to human serum albumin. From the peak height of the zonal peak, the unbound drug concentration was calculated. By Scatchard analysis, we obtained the binding constant (K) and binding affinity (nK), $K=1.32\times10^4$ (L mol^{-1}), $nK=0.47\times10^4$ (L mol^{-1}) for catechin hydrate, and $K=5.17\times10^4$ (L mol^{-1}), $nK=2.14\times10^4$ (L mol^{-1}) for genistein. This method is sample, precise and may provide useful information for clinical application of these drugs.

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