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A novel lophine-based fluorescence probe and its binding to human serum albumin

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Abbreviations: DAPIM, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1H-imidazol-2-yl]benzoic acid methyl ester; HSA, human serum albumin; BCG, bromocresol green;
Abstract

The binding of a lophine-based fluorescence probe, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1H-imidazol-2-yl]benzoic acid methyl ester (DAPIM) with human serum albumin (HSA) was investigated by fluorescence spectroscopy under physiological conditions. While DAPIM shows extreme low fluorescence in aqueous solution, DAPIM binding with HSA emits strong fluorescence at 510 nm. The binding constant and binding number determined by Scatchard plot was $3.65 \times 10^6$ M$^{-1}$ and 1.07, respectively. Competitive binding between DAPIM and other ligands such as warfarin, valproic acid, diazepam and oleic acid, were also studied fluorometrically. The results indicated that the primary binding site of DAPIM to HSA is site II at subdomain IIIA. DAPIM can be a useful fluorescence probe for the characterization of drug-binding sites. In addition to the interaction study, because the fluorescence intensity of DAPIM increased in proportion to HSA concentration, its potential in HSA assay for serum sample was also evaluated.

Keywords: 4-[4-(4-dimethylaminophenyl)-5-phenyl-1H-imidazol-2-yl]benzoic acid methyl ester; human serum albumin; binding constant; binding site
**Introduction**

Human serum albumin (HSA) is the most abundant protein constituent of blood and serves as a protein storage component. Its principal function is to bind and transport a wide variety of bioactive molecules such as fatty acids, hormones, vitamins and numerous pharmaceuticals [1, 2].

The main regions of HSA for ligand binding are located in hydrophobic cavities in the subdomains IIA (site I) and IIIA (site II) [3-5]. The binding affinity of a drug to HSA affects the distribution, pharmacokinetics, toxicity and rate of excretion of the drug [4]. Therefore, information on the binding affinity of a drug and biomolecule to HSA is particularly useful for solving *in vivo* pharmaceutical problems [6].

The spectroscopic techniques are of great help in the study of interactions between small molecules and HSA. Among them, fluorescence spectroscopy can provide an important information for the structure and the microenvironment based on the characteristics of emission, fluorescence polarization and energy transfer. A variety of fluorescent probes have been used for studies of the characteristics of ligand binding and binding sites of HSA, competitive binding of other ligands, and the spatial relationship between Trp-214 and the probe-binding [7-15].

On the other hand, there is a connection between the content of HSA in urine or blood and some diseases, such as nephropathy, so the determination of HSA is very important in clinical diagnosis.

Many methods have been described for the determination of HSA. Various compounds such as bromocresol green (BCG) and bromocresol purple (BCP) have been reported as analytical reagents based on changes in their colors by binding to HSA [16-18]. BCG and BCP methods are most widely used for HSA assay in clinical laboratory [19, 20].

Until now, we reported several analytical methods for biologically important compounds by
employing the fluorescence and chemiluminescence properties of lophine derivatives mainly as a labeling reagent [21-27]. During these studies, we discovered that a lophine derivative, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1H-imidazol-2-yl]benzoic acid methyl ester (DAPIM, Fig. 1) has almost no fluorescence, but the fluorescence intensity of DAPIM solution was drastically enhanced by the addition of HSA in aqueous solvent [21], which may be a result of the interaction between DAPIM and HSA. In this paper, in order to systematically explore the binding mechanism of DAPIM with HSA, the binding characteristics were discussed by determining the binding constant and binding sites under physiological conditions. Also, we evaluated its potential as a fluorescence probe for determination of HSA in human serum.

Materials and methods

Reagents and apparatus

HSA and warfarin were purchased from Sigma (St. Louise, MO, USA). Diazepam, valproic acid, BCG and oleic acid were obtained from Wako (Osaka, Japan). DAPIM was synthesized according to our previous report [21]. Fluorescence was measured with a Shimadzu RF-1500 spectrofluorometer (Kyoto, Japan).

General procedure

Under the optimum experimental conditions, 20 µL of HSA solution and 3.0 mL of 2 mM DAPIM in pH 7.4 phosphate buffered saline (PBS, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl) containing 0.025% sodium dodecyl sulfate (SDS) were mixed, then were incubated at room temperature for 20 min. The fluorescence intensity was measured with the
following settings of spectrofluorometer: excitation wavelength ($\lambda_{ex}$), 370 nm; excitation slit, 10 nm; emission wavelength ($\lambda_{em}$), 510 nm; emission slit, 10 nm.

Circular dichroism measurement

The alterations in the secondary structure of the HSA in the presence of DAPIM with different concentrations were studied by monitoring circular dichroism (CD) spectra on a Jasco J-725 CD spectrophotometer using a rectangular quartz cuvette of path length 2 mm at 0.2 nm data pitch intervals. All CD spectra were taken in a wavelength from 200 to 240 nm. The spectrophotometer was sufficiently purged with 99.9% nitrogen before starting the measurement. The spectra were collected at a scan speed of 200 nm/min and a response time of 1 s. The final plot was taken as an average of three accumulated plots.

Serum samples

Sera from healthy donors (n=10; 21-30 years; 5 female) were collected in our laboratory. The whole blood was collected by tubes containing coagulation accelerator. After removing the clot by centrifuging 1300 g for 10 min at 4°C, the resulting supernatant (serum) was stored at -80°C. The samples were diluted 10-fold with PBS (pH 7.4) and the diluents were used for HSA determination. According to the procedures presented in previous literatures [20], 5.0 mL of BCG (150 µM) in citrate buffer (pH 4.0) was added to 25 µL of serum and then, stand at room temperature for 10 min. An UV absorbance at 628 nm was measured by Shimadzu UV-265FS. All the experiments were performed with approval from the institutional ethics committee of the Graduate School of Biomedical Sciences, Nagasaki University.
**HPLC system and conditions**

The HPLC system consisted of two LC-6A liquid chromatographic pumps (Shimadzu, Kyoto), a F1000 fluorescence detector (Hitachi, Tokyo), a 7125 injector with a 5-µL loop (Rheodyne, Cotati, CA, USA), and a R-02A recorder (Rikadenki, Tokyo). Chromatographic separation was performed on a Asahipak GS-520 7E (250 x 4.6 mm, i.d., Shodex, Tokyo) with a mobile phase of PBS at a flow rate of 0.5 mL/min. The column eluent was mixed with 5 µM DAPIM in PBS as a post-column reagent at a flow rate of 0.5 mL/min, and the fluorescence was monitored at 510 nm with excitation at 370 nm.

**Results and discussion**

Fluorescence spectra characteristics of DAPIM binding to HSA

To investigate the fluorescence change upon binding of DAPIM to HSA, fluorescence titration was carried out in HSA solution with 0.2-1.0 g/dL. Excitation and emission spectra of DAPIM in blank and HSA solution are given in Fig. 2. The enhancement of the fluorescence of DAPIM was in proportion to the concentration of HSA. It was reported that the fluorescence intensity was 230 times higher in n-hexane than that in methanol [21]; therefore, DAPIM may bind to hydrophobic cavities in HSA and exhibit a remarkable fluorescence.

Binding constant and binding site number

In order to study the interaction of small molecules with macromolecules, the Scatchard plot is commonly used to characterize the binding properties such as binding constant and number of
binding sites [28]. From the recorded fluorescence titration data, the binding constant and binding number of DAPIM with HSA were determined as $3.65 \times 10^6 \text{M}^{-1}$ and 1.07, respectively (Fig. 3). The binding number indicates that DAPIM-HSA complex may have one binding site.

Identification of the binding site

To obtain the information about the binding site of DAPIM in HSA, competitive binding between DAPIM and other ligands (i.e., warfarin, valproic acid, diazepam and oleic acid) was studied. Warfarin, valproic acid, diazepam, and oleic acid are reported to bind to HSA at site I, site I and II, site II, and site II, respectively [29-32]. The fluorescence of DAPIM (0.05-5 µM) plus HSA (75.8 µM) was measured in the presence and the absence of other ligands (0.5 µM). As illustrated in Fig. 4a, DAPIM was moderately displaced by diazepam, whereas the fluorescence of DAPIM bound to HSA was not affected by the other ligands. Therefore, the site II at subdomain IIIA is the specific binding site for DAPIM. Furthermore, the displacement by diazepam was enhanced according to an increase in its concentration (Fig. 4b). As mentioned above, the main regions of HSA for ligand binding are located in subdomains II A (site I) and IIIA (site II); that is, DAPIM can be used as a fluorescent probe for many studies of drug-binding sites on HSA.

Effect of DAPIM concentration, temperature and incubation time on the HSA assay

In order to develop HSA assay using DAPIM, several measurement conditions were optimized. The effect of the concentration of DAPIM on fluorescence intensity was investigated (Fig. 5). At more than 1.5 µM, the maximum fluorescence intensity was obtained and 2.0 µM was chosen for further study. Because the DAPIM-HSA complex, which provides fluorescence, might become
more unstable as temperature increased, the effect of temperature on fluorescence intensity was also studied under 298, 305, 310 and 316 K (data not shown). As expected, temperature had a great influence on the fluorescence intensity. The fluorescence intensity gradually decreased with increasing temperature; thus, we selected room temperature (298 K). The incubation time of more than 20 min provided the maximum and stable fluorescence intensity.

Studies on CD spectra of HSA in the presence of DAPIM

As shown in Fig. 6, the CD spectrum of HSA exhibits two negative peaks at 208 nm and 222 nm which are contributed from \( n \rightarrow \pi^* \) transition of the peptide inter linkage of \( \alpha \)-helix [33]. The results of CD studies indicated that with the addition of DAPIM, the intensities of both the peaks slightly increased. This indicates that certain conformational changes of HSA were occurred by the addition of DAPIM. In addition, no change in band shape and induction of a new peak suggest that DAPIM leads to conformation structural changes but no conformational transition of HSA.

Analytical characteristics

Calibration curves for determination of HSA were linear over the concentration of 0.1-0.8 g/dL (\( r=0.999 \)) and the corresponding regression equation was \( Y=36.7X+2.2 \), where \( Y \) is the fluorescence intensity of DAPIM-HSA and \( X \) is the HSA concentration. The correlation efficient \( (r) \) was greater than 0.999. The assay parameters consisting of calibration range, slope (36.7), intercept (2.2) and the limit of detection (0.0067 g/dL, defined as the concentration corresponding to three times of the standard deviation of the background signal) were obtained. This method exhibited good repeatability with a relative standard deviation of 1.7% obtained from six separate
determinations for 0.2 g/dL HSA.

Determination of HSA in human serum

The proposed method was employed to determine HSA in human serum. Sera obtained from healthy volunteers (n=10) were tested without pre-treatment except dilution (10-fold) by PBS. The analytical results ranged from 6.10 to 7.4 g/dL (mean ± standard deviation = 6.6 ± 0.4 g/dL). Although there have been a lot of criticism to BCG method, BCG method is still a standard method for HSA assay and is most widely used in clinical settings [19, 20, 34, 35]. Therefore, it was employed as a reference method in present study. As shown in Fig. 7a, comparison of the proposed method with BCG assay is performed using a nonparametric Passing-Bablok analysis [36, 37]. The 95% confidence interval (CI) was calculated for the slope and intercept by nonparametric Bootstrap. The Blank-Altman approach [38-41] was used as an alternative to correlation and regression models for further assessing the difference between both methods by plotting the relative difference between the two assays versus the determined mean concentration (Fig. 7b).

Regression analysis of the data yielded the following equations: proposed method = 1.05 (BCG) + 0.62 [g/dL] (95% CI for slope, 0.69-1.48; 95% CI for intercept, -2.1-2.5). In the usual linear regression model, the line of best-fit equation is calculated by minimizing the $y$-squared residuals. This approach assumes that there is no error on the $x$ variable and that the $y$ variable has a constant analytical precision. Contrastingly, nonparametric procedures including the Passing-Bablok regression are based on the rank principle [36, 37]. This approach assumes an error on both $x$ and $y$ variables, a constant ratio of the variances and no special assumptions regarding the distribution of
the values. Underestimation of the concentrations with BCG was described by some groups [42, 43]. These observations were confirmed in the present study with a mean underestimation of 0.86 g/dL.

HPLC analysis of HSA by post-column reaction with DAPIM

HSA eluted from the gel-filtration column was mixed with DAPIM, and the generated fluorescence was monitored. Fig. 8a shows a typical chromatogram of standard HSA solution, and the peak of HSA was detected at 20 min on the chromatogram. Also, as shown in Fig. 8b, the peak of HSA in serum could be clearly detected without any interference from other biological components. This result also demonstrates the excellent selectivity of DAPIM for HSA detection. Therefore, the application of DAPIM in HPLC analysis should be useful to investigate HSA analogues in complicated matrices.

Conclusions

In this paper, the interaction between DAPIM and HSA has been investigated by utilizing fluorescence spectroscopy. The binding constant to HSA is 3.65 x 10^6 M^{-1} and the primary binding site on HSA is site II at subdomain IIIA. With its site specificity to HSA, DAPIM will be useful as fluorescence probes to elucidate the interaction between HSA and other molecules including drugs. Based on the phenomenon that the fluorescence intensity of DAPIM was enhanced in proportion to the concentration of HSA, a novel fluorescence assay of HSA can be developed although further optimization will be needed.
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References


**Figure captions**

**Fig. 1** Structure of 4-[4-(4-dimethylaminophenyl)-5-phenyl-1H-imidazol-2-yl]benzoic acid methyl ester (DAPIM).

**Fig. 2** Excitation and emission spectra of DAPIM in the presence of various concentration of HSA (0-1.0 g/dL). The concentration of DAPIM is 5 µM.

**Fig. 3** Scatchard plot of DAPIM binding to HSA.

**Fig. 4** Effect of site marker probe on the fluorescence of DAPIM (0.05-5 µM). a) several marker probes. b) using diazepam with different concentrations.

**Fig. 5** Effect of DAPIM concentration on the relative fluorescence intensity. The concentration of HSA is 0.5 g/dL.

**Fig. 6** CD spectra of HSA in the presence of DAPIM. Conditions: HSA, 0.3 g/dL; DAPIM, (a) 0, (b) 2.5 µM, (c) 5 µM.

**Fig. 7** a) comparison of HSA results obtained by BCG method and our proposed method by Passing-Bablok regression. b) Bland-Altman plot for the comparison of BCG method versus our proposed method. The mean value (n=10) of the two method is plotted against the difference the
two values (our proposed method-BCG method). The mean difference between the two methods was 0.86 g/dL. The mean difference and the mean ± 2 SD difference were shown by solid line and dashed lines, respectively.

Fig. 8 Chromatograms for a) standard solution of 0.5 g/dL HSA and b) human serum obtained by the proposed HPLC system. The human serum was diluted 20 times with PBS before injection.

The human serum was diluted 20 times with PBS before injection.
Fig. 1
Fig. 2

Excitation spectra

Emission spectra

HSA, g/dL

$\lambda_{em} = 510 \text{ nm}$

$\lambda_{ex} = 370 \text{ nm}$

Wavelength, nm
Fig. 3

\[ y = -3.65 \times 10^6 x + 3.91 \times 10^6 \]

\[ r = 0.983 \]
Fig. 4

(a) Graph showing relative fluorescence intensity against DAPIM (µM) for various ligands: diazepam, valproic acid, warfarin, no ligand, oleic acid.
Fig. 4

b)

Without diazepam
0.5 μM diazepam
2.5 μM diazepam

Relative fluorescence intensity vs. DAPIM, μM
Fig. 6
Fig. 7

(a)

(b)
Fig. 8

(a) HSA

Relative fluorescence intensity

Retention time, min

(b) HSA

Relative fluorescence intensity

Retention time, min
**Highlights**

1. Lophine-based probe (DAMIP) shows strong fluorescence when it binds with albumin.
2. The binding constant of DAPIM to albumin is $3.65 \times 10^6$ M$^{-1}$.
3. The primary binding site of DAPIM to HSA is site II at subdomain IIIA.
4. DAPIM was successfully applied to the determination of albumin in human serum.