Full Length Research Paper

Determination of the ratio between mercaptalbumin and nonmercaptalbumin by HPLC with fluorescence probe specifically binding to albumin

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Abstract

A simple and selective HPLC-fluorescence (FL) method with FL probe, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1*H*-imidazol-2-yl]benzoic acid methyl ester (DAPIM), for simultaneous determination of mercaptalbumin (HMA) and nonmercaptalbumin (HNA1) was developed. After HMA and HNA1 were separated on an ion-exchange column, they were on-line and post-column mixed with DAPIM. The DAPIM-albumin complex produces FL (λex 370 nm and λem 510 nm); however, DAPIM solution never gives the FL. Based on this mechanism, selective determination of HMA and HNA1 were achieved without any pretreatment and interfering peak. The proposed method was applied to the measurement of HMA and HNA1 in human serum of healthy volunteers and diabetes mellitus patients.

Keywords: 4-[4-(4-dimethylaminophenyl)-5-phenyl-1*H*-imidazol-2-yl]benzoic acid methyl ester; fluorescence probe; mercaptalbumin; nonmercaptalbumin; redox state

Introduction

Human serum albumin (HSA) is composed of mercaptalbumin (HMA) with a free thiol group on Cys-34, nonmercaptalbumin 1 (HNA1) with Cys-34 bound to cystein or glutathione by a disulfide bond, and nonmercaptalbumin 2 (very small amount) with Cys-34 oxidized to sulfenic, sulfinic or sulfonic [1-4]. HMA is the major extracellular source of reduced sulfhydryl groups, which are potent scavengers of reactive oxygen and nitrogen species [8]. It is known that HMA is reversibly converted to HNA1 accompanied with increasing of oxidative stress by reactive oxygen and nitrogen species. Oxidative stress is believed to play a major role in the pathogenesis of several diseases [5-7]. Therefore, in order to observe the redox state of circulatory system, HMA and HNA were extensively determined in serum or other biological fluids of the elderly and the patients with renal and liver dysfunctions, endocrine diseases, eye diseases [1, 9-16].

Several HPLC methods with ultraviolet (UV) or fluorescence (FL) detection were developed for the determination of HMA and HNA1 [9, 10, 13-17]. Because of many interfering peaks observed by UV detection, most of them were based on FL detection using the native FL of tryptophan residue in HSA. The use of native FL would be ideal but it still suffers from interfering substances from biological extracts [18]; indeed, large peaks except for HMA and HNA1 were found in chromatograms obtained by above HPLC-FL methods [14, 15, 17]. In this article, HPLC-FL method using an FL probe specifically binding to HSA must be a useful technique for selective and sensitive determination of HMA and HNA1; however, such a method has yet to be developed.

Previously, we discovered that a lophine derivative, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1*H*-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 [19], which was a result of the interaction between DAPIM and HSA. Furthermore, we revealed that DAPIM specifically binds to the site II in subdomain IIIA of HSA with higher binding constant than other FL probes and is successfully applied for fluorometric measurement of total albumin in human serum [20].

The aim of this study is developing a highly selective HPLC-FL method using DAPIM for simultaneous determination of HMA and HNA, and applying it for human serum samples of healthy volunteers and diabetes mellitus (DM).

Materials and Methods

Reagents

HSA was purchased from Sigma (St. Louise, MO, USA). Sodium tetraborate, sodium hydroxide, sodium acetate, and sodium sulfate were from Nacalai Tesque (Kyoto, Japan). L-Cystine was purchased from Ajinomoto Co., Inc. (Tokyo, Japan). An HPLC grade of ethanol was obtained from Wako Pure Chemicals (Osaka, Japan). DAPIM was synthesized according to our previous paper [19].

HPLC system

An HPLC system (Fig. 2) consisted of two HPLC pumps (L-7100, Hitachi, Tokyo), a Hitachi L-7480 FL detector, a Rheodyne 7125 injector (Cotati, CA, USA) with a 10-μL sample loop, Asahipak ES-502N 7C column (100 x 7.6 mm i.d., Shodex, Tokyo), a Shimadzu CTO-6A column oven (Kyoto), reaction coil (2.0 m x 0.5 mm i.d.), and a Rikadenki Multi-pen recorder (Tokyo).

HMA and HNA were isocratically separated at 35 ºC with a mobile phase of 50 mM sodium acetate and 400 mM sodium sulfate (pH 4.8) containing 0.8% ethanol at a flow rate of 0.8 mL/min. The column eluent was mixed with 4 μM DAPIM in 70 mM borate buffer (pH 10.7) as a post-column reagent at a flow rate of 0.4 mL/min and the FL was monitored at 510 nm with excitation at 370 nm. The values for fractions of HMA (f(HMA)) and HNA1 (f(HNA1)) were obtained by the following equations:

$$f(HMA) = [HMA/(HMA+HNA1)] \times 100$$

$$f(HNA1) = [HNA1/(HMA+HNA1)] \times 100$$

Oxidation of HSA

Usually, HSA is commercially available as a mixture of HMA and HNA1. In order to identify peaks on a chromatogram, HMA in commercially available HSA was forcibly oxidized to HNA1 by the incubation with L-cystine. HSA (50 mg) was dissolved in 1.25 mL L-cystine solution (2 mM in phosphate buffer (pH 6.87)) and mixed by shaking at 20 °C for 3 h. Then, the mixture was filtered and incubated at 35 °C for 108 h.

Serum sample analysis

Serum samples were collected from healthy volunteers (n = 10; 22-30 years; 5 females) and DM patients (n = 10; 39-59 years; 4 females) with the approval from Ethics Committee of Graduate School of Biomedical Sciences, Nagasaki University. Whole blood was collected into glass tubes containing coagulation accelerator. The coagulated blood is left to clot at room temperature for 30 min. After removing the clot by centrifugation at 1300 x *g* for 10 min at 4 °C, the resulting

supernatant (serum) was stored at -35 °C. Serum samples were 20-fold diluted with phosphate buffered saline, and then were injected into the HPLC system.

Result and Discussion

Optimization of analytical conditions

Fig. 3 A) and B) show the chromatogram of standard HSA solution before and after the incubation with L-cystine, respectively. Two major peaks were detected at 13 and 23 min on the chromatogram before the reaction with L-cystine. After the reaction, the peak at 13 min was disappeared while the peak at 23 min increased. These results indicated that the component of the peak at 13 min was converted to the component of the peak at 23 min by the oxidation with L-cystine. Therefore, the peaks at 13 and 23 min could be identified to HMA and HNA1, respectively.

In order to make the analytical performance better, the analytical conditions including the pH and concentration of buffer as well as the concentration of DAPIM in the post-column derivatization reagent solution were optimized using HSA standard solution.

In previous study, it was found that higher pH provided stronger fluorescence of DAPIM binding to HSA [20]. Based on this finding, the effect of pH of borate buffer in post-column derivatization reagent was studied, ranging from 9.3 to 10.7. As the pH increases, the peak area of HMA and HNA1 increased. Different concentrations of borate buffer ranging from 20 to 120 mM were studied. Maximum and constant peak area could be obtained at 70 mM or more; 70 mM was selected for subsequent work. Subsequently, DAPIM concentration was examined over the range of 1 to 5 μM in 70 mM borate buffer (pH 10.7) (Fig. 4). The peak area increased as DAPIM

concentration increased and the highest and constant peak area was provided at concentration higher than 4 μM; 4 μM was chosen as an optimum.

Methods validation

The precision and accuracy of the method were determined by using healthy serum samples. Precision (%) is expressed as relative standard deviation (RSD). During the course of method validation, intra- and inter-day precision were 1.9% and 4.0% for HMA and 4.3% and 7.5% for HNA1. These data show that the method has sufficient reproducibility in human serum analysis. As reported in our previous study [20], site II-binding drugs such as diazepam can competitively bind to HSA against DAPIM, resulting in underestimation. However, such underestimation occurs only when the concentration of diazepam is 4-fold higher than its therapeutic concentration. Also, site-II binding drug should equally bind with HMA and HNA1; therefore, the ratio of HMA and HNA1 is not affected.

Each fraction of HMA and HNA in serum of healthy volunteers and DM patients

Fig. 3 C) shows the chromatograms obtained from serum of healthy volunteers. Different from other HPLC-FL methods [14, 15, 17] and HPLC-UV method (Fig. 3 D), there were no any peak around the peaks of HMA and HNA1. This advantage in our proposed method can realize highly selective analysis of HMA and HNA1. In the same way, peaks of HMA and HNA1 in serum of DM patients could be clearly detected without any interference from other biological components (Fig. 3 E). Also, similarly as previous study [9], the concentration of HNA1 in serum was smaller than that of HMA.

The values of f(HMA) and f(HNA1) were calculated by measurement of HMA and HNA1 are summarized in Table 1. In this study, age- and sex-matched healthy volunteers could not be enrolled and so, the statistical analysis of f(HMA) and f(HNA1) between healthy donors and DM patients could not be performed. However, when f(HMA) and f(HNA1) were statistically compared in each group by un-paired *Student*'s t-test, P value of <0.001 was obtained in healthy donors [f(HMA)/f(HNA1) = 3.27], while that of 0.03 was obtained in DM patients [f(HMA)/f(HNA1) = 1.28]. Therefore, it was obvious that f(HNA1) of DM patients drastically increased, compared with that of healthy donors, which clearly agreed with the finding in previous reports [13, 21]. The biological monitoring presented here suggests that our proposed method is useful to investigate of dynamic change in the redox state of HSA.

Conclusion

We developed a selective method for the simultaneous determination of HMA and HNA1 by HPLC-FL using DAPIM as an FL probe. Because DAPIM specifically binds to HSA and provides FL signal, there are no interfering peak, resulting in highly selective determination. The proposed method was successfully applied for determination of HMA and HNA1 in serum of healthy donors and DM patients. This method will be a useful technique to observe the redox state of HSA.

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Figure captions

Fig. 1 Fluorescence probe, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1*H*-imidazol-2-yl]benzoic acid methyl ester (DAPIM), specifically bind to human serum albumin.

Fig. 2 HPLC-FL system for the determination of HMA and HNA. P, pump 2; I, injector; Column, Shodex Asahipak ES-502N 7C (100 mm x 7.6 mm, i.d.); RC, reaction coil (2.0 m x 0.5 mm, i.d.); D, FL detector; R, recorder.

Fig. 3 HPLC chromatograms of HSA standard solution (A) before and (B) after incubation with L-cystine, and HSA in serum of a healthy volunteer detected by (C) FL detection and (D) UV detection, and (E) HSA in serum of DM patient. Peaks: 1, HMA; 2, HNA1.

Fig. 4 Effect of DAPIM concentration on relative peak area. Peak area at 4 μM was defined as 100%.

Fig. 1

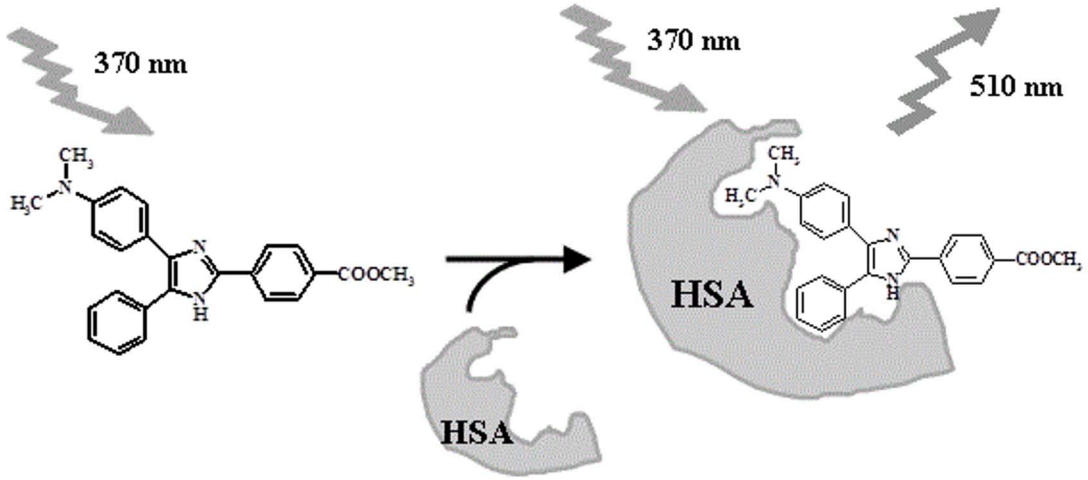
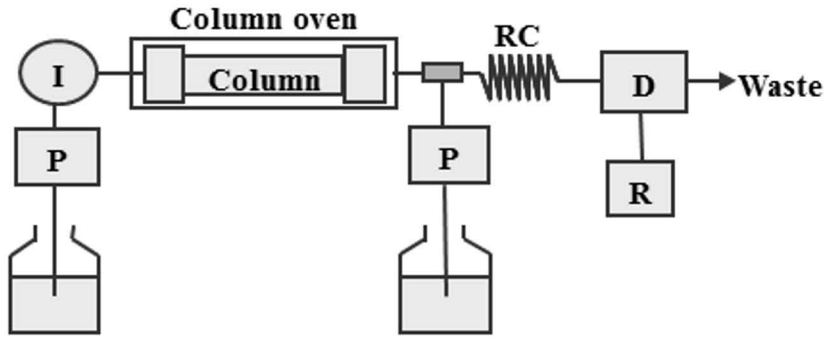


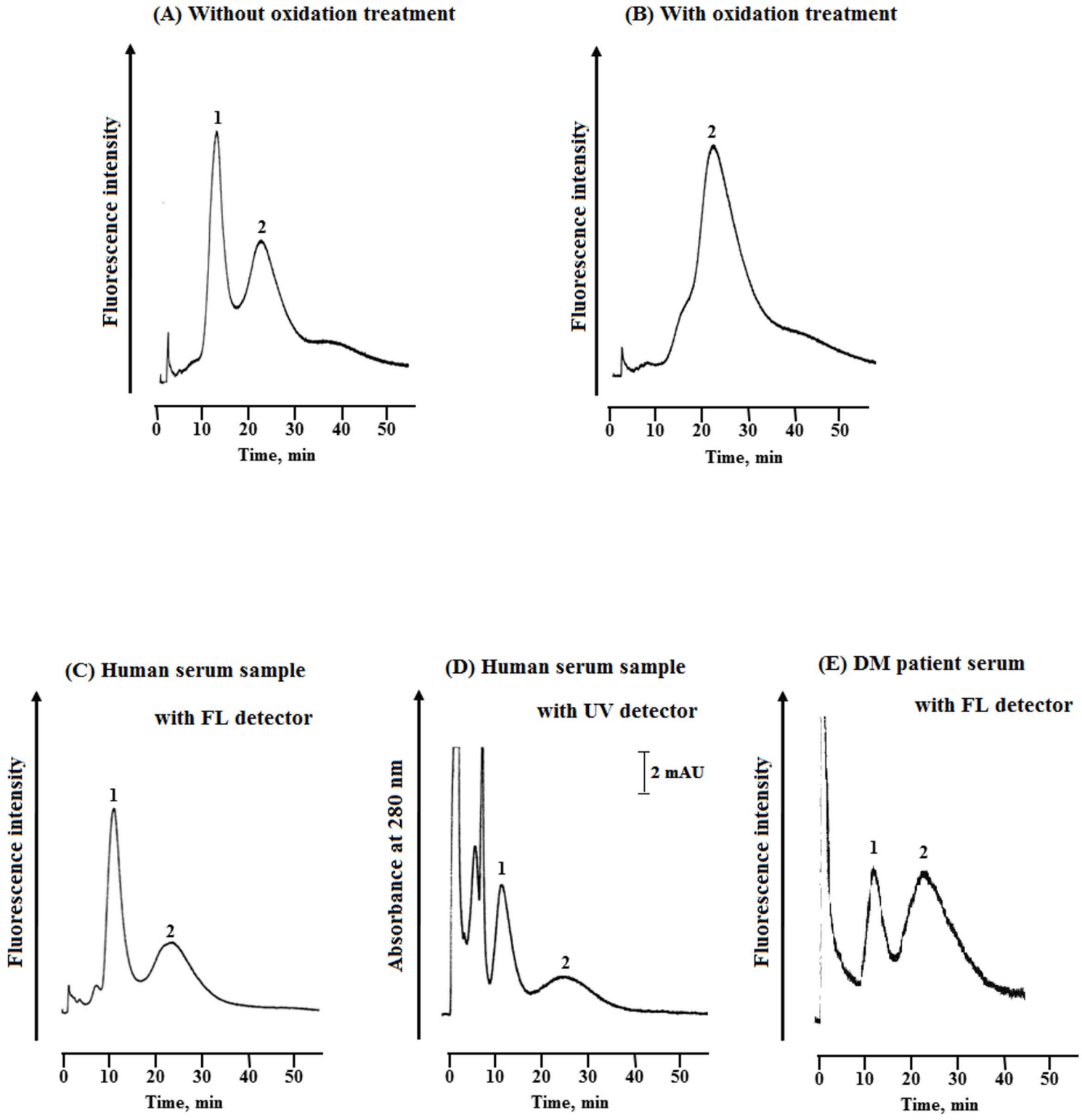
Fig. 2



Mobile phase:
50 mM sodium acetate - 0.4 M sodium sulfate (pH 4.8) / ethanol = 99.2 / 0.8 (v/v, %)

FL reagent:
4 μM DAPIM in borate buffer (pH 10.7)

Fig. 3


(A) Without oxidation treatment
Fluorescence intensity
1
2
0
10
20
30
40
50
Time, min
(B) With oxidation treatment
Fluorescence intensity
2
0
10
20
30
40
50
Time, min
(C) Human serum sample
with FL detector
Fluorescence intensity
1
2
0
10
20
30
40
50
Time, min
(D) Human serum sample
with UV detector
Absorbance at 280 nm
2 mAU
1
2
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20
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Time, min
(E) DM patient serum
with FL detector
Fluorescence intensity
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Time, min

Fig. 4

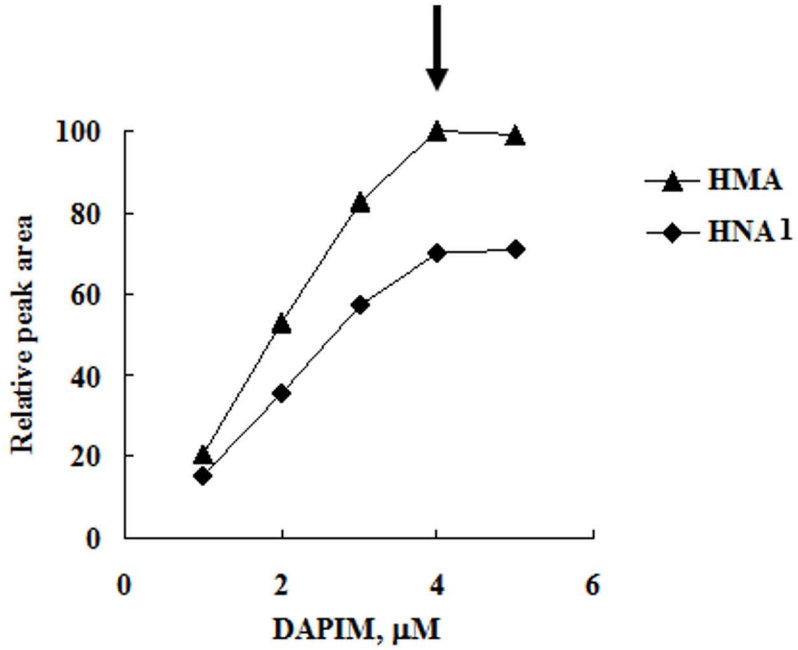

Table 1 f(HMA) and f(HNA1) values (%) for healthy volunteers and DM patients

	age (mean ± SE)	**f(HMA) (mean ± SE)**	**f(HNA) (mean ± SE)**
Healthy volunteers (n = 10)	**24.3 ± 0.75**	**76.6 ± 1.11**	**23.4 ± 1.11**
DM patients (n = 10)	**51.3 ± 2.19**	**56.1 ± 3.65**	**43.9 ± 3.65**