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

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17

18 Abbreviations: DAPIM, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1*H*-imidazol-2-yl]benzoic acid

19 methyl ester; HSA, human serum albumin; BCG, bromocresol green;

Abstract

The binding of a lophine-based fluorescence probe, 4-[4-(4-dimethylaminophenyl)-5-phenyl-1*H*-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 3.65×10^{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-1*H*-imidazol-2-yl]benzoic acid methyl ester; human serum albumin; binding constant; binding site

36 **Introduction**

37 Human serum albumin (HSA) is the most abundant protein constituent of blood and serves as a
38 protein storage component. Its principal function is to bind and transport a wide variety of
39 bioactive molecules such as fatty acids, hormones, vitamins and numerous pharmaceuticals [1, 2].
40 The main regions of HSA for ligand binding are located in hydrophobic cavities in the subdomains
41 IIA (site I) and IIIA (site II) [3-5]. The binding affinity of a drug to HSA affects the distribution,
42 pharmacokinetics, toxicity and rate of excretion of the drug [4]. Therefore, information on the
43 binding affinity of a drug and biomolecule to HSA is particularly useful for solving *in vivo*
44 pharmaceutical problems [6].

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

51 On the other hand, there is a connection between the content of HSA in urine or blood and some
52 diseases, such as nephropathy, so the determination of HSA is very important in clinical diagnosis.
53 Many methods have been described for the determination of HSA. Various compounds such as
54 bromocresol green (BCG) and bromocresol purple (BCP) have been reported as analytical reagents
55 based on changes in their colors by binding to HSA [16-18]. BCG and BCP methods are most
56 widely used for HSA assay in clinical laboratory [19, 20].

57 Until now, we reported several analytical methods for biologically important compounds by

58 employing the fluorescence and chemiluminescence properties of lophine derivatives mainly as a
59 labeling reagent [21-27]. During these studies, we discovered that a lophine derivative,
60 4-[4-(4-dimethylaminophenyl)-5-phenyl-1*H*-imidazol-2-yl]benzoic acid methyl ester (DAPIM, Fig.
61 1) has almost no fluorescence, but the fluorescence intensity of DAPIM solution was drastically
62 enhanced by the addition of HSA in aqueous solvent [21], which may be a result of the interaction
63 between DAPIM and HSA. In this paper, in order to systematically explore the binding
64 mechanism of DAPIM with HSA, the binding characteristics were discussed by determining the
65 binding constant and binding sites under physiological conditions. Also, we evaluated its potential
66 as a fluorescence probe for determination of HSA in human serum.
67
68 **Materials and methods**
69 *Reagents and apparatus*
70 HSA and warfarin were purchased from Sigma (St. Louise, MO, USA). Diazepam, valproic
71 acid, BCG and oleic acid were obtained from Wako (Osaka, Japan). DAPIM was synthesized
72 according to our previous report [21]. Fluorescence was measured with a Shimadzu RF-1500
73 spectrofluorometer (Kyoto, Japan).
74
75 *General procedure*
76 Under the optimum experimental conditions, 20 μL of HSA solution and 3.0 mL of 2 mM
77 DAPIM in pH 7.4 phosphate buffered saline (PBS, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM
78 NaCl, 2.7 mM KCl) containing 0.025% sodium dodecyl sulfate (SDS) were mixed, then were
79 incubated at room temperature for 20 min. The fluorescence intensity was measured with the

80 following settings of spectrofluorometer: excitation wavelength (λ_{ex}), 370 nm; excitation slit, 10
81 nm; emission wavelength (λ_{em}), 510 nm; emission slit, 10 nm.
82
83 *Circular dichroism measurement*
84 The alterations in the secondary structure of the HSA in the presence of DAPIM with different
85 concentrations were studied by monitoring circular dichroism (CD) spectra on a Jasco J-725 CD
86 spectrophotometer using a rectangular quartz cuvette of path length 2 mm at 0.2 nm data pitch
87 intervals. All CD spectra were taken in a wavelength from 200 to 240 nm. The
88 spectrophotometer was sufficiently purged with 99.9% nitrogen before starting the measurement.
89 The spectra were collected at a scan speed of 200 nm/min and a response time of 1 s. The final
90 plot was taken as an average of three accumulated plots.
91
92 *Serum samples*
93 Sera from healthy donors (n=10; 21-30 years; 5 female) were collected in our laboratory. The
94 whole blood was collected by tubes containing coagulation accelerator. After removing the clot
95 by centrifuging 1300 *g* for 10 min at 4°C, the resulting supernatant (serum) was stored at -80°C.
96 The samples were diluted 10-fold with PBS (pH 7.4) and the diluents were used for HSA
97 determination. According to the procedures presented in previous literatures [20], 5.0 mL of BCG
98 (150 μM) in citrate buffer (pH 4.0) was added to 25 μL of serum and then, stand at room
99 temperature for 10 min. An UV absorbance at 628 nm was measured by Shimadzu UV-265FS.
100 All the experiments were performed with approval from the institutional ethics committee of the
101 Graduate School of Biomedical Sciences, Nagasaki University.

102

103 *HPLC system and conditions*

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

111

112 **Results and discussion**

113 *Fluorescence spectra characteristics of DAPIM binding to HSA*

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

120

121 *Binding constant and binding site number*

122 In order to study the interaction of small molecules with macromolecules, the Scatchard plot is
123 commonly used to characterize the binding properties such as binding constant and number of

124 binding sites [28]. From the recorded fluorescence titration data, the binding constant and binding
125 number of DAPIM with HSA were determined as 3.65 x 10^6 M^{-1} and 1.07, respectively (Fig. 3).
126 The binding number indicates that DAPIM-HSA complex may have one binding site.
127

128 *Identification of the binding site*

129 To obtain the information about the binding site of DAPIM in HSA, competitive binding
130 between DAPIM and other ligands (*i.e.*, warfarin, valproic acid, diazepam and oleic acid) was
131 studied. Warfarin, valproic acid, diazepam, and oleic acid are reported to bind to HSA at site I,
132 site I and II, site II, and site II, respectively [29-32]. The fluorescence of DAPIM (0.05-5 μM)
133 plus HSA (75.8 μM) was measured in the presence and the absence of other ligands (0.5 μM). As
134 illustrated in Fig. 4a, DAPIM was moderately displaced by diazepam, whereas the fluorescence of
135 DAPIM bound to HSA was not affected by the other ligands. Therefore, the site II at subdomain
136 IIIA is the specific binding site for DAPIM. Furthermore, the displacement by diazepam was
137 enhanced according to an increase in its concentration (Fig. 4b). As mentioned above, the main
138 regions of HSA for ligand binding are located in subdomains II A (site I) and IIIA (site II); that is,
139 DAPIM can be used as a fluorescent probe for many studies of drug-binding sites on HSA.
140

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

142 In order to develop HSA assay using DAPIM, several measurement conditions were optimized.
143 The effect of the concentration of DAPIM on fluorescence intensity was investigated (Fig. 5). At
144 more than 1.5 μM, the maximum fluorescence intensity was obtained and 2.0 μM was chosen for
145 further study. Because the DAPIM-HSA complex, which provides fluorescence, might become

146 more unstable as temperature increased, the effect of temperature on fluorescence intensity was also

147 studied under 298, 305, 310 and 316 K (data not shown). As expected, temperature had a great

148 influence on the fluorescence intensity. The fluorescence intensity gradually decreased with

149 increasing temperature; thus, we selected room temperature (298 K). The incubation time of more

150 than 20 min provided the maximum and stable fluorescence intensity.

151

152 *Studies on CD spectra of HSA in the presence of DAPIM*

153 As shown in Fig. 6, the CD spectrum of HSA exhibits two negative peaks at 208 nm and 222 nm

154 which are contributed from $n \rightarrow \pi^*$ transition of the peptide inter linkage of α-helix [33]. The

155 results of CD studies indicated that with the addition of DAPIM, the intensities of both the peaks

156 slightly increased. This indicates that certain conformational changes of HSA were occurred by

157 the addition of DAPIM. In addition, no change in band shape and induction of a new peak suggest

158 that DAPIM leads to conformation structural changes but no conformational transition of HSA.

159

160 *Analytical characteristics*

161 Calibration curves for determination of HSA were linear over the concentration of 0.1-0.8 g/dL

162 (r=0.999) and the corresponding regression equation was Y=36.7X+2.2, where Y is the

163 fluorescence intensity of DAPIM-HSA and X is the HSA concentration. The correlation efficient

164 (r) was greater than 0.999. The assay parameters consisting of calibration range, slope (36.7),

165 intercept (2.2) and the limit of detection (0.0067 g/dL, defined as the concentration corresponding

166 to three times of the standard deviation of the background signal) were obtained. This method

167 exhibited good repeatability with a relative standard deviation of 1.7% obtained from six separate

168 determinations for 0.2 g/dL HSA.

169

170 *Determination of HSA in human serum*

171 The proposed method was employed to determine HSA in human serum. Sera obtained from
172 healthy volunteers (n=10) were tested without pre-treatment except dilution (10-fold) by PBS.
173 The analytical results ranged from 6.10 to 7.4 g/dL (mean ± standard deviation = 6.6 ± 0.4 g/dL).
174 The analytical results ranged from 6.10 to 7.4 g/dL (mean ± standard deviation = 6.6 ± 0.4 g/dL).
175 Although there have been a lot of criticism to BCG method, BCG method is still a standard method
176 for HSA assay and is most widely used in clinical settings [19, 20, 34, 35]. Therefore, it was
177 employed as a reference method in present study. As shown in Fig. 7a, comparison of the
178 proposed method with BCG assay is performed using a nonparametric Passing-Bablok analysis [36,
179 37]. The 95% confidence interval (CI) was calculated for the slope and intercept by nonparametric
180 Bootstrap. The Blank-Altman approach [38-41] was used as an alternative to correlation and
181 regression models for further assessing the difference between both methods by plotting the relative
182 difference between the two assays versus the determined mean concentration (Fig. 7b).
183 Regression analysis of the data yielded the following equations: proposed method = 1.05 (BCG) +
184 0.62 [g/dL] (95% CI for slope, 0.69-1.48; 95% CI for intercept, -2.1-2.5). In the usual linear
185 regression model, the line of best-fit equation is calculated by minimizing the y-squared residuals.
186 This approach assumes that there is no error on the x variable and that the y variable has a constant
187 analytical precision. Contrastingly, nonparametric procedures including the Passing-Bablok
188 regression are based on the rank principle [36, 37]. This approach assumes an error on both x and
189 y variables, a constant ratio of the variances and no special assumptions regarding the distribution of

190 the values. Underestimation of the concentrations with BCG was described by some groups [42,
191 43]. These observations were confirmed in the present study with a mean underestimation of 0.86
192 g/dL.
193

194 *HPLC analysis of HSA by post-column reaction with DAPIM*

195 HSA eluted from the gel-filtration column was mixed with DAPIM, and the generated
196 fluorescence was monitored. Fig. 8a shows a typical chromatogram of standard HSA solution, and
197 the peak of HSA was detected at 20 min on the chromatogram. Also, as shown in Fig. 8b, the
198 peak of HSA in serum could be clearly detected without any interference from other biological
199 components. This result also demonstrates the excellent selectivity of DAPIM for HSA detection.
200 Therefore, the application of DAPIM in HPLC analysis should be useful to investigate HSA
201 analogues in complicated matrices.
202

203 **Conclusions**

204 In this paper, the interaction between DAPIM and HSA has been investigated by utilizing
205 fluorescence spectroscopy. The binding constant to HSA is 3.65 x 10^6 M^{-1} and the primary
206 binding site on HSA is site II at subdomain IIIA. With its site specificity to HSA, DAPIM will be
207 useful as fluorescence probes to elucidate the interaction between HSA and other molecules
208 including drugs. Based on the phenomenon that the fluorescence intensity of DAPIM was
209 enhanced in proportion to the concentration of HSA, a novel fluorescence assay of HSA can be
210 developed although further optimization will be needed.
211

212 **Acknowledgement**

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218

218 **References**

219 [1] W.E. Muller, U. Wollert, Pharmacology 19 (1979) 56-59.

220 [2] K. Yamasaki, T. Maruyama, U. Kragh-Hansen, M. Otagiri, Biochim. Biophys. Acta 1295
221 (1996) 147-157.

222 [3] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153-203.

223 [4] X.M. He, D.C. Carter, Nature 358 (1992) 209-215.

224 [5] J. Ghuman, P.A. Zunszain, I. Petitpas, A.A. Bhattachary, M. Otagiri, S. Curry, J. Mol. Biol. 353
225 (2005) 38-52.

226 [6] G. Colmenarejo, A. Alvarez-Pedraglio, J.-L. Lavandera, J. Med. Chem. 44 (2001) 4370-4378.

227 [7] K. Takehara, K. Yuki, M. Shirasawa, S. Yamasaki, S. Yamada, Anal. Sci. 25 (2009) 115-120.

228 [8] F. Moreno, J. González-Jiménez, Chem-Biol. Inter. 121 (1999) 237-252.

229 [9] A. Douhal, M. Sanz, L. Tormo, Proc. Natl. Acad. Sci. USA 102 (2005) 18807-18812.

230 [10] K. K. Park, J.W. Park, A.D. Hamilton, Org. Biomol. Chem. 7 (2009) 4225-4232.

231 [11] D.-H. Li, H.-H. Yang, H. Zhen, Y. Fang, Q.-Z. Zhu, J.-G. Xu, Anal. Chim. Acta 401 (1999)
232 185-189.

233 [12] C. Q. Ma, K.A. Li, W.T. Hu, S.Y. Tong, Microchem, J. 59 (1998) 417-426.

234 [13] Z. Xu, W. Yang, C. Dong, Bioorg. Med. Chem. Lett. 15 (2005) 4091-4096.

235 [14] Y.-J. Hu, W. Li, Y. Liu, J.-X. Dong, S.-S. Qu, J. Pharm. Biomed. Anal. 39 (2005) 740-745.

236 [15] F. Cui, J. Wang, Y. Cui, J. Li, X. Yao, Y. Lu, J. Fan, J. Luminesc. 127 (2007) 409-415.

237 [16] F. L. Rodkey, Clin. Chem. 11 (1965) 478-487.

238 [17] H. Schenck, K. Rebel, Clin. Chem. 28 (1982) 1408-1409.

239 [18] A.E. Pinnell, B.E. Northam, Clin. Chem. 24 (1978) 80-86.

240 [19] B.T. Doumas, T. Peters Jr., Clin. Chim. Acta 258 (1997) 3-20.

241 [20] B.T. Doumas, W. Ard Watson, H.G. Biggs, Clin. Chim. Acta 258 (1997) 21-30.

242 [21] K. Nakashima, Y. Fukuzaki, R. Nomura, R. Shimoda, Y. Nakamura, N. Kuroda, S. Akiyama,

243 K. Irgum, Dyes and Pigments 38 (1998) 127-136.

244 [22] K. Nakashima, Biomed. Chromatogr. 17 (2003) 83-95.

245 [23] N. Kuroda, R. Shimoda, M. Wada, K. Nakashima, Anal. Chim. Acta 403 (2000) 131-136.

246 [24] O.Y. Al-Dirbashi, N. Kuroda, M. Wada, M. Takahashi, K. Nakashima, Biomed. Chromatogr.

247 14 (2000) 293-300.

248 [25] N. Kuroda, S. Sugihara, Y. Sugihara, M. Wada, N. Kishikawa, Y. Ohba, K. Nakashima, J.

249 Chromatogr. A 1066 (2005) 119-125.

250 [26] T. Ichibangase, C. Hamabe, Y. Ohba, N. Kishikawa, K. Nakashima, Y. Kayamori, D. Kang, N.

251 Hamasaki, N. Kuroda, Luminescence 21 (2006) 62-66.

252 [27] S. Yamaguchi, N. Kishikawa, K. Ohyama, Y. Ohba, M. Kohno, T. Masuda, A. Takadate, K.

253 Nakashima, N. Kuroda, Anal. Chim. Acta 665 (2010) 74-78.

254 [28] G. Scathard, Ann. NY Acad. Sci. 51 (1949) 660-672.

255 [29] U. Kragh-Hansen, V.T. Chuang, M. Otagiri, Biol. Pharm. Bull. 25 (2002) 695-704.

256 [30] J. Ghuman, P.A. Zunszain, I. Petitpas, A.A. Bhattacharya, M. Otagiri, S. Curry, J. Mol. Biol.

257 353 (2005) 38-52.

258 [31] H.S. Kim, D.S. Hage, J. Chromatogr. B 816 (2005) 57-66.

259 [32] R. Artali, G. Bombieri, L. Calabi, A. Del Pra, IL Farmaco 60 (2005) 485-95.

260 [33] P. Yang, F. Gao, The Principle of Bioinorganic Chemistry, Science Press, Marrickville, 2002.

261 [34] W. Zeng, X. Meng, N. Li, S. Tong, Anal. Chim. Acta 316 (1995) 387-389.

262 [35] D.H. Li, H.H. Yang, H. Zhen, Y. Fang, Q.Z. Zhu, J.G. Xu, Anal. Chim. Acta 401 (1999)

263 185-189.

264 [36] H.A. Passing, J. Clin. Chem. Clin. Biochem. 21 (1983) 709-720.

265 [37] H.A. Passing, W. Bablok, J. Clin. Chem. Clin. Biochem. 22 (1984) 431-435.

266 [38] J.M. Bland, D.G. Altman, Lancet 1 (1986) 307-310.

267 [39] J.M. Bland, D.G. Altman, Lancet 346 (1995) 1085-1087.

268 [40] K. Dewitte, C. Fierens, D. Stockl, L.M. Thienpont, Clin. Chem. 48 (2002) 799-801.

269 [41] N. Ansermot, M. Fathi, J.L. Veuthey, J. Desmeules, S. Rudaz, D. Hochstrasser, Clin. Biochem.

270 41 (2008) 910-913.

271 [42] H.A. Assink, B.G. Blijenberg, G.J.M. Boerma, B. Leijnse, J. Clin. Chem. Clin. Biochem. 22

272 (1984) 685-692.

273 [43] R. Calvo, R. Carlos, S. Erill, Int. J. Clin. Pharmacol. Ther. Toxicol. 23 (1985) 76-78.

274

274 **Figure captions**

275

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

277 methyl ester (DAPIM).

278

279 **Fig. 2** Excitation and emission spectra of DAPIM in the presence of various concentration of

280 HSA (0-1.0 g/dL). The concentration of DAPIM is 5 μM.

281

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

283

284 **Fig. 4** Effect of site marker probe on the fluorescence of DAPIM (0.05-5 μM). a) several marker

285 probes. b) using diazepam with different concentrations.

286

287 **Fig. 5** Effect of DAPIM concentration on the relative fluorescence intensity. The concentration

288 of HSA is 0.5 g/dL.

289

290 **Fig. 6** CD spectra of HSA in the presence of DAPIM. Conditions: HSA, 0.3 g/dL; DAPIM, (a)

291 0, (b) 2.5 μM, (c) 5 μM.

292

293 **Fig. 7** a) comparison of HSA results obtained by BCG method and our proposed method by

294 Passing-Bablok regression. b) Bland-Altman plot for the comparison of BCG method versus our

295 proposed method. The mean value (n=10) of the two method is plotted against the difference the

296 two values (our proposed method-BCG method). The mean difference between the two methods

297 was 0.86 g/dL. The mean difference and the mean ± 2 SD difference were shown by solid line and

298 dashed lines, respectively.

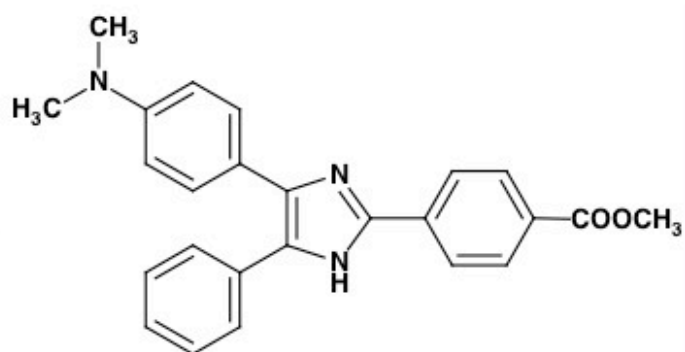
299

300 **Fig. 8** Chromatograms for a) standard solution of 0.5 g/dL HSA and b) human serum obtained by

301 the proposed HPLC system. The human serum was diluted 20 times with PBS before injection.

302

Fig. 1



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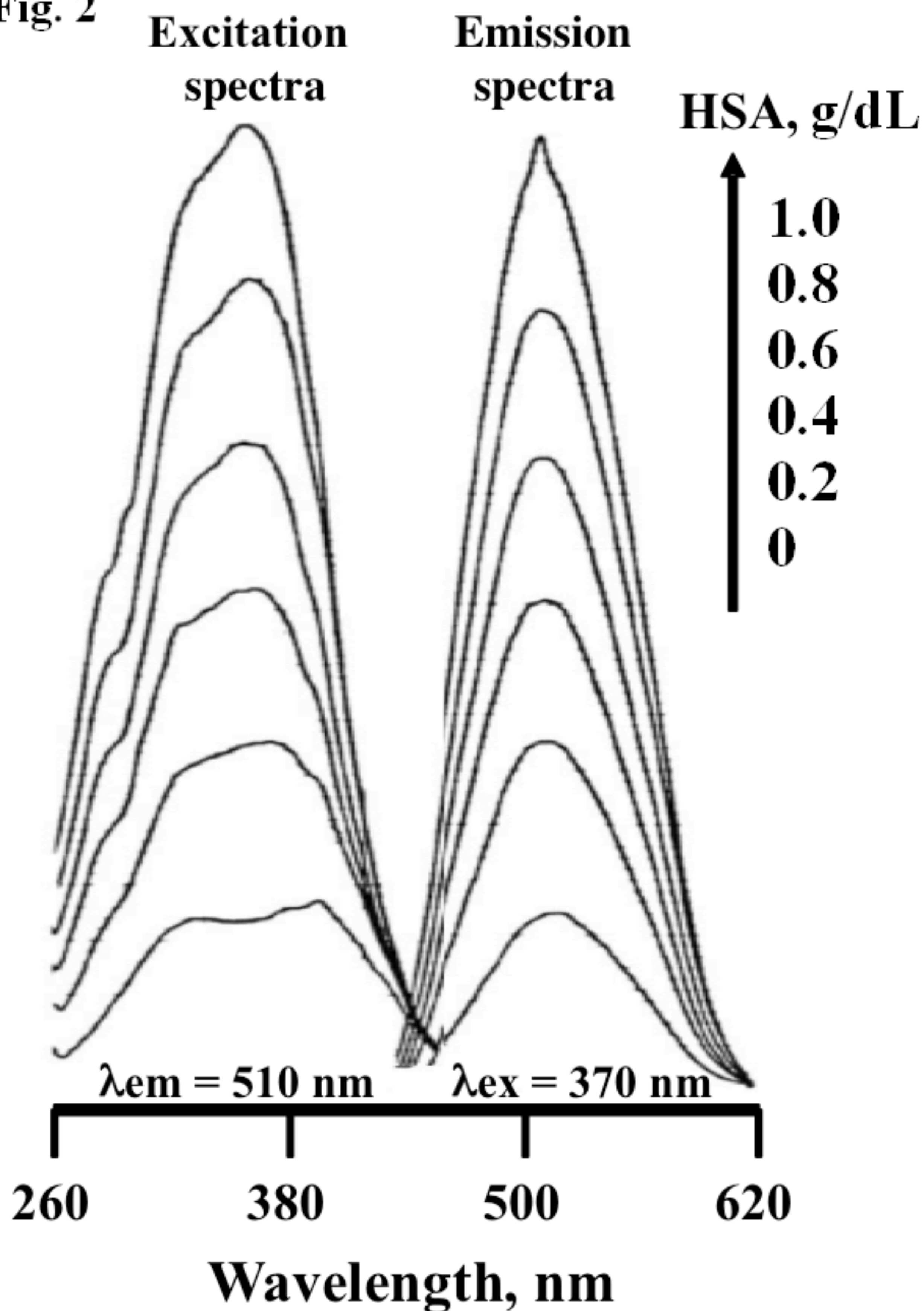

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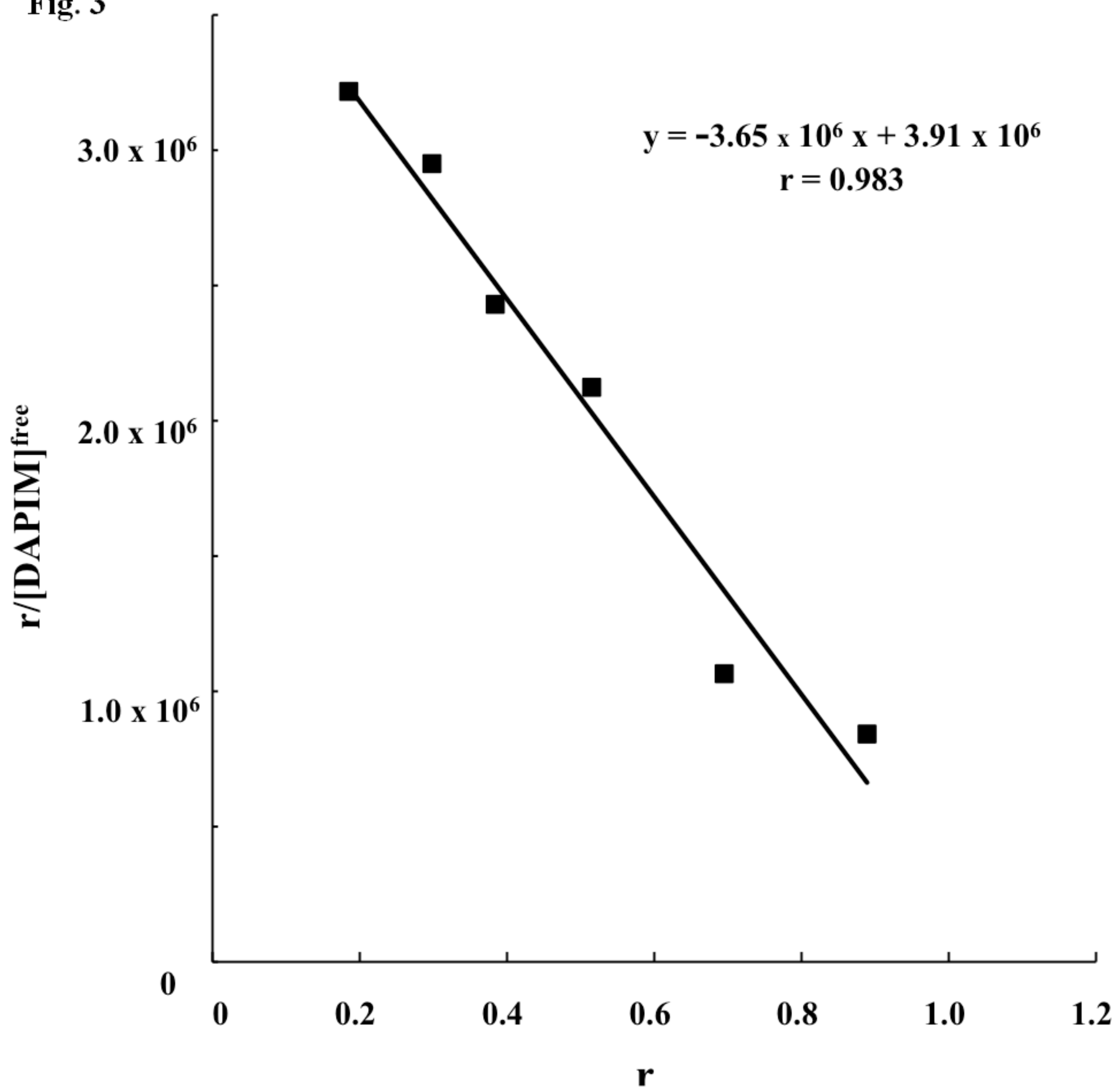

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Fig. 4

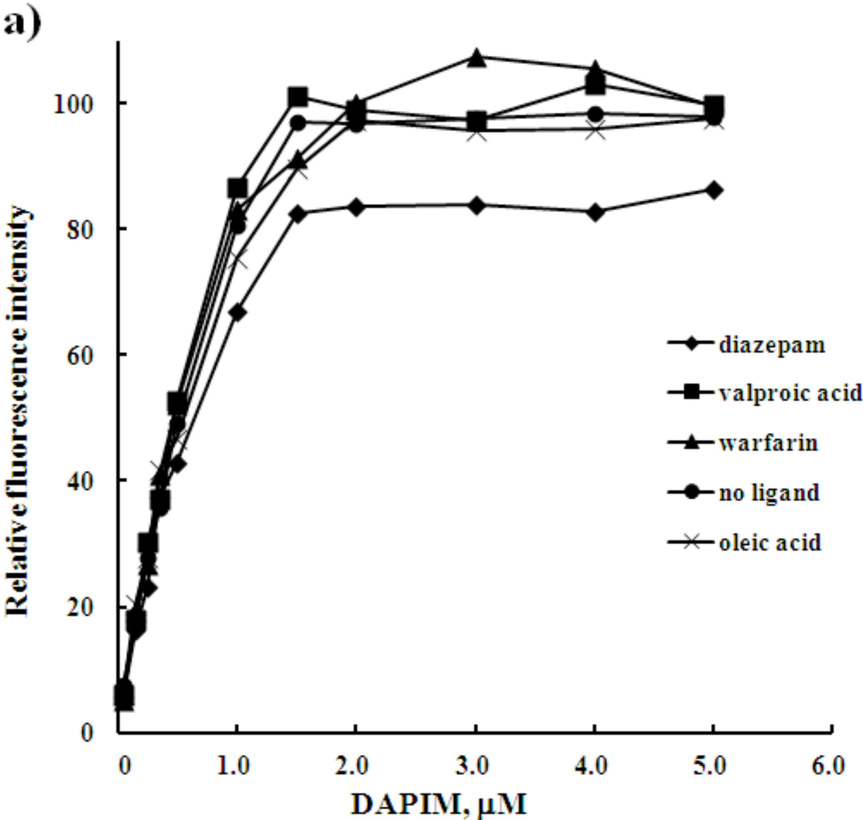

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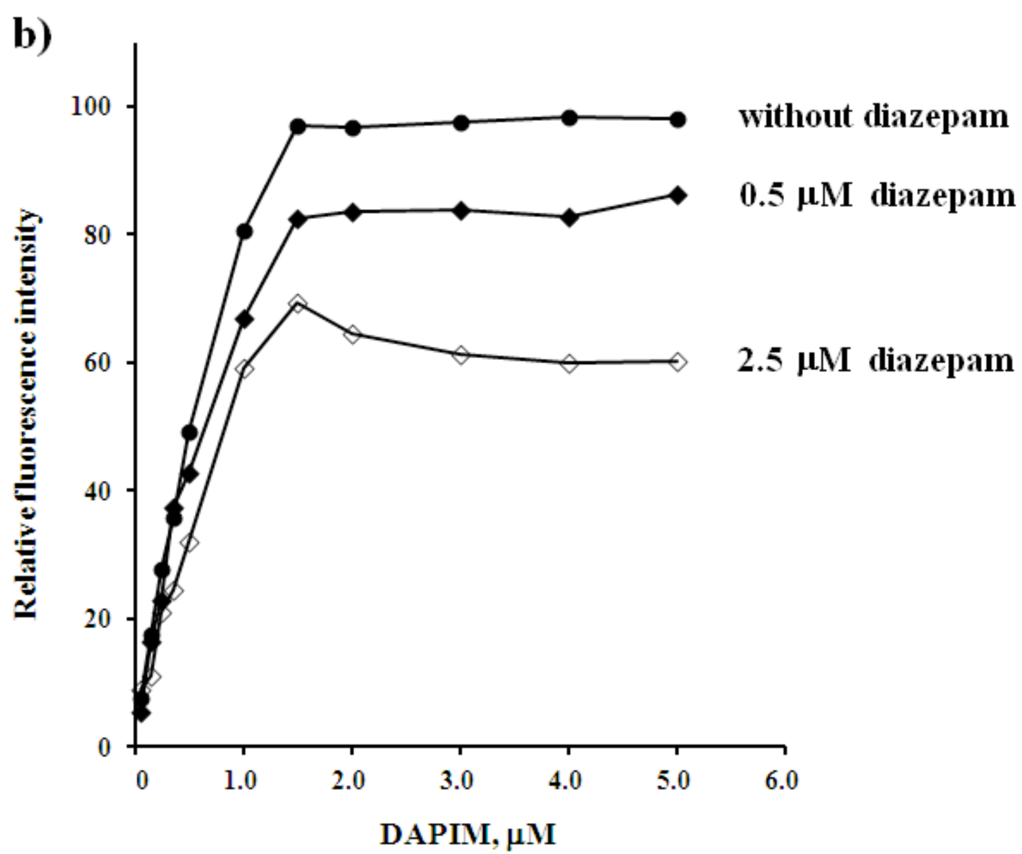
Fig. 4



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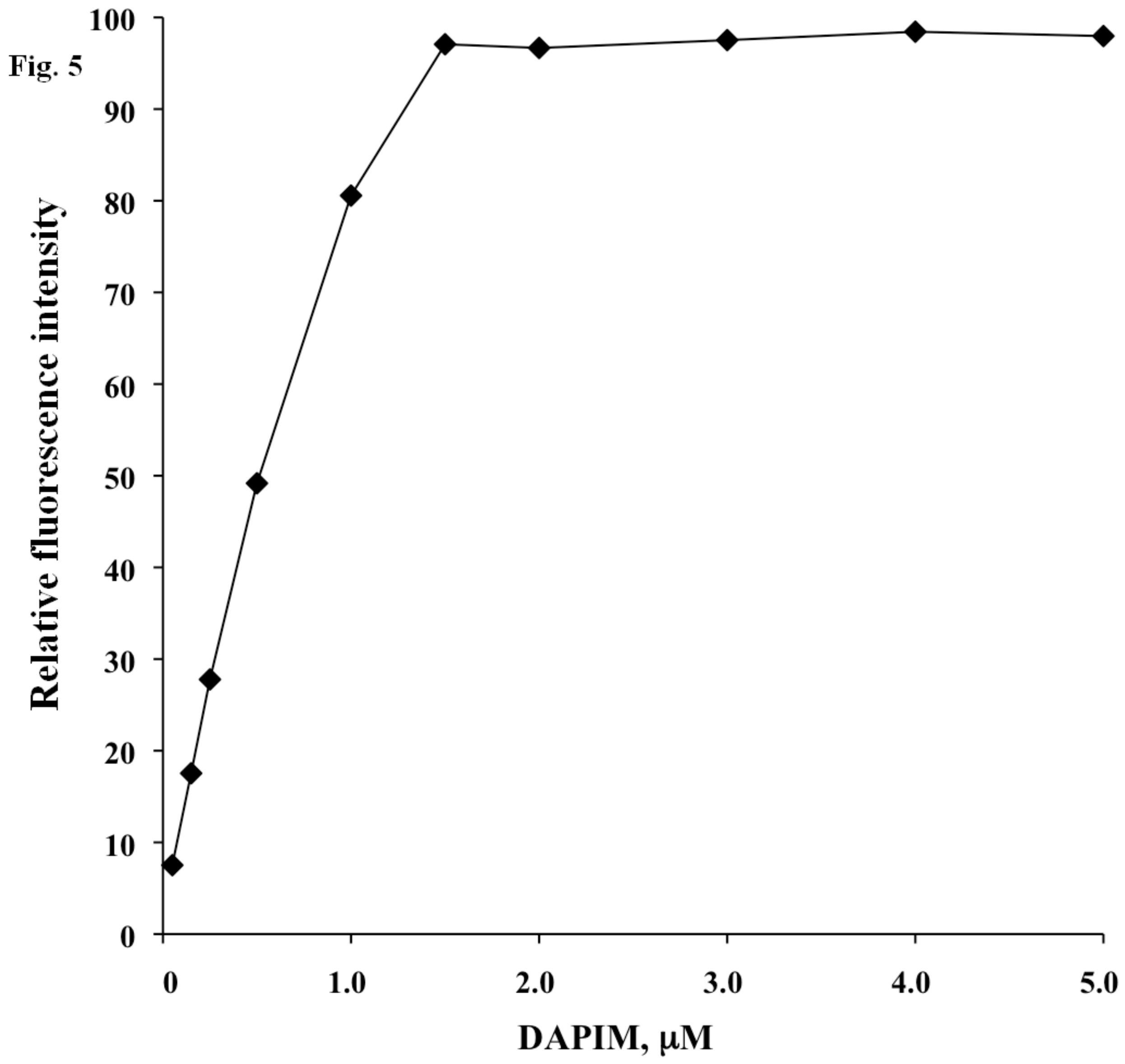

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Fig. 6

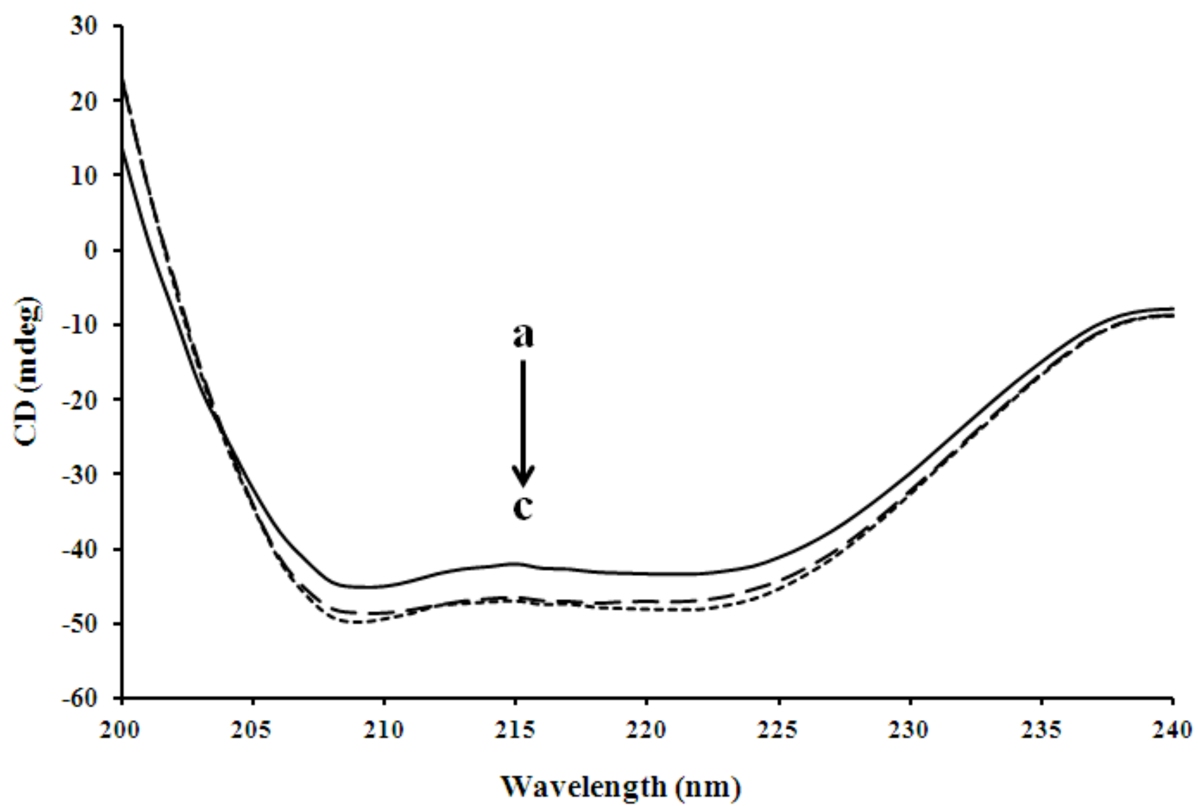

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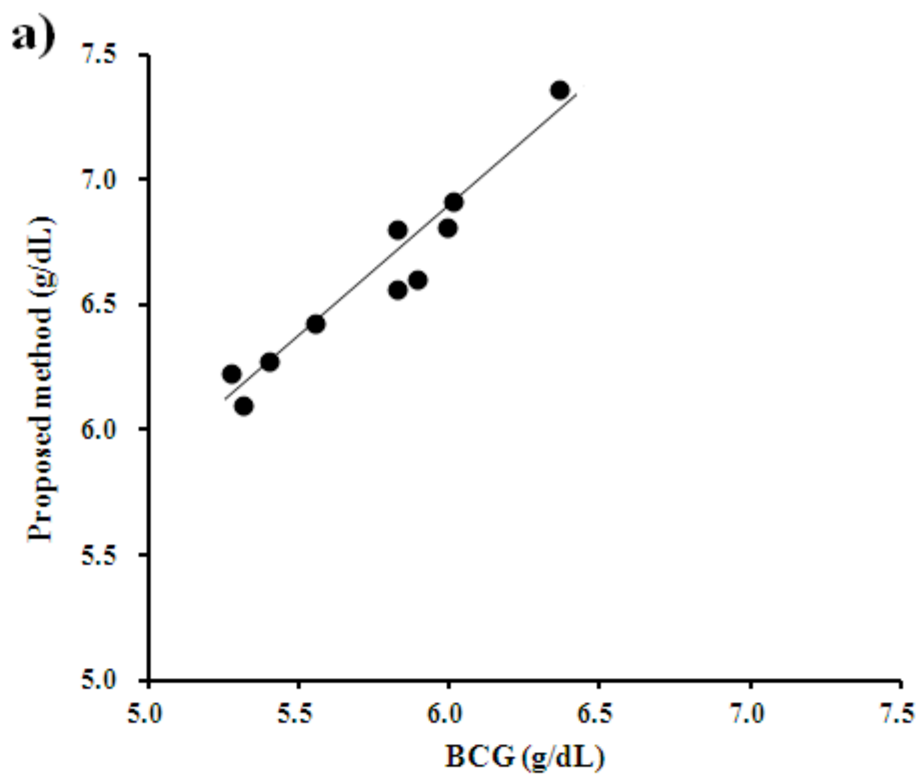
Fig. 7



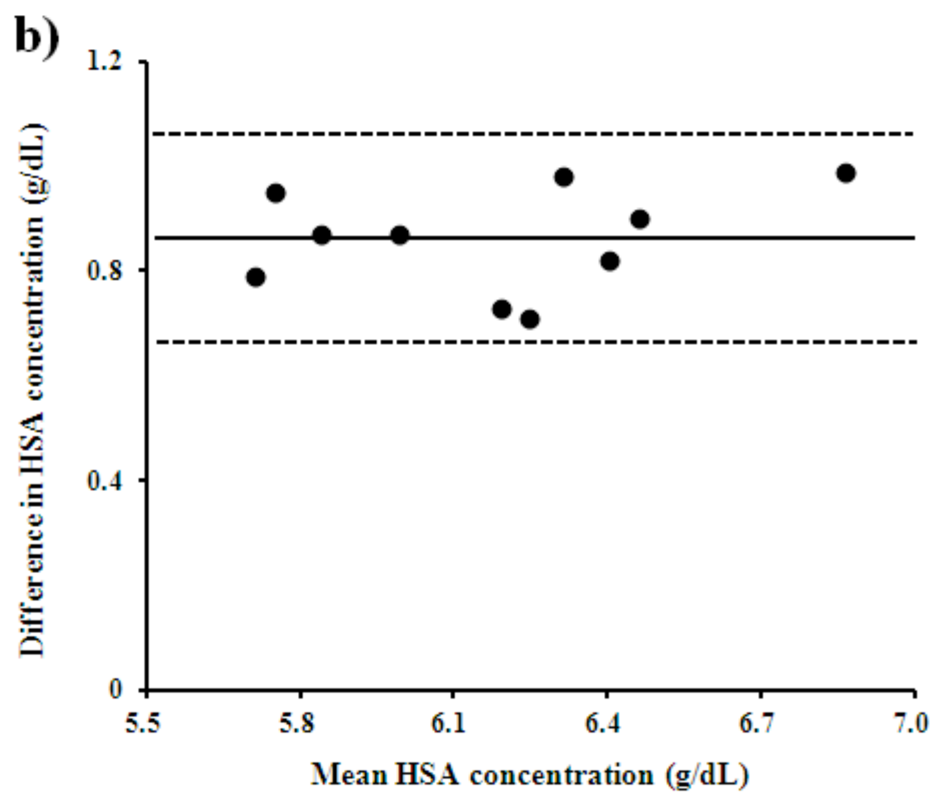


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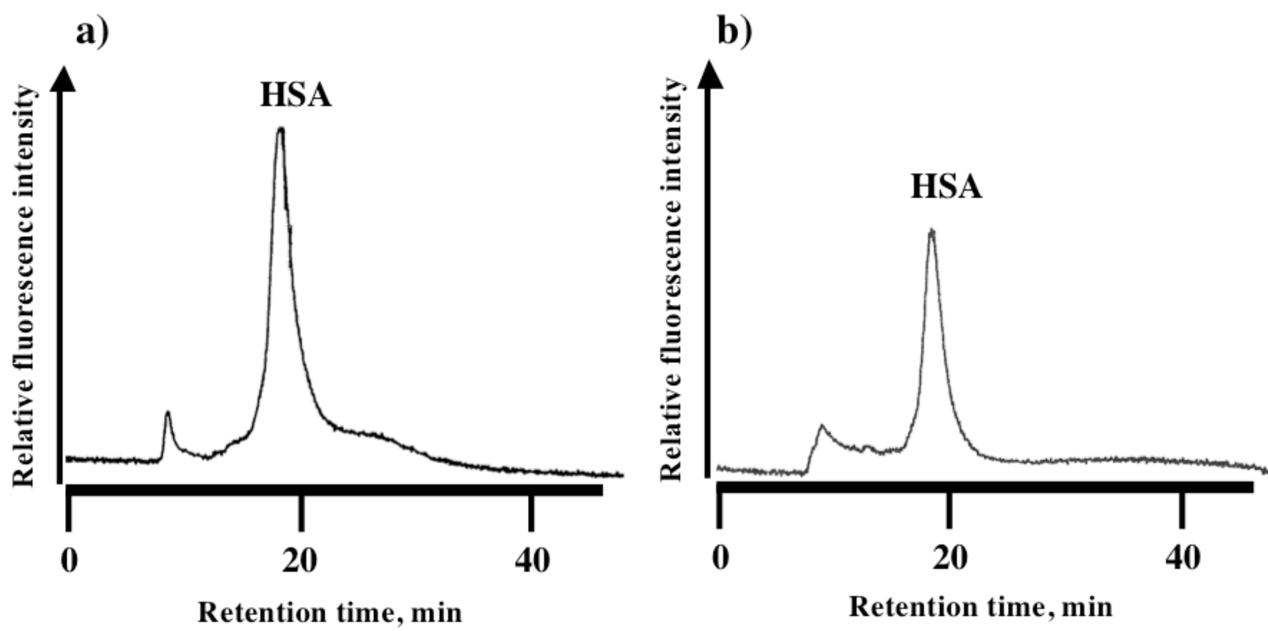
Fig. 8



310

311

311 **Highlights**

312

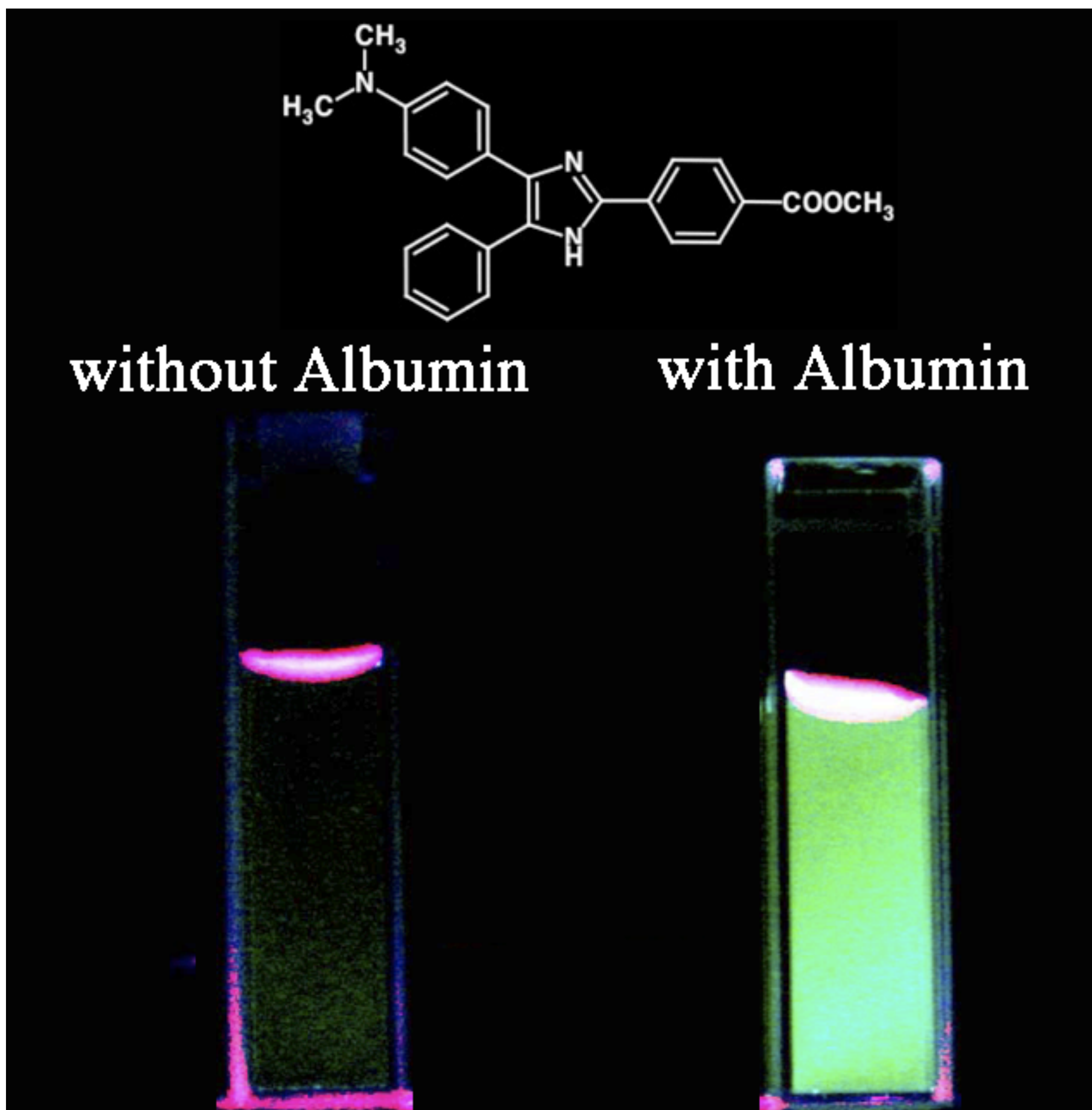
313

314 1. Lophine-based probe (DAMIP) shows strong fluorescence when it binds with albumin.

315 2. The binding constant of DAPIM to albumin is 3.65 x 10^6 M^{-1}.

316 3. The primary binding site of DAPIM to HSA is site II at subdomain IIIA.

317 4. DAPIM was successfully applied to the determination of albumin in human serum.



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