J Fluoresc (2011) 21:1825–1830
DOI 10.1007/s10895-011-0878-3

ORIGINAL PAPER

Displacement Reaction Using Ibuprofen in a Mixture of Bioactive Imidazole Derivative and Bovine Serum Albumin—a Fluorescence Quenching Study

Jayaraman Jayabharathi · Venugopal Thanikachalam · Marimuthu Venkatesh Perumal · Kanagarathinam Saravanan

Received: 6 January 2011 / Accepted: 23 February 2011 / Published online: 18 March 2011

Abstract The mutual interaction of imidazole derivative (PIPP) with bovine serum albumin (BSA) was investigated using photoluminescent studies. The fluorescence quenching mechanism of BSA by PIPP was analyzed and the binding constant was calculated. The binding distance between PIPP and BSA was obtained based on the theory of Forester's non-radiation energy transfer. Displacement experiments were performed by using ibuprofen to identify PIPP binding site in BSA. The effect of some common ions on the binding constant between PIPP and BSA was also examined.

Keywords Bioactive imidazole · FRET · Forester distance · Stern-Volmer Plot · Ibuprofen

Introduction

Recently, heterocyclic imidazole derivatives have attracted considerable attention because of their unique optical properties [1]. These compounds play very important role in chemistry as mediators for synthetic reactions, primarily for preparing functionalized materials [2]. Imidazole nucleus forms the main structure of some well-known components of human organisms, i.e., the amino acid histidine, Vitamin B_{12}, a component of DNA base structure, purines, histamine and biotin and present in structure of many natural or synthetic drug molecules, i.e., azomycin, cimetidine and metronidazole and also have significant analytical applications utilizing their fluorescene and chemiluminescence properties [3].

J. Jayabharathi (✉) · V. Thanikachalam · M. V. Perumal · K. Saravanan
Department of Chemistry, Annamalai University, Annamalainagar, Tamilnadu 608002, India
e-mail: jtchalam2005@yahoo.co.in

Investigations related to proteins are hot research subject in life sciences, pharmacology, chemistry and so on [4–8]. The most abundant protein in blood plasma is serum albumin. One of the significant way of transport of drugs in the blood is binding with serum albumin. The binding ability of drug to the serum albumin in blood may have a considerable impact on distribution, free concentration and metabolism of drugs. Therefore, drug–protein interaction has a considerable significance in pharmacology and the binding parameters are helpful in the study of pharmacokinetics [9]. BSA is highly stable and comparatively cheap and its structure is similar to human serum albumin in 76% [10]. So it is often used to replace human serum albumin in drug–protein studies [4–6].

BSA is composed of a single chain of 582 amino acid residues and can be divided into three homologous domains. Each domain can be subdivided into two subdomains (A and B). The binding sites of BSA for endogenous and exogenous ligands may be in these domains and the principal region of drug binding sites of albumin are often located in hydrophobic cavities in subdomains IIA and IIIA. So called sites I and II are located in subdomain IIA and IIIA of albumin, respectively. Many ligands bind specifically to serum albumin, for example, ketoprofen in site I [11], flufenamic acid and ibuprofen in site II [12]. After having thorough literature survey, we have interested to identify the binding interaction behaviour of a newly designed imidazole derivative namely, 2-(1-phenyl-1 H-imidazo[4,5-f][1, 10]phenanthrolin-2-yl)phenol (PIPP) with BSA. Due to the presence of phenanthroline function with two diiminic nitrogen atoms in PIPP, this imidazole derivative behaves as a highly active Lewis base. We have also exploited the fluorescence resonance energy transfer (FRET) from PIPP to the BSA. The binding characteristic between PIPP and BSA was investigated in

detail by using fluorescence and UV–Vis absorption. The aim of this work is to explore the interaction mechanism, the specific binding site and the effect of PIPP on the conformational changes of BSA.

Experimental

Materials and Methods

1,10-phenanthroline-5,6-dione (Sigma-Aldrich Ltd.), salicylaldehyde (S.D. fine.), aniline (S.D. fine) and all the other reagents used without further purification. Bovine Serum Albumin (BSA) was obtained from Sigma-Aldrich Company, Bangalore. All BSA solution was prepared in the Tris–HCl buffer solution (0.05 mol L^{-1} Tris, 0.15 mol L^{-1} NaCl, pH 7.4) and was kept in the dark at 303 K. Tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol) had a purity of no less than 99.5% and NaCl, HCl and other starting materials were all of analytical purity and doubly distilled water was used throughout.

Optical Measurements and Composition Analysis

NMR spectra were recorded for PIPP on a Bruker 500 MHz The ultraviolet–visible (UV–Vis) spectra were measured on UV–vis spectrophotometer (Perkin Elmer, Lambda 35) and corrected for background due to solvent absorption. Photoluminescence (PL) spectra were recorded on a (Perkin Elmer LS55) fluorescence spectrometer. An MS spectrum was recorded on a Varian Saturn 2200 GCMS spectrometer.

General Procedure for the Synthesis of 2-(1-phenyl-1 H-imidazo[4,5-f][1,10]phenanthro- lin-2-yl)phenol (PIPP)

The experimental procedure was used as the same as described in our recent papers [13–22]. The imidazole derivative, PIPP was synthesized from an unusual four components assembling of 1,10-phenanthroline-5,6-dione, ammonium acetate, aniline and salicylaldehyde (Scheme 1).

2-(1-Phenyl-1 H-imidazo[4,5-f][1,10]phenanthro- Lin-2-yl) phenol (PIPP)

Yield: 55%. mp=289°C, Anal. calcd. for $C_{25}H_{16}N_4O$: C, 77.3; H, 4.15; N, 14.42. Found: C, 76.65; H, 4.08; N, 13.98. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.90 (s, 1 H), 6.63 (d, 1 H), 6.81 (m, 2 H), 7.01–7.16 (m, 1 H), 7.29 (d, 2 H) [*o*-hydroxy phenyl ring], 7.59–7.75 (Aromatic protons of aniline ring), 9.04 (d, 2 H, H-aryl, J=4.5 Hz), 9.10 (d, 2 H, J=8.0 Hz), 9.20 (d, 2 H, J=5.6 Hz). ^{13}C (100 MHz, $CDCl_3$): δ 115.86, 119.42, 121.50, 121.85, 122.40, 122.91, 127.53, 127.61, 128.30, 129.05, 129.72, 130.67, 135.20, 136.40, 137.40, 149.4, 150.00, 154.1, 156.02. MS: m/z 388.13,calcd 388.42.

Results and Discussion

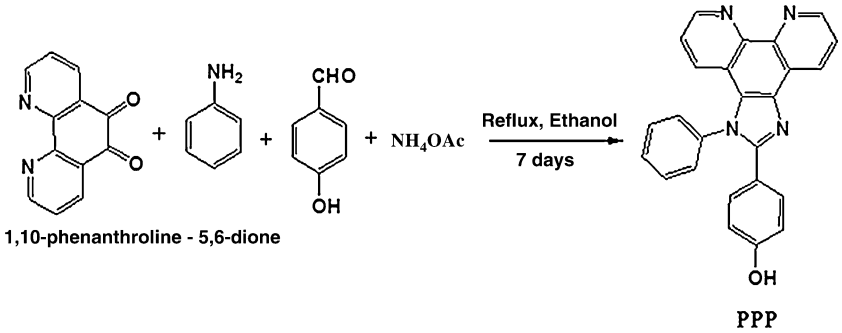
Analysis of Fluorescence Quenching of BSA by PIPP

The fluorescence intensity can be corrected for absorption of exciting light and re-absorption of emitted light using the following relationship [23]:

$$F_{cor} = F_{obs} \times e^{(Aex/Aem/2)} \quad (1)$$

where F_{cor} and F_{obs} are the fluorescence intensity corrected and observed, respectively and A_{ex} and A_{em} are the absorption and emission wavelength, respectively. There exists obvious absorption for PIPP in the range of 270–400 nm. So the intensity of fluorescence used is corrected in this study. There are three types of fluorophores in BSA, namely tryptophan residues (Trp), tyrosine residues (Tyr) and phenylalanine (PA) residues. Although both Trp and Tyr residues can be excited at 280 nm, because of energy transfer effect between amino acid residues, the recorded intrinsic fluorescence of BSA excited at 280 nm mostly comes from Trp residues [24]. In order to determine whether both Trp and Tyr residues are involved in the interaction with PIPP, the fluorescence of BSA excited at 280 and 295 nm, respectively and compared in the presence of PIPP. When 280 nm is used as excitation wavelength, fluorescence of the albumin comes from both Trp and Tyr residues, whereas 295 nm wavelength only excites Trp residues [12]. The plot of F/F_0 against [PIPP]/[BSA] is shown in Fig. 1, which indicates that the fluorescence of BSA excited at 280 nm obviously differs from BSA exciting at 295 nm in the presence of PIPP. This significant difference between quenching of serum albumin fluorescence shows that both Tyr and Trp residues participated in the molecular interaction between PIPP and BSA. At the excitation wavelength of 280 nm, the fluorescence quenching spectra of BSA with growing amounts of PIPP are shown in Fig. 2. The intensity of BSA fluorescence regularly decreased but the maximum emission wavelength of BSA did not apparently shift with the increase of PIPP concentration. Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecules. Under the conditions of fixed pH, temperature and ionic strength, fluorescence quenching may result from ground complex formation, energy transfer and dynamic quenching processes [25]. Dynamic quench-

Scheme 1



ing refers to a process that the fluorophore and the quencher come into contact during the lifetime of the excited state, whereas static quenching refers to fluorophore- quencher complex formation. To shed light on the fluorescence quenching mechanism, the fluorescence quenching data are analyzed by the Stern–Volmer equation [26]:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. K_q is the bimolecular quenching rate constant, τ_0 is the average lifetime of the biomolecule in the absence of quencher, [Q] is the concentration of quencher, and $K_{sv}=K_q/\tau_0$ is the Stern–Volmer quenching constant. The fluorescence lifetime of biopolymer is about 10^{-8} s [27]. The Stern–Volmer plot is shown in Fig. 3, based on the measured fluorescence data determined at room temperature and the corresponding K_{SV} value was calculated as 3.816 x 10^3 (r=0.997). The value of K_q was also obtained in the range of 10^{12} to 10^{13} $M^{-1}s^{-1}$. Obviously, the rate constant of protein quenching procedure initiated by PIPP is much greater than the maximum scatter collision quenching constant of the biomolecule ($K_q=2.0\times10^{10}$ $M^{-1}s^{-1}$) [28]. This means that the possible quenching mechanism of fluorescence of BSA by PIPP is not initiated by dynamic collision but from the formation of a complex.

Binding Constants and Binding Points

The apparent binding constant, K_a, and the number of binding sites, n, can be calculated using the following equation [29]:

$$\log\frac{F0-F}{F} = n\log K_a + n\log\left\{[Q]_t - [P]_t\left(\frac{F0-F}{F0}\right)\right\} \quad (3)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, $[Q]_t$ and $[P]_t$ are the total quencher concentration and the total protein concentration, respectively. The main advantage of this equation is that it uses the total concentration of quencher instead of the free concentration of quencher. K_a and n values were calculated as 1.224×10^4 and 1.13. The correlation coefficient is larger than 0.999 indicating that the interaction between PIPP and BSA agrees well with the site-binding model underlined in the above equation. The value of n (1.13) indicates the existence of one association site.

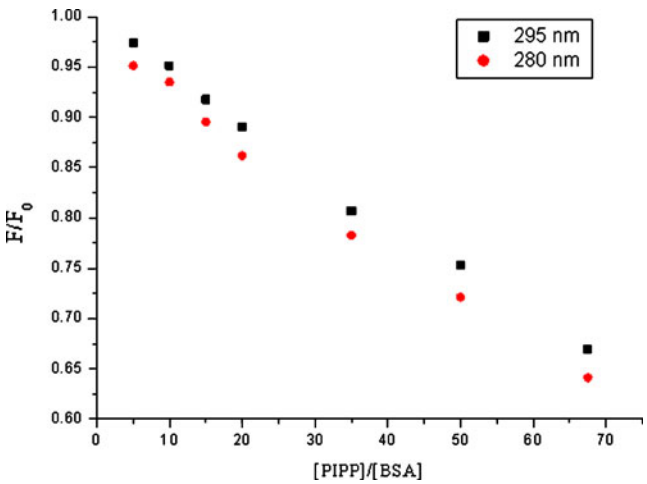


Fig. 1 Plot of F/F_0 versus [PIPP]/[BSA]

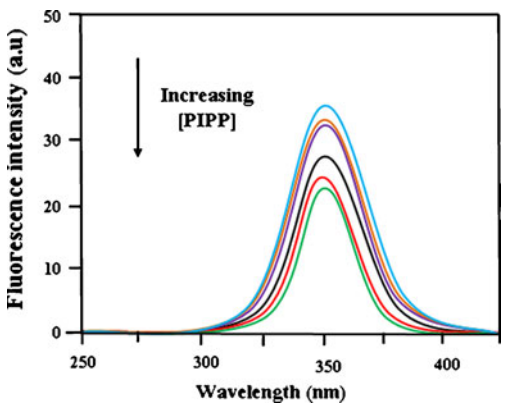


Fig. 2 Fluorescence quenching spectra of BSA with increasing concentration of PIPP

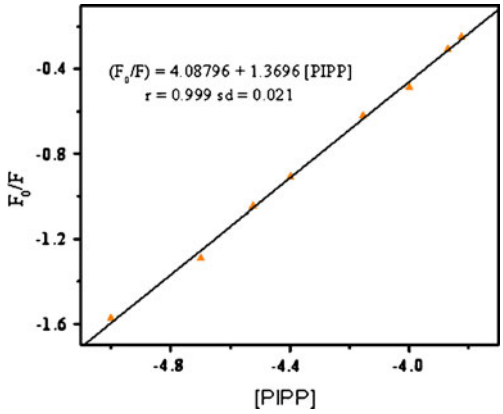


Fig. 3 Stern-Volmer plot of F_0/F versus [PIPP]

Forester Energy Transfer Efficiency from BSA to PIPP

The overlap of the UV–Vis absorption spectrum of PIPP with the fluorescence emission spectrum of BSA is shown in Fig. 4. The importance of the energy transfer in biochemistry is that the efficiency of transfer can be used to evaluate the distance between the PIPP and the tryptophan residues in BSA. According to Forster non-radiative energy transfer theory [30], the efficiency of energy transfer mainly depends on the fluorescence of donor, overlap emission spectrum of donor and absorption spectrum of acceptor and the distance between the donor and the acceptor (usually shorter than 8 nm). Based on the theory of Forster energy transfer, the distance between PIPP and BSA can be calculated by the equation:

$$E = 1 - \frac{F}{F_0} = R_0^6/\left(R_0^6 + r_0^6\right) \quad (4)$$

where r_0 is the distance between the donor and acceptor, R_0 is the Forster's distance or critical energy transfer distance,

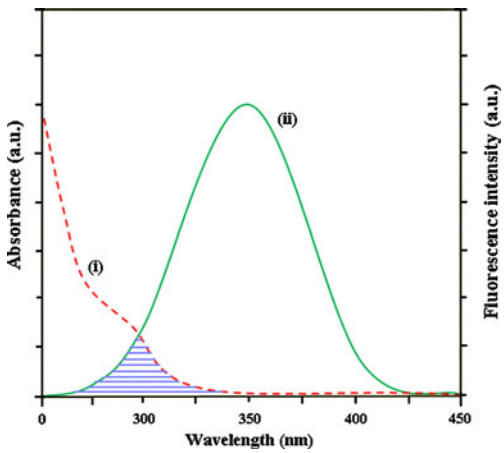


Fig. 4 Overlap of absorption spectrum of PIPP (*i*) with the emission spectrum of BSA (*ii*)

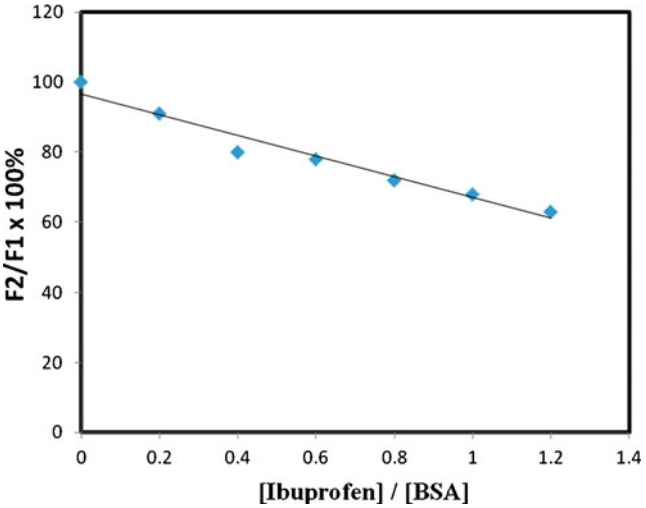


Fig. 5 Plot of F2/F1×100% versus [Ibuprofen]

at which the efficiency of transfer is 50% and R_0 can be calculated as follows:

$$R_0^6 = 8:8 \times 10^{23}\left[\kappa^2 n^{-4}\Phi_D J(\lambda)\right] \text{ in } \text{Å}^6, \quad (5)$$

where κ^2 is the relative orientation of the donor and acceptor molecule, n is the refractive index of the medium, Φ_D is the quantum yield of the donor in absence of the acceptor and $J(\lambda)$ is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. The overlap integral $J(\lambda)$ for a donor-acceptor pair is defined as

$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad (6)$$

where $F_D(\lambda)$ is the corrected fluorescence intensity of the donor at wavelength λ to $(\lambda+\Delta\lambda)$, with the total intensity normalized to unity and $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor at wavelength λ. The Forster distance (R_0) has been calculated assuming random orientation of the donor and acceptor molecules. In the present case, $\kappa^2=$

Table 1 Effects of metal ions on the binding constant of BSA–PIPP

System	Binding constants (-10^4 L mol^{-1})	Error (±) (-10^5 L mol^{-1})
BSA+PIPP	1.224	0.20
BSA+PIPP+Cu^{2+}	1.200	0.26
BSA+PIPP+Zn^{2+}	1.194	0.25
BSA+PIPP+Ca^{2+}	1.163	0.23
BSA+PIPP+Mg^{2+}	1.146	0.18

Number of measurements: 4 times; Temperature: 303 K

2/3, n=1.422, Φ_D=0.62 and from the available data, it results that J(λ)=4.315×10^{-16}, E=0.18, R_0=3.8 nm and r=1.8 nm were calculated. The binding distance r=1.8 nm is less than 8 nm and 0.5 R_0<r<1.5 R_0 which indicates that the energy transfer from BSA to PIPP occurs with high probability [31].

The Displacement Experiment Using Ibuprofen

BSA has a large hydrophobic cavity that can accommodate two or more ligands. When two ligands (denoted with Ligand1 and Ligand2) bind to BSA simultaneously, two types of interaction can occur [32]:

(a) Competitive binding

$$\text{Ligand 1} \xrightarrow{+\text{BSA}} \text{Ligand 1-BSA} \xrightarrow{+\text{Ligand 2}} \text{Ligand 2-BSA} + \text{Ligand 1} \quad (7)$$

(b) Non-Competitive binding

$$\text{Ligand 1} \xrightarrow{+\text{BSA}} \text{Ligand 1-BSA} \xrightarrow{+\text{Ligand 2}} \text{Ligand 2} + \text{BSA-Ligand 1} \quad (8)$$

In order to identify the location of the PIPP binding site on BSA, the displacement experiments were carried out using ibuprofen. PIPP to BSA ratio was kept at 5:1 in order to keep non-specific binding of ibuprofen to a minimum. The percentage of fluorescence probe displaced by the drug was determined by measuring the changes in fluorescence intensity according to the method proposed by Sudlow et al. [33]:

$$F_2/F_1 \times 100\% \quad (9)$$

where F_1 and F_2 denote the fluorescence of the PIPP plus BSA without the ibuprofen and with the ibuprofen, respectively. The fluorescence spectra of the BSA+PIPP mixture solution upon addition of ibuprofen was recorded in the range from 300 to 500 nm upon excitation at 280 nm. The plot of F_2/F_1 against site ibuprofen concentration (Fig. 5) reveals that the fluorescence was remarkably affected by adding ibuprofen to the same solution. This result indicates that ibuprofen displaced PIPP from the binding site.

Effect of Common Ions on the Binding Constants of PIPP–BSA Complex

There exists many metal ions in the blood system and these ions can directly influence the binding force of drug with protein. The effect of metal ions Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} on the binding constants was investigated at 287 K by recording the fluorescence intensity in the range from 300 to 500 nm upon excitation at 280 nm and the concentrations of BSA and metal ions were fixed at 2 μM (Table 1). The competition between the common metal ions and PIPP makes the binding constant of PIPP–BSA decrease as compared to the binding constant without the metal ions. Therefore, the common metal ions will shorten the storage time of the drug in blood plasma, which may lead to need for more doses of PIPP to achieve the desired therapeutic effect [5].

Conclusions

In this paper, the interaction of PIPP with BSA has been investigated by fluorescenc spectrum and UV–vis spectrum and the experimental results indicated the energy transfer from BSA to PIPP occurred. The fluorescence quenching results show that both of *Trp* and *Tyr* residues participated in the interaction between PIPP and BSA and the probable quenching mechanism of fluorescence of BSA by PIPP is a static quenching process. The results also reveal the presence of a single binding site and a complex formation is in 1:1 mole ratio; hydrophobic interaction played a major role in stabilizing the complex. The distance (r) between BSA and PIPP was calculated to be 1.8 nm which indicated the energy transfer with high probability. All the experimental results clarified that PIPP could bind with BSA effectively which could be a useful guideline for imidazole drug design.

Acknowledgment One of the authors Dr. J. Jayabharathi, Associate professor, Department of Chemistry, Annamalai University is thankful to Department of Science and Technology [No. SR/S1/IC-07/2007] and University Grants commission (F. No. 36-21/2008 (SR)) for providing fund to this research work.

References

1. Santos J, Mintz EA, Zehnder O, Bosshard C, Bu XR, Gunter P (2001) New class of imidazoles incorporated with thiophenevinyl conjugation pathway for robust nonlinear optical chromophores. Tetrahedron Lett 42:805–808
2. Kamidate T, Segawa T, Watanabe H, Yamaguchi K (1989) Lophine chemiluminescence for determination of chromium(VI) by continuous flow method. Anal Sci 5:429–433
3. Ucucu U, Karaburun NG, Isikdag I (2001) Synthesis and analgesic activity of some 1-benzyl-2-substituted-4,5-diphenyl-1 H-imidazole derivatives. I L Farmaco 56:285–290
4. Kandagal PB, Ashoka S, Seetharmappa J (2006) Study of the interaction of an anticancer drug with human and bovine serum albumin: Spectroscopic approach. J Pharmaceut Biomed 41:393–399
5. Cheng Z, Zhang Y (2008) Spectroscopic investigation on the interaction of salidroside with bovine serum albumin. J Mol Struct 889:20–27
6. Ni Y, Liu G, Kokot S (2008) Fluorescence spectrometric study on the interactions of Isoprocarb and sodium 2-isopropylphenate with bovine serum albumin. Talanta 76:513–521
7. Timerbaev AR, Hartinger CG, Aleksenko SS, Keppler BK (2006) Interactions of antitumor metallodrugs with serum proteins: Advances in characterization using modern analytical methodology. Chem Rev 106:2224–2248
8. Gentili PL, Ortica F, Favaro G (2008) Static and dynamic interaction of a naturally occurring photochromic molecule with bovine serum albumin studied by UV-visible absorption and fluorescence spectroscopy. J Phys Chem B 112:16793–16801
9. Leslie C, Scott CJW, Caird FI (1992) Principal alterations to drug kinetics and dynamics in the elderly. Med Lab Sci 49:319–325
10. El Kadi N, Taulier N, Le Huerou JY, Urbach W, Nwigwe I, Kahn PC, Waks M (2006) Unfolding and refolding of bovine serum albumin at acid pH: ultrasound and structural studies. Biophys J 91:3397–3404
11. He XM, Carter DC (1992) Atomic structure and chemistry of human serum albumin. Nature 358:209–215
12. Sjoholm I, Ekman B, Kober A, Ljungstedt-Pahlman I, Seiving B, Sjodin T (1979) Binding of drugs to human serum albumin: XI. Mol Pharmacol 16:767–777
13. Jayabharathi J, Thanikachalam V, Saravanan K, Srinivasan N (2010) Iridium(III) complexes with orthometalated phenylimidazole ligands subtle turning of emission to the saturated green colour. J Fluoresc. doi:10.1007/s10895-010-0737-7
14. Saravanan K, Srinivasan N, Thanikachalam K, Jayabharathi J (2010) Synthesis and photo physics of some novel imidazole derivatives used as sensitive fluorescent chemisensors. J Fluoresc. doi:10.1007/s10895-010-0690-5
15. Gayathri P, Jayabharathi J, Srinivasan N, Thiruvalluvar A, Butcher RJ (2010) 2-(4-Fluorophenyl)-4,5-dimethyl-1-(4-methylphenyl)-1 H-imidazole. Acta Crystallogr E 66:o1703
16. Gayathri P, Thiruvalluvar A, Saravanan K, Jayabharathi J, Butcher RJ (2010) 4,5-Dimethyl-1,2-diphenyl-1*H*-imidazole monohydrate. Acta Crystallogr E 66:o2219
17. Gayathri P, Jayabharathi J, Srinivasan N, Thiruvalluvar A, Butcher RJ (2010) 2-(4-Fluorophenyl)-1,4,5-triphenyl-1*H*-imidazole. Acta Crystallogr E 66:o2519
18. Jayabharathi J, Thanikachalam V, Srinivasan N, Saravanan K (2010) Synthesis, structure, luminescent and intramolecular proton transfer in some imidazole derivatives. J Fluoresc. doi:10.1007/s10895-010-0747-5
19. Jayabharathi J, Thanikachalam V, Saravanan K, Srinivasan N, Venkatesh Perumal M (2010) Physiochemical properties of organic nonlinear optical crystal from a combined experimental and theoretical study. Spectrochim Acta A. doi:10.1016/j.saa.2010.12.026
20. Jayabharathi J, Thanikachalam V, Jayamoorthy K, Venkatesh Perumal M (2010) A physiochemical study of excited state intramolecular proton transfer process Luminescent chemosensor by spectroscopic investigation supported by ab initio calculations. Spectrochim Acta A. doi:10.1016/j.saa.2010.12.027
21. Gayathri P, Jayabharathi J, Srinivasan N, Thiruvalluvar A, Butcher RJ (2010) 4,5-Dimethyl-2-phenyl-1-(*p*-tolyl)-1*H*-imidazole. Acta Crystallogr E 66:o2826
22. Jayabharathi J, Thanikachalam V, Saravanan K (2009) Effect of substituents on the photoluminescence performance of Ir(III) complexes: Synthesis, electrochemistry and photophysical properties. J Photochem Photobiol A Chem 208:13–20
23. Anbazhagan V, Renganathan R (2008) Study on the binding of 2,3-diazabicyclo[2.2.2]oct-2-ene with bovine serum albumin by fluorescence spectroscopy. J Lumin 128:1454–1458
24. Sulkowska A, Maciazek-Jurczyk M, Bojko B, Rownicka J, Zubik-Skupien I, Temba E, Pentak D, Sulkowski WW (2008) Competitive binding of phenylbutazone and colchicines to serum albumin in multidrug therapy: A spectroscopic study. J Mol Str 881:97–106
25. Bhattacharyya M, Chaudhuri U, Poddar RK (1990) Evidence for cooperative binding of chlorpromazine with haemoglobin. Biochem Biophys Res Commun 167:1146–1153
26. Lakowicz JR (1999) Principles of Fluorescence Spectroscopy, 2nd edn. Plenum Press, New York, pp 237–265
27. Dewey TG (1991) Biophysical and Biochemical Aspects of Fluorescence Spectroscopy. Plenum Press, New York, pp 1–41
28. Ware WR (1962) Oxygen quenching of fluorescence in solution: an experimental study of the diffusion process. J Phys Chem 66:455–458
29. Rasoulzadeh F, Jabary HN, Naseri A, Rashidi MR (2009) Fluorescence quenching study of quercetin interaction with bovine milk xanthine oxidase. Spectrochim Acta A 72:190–193
30. Forster T, Sinanoglu O (1966) Modern Quantum Chemistry, Vol. 3. Academic, New York, p 93
31. Valeur B, Brochon JC (1999) New Trends in Fluorescence Spectroscopy, 6th edn. Springer, Berlin, pp 25–28
32. Ni Y, Zhang X, Kokot S (2009) Spectrometric and voltammetric studies of the interaction between quercetin and bovine serum albumin using warfarin as site marker with the aid of chemometrics. Spectrochim Acta A 71:1865–1872
33. Sudlow G, Birkett DJ, Wade DN (1976) Further characterization of two specific binding sites of human serum albumin. Mol Pharmacol 12:1052–1061