



Binding Study of Naturally Occurring Flavonoids with Bovine Serum Albumin: A Fluorescence Quenching study

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Accepted on: 15-12-2013; Finalized on: 28-02-2014.

ABSTRACT

We present here the binding behavior of pharmaceutically important naturally occurring flavonoids Biochanin A, morin hydrate and 3,6-dihydroxy flavone with two homologous serum albumin, bovine (BSA), in aqueous pH 7.4 buffer using optical spectroscopic techniques. Protein intrinsic fluorescence quenching by flavonoids occur through ground-state static interaction and strong binding occurs. Site-marker competitive binding shows that flavonoids bind primarily to site I subdomain II of serum albumin.

Keywords: Bovine serum albumin, Flavonoids, Fluorescence, Quenching.

INTRODUCTION

Plants are good source of pharmaceutically and dietary important polyphenols such as flavonoids, which are used as a food supplement since ancient times. They are commonly present in fruits, wine, vegetables, nuts, beverages including green tea and contain wide range of therapeutic activities such as anticancer, antimicrobial, anti-allergic, anti-inflammatory, antibacterial, anticancer and antioxidant properties are widely studied.¹ The basic scaffold of flavonoids contains 2,3-double bond in conjugation with a 4-oxo group and 1,2-dihydroxybenzene (the catechol unit) is a critical for its antioxidant properties.² Further, presence of hydroxyl groups on various positions in different rings help in protein interaction which plays key role in various biological activities. Recently, sincere efforts have been made to understand the interactions between flavonoids and plasma proteins.³

Serum albumin is the most abundant plasma proteins in the circulatory system which maintains pH of body liquid and interacts reversibly with biomolecules such as lipids, amino acids, drugs and inorganic ions.⁴ BSA is homologous to HSA, having about 88% sequence homology, and consists of three linearly arranged domains (I–III) that are composed of two subdomains (A and B).⁵ There are two tryptophan residues (Trp134 and Trp213), of which Trp134 is located on the surface of the molecule and Trp213 resides in the hydrophobic pocket/fold. Similarly, the only tryptophan residue (Trp214) of HSA is also located in the hydrophobic pocket.⁶ The environments of two tryptophan residues in BSA are different from each other, and thus the study of their interactions with small molecules can provide useful insights to understanding the environment-dependent molecular interactions. Moreover, there is evidence of conformational changes of serum albumin induced by its interaction with low molecular weight dyes and drugs, which appear to affect secondary and tertiary structures of proteins.⁷

Researches on flavonoids are largely focused on two aspects: (a) first concerns with their biological and relevant therapeutic applications and (b) investigation of unusual properties of flavonoids e.g. fluorescence, extreme sensitivity of emission parameters.

Our laboratory involves design, synthesis and characterization of antimalarial,⁸ antifilarial⁹ and X-rays analysis of small molecules.¹⁰ To extend the diversification in research work, we choose three flavones viz. 3,6-dihydroxy flavones, Biochanin A (O-methylated isoflavone) and morin hydrate having different number of hydroxyl groups and studied their interaction with bovine serum albumin. This study may provide valuable information related the biological effects and importance of biologically active and therapeutic effect of flavonoids in pharmacology and pharmacodynamics.

MATERIALS AND METHODS

Apparatus and Chemicals

All fluorescence measurements were carried out on Fluoromax 4 (Spex) spectrofluoro photometer equipped with a xenon lamp source and quartz cells (1.0 cm). UV spectra were recorded on GBC Cintra UV-vis spectrophotometer (Australia) equipped with quartz cells.

Bovine serum albumin, tryptophan, 3,6-dihydroxyflavone, 5,7-dihydroxy-4'-methoxyflavone (Biochanin A) and Morin Hydrate (purity 99%, crystallized and lyophilized) were obtained from Sigma-Aldrich, USA and were used in the experiment without further purification. HPLC grade solvents were procured from the Spectrochem Limited India, and used directly in the experiments. All other reagents and solvents were of analytical grade and used without further purification unless otherwise stated. All aqueous solutions were prepared in freshly double-distilled water.



UV-Vis Spectra

UV-vis studies were performed on a GBC Cintra UV-vis spectrophotometer (Australia) at 25°C in the range of 250-500 nm using quartz cells with 1cm path length.

Fluorescence Spectra

The three flavones viz. 3,6-dihydroxyflavone, 5,7-dihydroxy-4'-methoxyflavone (Biochanin A) (figure 1) and morin hydrate were insoluble in water. A stock solution of

10mM of each flavones in methanol were first prepared and then diluted with 5mM aqueous Na₂HPO₄ solution till reached a clear solution of 100µM so that the methanol content in the solution did not exceed 1%. The stock solution of 0.1 Malbumin was prepared by using 0.1 M phosphate buffer. The fluorescence emission spectra were recorded in the range of 300-600 nm using a slit width of 5 nm. The BSA was excited at 280 nm and the fluorescence intensity at 350 nm was determined.

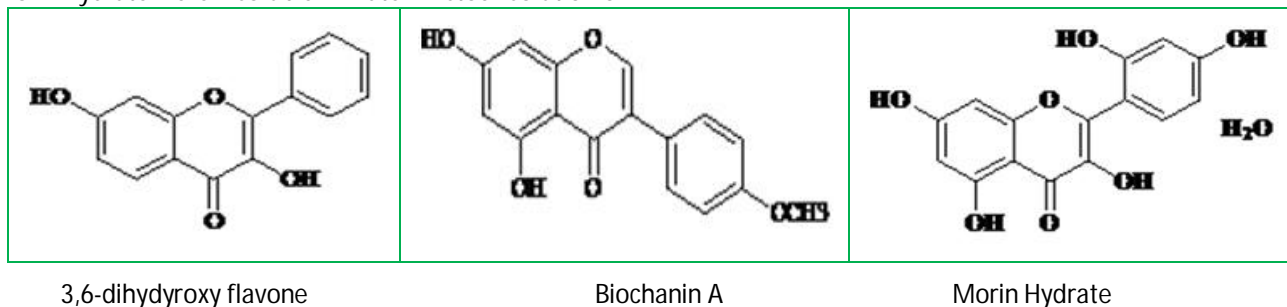


Figure 1: Chemical structure of flavonoids

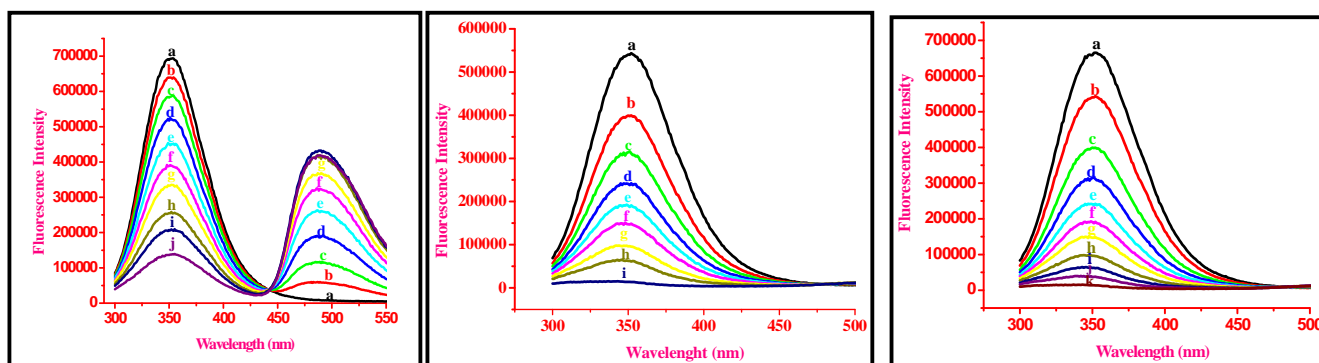


Figure 2 a, b & c: The quenching effect of flavonoids on BSA fluorescence intensity $\lambda_{ex} = 280$ nm; BSA, 1.00×10^{-6} M

RESULTS AND DISCUSSION

Absorption Spectroscopy

UV-Vis absorption measurement is a simple but effective method of detecting complex formation. In the case of static quenching, a dark complex is formed between ground state of fluorescent substance and the quencher, and thereby the fluorescence quantum yield is reduced.¹¹ Therefore, the absorption spectra of fluorophore would be affected as a result of complexation. The λ_{max} of BSA is 271 nm, absorbance intensity increased upon addition of Biochanin A without showing any peak shift. Also, isosbestic point at 281 nm is indicative of a ground-state equilibrium system. Similar ground-state protein-flavonoid has also been observed with morin hydrate-BSA and 3,6-DHF-BSA (data is not shown).

Fluorescence Studies

Fluorescence experiment was carried out to study interaction between BSA and various flavonoids. To study this, BSA (10µM, 2mL) concentration was kept constant while concentration of three flavonoids viz. 3,6-dihydroxyflavone (3,6-DHF) (0.05mL, 2mM), morin hydrate (0.05mL, 2mM) and Biochanin A (0.05mL, 2mM)

were added gradually to make the final concentration of BSA and substrate 1:1 ratio. In absence of flavones, BSA was excited at 280 nm and emission was recorded at 350 nm.¹² On gradual addition of 3,6-dihydroxyflavone to BSA showed decrease in fluorescence intensity while appearance of new peak at 500nm. Quenching of protein intrinsic fluorescence was employed in this study for more detailed study of flavonoid-BSA binding. Emission spectra were recorded upon excitation at 280 nm which is attributed to tryptophan residues only.¹³

Flavonoid-BSA systems were read at emission wavelength of 350 nm which is the emission maximum of BSA. Protein solution was titrated by the successive addition of three flavones, (3,6-dihydroxyflavone (3,6-DHF) (figure 2a) morin hydrate (figure 2b) and Biochanin A (figure 2c). It was observed that fluorescence intensity gradually decreased with increasing concentration of flavonoids as shown in figure 2. This observation suggested that the microenvironment around the tryptophan residues of BSA was changed due to the interaction with flavonoids.

Further, emission spectra of 3,6 dihydroxy flavone showed isosbestic point which might indicate that the flavone exists both in bound and free form with BSA and

are in equilibrium at excited state. The bound form exerts fluorescence whereas the unbound form does not as shown in figure 2a. The 3,6-hydroxyflavone. Moreover, a red shift of tryptophan emission maximum at 340nm was found on increasing the concentration of biochanin A in BSA solution as shown in figure 2c. Here, emission maximum was slightly shifted towards longer wavelength by 4 nm for Biochanin A-BSA system. This study showed that efficient binding of flavones with BSA result non-fluorescence co-complex. Considering the effect of Biochanin A on the fluorescence spectra of BSA, there was weak blue shift of λ_{em} was observed. This observation suggests that flavonoid interacts with BSA which changes the local environment of tryptophan that resulted in BSA quenching. Similar quenching results were obtained in case of other flavonoid under study.

Quenching Of BSA Fluorescence By Flavones

The quenching of BSA fluorescence by three flavones is shown in figure 3. It is evident from the graph that at the same concentration (1.0×10^{-5} M), MH and BiochaninA showed 90 % while 3,6-DHF showed 75% quenching of BSA at room temperature. We concluded from these results that the intensity of BSA tryptophan fluorescence decreased rapidly with the increase concentration of morin hydrate and Biochanin A, while decreased slowly in case of 3, 6-DHF.

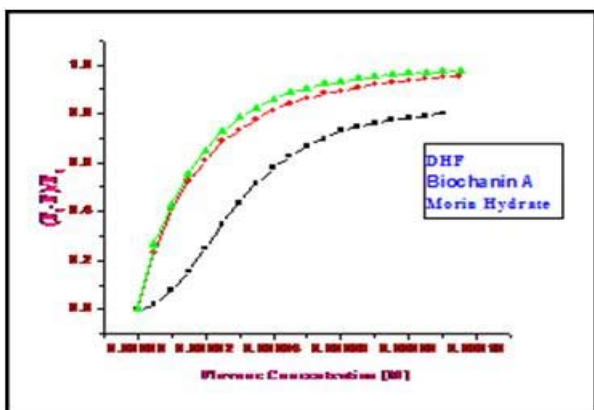


Figure 3: Quenching ratio ((F₀-F)/F₀) of BSA with addition of morin hydrate, biochanin A and 3,6-dihydroxy flavone.

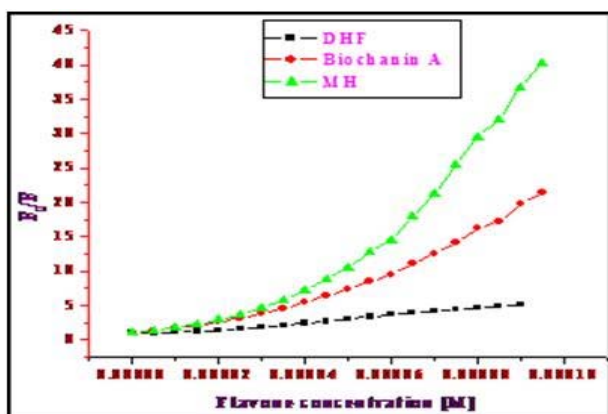


Figure 4: The Stern-Volmer plots for the BSA fluorescence quenching by 3,6Dihydroxy flavone, Biochanin A, Morin hydrate.

A deviation from linearity towards x-axis indicates the presence of two fluorophores, and one class is not accessible to quencher.¹⁴ Figure 4 shows the Stern-Volmer plots for the BSA fluorescence quenching by morin hydrate, biochanin A and 3,6-DHF. An upward curvature i.e. concave towards y-axis at high quencher concentration in Stern-Volmer plot is common in this case. A modified Stern-Volmer equation is used to relate F/F₀ with [M].

$$F_0/F = (1 + K_D[M]) (1 + K_S[Q]) \dots \dots \dots (1)$$

Where K_D and K_S are dynamic and static quenching constants, respectively.

In our research, both dynamic and static quenching were involved for morin hydrate and biochanin A, which was demonstrated by the fact that Stern-Volmer plot deviated from linearity towards y-axis at high flavone concentrations.

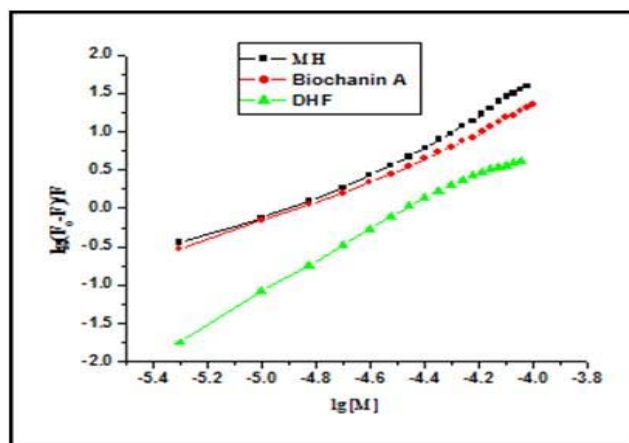


Figure 5: Double-log plot of MH, Biochanin A & DHF quenching effect on BSA

It can be concluded from these results that number and position of hydroxyl group play an important role in binding of flavonoids and BSA. But 3,6-DHF, having a different structure from morin hydrate and biochanin A in terms of B ring substitution had different binding model with BSA i.e. deviates from linearity towards x-axis at high concentration. From these results we can conclude that in both morin hydrate and biochaninA complex and collision formation occur. Also, presence of hydroxyl and methoxy group in ring B in morin hydrate and biochanin A respectively play an important role in binding with BSA. Dangles and his group also perform the same experiment with quercetin an isomer of morin hydrate and found similar results.¹⁵ In case of 3, 6-DHF, A ring hydroxylation play crucial role in binding.¹⁶ Thus, both A and B ring hydroxylation are important for quenching of BSA.

Evaluation of Binding Constant and Binding Site Number

It is well documented that binding of quencher to protein can be determined both theoretically and practically The fraction of drug bound, θ , was determined using the equation,¹⁷

$$\theta = (F_0 - F) / F \dots \dots \dots (2)$$

Where, F and F_0 denote the fluorescence intensities of serum protein in a solution with a given concentration of drug and without drug, respectively. The θ represents the fraction of the site on the protein occupied by drug molecule. Figure 5 shows the double-logarithm curve of flavones quenching BSA fluorescence and Table 1 summarizes the corresponding calculated results. From Table 1, the apparent binding constant and binding site value increases on hydroxylation on the ring A and B in morin hydrate.

Table 1: The binding constant and binding site number of flavones on BSA

Flavones	$\log_{10}K_a$	n
3,6 DHF	3.05	0.6
Biochanin A	4.7	0.84
Morin Hydrate	4.7	0.77

In biochaninA binding constant is equal to morin hydrate but binding site number increases in presence of methoxy group in B-ring. Xiao et al also studied the influence of hydroxylation on B-ring on flavonols with BSA and also found similar results.¹⁸ This, suggest that in native protein hydrophobic groups are present in the interior of the tertiary structure and hydrophilic groups on the outer surfaces. In case of morin hydrate hydrogen bonding takes place between the –OH groups in flavonols and the BSA polar groups on surfaces.¹⁹

CONCLUSION

In this article the binding of three flavones viz. 3,6-DHF, Biochanin A and morin hydrate to bovine serum albumin at pH 7.4 has been investigated by employing different optical spectroscopic techniques. The natures of quenching curve differ with respect to flavonoids used. In BSA near saturation behavior was observed at higher flavonoid concentration after initial quenching. Difference in accessibility of two tryptophans (Trp 213 and Trp 134) for quenching by flavonoids is found to be responsible for such biphasic binding nature for the flavonoid-BSA system. The upward curvature of Sten-Volmer plot in BSA is most likely due to large extent of quenching. Absorption spectroscopy and the magnitude of bimolecular quenching constant indicate that the primary mode of binding is static.

Acknowledgements: SKA is thankful to Department of Science and technology (DST), University Scientific Instrumentation Centre (USIC) University of Delhi for financial support. SS is thankful to UGC (Scheme no 37-410/2009) for financial assistance.

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Source of Support: Nil, Conflict of Interest: None.

