DETERMINATION OF THE BINDING PARAMETERS OF FLURBIPROFEN TO HUMAN SERUM ALBUMIN BY MULTIWAVELENGTH MOLECULAR FLUORESCENCE AND COMPUTATIONAL ANALYSIS

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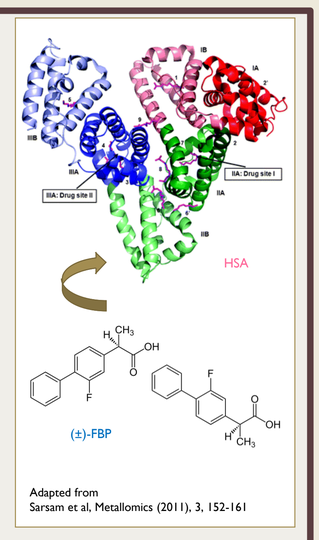
INTRODUCTION

(±)-Flurbiprofen (FBP) is a non-steroidal anti-inflammatory (NSAID) drug used to treat pain or inflammation caused by osteoarthritis or rheumatoid arthritis, between others. The bioavailability of this drug resides in its affinity for human serum albumin (HSA), the main drug transport protein. Ideally, drug-albumin interaction must be strong enough for allowing the transport through the bloodstream and weak enough to release the drug in the target where the therapeutic effect is intended. There are numerous binding sites on albumin, but drugs and other exogenous compounds bind, mainly, two of them: Sudlow I, placed on subdomain IIA, and Sudlow II, located on subdomain IIIA. Previous works with NSAIDs indicate that the main binding site for this molecules is Sudlow II, although they have also shown weaker affinities for other regions of the protein [1]. Crystallographic data and molecular docking have provided relevant information to support experimental results [2].

One of the most widespread techniques for determining the binding parameters between drugs and albumin is molecular fluorescence (FS). Usually, albumin fluorescence quenching is modeled by the double logarithm Stern Volmer (DLSV) equation and binding parameters (n, K_b) are obtained [3]. However, this model assumes that the sole fluorophore is the protein.

$$\log \frac{F_0 - F}{F} = \log K_b + n_H \log[Q]$$

The extended version of the STAR program allows the data treatment of the full fluorescence spectra at the different fluorescence modes of work simultaneously (emission and/or synchronous modes), calculating the binding constants and the unitary spectrum of the species formed [4]. In this way, the drawbacks owed to the application of the DLSV equation can be overcome. On the other hand, the fluorescence experimental data have been treated by Parallel Factor Analysis (PARAFAC) [5] in order to compare the spectra obtained with this tool and those given by STAR. Additionally, crystallographic data available for other NSAIDs and molecular docking of this family of compounds will be helpful to support the results and to evaluate the most probable binding sites.



EXPERIMENTAL METHODS AND DATA TREATMENT

(±)-FBP (300 μM, phosphate buffer pH 7.4 I=150 mM)
Successive volume additions

HSA (3 μM, phosphate buffer pH 7.4 I=150 mM)

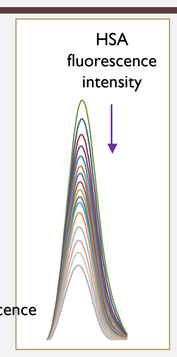
Stirring for 3 min

25/32/37 ºC

Cary Eclipse fluorescence spectrometer (Agilent)

- 1 cm path length quartz cuvette
- Scan: Emission (λ_{exc}=285 nm) and synchronous at $\Delta\lambda$ = 15 and 60 nm modes

Theoretical spectra due to HSA fluorescence quenching after drug additions



	n ?	K_b ?
DLSV approach	✗	✗
STAR program (extended version)	✓	✓
PARAFAC (Parallel Factor Analysis)	✓	✗

Binding sites? → NSAIDs crystallographic data; FBP – HSA interaction modelling

RESULTS AND DISCUSSION

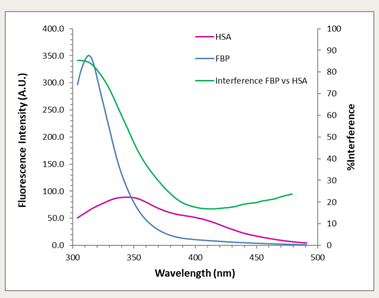


Fig 1. Spectral inferences of FBP over HSA fluorescence signal

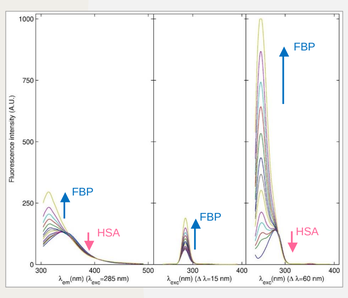


Fig 2. Spectra obtained after the successive additions of FBP over HSA

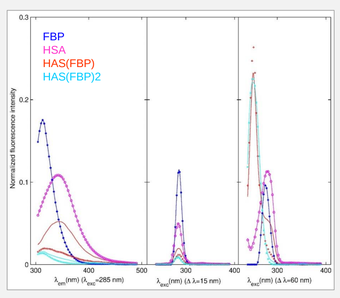


Fig 3. Calculated unitary spectra of the four species detected using STAR (lines) and PARAFAC (symbols)

Reference	n_1	log K_{b1}	n_2	log K_{b2}	Medium and temperature	Technique
This work	1	4.91 (0.01)	2	5.53 (0.01)	PBS 10 mM pH 7.4, 20ºC	FS
This work	1	4.96 (0.02)	2	5.52 (0.01)	PBS 10 mM pH 7.4, 25ºC	FS
This work	1	4.98 (0.02)	2	5.22 (0.01)	PBS 10 mM pH 7.4, 37ºC	FS
J. Chromatogr. B, 661 (1994), 335-340	-	5.11	-	3.83	PBS 10 mM pH 7.0	CE
J. Pharm. Biomed. Anal., 16 (1997), 223-229	0.97	5.94	5.53	4.28	PBS pH 7.4	ED
Mol. Pharmacol., 24 (1983), 458-463	0.90	4.53	-	-	PBS 100 mM pH 7.4	ED
Mol. Pharmacol., 18 (1980), 421-426	1.30	6.61	-	-	PBS 50 mM pH 7.4	ED
Mol. Pharmacol., 18 (1980), 421-426	1.00	6.70	-	-	PBS 50 mM pH 7.4	PAM
Mol. Pharmacol., 25 (1984), 137-150	-	6.08	-	5.15	PBS 66 mM pH 7.4	ED
Int. J. Pharm., 62 (1990), 21-25	1.19	6.14	7.69	3.95	PBS 67 mM pH 7.4	ED
Talanta (2014), 241-250	0.71	6.70	4.8	4.78	HEPES 50 mM pH 7.4	ITC
Talanta (2014), 241-250	-	-	5.0	4.54	HEPES 50 mM pH 7.4	CE

Table. HSA-flurbiprofen binding parameters reported using different experimental conditions and techniques.
CE: Capillary electrophoresis; ED: Equilibrium dialysis; PAM: partition/HSA microparticles, ITC: Isothermal titration calorimetry

(±)-FBP is a fluorophore and interferes with HSA signal (Fig. 1, 50-85% depending on the fluorescence mode at HSA λ_{max}). When performing the binding experiments (i.e. Fig. 2) it is observed how FBP intensity increases after successive additions and overlaps with HSA signal that would decrease, as observed for other non-interfering NSAIDs.

As interferences are over 10%, DLSV approach for FBP-HSA binding study is not applicable.

Program STAR calculates the equilibrium constants and the unitary spectra of each species from spectrometric data by using a Gauss-Newton iterative procedure. It is based on a given model (number of chemical species, stoichiometric coefficients and guessed binding constants) and several constraints (mass balances of each component and nonnegative unitary spectra). PARAFAC is a chemometric procedure, which decomposes the data matrix (spectral data) in two matrices (loadings and scores) related with the concentration and unitary spectra of each species.

The results of both procedures (STAR and PARAFAC) indicate that the system could be described by four fluorescent species: HSA, FBP and two additional species. The additional species were assigned to HSA(FBP) and HSA(FBP)2 by using STAR, together with the binding constants and the corresponding unitary spectra. The PARAFAC results (Fig. 3) are compatible with those obtained by STAR, where the normalized spectra obtained by both procedures are compared. The spectra of HSA, FBP and HSA(FBP)2 are in good agreement, but not in the case of HSA(FBP); this fact can be explained because the low formation of this species and the high spectral overlapping.

The two different interactions detected show very similar affinity for HSA sites (Table). Temperatures tested have not shown significant differences and hence other thermodynamic parameters could not be calculated using the van't Hoff equation. Literature reports similar interaction values by using complementary techniques (Table). Also, it indicates that other binding sites with higher (log K_b~6.5) and lower (log K_b~4) affinity for FBP than the ones found by fluorescence would also exist.

Docking computations reveal that (*S*)-FBP (Fig. 4 left) binds to site IIIA matching the arrangement observed for (*S*)-naproxen and (*S*)-ibuprofen in this cavity, enabling the formation of a hydrogen bond between the carboxylate group of (S)-FBP and the hydroxyl group of Tyr411. This interaction should have little quenching effect on the fluorescence of HSA, due to the large distance from Trp214. On the other hand, docking in IIA and IIC leads to poses with lower scores. It is worth noting that (*S*)-FBP exhibits a large overlap with the X-ray crystallographic pose of (*S*)-naproxen at site IIC, and diflunisal at site IIA (Figure 6). Finally, docking of (S)-FBP in site IIA-IIB leads to a binding mode that superposes well with diflunisal. With regard to (*R*)-FBP (Fig. 4 right) , site IIC appears to be the best binding site, leading to a close overlap of both the carboxylate moieties and aromatic rings of both (*R*)-FBP and (*S*)-naproxen. Lower scores are obtained for the binding to sites IIIA, IIA and IIA-IIB.

Taking into account the analysis reported for other NSAIDs, present results suggest that the strongest binding site observed from complementary techniques (log K_b~6.5) may primarily correspond to the binding of FBP to site IIIA. The two binding signals determined by FS and others, may arise from the interaction of the two enantiomers at sites IIA or IIA-IIB. Docking calculations suggest a favorable binding to site IIC. Probably, FBP is preliminarily bound to site IIA and next, with slower kinetics, would migrate to site IIC.

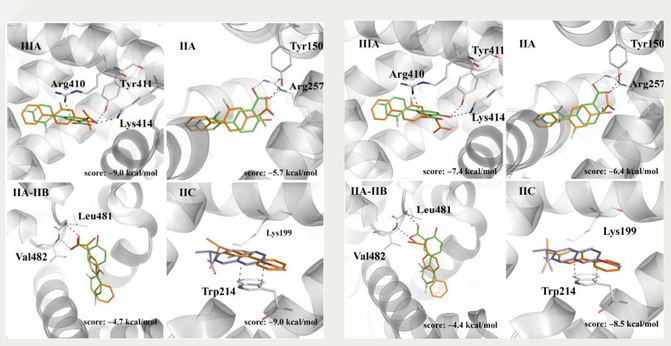


Fig 4. Predicted poses of (*S*)-FBP (left) and (*R*)-FBP (right) bound to sites IIIA, IIA, IIA-IIB, and IIC. The crystallographic poses of diflunisal (bound at sites IIIA, IIA, and IIA-IIB; PDB entry 2BXE), and (*S*)-naproxen (bound at site IIC; PDB entry 4OR0) are shown as sticks (C atoms in green and dark blue, respectively). The docked pose of (*S*) and (*R*)-FBP is shown with C atoms as orange sticks. The protein backbone is displayed as a gray cartoon. Selected interactions with HSA residues are represented as dashed lines.

CONCLUSIONS

- FBP-HSA binding secondary sites can be selectively evaluated by FS and using the STAR program to deal with the interferences caused by the drug itself. Two different binding events log K_b~5 detected may correspond to the interaction of each of the isomers with HSA sites.
- Docking indicates that there would be a primary binding site common with other NSAIDs, site IIIA, and other secondary ones. Due to the flexibility of HSA, those evaluated by FS may correspond to the binding to sites IIA, IIA-IIB or IIC.

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