MOLECULAR MODELING AND EXPERIMENTAL INVESTIGATIONS OF THE INTERACTION BETWEEN FLUOXETINE AND BETA-CYCLODEXTRIN IN AQUEOUS SOLUTION

CARMEN N. PURDEL#, MARINELA FLOREA*, CLAUDIA M. GUȚU*, RUCSANDRA DĂNCIULESCU MIULESCU**, MIHAELA ILIE*, CORINA CRISTINA ARAMĂ**

*Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania,
#e-mail: carmenpurdel@gmail.com
**"N.C. Paulescu" National Institute of Diabetes, Nutrition and Metabolic Diseases, Bucharest, Romania

Abstract. Cyclodextrin and their derivatives have the property of forming inclusion complexes with guest molecules having suitable characteristics of polarity and dimension. The paper presents two approaches to explore the possible interaction between fluoxetine and beta-cyclodextrin in aqueous solution: the molecular analysis *in vacuo* and the experimental characterization of the inclusion complex based on its stability and luminescence characteristics. The molecular modeling predicted the interaction, while fluorescence experiments confirmed it. The spectral characteristics, investigated by fluorescence spectrometry, revealed that the molecular aggregate presents an enhancement of the fluorescence emission at 292 nm, compared with the free ligand or cyclodextrin. The fluorescence intensity of the molecular aggregate depends on the beta-cyclodextrin concentration, but it is not affected by the presence of the buffer or its pH value. The molar ratio of the molecular aggregate 1:1 was established using the continuous variation method, while the association constant calculated using Benesi-Hildebrand's method corresponds to 17.67 mol^{-1}. Both approaches proved to be useful tools for this type of physical interaction.

Key words: Fluoxetine, molecular aggregate, beta-cyclodextrin, molecular modeling, spectrofluorimety.

INTRODUCTION

Cyclodextrins are cyclic oligosaccharides composed of six, seven or eight α-1,4-linked glucose residues and are characterized by a truncated cone shape. In their cavity, the cyclodextrins can include guest molecules (so-called ligands) having suitable characteristics of polarity and dimension [18, 19]. The inclusion of the ligands within the cyclodextrins cavity was extensively investigated [13, 14, 16].

Received: June 2014;
in final form July 2014.

ROMANIAN J. BIOPHYS., Vol. 24, No. 2, P. 75–87, BUCHAREST, 2014

Rather than being a classical hydrophobic effect, complexation by cyclodextrins is governed by several factors, the most important of which are van der Waals and hydrophobic interactions, hydrogen bonding interactions and conformational changes or strain release of the cyclodextrin molecule upon molecular encapsulation [1, 5, 7, 9].

Moreover, the non-radiative decay processes of the molecules introduced into their hydrophobic internal cavity are often significantly attenuated, and the fluorescence emission increases [6].

The interaction between cyclodextrins and ligands may occur in the solid state or in solution and can be predicted using molecular modeling approach (docking algorithm). The generation of molecular conformations and the evaluation of interaction potentials are common tasks in molecular modeling applications, particularly in protein−ligand or protein−protein docking programs, but can be extrapolated to other classes.

Fluoxetine hydrochloride (FLU) is an important antidepressant drug for the treatment of unipolar mental depression, obsessive-compulsive disorder, bulimia and panic disorder [3, 11, 15]. It enhances serotoninergic neurotransmission through potent and selective inhibition of presynaptic serotonin reuptake.

The interactions between fluoxetine and different cyclodextrins were investigated in the field of pharmacology [4, 10] or analytical chemistry [8, 17].

In the present article, we used two approaches to investigate the possible interaction between fluoxetine and beta-cyclodextrin (b-CD) in aqueous solution: the molecular modeling and the experimental characterization of the molecular aggregate based on its stability and luminescence characteristics. Both approaches are useful to get more insight into this type of physical interaction.

MATERIALS AND METHODS

MOLECULAR MODELING

In order to obtain the most stable conformations of fluoxetine and beta-cyclodextrin (with minimum internal energy), conformational studies for both structures were conducted by using the Conformational Search program from HyperChem Professional package 7.5. [20].

The following steps were run over: (1) for the FLU and b-CD structures in a random conformation, but with defined bond length and angles, all flexible bonds and rings were set up and used in the conformational analysis; (2) to minimize the energy, in order to obtain a more stable conformation of the molecules AMBER (Assisted Model Building and Energy Refinement) force field with distance-dependent dielectric constant was used, electrostatic and van der Waals 1–4 scale factors were set to 0.5 and cutoffs set to none; (3) the minimizing of the conformation energy was conducted until the total root-mean-square (RMS)

gradient was lower than 0.1 kcal/mol; (4) the most stable conformations were retained for the interaction studies.

The geometry optimization parameters were: Polak-Ribiere (conjugate gradient) algorithm, RMS gradient of 0.1 kcal·mol^{-1}·Å^{-1}, *in vacuo*, without periodic boundary conditions.

SPECTROPHOTOMETRIC ASSAY

Materials

All experiments were performed with analytical reagent grade chemicals, pure solvent and Milli-Q water. Fluoxetine hydrochloride (FLU) was supplied by Sigma-Aldrich and the cyclodextrins by Sigma Chemie GmbH.

A stock solution of FLU (12.5 mg fluoxetine hydrochloride dissolved in 100 mL water) was diluted to prepare standard working solutions. The stock standard solution was stored away from light and kept at 4 °C. Under these conditions it was stable for at least 2 weeks. Solution of 5×10^{-3} M b-CD was prepared in water. The 0.6 M pH = 7.2 phosphate buffer solution was prepared by mixing appropriate amounts of sodium dihydrogen phosphate with sodium hydroxide.

In order to study the formation of a molecular aggregate based on charge transfer between FLU and b-CD, two standard stock solutions were prepared: (A) 0.01 mg/mL FLU and (B) 5×10^{-3} M b-CD. An aliquot (200 μL) of the (A) solution and 1000 μL of 0.6 M phosphate buffer were transferred into a 10 mL volumetric flask and different volumes of the (B) solution were further added. Water was added to a final volume of 10 mL. By this way the concentration of b-CD was varied between 0 and 3.125×10^{-3} M, while the FLU concentration was kept constant (200 ng/mL). The solutions were mixed using vortex for 4 hours.

Method

The molar ratio of the molecular aggregate was performed using Job's method of continuous variation [11]. To study the interaction between FLU and b-CD, six different standard solutions were prepared: (A) 5.7×10^{-4} M, 3.8×10^{-4} M and 2.9×10^{-4} M FLU and (B) 5.7×10^{-4} M; 3.8×10^{-4} M and 2.9×10^{-4} M b-CD. For each standard solution, the following five ratios between A and B solutions were prepared: 3:7 (v/v); 4:6 (v/v); 5:5 (v/v); 6:4 (v/v) and 7:3 (v/v).

All the measurements were performed on a Cary 100 Bio (Varian Inc., USA) spectrophotometer, at 275 nm *versus* a blank (water).

SPECTROFLUORIMETRIC ASSAY

For preparation of the calibration graph (three replicates per point) aliquots of FLU standard solution were pipetted into 10 mL volumetric flasks to give a final

concentration of 50–1000 ng/mL, then b-CD as required to give 1.5×10^{-3} M and the volume of buffer solution required to give 10^{-3} M. The solution was diluted to final volume with water and mixed using vortex for 4 hours. A portion of the solution was transferred to a 1 cm pathlength fluorescence cuvette and the fluorescence spectra were plotted using the following parameters: 224 nm excitation, 250 – 400 nm emission, 5 nm spectral bandwidth for both excitation and emission monochromators, 540 nm/min scan rate. The fluorescence emission intensity was measured at 292 nm.

All fluorimetric measurements were performed on a LS 50 B Perkin Elmer device connected to a computer equipped with the Perkin Elmer FLDataManager software.

The association constant of the complex was calculated by Benesi-Hildebrand's method [2]. Using the fact that b-CD forms a 1:1 molecular aggregate with FLU, the relationship between the observed fluorescence intensity enhancement ($F - F_0$) and the b-CD concentration is given using equation [1]:

$$\frac{1}{(F - F_0)} = \frac{1}{(F_\infty - F_0) K_1 [b - CD]_0} + \frac{1}{(F_\infty - F_0)} \qquad [1]$$

where F_0 is the fluorescence intensity of fluoxetine in the absence of b-CD; F_∞ – the fluorescence intensity when all of the fluoxetine molecules are essentially complexed with b-CD; F – the observed fluorescence at each b-CD concentration tested; K_1 – the association constant, and $[\text{b-CD}]_0$ – the b-CD concentration tested.

The association constant was determined by dividing the intercept to the slope of the straight line obtained in the plot $1/(F - F_0)$ *versus* $1/[\text{ b-CD}]_0$.

RESULTS AND DISCUSSION

MOLECULAR MODELING

The diameter of the cavity of b-CD is estimated at 6.8 Å [14, 16], while the diameter of the benzene ring in fluoxetine hydrochloride is of about 6.7 ~ 6.8 Å. Therefore, at least theoretically, FLU enters the cavity of b-CD and forms a steady inclusion complex. There are three hypothetical distinct modes of inclusion: through the ring, with or without $–CF_3$ group or through amino radical ($–CH_2–CH_2–NH–CH_3$).

Molecular modeling was used in order to gain information on the interaction mechanism at molecular level. Using the geometry optimization method the most stable conformations were obtained.

The partial surface charges and the molecular energy of the most stable conformation were calculated for FLU and schematically represented in Fig. 1.

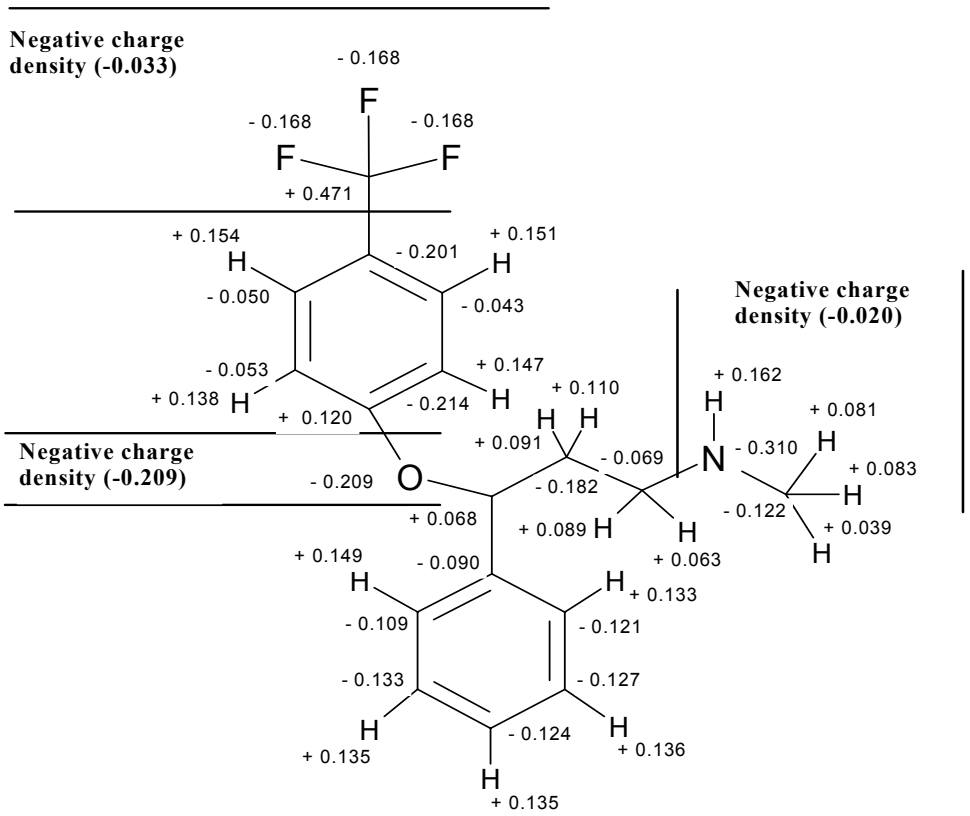

Fig. 1. Partial charges calculated on fluoxetine surface.

Three areas with negative charge density can be distinguished on the FLU surface (Fig.1.): on the $–CF_3$ group (–0.033), on the oxygen from the phenoxy group –O– (–0.209), and on the $–NH–CH_3$ (–0.020) group. The calculated energy of the most stable conformation corresponds to 8.801 kcal/mole.

The value of logarithm of the octanol/water partition coefficient, (log P = 1.93), the partial charges on the FLU surface and the calculated diameter of both benzene rings (5 Å) and of the amino radical (2.94 Å) support the hydrophobic interaction (*i.e.* charge transfer) between the hydrophobic moiety of the FLU and the inner cavity of b-CD, that corresponds to 6.67 – 5.96 Å.

The partial charges on FLU surface and the molecular energy were also calculated after the interaction with b-CD (Fig. 2). If the interaction with b-CD is considered, in the molecular aggregate based on charge transfer, FLU is engaged with the amino radical ($–CH_2–CH_2–NH–CH_3$). An increase in the density of negative charges for all three areas was observed on the FLU surface (Fig. 2): (1) on

$–CF_3$ group, from –0.033 to –0.247; (2) on oxygen from the phenoxy group from –0.209 to –0.478 and (3) on $–NH–CH_3$, from –0.020 to –0.127.

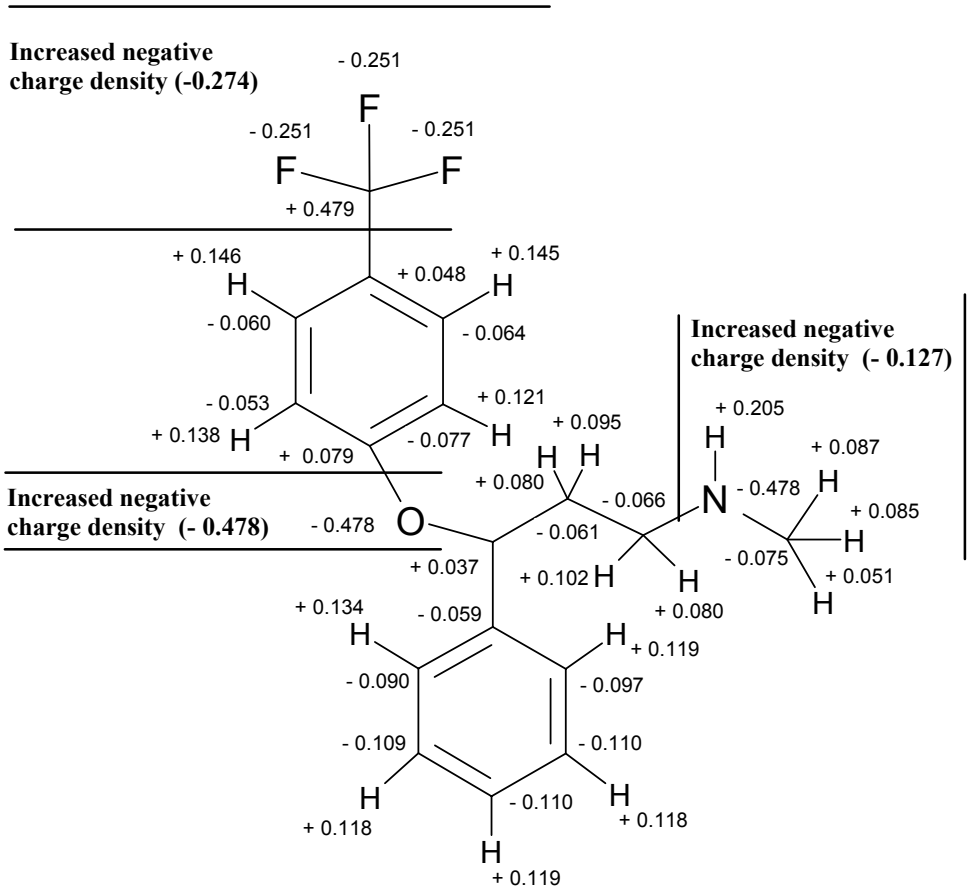

Fig. 2. Partial charges calculated on fluoxetine surface after interaction with b-CD.

The estimated binding energy of the aggregate (meaning relative enthalpy) also calculated *in vacuo* using Hyperchem program was of –53.622 kcal/mole.

Up-to-date other researchers investigated the interaction between FLU and b-CD in methanolic solution [8] or D_2O [17], but in both cases the most stable complex (due to less energy value) has the ring with the $–CF_3$ group enclosed into the hydrophobic cavity of b-CD. Taking into account that different experimental conditions (such as the nature of the solvent or the pH value) have a major influence on the charges on fluoxetine surface, the differences between their results and our findings are justified.

SPECTROPHOTOMETRIC CHARACTERIZATION OF THE MOLECULAR AGGREGATE

The interaction between b-CD and FLU was assessed in solution taking into account that FLU may change its physical chemical properties when interacts with b-CD and the changes can be followed by different techniques.

The UV absorption spectra in the range of 220–320 nm, performed on 5.76×10^{-4} M FLU, with or without 5.76×10^{-4} M b-CD, revealed that the absorbance was increased by adding b-CD in the FLU solution, the molecular aggregate based on charge transfer having two peaks, at 275 and 264 nm (Fig. 3).

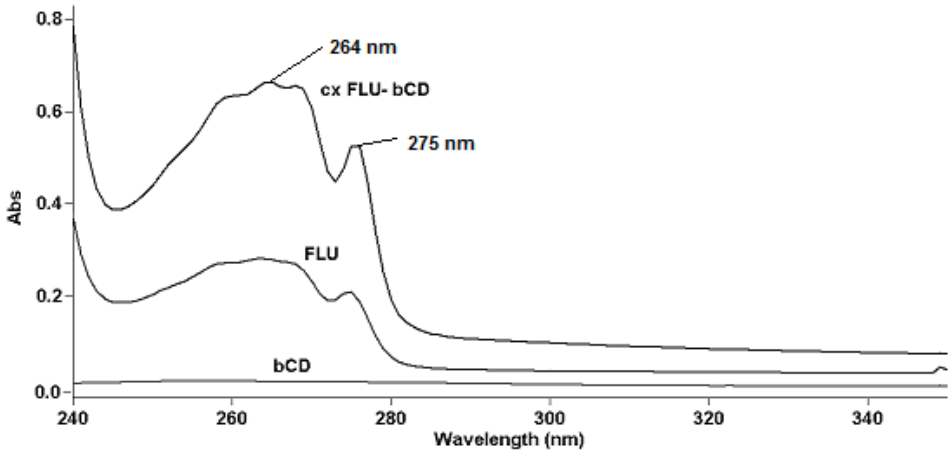

Fig. 3. UV spectra of 5.76×10^{-4} M FLU, 5.76×10^{-4} M b-CD and the FLU-b-CD complex.

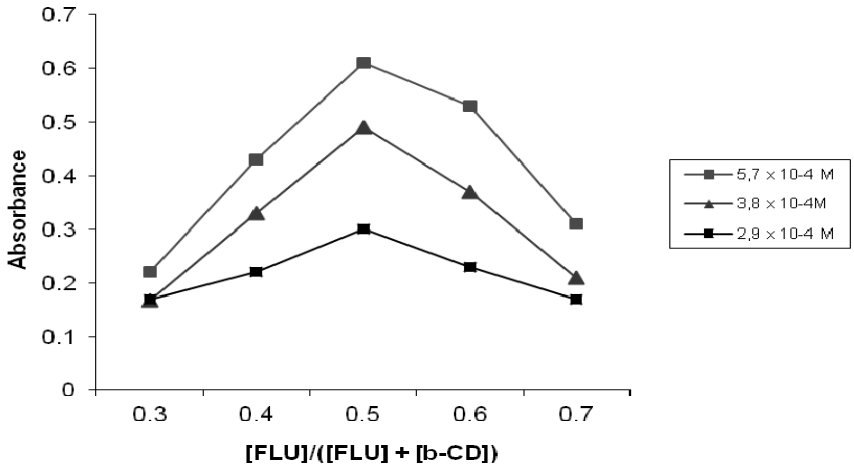

Fig. 4. Absorbance *vs*. [FLU] / ([FLU] + [b-CD]) ratio.

The changes in the spectral properties of the fluoxetine upon inclusion into the b-CD cavity are due to the hydrophobic character of the cavity and to the restrictions imposed by the host upon the fluoxetine's movement.

The molar ratio of the molecular aggregate was performed using Job's method of continuous variation [11]. The UV absorption spectra were recorded for each mixture between FLU and b-CD at 275 nm. Plotting the absorbance at 275 nm *vs*. [FLU]/ ([FLU] + [b-CD]) ratio (Fig. 4), the maximum corresponds to 0.5, which indicated that the aggregate had a 1:1 stoichiometry [12].

SPECTROFLUORIMETRIC CHARACTERISATION OF THE MOLECULAR AGGREGATE

Spectral characteristics

Several preliminary studies were performed to investigate the possible formation in solution and fluorescence characteristics of the molecular aggregate based on charge transfer between FLU and b-CD.

The emission spectrum of FLU solution (0.4 μg/mL) at pH 7.2 exhibits a strong fluorescent signal at 292 nm. Addition of 5×10^{-3} M b-CD to FLU solution resulted in an enhancement of the fluorescence intensity. Compared with the FLU or b-CD spectra, the complex showed some significant changes in the shape, or peaks, or wavelengths (Fig. 5).

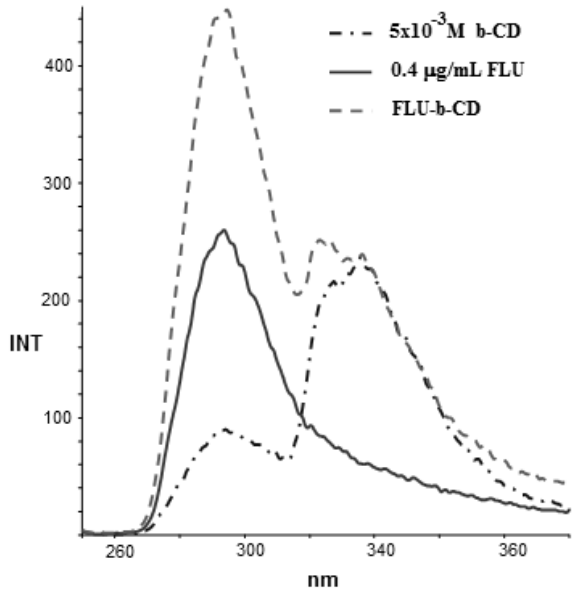

Fig. 5. Influence of b-CD on the fluorescence intensity.

Using the molecular modeling on the surface charge density of FLU we observed changes of partial charges of groups $–CF_3$ and –O–, after interaction with b-CD. Taking into account that these groups serve to enhance the fluorescence signal of FLU, changes observed through modeling can be corroborated with the changes observed in the fluorescence signal of fluoxetine: the signal intensity at 292 nm increases from 262 (a.u.) for free FLU to 448 (a.u.) for FLU included in the complex with b-CD.

It is important to underline that, after interaction, the b-CD spectrum is also changed (hence the ratio of peak intensities at 337 and 348 is amended). It should also be noted that b-CD has a weak fluorescent signal at 292 nm, where fluoxetine exhibits the strongest fluorescent signal. However, the existence of interaction is demonstrated by the fact that the maximum intensity at 292 nm is higher than the simple sum of the intensity components, which shows that the maximum of 292 nm is not a simple convolution of the spectral components.

Berzas *et al.* [3] revealed a similar change in the fluorescence intensity in the case of FLU interaction with other cyclodextrins, like methyl-b-CD. But in this case the ring of FLU with $–CF_3$ group is enclosed into the hydrophobic cavity of methyl-b-CD, forming an inclusion complex. However, this complex is completely different from our molecular aggregate, where FLU is engaged on charge transfer with the amino radical ($–CH_2–CH_2–NH–CH_3$).

These results revealed that the type of interaction (physical inclusion or molecular aggregate based on charge transfer) is strongly dependent on the characteristics of cyclodextrins and the solvents used. Therefore, the pH value and b-CD concentration that influence the formation and stability of the molecular aggregate based on charge transfer were also investigated.

Influence of pH on luminescence characteristics of the molecular aggregate

The relationship between the pH value or the reaction media and the fluorescence intensity of FLU or FLU-b-CD complex was studied; results are illustrated in Fig. 6.

As can be seen, the fluorescence intensity of FLU decreases with the pH value, especially at values higher than 9, whereas it remains constant in the case of the molecular aggregate. An optimum value of 7.2 was selected for further experiments, and sodium dihydrogen phosphate/sodium hydrogen phosphate buffer was used to adjust the pH value. The fluorescence intensity of the molecular aggregate was not affected by the buffer concentrations; therefore a 10^{-3} M concentration of the buffer was selected to achieve adequate intensity and buffering capacity.

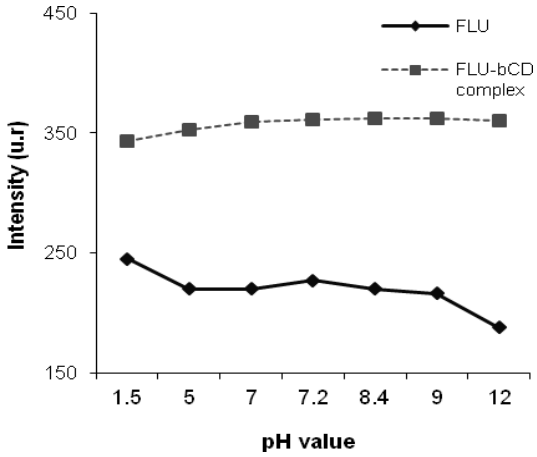

Fig. 6. Influence of the pH value of the reaction medium on the fluorescence intensity.

Influence of b-CD concentration

The influence of b-CD concentration on the fluorescence intensity of the molecular aggregate was investigated in the range 0 – 3.125 $\times 10^{-3}$ M.

As can be seen from Fig. 7, the relative fluorescence emission increases with increasing b-CD concentration up to about 1.5 $\times 10^{-3}$ M, and then decreases. The maximum increase is of about 1.4 times. Our experimental results indicate that adding b-CD at a concentration higher than 1.5 $\times$ 10^{-3} M results in an inhibition effect on the fluorescence of the molecular aggregate induced by the free b-CD and therefore the excess of cyclodextrin should be avoided.

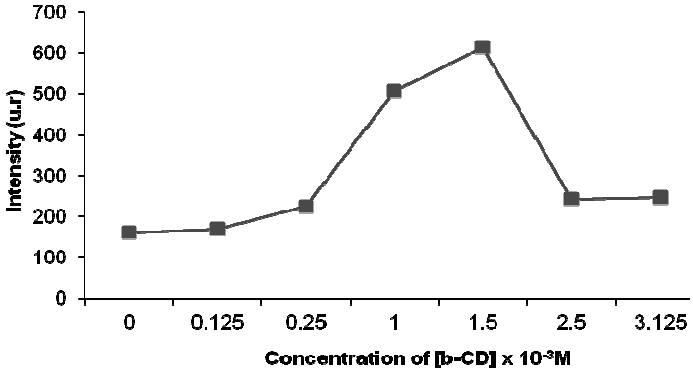

Fig. 7. Influence of the b-CD concentration on the fluorescence intensity of 200 ng/mL FLU.

Therefore 1.5 $\times$ 10^{-3} M b-CD concentration was selected as the optimum for the analytical procedure, as it gave maximum and constant fluorescence emission.

The data collected in this experiment were subsequently used to determine the association constant of the molecular aggregate.

The association constant of the molecular aggregate

For an aggregate with the stoichiometry 1:1, a plot of $1/(F - F_0)$ *versus* $1/[\text{b-CD}]_0$ should give a straight line. The experimental results showed a linear relationship between the two computed parameters (Fig. 8), thus confirming the results obtained by Job's method.

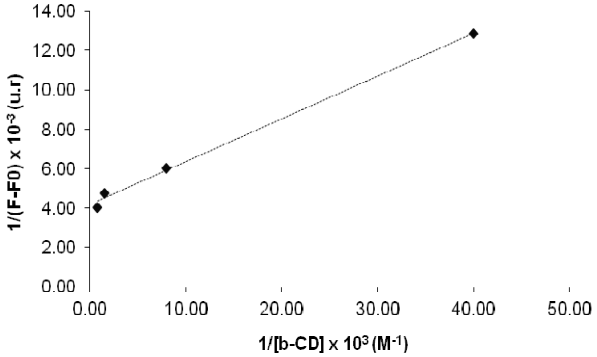

Fig.8. Benesi-Hildebrand plot for FLU-b-CD molecular aggregate: $1/(F - F_0)$ *vs*. 1 / [b-CD]$_0$.

Once the stoichiometry of the system is demonstrated to be 1:1, the association constant can also be calculated, in order to reveal the magnitude of the FLU-b-CD interaction. The association constant was determined using the Benesi-Hildebrand's method, by dividing the intercept to the slope of the straight line obtained in the plot $1/(F - F_0)$ *versus* 1 / [b-CD]$_0$.The obtained results are presented in Table 2.

The association constant found experimentally suggests a pronounced interaction between FLU and b-CD, supporting also the estimated binding energy (–53.622 kcal/mole).

Table 2

Statistical analysis data from Benesi-Hildebrand's method

Parameter	**Value**
Intercept	4.1864
Slope	0.2368
Squared correlation coefficient (R^2)	0.9967
Association constant	17.67 mol^{-1}

CONCLUSIONS

Molecular modeling was used to explore the interaction between fluoxetine and beta-cyclodextrin in aqueous solution, thus demonstrating the possibility to obtain a molecular aggregate based on the charge transfer between fluoxetine and beta-cyclodextrin. Fluorescence experiments confirmed the interaction and the formation of an aggregate based on charge transfer. The spectral characteristics revealed that the molecular aggregate presents an enhancement of the fluorescence emission at 292 nm, compared with the free ligand or cyclodextrin. The fluorescence intensity of the molecular aggregate depends on the beta-cyclodextrin concentration, but is not affected by the presence of the buffer or its pH value. The molar ratio of the molecular aggregate of 1:1 was established using Job's method, while the association constant was calculated using Benesi-Hildebrand's method and corresponds to 17.67 mol^{-1}. Experimental results confirmed that modeling is a useful tool for this type of physical interaction.

REFERENCES

1. ARIGA, K., T. KUNITAKE, *Supramolecular Chemistry – Fundamentals and Applications*, Springer-Verlag, Heidelberg, Leipzig, 2006.
2. BENESI, H., J.A. HILDEBRAND, Spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons, *J. Am. Chem. Soc*., 1949, **71**, 2703–2707.
3. BERZAS, J.J, A. ALANON, J.A. LAZARO, Cyclodetrin enhanced spectrofluorimetric determination of fluoxetine in pharmaceuticals and biological fluids, *Talanta* , 2002, **58**, 301–309.
4. BONEFANT, D., M. MIMEAULT, P. NIQUETTE, R. HAUSLER. Adsorption study of a commonly used antidepressant drug, fluoxetine hydrochloride, onto a crosslinked β-cyclodextrin-carboxymethylcellulose polymer, *Water Sci. Technol.,* 2012, **66,** 224–230.
5. BREWSTER, M.E., T., LOFTSSON, Cyclodextrins as pharmaceutical solubilizers, *Advanced Drug Delivery Reviews*, 2007, **59**, 645–666.
6. CONNORS, K.A., *Binding Constants. The Measurements of Molecular Complex Stability*, Wiley, New York, 1987.
7. DENADAI, A.M.L., M.M. SANTORO, L.H.D. SILVA, A.T. VIANA, R.A.S. SANTOS, R.D. SINISTERRA, Self-assembly characterization of the β-cyclodextrin and hydrochlorothiazide system: NMR, phase solubility. ITC and QELS, *J. Incl. Phenom. Macro. Chem*., 2006, **55**, 41–49.
8. DE SOUSA, F.B., A.M.L. DENADAI, I.S. LULA, J.F. LOPES, H. F. DOS SANTOS, B.W. DE ALMIEDA, R. D. SINISTERRA, Supramolecular complex of fluoxetine with β-cyclodextrin: An experimental and theoretical study*, International Journal of Pharmaceutics*, 2008, **353**, 160–169.
9. FUNG, S.Y., H. YANG, P. CHEN, Formation of colloidal suspension of hydrophobic compounds with an amphiphilic self-assembling peptide, *Colloid Surf.*, 2007, **55**, 200–211.
10. GECZY, J., J. BRUHWYLER, J. SCUVEE-MOREAU, V. SEUTIN, H. MASSET, J.C. VAN HEUGEN, A. DRESSE, C. LEJEUNE, E. DECAMP, L. SZENTE, J. SZEIJTLI, J.F. LIEGEOIS, The inclusion of fluoxetine into γ-cyclodextrin increases its bioavailability: behavioural, electrophysiological and pharmacokinetic studies, *Psychopharmacology*, 2000, **151**, 328–334.

11. HEIKKINEN, T., U. EKBLAD, P. PALO, K. LAINE, Pharmacokinetics of fluoxetine and norfluoxetine in pregnancy and lactation. *Clin. Pharmacol. Ther*., 2002, **73**, 330–337.
12. JOB, R., Stoichiometry of Molecular Complexes, in: J. Rose (ed.) *Advanced Physiochemical Experiments*, Ed. Pitman Publishing, London, UK, 1964, pp. 65–79.
13. NITALIKAR, M.M., D.M. SAKARKAR, P.V. JAIN, The cyclodextrins: A Review, *Journal of Current Pharmaceutical Research* , 2012, **10**, 1–16.
14. PINJARI, R.V., J.K. KHEDKAR, S.P. GEIJI, Cavity diameter and height of cyclodextrins and cucurbit[*n*]urils from the molecular electrostatic potential topography, *J. Incl. Phenom. Macrocycl. Chem.*, 2010, **66**, 371–380.
15. ROSSI, A., A. BARRACO, P. DONDA, Fluoxetine: a review on evidence based medicine, *Ann. Gen. Hosp. Psychiatry*, 2004, 3:2. doi: 10.1186/1475-2832-3-2.
16. SINGH, R., N. BHARTI, J. MADAN, S. N. HIREMATH, Characterization of cyclodextrin inclusion complexes – A Review, *Journal of Pharmaceutical Science and Technology* , 2010, **2,** 171–183.
17. SYED, M.A., F. ASMAT, A. MAHESHWARI, M. KOKETSU, Complexation of fluoxetine hydrochloride with β-cyclodextrin. A proton magnetic resonance study in aqueous solution, *Il Farmaco*, 2005, **60**, 445–449.
18. SZEJTLI, J., Cyclodextrins in pharmaceuticals, In: J. Szejtli (ed.) *Topics in inclusion science. Cyclodextrin technology*, Kluwer, Dordrecht, The Netherlands, 1988, pp. 186–306.
19. SZEJTLI, J., Introduction and general overview of cyclodextrin chemistry, *Chem. Rev*., 1998, **98**, 1743–1754.
20. *** HyperChem 7.5 Release for Windows, HyperCube, Inc., Gainsville, Florida, USA.