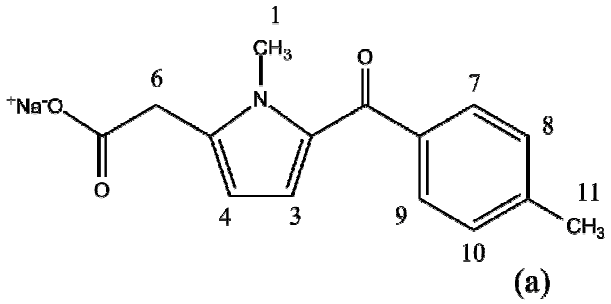
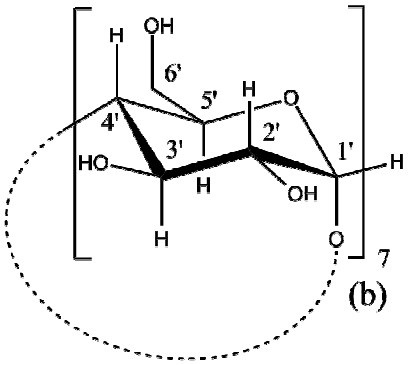
Summary report 2016

The study of the interaction between drugs and proteins is an important issue from a biochemical and clinical point of view. Transport of drugs in the body, take place via the circulatory system. Human serum albumin (HSA), the most abundant protein in the circulatory system, has been one of the most extensively studied of all the proteins. It is well known that the disposition, transportation and efficacy of drugs are strongly affected by the binding of drugs with serum albumin. Therefore is important to study the interaction of the drug with serum albumin, which plays a major role in pharmacology and pharmacodynamics. Although the studies on protein binding of drugs are commonly found in the literature, reports on the influence of β-cyclodextrin (β-CD), on drug –serum albumin binding remains rare. More than that, according to our knowledge, the influence of HSA on the drug-β-CD complex formation was not reported until now in the literature. We report in the following,the spectroscopic investigation on the interaction of tolmetin (TOL) a nonsteroidal anti-inflamatory drug (NSAID), with β-cyclodextrin in the absence and presence of human serum albumin. The complexation process between TOL**(a)** and β-CD**(b)** in solution, and the influence of HSA on the stability of the inclusion complex was determined using ^{1}H NMR spectroscopy. The stoichiometry and the binding constant were determined using the induced chemical induced shifts data of both host and guest protons. We also analyzed in this paper using fluorescence methods, the binding process between tolmetin and human serum albumin in the absence and presence of β-CD. The obtained differences in the binding strengths are discussed.



(a)



(b)

Aparatus

All ^{1}H NMR measurements were carried out on a Bruker AVANCE III spectrometer operating at 500.13 MHz for protons. The NMR spectra were recorded in buffered D2O solution at 298 K and all chemical shifts were measured relative to TMS. For each ^{1}H NMR experiment, 32 transients were collected into 65 K data points over 4000 Hz spectral window using a 5 s relaxation delay.

Steady-state fluorescence spectra were acquired on a JASCO-6500 fluorescence spectrophotometer equipped with a xenon lamp and 1.0 cm quartz cells. The fluorescence emission spectra were recorded in the wavelength range of 310 – 500 nm, upon excitation at λ_{ex} = 295 nm. Excitation and emission bandwidth was set to 3nm/5nm, and the fluorescence intensity was corrected, for the inner filter effect. The UV-vis absorption spectra were recorded in the range 250 - 500 nm, on a double beam JASCO-550 spectrophotometer equipped with 1.0 cm quartz cells.

Preparation of test solutions

In order to study the complexation process between TOL and β-CD in solution by NMR spectroscopy, two 10 mM stock solutions of tolmetin and β-CD in buffered D_2O were prepared. Based on these equimolar solutions, a series of samples containing β-CD and TOL were prepared. This was accomplished by mixing the two solutions to constant volume at varying proportions so that a complete range ($0 < r < 1$) of the mole fraction $r = [X]/([G]+[H])$ was sampled. X = H or G and [H] and [G] are the concentrations of the host (β-CD) and guest (TOL), respectively. Thus the total concentration $[H]_t + [G]_t$ = 10 mM was kept constant for each sample. This set of samples was used both for determination of stoichiometry and the evaluation of the association constant, K_a. In order to study the influence of HSA on TOL – β-CD inclusion complex stability, two sets of samples were prepared. The first set contained 4μM HSA, 1mM of TOL and the concentration of β-CD was varied between 0 and 8mM. In the second set the concentration of HSA and TOL was kept constant at 10μM and 1mM respectively, while the concentration of β-CD was varied between 0 and 10mM. For fluorescence spectroscopy measurements, the solutions were prepared using distilled water and Tris-HCl as buffer. The working solutions were prepared by appropriate dilutions of the stock solutions of TOL, β-CD and HSA. A stock solution of 4μM HSA was prepared in 20 mM Tris-HCl buffer solution. The binding titration of HSA against TOL was carried out by successive addition of different concentrations of TOL between 0 and 24μM. Similarly, three sets of samples were prepared for the titration of HSA with TOL- β-CD. The samples were prepared by keeping the concentration of HSA fixed (4μM) and adding successively different concentrations of TOL between 0 and 24μM, in the presence of β-CD at 0.035mM, 0.7mM and 1.4mM.

Inclusion complex of tolmetin with β-cyclodextrin

Inclusion of TOL in β–CD cavity is shown by the change in chemical shifts of some of the guest and host protons, in comparison with the chemical shifts of the same protons in the free components. The ^1H NMR observations allow us to use the continuous variation method to

determine the stoichiometry of the formed complex. In our case, the continuous variation method is based on the induced chemical shift variation, $\Delta\delta = \delta_{free} - \delta_{obs}$, which is directly related to the concentration of the complex. Thus if a physical quantity, containing $\Delta\delta$, is plotted as a function of the mol fraction of the host or guest, r, (Job's plot), its maximum value will occur at $r_{drug} = m/(m+n)$ or $r_{CD} = n/(m+n)$, where *m* and *n* are, the molar ratios of drug and CD in the complex. The continuous variation method was applied for protons belonging both to guest and host molecules and yielded identical results. For the sake of conciseness, only the most markedly affected protons have been selected and reported in Fig.1.

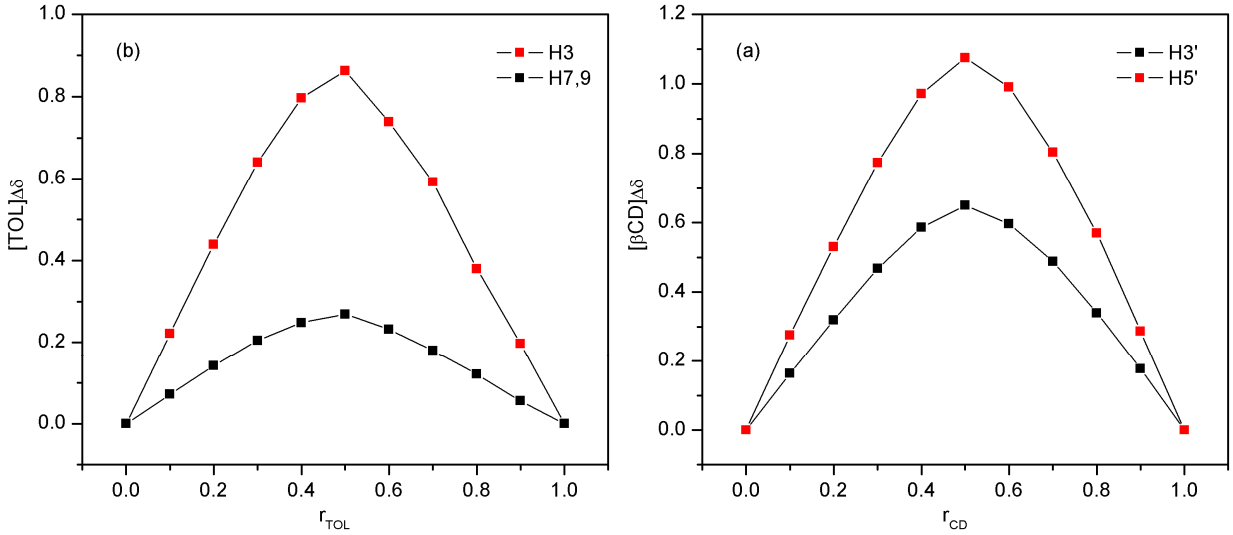


Fig.1 Job's plots corresponding to the induced chemical shift variation of some (a) β-CD and (b) TOL protons, for the β-CD-TOL system

In all cases, Job's plots show a maximum at *r* = 0.5 and a highly symmetrical shape, indicating **the existence of a complex with 1:1 stoichiometry**, within the range of the investigated concentrations. In order to determine the extent of the intermolecular binding between TOL and β-CD, the association constant has been evaluated. The association constant, K_a, for the 1:1 complex was evaluated by a nonlinear least-squares curve fitting regression analysis of the observed chemical shift changes of the TOL and β-CD NMR lines, as a function of concentration according to the following equation;

$$\Delta\delta^{(i,j)} = \frac{\Delta\delta_c^{(j)}}{2[X]_t^{(i)}}\left\{[G]_t^{(i)} + [H]_t^{(i)} + \frac{1}{K_a} - \left[\left([G]_t^{(i)} + [H]_t^{(i)} + \frac{1}{K_a}\right)^2 - 4[H]_t^{(i)}[G]_t^{(i)}\right]^{1/2}\right\} \quad .$$

where *i* counts the sample number and *j* the studied proton. If the studied proton belongs to the guest or host molecule, then X=G or H, respectively. $\Delta\delta_c^{(j)}$ represents the chemical shift difference (for a given proton, *j*) between the free component and the pure inclusion complex. In our case, we applied the above equation for a set of protons consisting in H(3') and H(5') of β-CD and H(3); H(1) and H(7,9) of TOL. The obtained association constant, using the above described procedure

is **K_a = 2164.5 M^{-1}** with the error function ***E* = 3.06·10^{-4}** and a correlation factor ***R* = 0.9992.** The calculated chemical shift differences, $\Delta\delta_c$, between the free and the pure inclusion complex are:

$$\Delta\delta_c H(3) = 0.2328\ ppm \qquad \Delta\delta_c H(1) = -0.1073\ ppm \qquad \Delta\delta_c H(7,9) = 0.0741\ ppm$$

$$\Delta\delta_c H(3') = 0.1734\ ppm \qquad \Delta\delta_c H(5') = 0.2877\ ppm$$

The influence of HSA on TOL-β-CD complex formation

In order to understand the role of HSA in the complexation process of TOL with β-CD the NMR titration procedure was applied for two sets of samples in which the concentration of HSA was maintained constant at 4μM and 10μM respectively. For the determination of TOL-β-CD binding constant K_a, the TOL concentration was kept constant (1mM) and the resonances of TOL protons are monitored as a function of β-CD concentration. As an example, the variation of $\Delta\delta_{obs} = \delta_{free} - \delta_{obs}$ as a function of β-CD concentration for some TOL proton signals is presented in Fig.2 for the sample set with [HSA] = 10μM.

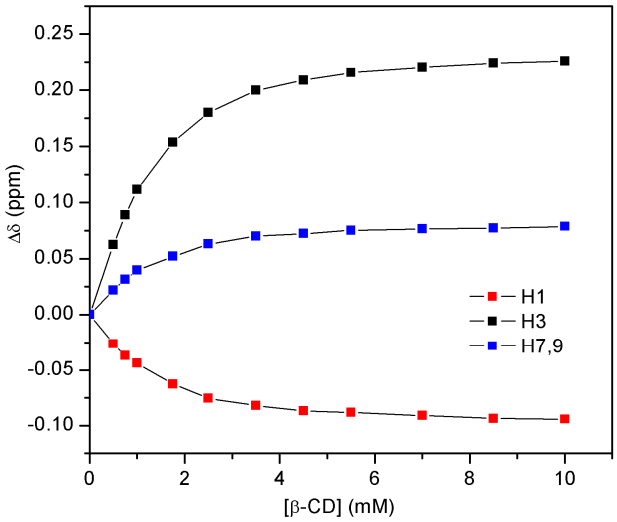


Fig.2 Variation of Δδ's function as a function of β-CD concentration for some proton signals of TOL

The solid lines were obtained by a nonlinear least-squares fitting, on the assumption that TOL forms a 1:1 inclusion complex with β-CD. The fitting parameters K_a and $\Delta\delta_c$ in the presence of HSA, are summarized below.

[HSA] (μM)	**K_a** ($10^3 Lmol^{-1}$)	**$\Delta\delta_c$H(3)** (ppm)	**$\Delta\delta_c$H(1)** (ppm)	**$\Delta\delta_c$H(7,9)** (ppm)	**E(10^{-4})**	**R**
4	2.033	0.2494	- 0.1001	0.0884	0.294	0.9999
10	1.493	0.2466	- 0.1016	0.0853	0.477	0.9995

Data analysis showed that the addition of HSA weakened the strength of TOL binding to β-CD but did not affect the stoichiometry and the binding mode. This finding may be caused by the adsorption of some TOL molecules on HSA and thus decreasing the concentration of free drug in the solution available for complexation with β-CD.

Binding of TOL – β-CD complex with HSA

In a previous report (2013), the interaction of TOL with HSA was studied by fluorescence and UV-vis absorption spectroscopy at different temperatures, combined with time-resolved fluorescence measurements. Using the continuous variation method, a single class of binding sites for TOL on HSA was put in evidence, and the binding constants K_a at different temperatures were calculated. The TOL molecule is capable of binding to β-CD with a significant binding strength, as discussed in a previous section. This encapsulation process can modulate the HSA-TOL binding interaction as the β-CD molecule acts like a sheath that cover up the TOL molecule, preventing it from freely binding to HSA. In order to understand the role of β-CD in the TOL- HSA interaction, the fluorescence spectra of HSA were recorded at three increasing β-CD concentrations. For example, the fluorescence spectra of HSA in the presence of 0.35 mM of β-CD with varying amounts of TOL are shown in Fig.3

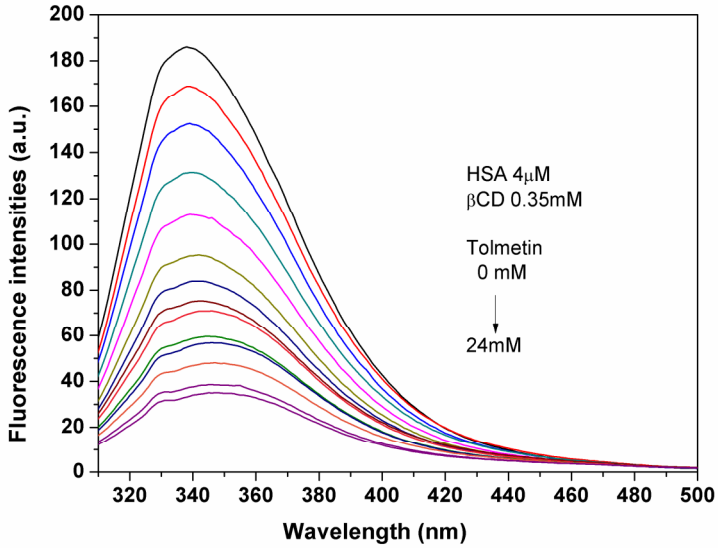


Fig.3 The fluorescence spectra of HSA in the presence of 0.35mM of β-CD with varying concentrations of TOL between 0 and 24μM.

It illustrates that TOL quenched the fluorescence of HSA. The fluorescence quenching is often described by the Stern-Volmer equation, namely $F_0/F = 1 + K_{SV}[Q]$ where F_0 and F are the fluorescence intensities in the absence and in the presence of quencher (TOL) respectively, [Q] is the concentration of the quencher, and K_{SV} is the Stern-Volmer quenching constant. The Stern-Volmer plots for the observed quenching process are linear as shown in Fig.4.

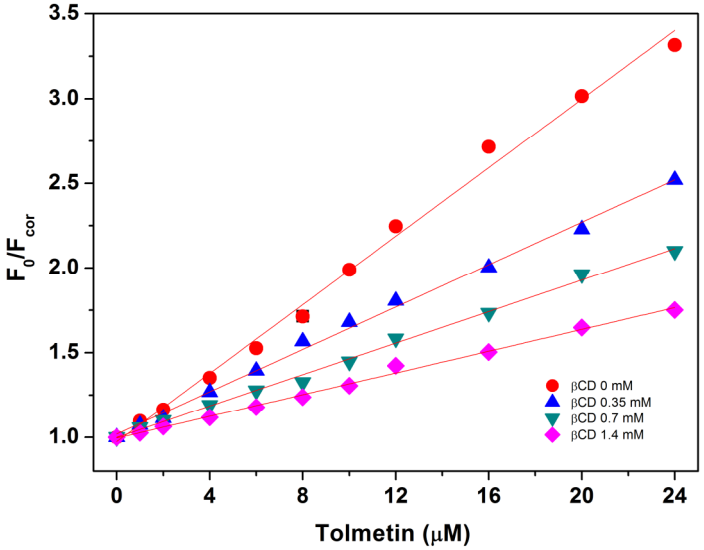


Fig.4 Stern-Volmer plots for the quenching of HSA fluorescence by TOL, in the presence of different β-CD concentrations.

It can be seen that all the ternary investigated systems showed the same linear Stern-Volmer plots as the HSA-TOL system, but with weaker quenching compared with the absence of β-CD. This suggests that the addition of different amount of β-CD did not influence the quenching mechanism of HSA-TOL binding. The K_{SV} values for TOL - HSA interaction in the absence and presence of different β-CD concentrations were determined by a linear regression of the plot of F_0/F against [TOL] and the obtained results are presented in the table below. These values are smaller than that observed in the case of TOL-HSA binding in the absence of β-CD. This means that β-CD hinders the collision of the TOL molecules with HSA due to the formation of an inclusion complex.

[HSA] (μM)	**[β-CD]** (mM)	**K_{SV}** (10^4 $Lmol^{-1}$)	**S.D.**[a]	**R**[b]
4	0.00	10.1	0.25	0.993
	0.35	6.2	0.12	0.996
	0.70	4.6	0.09	0.996
	1.40	3.2	0.69	0.995

[a] S.D. is standard deviation; [b] R is the correlation factor

In order to determine the binding constants K_a for the binding of TOL with HSA in the absence and presence of β-CD, we used the most generally valid equation to analyze fluorescence changes upon formation of a 1:1 complex, which in normalized version can be expressed as:

$$\frac{F_{corr}}{F_0} = 1 + \left(\frac{F_c}{F_0} - 1\right) \frac{[P]_t + [Q]_a + K_d - \sqrt{([P]_t + [Q]_a + K_d)^2 - 4[P]_t[Q]_a}}{2[P]_t}$$

where F_{corr} is the measured fluorescence corrected for the inner-filter effect, F_0 the fluorescence in the absence of the TOL, F_c the residual fluorescence of the fully complexed HSA, K_d the

dissociation constant, $[P]_t$ the concentration of HSA, and $[Q]_a$ the concentration of added TOL. The values of $\mathbf{K_d}$ and $\mathbf{F_c}$ were obtained in the absence and presence of various β-CD concentrations (Fig. 5) by fitting the experimental data with the above equation.

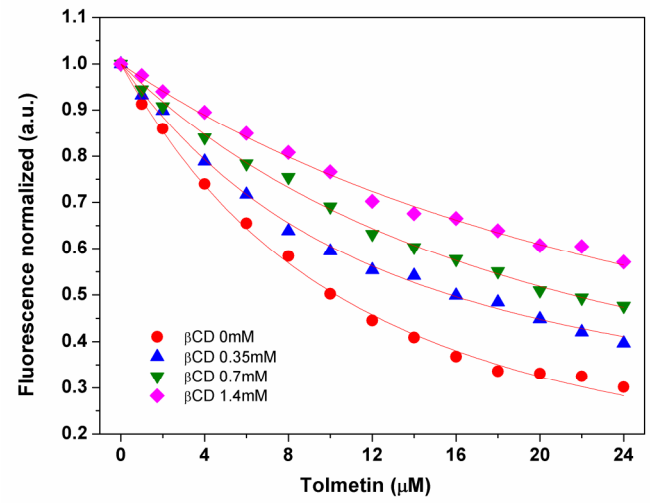


Fig.5 The normalized fluorescence of HSA versus added TOL concentration in the absence and presence of various β-CD concentrations. The continuous lines were calculated with K_d and F_c as fitting parameters.

The obtained results are presented below. It can be observed that we have practically no residual fluorescence, the interaction between HSA and TOL weakened in the presence of β-CD, the $K_a = 1/K_d$ decreasing with increasing β-CD concentration. This means that the presence of small amounts of β-CD in solution, are very efficient in hindering the TOL-HSA specific interaction

System	**[β-CD]** (mM)	**F_c** (a.u)	**K_a** (10^5 $Lmol^{-1}$)	**R**
HSA - TOL	0.00	0.0056	1.223	0.9977
	0.35	0.1527	1.075	0.9949
	0.70	0.0503	0.572	0.9965
	1.40	0.0213	0.361	0.9953

Conclusions

The inclusion process of β-CD with tolmetin was examined by NMR spectroscopy. The stoichiometry of the inclusion complex is 1:1 and the binding constant is 2164.5 M^{-1}. The titration of TOL with β-CD in the presence of HSA showed that the addition of HSA weakened the strength of TOL binding to β-CD. This effect may be caused by the adsorption of some TOL molecules on HSA and thus decreasing the concentration of free TOL in the solution. The binding of TOL with HSA in the absence and presence of β-CD were studied by fluorescence spectroscopy. Data analysis showed that the addition of β-CD weakened the quenching and binding of TOL with HSA. These results indicate that the competition between β-CD and HSA in binding TOL can modulate the free TOL in solution, having an impact on the drug bioavailability.