2013 - Summary report

A1. Spectroscopic investigation of tolmetin interaction with human serum albumin

The interaction of tolmetin (TOL) with human serum albumin(HSA) in physiological buffer solution (pH 7.4) was studied by fluorescence and UV–vis absorption spectroscopy at different temperatures, combined with time-resolved fluorescence measurements. The experimental results showed that there was a strong fluorescence quenching of HAS by tolmetin. Using the continuous variation method, a single class of binding sites for TOL on HSA was put in evidence. The binding constants Ka, were calculated at different temperatures with the most generally valid equation for a 1:1 complex, using a non linear fit to the experimental data. In addition the changes in the thermodynamic parameters ΔH^0, ΔS^0 and ΔG^0 were calculated according to the reaction isotherm equation. The obtained thermodynamic signature suggests that at least van der Waals and electrostatic type interactions are present. Quenching efficiency calculations, based on steady state and time-resolved spectroscopy, indicate that both static and dynamic quenching mechanisms are present.

A2. Characterization of β-cyclodextrin inclusion complex with procaine hydrochloride by ^{1}H NMR and ITC

The inclusion of local anesthetic drug procaine hydrochloride by β-cyclodextrin was investigated by 1D and 2D proton NMR spectroscopy and isothermal titration calorimetry (ITC) at 298 K. The stoichiometry of the complex was determined by the method of continuous variation, using the chemical induced shift of both host and guest protons. The association constant K, of the obtained complex was calculated and found to be 293.17 M^{-1}.Rotating frame NOE spectroscopy, was used to ascertain the solution geometry of the host–guest complex. The result reveals that the procaine molecule penetrates into the β-cyclodextrin cavity with the aromatic ring. The energetics of complexation process is investigated by ITC technique. The analysis indicates that the complexation of procaine by β-CD is an exothermic process and shows that both enthalpy and entropy contribute to the binding process. The obtained value for the association constant is in good agreement with that obtained from NMR and both methods sustain a 1:12 stoichiometry.

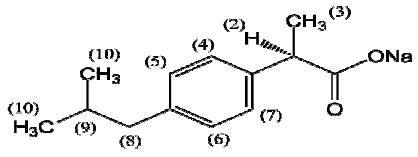
A3. Pulse sequences implementation on the existing NMR spectrometer for spin-lattice relaxation rates determination.

The spin-lattice relaxation rate R_1 is one of the most adequate NMR parameter used to study the ligand – receptor interaction. These kind of studies are based on the comparison of selective ($R_{1,sel}$) and non-selective ($R_{1,ns}$) spin lattice relaxation rate in the presence and absence of macromolecular receptor. The formation of ligand – receptor complex affects $R_{1,ns}$ and $R_{1,sel}$ to different extents depending on the dynamical parameters (i.e. molecular rotational correlation time τ_c) assuming fast chemical

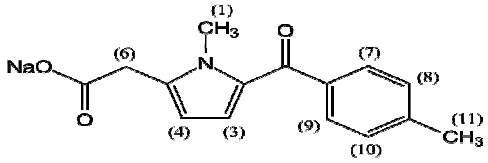
exchange between the bound and the free environments. The implemented sequences, for spin-lattice relaxation rates determination was the well known "inversion recovery" sequence, $(180^{0} - \tau_v - 90^{0} - t_r)_n$. For non – selective $R_{1,ns}$ determinations the 180^{0} pulse has 20.2 μs. For selective inversion of a certain proton the 180^{0} pulse we used a Gauss 1_180i_1000 soft pulse with a length of 18.35 ms and a power of 51 dB, corresponding to an excitation width of 40 Hz. The relaxation time, $T_1 = 1/R_1$, was calculated by exponential regression analysis of the recovery curve using the equation $A(t) = A(0)\{1 - 2\exp(-\tau_v/T_1)$, where A(t) is the NMR signal area at $\tau_v = t$ and A(0) is the area of the same signal after the 180^{0} pulse. The τ_v table used in these experiments has 15 values starting with 0.01s and ending with 10s in the presence of the macromolecule, and 25 – 30s in the case of pure ligand. The value of t_r fulfill the condition $t_r \geq 5T_1$. The number or sequence repetition was *n* = 8.

A4. Spin lattice relaxation rates determination for a series of bio-ligands.

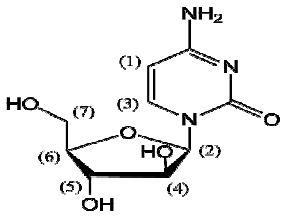
In order to study the bioligand – macromolecule interaction based on relaxation rate variation as a function of bioligand concentration, the R_1 for the bioligand protons in the absence of macromolecule is mandatory. For this reason we determined $R_{1,ns}$ for Ibuprofen, tolmetin, paracetamol, citarabineand 5-fluorouracil.



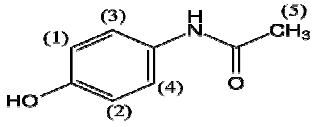
Ibuprofen



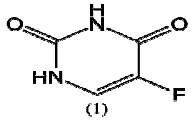
Tolmetin



Citarabine



Acetaminofen



5-Fluoro-uracil

R_1^{ns} **- Ibuprofen (s^{-1})**

H(4,7)	H(5,6)	H(2)	H(8)	H(9)	H(3)	H(1)
0.4673± 0.002	0.5192± 0.003	0.4369± 0.002	1.1236± 0.004	0.5907± 0.002	1.3986± 0.005	1.018± 0.003

R_1^{ns} **- Tolmetin (s^{-1})**

H(7,9)	H(8,10)	H(3)	H(4)	H(6)	H(1)	H(11)
0.531±0.004	0.550±0.004	0.540± 0.005	0.435± 0.003	0.855± 0.004	1.530± 0.006	1.030± 0.005

R_1^{ns} - Citarabine (s^{-1})

H(1)	H(2)	H(3)	H(4)	H(5)	H(6)	H(7)
0.4803± 0.001	0.4294± 0.002	0.2944± 0.006	0.4662± 0.002	0.4378± 0.001	0.4751± 0.003	1.5244± 0.006

R_1^{ns} - Acetaminofen (s^{-1})

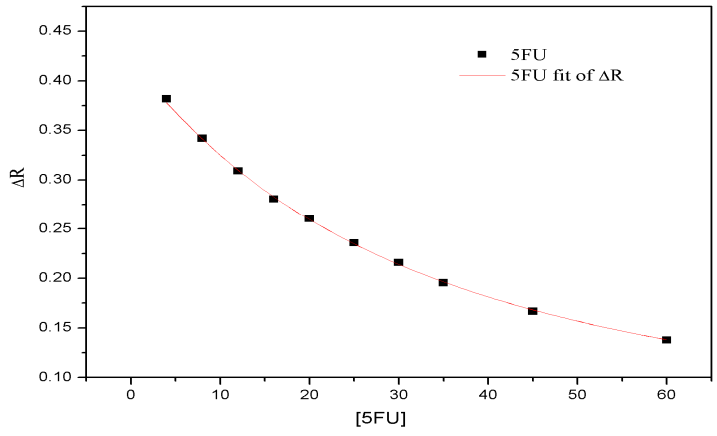
H(1,2)	H(3,4)	H(5)
0.2625± 0.003	0.2476± 0.003	0.6010 ±0.003

R_1^{ns} - 5-fluorouracil (s^{-1})

$$H(1) = 0.0924 \pm 0.0005$$

A5 ^1H NMR relaxation study of 5-fluorouracil – human serum albumin interaction

HSA can bind bioligands in two distinct ways. One is the high-affinity binding with one or two specific binding sites and with the association constant in the order of 10^4 – 10^6 M^{-1}. The other is the low-affinity binding with tens of nonspecific binding sites. When the ligand concentration is much higher than that of HSA in solution, the high-affinity binding sites (*n*), are fully saturated and the interactions between the ligand in excess and HSA are governed by the low-affinity and high-capacity binding.



We have studied the binding of 5 fluorouracil (5FU) to HSA, in the low affinity binding sites, by NMR relaxometry. The experimental condition were: the concentration of HAS

was maintained constant at 0.2 mM and that of 5 FU varied in the range 4 – 60 mM. The $\Delta R = R_{1,obs} - R_{1free}$ was monitored as a function of 5FU concentration. The obtained dependence is presented in the above figure. The fitting function has the following expression:

$$f_b = \left(\frac{R_{1,obs} - R_{1f}}{R_{1b} - R_{1f}} \right) = \alpha - \sqrt{\alpha^2 - \beta} \quad \text{where} \quad \alpha = \left(C_L + nC_P + K_d\right)/2C_L \text{ si } \beta = nC_P / C_L$$

The obtained results are:

$R_{1bound} = 1.531 \pm 0.34\ s^{-1}$
$n = 37 \pm 3$
$K_{diss} = 19.2 \pm 1mM$