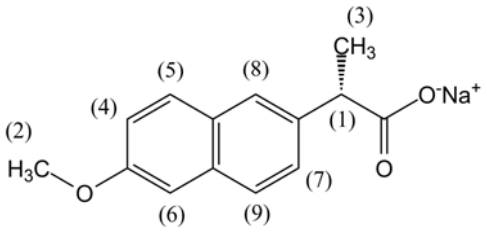
Summary report 2015

Studies on the binding of drugs to protein are of great importance in biological, biomedical and pharmaceutical sciences. The binding affinity and capacity can be described by the dissociation constant of the molecular complex and the number of the binding sites on the protein. Any interaction that interferes with the binding of a drug to its receptor, such as competitive binding, may affect the pharmacological activity of the drug. HSA interacts with drugs through its binding sites. There are mainly two classes of binding sites on the HSA molecule. One is the high-affinity binding sites and the other is the low-affinity binding sites. The high-affinity binding sites, which have the properties of low capacity, have been the main topic of study for a long time by various analytical techniques including HPLC, capillary electrophoresis and some spectroscopy methods, such as circular dichroism and fluorescence.

The low affinity binding sites have the properties of high capacity. It means that in this type of binding, one HSA molecule can bind many drug molecules but the interaction is very weak. It has been found that many drugs are involved in this type of low-affinity binding interaction, especially when the drug concentration is much higher than that of HSA in blood plasma. In this case the drug is bound first to the high-affinity binding sites and then the drug in excess is bound to the low-affinity binding sites. Both mechanisms of binding contribute to the biological effect of drugs. NMR has been extensively used as a useful method for obtaining information on the interactions between macromolecules and small ligand molecules. However, in studies on the high-affinity binding between proteins and drug molecules, the application of this spectroscopic method has been limited to smack proteins. When the drug molecules are tightly bound to the high-affinity sites of a large protein as HSA, the line-broadening effect makes the drug signal non-observable. However, NMR is suitable for studies of the weak low-affinity interaction, where the drug molecules in free and bound states are in fast exchange on the NMR time scale. In such a case, NMR parameters of drug molecules, such as chemical shifts, relaxation rates and self-diffusion coefficients are the weighted-average of the free and bound states. By the measurements of the variation of one of these NMR parameters both the binding position of a drug to albumin and the dynamic parameter of this interaction can be determined. Thus we studied the low-affinity interaction between HSA and a series of drug molecules by measuring the selective spin lattice relaxation rate of certain drug protons at constant HSA concentration, with the drug concentration well in excess. We have investigated the interaction of **HSA** with: **Naproxen, Acetaminophen, Na salicylate, 5-fluorouracil**, **cytarabine** and **clonidine HCl**. All the measurements were made with a 500 MHz Bruker AVANCE III NMR spectrometer. The temperature was maintained at 298 K and the samples were prepared in phosphate buffer

(pH=7,4) with D_2O. Selective spin lattice relaxation times were measured by a, $180^0 - \tau - 90^0$ inversion recovery method with a selective 180^0 Gauss1_180i_1000 soft pulse. In the following we present briefly the obtained results.

a) Naproxen – HSA system



Naproxen

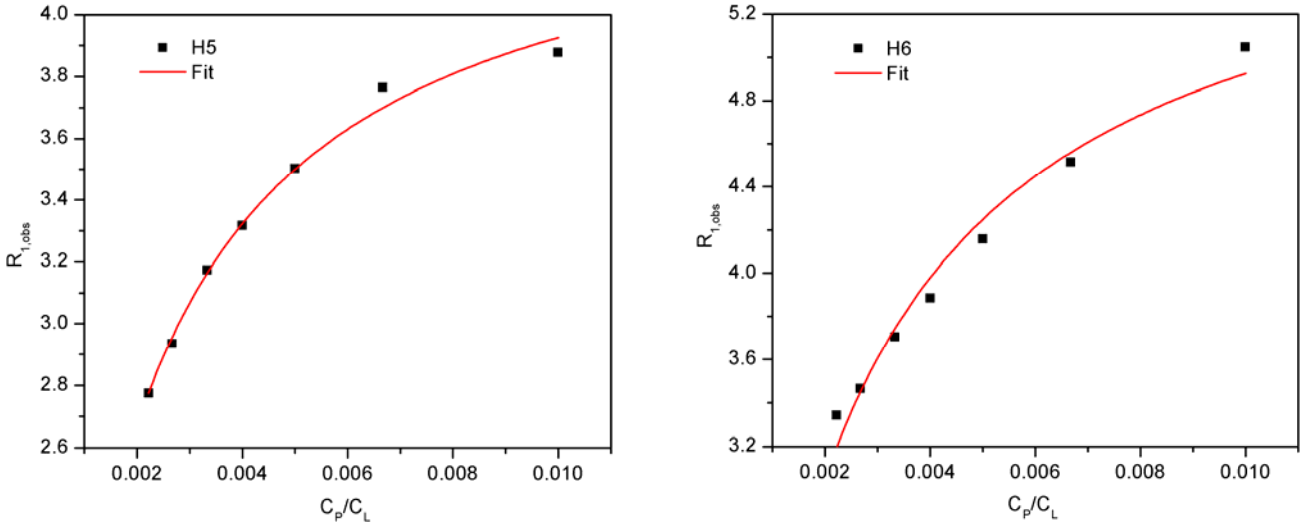


Fig 1. Plots of the observed selective relaxation rates versus the concentration ratios C_P/C_L of H5 and H6 of naproxen

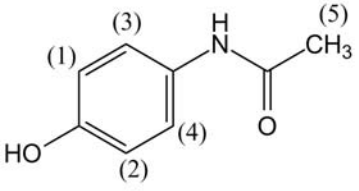
The fitting equation is:

$$\frac{2\left(R_{1,obs}-R_{1,free}\right)}{\left(R_{1,bound}-R_{1,free}\right)}=1+\left(\frac{K_D}{C_P}\right)\left(\frac{C_P}{C_L}\right)+n\left(\frac{C_P}{C_L}\right)-\left(\left[1+\left(\frac{K_D}{C_P}\right)\left(\frac{C_P}{C_L}\right)+n\left(\frac{C_P}{C_L}\right)\right]^2-4n\left(\frac{C_P}{C_L}\right)\right)^{1/2} \quad (1)$$

The obtained results are:

Proton	$R_{1,bound}$ (sec^{-1})	n	K_D (mM)	r^2
H(5)	73,3±3	34±4	57,4±3,3	0,992
H(6)	69,4±5,6	34±2	40,7±4,8	0,969

b) Paracetamol – HSA system



Paracetamol

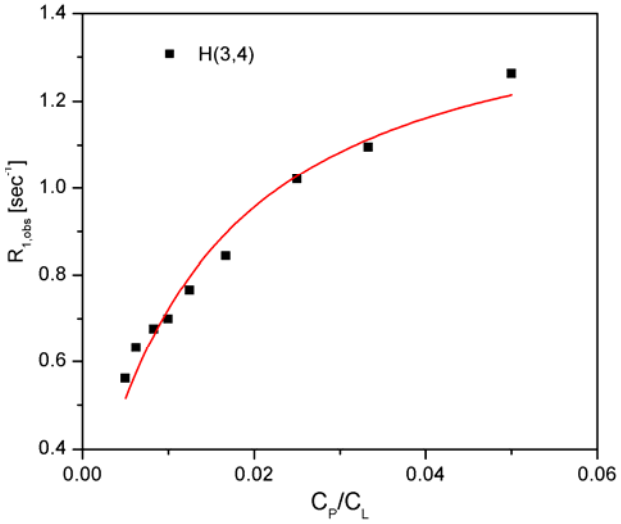
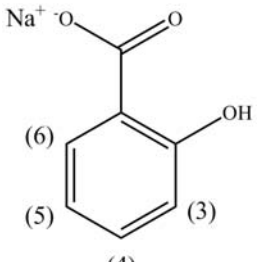


Fig.2 The observed selective relaxation rates of H(3,4) of paracetamol versus C_P/C

Equation (1) was used for fitting the experimental data. The obtained results are:

$$\mathbf{K_D = 5{,}25 \pm 0{,}81\ mM} \qquad \mathbf{n = 12 \pm 1}$$
$$\mathbf{R_{1,bound} = 7{,}117 \pm 0{,}57\ sec^{-1}} \qquad \mathbf{r^2 = 0{,}969}$$

c) Na salicylate – HSA system



Na Salicylate

Changes of the selective relaxation rate $R_{1,obs}$, as a function of the concentration ratio C_P/C_L for H(4) proton of Na salicylate is presented in figure 3.

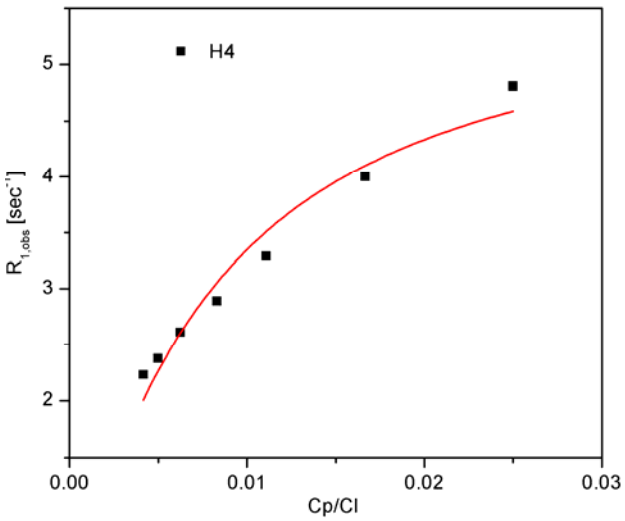


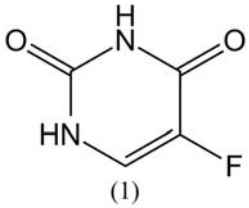
Fig.3 The observed selective relaxation rate versus C_P/C_L for the H(4)proton of Na salicylate

Eq.(1) was used to fit the experimental data, using the determined value of the selective relaxation rate for the free salicylate ($\mathbf{R_{1,\,free} = 0125{\pm}0{,}003\ sec^{-1}}$). The obtained results are:

$\mathbf{K_D = 6{,}80 \pm 1{,}60\ mM}$ $\qquad$ $\mathbf{n = 36{,}5 \pm 1{,}5}$

$\mathbf{R_{1,bound} = 16{,}41 \pm 1{,}75\ sec^{-1}}$ $\qquad$ $\mathbf{r^2 = 0{,}955}$

d) 5-fluorouracil – HSA system



(1)

5-Fluoro-uracil

Changes of the selective relaxation rate variation $\Delta R_{1,obs} = R_{1,obs} - R_{1,\,free}$ of the H(1) proton of 5-fluorouracil as a function of 5-Fu concentration is presented in figure 4.

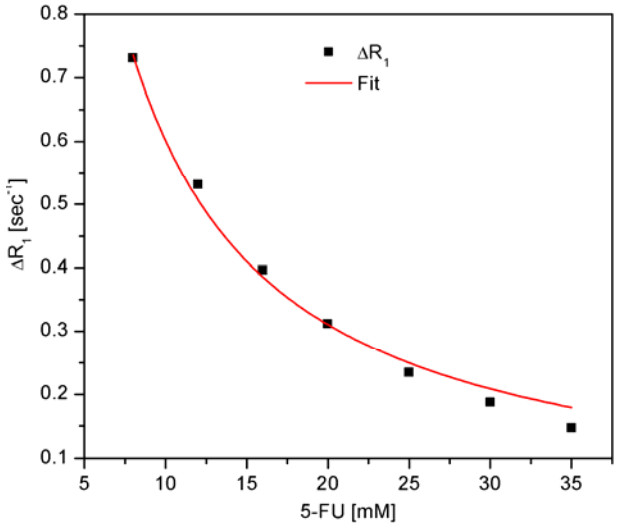


Fig.4. The selective transverse relaxation rate variation (ΔR_1) for 5-Fu proton as a function of its concentration

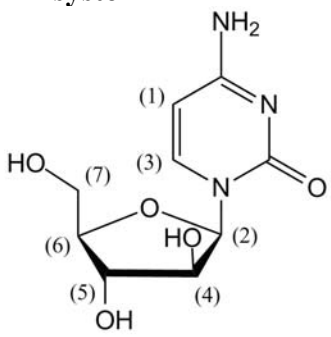
The experimental data presented in fig.4, were fitted with the equation:

$$\Delta R_1 = R_{1,obs} - R_{1,\,free} = \left(R_{1,bound} - R_{1,\,free}\right)\left[\frac{C_L + 0{,}2n + K_D}{2x} - \sqrt{\left(\frac{C_L + 0{,}2n + K_D}{2x}\right)^2 - \frac{0{,}2n}{C_L}}\right] \qquad (2)$$

From a separate experiment we determined for 5-Fu in the free state a selective relaxation rate value of $\mathbf{R_{1,free} = 0{,}0978 \pm 0{,}00003\ sec^{-1}}$**.** The obtained fitting parameters are:

$\mathbf{R_{1,bound} = 1{,}871\ sec^{-1}}$ $\qquad$ $\mathbf{n = 17{,}9 \pm 0{,}3}$ $\qquad$ $\mathbf{K_D = 0{,}372\ mM}$ $\qquad$ $\mathbf{r^2 = 0{,}991}$

e) Cytarabine – human serum albumin system



Citarabine

To study the direct binding of cytarabine in low-affinity binding sites of HSA, we prepared a set of 9 samples in which [hsa] = 0,2 mM = const. and [Cyt] varied between 4 and 45 mM. Experimentally we recorded the selective relaxation rate of H(5) and H(6) protons of cytarabine as a function of its concentration. Changes of the selective relaxation rate variation $\Delta R_{1,obs} = R_{1,obs} - R_{1,\,free}$ of the H5) and H(6) protons of cytarabine as a function of cytarabine concentration is presented in figure 5.

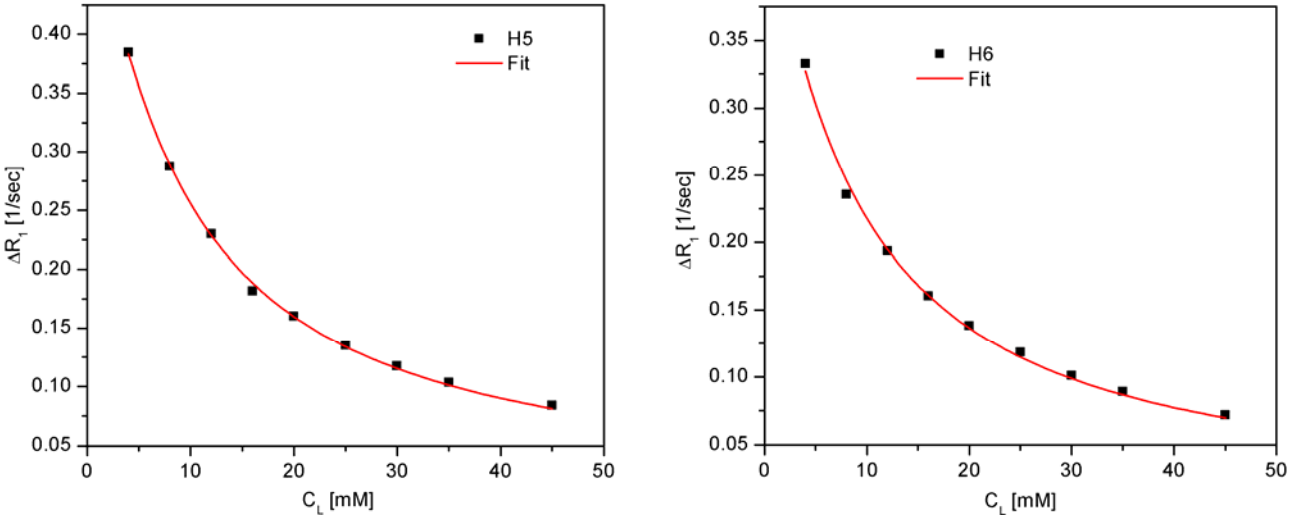


Fig. 5 The selective transverse relaxation rate variation (ΔR_1) for H(5) and H(6) protons of cytarabine as a function of its concentration

From a separate experiment we determined for cytarabine in the free state, the selective relaxation rate values of H95) and H(6) as:.

$$\mathbf{R_{1,free}(H5) = 0{,}322 \pm 0{,}001\ sec^{-1}} \qquad \mathbf{R_{1,free}(H6) = 0{,}373 \pm 0{,}001\ sec^{-1}}$$

Using eq.(2) to fit the experimental data we obtained the following results:

Proton	K_D (mM)	n	$R_{1,bound}$ (sec^{-1})	r^2
H(5)	4,91±0,16	13,8±0,2	1,80	0,999
H(6)	5,20±0,31	12,3±0,3	1,80	0,996

Because the obtained values for H(5) and H(6) are very close , we take into consideration their average values :

$$\mathbf{n = 13{,}05 \pm 0{,}25} \qquad \mathbf{K_D = 5{,}06 \cdot \pm 0{,}23\ mM}$$

f) Cytarabine – 5 fluorouracil – HSA system

An NMR investigation of this ternary system was used **to describe quantitatively the competitive binding** between 5-fluorouracil and cytarabine with human serum albumin. By following the selective relaxation rates variation of one ligand L (3-Fu) proton, as a function of the competitor ligand C (Cyt) concentration, we can determine the dissociation constant that characterize the competitor ligand C, in the presence of L. To realize such an experiment, first it is necessarily to determine in a **non-competitive binding experiment** the following parameters**:** K_D(L), $R_{1,free}$ (L) and $R_{1,bound}$(L). In a second experiment, we keep constant the ligand L and human serum albumin concentrations, for different competitor C, concentrations.. In our particular case, the first experiment consisted in the determination of $R_{1,free}$(5-Fu), $R_{1,bound}$(5-Fu) and K_D(5-Fu). The obtained results were presented in section (d). For the second experiment we prepared a set of 7 samples in which:

[hsa] = $[P_0]$ = 0.2 mM = ct.
$[L_0]$ = [5-Fu] = 10 mM = ct.
$[C_0]$ = [Cyt] varying between 4mM si 30 mM

In the Scientific Report 2015, we deduced the theoretical expression for the selective relaxation rate variation of L_0 = 5-Fu, as a function of the competitive ligand C_0 = Cyt., at a constant concentration of HSA. Working with both ligands concentration in excess we obtained:

$$\frac{[P_0]}{(R_{1,obs} - R_{1,free})} = \frac{K_D(L)}{K_D(C)(R_{1,bound} - R_{1,free})}[C_0] + \frac{K_D(L) + [L_0]}{(R_{1,bound} - R_{1,free})} \qquad (3)$$

A plot of $[P_0]/ (R_{1,obs} - R_{1,free})$ as a function of $[C_0]$ has a slope **m** = $K_D(L)/K_D(C)(R_{1,bound} - R_{1,free})$. In our case K_D(L) = K_D(5-Fu) = 0,372 mM and $R_{1,bound}$ (5-Fu) = 1,871 sec^{-1} .These parameters were deduced in the first experiment (section (a)). The dependence of the term $[hsa]/(R_{1,obs} - R_{1,free})$ as a function of cytarabine concentration is presented in Fig.6.

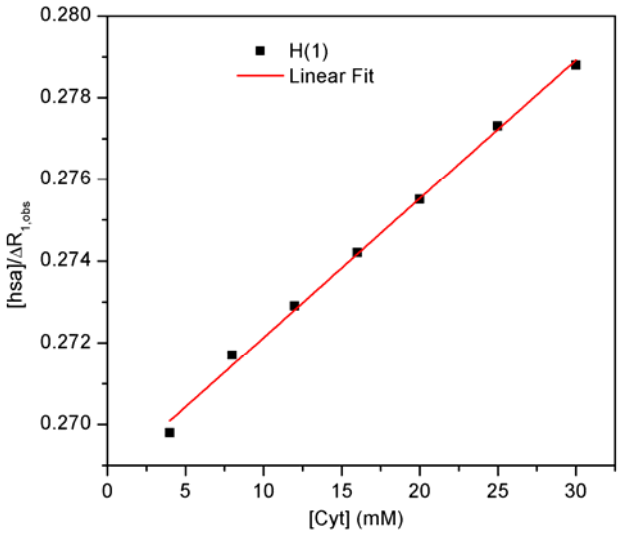


Fig.6. Plot of the 5-Fu H(1) proton relaxation data according to Eq.(2) for competitive titration with cytarabine

Fitting the experimental data with a linear equation (Y = mx + n) gave the following results:

m = 3,3946·10^{-4} sec **n = 0,2687 mM·sec** **r^2 = 0,9964**

From the value obtained for **m,** we deduced the dissociation constant of cytarabine, in the presence of 5-fluorouracil : **K_D(cyt) = 616,7 mM**

In the case of direct binding experiments (section **d** and **e**), the obtained results for $\mathbf{K_a = 1/K_D}$ were:

$$\mathbf{K_a(5\text{-}Fu) = 2688{,}2\ M^{-1}} \qquad \mathbf{K_a(cyt) = 197{,}6\ M^{-1}}$$

The conclusion, in the case of these two ligands, is that 5-fluorouracil is much stronger bound to human serum albumin than cytarabine. In a competitive binding, in the presence of 5-fluorouracil, cytarabine binds very weakly to HSA, its dissociation constant being almost two order of magnitude bigger then the value which characterize the direct binding. Due to the fact that both ligands bounds in subdomain IIA of HSA, we conclude that cytarabine is not able to replace 5-fluorouracil from its binding sites, only to a very small extent.

g) Clonidine- HSA system

In the case of clonidine –hsa system we have investigated the selective relaxation rate of clonidine H(2) proton as a function of its concentration, which varied between 8 and 50 mM. The human serum albumin concentration was kept constant at 0.1 mM. For the whole investigated concentration range, the differences between the measured values of $\mathbf{R_{1,obs}}$ were included in the experimental error. In conclusion, based on NMR selective relaxation rates measurements is was not possible to obtain quantitative information about the number of low-affinity binding sites and the association constant.