Research Article

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Investigation of relaxation times in 5-fluorouracil and human serum albumin mixtures

5-Fluorouracil ve İnsan Serum Albümin Karışımlarında Rölaksasyon Zamanlarının İncelenmesi

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Abstract

Background: Human serum albumin (HSA) is often selected as a subject of any study because albumin is the most abundant protein in human blood plasma. NMR is recognized as a valuable method to determine the structure of proteins-ligand and protein-drug complexes.
Objective – Aim of the study: In this study, protein drug interactions were investigated using 5-Fluorouracil anticancer drug and human serum albumin protein.
Materials and methods: In this context 400 MHz NMR spectrometry was used and NMR relaxation rates in drug-albumin complex were investigated with respect to increase albumin concentration and increase in 5-Fluorouracil (5-FU)-albumin solution temperature.
Results: The results of this study indicated that 5-FU had a weak association with albumin, and it easily dissociated from the protein to which it was attached.
Conclusion: The obtained results also gave us useful information about molecular dynamics of drug-albumin interactions.

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Keywords: Human serum albumin; 5-Fluorouracil; NMR; Relaxation rates; Temperature.

Öz

Geçmiş: İnsan serum albümini (HSA) sıklıkla herhangi bir çalışmanın konusu olarak seçilir çünkü albümin, insan kan plazmasında en bol bulunan proteindir. NMR, protein-ligand ve protein-ilaç komplekslerinin yapısını belirlemek için değerli bir yöntem olarak kabul edilmektedir.
Amaç – Aim of the study: Bu çalışmada, 5-Fluorourasil anti-kanser ilacı ve insan serum albümin proteini kullanılarak protein ilaç etkileşimleri araştırılmıştır.
Araç ve Yöntemler: Bu bağlamda, 400 MHz NMR spektrometresi kullanılmış ve ilaç-albümin kompleksi içindeki NMR rölaksasyon (gevşeme) oranları, albümin konsantrasyonunun arttırılması ve 5-Flüoroürasil (5-FU)-albümin çözelti sıcaklığındaki artış açısından incelenmiştir.
Bulgular: Bu çalışmanın sonuçları, 5-FU'nun albümin ile zayıf bir ilişkisinin olduğunu ve ekli olduğu proteinden kolayca ayrıldığını göstermiştir.
Sonuç: Elde edilen sonuçlar bize ilaç-albümin etkileşimlerinin moleküler dinamikleri hakkında da faydalı bilgiler vermiştir.

Anahtar Kelimeler: İnsan serum albumin; 5-Fluorouracil; NMR; Rölaksasyon (gevşeme) oranları; Sıcaklık.

Introduction

NMR has been a powerful tool for physicochemical analysis over almost 50 years, and NMR Spectroscopy is known as a valuable method for determining the structure of proteins,

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protein-ligand and protein-drug complexes [1–19]. Since a drug is usually transported as a complex with serum albumins in a circulatory system, the binding affinity of drugs to albumin plays a very important role in the determination of therapeutic dosage of drugs. Such binding affinities can be determined by NMR [20, 21]. During long-term treatment such as chemotherapy, a combination of different drugs is often required [22, 23]. The effect of the secondary drug used in the combination chemotherapy can also be analyzed in terms of NMR parameters such as chemical shifts and the line width of single proton and carbon resonances [24–32]. Such studies will always be the interest of research due to strong need for new generation drugs.

In the present work, HSA is selected as an object of study because albumin is the most abundant protein in human blood plasma. Albumin also plays an important role while storing and transporting drugs in human blood. 5-Fluorouracil (5-FU) is selected as drug since it is more often used in cancer therapy. To our best knowledge, the interaction between albumin and 5-FU has not been studied by NMR so far.

This work aims to gather information on binding affinity of the drug to albumin and also on the molecular dynamics of the drug-albumin complex. For these reason, the T_1 and T_2 relaxation times in D_2O solutions of albumin, in the presence and absence of 5-FU, were measured by 400 MHz NMR at different temperatures.

Materials and methods

5-FU, HSA, and deuterium oxide (D_2O) were obtained from Sigma-Aldrich Chemical Co. Six mixing bowls were prepared and 0.05 M 5-FU was added into each of them. One milliliter D_2O is added into each bowl and dissolution of the drug is provided in D_2O. Albumin is added to the D_2O-5-FU solution in each bowl in the amounts of 0, 0.01, 0.02, 0.03, 0.04, and 0.05 g, respectively, provided that albumin is thoroughly dissolved in the solutions. Similar concentrations of albumin in D_2O were also prepared in the absence of 5-FU. After complete dissolution of albumin, six different samples for 5-FU with varying concentrations of albumin were transferred to the NMR tube and measurements were then taken. Similar relaxation measurements were made for pure albumin concentrations (5-FU free samples), too.

To examine the effect of temperature in protein-drug interactions, 0.05 M 5-FU is added into a mixing vessel, 1 mL D_2O is added into the vessel, and dissolution of the drug is provided. Then 0.05 g of albumin is added into a D_2O-5-FU solution, provided that albumin is thoroughly dissolved in the solutions. Consequently, the resulting solution is transferred to the NMR tube. The ^1H NMR spectrum and spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) were measured for this sample at 298, 303, 308, 313, and 318 K.

Relaxation measurements were carried out with a Bruker Avance 400 spectrometer using 5 mm tubes. For proton measurements the probe was tuned at 400 MHz. For water suppression, the presaturation method was used. T_1 relaxation times measurements were carried out using Inversion Recovery Technique [the (180-τ-90) pulse sequence.] T_2 relaxation times measurements were carried out using CPMG (Carr-Purcell-Meiboom-Gill) technique [the (90-τ-180) pulse sequence]. In order to get the most suitable recovery curve for T_1 and decay curves for T_2, the more suitable set of τ delay times was chosen for each peak in the observed spectrum. A representative T_1 and T_2 curves of the HDO solution containing 0.05 M 5-FU + 0.01 g albumin are shown in Figure 1.

Results

Figure 2 shows 400 MHz $_1H$ NMR spectrum of the solution with 0.05 M 5-FU. In this spectrum, the HDO signal was observed at 4.70 ppm, whereas the doublet peak of CH belongs to 5-FU was seen at 7.59 and 7.58 ppm. The CH

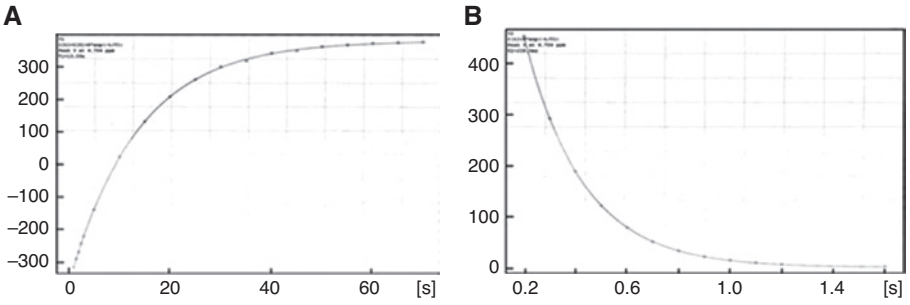


Figure 1: T_1 (A) and T_2 (B) profile of HDO signal in D20 with 0.05 M 5-FU + 0.01 g albumin sample.

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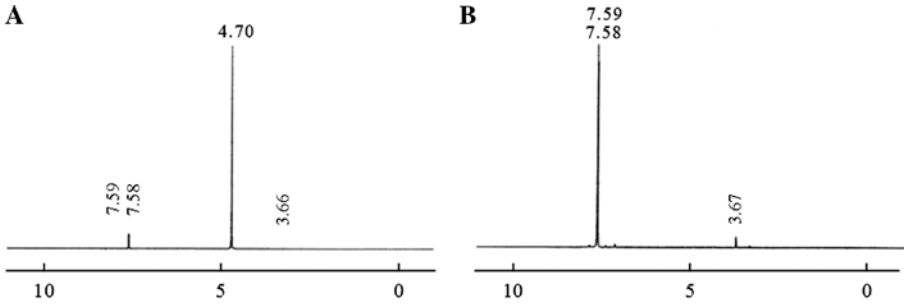


Figure 2: 400 MHz ^{1}H NMR spectrum of 5-FU single pulse (A), pre-saturation (B).

molecule peak splits into two parts and forms a doublet due to the neighborhood with HN molecule. The NMR spectrum of pure albumin in D_20 was not given here for the sake of brevity.

The HDO signal in the NMR spectrum, shown in Figure 2, was suppressed using the pre-saturation technique, and in this case the resulting $_1$H NMR spectrum is shown in Figure 2B. Referring to Figure 2B, the HDO signal is completely eliminated, whereas the intensity of the signal shown in 3.67 ppm for CH was observed to increase. The two spectra shown in Figure 2A, B have been selected in the same vertical axis scale and the corresponding comparisons have been discussed for this case.

The T_1 and T_2 relaxation rates in the solutions with different albumin contents are shown in Table 1. The relaxation rates of the signal in 3.66 ppm could not be calculated because of T_1 and T_2 relaxation times measurements did not yield any results. The plots for the $1/T_1$ and $1/T_2$ rates of albumin, HDO and the doublet versus albumin concentrations are given in Figure 3A, B and C, respectively. As shown in Figure 3B, C; $1/T_1$ and $1/T_2$ rates of HDO and CH signal in the solution with 5-FU increase linearly with the concentration of albumin. The relaxation enhancements for T_2 are much higher than those for T_1.

The relaxation rates in the solution at different temperatures are shown in Table 2, while the temperature

Table 1: The $1/T_1$ and $1/T_2$ relaxation rates of each peak in ^{1}H NMR spectrum of six different samples obtained by adding 0, 0.01, 0.02, 0.03, 0.04, and 0.05 g albumin into 1 mL D2O-0.05 M fixed concentration 5-FU at 250 C (298 K) constant temperature.

Albumin concentration (g/mL)	$1/T_1$ (s^{-1})			$1/T_2$ (s^{-1})		
	HDO with albumin	HDO with albumin + 5-FU	Doublet (CH)	HDO with albumin	HDQ-ALB + 5-FU	Doublet (CH)
0	0.0635	0.0563	0.0771	0.3224	0.5353	0.1254
0.01	0.0871	0.0735	0.2142	0.8865	4.3764	1.7870
0.02	0.0986	0.0877	0.3446	1.0995	4.7328	3.670
0.03	0.1055	0.0987	0.4384	1.1477	6.2657	5.2970
0.04	0.1242	0.1112	0.5333	1.4524	7.1994	7.0670
0.05	0.1285	0.1279	0.5787	1.7401	8.4317	8.7570

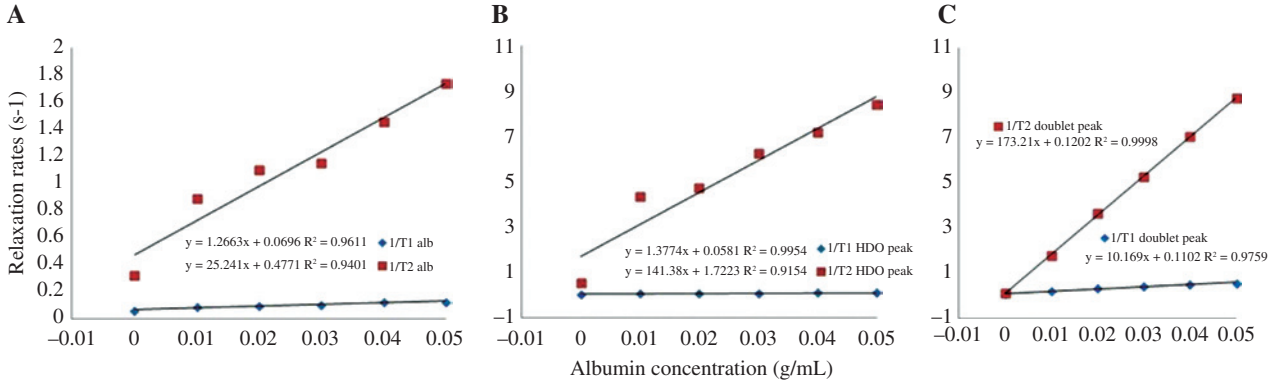


Figure 3: Graph for the relaxation rates of 5-FU peaks versus six different concentrations of albumin.

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Table 2: The $1/T_1$ and $1/T_2$ relaxation rates of each peak of ^{1}H NMR spectrum for the sample prepared by adding 0.05 g albumin into 1 mL D2O-0.05 M fixed concentration 5-FU at 298, 303, 308, 313, and 318 K.

Temperature (K)	$1/T_1$ (s^{-1})		$1/T_2$ (s^{-1})	
	HDO + albumin + 5-FU	Doublet (CH)	HDO + albumin + 5-FU	Doublet (CH)
298 K	0.1279	0.5787	8.432	8.756
303 K	0.1251	0.4847	9.009	6.784
308 K	0.1178	0.4353	9.107	5.171
313 K	0.1125	0.3886	9.208	3.825
318 K	0.1256	0.3859	8.865	2.940

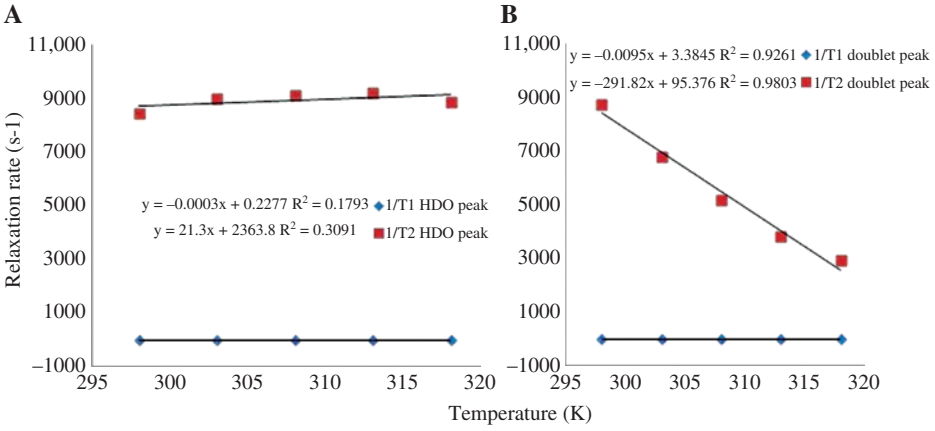


Figure 4: Graph of the relaxation rates of 5-FU peaks versus five different temperatures.

dependence of the $1/T_1$ and $1/T_2$ for HDO and CH signal are given in Figure 4A and B, respectively. It is seen that $1/T_1$ values of each peak and $1/T_2$ values of the HDO increased slightly with temperature but $1/T_2$ of the CH decreased strongly.

Discussion

Fielding and his collaborators discussed the advantage of using bovine serum albumin (BSA) as a model protein to test NMR techniques for the investigation of protein–ligand interactions. In their work, the binding affinity and stoichiometry of the specific binding site for some drugs such as L-tryptophan, D-tryptophan, naproxen, ibuprofen, salicylic acid, and warfarin have been established. In contrast to other techniques used in structural biology, NMR enables the determination of binding constants, too [33, 34]. Determination of the binding constants or binding affinity is involved in proton T_1-relaxation enhancement which is difference of the relaxation rates between drug-bound and -unbound cases [35, 36]. In the present case such difference can be calculated from extraction of values in third column from those of first one in Table 2. The small difference indicates binding of drug to albumin. This is consistent with previous literature given above. On the other hand, crystal structure analyses have showed that the drug binding sites are located in subdomains IIA and IIIA. The IIA subdomain has a large hydrophobic cavity [37, 38]. The present study is also convenient to the presence of drug-binding sites of albumin.

Chemotherapy is a treatment used to treat cancer. Surgical intervention and radiation therapy can kill or damage cancer cells in a particular region, but chemotherapy is used for working on the entire body. Over 100 chemotherapy drugs and their different combinations are used to treat cancer. Chemotherapy drugs can be used individually to treat cancer, but there are also cases of more than one drug being used in combination (called combination therapy). In combination therapy, several drugs with different effects work together to damage or kill large amounts of cancer cells [39, 40]. 5-FU is one of the most commonly used chemotherapy drugs to treat cancer. It has played a major role in the treatment of different types of cancer such as colorectal and breast cancers. As a result of understanding the mechanism of 5-FU action, different methods have been developed to enhance its anticancer activity since the last 50 years. In contrast to all these advances, the clinical

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use of 5-FU has important limitations due to drug resistance [41, 42]. Many articles contain information related to the chemical structure of 5-FU [43–46]. The molecular weight of 5-FU is 130.08 g/mol and its chemical formula is $C_4H_3FN_2O_2$. The binding of 5-FU to native and modified HSA was studied by several spectroscopic techniques. In one of these studies Bertucci et al. investigate the binding of 5-FU to native and modified human serum albumin by UV, CD, and ^1H and ^{19}F NMR [47]. Dissociation of FU from albumin is also important since albumin is just a carrier to transport drug to cancer tissue. The small relaxation increase in Table 1 indicates that binding is week. As a result, and that 5-FU dissociates from albumin easily. This is also consistent with previous results [48, 49].

On the other hand, the linear relationship between the relaxation rates and albumin concentration, given in Figure 1, indicates the presence of a fast chemical exchange between protons of HDO and albumin. This also consistent with data in Table 2 and Figure 4. However decrease in the $1/T_1$ of HDO + albumin + 5-FU and the relaxation rate of CH versus T indicate dipolar relaxation mechanism. On the other hand, the increase in $1/T_2$ of HDO + albumin + 5-FU versus T indicates a different relaxation mechanism, so called spin rotation mechanism. The spin-rotation theory for the relaxation mechanism of chemical molecules has already been given in several studies. The spin rotation contribution to $1/T_2$ is given by

$$\frac{1}{T_2}=\left(\frac{2}{3}\right)IC2h^{-2}kT\tau_{sr} \quad (1)$$

where k is the Boltzmann constant, T is the sample temperature, τ_{sr} is the correlation time for spin rotation, C is the spin-rotation interaction constant and I is the moment of inertia of the molecule [50]. Linear dependence of $1/T_2$ on T in Eq. 1 is consistent with T_2 data in Table 2 and the plot of $1/T_2$ in Figure 4A. It is also consistent with T_2 data obtained for albumin in D_2O.

Conclusions

Our results suggest that 5-FU is weekly associated to albumin, and easily dissociates from protein. The data also suggest that the relaxation mechanisms of CH and T_1 mechanism of D_2O + albumin + 5-FU are dipolar, while $1/T_2$ mechanism of D_2O + albumin + 5-FU is spin rotational [50, 51] since probability of bound phase is nearly 1 bound phase dominates the relaxation. Temperature dependence of data suggests that the relaxation of bound phase is caused by spin rotational relaxation mechanism [50].

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Conflict of interest: The authors declare that there is no conflict of interests regarding the publication of this article.

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