Journal of Luminescence 192 (2017) 424–427

Contents lists available at ScienceDirect

Journal of Luminescence

journal homepage: www.elsevier.com/locate/jlumin

The «*in vivo*» study of blood 5-fluorouracil content by quenching of intrinsic protein fluorescence

Regina R. Kayumova[a], Aleksandr V. Sultanbaev[b], Sergey S. Ostakhov[a], Sergey L. Khursan[a,*], Marat F. Abdullin[a], Shamil Kh. Gantsev[c], Dina D. Sakaeva[b]

[a] *Ufa Institute of Chemistry of the Russian Academy of Sciences, 71, Oktyabrya Prosp., 450054 Ufa, Russia*
[b] *Bashkir Republican clinical oncologic dispensary, 73/1, Oktyabrya Prosp., 450054 Ufa, Russia*
[c] *Bashkir State Medical University, 3, street Lenin, 450000 Ufa, Russia*

ARTICLE INFO

Keywords:
5-fluorouracil
Fluorescence
Tryptophan
Blood
Oncology
Photoinduced electron transfer

ABSTRACT

The dynamics of blood fluorescence (FL) after intravenous injection of 5-fluorouracil (FU) to oncology patients taking chemotherapy were investigated. It was demonstrated that FU quenches FL of water solutions of tryptophan (Trp) (total concentration is 1.0×10^{-5} mol L^{-1}, λ_{max} = 353 nm) and plasma protein – albumin (λ_{max} = 340 nm) with the Stern–Volmer constants 16.4×10^{3} L mol^{-1} and 52 L mol^{-1}, accordingly. The quenching of both Trp and albumin FL by 5-fluorouracil was concluded to proceed *via* the static mechanism of reversible photoinduced electron transfer (PET).

1. Introduction

5-Fluorouracil (FU) is a broad spectrum anti-cancer medication used in oncology to treat the tumors of stomach, colon, pancreas, lungs and metastases to these organs [1]. The mechanism of action of FU active metabolites: 5-fluorodeoxyuridine monophosphate, 5-fluorodeoxyuridine triphosphate, 5-fluorouridine triphosphate, and 5,6-dihydro-FU is based on the disturbance of DNA synthesis, formation of structurally deficient RNA and, as a consequence, decrease in cancer cell division [2].

One of the main assessment parameters of the medication efficiency is elimination half-life of a drug. From this perspective, the search and development of analytical tests on FU and its metabolites content in biological media are of current interest. It is known different analytical methods in the literature like the capecitabine (fluoropyrimidine carbamates) tests in the blood plasma using high-performance liquid chromatography (HPLC) with UV detector [3,4], or the detection of capecitabines, FU and other active metabolites in the blood plasma and human tumor cells by tandem mass spectrometry [5]. The NMR spectroscopy with fluorine tracers (F^{19}) is shown to be promising for the investigating of FU metabolism in the living tissue [6]. Unfortunately, an apparent significance and sensitivity of above-mentioned analytical methods to study FU pharmacokinetics is associated with a labor-consuming preparation of analyzed samples [7,8] as well as with expensive equipment.

It is common knowledge that proteins obtain a bright UV luminescence which is caused by the radiative deactivation of excited states of aromatic amino acids – tyrosine, phenylalanine and, most of all, tryptophan [9–11]. Considering the fact that the FL intensity (I_{fl}) of Trp is highly sensitive to the changes of Trp environment [9], the method of intrinsic protein luminescence [12] may be worthwhile for studying properties of biological objects, and this may be the simple and economical way to solve a lot of problems of physicochemical analysis in medical and biochemical investigations as well as in clinical diagnosis [13,14].

It was demonstrated earlier that FU quenches FL of tryptophan component of water solutions of bovine serum albumin (BSA) [15], Trp and blood [16,17]. In this regard, the objective of this work is to study the FU content in the blood of oncology patients taking chemotherapy using quenching of blood FL that involves tracking changes of I_{fl} with the time after medication administration.

2. Experimental section

The corrected FL spectra were registered using spectrofluorometer "CM-2203" in quartz cells (l = 5 mm) by photoexcitation at the angle of 45°. The photoexcitation of water solutions of Trp and blood was followed into the $S_2 \leftarrow S_0$ amino acid transition band on the wavelength (λ_{ex}) of 220 nm; then the FL spectra in the emission wavelength diapason (λ_{em}) of 300 ÷ 400 nm were recorded. 5-Fluorouracil (99.5%)

* Corresponding author.
E-mail address: khursansl@gmail.com (S.L. Khursan).

http://dx.doi.org/10.1016/j.jlumin.2017.07.023
Received 22 February 2017; Accepted 14 July 2017
Available online 15 July 2017

was used without a preliminary purification. The Trp solutions were prepared with bi-distilled water.

The blood was taken from the median cubital vein before and after the 2 min stream infusion of the medication "5-Fluorouracil EBEWE", produced by EBEWE Farma (Austria). The blood specimens (1 ml) after the injection were diluted with the solution of anticoagulant – sodium citrate at the ratio of 1:1.

The mass-spectra of electrospray ionization (ESI) of the blood plasma specimen were obtained using quadrupole liquid chromatograph/mass spectrometer LCMS-2010 EV Shimadzu (syringe injection of a specimen, 0.1 ml/min, eluent – acetonitrile/water at the ratio of 75/25) in the registration mode of negative ions by the capillary potential of 3.5 kV. Heater temperature was 200 °C, evaporator temperature was 230 °C. The spraying gas flow velocity (nitrogen) was 1.5 l/min. The specimen of the blood plasma taken in equal amounts before the injection, after 1 min and 12 min after the FU stream infusion, diluted with equal amounts of bi-distilled water, were introduced into the mass spectrometer.

3. Results and discussion

The Fig. 1 shows the spectra of FL of Trp water solution (spectrum 1, λ_{max} = 353 nm) and blood (spectrum 2, λ_{max} = 340 nm); the latter is caused by luminescence of the superficial Trp residues in the serum albumin [9,15]. The adding of FU leads to FL quenching of Trp water solution (Fig. 1, spectrum 3) or blood (Fig. 1, spectrum 4). The effect of blood FL quenching by 5-fluorouracil enables the FL control of FU content in the native specimens without their preliminary treatment, and this determines the rapidity and easiness of studying the pharmacokinetics of an anti-cancer medication.

The I_{fl}*vs.* concentration dependence of the quenching of Trp and blood FL with 5-fluorouracil is well obeyed to the Stern-Volmer equation (Fig. 2) [18]:

$$I_0/I - 1 = K[\mathrm{FU}] = k_{bi}\cdot\tau_0[\mathrm{FU}] \quad (1)$$

Here, K is the Stern-Volmer quenching constant; [FU] – quencher concentration; I_0, I –intensity of Trp or blood FL without and with FU, correspondingly; k_{bi} – an effective bimolecular quenching rate constant; τ_0 – lifetime of electron-excited state of Trp in the absence of quencher.

The Stern-Volmer FL quenching constant of Trp with 5-fluorouracil (Fig. 2, dependence 1) was found to be 16.4 × 10^3 L mol^{-1}, which is in

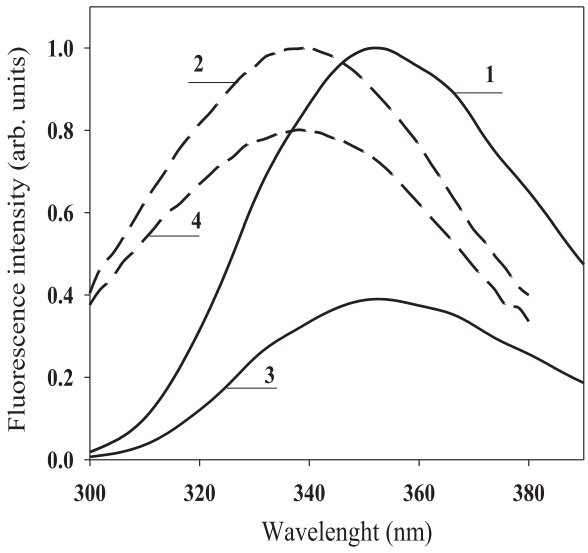

Fig. 1. The corrected normalized FL spectra of: 1 – Trp (1.0 × 10^{-5} mol L^{-1}, H_2O), 2 – blood, 3 – Trp (1.0 × 10^{-5} mol L^{-1}, H_2O) in the presence of FU (1.0 × 10^{-4} mol L^{-1}), 4 – blood in the presence of FU (8.0 × 10^{-3} mol L^{-1}). Experimental conditions: λ_{ex} = 220 nm, T = 298 K.

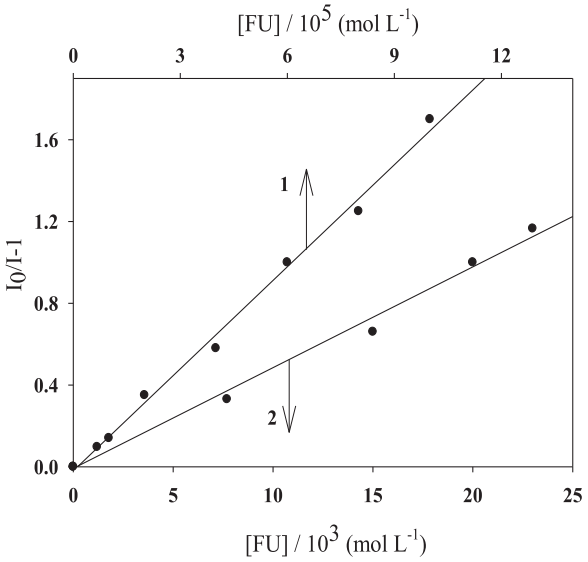

Fig. 2. The FL intensity *vs.* FU concentration dependence in the Stern-Volmer equation coordinates (l): 1 – Trp (1.0 × 10^{-5} mol L^{-1}, H_2O), 2 – blood. Experimental conditions: λ_{ex} = 220 nm, T = 298 K.

reasonable consistence with that of FL quenching of BSA water solutions (1.0 × 10^{-5} mol L^{-1}, 301 K) [15], K = 2.6 × 10^3 L mol^{-1}. The observed difference in the K values is apparently caused by the hindered accessibility of Trp for the quencher in the albumin protein.

Since the FL Trp lifetime in water is 3.1 ns [9], the effective bimolecular quenching rate constant may be estimated as $k_{bi} = K/\tau_0$ = 5.3 × 10^{12} L mol^{-1} s^{-1}. The obtained value is 1000-fold greater than the diffusion rate constant limit in water (6.5 × 10^9 L mol^{-1} s^{-1}), 293 K [18]. On this basis it is assumed that the quenching of FL Trp is followed to the static mechanism [19] as a consequence of the reversible intracomplex photoinduced electron transfer (PET) (equation 2).

$$\mathrm{Trp} + \mathrm{FU} \overset{h\nu}{\rightleftarrows} [\mathrm{Trp}^{\bullet+}\cdots\mathrm{FU}^{\bullet-}] \quad (2)$$

It is worth pointing out that the static mechanism of FL quenching of Trp and BSA water solutions through the PET mechanism was postulated previously in the Refs. [15–17]. According to the Weller equation [20], the change in the Gibbs free energy ΔG of the PET reaction from the electron-excited donor D (Trp) to the acceptor of electron A (FU):

$$\Delta G = E^{\circ}(\mathrm{D}^{\bullet+}/D) - E^{\circ}(A/A^{\bullet-}) - \Delta E_{0-0}{}^{*} + W \quad (3)$$

is defined by the difference of the redox potentials of the donor $E(\mathrm{D}^{\bullet+}/\mathrm{D})$ and acceptor $E(\mathrm{A}/\mathrm{A}^{\bullet-})$, as well as by the excitation energy of tryptophan molecules. The standard redox potential (E) of tryptophan and its 0–0 transition excitation energy ($\Delta E_{0-0}{}^{*}$) were taken from the literature data: $E^{\circ}(\mathrm{Trp}^{\bullet+}/\mathrm{Trp})$ = 1.015 V [21], $\Delta E_{0-0}(\mathrm{Trp}^{*})$ = 3.9 eV [22]. The last term W is negative and counts for the Coulomb interaction in the donor/acceptor radical-ion pair. In our particular case this energy is too small in aqueous solutions (ε = 78): $W \approx -0.03$ eV under simplified approximation of the donor–acceptor pair ($\mathrm{D}^{\bullet+}\ldots\mathrm{A}^{\bullet-}$) as point charges e and taking the distance between reactants about 10 Bohr radii. Thus, in our case, the W value in the Weller equation can be neglected. Since the redox potential of FU is unknown, the thermodynamic probability of PET ΔG_I was calculated in the G3MP2B3 + IEFPCM approximation. The Weller equation can be rewritten in terms of Gibbs free energy as follows:

$$\Delta G \le \Delta G_{solv}(\mathrm{D}^{\bullet+}/D) - \Delta G_{solv}(A/A^{\bullet-}) - \Delta E_{0-0}{}^{*} \quad (4)$$

where ΔG_{solv} is the difference between the Gibbs free energies of a neutral and ionized molecule of the donor or acceptor with account of solvation of species participating in the electron transfer. The values of absolute G_{solv} values for 5-fluorouracil and its anion-radical are

Table 1
G3MP2B3 + IEFPCM calculation of the Gibbs free energies of uracils and their ionized forms.

Parameter	Uracil	Thymine	5-Fluorouracil
G_{gas}(A), Hartree	− 414.305233	− 414.305233	− 513.457382
ΔG_{PCM}(A), Hartree	–	–	− 0.019044
G_{solv}(A), Hartree	–	–	− 513.476426
G_{gas}(A$^{\bullet-}$), Hartree	− 414.307004	− 414.307004	− 513.468014
ΔG_{PCM}(A$^{\bullet-}$), Hartree	–	–	− 0.094478
G_{solv}(A$^{\bullet-}$), Hartree	–	–	− 513.562492
EA, eV	0.048	0.010	0.29
EA, eV [24]	0.075	0.069	–

specified in the Table 1. It was found that $\Delta G_{solv}(A/A^{\bullet-}) = 2.34$ eV. The reliability of theoretical value was illustrated by the calculation of electron affinity (*EA*) of uracil and thymine, $EA = G_{gas}(A^{\bullet-}) - G_{gas}(A)$, see Table 1. The $\Delta G_{solv}(D^{\bullet+}/D)$ value of tryptophan was calculated based on known values of Trp redox potential (see above) and an absolute redox potential of the standard hydrogen electrode (*SHE* = 4.281 V [23]). Since $\Delta G = -n{\cdot}F{\cdot}\Delta E^{\circ}$ (where $n = 1$ is a number of transferred electrons, $F = 1$ eV/V is Faraday constant), it was obtained $\Delta G_{solv}(D^{\bullet+}/D) = -n{\cdot}F{\cdot}(1.015 + 4.281) = 5.30$ eV. Thus, the Eq. (4) allows to calculate the value of PET ΔG_I from Trp to 5-fluorouracil. The obtained result ($\Delta G_I = -0.94$ eV) shows a high efficiency of PET in the electron-excited tryptophane – 5-fluorouracil donor-acceptor pair.

The efficiency of blood FL quenching by 5-fluorouracil (Fig. 2, dependence 2) was found to be 52 L mol^{-1} which is significantly lower than that in water solutions of Trp and BSA. We tentatively explained this observation by the higher viscosity of blood, which retards the diffusion of donor and acceptor to each other.

The Fig. 3 shows the dependence of I_{fl} of blood specimen from the time after the FU stream infusion to the patient No 1. According to the known pharmacokinetic data, the FU elimination half-life period is 8 ÷ 22 min [25] and a sharp blood FL quenching immediately after the injection with the following monotonous luminescence intensity growth would be logical. But, contrary to our expectations, two minima for the dependence of blood I_{fl}*vs.* time are registered (Fig. 3). The FL minima were observed for the patient No 1 in 1 and 16 min after the FU medication injection.

This unexpected observation cannot be associated with an artefact, since the same blood I_{fl}*vs.* time profile with two minima was obtained

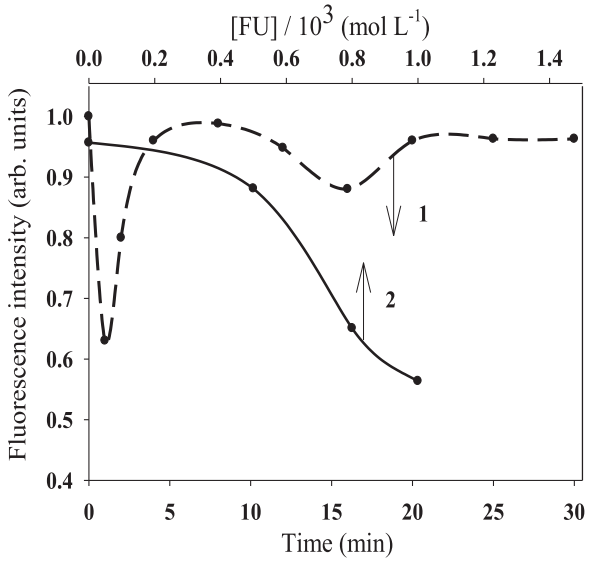

Fig. 3. The blood I_{fl}*vs.* time profile for the patient No. 1 after the stream intravenous injection of 0.5 g of FU, $T = 298$ K (1); the plot of blood I_{fl} against the FU concentration, $T = 310$ K (2).

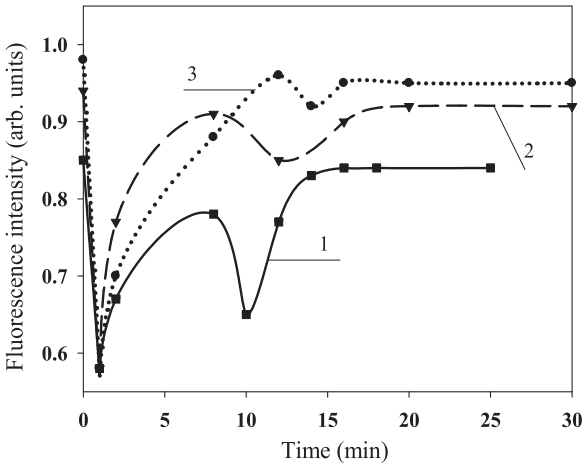

Fig. 4. The blood I_{fl}*vs.* time profile for three patients after the stream intravenous injection of FU, normalized to the first minimum, $T = 298$ K.

for the whole group of patients (in total, 19 persons) irrespective of introduced medication dose, sex, age, grade and localization of malignancy. By the heart rate of 70 ÷ 80 strokes per minute, the blood circulation takes about 20 ÷ 23 s [26]. The first minimum is observed for all patients strictly after 1 min after the FU injection and is supposed to correspond to the maximal FU concentration in blood. In order to calculate the *in vivo* FU concentration, we have obtained the calibration plot of blood I_{fl} (310 K) against the *in vitro* concentration of medication (Fig. 3, dependence 2). The blood volume depends on the human body weight and amounts to 6 ÷ 8% from its value, in average [24]. For the patient No 1 the FU concentration in blood after its injection in the dose of 0.5 g (weight = 68 kg, blood volume is *ca.* 4.1 ÷ 5.4 l) varies within the range of 0.7 ÷ 1.0 × 10^{-3} mol L^{-1}. It is obvious from the plots 1 and 2 (Fig. 3) that within 1 min after the injection, the FU concentration in blood is as high as 0.8 × 10^{-3} mol L^{-1}. Similarly, it is worth noting that for all 19 patients the FU concentration in blood after 1 min is corresponded to the dose of introduced medication.

Contrary to the first minimum, the appearance and depth of the second minimum on the time dependence of blood I_{fl} after the FU injection is different for all patients. The Fig. 4 illustrates the I_{fl}*vs.* time curves for three patients obtained the equal injected FU dozes. All plots are normalized to the first minimum. Apparently, the individual constitutional features (metabolism, tumor localization, stage of oncological disease, presence of metastases, liver and kidney function *etc.)* are responsible for the time of appearance and I_{fl} level for the second minimum, which was observed within 10 ÷ 16 min interval after FU injection.

In order to assign extrema on the dependence 1 (Fig. 4) to the action of FU or its active metabolites [2], a mass-spectrometry analysis of the blood plasma taken at the different time after the medication injection was performed. The mass-spectra of the studied specimen after both 1 and 12 min of post-injection period show the only one ion peak typical for FU $(M\text{-}H)^-$ with *m/z* 129. The peak height after 12 min in the mass-spectra is significantly lower than that after 1 min. Thus, we can state that both minima on the time dependence of blood I_{fl} are caused by the quenching of blood intrinsic FL by 5-fluorouracil solely, not by its active metabolites. At present it is hard to interpret unambiguously such an unusual dynamics of intrinsic blood FL quenching after the 5-fluorouracil injection. As a working hypothesis we can suggest that the presence of the second minimum on the time dependence of blood I_{fl} after the FU injection is due to the fact that, according to the main rule of lymphology [27], plasma proteins getting from blood into interstitial space may return again to the blood flow.

4. Conclusion

The obtained results open a prospect for developing an analytical express FU test by means of FU quenching of intrinsic blood FL and, in broader terms, fluorescence titration of multicomponent solutions of biological fluids after injecting of various medications quenching tryptophan FL.

Acknowledgement

All calculations were performed using the supercomputer cluster of Ufa Institute of Chemistry of Russian Academy of Sciences. The facilities of the center of collective use “Chemistry” (Ufa Institute of Chemistry of RAS) were used for spectral measurements.

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