FLUORESCENCE AND DSC STUDY OF INTERACTION OF TWO ANTIRETROVIRAL DRUGS WITH HUMAN METHAEMOGLOBIN

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Abstract: Fluorescence and calorimetric measurements were performed to characterise interaction of methaemoglobin with two antiretroviral drugs (nevirapine NVP and azidothymidine AZT) in water solution. Fluorescence quenching was used to determine the binding constant K_D, association constant K_a and the number of binding sites. Moreover, calorimetric measurements were done to describe the thermal stability of methaemoglobin in presence of NVP and AZT. From DSC data, first order rate constant k, activation energie E_a and half life time τ were calculated. The results point at the weak interaction between drugs and mHb and reduction in stability of mHb in presence of ligands.

Introduction

Methaemoglobin (mHb) is formed by oxidation of ferrous iron of haemoglobin to ferric form, which normally takes place in erythrocytes at rate 3% per day [1]. It is maintained at minimal level by constant reduction to haemoglobin by NADH dehydrogenase. Methaemoglobinemia can occur if oxidant stress exceeds the reverse capacity of the reducing enzyme, if enzyme is deficient or inhibitiend, or if supply of NADH is reduced [2]. It is well known that many of drugs are bound to serum proteins. So, the studies on the binding drugs to these proteins may provide information of the structural features that determine the therapeutic effectiveness of drugs and become an important research field in life science, chemistry and clinical medicine. Newborns with HIV are especially open on pulmonary hypertension which is treated by drugs caused high level of mHb. Moreover, antiretroviral drugs through interactions with mHb could slow down its reduction to haemoglobin and in consequence lead to methaemoglobinemia.

Azidothymidine (AZT) and nevirapine (NVP) belong to nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), respectively. Combination of these two drugs reduce mother to child transmission of the human immunodeficiency virus (HIV).

In series of study methods concerning the interaction of drugs and protein, fluorescence and calorimetric techniques are vary useful because of their high sensitivity, rapid and easy implementation. To our knowledge there are no paper concerned interaction between NVP or AZT and mHb.

Materials and methods

Human methaemoglobin (Lot 103H9341, crystallized, lypophylized and dialyzed) was purchased from Sigma Chemical Co. and used as received. Azidothymidine come from GlaxoSmithKline and nevirapine from Boehringer Ingelheim Pharmaceuticals Inc.

Methaemoglobin was dissolved in water pro injection. The protein concentration was $1.55 \cdot 10^{-5}$M in all experiments. The AZT and NVP were added to the mHb solutions to obtain final concentration 0.1-1mg mL^{-1}

The binding of drugs to mHb was studied by monitoring changes in emission fluorescence spectra of protein in the presence of drugs using Hitachi F-2500 Spectrofluorimeter.

The binding of the drugs to mHb was studied at excitation wavelength of 274 nm and the data sampling interval of 1 nm. The emission of the fluorescence was measured in the range 284 – 535 nm. All spectra were recorded at speed 60 nm min^{-1}. Both excitation and emission side slit widths were 2.5 nm with photomultiplier voltage 700 V.

Spectrophotometer UV/VIS Jasco V-530 was used as an assistant technique.

Calorimetric measurements were caried out with the VP DSC ultrasensitive microcalorimeter (Microcal Inc., Northampton, MA). The DSC curves were obtained in the temperature range 298 - 363 K at scanning rates $0.8 \cdot 10^{-2}$, $1.2 \cdot 10^{-2}$, $1.6 \cdot 10^{-2}$ and $2.5 \cdot 10^{-2}$ K s^{-1}. The data were corrected for the instrumental baseline and for the difference in heat capacity between the initial and final state by using a linear baseline. DSC curves were analyzed with MicroCal Origin software.

The results were obtained from seven independent experiments. The estimated uncertainties did not exceed 5%.

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Results

a) Fluorescence studies

Figure 1 and Figure 2 present fluorescence emission spectra between 300 and 400 nm where peak with maximum about 323 (±2) nm for mHb occurs. The decrease in the fluorescence intensity for all studied samples tritrated drugs was observed.
The addition of both drugs changed the fluorescence intensity of mHb-drug mixtures. When mHb was excited with 274 nm the fluorescence quenching effect was accompanied with the red shift 1 - 10 (±2) nm for nevirapine (Figure 1), while for azidothymidine any shift of emission peak was observed (Figure 2). The biggest shift was obtained at $9.30 \cdot 10^{-4}$M NVP concentration. The fluorescence quenching was stronger for mHb with NVP than for AZT.

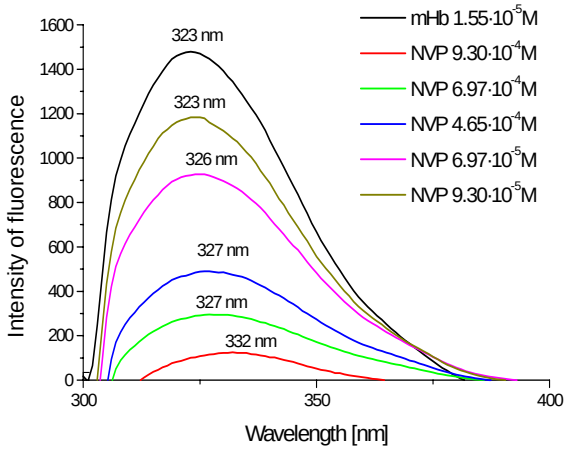

Figure 1: Fluorescence emission spectra of human methaemoglobin in presence of different nevirapine NVP concentration excited at 274 nm.

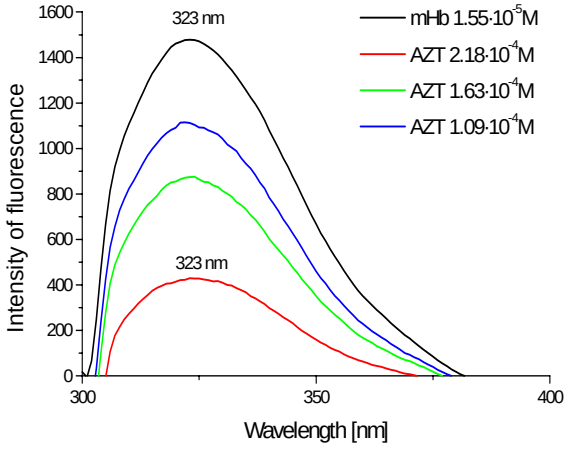

Figure 2: Fluorescence emission spectra of human methaemoglonin in presence of different azidothymidine AZT concentration excited at 274 nm.

Using fluorescence spectroscopy the interaction of drugs with methaemoglobin (mHb) could be studied.

The binding constants were determined from the modified Stern–Volmer equation (1) for protein as a macromolecule [3]:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_D} \cdot \frac{1}{[Q]} + \frac{1}{f_a} \qquad (1)$$

where: F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the concentration of quencher.

Moreover, the association constants (K_a) and the number of binding sites (n) for both drugs were determined from the Scatchard equation (2) [4]:

$$\frac{[L_b]}{[L_f][protein]} = nK_a - \frac{K_a[L_b]}{[protein]} \qquad (2)$$

where:

$$[L_b] = \frac{F_0 - F}{(F_0 - F)_{\max}} \cdot [protein] \qquad (3)$$

and

$$[L_f] = [L] - [L_b] \qquad (4)$$

[L] – total concentration of drug fraction, *[L_f]* – concentration of free fraction, *[L_b]* – concentration of bound fraction.

Obtained measurements allow us to calculate characteristic parameters of interaction between mHb and drugs according to equations (1 – 4).

The values of quenching constants (K_D), K_D^{-1} (quencher concentration at which 50 % of the fluorescence intensity is quenched), the values of association constant (K_a) and n for both drugs are presented in Table 1.

Table 1: The values of quenching constants (K_D), K_D^{-1} (quencher concentration at which 50 % of the fluorescence intensity is quenched), association constant (K_a) and number of binding sites (n) for NVP and AZT in mHb solution.

Parameters	NVP + mHb	AZT + mHb
K_D [M^{-1}]	$(2.6 \pm 0.4) \cdot 10^{3}$	$(1.9 \pm 0.2) \cdot 10^{3}$
K_D^{-1} [M]	$(3.8 \pm 0.5) \cdot 10^{-4}$	$(5.2 \pm 0.5) \cdot 10^{-4}$
K_a [M^{-1}]	$(3.9 \pm 0.3) \cdot 10^{3}$	$(2.5 \pm 0.4) \cdot 10^{3}$
n	1.2 ± 0.1	1.0 ± 0.1

mean ± SEM

b) DSC studies

DSC measurements confirm the occurrence interaction between drugs and human mHb (Figure 3, and 4). Figure 3 presents a typical curves apparent heat

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capacity C_p profile obtained at different scanning rate for the thermal denaturation of mHb water solution. The transition temperature T_m shifts towards higher temperatures with scan rate increasing.

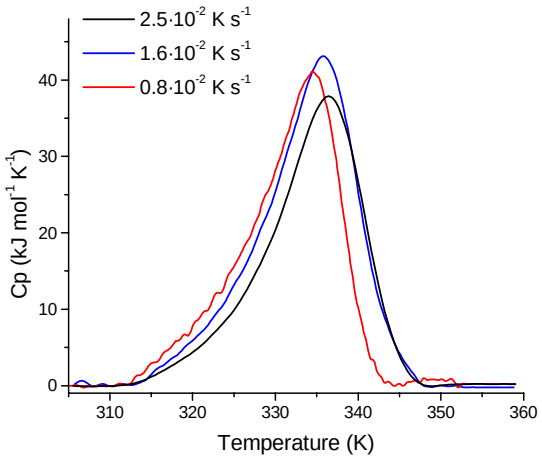

Figure 3: DSC curves of methaemoglobin obtained at different heating rates.

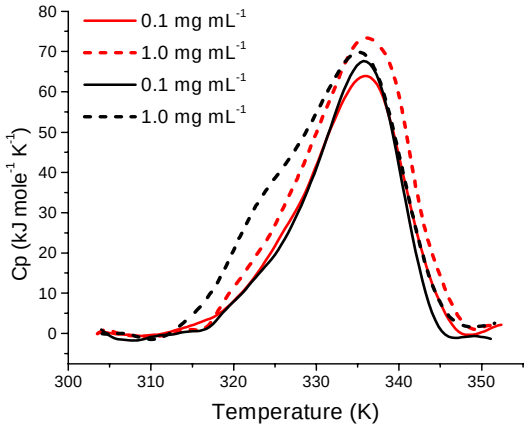

Figure 4: The effect of AZT (red lines) and NVP (black lines) concentration L on the DSC curves of mHb at the heating rate $2.5 \cdot 10^{-2}$ K s^{-1}.

The effect of AZT and NVP concentration on mHb DSC curves at the heating rate $2.5 \cdot 10^{-2}$ K s^{-1} is shown on Figure 4. The peak of denaturation heat absorption is a little shifted to lower temperatures and its height increase with drugs concentration. Moreover, some wideness and change of DSC curve shape were observed in the presence of drugs.

Concentration dependence of denaturation temperature T_d as well as denaturation enthalpy $\Delta_d H$ are well approximated by linear functions (Figure 5). Average parameters of fitting function are presented in Table 2. Small deviations from average values indicates an independence of slope factors B_T for $T_d(L)$ and B_H for $\Delta_d H(L)$ on the heating rate.

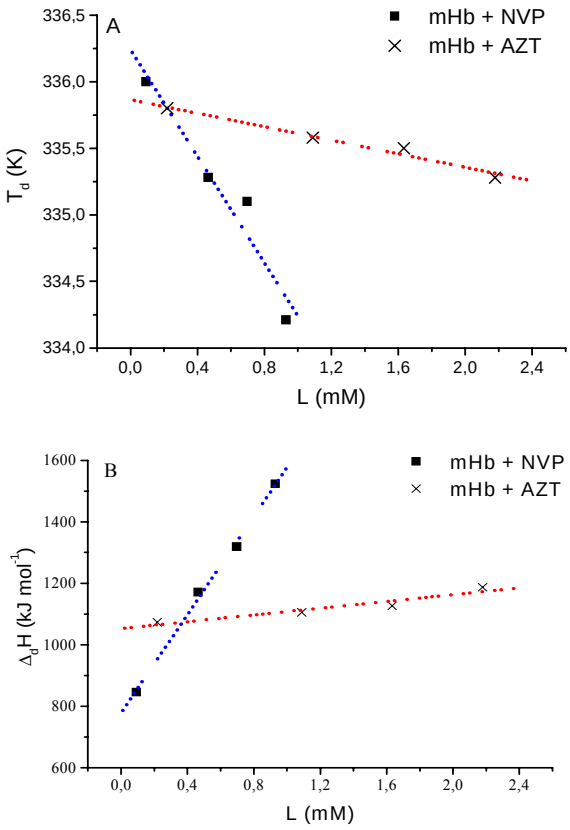

Figure 5: The effect of drugs concentration on the denaturation (A) temperature and (B) enthalpy of mHb at heating rate 1.5 K min^{-1}.

Table 2: The slope factors B_T for $T_d(L)$ and B_H for $\Delta_d H(L)$ averaged over all heating rates.

Parameters	NVP + mHb	AZT + mHb
B_T [K M^{-1}]	-2.0 ± 0.03	-0.2 ± 0.01
B_H [J mol^{-1} M^{-1}]	796.4 ± 3.0	55.1 ± 0.3

mean ± SEM

The scan rate dependence indicate that degradation process of mHb could be treated as kinetically controlled. The rate constant k of mHb, mHb–NVP and mHb–AZT solutions degradation process can be calculated with use three different equation (5 – 7) where Q_t is total heat of process (equivalent to $\Delta_d H$), Q - heat evolved at given temperature T, C_p^E excess heat capacity [5]:

$$k = VC_p^E (Q_t - Q)^{-1} \quad (5)$$

and the energy of activation E_a is estimated from the slope of the Arrhenius plot, ln k versus T^{-1} :

$$\ln[\ln Q_t (Q_t - Q)^{-1}] = \frac{E_a}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right) \quad (6)$$

and a plot of $\ln[\ln Q_t (Q_t\text{-}Q)^{-1}]$ versus T^{-1}.

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$$E_a = 2.718RC_p^m T_m^2 Q_t^{-1} \quad (7)$$

The results do not depend on the calculation way. Average values of activation energy, rate constant k and half life time obtained with use of three methods are presented in Table 3.

Table 3: The activation energies E_a, first order rate constant k and half - live time $\tau_{1/2}$ for mHb water solution in absence and presence of AZT or NVP.

Sample	L [mg mL^{-1}]	E_a [kJ mol^{-1}]	k_{25} 10^{-4} [min^{-1}]	$\tau_{1/2}$ [days]
mHb	1.0	170.0 ± 6.2	3.0 ± 0.5	1.5 ± 0.1
NVP + mHb	0.1	158.5 ± 12.4	3.1 ± 0.8	1.4 ± 0.3
	0.5	150.5 ± 9.7	4.4 ± 1.0	1.1 ± 0.2
	0.7	142.1 ± 6.8	6.1 ± 0.7	0.7 ± 0.1
	1.0	128.0 ± 8.8	10.0 ± 2.5	0.5 ± 0.1
AZT + mHb	0.1	168.2 ± 6.4	2.6 ± 0.7	1.8 ± 0.4
	0.5	153.2 ± 13.0	3.4 ± 0.8	1.4 ± 0.5
	0.7	147.0 ± 6.3	3.0 ± 0.8	1.6 ± 0.5
	1.0	147.3 ± 9.0	3.3 ± 1.0	1.4 ± 0.3

mean ± SEM

Discussion

Spectrofluorimetric as well as microcalorimetric measurements allow us to estimate magnitude of interaction between antiretroviral drugs and human methaemoglobin.

A decrease of fluorescence intensity with drug concentration for methaemoglobin solutions was obtained. It is known, that collisional quenching of fluorescence (the absorption spectra did not change in the presence of the quenchers, which were nonfluorescent) is connected with interaction of drugs with protein in water solution [3, 4, 7-10]. A red shift of maximal emission wavelength with concentration was observed for mHb – NVP mixture unlike mHb – AZT. Such shift is caused by fact that during protein unfolding the environments of tryptophan and tyrosine residues become less hydrophobic and more exposed to the solvent [3, 6]. It confirms the drug-protein interaction.

The observed endothermic transition on DSC curves is connected with the unfolding of mHb molecules but the changes in shape of curves in the presence of drugs seems to be caused by interaction between components as well as aggregation process. Shift of denaturation temperature in mHb – drug mixtures does not depend on the heating rate and reflects their thermodynamic nature. It suggest that drugs interact with mHb [11].

Moreover the estimation in kinetic model showed that activation energy and half - life time decrease with ligand concentration increase. It follows that stability of mHb in presence of drugs decreases, especially for nevirapine.

Conclusions

Fluorescence and microcalorimetric data analysis showed that nevirapine and azidothymidine can be bounded to methaemoglobin. This bounding is more marked for mHb – NVP mixture than for mHb – AZT. Thermal stability of mHb solution in drugs presence decreases with increasing drug concentration.

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