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BINDING OF CAFFEINE WITH NICOTINAMIDE: A STUDY BY MEANS OF FLUORESCENCE QUENCHING AND UV-Vis SPECTROSCOPIC TECHNIQUES

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Abstract. In this work, the binding of caffeine (CAF) with nicotinamide (NIC) has been investigated by means of fluorescence quenching and UV-Vis spectroscopic techniques. The results showed that CAF could effectively quench the intrinsic fluorescence of NIC due to a static quenching mechanism rather than a dynamic one. The key parameters of the process: the quenching constant and the bimolecular quenching rate one, the number of binding sites ($n \approx 1$), as well as the thermodynamic properties of NIC with CAF were calculated. The binding constants were 6.500 and 5.577 kL/mole at 295 and 303 K respectively. The thermodynamic parameter values determined using the Van't Hoff's equation ($\Delta H = -14.220$ kJ/mole, $\Delta S = 22.764$ J/(mole·K)) indicated that the binding process was continuous and electrostatic forces had a major role in the reaction of CAF with NIC molecules. Similarly, the UV-Vis absorption spectra of the interaction were studied and used to confirm the fluorescence quenching mechanism of the molecules.

Keywords: fluorescence quenching, binding constant, nicotinamide, caffeine

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ИЗУЧЕНИЕ СВЯЗЫВАНИЯ КОФЕИНА С НИКОТИНАМИДОМ С ПОМОЩЬЮ МЕТОДОВ ТУШЕНИЯ ФЛУОРЕСЦЕНЦИИ И СПЕКТРОСКОПИИ В УЛЬТРАФИОЛЕТОВОЙ И ВИДИМОЙ ОБЛАСТЯХ

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Аннотация. В работе исследовано связывание кофеина (CAF) с никотинамидом (NIC) с помощью методов тушения флуоресценции и спектроскопии оптического поглощения в УФ- и видимой областях. Установлено, что при связывании CAF может эффективно

подавлять у NIC собственную флуоресценцию вследствие механизма статического (а не динамического) тушения. Были рассчитаны значения основных параметров процесса: констант тушения и скорости бимолекулярного тушения, число сайтов связывания (составило около 1), а также термодинамические свойства NIC с CAF. Полученные константы связывания составляют 6,500 и 5,577 кл/моль при температурах 295 и 303 K соответственно. Значения термодинамических параметров, найденных с использованием уравнения Вант-Гоффа (ΔH = −14,22 кДж/моль и ΔS = 22,764 Дж/(моль·K)), показали, что процесс связывания является непрерывным и электростатические силы играют важную роль в реакции CAF с молекулами NIC. Аналогичным образом были изучены спектры оптического поглощения в УФ- и видимой областях при взаимодействии молекул, которые были использованы для подтверждения механизма тушения флуоресценции молекул.

Ключевые слова: тушение флуоресценции, константа связывания, никотинамид, кофеин

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Introduction

Improving the physicochemical properties of active pharmaceutical ingredients is the key issue in the field of pharmaceuticals. Human health strongly depends on the presence and level of different vitamins in their diet [1]. Nicotinamide (3-pyridine-carboxamide) is a pyridine derivative bearing a carboxamide group at the *b* position. Nicotinamide is the derivative of vitamin B3 and is involved in many aspects of biological activity [2 − 9] including diabetes treatment and prevention [2], improving the skin's appearance [10 − 12], energy metabolism, synthesis of fatty acids, growth and development, signal transduction, and maintenance of the integrity of the genome [6 − 8]. Moreover, it serves as an important functional group of coenzymes NAD+ and NADP+ [13, 14] which are involved in various chemical reactions, including the production of energy in all types of cells [15, 16], exhibit antioxidant, anti-inflammatory and anticarcinogenic activities [3, 4], prevent immune suppression caused by UVA and UVB radiation [17], and have cytoprotective effects on neural and vascular tissues as well as their anti-inflammatory activity [17, 18]. Due to its application in dietary foods, nutritional ingredients and cosmetics [5] and the structural and biochemical importance of the combination of pyridine-ring and carboxamide moieties, investigations on nicotinamide are ongoing [19 − 22]. Its deficiency leads to pellagra, which is characterized by the triad of diarrhea, dermatitis, and dementia [23].

Bioactive components that make up popular drinks (such as coffee, tea, cola beverages) and foods (e. g., chocolates) are the most useful compounds for human health [24, 25]. Besides, they are the most widely consumed of all behaviorally active drugs in the world [26, 27]. Caffeine (1,3,7-trimethylxanthine) is one of the most widely consumed compounds in the form of caffeinated beverages throughout the entire world. It can be used as an ingredient in anesthetics, antifever, and dietary medicines [28 − 32]. The richness of caffeine in the human diet and in drugs and its biological properties have attracted the interest of many researchers.

Drugs should undergo different chemical reactions before they reach their target sites to interact with biomolecules [33]. Accordingly, understanding their behaviour in a solution is very important in order to improve their pharmacological and biological activity. Thus, drugs may bind to different compounds either by a direct reaction or by weak interactions involving intermolecular bonds such as hydrogen bonding, hydrophobic interactions, etc. [34]. Thereby, as nicotinamide is pharmacologically and physiologically active compound [35 − 38], it interacts with different types of compounds, such as metal precursors (Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II) and Cd(II)) [39], bipyridyl ruthenium(II) [40], moricizine [41], indomethacin [42], caffeine [43],

chlorogenic acid [44], ascorbic acid [45], ibuprofen [46], etc. Moreover, many researchers have used various methods in binding reactions, such as equilibrium dialysis, ion-selective electrodes [47, 48], as well as UV-Vis [49], fluorescence [50, 51] and Fourier transform infrared (FTIR) spectroscopy. Among these methods, spectroscopic methods are most sensitive, easy to use, and have a short analysis time that can be preferable for studies of this kind. And, to the best of our knowledge, the effects of caffeine on the solution of nicotinamide, the thermodynamic aspects of the binding process, and the characterization of the binding sites, have not yet been investigated using these spectroscopic techniques.

The interest arises to study the binding of nicotinamide (here(in)after referred as NIC) with caffeine (CAF) is because the compounds could be found in many natural products, food/drug staffs, and used in many patients for the purpose of treating different diseases. Thus, it is an important aspect to study the compounds in order to improve their efficiency in pharmaceutical and biological activities, understanding their binding in biological system, controlling the effect of physicochemical properties, and to characterize their optoelectric properties. Therefore, in this research the interaction mechanism for CAF with NIC was investigated using fluorescence quenching and UV-Vis spectroscopy.

Experimental methods

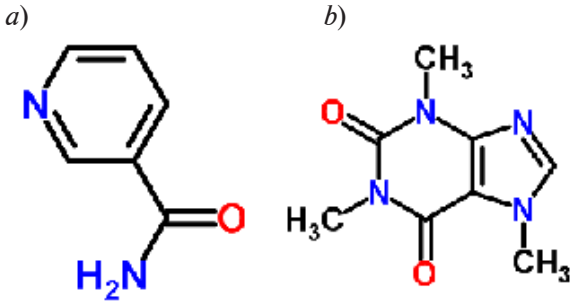

Fig. 1. Chemical structures of NIC (*a*) and CAF (*b*)

Nicotinamide (NIC, Fig. 1, *a*) and caffeine (CAF, Fig. 1, *b*) were purchased from Sigma-Aldrich Co. and used without any further purification. A sodium phosphate buffer solution was used to control the pH value at 6.85. All other reagents used were of analytical grade purity, and double distilled water was used throughout the experiment.

All fluorescence spectra were recorded on a Fluoromax-4 spectrophotometer (Horiba) equipped with a 1.0 cm quartz cell. The UV-Vis absorption measurements were carried out using a Perkin Elmer Lambda 19 UV-Visible/NIR spectrophotometer. Stock solutions of NIC (1.250 mM) were prepared in the sodium phosphate buffer solution for stead-state fluorescence and UV-Vis absorption measurements. Also, the stock solution of CAF (1.287 mM) was prepared in the deionized water.

The fluorescence spectra measurements were carried out by successive addition of CAF (0.2925 – 0.9365 μM) to a fixed amount of NIC (0.2841 μM) solution. The spectra of these series of solutions containing different amounts of CAF and definite amounts of NIC were obtained. The excitation wavelength was λ_{ex} = 250 nm for NIC, and corresponding emission ones were recorded over a range of 300 – 500 nm. The excitation and emission widths of slits were set to 10 nm, and the scan speed to 240 nm/min. The bimolecular quenching rate constant k_q, the Stern – Volmer constant K_{SV} and the number of binding sites n were calculated from the emission spectral analysis. To evaluate the effect of temperature on the interactions, the fluorescence quenching experiments were carried out at two different temperatures: 295 and 303 K.

Similarly, the UV-Vis absorption spectra of NIC were recorded in the absence and presence of CAF in the wavelength range from 200 to 500 nm and in the concentration range from 0.1897 to 0.2010 μM at a fixed amount of NIC 0.2275 μM. The absorption spectra of pure NIC solutions were also obtained in the same wavelength range.

Results and discussion

Fluorescence quenching mechanism of NIC. The observed fluorescence quenching of the

compound can be reduced by a variety of molecular interactions, such as excited state reactions, rearrangement of molecules, energy transfer, ground-state complex formation, and collisional quenching [52, 53]. Furthermore, the static quenching and dynamic one are major quenching mechanisms that differ in their dependences on temperature or viscosity [53, 54]. The dynamic quenching is due to collisions between the fluorophore and the quencher, and the quenching constant is expected to increase with temperature since higher temperatures result in larger diffusion coefficients. Whereas, the static quenching is the formation of a ground-state complex of the fluorophore with the quencher [53, 54], in the presence of which a higher temperature may bring about a decrease in the stability of the complexes, resulting in a lower quenching constant.

Fig. 2 shows the fluorescence spectra of NIC in the presence of CAF. The fluorescence intensities of NIC decreased gradually with increasing the CAF concentration. Peaks in the fluorescence spectra of NIC were observed at a wavelength of 424 nm in both the absence and presence of CAF. The addition of CAF to NIC produced significant quenching of the fluorescence intensity in a concentration-dependent manner. Furthermore, CAF did not show fluoresce near the emission maximum of NIC; this indicated that CAF could interact with NIC.

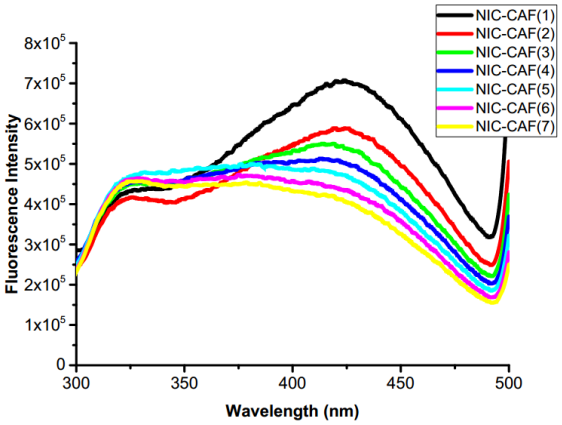

Fig. 2. Emission spectra of NIC (λ_{ex} = 250 nm) in the presence of CAF at 295 K. The CAF concentration, μM: 0.2925 (*1*), 0.4058 (*2*), 0.5171(*3*), 0.6264 (*4*), 0.7338 (*5*), 0.8393 (*6*), 0.9365 (*7*), and the NIC one being fixed at 0.2841 μM

The data indicated that CAF quenched the intrinsic fluorescence intensity of the NIC molecules. The strong quenching effects clearly indicated the existence of binding among the molecules.

In order to confirm the quenching mechanism of the molecules, the fluorescence quenching data were analyzed according to the Stern – Volmer equation [55, 56]:

$$F_0/F = 1 + k_q\tau_0\,[Q] = 1 + K_{SV}\,[Q], \quad (1)$$

where F_0, F are the fluorescence intensities in the absence and presence of the quencher, respectively; $[Q]$ is the initial concentration of CAF; K_{SV} is the Stern – Volmer quenching constant; k_q is the rate constant of quenching controlled by diffusion; $K_{SV} = k_q\tau_0$; τ_0 is the average fluorescence lifetime of NIC in the absence of CAF.

The K_{SV} value was determined by linear regression of the Stern – Volmer equation, and the plot of F_0/F versus $[Q]$ would be linear for a single static or dynamic quenching within a certain concentration [52, 53, 55].

Fig. 3 is the Stern – Volmer plots for the quenching of NIC by CAF at temperatures of 295 and 303 K. The curves showed good linear relationships within the investigated concentrations at the two different temperatures. The linear Stern –Volmer plots show the possible existence of a single type of quenching, and the quenching process is static rather than dynamic one. The fluorescence lifetime τ_0 of NIC was assumed to be 0.3 ns [57], and the K_{SV} values obtained from the Stern – Volmer equation are presented in Table 1. Values of the quenching rate constant k_q determined at the two temperatures are given there. It is clear that the k_q values are in the range

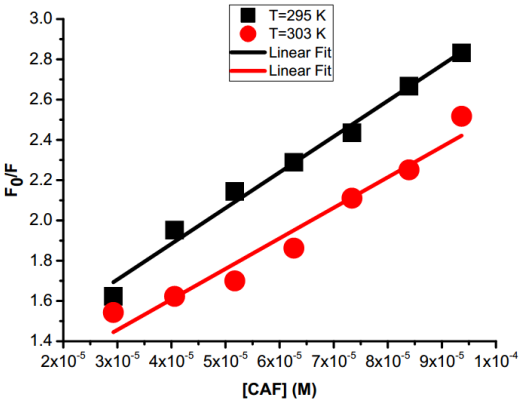

Fig. 3. Stern – Volmer plots for the quenching of NIC by CAF at 295 (black) and 303 K (red)

10^{13} L/(mol·s) at both temperatures which exceed by far the diffusion controlled rate constant $2 \cdot 10^{10}$ L/(mol·s) in an aqueous solution [58, 59], confirming that the quenching does not involve a dynamic diffusion process, but rather occurs due to the formation of CAF-NIC complexes.

Table 1

The values of fluorescence quenching parameters obtained for NIC-CAF binding at two temperatures

T, K	K_{SV}, 10^4 L/mol	k_q, 10^{13} L/(mol·s)	R_c
295	1.775	5.920	0.98
303	1.515	5.052	0.97

Notations: K_{SV} is the Stern – Volmer constant, k_q is the quenching rate constant, R_c is the correlation coefficient.

Binding constant and binding sites

The fluorescence data was further examined for static quenching process. When molecules are bound independently to a set of equivalent sites on macromolecules, the equilibrium between free and bound molecules is given by the following equation [60]:

$$\text{Log}\,[(F_0 - F)/F] = \text{Log}\,K_c + n \cdot \text{Log}[\text{CAF}], \quad (2)$$

where K_c is the binding constant, n is the number of binding sites per molecule, [CAF] is the CAF concentration.

The values of K_c and n were determined from the slope and intercept of the linear fit of Eq. (2) to the experimental data of Fig. 4. Values of the association constant of CAF with NIC at 295 and 303 K are listed in Table 2. As the data in Table 2 show, the values of K_c decreased with increasing temperature, which suggested that the binding reaction between the NIC and CAF was exothermic [61].

Strong binding constants were observed between NIC and CAF. Moreover, the values also decreased with increasing temperature, which indicated the formation of unstable compounds that partly decompose at relatively higher temperatures. The calculated binding site number n is about 1, indicating the existence of a single site for the binding of NIC to CAF.

Thermodynamic parameters and nature of the binding forces

The thermodynamic parameters of binding reaction are the main evidence for confirming the binding mode. The formation of binding can be described by several biophysical parameters such as the association constant, and other thermodynamic properties. The forces acting between small

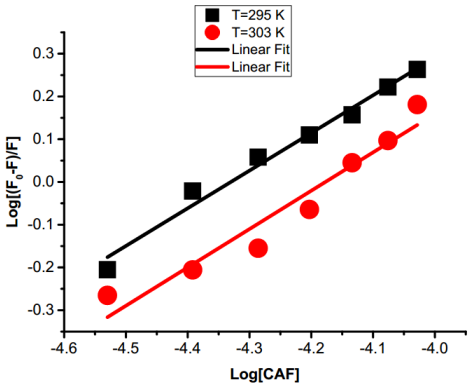

Fig. 4. The plots of Log $[(F_0 - F)/F]$ versus Log[CAF] at 295 (black) and 303 K (red)

Table 2

The values of NIC-CAF binding parameters obtained at two temperatures

T, K	K_c, 10^3 L/mol	n	R_c
295	6.500	0.880	0.98
303	5.577	0.897	0.99

Notations: K_c is the binding constant, n is the number of binding sites per molecule, R_c is the correlation coefficient.

molecules and macromolecules are mainly hydrogen bonds, electrostatic forces, the Van der Waal's force and hydrophobic interaction. To obtain further insight into the weak interactions associated with the complexation of NIC with CAF, we endeavored to determine the thermodynamic parameters using the Van't Hoff's equation. If the enthalpy change ΔH does not vary significantly over the temperature range of the study, then the thermodynamic parameters can be determined by the following equation:

$$\ln K_c = -\Delta H/RT + \Delta S/R, \qquad (3)$$

and the Gibbs free energy change ΔG can be calculated at each temperature using formula

$$\Delta G = -RT \cdot \ln K_c = \Delta H - T\Delta S, \qquad (4)$$

where ΔS is the entropy change, R is the gas constant.

Table 3

Thermodynamic properties determined by the fluorescence quenching of CAF with NIC at two temperatures

T, K	ΔH, kJ/mol	ΔS, J/(mol·K)	ΔG, kJ/mol
295	–14.220	24.764	–21.52
303	–	–	–21.72

Notations: ΔH, ΔS, ΔG are the changes in the enthalpy, the entropy and the Gibbs free energy, respectively.

From the linear relationship between $\ln K_c$ and the reciprocal of absolute temperature the values of the thermodynamic parameters were obtained as listed in Table 3. Accordingly, the negative values of ΔH and the positive value of ΔS indicate that an electrostatic force played a major role in the reaction between NIC and CAF, whereas the negative sign of ΔG indicates the spontaneity of the binding for NIC with CAF. Moreover, the negative value of enthalpy indicates that the absorption process of the compounds is an exothermic reaction. Besides, the positive value of entropy confirms the increasing randomness of the solution interface of the molecules of the compounds [61, 62].

UV-Vis absorption spectra

The UV-Vis absorption spectroscopy was used to verify the mechanism of binding of CAF to NIC. This measurement is very simple and the method is applicable to explore the structural changes and to know the complex formation of different compounds. In the dynamic quenching, the spectra of the molecule will not change, however, in the static one the spectral changes due to the formation of reaction were observed in the compound. Fig. 5 shows the absorption spectra of NIC in the presence and absence of CAF. In the absence of CAF, the UV-Vis absorption spectra of NIC was characterized by a single absorption band. With the addition of CAF solution, the interactions between CAF and NIC led to the red shift of the NIC spectra, and the intensity of the peak at a wavelength of 261 nm increased. The curve N (see Fig. 5) was different from the curves IP of the complexes of NIC-CAF and isosbestic points were observed at different wavelengths of the complexes. This change was a reasonable result to confirm the binding of CAF with NIC due to ground state complex formation, which is evidence that the static quenching existed in the binding process of the fluorescence quenching.

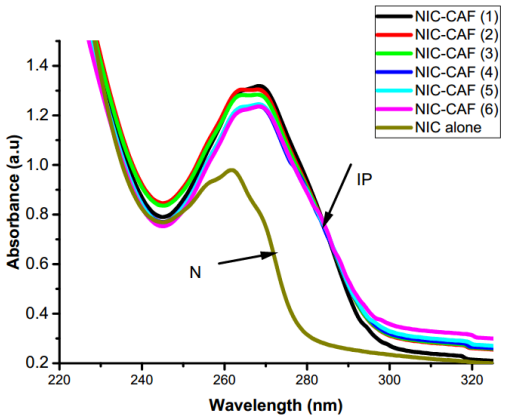

Fig. 5. UV-Vis absorption spectra of NIC alone (curve N) and in the presence of CAF with various concentrations (curves IP), μM: 0.2010 (*1*), 0.2189 (*2*), 0.1970 (*3*), 0.1950 (*4*), 0.1920 (*5*), 0.1897 (*6*); the concentration of NIC was 0.2275 μM

Conclusion

The binding of caffeine (CAF) with nicotinamide (NIC) was investigated using fluorescence quenching and UV-Vis spectroscopic techniques. The experimental results indicated that CAF quenched the fluorophore of NIC by forming the ground state complex or non-fluorescent NIC–CAF with high binding affinities. The thermodynamic parameters suggested that the binding reaction was exothermic and occured spontaneously, and the electrostatic force played a major role in the binding reaction. All these experimental results clarified that NIC can bind to CAF, which can be a useful guideline for further clinical study. The study results help us to understand the mechanisms of binding of the drug with the biologically active compound of a coffee bean that is naturally available in different plant types.

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