Mucin-drugs interaction: The case of theophylline, prednisolone and cephalexin

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1. Introduction

Mucus is a viscoelastic gel secreted by epithelial cells to protect the epithelium from environmental factors [1]. Accumulation of mucus with abnormal viscosity in the airways is a central pathological feature of cystic fibrosis, asthma, and chronic obstructive pulmonary disease [2, 3]. Mucus is composed of water, ions, lipids, and approximately 2% of protein: the protein mainly expressed is mucin, an high molecular O-glycosylated protein. It plays an important role in the defence of epithelia, forming a protective extracellular mucin gel, a steric barrier against assault. Qualitative and quantitative anomalies of mucin in many diseases like cancers or chronic airway diseases (BPCO broncho-pneumopathie chronique obstructive or cystic fibrosis) allowed to identify this protein as an important marker of adverse prognosis and attractive therapeutic target over the recent years [4, 5].

Therefore, protein-drug interactions play a key role in pharmacokinetics and pharmacodynamics of the drugs. In particular mucin-drug interaction may have important effects on the drug absorption, considering that mucus is the first barrier that drugs must overcome to be adsorbed and gain access to the circulatory system [6] and distribution, because only the free concentration of drug can get to the target and produce a biological response. This means that a high binding may reduce the drug's pharmaceutical effect.

Since mucus act as a barrier, there are two main mechanisms that limit diffusion through mucus gel: (a) interaction with mucus components (i.e., electrostatic and hydrophobic interactions with mucins), and (b) size filtering related to the size of the mesh spacing between the mucin fibers [7]. Many studies highlighted that no definitive picture of the nature of the molecular interactions between drug molecules and mucus components can be drawn. [6, 7]

Mucin-drugs complex has been studied by differents methods: a) chromatography using biomimetic stationary phases [8] and novel stationary phase based on covalently immobilized mucin [9] and b) ultrafiltration isothermal titration micro-calorimetry (ITC) and transmission electron microscopy (TEM) [10]. However, the interaction between mucin and some drugs used in the treatment of cystic fibrosis by spectroscopic approaches has never been investigated. Cystic fibrosis (CF) is a life-threatening genetic disorder. It severely affects the lungs and digestive system. There is no cure for cystic fibrosis, but treatment can ease symptoms and reduce complications. However, with improved medication and treatment to manage symptoms, life expectancy has been extended considerably. Among the most used drugs for the symptons treatment of CF we can cite antibiotics to treat and prevent lung infections, bronchodilators to help keep airways open by relaxing the muscles around your bronchial tubes and anti-inflammatory drugs to limit inflammatory processes.

In this paper we analyzed the interaction of mucin with some drugs used to treat the symptomatology of cystic fibrosis, in particular theophylline for airway obstructions and asthma conditions, cephalexin for infection and prednisolone for inflammation. (Fig.1)

The interaction between drugs and mucin has been investigated using fluorescence and UV-Vis absorption spectroscopy. The quenching mechanism was analysed by Stern-Volmer equation. The binding constants were obtained by Lineweaver-Burk equation and by a non linear fit equation. Lastly, the binding sites, thermodynamic parameters and binding distance were obtained.

2. Materials and methods

2.1. Materials

Mucin from porcine stomach (type III, bound sialic acid 0.5-1.5%, partially purified powder) was purchased from Sigma Aldrich. The stock solution (1 mg/mL) was prepared by dissolving mucin in PBS (phosphate buffer solution, 2 mM, pH = 7.4).

Theophylline (≥99%), cephalexin (CAS# 23325-78-2) and prednisolone (≥98%) were all purchased from Sigma Aldrich; the stock solutions (3 mM) of theophylline and cephalexin were prepared by dissolving drugs in PBS and the stock solution (3 mM) of prednisolone was prepared by dissolving drug in a solution 1:1 of ethanol 96% and PBS.

2.2. Apparatus

All fluorescence spectra were recorded with a Horiba Jobin Yvon Fluorolog3 TCSPC spectrofluorophotometer with 1.0 cm quartz cells. UV-Vis spectra were recorded on a UH5300 Hitachi spectrophotometer. The pH measurements were made with a Eutech Instruments pH2700.

2.3. Measurements of spectra

Mucin 0.05 mg/mL was titrated by successive additions of drugs solutions at different concentrations: over a range from 50 μM to 600 μM for theophylline, from 3 μM to 400 μM for cephalexin and over a range from 20 μM to 500 μM for prednisolone. Titrations were done manually using microinjectors. Fluorescence quenching spectra were measured in the range of 285-500 nm at the excitation wavelength of 265 nm. The excitation and emission slits were respectively 6 nm and 10 nm. The fluorescence spectra were performed at three temperatures (296 K, 303 K, 310 K).

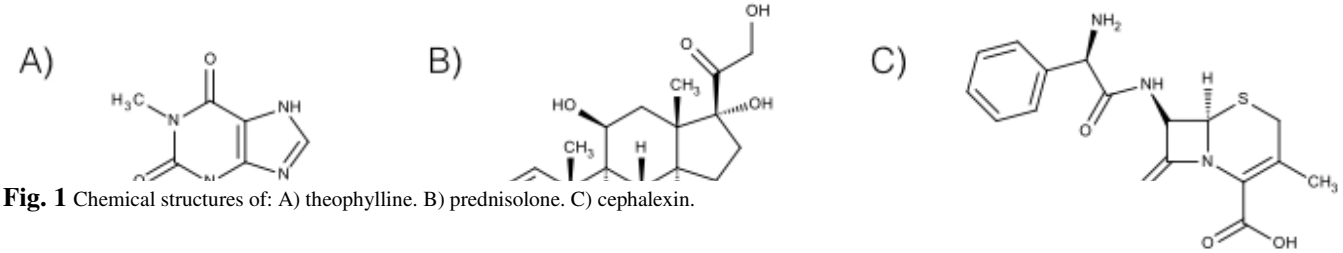

Fig. 1 Chemical structures of: A) theophylline. B) prednisolone. C) cephalexin.

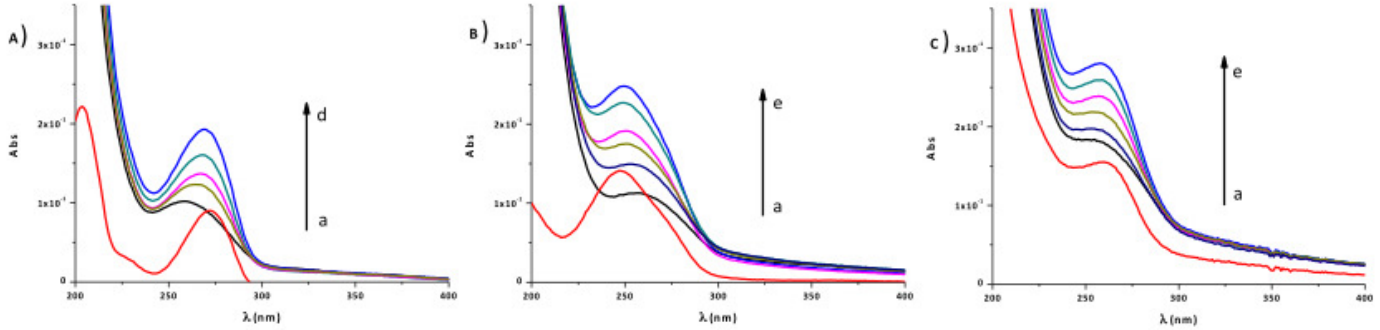

Fig. 2 UV-Vis spectra of mucin-drugs interaction (T=296 K). A) UV-Vis spectra of mucin-theophylline: [mucin]= 0.05 mg/mL; a-d [Theophylline]= (3.0, 5.0, 7.0, 10.0) µM. B) UV-Vis spectra of mucin-prednisolone: [mucin]= 0.05 mg/mL; a-e [Prednisolone]= (2.0, 4.0, 6.0, 8.0, 10.0) µM. C) UV-Vis spectra of mucin-cephalexin: [mucin]= 0.05 mg/mL; a-e [Cephalexin]= (2.0, 4.0, 6.0, 8.0, 10.0) µM.

3. Results and discussion

3.1. UV-Vis absorption studies

UV-Vis absorption measurement is a simple and applicable method to investigate the formation of a complex [11, 12]. The absorption spectra of mucin in absence and in presence of different concentrations of drugs were recorded and presented in Fig.2; the absorption intensity of mucin at around 260 nm increased with the addition of increasing concentrations of drugs. Moreover, the absorption spectrum of mucin-drug complex is different from that of mucin and drugs alone. The maximum peak position of mucin-drugs complex was slightly shifted towards lower wavelength region, as shown in the reported spectra (Fig.2).

An explanation for this phenomenon may come from the formation of a new drug–protein complex with a new structure. This behavior may also indicate a change in polarity around the tryptophan residue and a change in peptide strand of mucin and thus the change in hydrophobicity as described in literature [13].

3.2. Fluorescence quenching mechanism of mucin-drugs system

The fluorescence spectra of mucin in absence and in presence of drugs at different concentrations are shown in Fig.3. Mucin shows a strong fluorescence emission at 360 nm and its fluorescence intensity decreases gradually with the increase of drug's concentration; from the spectra it is possible to observe a hypsochromic effect for the complex mucin-theophylline (from 360 nm to 348 nm), a bathochromic effect for the complexes mucin-cephalexin (from 356 nm to 362 nm) and mucin-prednisolone (from 360 nm to 371 nm).

The quenching of mucin fluorescence by drugs was due to the formation of a protein-drug complex and the microenvironment of mucin was changed during the binding interaction. Binding studies were performed and the obtained steady-state maximum fluorescence intensity was recorded. Data were treated by two different methods, to obtain the evaluation of the equilibrium association (K_A) and dissociation (K_D) constants.

3.3. Stern-Volmer equation

The fluorescence quenching of mucin is analyzed by Stern-Volmer equation (1) [14]:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \qquad (1)$$

where F_0 is the fluorescence intensity of mucin alone and F is the fluorescence intensity of mucin in the presence of increased concentration of quencher. K_{SV} is the Stern–Volmer quenching constant, which describes a collisional quenching of fluorescence and [Q] is the quencher concentration. Quenching data are presented as plots of F_0/F vs. [Q], yielding an intercept of one on the y- axis and a slope equal to K_{SV}. Stern-Volmer plots at different temperatures are reported in Fig.4.

It is important to recognize that observation of a linear Stern-Volmer plot does not prove that collisional quenching of fluorescence has occurred. Static and dynamic quenching can be distinguished by their differing dependence on temperature: higher temperatures result in faster diffusion and hence larger

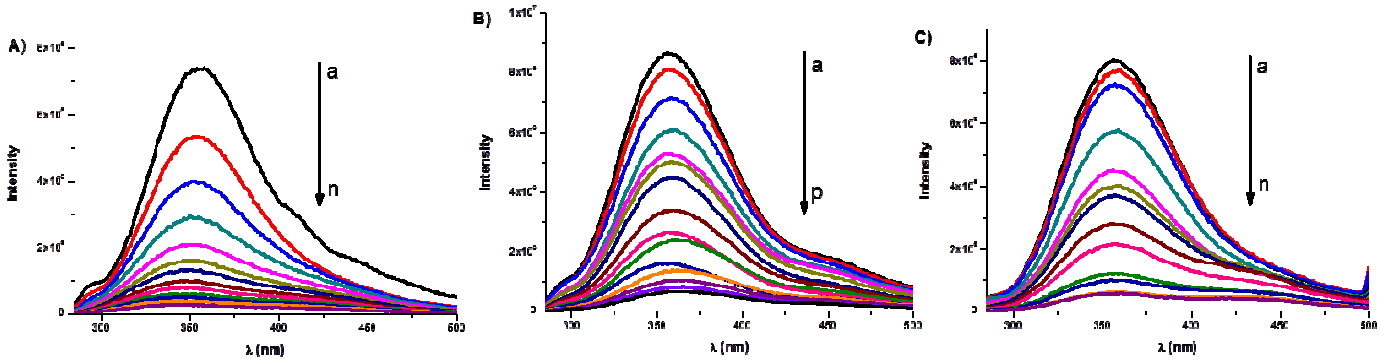

Fig. 3 Fluorescence spectra of mucin-drugs interaction (T=296 K). A) Fluorescence spectra of mucin-theophylline: [mucin]= 0.05 mg/mL; a-n [Theophylline]= (50.0, 100.0, 150.0, 200.0, 250.0, 300.0, 350.0, 400.0, 450.0, 500.0, 550.0, 600.0) µM. B) Fluorescence spectra of mucin-prednisolone: [mucin]= 0.05 mg/mL; a-p [Prednisolone]= (20.0, 35.0, 50.0, 60.0, 70.0, 100.0, 125.0, 150.0, 200.0, 225.0, 250.0, 300.0, 350.0, 400.0) µM. C) Fluorescence spectra of mucin-cephalexin: [mucin]= 0.05 mg/mL; a-n [Cephalexin]= (3.0, 7.0, 10.0, 50.0, 100.0, 125.0, 150.0, 200.0, 250.0, 300.0, 350.0, 400.0) µM.

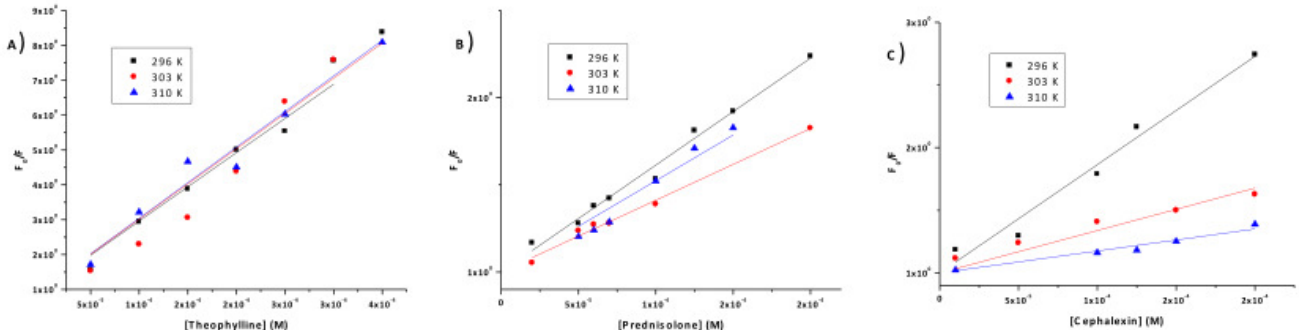

Fig. 4 The Stern Volmer plots of the fluorescence quenching of mucin by drugs at different temperatures; A) theophylline; B) prednisolone; C) cephalexin.

amounts of collisional quenching. Higher temperature will typically result in the dissociation of weakly bound complexes, and hence smaller amounts of static quenching. [14]

As shown in Table 1, the K_{SV} of the complex mucin-cephalexin decreases with increasing temperature. This indicates a static quenching interaction between protein and drug. [12] The K_{SV} of theophylline-mucin and prednisolone-mucin complexes are similar with negligible variations by changing the temperature.

Table 1. The quenching constants of mucin and drugs: A) theophylline, B) prednisolone and C) cephalexin at different temperatures.

	296 K	**303 K**	**310 K**
	K_{SV} (M^{-1})	K_{SV} (M^{-1})	K_{SV} (M^{-1})
A	20415	19628	20195
B	6136	4120	5332
C	9262	3385	1753

3.4. Non linear least squares

Fluorescence data at different temperatures were also analysed with a non- linear least-squares fit procedure, [15] based on equation 2:

$$y = \frac{B_{max}[Q]}{K_D+[Q]} \qquad (2)$$

where, acting as a quencher, [Q] is the drug concentration, y is the specific binding derived by measuring fluorescence intensity, B_{max} is the maximum amount of the complex protein/drug formed at saturation and K_D is the equilibrium dissociation constant. The binding curves are reported in Fig.5; the percentage of bound mucin, i.e. y, derived from the fluorescence intensity emission maximum, is plotted against the drug concentration. The corresponding K_D and K_A at different temperatures are shown in Table 2.

3.5. Lineweaver-Burk

The other method used to analyse fluorescence data is the Lineweaver-Burk equation [14] based on Eq. (3):

$$\frac{1}{F_0-F} = \frac{1}{F_0} + \frac{K_D}{F_o\ [Q]} \qquad (3)$$

where F_0 is the steady-state fluorescence intensity of mucin alone, F is the steady-state fluorescence intensity of the complex at the increasing quencher concentration, [Q] is the drug concentration in solution and K_D is the equilibrium dissociation constant. As shown in Fig.6 reciprocals of F_0-F are plotted against reciprocals of [Q]. The slope of the line is the K_d/F_0 ratio while the intercept is the reverse of F_0. The equilibrium dissociation (K_D) and association (K_A) constants at different temperatures are thus easily calculated and reported in Table 2.
The results can be compared with the data obtained with the non linear fit equation.

As shown in figures 4, 5 and 6, for all the equations used, the difference of binding at different temperatures is greater for cephalexin, while the binding constants remain nearly unchanged for theophylline and prednisolone. This can mean that the binding of cephalexin is more…

3.6. Binding parameters

To obtain the number of binding sites (n), it is possible to use the double logarithm regression curve (shown in equation 4) [14], which describes the relationship between the fluorescence intensity and the concentration of the quencher.

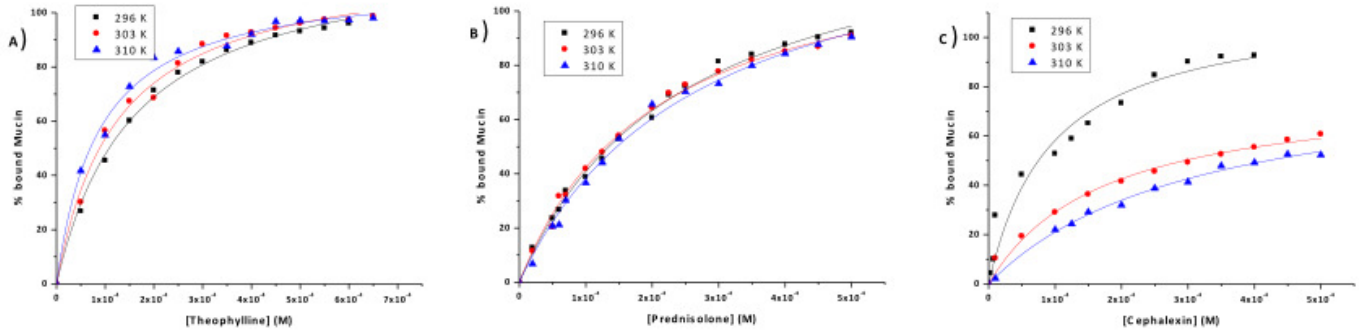

Fig. 5 The binding curves of mucin-drugs complex at different temperatures. A) Theophylline; B) Prednisolone; C) Cephalexin.

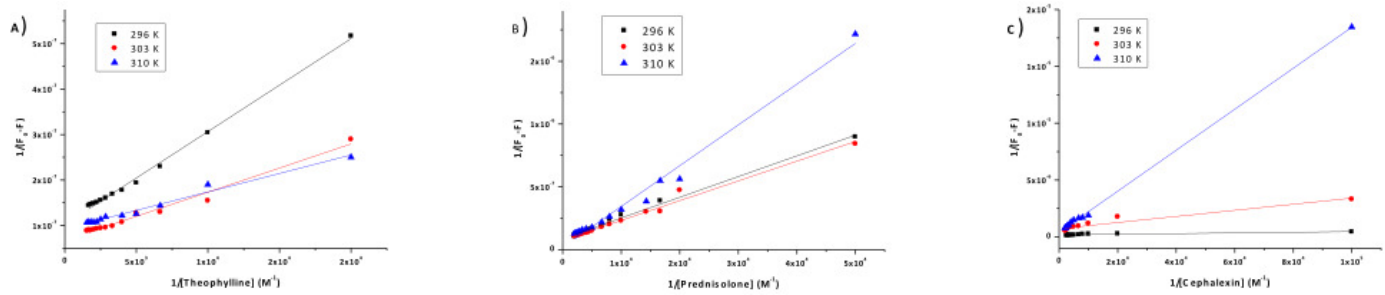

Fig. 6 The Lineweaver-Burk plots of mucin-drugs complex at different temperatures. A) theophylline; B) prednisolone; C) cephalexin.

$$\log \frac{(F_0-F)}{F} = \log K_A + n \log[Q] \qquad (4)$$

where F_0 is the fluorescence intensity of mucin alone, F is the fluorescence intensity after the addition of the quencher and [Q] is the quencher concentration. The slope of the line is the n value. If the value of n is equal to 1, it means that a strong binding exists between the protein and the drugs. [13]

The number of binding sites (n) for mucin-cephalexin complex is 1.65 (296 K), 0.70 (303 K), 0.99 (310K), for mucin-theophylline is 1.67 (296 K), 1.78 (303K), 1.66 (310 K) and for mucin-prednisolone is 1.80 (296 K), 1.51 (303 K) 0.90 (310 K). Almost all values are approximately equals to 1, indicating that there is one independent binding site on mucin for every analyzed drug. [16]

Table 2 Values of the equilibrium dissociation and association constants of mucin-drugs complex at different temperatures obtained by non linear fit equation and by Lineweaver-Burk equation. A) theophylline. B) prednisolone. C) cephalexin

	296 K		303 K		310 K	
	K_A (M^{-1})	K_D ($\cdot 10^{-4}$M)	K_A (M^{-1})	K_D ($\cdot 10^{-4}$M)	K_A (M^{-1})	K_D ($\cdot 10^{-4}$M)
			Non linear fit			
A	6329	1.50	8130	1.23	11494	0.87
B	4098	2.44	4760	2.07	3846	2.67
C	10482	0.96	5780	1.73	3144	3.18
			Lineweaver-Burk			
A	6808	1.47	8203	1.22	14721	0.68
B	7049	1.42	6156	1.62	3582	2.79
C	14902	0.23	12730	0.78	2300	4.35

3.7. Thermodynamic parameters

The interaction forces between small molecules and macromolecules include four binding modes: H-bonding, Van der Waals, electrostatic, and hydrophobic interactions [17]. According to the data of enthalpy change (ΔH) and entropy change (ΔS), the model of interaction between drug and biomolecule can be concluded [18]: 1- $\Delta H > 0$ and $\Delta S > 0$, hydrophobic forces; 2- $\Delta H < 0$ and $\Delta S < 0$, van der Waals interactions and hydrogen bonds; 3- $\Delta H < 0$ and $\Delta S > 0$, electrostatic interactions. The thermodynamic parameters, enthalpy (ΔH) and entropy (ΔS) of the reaction, of mucin and drugs complex are important to confirm binding modes. The temperature-dependence of the binding constant was studied at 296, 303, and 310 K and was calculated from the following Van't Hoff equation [14]:

$$\int K_A = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad (5)$$

$$\Delta G = -RT\, In\, K_A \qquad (6)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \qquad (7)$$

where K_A is the binding constant, R is the gas constant and T is the experimental temperatures The values of ΔH and ΔS obtained for the binding site are shown in Table 3.

Table 3. Thermodynamic parameters for mucin-theophylline, mucin-prednisolone and mucin-cephalexin complexes at different temperatures.

	ΔH (kj mol^{-1})	ΔG (kj mol^{-1})	ΔS (J mol^{-1} K^{-1})
		Theophylline	
296 K	41.85	-21.55	214.18
303 K		-23.05	
310 K		-24.55	
		Prednisolone	
296 K	-36.70	-21.97	-49.78
303 K		-21.62	
310 K		-21.27	
		Cephalexin	
296 K	-31.11	-24.29	-259.66
303 K		-22.48	
310 K		-20.66	

From Table 3 it can be seen that for mucin-theophylline complex both ΔH and ΔS have a positive value. For this drug–protein interaction, positive entropy is frequently taken as evidence for hydrophobic interaction, but it has been pointed out that positive entropy may also be a manifestation of electrostatic interaction. Conversely, it can be seen that for mucin-prednisolone and mucin-cephalexin complexes both ΔH and ΔS have a negative value. This indicates that van der Waals interactions and hydrogen bonds may play a major role in the binding [17]. The negative sign for ΔG means that the binding process is spontaneous.

3.8. Energy transfer

FRET (Fluorescence Resonance Energy Transfer) is a simple method to measure the distance between protein and drug [19] According to Föster's non-radiative energy transfer theory, energy efficiency E, critical energy- transfer distance R_0 (E= 50%), the energy donor and the energy acceptor distance r and the overlap integral between the fluorescence emission spectrum of donor and the absorption spectrum of the acceptor J can be calculated by the following equations [14]:

$$E = 1 - \left(\frac{F}{F_0}\right) = \frac{R_0^6}{R_0^6 + r^6} \qquad (8)$$

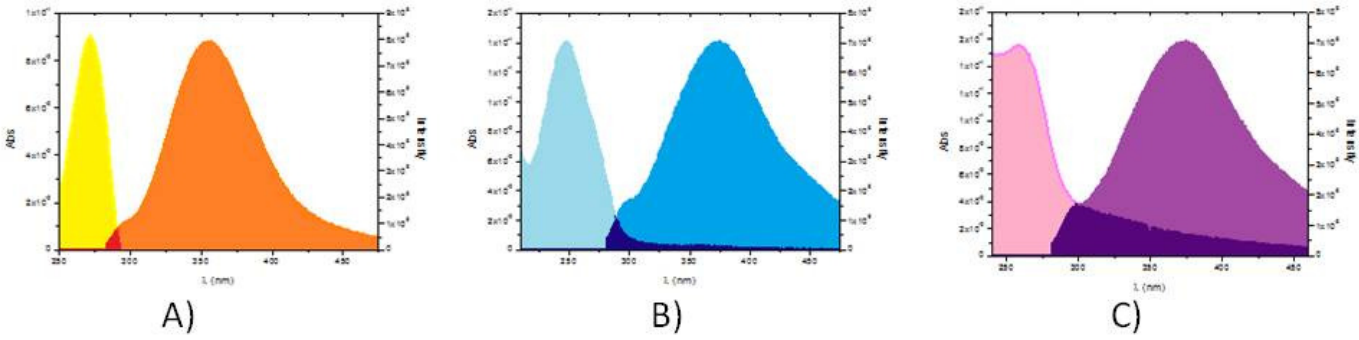

Fig. 7 The overlaps of emission spectra of mucin and absorption spectra of theophylline (A), prednisolone (B) and cephalexin (C) at 296 K.

$$R_0^6 = 8{,}79 \times 10^{-5}\,[\kappa^2 n^4 \varphi\, J(\lambda)] \qquad (9)$$

$$J = \frac{F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \qquad (10)$$

where k^2 is the orientation factor, Φ is the fluorescence quantum yield of the donor, N is the refractive index of the medium, F(λ) is the fluorescence intensity of the donor at wavelength λ and ε(λ) is the molar absorption coefficient of the acceptor at wavelength λ. In this case, k^2=2/3, N= 1.336 and Φ= 0.118 [11]. The overlaps of emission spectra of mucin and absorption spectra of theophylline, cephalexin and prednisolone at 296 K were obtained (Fig.7).

Using the data equations, J, E, R_0 and r were obtained for every interaction and they were reported in Table 4.

Table 4. Parameters of J, E, R_0 and r of mucin-drugs complexes at 296 K.

	J (cm^3 L mol^{-1})	E (%)	R_0 (nm)	r (nm)
A	$1.91 \cdot 10^{11}$	0.96	0.85	0.5
B	$2.51 \cdot 10^{13}$	0.93	1.93	1.26
C	$3.10 \cdot 10^{12}$	0.92	1.36	0.90

The distance r<7 nm indicates that the energy transfer between protein and drugs occurred with a high probability. [11, 20]

4. Conclusions

In this paper, the interaction of mucin with three different classes of drugs (theophylline, prednisolone and cephalexin) was investigated at different temperatures by different spectroscopic methods.

UV–Vis spectroscopy showed that all the three investigated drugs can bind to mucin to form a protein-drug complex. Fluorescence data proved that mucin fluorescence can be quenched by the studied drugs and that the quenching is governed by a static quenching for mucin and cephalexin interaction. According to thermodynamic parameters (positive ΔH and ΔS value) hydrophobic forces played a major role in the binding process between mucin and theophylline, while the hydrogen bonds and van der Waals forces may play a major role in stabilizing mucin-prednisolone and mucin- cephalexin complex.

The evaluation of the equilibrium association (K_A) and dissociation (K_D) constants was obtained by two different methods (comparable to each other) at different temperatures. The data showed that temperature does not influence the formation of mucin-theophylline and mucin-prednisolone complex, while it can influence the interaction between mucin and cephalexin. However, the difference in the K_A and K_D values observed at different temperatures is not due to degradation of mucin, that results thermally stable at the temperatures used in the experiment. [21]

The obtained results on the investigation of mucin-drugs interaction can facilitate the interpretation of absorption and distribution process of the drugs used in CF and could be helpful in the future to explain the structure-activity relationship (SAR) of new therapeutic molecules to identify mucin as a therapeutic target.

5. Acknowledgements

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