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Interaction of norfloxacin and sparfloxacin with melanin in vitro in relation to phototoxic reactions

Oddziaływanie norfloksacyny i sparfloksacyny z melaniną *in vitro* w odniesieniu do niepożądanych reakcji fototoksycznych

Fluoroquinolones are a group of modern, synthetic, broad spectrum bactericidal antibiotics used to treat various infections of urinary and respiratory systems, as well as in ophthalmology and dermatology [9]. They act through inhibition of two types DNA topoisomerase enzymes: DNA gyrase (topoisomerase II) and topoisomerase IV [9, 11]. One of the important and common side-effects of these drugs is photosensitivity. Ultraviolet (UV) light is the main action spectrum to elicit photosensitive reactions in patients medicated with fluoroquinolones [5, 9]. Photosensitivity includes phototoxicity and photoallergy, and many cases of fluoroquinolones-induced photosensitivity have phototoxic nature [5, 8]. Phototoxicity is mostly influenced by halogenation of the 8-position in concert with the fluorination of the 6-position [7] and is associated with the formation of reactive oxygen species (ROS) – mainly singlet oxygen and superoxides. ROS are propably responsible for lipid peroxidation and also mitochondria and DNA damage [9, 11].

Melanin pigments are multifuncional polymers. The physiological function of melanin is to buffer against photochemical stress through absorption and dispersing UV radiation and trapping free radicals [3, 10]. Fluoroquinolones have been reported to bind well to melanin rich tissues such as eyes and skin [2]. The protective role of melanin pigments may avoid initiation of the phototoxic process [9]. On the other hand, slow release of fluoroquinolones from bonds may build up high and long-lasting levels of drugs stored by melanin and lead to the prolonged exposure of melanin containig cells and surrounding tissues to the toxicity of ROS [2].

The purpose of the studies was to examine *in vitro* the binding capacity of two fluoroquinolone derivatives: norfloxacin and sparfloxacin to synthetic DOPA-melanin.

MATERIAL AND METHODS

Chemicals. L-3,4-dihydroxyphenylalanine (L-DOPA), norfloxacin and sparfloxacin were obtained from Sigma Chemical Co. The remaining chemicals were produced by POCH S.A., Poland.

Synthetic dopa-melanin. DOPA-melanin was obtained by oxidative polymerization of L-3,4-dihydroxyphenylalanine (L-DOPA) in 0.067M phosphate buffer at pH 8.0 according to the Binns method [1].

Drug-melanin complex formation. Drug-melanin complexes were obtained by suspending 5 mg of synthetic melanin in 5 ml of norfloxacin or sparfloxacin solution prepared in

phosphate buffer pH = 7.0. The initial concentration of drugs ranged from 5 x 10^{-5} M to 5 x 10^{-3} M. For each drug concentration and time of complex formation three independent samples were prepared. A mixture of melanin and drug solution was incubated at room temperature and then filtered. Control samples, containing melanin suspended in buffer (without drug) were treated in the same way.

Determination of the amount of drugs bound to melanin. The UV spectrophotometric method was used for quantitative determination of the analyzed drugs [6]. Analytical wavelengths (λ_{max}) for the compounds studied were chosen as follows: 272 nm for norfloxacin and 290 nm for sparfloxacin. The sensitivity of the spectrophotometric methods was investigated by measuring absorbance of different concentrations of drugs: from 2.5 x 10^{-6} M to 5 x 10^{-5} M. All the data points produced a strong (R^2>0.99) linear correlation. Linear regression analysis gave equation y=ax+b, where constant b was found to be insignificant. The calculated values of the molar absorption coefficient ($\varepsilon_{\lambda max}$): 35361 for norfloxacin and 27430 for sparfloxacin were used to estimate the amount of drug bound to the polymer. All spectrophotometric measurements were performed with the use of JASCO model V-530, UV-VIS spectrophotometer.

Kinetics of drug-melanin complex formation. Kinetics of formation of melanin complexes with norfloxacin and sparfloxacin were evaluated on the basis of the relationship between the amount of drug bound to the polymer (μmol/mg) and the time of complex formation. In the studies, the following initial drug concentrations were used: 1 x 10^{-4} M, 5 x 10^{-4} M, 1 x 10^{-3} M and 5 x 10^{-3} M. Complex formation lasted for 0.5, 1, 3, 6, 12, 24 and 48 hours.

Binding parameters of drug-melanin complexes. The number of strong (n_1) and weak (n_2) binding sites and the association constants (K) of the synthetic melanin complexes with norfloxacin and sparfloxacin were calculated *via* Sctachard plots [4]. Experimental binding isotherms were used to construct these plots. They show the relationship between the amount of drug bound to melanin and its initial concentration after reaching equilibrium state, i.e. after 24 hours.

Statistical analysis. In all experiments, the mean values for three independent experiments ± standard deviations (S.D.) were caltulated.

RESULTS AND DISCUSSION

Kinetics of norfloxacin and sparfloxacin-melanin complexes formation shown as the relation between the amount of drug bound to the polymer and the incubation time for four given initial drug concentrations (c_0) is presented in Fig.1. It can be seen that the amount of drug bound to melanin increases with the prolongation of incubation time and after about 24 h it attains the equilibrium state. With an increase in the initial drug concentration, complex formation efficiency (being the ratio of the amount of drug bound to DOPA-melanin and the amount of drug added to complex formation expressed in %) decreased.

The relation between the amount of norfloxacin and sparfloxacin bound to melanin after 24 h of incubation and the initial drug concentration is presented in Fig. 2A as binding isotherms.

It can be seen from the binding curves that with an increase in the initial concentration of drugs the amount of drug bound to melanin increased. Moreover, the amount of drug bound to a constant amount of DOPA-melanin reaches a plateau at about 0.8 μmol norfloxacin per 1 mg melanin, which reflects the initial norfloxacin concentration 3 x 10^{-3}M, and about 0.85 μmol sparfloxacin/mg melanin for the initial sparfloxacin concentration: 3.5 x 10^{-3}M.

The use of the Scatchard method can provide information on binding parameters, i.e., the association constants and the number of binding sites for drug-melanin complexes. For the analyzed complexes of norfloxacin and sparfloxacin with synthetic melanin the Scatchard plots (Fig. 2B) are

curvilinear with an upward concavity indicating that at least two classes of independent binding sites are implicated in the formation of these complexes. The calculated binding parameters for the interaction of the analyzed drugs with DOPA-melanin are listed in Table 1. When comparing the number of strong (n_1) and weak (n_2) binding sites it can be observed that weak binding sites (n_2) are prevailing. The total number of binding sites (n_1+n_2) in the tested drug-melanin complexes indicates that norfloxacin and sparfloxacin have similar affinity to synthetic melanin ($n_1+n_2 \sim 1.1$ μmol drug/mg melanin). Simultaneously, the sparfloxacin-melanin complexes are characterized by greater stability ($K_1 \sim 10^5$ M^{-1}, $K_2 \sim 10^2$ M^{-1}) in regard to norfloxacin-melanin complexes ($K_1 \sim 10^4$ M^{-1}, $K_2 \sim 10^2$ M^{-1}).

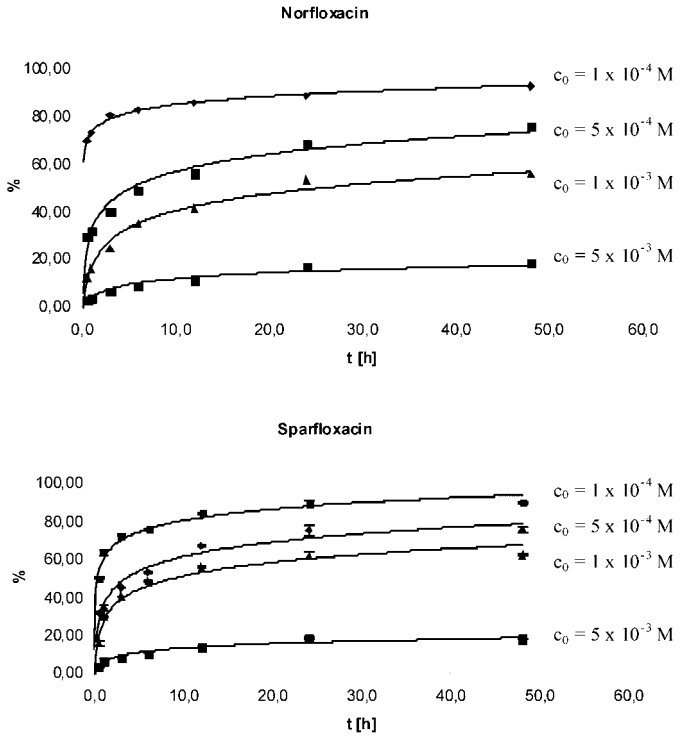

Fig. 1. Effect of incubation time (t) and initial drug concentration (c_0) on the amount of norfloxacin and sparfloxacin bound to DOPA-melanin (in %). Mean values ±SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

Table 1. Binding parameters for norfloxacin and sparfloxacin complexes with DOPA-melanin

Analyzed complex	Association constants K (M^{-1})	Number of binding sites n (μmol drug/mg melanin)
Norfloxacin – melanin	$K_1 = 6.71 \times 10^4$ $K_2 = 5.89 \times 10^2$	$n_1 = 0.360$ $n_2 = 0.744$ $n_1+n_2 = 1.104$
Sparfloxacin – melanin	$K_1 = 7.00 \times 10^5$ $K_2 = 8.49 \times 10^2$	$n_1 = 0.268$ $n_2 = 0.851$ $n_1+n_2 = 1.119$

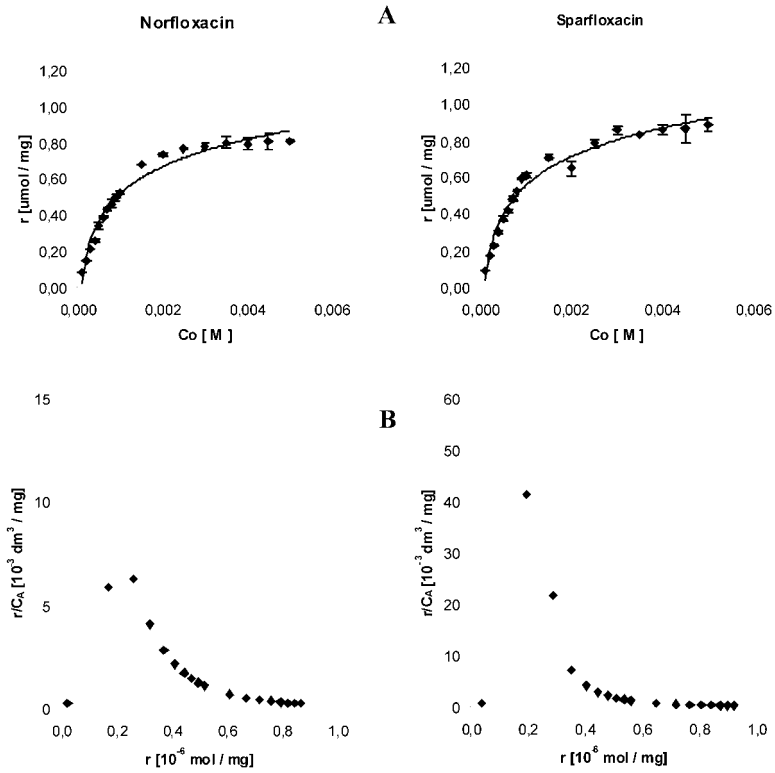

Fig. 2. Binding isotherms (A) and Scatchard plots (B) for norfloxacin and sparfloxacin complexes with DOPA-melanin; r – amount of drug bound to melanin, c_0 – initial drug concentration, c_A – concentration of unbound drug. Mean values ±SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

It has been reported that fluoroquinolones show affinity for melanin and pigmented tissues [2, 6]. Moreover, it has been sugested that electrostatic forces can play an important role in the binding of drugs to melanin. Simultaneously, non electrostatic contributions, including hydrophobic and van der Waals interactions and charge-transfer reactions would also contribute to the binding of drugs [6].

In our studies, the binding capacity of norfloxacin and sparfloxacin to melanin, was demonstrated. The ability of the analyzed fluoroquinolone derivatives to form complexes with synthetic melanin *in vitro*, may be one of the reasons for their phototoxicity *in vivo* as a result of their interaction with melanin in the pigmented structures of the eye and skin.The obtained results indicate that sparfloxacin forms more stable complexes with melanin, which may explain the higher capacity of sparfloxacin to induce phototoxic reactions as compared with norfloxacin. However, the question of association between fluoroquinolones accumulation in melanin rich tissues and phototoxic action remains open.

CONCLUSIONS

1. Norfloxacin and sparfloxacin form stable complexes with DOPA-melanin.

2. The analysis of drugs binding to melanin has shown that at least two classes of independent binding sites are implicated in these complexes formation: strong binding sites (n_1) with the association constant $K_1 \sim 10^4 – 10^5$ M^{-1} and weak binding sites (n_2) with the association constant $K_2 \sim 10^2$ M^{-1}.

3. Sparfloxacin-melanin complexes are characterized by greater stability in regard to norfloxacin-melanin complexes, which may explain the higher capacity of sparfloxacin to induce phototoxic reactions.

4. The ability of the analyzed fluoroquinolone derivatives to form complexes with synthetic melanin *in vitro* may be one of the reasons for their phototoxicity *in vivo* as a result of their interaction with melanin in the pigmented structures of the eye and skin.

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SUMMARY

The aim of this study was to examine *in vitro* the binding capacity of fluoroquinolone derivatives: norfloxacin and sparfloxacin, causing adverse phototoxic effects in the eye and skin structures, to DOPA-melanin. The analysis of results concerning the kinetics of drug-melanin complexes formation showed that the amount of drug bound to melanin increases with increasing initial drug concentration and prolongation of incubation time. The analysis of drugs binding to melanin has shown that at least two classes of independent binding sites must be implicated in these complexes formation: strong binding sites (n_1) with the association constant $K_1 \sim 10^4\ M^{-1}$ for norfloxacin and $K_1 \sim 10^5\ M^{-1}$ for sparfloxacin complexes and weak binding sites (n_2) with $K_2 \sim 10^2\ M^{-1}$. The demonstrated ability of norfloxacin and sparfloxacin to interact with DOPA-melanin *in vitro* is discussed as one of the risk factors of their phototoxicity *in vivo*.

STRESZCZENIE

Celem badań była ocena zdolności wiązania z melaniną wybranych pochodnych fluorochinolonów: norfloksacyny i sparfloksacyny, wywołujących niepożądane działania fototoksyczne skierowane na struktury skóry i oka. Na podstawie uzyskanych wyników kinetyki tworzenia kompleksu lek-melanina stwierdzono, iż ilość leku związanego z melaniną wzrasta wraz ze wzrostem stężenia początkowego leku i wydłużaniem czasu inkubacji. Analiza wiązania leków do melaniny wykazała, że w tworzeniu tych kompleksów uczestniczą co najmniej dwie klasy niezależnych miejsc wiążących: miejsca silnie wiążące (n_1) o wartości stałej trwałości $K_1 \sim 10^4\ M^{-1}$ dla kompleksów norfloksacyny i $K_1 \sim 10^5\ M^{-1}$ dla kompleksów sparfloksacyny oraz miejsca słabo wiążące (n_2) z $K_2 \sim 10^2\ M^{-1}$. Wykazana zdolność norfloksacyny i sparfloksacyny do oddziaływania z DOPA-melaniną w warunkach *in vitro* może być jednym z czynników ryzyka rozwoju reakcji fototoksycznych *in vivo*.