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Interaction of quinidine, disopyramide and metoprolol with melanin *in vitro* in relation to drug-induced ocular toxicity

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The aim of this work was to evaluate binding capacity of quinidine, disopyramide and metoprolol to melanin *in vitro*. The antiarrhythmics studied cause adverse reactions to the eye. Synthetic DOPA-melanin was used in the studies and a UV spectrophotometric method was employed to determine the drugs. The studies of the kinetics of the formation of quinidine-melanin, disopyramide-melanin and metoprolol-melanin complexes indicate that for all the complexes investigated the maximum time to reach reaction equilibrium is 24 h. Binding parameters, i.e., the numbers of independent binding sites and the association constants were determined on the basis of the Scatchard plots. An analysis of the binding curves obtained supports our conclusion that both strong (n_1) and weak (n_2) binding sites are involved in the formation of the complexes investigated. The total numbers of binding sites in synthetic DOPA-melanin complexes with quinidine, disopyramide and metoprolol were 0.525, 0.493 and 0.387 μmol/mg, respectively. The quinidine-melanin complex is characterized by greater stability ($K_1 = 3.00 \times 10^5$ M^{-1}, $K_2 = 1.75 \times 10^3$ M^{-1}) in comparison with biopolymer complexes with disopyramide ($K_1 = 1.12 \times 10^4$ M^{-1}, $K_2 = 6.04 \times 10^2$ M^{-1}) and metoprolol ($K_1 = 1.42 \times 10^4$ M^{-1}, $K_2 = 7.89 \times 10^2$ M^{-1}). The ability of these drugs to form complexes with melanin *in vitro* may be one of the reasons for their ocular toxicity *in vivo*, as a result of their accumulation in melanin in the eye.

1. Introduction

Melanins, natural pigments, are composed of highly heterogeneous polymers containing indole subunits as well as intermediates of melanogenesis. An analysis of their chemical structure indicates that the biopolymer is a poly-anion with a high content of carboxyl and semiquinone groups [1–5]. It is characterized by a three-dimensional, heterogeneous random amorphous structure, and particular biopolymer subunits are combined via various weakly hydrolyzing bonds [2, 6–8].

Melanin biopolymers are present in animals and plants. In the human body pigments are found in high concentrations in the basal layer of epidermis [9, 10], hair, hair follicles [11], eye (choroid, retina, iris, ciliary body) [12–14], inner ear [15], locus coeruleus and substantia nigra [16, 17]. Melanin is also found in human eosinophils and in leucocytes of mammals [18].

Melanin is synthesized in melanosomes of the melanocytes. Its biosynthesis is complex and is influenced by genetic, hormonal and metabolic factors [19–22]. Tyrosine is an endogenous substrate in melanin production. In the presence of molecular oxygen and the enzyme tyrosinase, the amino acid oxidizes to 3,4-dihydroxyphenylalanine and then, as a result of the subsequent reactions, it turns into eumelanin (brown or black pigment), and when combined with cysteine or glutathione it turns into phaeomelanin (yellow or red pigment) [3–5].

Melanin biopolymers are capable to bind many medicinal substances. By inhibiting or significantly restricting drug access to cell receptors, they protect the organism against drug side effects [10, 23]. Albinos are deprived of such protection, which is why some toxic effects of drug administration occur much faster [24, 25]. The drugs binding to melanin include sympathomimetic amines [26], antibiotics [27, 28], β-blockers [23, 29, 30], antimalarials [31–34], local anaesthetics [35, 36], and drugs acting on the central nervous system [31, 37]. Binding of a drug to melanin is either a reversible or an irreversible process and is directly related to the type of melanin-drug bond. Electrostatic forces, charge-transfer reactions, Van der Waals forces and hydrophobic interactions play an important role in binding [31–33, 38]. Drug-melanin complexes are characterized by various degrees of stability, and in most cases there are at least two classes of independent binding sites involved in complex formation. Depending on the value of the association constant, they are divided into strong and weak binding sites [28, 31, 33]. Drugs identified as binding to melanin display some basicity (most have $pK_a > 7$) and lipophilic characteristics [39, 40].

Unfortunately, drugs bound to melanin may accumulate in pigmented tissues (e.g. in the eye or ear) and may induce lesions or degeneration [10]. Slow release of drugs from bonds may prolong the exposure of tissues to potentially beneficial or adverse reactions [24]. Some drug-melanin binding may trigger free radical formation inducing lesions in cells and tissues [41].

Ocular side effects, disorder, impairment or loss of vision, may occur as a result of some drugs. In addition to the cornea, lens and optic nerve, the retina and choroid are highly susceptible to toxic effects of drugs. Drug accumulation in the uveal tract and retina brings about a disturbance of retinal metabolism and drug-induced retinopathy, which is manifested by colour vision defects, visual field changes and a pigmentary disturbance of the macula, and in some cases may result in blindness [24, 42, 43]. Careful review of studies on drug-induced retinal lesions indicates that these lesions may result from the intrinsic toxicity of the drug (independent of drug-melanin binding), or from biopolymer binding of drugs. The results of these two mechanisms inducing lesions have also been considered [40].

Drugs exerting adverse reactions on the eye include class IA antiarrhythmics, such as quinidine and disopyramide, and class II antiarrhythmics, such as β_1-selective blockers, e.g. metoprolol.

Quinidine-induced eye lesions are manifested by visual disturbances including scotomate, impaired colour vision and toxic amblyopia, blurred vision and photophobia [44–46]. The drug may accumulate as deposits in the corneal epithelium [45] and may cause acute iridocyclitis [47, 48].

Disopyramide side effects on the eye are manifested by blurred vision, pupillary dilation and dry eyes [49–52]. In some cases symptoms of glaucoma have been observed [51, 53]. The intensity of disopyramide side effects depends on the drug dosage and treatment duration as well as individual patient sensitivity [50, 53].

Metoprolol-induced eye lesions, usually occurring during long-term therapy, relate to a relatively small group of patients, and are usually manifested by headaches, pains and soreness in both eyes, a transitory burning sensation in the eyes, dry eyes and difficulties in opening the lids [54–57].

A drug's affinity to melanin is usually examined *in vitro* using synthetic or isolated polymer. The purpose of the present studies is to evaluate *in vitro* the affinity to melanin of antiarrhythmics displaying adverse reactions in the eye, that is, quinidine, disopyramide and metoprolol. To achieve the objectives, the kinetics of drug-melanin complex formation have been examined and the number of independent binding sites and the association constants were determined. Synthetic DOPA-melanin was used in the studies because of its similarity to natural eumelanins.

2. Investigations and results

Kinetics of formation of quinidine, disopyramide and metoprolol-melanin complexes shown as the relation between the amount of drug bound to the polymer and the incubation time for the four given initial drug concentrations (c_o) are presented in Fig. 1. It was demonstrated that prolonged incubation time results in an increased amount of drug bound to synthetic melanin. This tendency is especially noticeable during the initial hours of complex formation. On the basis of the results obtained it can be shown that the maximum time to achieve equilibrium state is 24 h for the complexes examined. With an increase in the initial drug concentration, complex formation efficiency (the ratio of the amount of drug bound to DOPA-melanin and the amount of drug added to complex formation, expressed as a %) decreases.

The relationship between the amount of drug bound to synthetic DOPA-melanin after 24 h of incubation and the
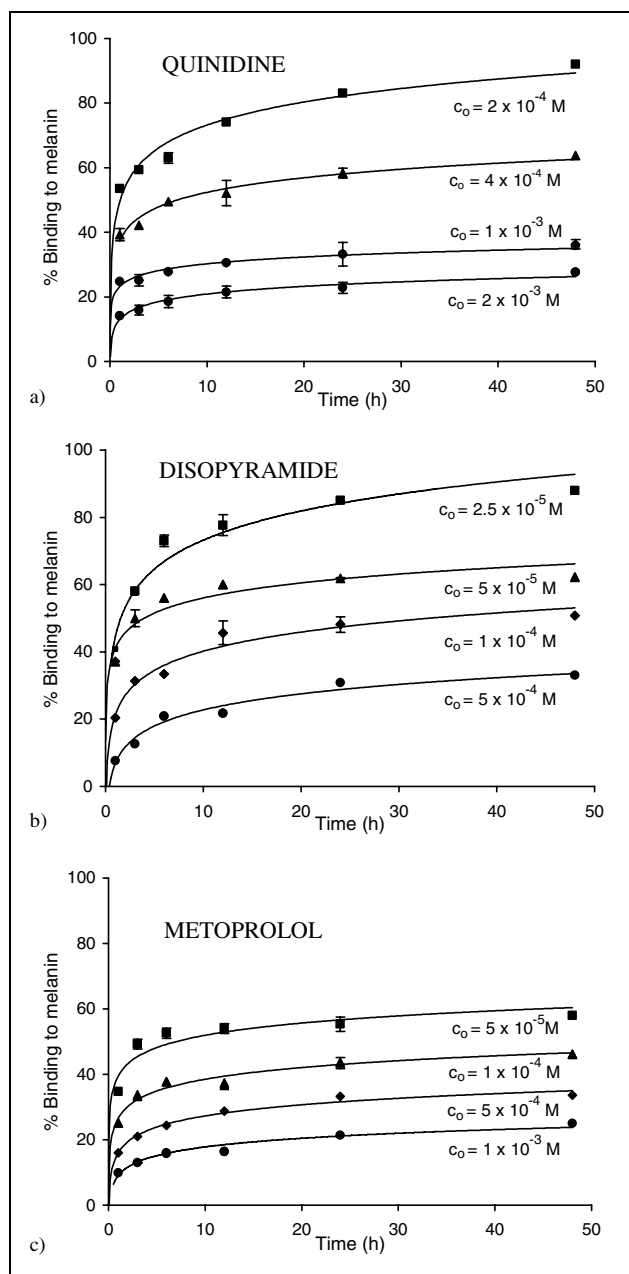

Fig. 1: Effect of incubation time and initial drug concentration (c_o) on quinidine, disopyramide and metoprolol binding to DOPA-melanin (in %). Results are means ± SD (N = 3). Points without error bars indicate that SD was less than the size of the symbol

initial drug concentration is presented in Fig. 2 as binding isotherms. It can be seen from the binding curves that with an increase in the initial concentration of quinidine, disopyramide and metoprolol the amount of drug bound to synthetic DOPA-melanin increases. In the quinidine concentration range studies (4×10^{-5}–5×10^{-3} M) the increase was 20-fold: from 0.0257 to 0.5285 µmol/mg. Similar observations can be made for disopyramide as an increase in the initial concentration from 2.5×10^{-5} M to 4×10^{-3} M results in an increase in the amount of drug bound to melanin from 0.0213 to 0.4306 µmol/mg, whereas an increase in the initial metoprolol concentration from 5×10^{-5} M to 3×10^{-3} M is accompanied by a 10-fold increase in the amount of drug bound to the biopolymer (from 0.0269 to 0.2735 µmol/mg). When comparing the amount of drug in µmoles bound to 1 mg of melanin it can be observed that quinidine binds most (about
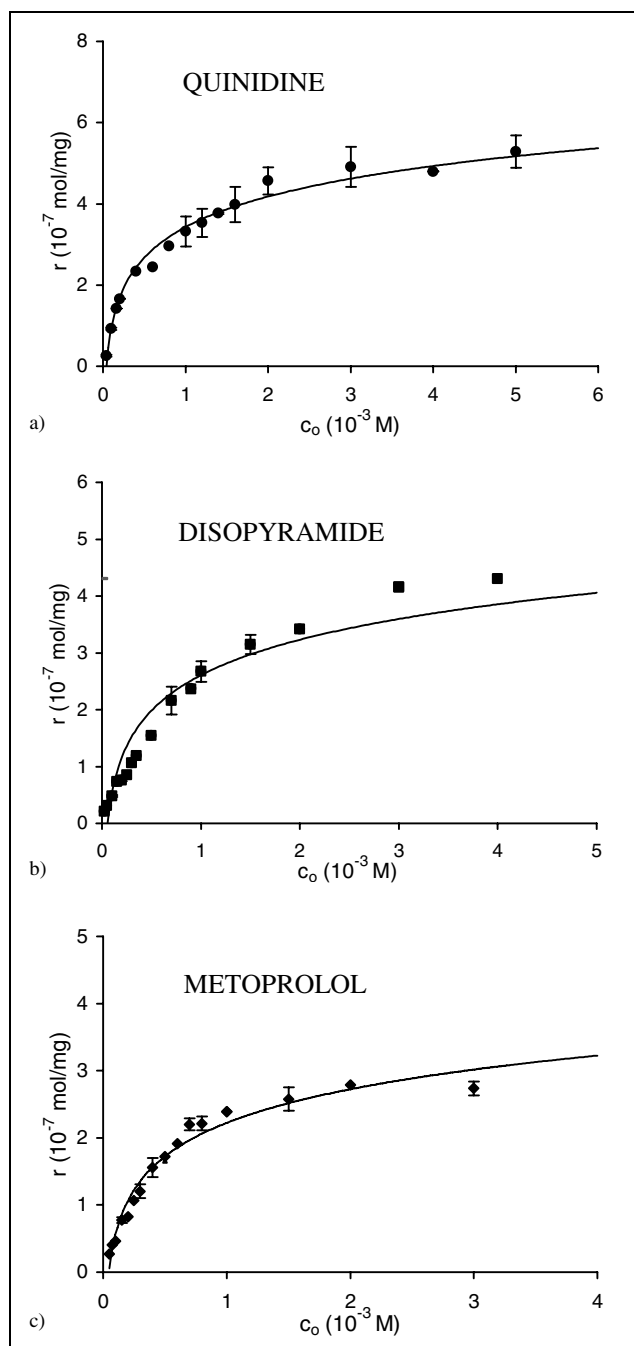
Fig. 2: Binding isotherms for quinidine-melanin, disopyramide-melanin and metoprolol-melanin complexes. r — amount of drug bound to melanin, c_o — initial drug concentration. Results are means ± SD (N = 3). Points without error bars indicate that SD was less than the size of the symbol

Fig. 3: Scatchard plots for quinidine-melanin, disopyramide-melanin and metoprolol-melanin complexes. r — amount of drug bound to melanin, c_o — initial drug concentration, c_A — concentration of unbound drug

0.53 μmol/mg) and metoprolol least (about 0.27 μmol/mg) to synthetic DOPA-melanin.

The use of the Scatchard method [58, 59] can provide information on binding parameters, i.e., the association constant and the number of binding sites for drug-melanin complexes. For all the complexes analyzed the Scatchard plots (Fig. 3) are curvilinear with an upward concavity indicating that at least two classes of independent binding sites are implicated in quinidine, disopyramide and metoprolol-melanin complex formation. When comparing the number of strong (n_1) and weak (n_2) binding sites (Table 1) in quinidine-melanin, disopyramide-melanin and metoprolol-melanin complexes it may be observed that weak binding sites predominate. For the quinidine-mela-
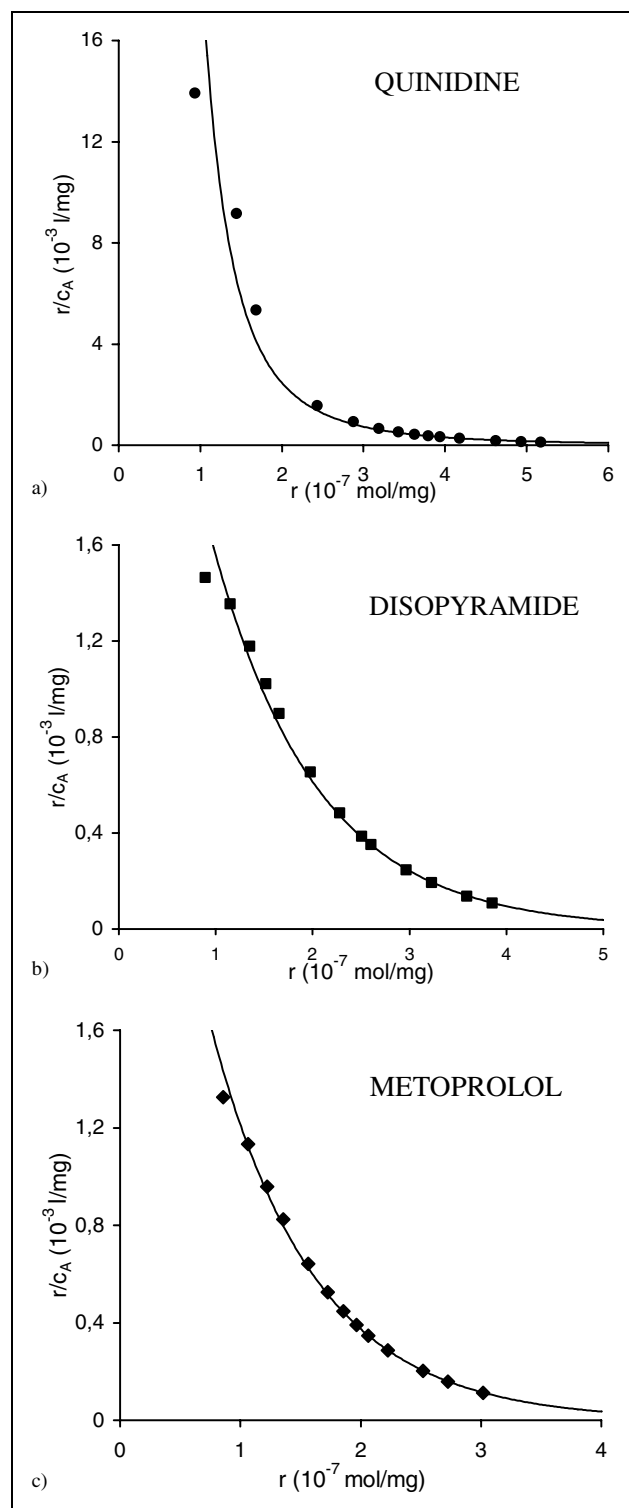
nin complex the ratio $n_2/n_1 = 2.4$, while for disopyramide-melanin and metoprolol-melanin complexes it is about 1.3. The greatest number of strong binding sites is present in the disopyramide-melanin complex and is about 1.5 times greater in comparison with the number of strong binding sites in other drug-melanin complexes. While analysing the data on the total number of binding sites it may be observed that it is greatest for quinidine (0.525 μmol/mg), slightly lower for disopyramide

Table: Binding parameters for quinidine-melanin, disopyramide-melanin and metoprolol-melanin complexes

Drug-melanin complex	Association constants K (M^{-1})	Number of binding sites n (μmol/mg)
Quinidine-melanin	$K_1 = 3.00 \times 10^5$ $K_2 = 1.75 \times 10^3$	$n_1 = 0.1541$ $n_2 = 0.3713$ $n_1 + n_2 = 0.5254$
Disopyramide-melanin	$K_1 = 1.12 \times 10^4$ $K_2 = 6.04 \times 10^2$	$n_1 = 0.2121$ $n_2 = 0.2806$ $n_1 + n_2 = 0.4927$
Metoprolol-melanin	$K_1 = 1.42 \times 10^4$ $K_2 = 7.89 \times 10^2$	$n_1 = 0.1667$ $n_2 = 0.2203$ $n_1 + n_2 = 0.3870$

(0.493 μmol/mg) and significantly lower for metoprolol (0.387 μmol/mg).

The quinidine-melanin complex is characterized by greater stability $(K_1 = 3.00 \times 10^5 \, M^{-1}$, $K_2 = 1.75 \times 10^3 \, M^{-1})$ in regard to disopyramide-melanin complex $(K_1 = 1.12 \times 10^4 \, M^{-1}$, $K_2 = 6.04 \times 10^2 \, M^{-1})$ and metoprolol-melanin complex $(K_1 = 1.42 \times 10^4 \, M^{-1}$, $K_2 = 7.89 \times 10^2 \, M^{-1})$.

3. Discussion

Because of their affinity for ring compounds, melanin biopolymers are said to play a protective role in shielding pigment cells and adjacent tissues against potentially harmful substances, including adverse reactions of drugs [23]. At the same time, melanin biopolymers ability to bind drugs may influence the drugs chemical, biological, photochemical and photobiological properties [33]. Drug accumulation in pigmented tissues (e.g. in the eye) may increase the risk of pathological states occurring there [10, 23, 32, 45, 60].

Although the blood-retinal barrier is very tight and usually restricts the passage of substances through the retinal pigment epithelium (RPE), the permeability of the membrane can occasionally be altered, leading to penetration of photosensitising compounds (e.g. drugs) through the RPE and retina [12]. Retinal lesions may be manifested by retinopathy, resulting from a high drug concentration in the pigment [24, 42, 43, 45]. Moreover, side effects on unpigmented structures are also manifested by keratopathy, as the result of drug accumulation. Drug-induced ocular toxicity may produce symptoms such as blurred vision, halos, photophobia and secondary colour blindness [45].

Like chloroquine and hydroxychloroquine, quinidine is a quinoline derivative and is known to have ocular toxic side effects [44–48]. The studies by Zaidman [45] suggest that the drug may accumulate within the corneal epithelium and the opacities observed are identical to those found in patients with chloroquine or hydroxychloroquine keratopathy. Additionally, colour vision defects and visual field changes are similar to those described in chloroquine retinopathy. Chloroquine, which is known for its phototoxicity, shows a high binding capacity for melanin biopolymer [33]. It has been demonstrated that at least two classes of independent binding sites participate in chloroquine-melanin complex formation: strong binding sites $n_1 = 0.103$ μmol/mg with the association constant $K_1 = 2.52 \times 10^5 \, M^{-1}$ and weak binding sites $n_2 = 0.742$ μmol/mg with $K_2 = 2.71 \times 10^3 \, M^{-1}$ [33]. When comparing the binding parameters for chloroquine-melanin complexes [33] with the results obtained for qui-

nidine-melanin complexes (Table 1), similar stability and prevalence of weak binding sites in both types of complex are observed. The total number of binding sites $(n_1 + n_2)$ is lower by about 40% in the case of quinidine, compared with the chloroquine-melanin complex.

However, the disopyramide-melanin and metoprolol-melanin complexes (Table 1) are significantly less stable in comparison to chloroquine and quinidine-melanin complexes (the association constants differ in their magnitude). Furthermore, the total number of binding sites is also lower, especially in the case of the metoprolol-melanin complex $(n_1 + n_2 = 0.387$ μmol/mg).

Among the drugs investigated, quinidine exerts the strongest ocular side effects and it shows the greatest affinity to melanin *in vitro* (Table 1). However, the affinity of quinidine as well as that of disopyramide and metoprolol is lower than that of chloroquine [33]. It is probably related to their weaker photosensitivity reactions and adverse effects on the eye, especially in case of metoprolol, in comparison with those of chloroquine [42, 46, 61].

Similar studies concerning the interaction of antimalarial drugs with melanin *in vitro* have been performed by Kristensen et al. [34]. They evaluated the complex formation of quinine (laevorotatory isomer of quinidine) with melanin from Sepia officinalis. The authors calculated the total number of binding sites $n = 2.89 \times 10^{-6}$ mol/5 mg of melanin (i.e. 0.58 μmol/mg), which is comparable with our results (the total number of binding sites in the quinidine-melanin complex $n_1 + n_2 = 0.53$ μmol/mg).

Our studies demonstrate that the antiarrhythmics (quinidine, disopyramide and metoprolol) are capable of forming complexes with synthetic DOPA-melanin using at least two classes of independent binding sites. The demonstrated ability of these drugs to interact with melanin *in vitro* may influence their ocular toxicity *in vivo*, as a result of their accumulation in melanin in the eye.

4. Experimental

4.1. Chemicals and apparatus

L-3,4-Dihydroxyphenylalanine (L-DOPA), quinidine (sulphate salt dihydrate), disopyramide (phosphate salt), and metoprolol (tartrate salt) used in the studies were obtained from Sigma Chemical Co. The remaining chemicals were produced by P.P.H. POCH, Poland. Spectrophotometric measurements were performed using a JASCO model V-530 UV-VIS spectrophotometer.

4.2. Melanin synthesis

Model synthetic melanin was obtained by oxidative polymerization of L-3,4-dihydroxyphenylalanine (L-DOPA) solution (1 mg/ml) in 0.067 M phosphate buffer (pH 8.0) for 48 h according to the Binns method [62].

4.3. Drug-melanin complex formation

Drug-melanin complexes were obtained by suspending 5 mg of synthetic DOPA-melanin in 5 ml of a solution of the drug under investigation. Complex formation was conducted in a neutral medium using an aqueous solution of quinidine and 0.067 M phosphate buffer (pH = 7.0) solutions of disopyramide and metoprolol. 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 the appropriate solvent, were treated in the same way.

4.4. Determination of the amount of drugs bound to melanin

A UV spectrophotometric method was used for quantitative determination of the drugs. Absorption spectra in the UV-VIS range were measured for an aqueous solution of quinidine and buffer solutions of disopyramide and metoprolol. Analytical wavelengths (λ_{max}) for the compounds studied were chosen as follows: 331 nm for quinidine, 261 nm for disopyramide and 274 nm for metoprolol. The calculated values of the molar absorption coef-

ficient ($\varepsilon_{\lambda max}$), 4.88×10^3 for quinidine, 4.22×10^3 for disopyramide and 1.41×10^3 for metoprolol, were used to estimate the amount of drug bound to the polymer.

4.5. Kinetics of drug-melanin complex formation

Kinetics of formation of melanin complexes with quinidine, disopyramide and metoprolol were evaluated on the basis of the relationship between the amount of drug bound to the polymer (μmol/mg) and the time for complex formation. In the studies the following initial drug concentrations were used: 2×10^{-4}, 4×10^{-4}, 1×10^{-3}, and 2×10^{-3} M for quinidine, 2.5×10^{-5}, 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} M for disopyramide and 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M for metoprolol. Complex formation lasted for 1, 3, 6, 12, 24 and 48 h.

4.6. 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 DOPA-melanin complexes with quinidine, disopyramide and metoprolol were calculated via Scatchard plots [58, 59]. Experimental binding isotherms were used to construct these plots. They show the relationship between the amount of the drug bound to melanin and its initial concentration after reaching equilibrium state, i.e., after 24 h. The initial drug concentration was 4×10^{-5} M to 5×10^{-3} M for quinidine, 2.5×10^{-5} M to 4×10^{-3} M for disopyramide and 5×10^{-5} M to 3×10^{-3} M for metoprolol.

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