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# *Interaction of ketoprofen and paracetamol with melanin in vitro*

Oddziaływanie ketoprofenu i paracetamolu z melaniną *in vitro*

Ketoprofen is a non-steroidal anti-inflammatory drug belonging to the arylopropionic acid derivatives. Paracetamol (acetaminophen) is an analgesic and antipyretic drug. Ketoprofen and paracetamol are widely used over-the-counter because of their relatively low toxicity. However, allergic and photoallergic dermatitis and eczema are the side-effects [3, 4, 12, 14]. Clinical experience has shown that people with red hair and light skin complexion have increased photosensitivity and a higher incidence of skin cancer than people with dark hair and skin. Skin colour differences between people are due to the amount, type, and arrangement of melanin in the skin [10]. Melanin is a complex mixture of biopolymers derived from tyrosine, produced in melanocytes and passed to surrounding keratinocytes [5]. Melanins play an important role in protecting skin against UV radiation [15]. They absorb UVR across a wide spectrum with the highest absorption in the shorter wavelengths that are most associated with DNA damage [6]. Melanins may also act as a biochemical dustbin, mopping up free radicals and other potentially toxic agents such as metal ions or drugs [11]. The ability of melanin biopolymer to bind various drugs is one of the most characteristic features of this pigment.

The aim of this study was to examine the interaction of ketoprofen and paracetamol with melanin.

## MATERIAL AND METHODS

Chemicals. L-3,4-dihydroxyphenylalanine (L-DOPA) used in the studies was obtained from Sigma Chemical Co. Ketoprofen was obtained in the form of solution – Ketonal (100mg/2ml) from Lek Pharmaceuticals d.d., Slovenia and paracetamol as Perfalgan (10mg/1ml) from Bristol-Meyers Squibb, Poland. The remaining chemicals were produced by POCH S.A., Poland.

Melanin synthesis. Model synthetic melanin was formed by oxidative polymerization of L-3,4-dihydroxyphenylalanine (L-DOPA) in 0.067M phosphate buffer at pH 8.0 for 48h according to the method described by Binns et al [2].

Drug-melanin complex formation. Drug-melanin complexes were obtained as follows: 5mg of melanin were placed in plastic test-tubes, where ketoprofen or paracetamol solutions were added to the final volume of 5ml. The initial concentration of drugs ranged from $1 \cdot 10^{-6}$M to $1 \cdot 10^{-4}$M. Control samples contained 5mg of melanin and 5ml of distilled water. All samples were incubated at room temperature, and then filtered.

Analysis of drug binding to melanin. The concentrations of ketoprofen and paracetamol remaining in each filtrate after incubation with melanin with respect to the control sample were determined spectrophotometrically according to the *Pharmacopoeia* method. All spectrophotometric measurements were made using the UV-VIS spectrophotometer JASCO model V-530, at wavelength 260nm for ketoprofen and 243nm for paracetamol. The sensitivity of the spectrophotometric methods was investigated by measuring absorbance of different concentrations of drugs (from $1 \cdot 10^{-6}$M to $1 \cdot 10^{-4}$M). All the data points produced 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 ($\varepsilon_{\lambda max}$) $1.61 \cdot 10^4$ for ketoprofen, and $9.62 \cdot 10^3$ for paracetamol, were used to estimate the amount of drug bound to the polymer.

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

The qualitative analysis of drug - melanin interaction was performed using the Scatchard plots of the experimental data according to Kalblitzer and Stehlik [9]. The number of binding sites (n) and the values of association constant (K) were calculated.

Statistical analysis. In all experiments, the mean values for three independent experiments ± standard deviation (SD) were calculated.

## RESULTS AND DISCUSSION

The effect of the incubation time and initial drug concentration on the amount of drugs bound to melanin 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 24 h it attains a plateau. It has been also shown that the amounts of drug bound to melanin increase with the increasing initial drug concentration. Simultaneously, the decrease of complex formation efficiency, expressed in % as the ratio of the amount of drug bound to melanin to the initial amount of drug added to melanin, was observed with the increase of the initial drug concentration (Fig. 1).

Dependence of the amount of ketoprofen and paracetamol bound to melanin after 24h of incubation as a function of the initial drug concentration is presented in Fig. 2A as binding isotherms. It can be seen from binding curves that the amount of drug bound to a constant amount of DOPA-melanin increases and reaches a plateau at about 8nmol ketoprofen per 1 mg melanin, which reflects the initial ketoprofen concentration $7 \cdot 10^{-5}$M, and about 7nmol paracetamol/mg melanin for the initial paracetamol concentration: $8 \cdot 10^{-5}$M.

Dependencies of the amount of drugs bound to melanin (r) to the concentration of unbound drugs ($c_A$), i.e., r/ $c_A$, versus r for ketoprofen and paracetamol complexes with DOPA-melanin are presented in Fig. 2B as Scatchard plots. The use of the Scatchard method can provide information about the number and nature of binding sites in the analyzed complexes. The analysis of drugs binding to melanin shows that Scatchard plots are curvilinear with an upward concavity, indicating that at least two classes of independent binding sites must be implicated in drug-melanin complexes formation. The calculated binding parameters are listed in Table 1. Strong binding sites ($n_1$) with the association constant $K_1$ about $4–6 \cdot 10^5 M^{-1}$ and weak binding sites ($n_2$) with the association constant $K_2$ about $2 \cdot 10^4 M^{-1}$ participate in ketoprofen and paracetamol interaction with melanin.

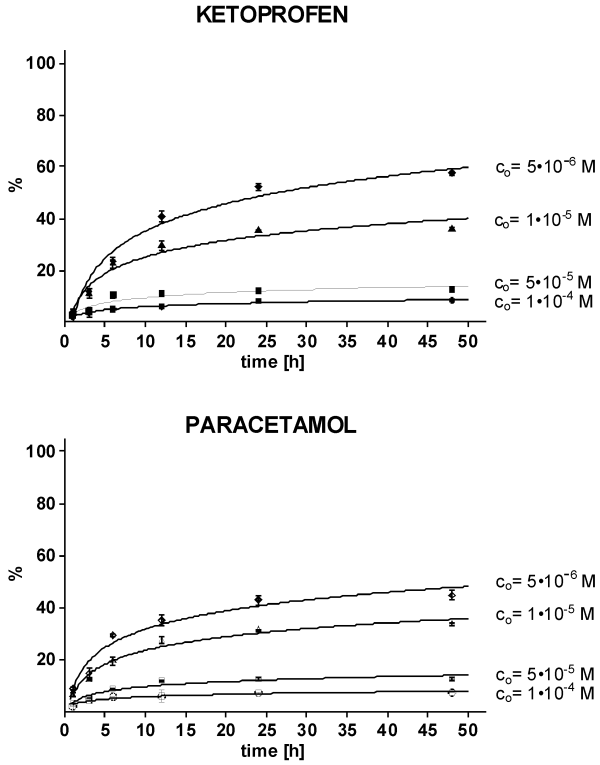


Fig 1. Effect of incubation time and initial drug concentration ($c_o$) on the amount of ketoprofen and paracetamol bound to DOPA-melanin (in %). Mean values ±SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

The biological impact of drugs-melanin interaction has received considerable attention for the past years [11], but the physiological meaning of melanin binding is still not fully understood. Chemical elucidation of the structure of melanin is difficult because it represents a range of biopolymers, and extraction damages primary structure. Nonetheless, two broad classes of melanins are commonly the basis for assay; eumelanin, which is brown and black, and pheomelanin, which is red or yellow [8, 13]. These two types of melanin vary in composition and properties: eumelanin is a nitrogeneous pigment mainly composed of 5,6-dihydroxyindole (DHI) (dark brown to black in colouration) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (light brown in colouration) [1]. Pheomelanin is like eumelanin a nitrogeneous compound, but it contains sulphur and is alkali soluble [8]. Melanins are polyanions with a relatively high content of carboxyl groups and o-semiquinones, which are negatively charged at physiological pH. Substances with cationic properties (eg. metal ions, some drugs) are thus bound to melanin probably by ionic interaction, which also may be strengthened by other forces such as van der Waals attraction, charge-transfer reactions and hydrophobic interactions [12].

Synthetic melanins prepared enzymatically or chemically from L-DOPA contain more carboxyl groups than natural melanins. However, it has been demonstrated that ion-exchange, redox and free radical properties established in synthetic melanins are also relevant for natural melanins. Both natural and synthetic melanin polymers have been used in binding studies of ligands and no significant differences in the affinity were observed [7].

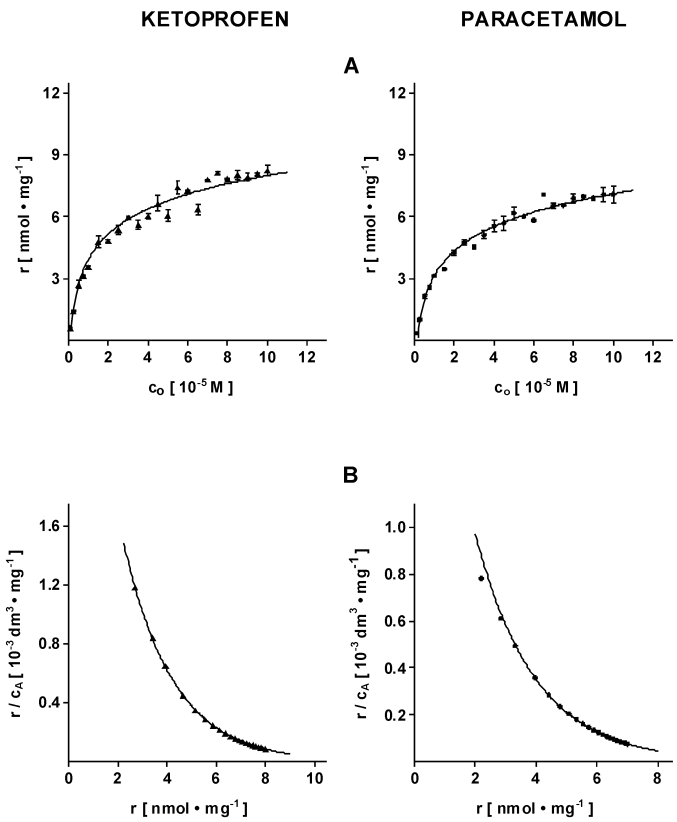


Fig 2. Binding isotherms (A) and Scatchard plots (B) for ketoprofen and paracetamol complexes with DOPA-melanin; r – amount of drug bound to melanin, $c_o$ – initial drug concentration, $c_A$ – concentration of unbound drug. Mean values ±SD from three independent experiments are presented. Points without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

The obtained results demonstrate that both analyzed drugs: ketoprofen and paracetamol form stable complexes with DOPA-melanin ($K_1$~$10^5 M^{-1}$, $K_2$~$10^4 M^{-1}$) *in vitro*. Melanin binding may lead to the accumulation of drug *in vivo* in melanin-rich tissues (e.g. skin), which may prolong the action of the drug and influence its phototoxic adverse effects.

Table 1. Binding parameters for ketoprofen and paracetamol complexes with DOPA-melanin

| Analyzed complex | Association constants K [$M^{-1}$] | Number of binding sites n [nmol drug/mg melanin] |
|---|---|---|
| Ketoprofen-melanin | $K_1=6.16•10^5$<br>$K_2=2.63•10^4$ | $n_1=4.2$<br>$n_2=5.7$<br>$n_1+n_2=9.9$ |
| Paracetamol-melanin | $K_1=4.50•10^5$<br>$K_2=2.45•10^4$ | $n_1=3.6$<br>$n_2=5.3$<br>$n_1+n_2=8.9$ |

## CONCLUSIONS

1. Ketoprofen and paracetamol 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 must be implicated in these complexes formation: strong binding sites ($n_1$) with the association constant $K_1$~ 4-6•$10^5$ $M^{-1}$ and weak binding sites ($n_2$) with $K_2$~2•$10^4$ $M^{-1}$
3. Melanin binding may lead to the accumulation of ketoprofen and paracetamol in melanin-rich tissues (e.g. skin) and influence their phototoxic effects.

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## SUMMARY

We have demonstrated that ketoprofen and paracetamol form stable complexes with melanin and the amount of drug bound to the polymer increases with the increase of the initial drug concentration and the 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 $K_1 \sim 4\text{-}6 \bullet 10^5\ M^{-1}$ and weak binding sites ($n_2$) with $K_2 \sim 2 \bullet 10^4\ M^{-1}$. The ability of the analyzed drugs to form complexes with melanin *in vitro* may be one of the reasons for their phototoxic side-effects *in vivo*, as a result of their accumulation in melanin in the skin.

## STRESZCZENIE

Wykazaliśmy, że ketoprofen i paracetamol tworzą stabilne kompleksy z melaniną, a ilość leku związanego z polimerem 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 $K_1 \sim 4\text{-}6 \bullet 10^5\ M^{-1}$ oraz miejsca słabo wiążące ($n_2$) z $K_2 \sim 2 \bullet 10^4\ M^{-1}$. Zdolność analizowanych leków do tworzenia kompleksów z melaniną *in vitro* może być jedną z przyczyn ich niepożądanych działań fototoksycznych *in vivo* w wyniku kumulacji w melaninie skóry.